The Handbook of Microbial Bioresources

Edited by V.K. Gupta, G.D. Sharma, M.G. Tuohy and R. Gaur

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The Handbook of Microbial Bioresources

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Preface

Microbes play an integral role in the development of biotechnology and biomedical sectors. It has become a subject of increasing importance as new microbial resources are identified. The interaction between microbes and their environment is central to many natural processes that occur in the biosphere. The hosts and habitats of these microorganisms are very diverse; microbes are present in every ecosystem on earth. The microbial branch of the tree of life is immensely diverse, consisting of several defined and still unresolved phyla, yet detailed knowledge is limited to relatively few taxa. The relationship between microbes and humans has been characterized by the juxtaposed viewpoints of microbes as infectious agents of much dread and their exploitation as highly versatile systems for a range of economically important biotechnological applications. Understanding the biology of different microbes in diverse ecosystems as well as their biotrophic interactions with other microorganisms, animals, humans and plants is essential to underpin effective and innovative technological developments. Microbes occupy an important place in the natural world because as non-photosynthetic organisms they obtain their nutrients from the degradation of organic material. They use many of their secondary metabolites to secure a place in a competitive natural environment and to protect themselves from predation. The diverse structures, biosyntheses and biological activities of microbial products or the microbe itself as a product have attracted chemists for many years. Microbes are ubiquitous and their activities affect many aspects of our daily lives, whether it be as sources of pharmaceuticals and food or as spoilage organisms and the causes of diseases in plants and man. Biosynthesis of biomolecules provides a unifying feature underlying the diverse structures of microbial metabolites. Microbes have the ability to transform chemicals in ways which can complement conventional reactions.

The book provides valuable information and recent developments on a wide range of microbes and their products involved in applications with plants, animals and humans. Plant topics include microbial resources for improved crop productivity, the potential of soil microbes in eco-friendly agriculture, the contributions of mycorrhizal fungi, *Trichoderma* biotechnology, *Bacillus* bacterium in plant defence, rhizobacteria-induced plant growth and biofilmed biofertilizers, microbial nanoformulation, *Bacillus thuringiensis* as insect pest control and microbial secondary metabolites from *Gibberella fujikuroi*. Microbial resources involved in human health that are covered include *Listeria monocytogenes*, bacteria as natural weapons against cancer, recent developments on *Giardia* and giardiasis, and bifidobacteria in food applications, probiotics and dental caries. There are chapters that focus on microbial technology employed in the industrial sector including functional enzymes for animal feed applications, microbial xylanases, microbial chitinase, characteristics of microbial inulinases, microbial cellulases, microbial resources for biopolymer production, microbial metabolites in

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cosmetic industries, *Pleurotus* as an exclusive eco-friendly modular biotool, microbial biotechnology for biosurfactants and probiotic carbohydrates, bio-flavones and biocolourants. Other topics covered include yeast modification for high expression of recombinant protein, actinomycetes in biodiscovery, molecular strategies for the studies of the expression of gene variation by real-time PCR and whole genome sequence typing for *Escherichia coli*. There are also chapters on applied aspects of microorganisms in the environmental sector, for example archaea for energy production, useful microorganisms for environmental sustainability and algal biofuel technology.

The present publication aims to provide a detailed compendium of work and information used to investigate different aspects of microbial resources and their products, as well as interdisciplinary interactions including biochemistry of metabolites and biomolecules as mentioned above, in a manner that reflects recent developments of relevance to researchers/scientists investigating the microbe.

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Foreword

Biological resources play a significant role in research and industry as the source of new biotechnology and medicines. The bioresources of developing countries, especially in tropical areas where there is great biodiversity, are particularly attractive as subjects for research. However, there has been a long debate over how best to apportion profits from the use of such resources to the developing country where they originated.

It is only now being fully realized by the chemical industry that microorganisms (bacteria, yeast and fungi, micro-algae) are an inexhaustible source of a wide range of useful chemical compounds. Indeed, an ever-increasing number of fine and bulk chemicals, solvents, food additives, enzymes, agrochemicals and biopharmaceuticals are now being produced based on microbial biotechnology via industrial fermentation or the process of biocatalysis. Also bioconversion reactions, based on the use of microbial biocatalysts (cells or enzymes), yield useful interesting molecules under mild reaction conditions. Furthermore, all these microbial processes have a positive environmental impact.

These microbial products generally are biodegradable and practically all are produced starting from renewable substrates. Agricultural practice, as well as this industrial processing, leads to agro-industrial residues, which should be considered now also as nutrient substrates, rather than as a waste. To maximize the bioresources' potential, several systems for systematic storage of biological extracts and chemical libraries, together with semi-high throughput capability in conducting various biological assays have been established.

In this context, *The Handbook of Microbial Bioresources* edited by one of the most renowned experts in bioprocesses, Dr Vijai Kumar Gupta, is extremely opportune not just to meet a growing demand in the sector, but also for its comprehensiveness and indisputable competence.

Each of the chapters presented in this book shows current and important information related to that mentioned above, which ensures high quality on each topic. One can certainly say that the relevance of the information brought by each of the authors in this publication will make a great contribution not only for professionals but also for students and scholars interested in the field of bioprocesses.

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1 Microbial Resources for Improved Crop Productivity

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Abstract
The ever-increasing human population and depletion of soil, nutrient and water resources make it necessary to ensure sustainability and genetic integrity of crops via exploitation of new technologies and through better agricultural practices. Many bacterial and fungal species may contain genes for plant resistance to biotic and abiotic factors and can produce metabolites that improve both the quality and the quantity of grains, fruits, fibre and nutritional energy. To ensure that beneficial microbes are available for commercial use, development of screening methods for identifying favourable traits is necessary. Recent advances in plant molecular biology, genomics and physiology using model plants and crop species together with improvements in microbial isolation, identification and culture techniques have provided the means to accelerate and strengthen the use of microbial formulations. This chapter describes recent advances in the field of plant–microbe biotechnology, such as the use of microorganisms for enhancing plant biomass production, reinforcing immunity and conferring tolerance to abiotic stress. These approaches should provide solutions to the current major problems, such as pollution and economic costs that threaten crop productivity and ecological sustainability.

1.1 Introduction

Current agricultural practices are becoming inadequate, with an imminent decline in crop productivity because of nutrient and water shortages and reduction of fertile arable soils. In contrast the global population increases at a rate of 1.2% per year and will double from 7×10^9 to 14×10^9 in less than 60 years (Pimentel, 2012). Thus, the demand placed upon farmers to supply enough plant products is one of the greatest challenges for modern biotechnology.

Since the start of the 'green revolution', the use of machinery, fertilizers and agrochemicals has played an essential role in sustaining a high yield, circumventing nutrient deficiencies, and in combating pathogens and pests. However, natural conditions or inadequate management causes salinization of soils and contamination of water ecosystems; these problems in turn negatively affect the productivity and sustainability of crop plants. In this regard, the role of naturally abundant yet functionally unexplored microorganisms – mainly bacteria and fungi – is of special significance in the provision of nutrients to commercial plantations at low cost and with a lesser environmental footprint (Ortiz-Castro *et al.*, 2009; Ortiz-Castro and López-Bucio, 2013).

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Microorganisms promote plant growth directly via fixation of nitrogen (N), solubilization of phosphorus (P) and iron (Fe), production of plant growth-regulating substances such as auxins and cytokinins, or via release of quorum-sensing (QS) signals or volatiles that are recognized by roots and allow for adjustment of morphogenesis (Ortiz-Castro and López-Bucio, 2013). Moreover, microorganisms can induce resistance to pathogens through systemic acquired resistance (SAR) or induced systemic resistance (ISR), and even increase tolerance to water scarcity (Shoresh *et al*., 2005; Dimpka *et al*., 2009).

The alternative to the extensive use of fertilizers containing N, P and Fe may be the development of bacterial formulations that increase nutrient uptake or efficiency of nutrient use by crops. The microbial communities inhabiting the rhizosphere – a soil zone in close contact with plant roots – play an important role in crop improvement in different agroecosystems, but our understanding of the composition of these microbial communities and the factors that determine specific root–microbe interactions is inadequate. Worldwide, salinity is an important abiotic stress that limits crop growth and productivity. Ion imbalance and hyperosmotic stress in plants caused by high concentrations of salt often lead to oxidative stress that restricts root growth (Krasensky and Jonak, 2012). Soil salinization may be due to natural causes and is common in the hot and dry regions of the world, or it may be a consequence of inadequate management of irrigation. In this context, the use of microorganisms that stimulate root growth either by increasing the amount of root hairs or lateral root expansion offers an attractive approach to the adaptation of crops to high-salt soils (Contreras-Cornejo *et al*., 2014).

Combining species of microorganisms within one formulation may take advantage of multiple beneficial mechanisms. An understanding of these mechanisms is likely to lead to the development of simple and practical approaches towards sustainable plant productivity. This chapter considers the roles of microbes in phytostimulation, plant defence and plant tolerance to abiotic stress and explains how these functions improve the efficiency of use of crop resources.

1.2 Microbes Promote Plant Growth and Nutrient Uptake

Sustained agricultural productivity is unlikely to come from farmers expanding their efforts into new territories; the availability of fertile arable land is more or less fixed and unlikely to increase significantly without negative effects on biodiversity. The current goal of the industry is to grow crops within smaller, more productive areas or on soils considered marginally useful for agricultural purposes. Not all such land is marginal in the same sense; some of it may be too dry or salty or may be limited in nutrients or contain high concentrations of metals, such as aluminium, often associated with acidity. Each situation requires crop plants with different adaptive mechanisms. Thus, researchers need to develop novel strategies to make plants more productive even when growth conditions are poor. To produce more fruits or grains, simply adding more and more fertilizer each season will not help. For instance, plants react in many ways to changes in nutrient provision, including the following: (i) regulation of root nutrient uptake; (ii) changes in root architecture; and (iii) fast modulation of shoot growth. All these responses are due to the combined action of external and internal nutrient levels. Levels of macronutrients such as N, P, potassium (K) and sulfur (S) and micronutrients such as Fe (required in low concentrations but essential for photosynthesis) are sensed locally by the root system (López-Bucio *et al*., 2003; Amtmann and Armengaud, 2009; Hindt and Guerinot, 2012; Nacry *et al*., 2013). Equally important is the sensing of levels of internal nutrients or metabolites, which circulate between stems and leaves and move downwards to the root system to activate or suppress the mechanisms of nutrient uptake.

During the course of evolution, plants and microbes have developed mutually beneficial or detrimental relationships, most of which involve nutrient use efficiency. Plants interact with endophytic and mycorrhizal fungi or with bacteria that form biofilms on root and leaf surfaces or live inside plant tissues; nitrogen-fixing bacteria are housed inside root nodules, and many pathogenic organisms can initiate active infection of leaves and roots (Ortiz-Castro *et al*., 2009). Therefore, colonization of plants by

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microbes is rather the norm and not an exception. The diversity of microbes associated with roots is huge: > 33,000 prokaryotic taxa (Mendes *et al.*, 2011). In some cases, plants have gained specific advantages from this intimate association with microbial partners, for example, an exchange of nutrients and protection from pathogens. Strong evidence exists that microorganisms affect plant fitness through direct or indirect effects on provision of nutrients. The best known microbes that help plants to deal with nutrient-poor soils are N-fixing rhizobia, which establish symbiosis with legume plants and arbuscular mycorrhizal fungi (AMF) that improves plant P supply in approximately 95% of land plants (Smith and Smith, 2011; Olivares *et al.*, 2013).

An interesting example of how the concept of the use of fungi as biofertilizers has been changing with time is AMF, which live within plant roots, from which they send out filaments or hyphae that collect phosphate, a critical nutrient for their host plants. These fungi, which microbiologists first recognized ~50 years ago, are found in all soils and form symbiosis with plant roots. Years ago, when scientists applied AMF to crops, they conducted field trials mostly in North America and Europe, where plants grow well with conventional phosphate fertilizers. Adding the fungi had little effect, and in consequence, only a few farmers started using AMF for cultivation of plants. Later, it was found that the high P level in most agricultural soils negatively affects the AMF–root interaction, thereby limiting any practical applications (Gu *et al.*, 2011).

Farming in the tropics is a different story because the soils there are often acidic. When working on acidic soils, farmers need to add large amounts of phosphates because P fertilizer combines with aluminium forming insoluble salts; therefore, P becomes unavailable for uptake by the roots. In accordance with the aim to increase yields and reduce the amounts of P used as a fertilizer, select isolates of AMF that thrive in tropical soils may improve the uptake of P by crops such as maize, sorghum and soybean grown in those regions; thus, it is important to test whether adding such fungi will improve yields (Cakmak, 2002).

Practical studies on various crop plants have been performed in developing countries of Latin America and Africa, probably as a result of the potential for cheaper production of inoculants by small companies or research groups and the low availability of fertilizers. These studies mostly involved plant growth-promoting rhizobacteria (PGPR) and yielded highly promising results (Bashan *et al.*, 2014). PGPR that are used for production of inoculants include species of the genera *Azospirillum*, *Azotobacter*, *Bacillus*, *Burkholderia*, *Enterobacter*, *Paenibacillus*, *Pseudomonas*, *Serratia* and *Stenotrophomonas*. Genera such as these allowed for reduced application rates of chemical fertilizers in greenhouses and in field trials, for which we present just a few recent examples (Table 1.1).

In a field trial that lasted 3 years, Adesemoye and associates (2008) evaluated a commercially available PGPR formulation, AMF, and their combination across two tillage systems. The inoculants promoted plant height, yield (dry mass of ears and silage) and nutrient content of grain and silage (Adesemoye *et al.*, 2008). Subsequently, in a greenhouse study tomato plants inoculated with a mixture of PGPR strains *Bacillus amyloliquefaciens* IN937a and *Bacillus pumilus* T4 consistently provided the same yield and show the same N and P uptake at 70% of the regular fertilizer amount as do plants with full fertilizer rate without inoculants, thus saving on the amount of fertilizer used. Addition of the AMF *Glomus intraradices* to the inoculant mixture produced an improvement only when P was a growth-limiting nutrient (Adesemoye *et al.*, 2009).

Enterobacter radicincitans is a promising plant growth-promoting bacterium isolated from the phyllosphere of wheat and known to increase the yield of crop plants such as wheat and maize as well as various horticultural plants from the families *Brassicaceae* (e.g. *Brassica napus*, *Brassica oleracea* and *Raphanus sativus*) and *Solanaceae* (e.g. *Solanum lycopersicum* and *Capsicum annuum*). To assess how reduced nitrogen fertilization modulates the growth-promoting capacity of *E. radicincitans*, its root colonization behavior and the regulation of nitrogen and phosphate marker genes were determined in tomato plants. When treated with *E. radicincitans*, dry weight of plant roots and shoots increases by 180% and 150%, respectively, under high nitrogen conditions and by 120% and 140%, respectively, under low nitrogen conditions. Interestingly, plant roots

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Table 1.1. Microbial traits that enhance nutrient uptake by plants.

Microorganism	Crop species	Nutrient	Mechanism	Reference
Rhizobium spp.	*Phaseolus vulgaris*	N	Symbiotic nitrogen fixation	Devi *et al*. (2013)
Bradyrhizobium japonicum	*Glycine max*	N	Symbiotic nitrogen fixation	Solomon *et al*. (2012)
Frankia spp.	*Discaria trinervis*	N	Symbiotic nitrogen fixation	Valverde and Wall (2003)
Frankia spp.	*Casuarina* spp.	N	Symbiotic nitrogen fixation	Zhang *et al*. (2012)
Azospirillum brasilense	*Oryza sativa*	N	Non-symbiotic nitrogen fixation	García de Salamone *et al*. (2010)
A. brasilense	*Triticum aestivum*	N	Non-symbiotic nitrogen fixation	Piccinin *et al*. (2013)
Azotobacter spp.	*Heliantus tuberosus*	N	Not determined	Hassan (2013)
Herbaspirillum spp. and *Burkholderia vietaminensis*	*O. sativa*	P and N	Phosphorous solubilization; promotion of N use efficiency	Estrada *et al*. (2013)
Bradyrhizobium	*Ipomoea batatas*	N	Non-symbiotic nitrogen fixation	Terakado-Tonooka *et al*. (2013)
Pseudomonas putida	*Hordeum vulgare*	P	Unknown	Mehrvarz *et al*. (2008)
Rhizobium spp.	*G. max*	P	Phosphorous solubilization by acidification	Qin *et al*. (2011)
Sinorhizobium meliloti	*Medicago truncatula*	P	Phosphorous solubilization by acidification and phosphatase activity	Bianco and Defez (2010)
Piriformospora indica	*Zea mays*	P	Phosphate transport	Yadav *et al*. (2010)
Bacillus mucilaginosus	*Sorgum vulgare*, *T. aestivum*, *Z. mays*	K	Potassium solubilization	Basak and Biswas (2009), Singh *et al*. (2010)
Arthrobacter agilis UMCV2	*P. vulgaris*	Fe	Iron reduction/ solubilization	Valencia-Cantero *et al*. (2007)
A. agilis UMCV2	*M. truncatula*	Fe	Plant strategy I induction	Orozco-Mosqueda *et al*. (2013b)

were more highly colonized by soil bacteria when grown in conditions of N availability than under low nitrogen conditions (Berger *et al*., 2013).

In different laboratory assays designed to evaluate possible promotion of plant growth by endophytic bacteria in maize, another *Enterobacter* species (strain FD17) showed both higher growth-promoting activity under axenic conditions and higher colonization capacity in two maize cultivars grown in pots until ripening and subjected to outdoor climatic conditions. Inoculation with FD17 increased plant biomass, the number of leaves, leaf area and grain yield up to 39%, 14%, 20% and 42%, respectively, compared with the uninoculated control plants. Similarly, the inoculation improved photosynthesis in maize plants and reduced the time needed for flowering (Naveed *et al*., 2014).

Bacillus species, such as *B. amyloliquefaciens*, contribute to biofertilization via production of extracellular phytases, which are special phosphatases that catalyse sequential hydrolysis of phytate to less-phosphorylated myo-inositol derivatives and inorganic phosphate (Jorquera *et al*.,

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2008). In addition, phytases eliminate chelate-forming phytate, which binds nutritionally important micronutrients. There are similar findings in the case of Fe. In the soil, the most prevalent form of iron is Fe^{3+}, which is almost completely insoluble, whereas the more reduced form Fe^{2+} is relatively soluble and taken up more readily by plants and microorganisms. *Arthrobacter agilis*, *Stenotrophomonas maltophilia* and *Bacillus* species such as *Bacillus megaterium* can potentially increase the bioavailability of Fe (Valencia-Cantero *et al*., 2007). In particular, *A. agilis* and *Sinorhizobium meliloti* increased Fe reduction mechanisms via emission of volatile organic compounds (VOCs) (Orozco-Mosqueda *et al*., 2013a, b).

AMF, *Trichoderma* and *Piriformospora indica* colonize the root surface or proliferate in internal plant tissues without causing any damage. Several of these fungal species are emerging as growth promoters. *P. indica* colonization of tobacco, sugarcane, strawberry and barley improves yields of crop plants because of increased shoot growth, greater number of inflorescences, flowers and seeds (Franken, 2012). For instance, the yield of barley increased by 10% depending on the cultivar because of a larger number of ears (Waller *et al*., 2005), whereas in tomato this approach increases the harvest yield and accelerates fruit maturation (Fakhro *et al*., 2010). *Trichoderma virens* and *Trichoderma atroviride* increase growth potential of plants and nutrient uptake, efficiency of fertilizer use, the percentage and rate of seed germination, and proliferation of lateral roots, thus leading to higher seedling fresh weight and foliar area (Contreras-Cornejo *et al*., 2013). These effects occur via the production of several classes of metabolites including auxins, volatiles, harzianolide and small peptides. These selected examples suggest that integration of microbes in nutrient management programmes can help to reduce but not eliminate the use of chemical fertilizers.

1.3 Microbes Produce Plant Growth-regulating Substances

Microorganisms are phytostimulators when they produce phytohormones such as auxins, cytokinins and gibberellins, which perform major functions in the configuration of root system architecture. Plastic root development enables plants to cope with abiotic stressors such as drought and salinity and is therefore important for adaptation to global climate changes. Plants increase the absorptive surface through proliferation of lateral roots and root hairs, which are important for anchorage to the soil and for acquisition of nutrients and water (Orman-Ligeza *et al*., 2013; Smith and Whatt, 2013).

Several bacterial and fungal species produce indole-3-acetic acid (IAA), auxin precursors or auxin mimics that might stimulate lateral root or root hair proliferation and nutrient uptake (Sukumar *et al*., 2013). For example, in *T. virens* biosynthesis of IAA is responsible for plant growth promotion, which in turn is dependent on the presence of the auxin precursor tryptophan (a major compound in plant root exudates) and on the signalling mechanism in plants involving auxin receptors and downstream components (Contreras-Cornejo *et al*., 2009). Similarly, inoculation of plants with cytokinin-producing *Bacillus subtilis* or *B. megaterium* strains has a beneficial effect on plant growth (Idris *et al*., 2007), whereas *Arabidopsis* mutants deficient in cytokinin receptors did not respond to *B. megaterium* inoculation in terms of enhanced growth and root branching (Ortiz-Castro *et al*., 2008b). AMF influence root architecture by enhancing lateral root formation probably because of the production and release of auxins, ethylene (ET) or other volatile compounds (Splivallo *et al*., 2009). Thus, various microbial species may influence common root developmental programmes by producing phytohormones.

VOCs and acyl-L-homoserine lactones (AHLs) from rhizobacteria trigger growth promotion in *Arabidopsis thaliana* by regulating auxin homeostasis, thus demonstrating a new paradigm as to how these bacteria promote plant growth (Zhang *et al*., 2007; Ortiz-Castro *et al*., 2008a, 2011). VOCs perform diverse and critical functions in plant–microbe interactions and can travel far from the point of production through the atmosphere, porous soils and water. Thus, VOCs are compounds highly active in both short- and long-distance intercellular communication. Gutiérrez-Luna and co-workers (2010) evaluated the effects of 12 bacterial strains isolated from the rhizosphere of lemon plants (*Citrus aurantifolia*) on plant growth and development. Several bacterial strains showed a plant growth-promoting effect stimulating

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biomass production, modulation of root system architecture and root hair production. VOC emission analysis identified aldehydes, ketones and alcohols as the most abundant compounds common to most rhizobacteria. Other VOCs, including 1-octen-3-ol and butyrolactone were strain specific (Gutiérrez-Luna *et al*., 2010). Characterization of the bacterial isolates by 16S rDNA analysis revealed the identity of these strains as *Bacillus cereus*, *Bacillus simplex* and other *Bacillus* spp. These data show that rhizospheric bacterial strains can modulate both plant growth and root system architecture via differential VOCs emission.

In bacteria, cell-to-cell communication occurs via QS signals. AHLs produced by Gram-negative bacteria are the best-known QS signals. AHLs enter the roots and are transported to shoots where they regulate gene expression and protein biosynthesis (von Rad *et al*., 2008; Sieper *et al*., 2014). Moreover, local sensing of AHL levels affects the growth rate, root development and resistance to microbial pathogens (Ortiz-Castro *et al*., 2008a; Schikora *et al*., 2011). Further research in this field will accelerate the development and application of new products that improve crop quality and yields.

1.4 Microbes Reinforce Plant Immunity

Plants live in close interaction with a broad range of pathogens and pests that affect roots or shoots; these noxious organisms use different infection strategies and include viruses, bacteria, fungi, oomycetes, nematodes and insects. By their parasitic relationships with plant hosts, pathogens can be necrotrophs or biotrophs. Necrotrophs usually destroy cells and tissues through the production of toxins and then feed on their contents. In contrast, biotrophs derive nutrients from living host tissues usually without destroying cells and tissues (Pieterse *et al*., 2009). Plants have well-developed defence strategies that include physical barriers, such as the cuticle and the cell wall, and produce antimicrobial compounds such as phytoalexins or pathogenesis-related (PR) proteins such as chitinases and glucanases, which degrade the fungal and oomycete cell wall.

The plant immune system recognizes conserved molecular characteristics of many microbes; these signatures are also known as microbe- or pathogen-associated molecular patterns (MAMPs or PAMPs). MAMPs include flagellin, peptidoglycan and lipopolysaccharides recognized by specific cell membrane receptors to activate MAMP-triggered immunity (Jones and Dangl, 2006). PAMPs may include strain-specific proteins from pathogens, which interact with plant resistance proteins to initiate SAR in parts of the plant distant from the site of the attack in order to induce immunity to subsequent infections by related or unrelated pathogens. SAR-induced defence responses include production of reactive oxygen species (ROS) or reactive nitrogen species such as nitric oxide (NO), alterations in the plant cell wall, production of antimicrobial compounds and the synthesis of host defence PR proteins (Pieterse *et al*., 2009).

Similar to the immunostimulatory properties of human probiotics, root colonization by certain strains of PGPR and plant growth-promoting fungi (PGPF) helps a plant to efficiently defend itself against a broad range of pathogens and even insects through ISR (Zamioudis and Pieterse, 2012). Colonization of roots by ISR-causing rhizobacteria and fungi does not directly activate the plant immune system but primes stems and leaves for an accelerated defence response after an attack by a pathogen or insect, thus providing cost-effective protection from plant diseases (Bulgarelli *et al*., 2013). The regulation of the defence network that translates the MAMP- or PAMP-induced early recognition events into activation of defence responses depends on the action of three plant hormones, namely salicylic acid (SA), jasmonic acid (JA) and ET. Necrotrophs are generally sensitive to defences controlled by JA and ET, whereas biotrophs are sensitive to SA-regulated defences. At certain developmental stages, such as leaf senescence, SA- and JA-dependent signalling effectively protect plants from potential attackers (Pieterse *et al*., 2012).

The preformed structural barriers such as cuticle protect plants from spread of pathogens. Nevertheless, some pathogenic fungi infect plants by penetrating the cell wall, and many bacterial pathogens invade plants primarily through stomata on the leaf surface. Entry of a foliar pathogen, *Pseudomonas syringae* pathovar tomato

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DC3000, into the plant body occurs through stomatal openings; consequently, a key innate immune response in plants is the transient closure of stomata, which delays disease progression. Kumar and associates (2012) provided evidence that the root colonization of *Bacillus subtilis* FB17 restricts the stomata-mediated pathogen entry of PstDC3000 in *A. thaliana* through a signalling mechanism involving abscisic acid (ABA) and SA that closes stomata (Kumar *et al.*, 2012). These findings underscore the importance of rhizospheric bacteria as an integral part of the plant innate immunity to foliar bacterial infections and open up new opportunities for adaptation of agriculture to climate changes.

Recent evidence suggests that plants actively recruit non-pathogenic root-associated microbes after an attack by pathogens or insects (Rudrappa *et al.*, 2008; Lakshmanan *et al.*, 2012). By regulating its root secretion of carbon-rich exudates such as organic acids (i.e. malate) or by detecting microbe signatures, plants can actually shape the root microbiome by changing microbial diversity, density and activity (Berendsen *et al.*, 2012).

Knowledge of AHL-signalling in bacteria led to identification of the compounds that activate plant immunity. The presence of AHL-producing bacteria in the rhizosphere of the tomato induces SA- and JA-dependent defence responses, conferring resistance to the fungal pathogen *Alternaria alternata* (Schuhegger *et al.*, 2006). Moreover, application of purified AHLs to *Medicago truncatula* and *Arabidopsis* plants results in differential transcriptional changes in roots and shoots, affecting expression of genes potentially involved in immune responses and development (von Rad *et al.*, 2008). Similarly, global analysis of gene expression changes in *A. thaliana* in response to *N*-isobutyl decanamide – a plant alkamide related to AHLs – revealed over-representation of defence-responsive transcriptional networks. In particular, genes encoding enzymes for JA biosynthesis are upregulated, and this activation occurs in parallel with accumulation of JA, NO and hydrogen peroxide (H_2O_2). The alkamides confer resistance to the necrotizing fungus *Botrytis cinerea* as shown by inoculating detached *Arabidopsis* leaves with conidiospores and by evaluating disease signs and fungal proliferation. Application of *N*-isobutyl decanamide significantly reduces necrosis caused by the pathogen and inhibits fungal proliferation (Méndez-Bravo *et al.*, 2011). These results are suggestive of a role of AHLs and alkamides in induction of plant immunity.

Volatile compounds recently emerged as important signals for plant–microbe and microbe–microbe signalling that activates plant immunity. In addition to the effects on plant volatiles emitted by leaves and flowers, several root-colonizing microbes can produce volatiles themselves. These microbial volatiles participate in ISR to pathogens (Zamioudis and Pieterse, 2012; Bulgarelli *et al.*, 2013; Farag *et al.*, 2013). For instance, the short-chain VOC 2,3-butanediol is produced by root-associated *B. subtilis* GB03 and *B. amyloliquefaciens* IN937a and can trigger ISR of *A. thaliana* to the pathogen *Erwinia carotovora* via the ET signalling pathway (Ryu *et al.*, 2004). Interestingly, application of synthetic 2,3-butanediol at different doses and a volatile extract from the strain GB03 causes similar disease protection, which is comparable to that induced by bacterial inoculation (Ryu *et al.*, 2004). The *Paenibacillus polymyxa* strain E681 is another PGPR; it was isolated from barley roots and acts as a promising biocontrol agent that can protect cucumber and sesame from damping-off caused by the soil-borne pathogens *Fusarium oxysporum*, *Rhizoctonia solani* and *Pythium ultimum*. Recent data from the *P. polymyxa* strain E681 reveal that long-chain bacterial VOCs, such as the C13 hydrocarbon tridecane, can also elicit ISR, as can C4 alcohols such as 2,3- butanediol (Lee *et al.*, 2012).

Root colonization by selected PGPF such as *Trichoderma* increases resistance to different types of pathogens in various plant species, both below and above ground (Shoresh *et al.*, 2005; Contreras-Cornejo *et al.*, 2013). This protective effect occurs through activation of the plant immune system via several mechanisms. Although a clear understanding of the *Trichoderma*–plant recognition process is lacking, several elicitors that activate plant basal immunity are in the fungus, including an ET-inducing xylanase, the proteinaceous elicitor Sm1, 18-mer peptaibols and cationic lipopeptides (Contreras-Cornejo *et al.*, 2013). Expression studies on marker genes linked to the main defence signalling pathways suggest that *Trichoderma*-induced systemic resistance is complex and may involve direct activation of both SA- and JA-related pathways as well as production of antimicrobial compounds

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(Shoresh *et al*., 2005; Contreras-Cornejo *et al*., 2011; Mathys *et al*., 2012). Interaction of plant roots with the mycelium of *T. virens* and *T. atroviride* induces growth and defence responses, indicating that both processes are not inherently antagonistic. Analysis of the pathogenesis-related reporter markers *pPr1a:uidA* and *pLox2:uidA* in response to *T. virens* or *T. atroviride* infection indicated that the defence signalling pathways activated by these fungi involves SA or JA depending on the amount of conidia used for inoculation. In *Arabidopsis* seedlings whose roots are in contact with *T. virens* or *T. atroviride* that are challenged with *B. cinerea* in leaves, disease severity is significantly lower than that seen for seedlings grown axenically (Contreras-Cornejo *et al*., 2011). To summarize, JA, SA and additional factors are essential for ISR to *B. cinerea* and other pathogens in *Arabidopsis*, but the specific requirements and degree of protection may depend on the *Trichoderma* strain and the degree of plant colonization. We speculate that the combined application of root-associated microbes acting via different signalling pathways may enhance plant defence against both pathogens and insect herbivores. Supporting this idea, in cucumber, co-inoculation with non-pathogenic *Trichoderma harzianum* and a *Pseudomonas* species contributes to significantly enhanced resistance after a challenge by the stem pathogen *F. oxysporum* via activation of both JA- and SA-dependent defence responses in comparison to individual treatments (Alizadeh *et al*., 2013). Whether activation of both JA- and SA-signalling pathways will also induce the biosynthesis of higher diversity of secondary metabolites remains to be determined, but it is increasingly clear that an analysis of different soil community members is important for a thorough understanding of their plant-mediated effects on plant defence.

A parallel case is the mutualistic relationship between the basidiomycete *Piriformospora indica* and a wide range of vascular plants. *P. indica* colonizes living root cells via direct penetration. This biotrophic colonization causes broad-spectrum suppression of root innate immunity. *P. indica* depends on JA for suppression of early immune responses as well as the SA defence pathway (Qiang *et al*., 2012). Inactivation of components of the ET pathway dramatically impairs root colonization by *P. indica*. Colonized plants acquire resistance to a variety of leaf and root pathogens. *P. indica* colonization helps the shoots and roots become preconditioned for infection via JA and ET signals from the roots (Unnikumar *et al*., 2013).

1.5 Microbes Provide Protection from Abiotic Stress

Inoculation of crops with beneficial PGPR or PGPF can help to better adapt these plant species to abiotic stressors such as drought or salinity because high temperatures and salinity affect more than 30% of arable land, reducing crop yields by up to 50% in certain regions. As a response to water deficit, plants increase synthesis of osmolytes, thereby increasing the osmotic potential within cells. Some naturally drought-tolerant plants produce trehalose, which stabilizes membranes and enzymes, protecting them from damage when cells need rehydration. Although the capacity for synthesis of trehalose is rather rare among plants, many microorganisms, including bacteria and fungi, can synthesize this simple disaccharide (Dimpka *et al*., 2009). Glycine betaine produced by osmotolerant bacteria possibly acts synergistically with plant-produced trehalose in response to the stress and increases drought tolerance in this manner. Consistent with this notion, the beneficial effects of osmolyte-producing rhizobacteria on rice are effective under drought stress with subsequent improvements in dry weight of shoots and roots and in the number of tillers in the inoculated rice plants compared with uninoculated controls (Yuwono *et al*., 2005). Another important factor in growth stimulation by these osmotolerant bacteria is their ability to produce IAA. Root proliferation is induced by this hormone in drought-stressed rice plants inoculated with bacteria and this increases water uptake (Yuwono *et al*., 2005). Additional studies have shown that inoculation with the endophytic bacteria such as *P. indica* and *Trichoderma* spp. can mitigate the effects of salt stress in various plant species. *Azospirillum*-inoculated seeds of lettuce, for instance, show better germination rates and vegetative growth than uninoculated control plants when exposed to NaCl (Barassi *et al*., 2006). *P. indica* has a strong growth-promoting effect during symbiosis with a broad spectrum of plants, conferring tolerance to salt stress on barley (Waller *et al*.,

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2005), whereas *T. virens* (Tv29.8) and *T. atroviride* (IMI 206040) promote *Arabidopsis* growth under both normal and high salt conditions (Contreras-Cornejo *et al.*, 2014). *Arabidopsis* seedlings grown under high salt conditions while inoculated with *Trichoderma* show increased levels of ABA, L-proline and ascorbic acid and enhanced elimination of Na^+ through root exudates (Contreras-Cornejo *et al.*, 2014). These data further confirm the critical role of auxin and root development to confer salt tolerance and suggest that these fungi may increase plant IAA levels as well as the antioxidant and osmoprotectant status of plants under salt stress.

1.6 Current Challenges for Agricultural Applications of Microbes

Much interest has arisen in relation to the use of microorganisms for stimulation of plant growth and development, and in some cases (e.g. *Rhizobia*, mycorrhizae and *Trichoderma*) these approaches are commercialized as part of so-called organic agriculture (Berg, 2009). Most farmers employing natural products increasingly utilize microbial technologies and other microbe-derived compounds to inoculate seeds or soils, in order to provide nutrients to the plants; however, often the farmer uses these products without proper knowledge and understanding and as a consequence, results can be variable and sometimes inconsistent. Microbes have also attracted worldwide attention because of their role in disease management and remediation of salinized/polluted land. Thus, microbial communities in general are promising tools for sustainable production of crops and are the trend of the future.

The success of microbial formulations for crop improvement will depend on the performance of microbes in the root/soil zone, on their consistent beneficial effects and on the acceptance by the growers (Bashan *et al.*, 2014). Current limitations necessitate concerted action by scientists, industry and farmers towards: (i) the establishment of microbial collections; (ii) testing of formulations based on robust scientific knowledge; (iii) greenhouse and field trials; (iv) dissemination of products for field use; and (v) communication of promising data. A major challenge is to ensure that microbial collections represent the existing genetic diversity relevant to agricultural applications. In this regard, the rhizospheres of locally adapted wild and crop plants are a rich source of bacterial and fungal species, and screening of thousands of local isolates for traits beneficial to plants is now possible (Ortiz-Castro *et al.*, 2013). These genetic resources need protection through propagation and exchange, and original isolates can be part of *ex situ* collections. Protocols for bacterial culture and preservation need to be continually developed and refined to maintain their biological properties and the genetic diversity present in the original sample. Microbial collections based on PGPR or PGPF have not been fully catalogued or developed. To facilitate the frequent and effective use of microbes in research and field use, the isolates as well as the associated information should be readily accessible and distributed rapidly and reliably among scientists and farmers. Once a promising formulation containing either a single or mixed microbial community is identified, seed treatment should be an effective way to deliver bioactive molecules directly to the seed surface to protect the seed while it lies dormant in the soil and later to enhance growth of the seedling (Bashan *et al.*, 2014).

Bioefficacy may be an additional desirable trait of microbe-based inoculants because the active microbe may control infestation by bacteria, fungi or insects that damage roots. This property certainly depends on various microbial traits, such as tolerance to desiccation, the root colonization potential and soil mobility. If such limitations are solved, the potential benefits are: (i) new collections of agriculturally important microorganisms; (ii) expanded exchange of microbial strains and relevant technical information; (iii) new cultivation protocols and techniques for enhancement of collection quality, genetic integrity and efficiency of management of genetic resources; (iv) protection of vulnerable or endangered microbial resources; and (v) more frequent use of bacteria and fungi for field trials. We hope that current basic and applied research will solve some of the existing problems and lead to an efficient agriculture based on highly valuable microbial resources.

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2 The Contributions of Mycorrhizal Fungi

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Abstract
There is mounting research on microbial diversity and their products, especially those associated with soil amendments such as biochar and compost. There is increasing recognition of the beneficial effects of biochar, which, applied in suitable amounts and forms, can stimulate soil microbial activity, soil microbes and symbiotic microorganisms, and mycorrhizal fungi. Mycorrhizas may have an important role in determining the impact of biochar on plant communities. Biochar influences carbon (C) sequestration in soils as a simple instrument to counterbalance C emissions related to the burning of fossil fuels. Among soil microorganisms, most plant species associate with mycorrhizas and/or other symbionts. Mycorrhizas permit the plant to perform better under unfavourable conditions, mostly in the soil superficial layers. Due to their key position in the soil–root interface, the study of mycorrhizas in combination with biochar/compost has the potential to make a significant contribution to the biochar effect. The appreciation of alternatives to cope with environmental constraints, such as the use of soil amendments such as biochar and compost, is also of interest for understanding the effect of drought on crops. This chapter explores current information on mycorrhizas in agroecosystems with respect to the benefits of biochar/compost amendments.

2.1 Introduction

The interest in climate change is rising with increased recognition that global changes can negatively affect biodiversity and ecosystem services (Sala *et al*., 2000; Pagano, 2013; van der Wal *et al*., 2013; Winfree, 2013). Similarly, the interest in ecologically sustainable plantations and agroforestry systems is also growing with a greater understanding of the adverse effects of agricultural intensification (Borie *et al*., 2006; Duarte *et al*., 2013) and the potential of terrestrial carbon (C) sequestration (see King, 2011). However, plantations and crops affect the soil's physical and chemical properties, modifying the number, diversity and activity of the soil microbiota, including both free and symbiotic fungal populations (Cardoso and Kuyper, 2006; Kahiluoto *et al*., 2009; Nyfeler *et al*., 2011; Pagano *et al*. 2011).

Legumes that host nitrogen-fixing bacteria and plant species associated with mycorrhizas, which enhance nutrient uptake, can offer a route for the return of C substrate directly to microbes and soil (Azcón and Barea, 2010). Additionally, the increased microbial nitrogen (N) inputs can intensify soil C stocks by influencing decomposition processes (reviewed by Stockmann *et al*., 2013). For example, the rhizobial and mycorrhizal symbioses with plants have been increasingly

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studied with respect to their benefits. Consequently, it is known that different arbuscular mycorrhizal fungi (AMF) communities can be found in degraded versus pristine forest (Haug *et al.*, 2010; Pagano *et al.*, 2011; De Souza *et al.*, 2013; Soteras *et al.*, 2013), which can consequently affect reforestation, plant production or plant succession.

In addition, there are some alternatives to cope with various environmental constraints, for example the use of soil amendments. Among them, the use of biochar and compost, which can stimulate soil microbial activity (Ogawa, 1994a, b; Nishio, 1996), have rapidly increased their inclusion in projects worldwide. Biochar application can also stimulate AMF colonization (Saito, 1990; Ogawa and Okimori, 2010) and rhizobia, which can fix atmospheric N_2 supply to leguminous vegetation (Nishio and Okano, 1991). Some field experiments in Indonesia have showed that biochar could increase yields of maize together with 500 kg/ha of NPK (15:15:15) fertilizer application (Yamato *et al.*, 2006). Benefits of biochar to plant nutrition and microbial activity have been found in the humid tropics (Lehmann and Rondon, 2006) as well as in temperate forest (Zackrisson *et al.*, 1996; Pietikäinen *et al.*, 2000). Furthermore, ancient anthropic soils found in the humid tropics show high fertility over long time usage, and serve as an historical model for biochar (Falcão *et al.*, 2003; Glaser and Birk, 2012; Jorio *et al.*, 2012).

This chapter explores current information on biochar and compost in plantations with respect to the benefits of AMF symbioses. Possible mechanisms through which biochar affects the relationship between crops and mycorrhizal fungi, as well as research paths that are necessary for the increased understanding of mycorrhizal benefits, are discussed.

2.2 Soil Amendments

Forests store ~45% of terrestrial C and can sequester large amounts of C (Bonan, 2008). Carbon uptake by forests contributes to the terrestrial C sink of anthropogenic C emissions from fossil fuels and land use change (Denman *et al.*, 2007). However, soil C sequestration can be four times higher: 2344 Gt of organic C is stored at the top 3 m of soil (54% of which is stored in the first metre), reducing global warming. This counteracts the 9 Gt of anthropogenic carbon dioxide (CO_2) liberated into the atmosphere (Stockmann *et al.*, 2013).

It is known that biochar may help to sequester C in soils as a simple instrument to counterbalance C emissions related to the burning of fossil fuels (Lehmann and Joseph, 2009a, b; Atkinson *et al.*, 2010; Powlson *et al.*, 2011), though it may also act as soil amendment to improve soil fertility.

Other indirect effects of biochar addition include the following: (i) increased water and nutrient retention; (ii) improvements in soil pH; (iii) increased soil cation exchange capacity; (iv) effects on phosphorus (P) and sulfur (S) transformations and turnover; (v) neutralization of phytotoxic compounds in the soil; (vi) improved soil physical properties; (vii) promotion of mycorrhizal fungi; and (viii) alteration of soil microbial populations and functions (the mechanisms are not yet understood). Biochar addition may alter the microorganism populations that stimulate plant health and resistance to biotic stresses (reviewed by Elad *et al.*, 2011).

Moreover, some authors have suggested that biochar induction of responses in systemic plant resistance results in controlling diseases (reviewed by Elad *et al.*, 2011). It is also known that biochar can be used to mitigate salt stress effects (Thomas *et al.*, 2013).

In addition, because black carbon (black-C) can be found in varied environments, it is crucial to elucidate alterations of its natural oxidation under different climatic and soil regimes. Black-C oxidation may be improved by biotic (Hamer *et al.*, 2004) and abiotic processes, such as greater temperature (Cheng *et al.*, 2006) and moisture. Conversely, black-C oxidation may diminish with better aggregate protection (Glaser *et al.*, 2000; Brodowski *et al.*, 2005), for example in fine-textured soils (see Cheng *et al.*, 2008).

Several studies reported experiments using laboratory-produced biochar, but only a few have showed the elemental concentrations and specific surface area of the samples (Baldock and Smernik, 2002; Czimczik *et al.*, 2002; Nguyen *et al.*, 2004; Zhu *et al.*, 2005; Brown *et al.*, 2006; Turney *et al.*, 2006). Nevertheless, it is known that the final formation temperature and gas composition during heating influence char properties (Brown *et al.*, 2006). Additionally, the

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temperature of formation is difficult to measure and is rarely known in natural chars (Hammes *et al.*, 2006).

Due to the relevance of this topic a conference on biochar took place in Italy – the 'International Conference BCD' 2013 – 'Biochars, Composts, and Digestates. Production, Characterization, Regulation, Marketing, Uses and Environmental Impact'. Among the topics discussed were recent scientific results including: (i) research from companies and current issues related to technological processes, analysis and characterization; (ii) sustainable uses and certification, regulation and marketing sides of biochar; and (iii) compost and digestate applications, waste, soil and water sciences, agriculture and environmental sciences and ecotoxicology (International Conference BCD, 2013). In this conference 80 oral presentations and 107 research posters were registered, including composting of olive mill waste, management of urban biodegradable waste, rice cropping systems amended with waste-derived material, and action of humic substances to name just a few.

The effects of biochar soil amendment on the different soil–plant–microbe interactions that may have a role in plant health have been little studied. Among the extended benefits of biochar is also improvement of plant responses to disease. Historical and new black-C samples have been examined worldwide, including samples collected from the remnants of historical charcoal (Cohen-Ofri *et al.*, 2006, 2007; Cheng *et al.*, 2008), black-C from Amazonian dark soils, also known as TPI (*Terra Preta de Índio*) (Falcão *et al.*, 2003; Glaser and Birk, 2012; Jorio *et al.*, 2012) and newly produced black-C (Sohi *et al.*, 2010).

Biochar has been compared to the historical black-C samples retrieved from Amazonian dark earth. However, evidence suggests that besides incorporating charcoal into the soil, the Amazonians practised intensive soil composting (reviewed by Briones, 2012). Lehmann and Joseph (2009a) give a detailed review of the effect of biochar for environmental management.

In parallel, there is greater availability of research on the compost as a peat substitute as well as on their known effects on biocontrol agents for plant disease management (see Martin and Brathwaite, 2012). The use of peat is critically viewed as a limited natural resource of unsustainable use (Joosten and Clarke, 2002).

Since 1898 approximately 14,752 peer-reviewed journal papers on compost have been reported all over the world (Scopus survey conducted in March 2014), of which 259 are due to be out in 2014. Composting is the controlled microbial aerobic decomposition and stabilization of organic substrates. The result is a stable product without pathogens and viable weed seeds that is employed in the cultivation of plants. Additionally, there is substantial interest on compost teas (the liquid obtained from compost) as plant disease suppressors (Martin and Brathwaite, 2012).

In order to improve access to technical information on compost and its potential to mitigate soil-borne diseases, more attention is nowadays paid to research and review papers compiling all this information (Martin and Brathwaite, 2012). Traditionally, compost has been created in rural farming areas of most developing tropical countries using small-scale, slow-rate, open composting methods, particularly heap and pit structures (Persad, 2000), whereas in urban areas and most developed countries, larger-scale techniques involving windrow or in-vessel composting have been adopted.

However, with growing rates of organic waste generation (in both rural and urban areas), technologies with higher turnovers, able to produce more stable end products, such as windrow and in-vessel systems, have been proposed (reviewed by Martin and Brathwaite, 2012). Nowadays, innovative technologies have increasingly won the market. In Italy, for example, companies such as Tersan Puglia Composting Plant and Biovegetal® Fertilizers Production Plant, transform urban organic waste, crop residues and agro-industrial waste into organic fertilizers (Fig. 2.1a). This is achieved in a composting plant in the industrial area of the city of Bari. In Brazil, compost (leaves, stems, grass and animal faeces) is transformed at the campus of the Federal University of Minas Gerais (Fig. 2.1b). After 6 months of maturity, the product is used for ornamental plant cultivation throughout the campus. In Europe and elsewhere, organic waste is generally separated from the rest of urban waste (glass, paper, plastic, woods, metals, etc.). The level of separate collection is increasing worldwide. In Italy, for example, ~38% of municipal waste is separated. The growing worldwide attention to the high levels of wastes will lead to a constant increase in their separation.

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Fig. 2.1. Large-scale composting techniques. (a) Compost produced in Italy at Biovegetal® production plant (www.biovegetal.it). (b) Compost produced on the campus of the Federal University of Minas Gerais, Belo Horizonte, Brazil. (Photographs by M. Pagano with permission.)

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Research on biochar is more recent. The first published reports on biochar in the Scopus database were in 2000, contrasting with the first report on compost (not in the Scopus) in 1898 from Germany. In the last years approximately 1,291 peer-reviewed journal papers on biochar were reported all over the world (Scopus survey conducted in March 2014) of which 16 are due to be out in 2014. Biochar may help in reforestation projects and reclamation of degraded rangelands (Stavi, 2012), improving our understanding of belowground ecology. Conservation practices such as agroforestry systems and application of biochar in soil are considered the most promising options to efficiently sequester large amounts of carbon in the long run (Stavi and Lal, 2013).

Nowadays biochar may also help to deal with wetland restoration practices. In the USA, Ballantine *et al.* (2012) found better soil properties, essential for ecological functioning, with biochar thus it appears it may be useful for restoration ecology. Both biochar and compost contain high levels of stable C and can be incorporated in the soil to improve soil quality. Thus, the use of biochar and compost can increase the soil organic C level compared with no amendment in long field experiments (D'Hose *et al.*, 2013).

2.3 Morphology and Composition of Historical Black-C

Black-C, the residue of incomplete combustion of biomass or fossil fuel, is considered a chemically and biologically very stable carbon that can persist in nature for a long time (Schmidt and Noack, 2000; Knicker, 2007). Research into active management of black-C as a means to sequester atmospheric CO_2 in soils is increasingly encouraged. However, the long-term persistence of black-C does not mean that the properties of black-C persist after its deposition. Cheng *et al.* (2006) showed rapid oxidation of black-C in short-term incubations, and altered black-C properties (formation of oxygen-containing functional groups). Moreover, black-C transport (Hockaday *et al.*, 2006), erosion (Rumpel *et al.*, 2006), stability (Bird *et al.*, 1999; Czimczik and Masiello, 2007) and cation retention (Liang *et al.*, 2006, 2008) will depend on the oxidation in soils. Up to now, detailed research on the natural oxidation of black-C in soils remains scarce and little is known about such aspects as to how fast or to what extent black-C is oxidized.

Ancient anthropic soils in the humid tropics provide an interesting model for a stable soil that exhibits unusually long fertility, being chemically and microbially stable. The high fertility has been ascribed to the millenary black-C found in these black earth soils (Falcão *et al.*, 2003; Glaser and Birk, 2012; Jorio *et al.*, 2012). By applying materials science tools, including scanning and transmission electron microscopy, energy dispersive X-ray, electron energy loss spectroscopy and Raman spectroscopy, it has been show that the stable carbon materials found in the Amazonian dark earth (named TPI-carbons, from *Terra Preta de Índio*) exhibit a complex morphology. Particles range in size from micrometres to nanometres from the core to the surface of the carbon grains, and they are rich in specific elements that are important for fertility and carbon stability (Jorio *et al.*, 2012; Ribeiro-Soares *et al.*, 2013; Archanjo *et al.*, 2014).

Scanning electron microscopy (SEM) images of TPI-carbon grains show they exhibit morphology in the micro-scale that is similar to that of charcoal, and this is consistent with the type of black-C appearing naturally in nature (Jorio *et al.*, 2012; Archanjo *et al.*, 2014). The TPI-carbon grains exhibit a 'fractal-like' structure, where a 'compact core surrounded by porous shell' structure repeats itself when zooming in at different scales, from hundreds of micrometres down to nanometres (Jorio *et al.*, 2012; Jorio and Cançado, 2012; Ribeiro-Soares *et al.*, 2013; Archanjo *et al.*, 2014).

The chemical composition at the microscopic level has been obtained for hundreds of grains, revealing that besides carbon, silicon and oxygen, which are common to all grains, elements such as Al, Fe, Cl, Ca, Mg, P, K Ti, Na, Mn, Ti and Pt can be found (Jorio *et al.*, 2012; Archanjo *et al.*, 2014, 2015). The main observations are: (i) the core is composed mostly of carbon, but oxygen and calcium are spread over the whole structure; and (ii) the other elements are located mostly in the grain shell. Metal oxide-rich nanoparticles were visible on a scale of 10–100 nm. The presence of Ca and P in the TPI-carbons has been attributed to bone fragments and animal residue.

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To probe the chemical state of both the core and the external regions of the TPI-carbon grains, spatially localized electron energy loss spectroscopy (EELS) has been performed, demonstrating a aromatic carbon structure (Jorio *et al.*, 2012). Raman spectroscopy measurements are consistent with aromatic carbon nanocrystallites (Jorio *et al.*, 2012b; Ribeiro-Soares *et al.*, 2013). EELS show four well-defined main peaks related to aromatic-C, phenol-C, aromatic carbonyl and carboxyl. The relative peak intensities for the EELS spectral peaks indicate that the core is more graphitic than the surface, the external region of the grain demonstrating a deeper level of oxidation, which caused an increase in the abundance of phenol, carbonyl and carboxylic groups. Spatially resolved mapping of each chemical group has been performed, and chemical maps confirm that the grain core has a tendency to be more aromatic (graphitic) than the grain surface (shell) (Archanjo *et al.*, 2014). In addition, the results clearly show the ageing-induced oxidation is not homogeneous in the sample, with the spatial resolution of 15 nm revealing chemical domains that go from 20 nm^2 up to hundreds of square nanometres (Archanjo *et al.*, 2014).

Theoretical calculations based on density functional theory have been performed to explore the role of oxygen and calcium for TPI-carbon stability (Archanjo *et al.*, 2014). Other theoretical studies address the stability dependence on the TPI-carbon nanocrystallite size (Jorio *et al.*, 2012). Insights about the in-plane dimension of the nanocrystalline structures composing these grains were obtained from Raman spectroscopy analysis, and the crystallite size distribution for TPI-carbons was found to be in the 3–8 nm range, while for charcoal it was typically found between 8 nm and 12 nm (Jorio and Cançado, 2012; Jorio *et al.*, 2012; Ribeiro-Soares *et al.*, 2013).

All these studies demonstrate the uniqueness of TPI-carbon morphology, scaling from microns to nanometres. Morphology and composition probably play important roles in the functionality of the historical black-C, providing a model of a chemically and microbially stable soil environment. It has been observed that increasing soil depth in a *Terra Preta de Índio* site, decreased soil nutrient content and associated abundance of microbiota (M.C. Pagano, J. Ribeiro-Soares, L.G. Cançado, N.P.S. Falcão, V.N. Gonçalves, L.H. Rosa, J.A. Takahashi, C.A. Achete and A. Jorio, 2014, unpublished data). Understanding the interplay between black-C morphology and microbiota might elucidate the importance of black-C in soil fertility.

2.4 Mycorrhizal Plants and Biochar

To date, only a few studies (e.g. Solaiman *et al.*, 2010; Warnock *et al.*, 2010) have focused on the effects of biochar on mycorrhizal fungi (Table 2.1). Other reports only mention mycorrhiza, but focus on biochar with regard to soil organic matter protection (Stockmann *et al.*, 2013) or to soil aggregation (George *et al.*, 2012).

Biochar application can have positive effects on beneficial soil microorganisms, improving the mycorrhizal root colonization, biological N_2 fixation by rhizobia (associated only with legumes) and activity of plant growth-promoting organisms in the rhizosphere (see Solaiman *et al.*, 2010; Quilliam *et al.*, 2013).

Table 2.2 illustrates reviews or papers dealing with biochar and AMF. For a review of some pioneering works in biochar research, see Ogawa and Okimori (2010). Ogawa (1998) was among those interested in the use of symbiotic microorganisms and charcoal. He observed that most symbiotic biota prefer to propagate in or around char. However, knowledge about how biochar affects soil aggregation and whether mycorrhizal fungi or an active C source might be needed to increase water stable aggregates in biochar-amended soils is still limited. It has been shown that the highest concentration of black-C was observed in the finest size fraction (< 0.53 μm) of soil aggregates (Brodowski *et al.*, 2006). Nevertheless, a preferential embedding of black-C particles has

Table 2.1. Database survey conducted in March 2014 (Scopus, 2014) for journal articles dealing with arbuscular mycorrhizal fungi (AMF) and biochar.

Key words	Number of journal articles
Biochar	1528
Microbes + biochar	21
Mycorrhizas + biochar	14
Biochar + ectomycorrhiza	3

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Table 2.2. Sample reviews and papers dealing with arbuscular mycorrhyzal fungi (AMF) and biochar.

Focus	Country/review	References
Biochar, mycorrhizal fungi and nitrogen fertilizer	North America/greenhouse experiment	LeCroy *et al*. (2013)
Charosphere	UK	Quilliam *et al*. (2013)
Biochar, ectomycorrhizal fungi	Canada	Robertson *et al*. (2012)
Terra Preta, biochar	Several countries	Briones (2012)
Carbon sequestration, soil microbes	Review	Ennis *et al*. (2012)
Biochar effect	Review	Elad *et al*. (2011)
Carbon remediation and microbes	Review	King (2011)
Biochar use in agriculture and forestry	Review	Ogawa and Okimori (2010)
Mycorrhizas in managed environments	Several countries	Solaiman *et al*. (2010)
Biochar effects on soil biota	Review	Lehmann *et al*. (2011)
Biochar effects on soil organisms, mycorrhizas	Review	Thies and Rillig (2009)
Arbuscular mycorrhizas	Field experiment in Colombia	Warnock *et al*. (2010)
Mycorrhizas	Review	Warnock *et al*. (2007)

been suggested compared with other organic compounds within soil aggregates (reviewed by Mukherjee and Lal, 2013).

Based on the scarce published research on biochars and mycorrhizas reviewed here, application of biochar stimulates arbuscular mycorrhizal colonization increasing P supply to plants. Solaiman *et al*. (2010) tested different rates of biochar addition to soils together with two types of commercial fertilizers (soluble and slow-release mineral fertilizers) on wheat in field conditions. Moreover, mineral fertilizers contain several beneficial and non-pathogenic fungi including mycorrhizas (*Glomus intraradices*) and bacteria (*Azospirillum* and *Azotobacter* among other beneficial microorganisms). They showed that at 30 kg/ha of soluble fertilizer and the highest rate of biochar (6 t/ha) increased the uptake of all nutrients by plants. With regard to root colonization by AMF, the inoculated treatment presented higher root colonization, which increases with biochar addition, especially in unirrigated samples. The presence of mycorrhizal fungi can increase the surrounding soil explored by hypha, which can cope with the drought stress during a dry period. Furthermore, soil amendments can influence the mycorrhizal colonization and establishment of plants. Biochar was shown to directly stimulate mycorrhizal root colonization resulting in better plant growth and yield of wheat. Additionally, a positive residual effect of biochar application on mycorrhizal root colonization, plant growth and nutrition of wheat was found after 2 years.

Quilliam *et al*. (2013) analysed the relation between biochar addition in agricultural soil and the associated microbiota. They proposed the term charosphere for the soil surrounding the biochar that is directly influenced by the chemical and physical properties of the char, which can in turn influence soil–plant–microbe interactions. The authors determined the level of microbial colonization of field-aged (3 years) biochar and showed there was only sparse colonization by microorganisms. They found that it was difficult to identify the advantages of colonizing fresh or newly applied biochar, as most microorganisms will be unable to utilize the limited resources. There was heterogeneity of colonization (microorganisms were frequently found in the remains of the vascular tissue of the feedstock) and this has implications when considering the addition of biochar to soils. The authors suggest that in time, microbial colonization of biochar can be improved through the influence of abiotic factors. Thus, partial microbial decomposition of biochar will provide both nutrient supply and habitat. Finally, they suggest that the physical breakdown of biochar may accelerate microbial colonization, and this can be achieved by amendment with biochar powder.

Some studies in Table 2.2 report that biochar addition can enhance soil properties and the early growth of pine and alder in sub-boreal forest soils, but ectomycorrhizal abundance does not change (Robertson *et al*., 2012). However, some cases showed that the combined treatment

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of biochar, mycorrhizal fungi and high nitrogen can decrease aboveground plant biomass, but it can promote mycorrhizal root colonization (LeCroy *et al.*, 2013). The authors ascribe the effect to an induced parasitism of the mycorrhizal fungus in the presence of nitrogen and biochar throughout the experiments. However, AMF colonization of biochar in soils with mycorrhizae and less nitrogen showed more surface oxidation. They conclude that soil nitrogen can act as a switch controlling the capacity of char to influence the AMF association and, consecutively, the degree to which the fungi oxidize the char surface.

Lehmann *et al.* (2011) compiled information on biochar effects on soil biota. They discussed biochar addition as an inoculant carrier, instead of peat. They highlighted the possible incorporation of mycorrhizal inoculum to biochar and the lack of debate on this topic. As biochar will remain in the soil it may affect the inoculated microorganisms. For instance, Ogawa (1989) reported the preservation of spores of *Gigaspora margarita* for more than 6 weeks in balls of biochar. Other studies (Rutto and Mizutani, 2006) showed reduced mycorrhizal symbiosis in combination with activated carbon. Nevertheless, the properties of biochar (pH, origin, production, etc.) should be appropriate for the inoculated microorganisms. In the future, a combination of approaches to design biochars as inoculant transporters may be very useful, but soil type should be also considered (Lehmann *et al.*, 2011).

2.5 Mycorrhizal Plants and Compost

We know that recycling organics from agricultural and urban wastes can help to deal with serious environmental challenges. However, our understanding of the factors that affect compost management is limited. Composting has developed rapidly in order to manage poultry, swine and cattle remains, but also to rehabilitate degraded soils. Moreover, due to the rising cost of peat and transmission of several soil-borne diseases, peat is frequently substituted by compost (see Raviv, 2011, 2013).

The benefits of compost to soils and vegetation are well researched. Compost (a source of organic matter) decreases bulk density and soil erosion, and intensifies aggregate stability, soil aeration, water infiltration and retention. Furthermore, compost carries micro- and macronutrients, providing a wider range of nutrients than inorganic fertilizers, with less nitrate leaching and water contamination (Gagnon *et al.*, 1997; Mamo *et al.*, 1999). However, better knowledge of the C and N turnover from composts and in the soil organic matter pools will be crucial for a specific control of the negative impact on the groundwater (see Amlinger *et al.*, 2003). It is worth mentioning that an international journal named *Compost Science & Utilization* has been published by Taylor & Francis (four issues per year) since 1995.

The positive effect of adding organic substrates and humic substances of particular concentrations on the growth of numerous plants is well documented (Nikbakht *et al.*, 2008; Verlinden *et al.*, 2009; Scharenbroch *et al.*, 2013; Thangarajan *et al.*, 2013). Copious reports on compost have been published; however, mycorrhizas, a special group of fungi, are still understudied. Mycorrhizas are not typically viewed in the same mode as compost addition. This can be due to the difficulties in inocula preparation as AMF are not culturable microorganisms (Smith and Read, 2008).

In the last few years approximately 112 peer-reviewed journal papers on compost and mycorrhizas were reported all over the world (Scopus survey conducted in March 2014), of which four were published in 2014. Of these journal papers 58% comprise research dealing with arbuscular mycorrhizas. Among them, Tanwar *et al.* (2013) tested different sugarcane bagasse substrates and one arbuscular mycorrhizal (AM) species (*Funneliformis mosseae*) in order to improve plant growth. Moreover, Daynes *et al.* (2013) tested sterilized compost (< 2 mm) inoculated with indigenous microbes and AMF (mostly *Glomus* sp.) in a controlled pot experiment. They showed that compost (at least 6%), living roots and AMF resulted in a more stable soil structure.

Bashan *et al.* (2012) showed that, in the Sonoran Desert, Mexico, small quantities of compost supplementation (cow manure and wheat straw) and bioinoculation helped the survival of three native leguminous trees planted to restore eroded soil. For inoculation they tested plant growth-promoting bacteria (*Azospirillum brasilense* and *Bacillus pumilus*) and native AMF (from sorghum plants inoculated with *Glomus*

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spp. of a consortium of native species collected under mesquite trees). These microorganisms can enhance drought resistance of plants. The compost was mixed with soil and added to each planting hole at a proportion of 1:8 (735 g: 10 kg, w/w). Interestingly, survival and growth of the trees depended on the plant species.

Marosz (2012) showed that green waste compost and AMF can improve the nutrient uptake of woody plants grown under salt stress (principal de-icing agents on roads). Application of compost enhanced the growth of certain species of trees and shrubs, and the inoculation of mycorrhizal fungi (AMF propagules), before planting trees sensitive to salinity, was useful in such stressful conditions.

The AMF inocula used in such experiments can consist of several fungal species, mainly from the genus *Glomus* or other species as yet not completely taxonomically identified (a mixture of site-specific species). The inoculum can consist of AMF propagules (e.g. spores or colonized roots) mixed with sphagnum peat. The inoculum is placed under the roots of trees or shrubs during planting. Knowledge about this issue is surprisingly scant, taking into account that inoculation with mycorrhizas greatly affects plant and soil health.

In 2010, Viti *et al.* tested the agricultural use of compost in both conventional and organic maize production showing that, while the use of the compost as an amendment may exert a restricted impact on AMF, it can significantly modulate the composition of plant growth-promoting rhizobacteria in the rhizosphere of maize. They found only species of *Glomus*.

All these reports show that *Glomus* spp. are generally associated with compost. This is not unexpected due to the prevalence of this genus in agricultural experiments and field trials (Oehl *et al.*, 2005; Mirás-Avalos *et al.*, 2011) that are highly susceptible to disturbance. This is due to the life history of *Glomus* spp., which is generally considered to be that of an r-strategist.[1] *Glomus* spp. belonging to the Glomeraceae 1 (GlGrA) b clade (Schwarzott *et al.*, 2001) show an opportunistic behaviour similar to r-strategists (Sykorová *et al.*, 2007). For instance, most different compost amendments are tested with *Glomus* as the inoculant (Üstüner *et al.*, 2009; Adewole and Ilesanmi, 2011). This is also because most crops, vegetables, fruits and cereals associate with *Glomus* spp. such as *Glomus mosseae* (nowadays named *Funneliformis mosseae*) (Miranda, 2008; Naher *et al.*, 2013). This also depends on the cultivation system, as shown by differences observed in rice cultivation systems (Watanarojanaporn *et al.*, 2013).

2.6 Conclusion

The large variety of organic matter types utilized as soil amendments, including composts, crop residues, peat and organic wastes, adds to the heterogeneity of organic C delivered into ecosystems. In the context of increasing rates of organic wastes, emissions of CO_2 and other gases from industrial and agricultural development which alter global C balance and climate C sequestration, strategies highlight the use of biochar. However, our understanding of the factors that affect biochar use and the notable variability of its biochemical qualities is limited. In the last decade, new throughput analytical techniques, such as Raman spectroscopy, have helped with biochar characterization, including the elucidation of the structure of historical black-C found in ancient anthropic soils. Understanding the interplay between black-C morphology and microbiota is an important direction for future research work.

Many studies have demonstrated that biochar can influence microbial activity by providing a favourable microhabitat, or that AMF can extend their extraradical hyphae into biochar, which helps nutrient conservation in soils. However, the weak alkalinity and strong dependence on pH of biochars may provide grounds for the variability of field observations. Since the interrelation of climate, land, water, vegetation and human activity affects ecosystems, increasing research on soil amendments and agroecosystem management is needed.

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Note

[1] A rapid colonizer of plant roots, which produces high numbers of spores in a brief period, traits that are favoured in unstable environments.

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3 *Trichoderma*: Utilization for Agriculture Management and Biotechnology

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Abstract

Plant diseases are the primary cause of reducing both the quality and the quantity of crop yields. Numerous synthetic products have been used to control plant infections; however, overuse of such products has favoured the development of strains of pathogens that are resistant to fungicides. Unfortunately, the more exact the impact of a synthetic product on a pathogen, the more likely it is that the pathogen will develop resistance to it. In addition, the widespread use of fungicides produces undesirable effects on non-target organisms. Concerns about nature, human well-being and other related hazards resulting from the overuse of synthetic chemicals have led to considerable interest in developing eco-friendly methods of biocontrol against plant pathogens. There have been many new discoveries that have identified biocontrol agents and this includes *Trichoderma*, a soil-borne fungus with green spores that is found worldwide; it has become one of the most popular biocontrol agents of plant pathogens. Natural control of a pathogen may be achieved in a variety of ways including: (i) the ability to colonize the rhizosphere and successfully compete with the pathogen for nutrients and space; (ii) antibiosis; (iii) mycoparasitism; (iv) dissolving cell walls; (v) improving plant development; and (vi) boosting plant immunity. *Trichoderma* spp. may produce distinctive antifungal mixtures of metabolites that attack and limit the development of fungal pathogens. *Trichoderma* spp. are readily available in soil where they can exist for a considerable time under a wide variety of natural conditions. The recognition of the usefulness of numerous *Trichoderma* strains as potential biocontrol agents has increased the urgency and importance of further work in order to commercialize strains. This chapter gives an overview of *Trichoderma* biology, the potential use of *Trichoderma* strains as biocontrol agents in agriculture, mechanisms of action and applications in biotechnological industries.

3.1 Introduction

In the present day, there is increasing pressure to grow and produce more food in order to feed the expanding world population. However, there are a wide range of pathogens that directly or indirectly affect crop production both pre- and postharvest. Such pathogens include phytoplasma, microorganisms, parasites, nematodes and parasitic plants. Plant diseases account for a significant proportion of the losses experienced in farming which prompts a need to combat disease-causing organisms in order to increase food security. Soil-borne pathogens such as *Pythium*, *Phytophthora*,

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Botrytis, *Rhizoctonia* and *Fusarium* stand out among the most damaging plant pathogens causing significant reductions in crop yields and resulting in considerable financial losses. They cause damage to standing harvests as well as to stored yields at risk of fungal diseases (Chet *et al.*, 1997). The requirement to increase horticultural production and quality has prompted overuse of synthetic compost which means there is a genuine risk of ecological contamination. It has been observed that the expansive range of fungicides that are used have created undesirable outcomes on non-target organisms (Tjamos *et al.*, 1992) and their overuse has favoured the advancement of pathogens that are resistant to fungicides. Plant researchers work towards keeping plants disease free to improve harvest yields while minimizing the use of pesticides. To minimize the use of synthetic compost their aim is to build use of bio-manures and biopesticides in order to maintain advances in agribusiness and to generate many eco-friendly products. This includes work on the traditional mycorrhizal organisms, rhizobium microorganisms, other plant growth-promoting rhizobacteria (PGPR) and potential biocontrol fungi, for example *Trichoderma* spp. and the root-colonizing species *Piriformospora indica* which can be used for strengthening plant development by stifling plant diseases (Van Wees *et al.*, 2008).

Many diverse soil-borne microscopic organisms and fungi have the capacity to colonize plant roots and may have helpful impacts on the plant. Members of the filamentous ascomycete genus *Trichoderma* are universal and are considered to be ordinary general saprotrophs. They are found in almost all soil types and biological systems, particularly those that are rich in natural matter, exhibiting great versatility in different environmental conditions (Klein and Eveleigh, 1998; Jaklitsch, 2009, 2011; Brotman *et al.*, 2010; Druzhinina *et al.*, 2011). The capability of *Trichoderma* as biocontrol specialists for plant infection was initially perceived as early as the 1930s; since then *Trichoderma* spp. have been used to control numerous infections (Elad and Kapat, 1999). An outline of the landmark discoveries in the biology and applications of *Trichoderma* are shown in Table 3.1. *Trichoderma* strains used as biocontrol agents against phytopathogens achieve control in a variety of ways including: (i) by successfully competing with the pathogen for nutrients and space; (ii) by changing the natural conditions; (iii) by improving plant development; (iv) by boosting plant immunity; (v) by antibiosis; and/or (vi) straightforwardly by mycoparasitism. Numerous types of *Trichoderma* are equipped to deliver catalysts that corrupt the target pathogenic organisms or produce various lethal mixtures which limit the development of pathogens. The adversarial properties of these are focused around numerous systems. These indirect and direct components may act in concert and their importance in terms of biocontrol depends upon the *Trichoderma* strain, the irritated fungus, the crop plant and the ecological conditions, alongside the nutrient supply, pH, temperature and iron levels. Initiation of each system suggests the gathering of particular mixtures and metabolites, in the same way as plant development elements, hydrolytic compounds, siderophores, antimicrobials and carbon and nitrogen permeases. These metabolites can be either overproduced or consolidated with proper biocontrol strains so that new methods of use are developed that are more effective in controlling plant diseases and with applications for preventing losses during post-harvest storage (Benitez *et al.*, 2004).

Trichoderma strains are ideal as biocontrol specialists because of: (i) their high regenerative limit; (ii) their capacity to survive under unfavourable conditions; (iii) their effectiveness in acquiring nourishment and adjusting to conditions in the rhizosphere; (iv) their hostility to growth of phytopathogenic organisms; and (v) their proficiency to advance plant development and a plant protection framework. These properties have made *Trichoderma* spp. widespread worldwide, being present in the soil of a variety of environments in high numbers. *Trichoderma* species are more abundant in acidic soil than alkaline soils. The most widely recognized biocontrol specialists of *Trichoderma* are strains of *Trichoderma virens*, *Trichoderma viride* and *Trichoderma harzianum*. Incredible effects of control have been accomplished with strains of *T. virens* and metalaxyl against *Pythium ultimum* infecting cotton (Chet *et al.*, 1997). *Trichoderma* species create various proteins, for example cellulases and hemicellulases, chitinases and glucanases. The chitinases and glucanases can control the spread of pathogens. Under field conditions *Trichoderma* has been recorded to improve plant development too. Nowadays, these readily accessible fungi are

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Table 3.1. Landmark discoveries in the biology and applications of *Trichoderma* in biotechnology (Mukherjee *et al*., 2013).

Year	Landmark discoveries	References
1932	*Trichoderma lignorum* (*Hypocrea virens*) is a mycoparasite. First evidence that *Trichoderma* spp. are mycoparasitic and have biocontrol potential	Weindling (1932)
1934	Discovery of gliotoxin. First antimicrobial from *Trichoderma*	Weindling (1934)
1957	Discovery of an effect of light on conidiation (in *Trichoderma viride*; syn. *Trichoderma reesei*)	Gutter (1957)
1972	Demonstration of biocontrol in the field. Biological suppression of *Sclerotium rolfsii* by *Trichoderma harzianum*	Wells *et al*. (1972)
1979	Cellulase hyperproducing mutant RutC30 of *T. reesei* isolated	Montenecourt and Eveleigh (1979)
1983	Cloning of the first cellulase of *T. reesei*	Shoemaker *et al*. (1983)
1986	Demonstration of plant growth promotion by *Trichoderma*	Chang *et al*. (1986)
1987	Successful transformation of *T. reesei*	Penttila *et al*. (1987)
1989	Heterologous protein expression in *T. reesei*	Harkki *et al*. (1989)
1992	Lectin-coated fibres are coiled by *Trichoderma*: biomimetics of fungal–fungal interactions	Inbar and Chet (1992)
1993	Cloning of the first mycoparasitism-related gene (*prb1*) and its induction by cell walls	Geremia *et al*. (1993)
1997	Ability of *Trichoderma* spp. to boost plant immunity at a site away from site of application (induced resistance)	Bigirimania *et al*. (1997)
1998	Diffusible factors induce mycoparasitism-related genes before contact	Cortes *et al*. (1998)
1999	Internal colonization of plant roots by *Trichoderma* demonstrated	Yedidia *et al*. (1999)
2002	Signalling pathways involved in biocontrol and conidiation; role of G-protein pathways established	Rocha-Ramirez *et al*. (2002)
2002, 2011	Genetics of peptaibols production. Cloning of peptaibol synthase genes and demonstration of one non-ribosomal peptide synthetase catalysing multiple peptaibols	Wiest *et al*. (2002), Mukherjee *et al*. (2011)
2003	Mitogen-activated protein kinase (MAPK) negatively regulates conidiation in *T. virens*	Mendoza-Mendoza *et al*. (2003), Mukherjee *et al*. (2003)
2004	Photoreceptors (Brl1 and Brl2) discovered in *Trichoderma atroviride*	Casas-Flores *et al*. (2004)
2005	Role of *Trichoderma* MAPK in induced systemic resistance (ISR)	Viterbo *et al*. (2005)
2006	The first true elicitor protein (Sm1/EplI) purified	Djonovic *et al*. (2006), Seidl *et al*. (2006)
2006	Master regulator of cellulases and xylanases identified in *T. reesei* (XYR1)	Stricker *et al*. (2006)
2008	*T. reesei* genome published. First *Trichoderma* genome sequenced	Martinez *et al*. (2008)
2009	Endophytic *Trichoderma* imparts biotic and abiotic stress tolerance in plants	Bae *et al*. (2009)
2009	Successful crossing in *T. reesei*. First time sexual crossing was achieved for any *Trichoderma* species under laboratory conditions	Seidl *et al*. (2009)
2009	Role of sucrose metabolism established. First clue to source–sink dynamics in the *Trichoderma*–plant interaction	Vargas *et al*. (2009, 2011)

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Table 3.1. Continued.

Year	Landmark discoveries	References
2009	Ability of mutualistic *Trichoderma* to modify plant root architecture through the production of auxins demonstrated	Contreras-Cornejo *et al*. (2009)
2010	Master regulator (*Vel1*) of morphogenesis and biocontrol identified	Mukherjee and Kenerley (2010)
2010	Peptide pheromone precursor genes described for *Trichoderma* spp. *T. reesei* HPP1 represents a novel type of pheromone	Schmoll *et al*. (2010)
2011	Comparative genome analysis of three *Trichoderma* species. Molecular support for the different lifestyles of *Trichoderma* spp.	Kubicek *et al*. (2011)
2012	*T. harzianum*, *Trichoderma asperellum*, *Trichoderma longibrachiatum* and *Trichoderma citrinoviride* genomes sequenced using next generation sequencing (Joint Genome Institute)	Mukherjee *et al*. (2013)
2012	High-throughput procedure for gene deletion developed for *T. reesei*. Provides a basis for knockout programmes in *Trichoderma*	Schuster *et al*. (2012)

widely used in a variety of commercial ways. Currently more than 60% of listed biofungicides used in agriculture worldwide are *Trichoderma*-based (Verma *et al*., 2007). This has brought about the creation of business based on the use of *Trichoderma* spp. to protect crops against a few plant diseases and to develop increased crop yields in India, Israel, New Zealand and Sweden.

The importance of *Trichoderma* strains as biocontrol agents and makers of important metabolites and proteins has confirmed their status among distinctive genera of organisms. The utilization of morphological and taxonomic characters alone to separate species inside the genus *Trichoderma* has been troublesome because of an absence of reliable characters. The next section provides an outline of the biology of this genus, including morphological characters and phylogeny, and this is followed by sections on the significance of *Trichoderma* species as biocontrol specialists and the biocontrol mechanism, progress in identification of *Trichoderma* genes and finally a section on commercial utilization.

3.2 Biology of Genus *Trichoderma*

Types of the contagious sort of *Trichoderma* are commonly soil inhabitants, existing as anamorphs fitting in with the sub-division Deuteromycotina (fungi imperfecti) (Hawksworth *et al*., 1983). Types of the mycotrophic filamentous ascomyceteous class *Trichoderma* (hypocreales, hypocreaceae, telemorph hypocrea) are among the most normally experienced growths (Druzhinina *et al*., 2011). *Trichoderma* are every now and again disconnected from soil and are discovered developing on dead wood, bark, other plants, building materials and creatures, including people, showing a high entrepreneurial potential and versatility to environmental conditions (Klein and Eveleigh, 1998; Druzhinina *et al*., 2011). Numerous *Trichoderma* species are of considerable financial value, creating hydrolytic compounds (e.g. chitinases, cellulases and xylanases) (Schirmbock *et al*., 1994; Ahamed and Vermette, 2008; Alvira *et al*., 2013), biochemicals and antibiotics (Ghisalberti and Sivasithamparam, 1991), products which have been applied to the fields of, for example, food processing and pulp bleaching (Nigam, 1994). Furthermore, a few species produce heterologous proteins (Nevalainen *et al*., l99l) and others have been effectively utilized as natural control specialists against a range of phytopathogens (Chet and Inbar, 1994). On the other hand, a few species of *Trichoderma* are detrimental to plants. For instance, decreases in mushroom yields have been ascribed to *Trichoderma* disease (Fletcher, 1990). *Trichoderma aggressivum* is the causal organism of green mould,

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a disease of the edible mushroom *Agaricus bisporus* and *T. viride* is the causal organism of green mould decay of onion (Samuels *et al*., 2002; Beyer *et al*., 2007). Besides, phytopathogenic strains of species have likewise been discovered, in spite of the fact that these appear to be moderately uncommon (Menzies, 1993).

3.2.1 Morphological characters of *Trichoderma* spp.

Rifai (1969) and Bissett (1991) provide details of the morphological characters they used to describe and separate species of *Trichoderma*. They stress the difficulties in characterizing morphological types of *Trichoderma*. Samuels (1996) additionally remarked on the utility of morphological characters to characterize species in *Trichoderma*. Morphological characters helpful for characterization and identification in other hyphomycete genera are often not as valuable in separating *Trichoderma* species. However, despite this, observed morphological characters remain the essential technique for identification and confirmation of species in *Trichoderma*.

Trichoderma strains can often be recognized by a unique morphology that incorporates rapid development, brilliant green or white conidial colours, and repeatedly branched conidiophores. Figure 3.1 shows classification of *Trichoderma* based on conidia and conidiophores. Bissett (1991) proposed to incorporate all the anamorphs of *Hypocrea* in the genus *Trichoderma*. On the off chance that this idea is acknowledged, then an acceptable morphological definition for the class *Trichoderma* would be dangerous (Samuels, 1996), since the conidiophore fanning structure

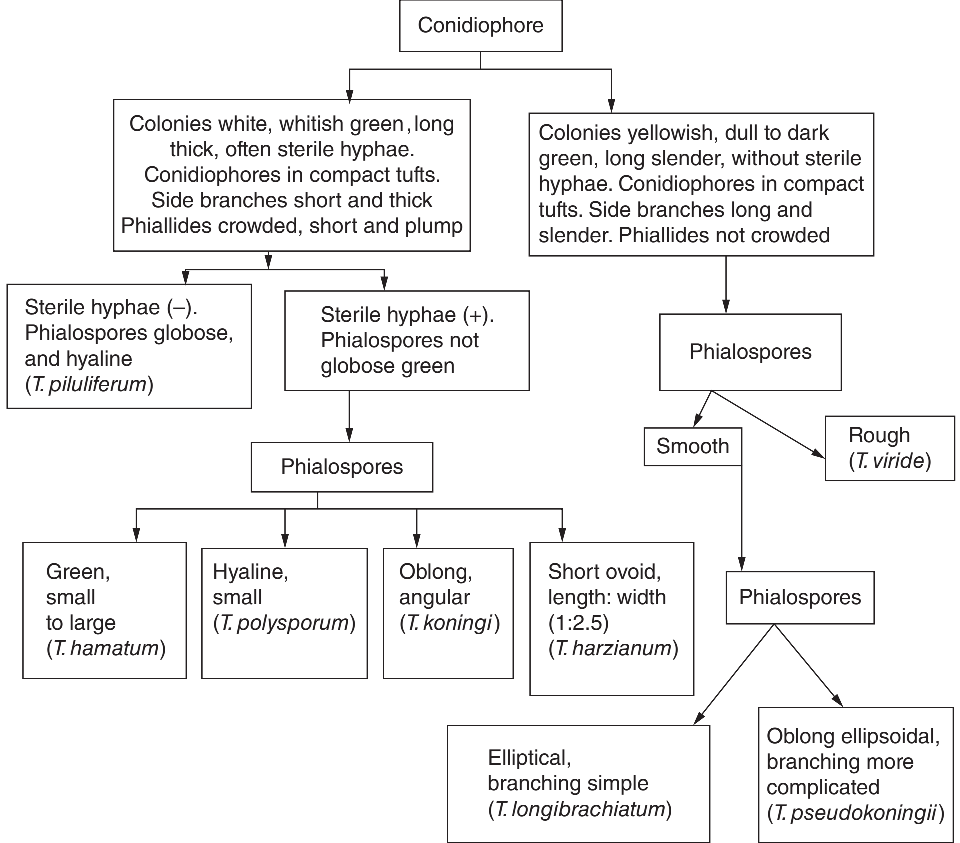

Fig. 3.1. Classification of *Trichoderma* based on conidia and conidiophore (Ranasingh *et al*., 2006).

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is very variable, and more or less looks somewhat like that of less significant genera, for example *Verticillium* and *Gliocladium*. By and large, *Trichoderma* anamorphs of *Hypocrea* can be separated from these two genera in having conidiophores that are more variable in that they fan out at an inconclusive number of levels – contrasting with the more consistent verticillate or biverticillate fanning in *Verticillium*; and being distinguished from *Gliocladium* which has a characteristic penicillate conidiophore structure with terminal penicillate extensions and more regularly aculeate phialides on a generally decently sized stipe.

Colony characters such as the development rate in culture, pigmentation, the conidiophores and conidia are important morphological characters used to identify diverse types of *Trichoderma*. Development rates in culture can be valuable to recognize generally comparative species. Colonies of strains of *T. viride* are usually an arresting brilliant greenish-yellow colour, at least when the colonies are small and restricted in terms of space. Dull yellowish shades are characteristic of numerous species so this is not a feature that can be used to distinguish different species. A few species are white while others are ruddy in colour. Some colonies have distinctive smells, for example *Trichoderma aureoviride* and the trademark sweet-smelling coconut smell produced by strains of *T. viride*, *T. harzianum* and *Trichoderma koningii*. A few strains of *Trichoderma* produce unpleasant smells. The fanning of the conidiophore is either consistently verticillate or unpredictable. Conidiophores are erect or straggling, expanded at standard intervals, with singular extensions or several branches collected into floccose tufts. Every progressive limb is apically and distally dynamically shorter and narrower than its predecessor giving the fan a pyramidal appearance. Conidiogenous cells are phialides, borne separately or in groups terminally, and these may be lageniform, ampulliform, sub-globose or barrel-shaped, with a conidium-bearing neck. Phialides are naturally short and stout in *T. harzianum*, though in *T. viride* they are elongated and lageniform to almost round and hollow. Terminal phialides in many species have a tendency to be longer and narrower and habitually pretty much subulate. Subterminal cells of the conidiophore may deliver conidia through a short parallel neck, hence intercalary phialides or what Gams and Bissett (1998) called aphanophialides are somewhat ordinarily seen in *Trichoderma*. The single-celled conidia may be dreary green, ash or tannish in colour; conidial shape changes from globose to ellipsoidal, obovoidal or short-tube shaped, with the basal end pretty much decreasing and truncate. There is not a great variety in conidial measurements in *Trichoderma*; however, related species can regularly be separated by slight yet steady contrasts in size. In many species the conidial surface appears to be smooth when examined under the light microscope, however, inspection using a scanning electron microscope reveals these apparently smooth conidia to be lightly ornamented. Conidia are either roughened or verrucose in *T. viride* and there are wing-like projections on the outer walls in *Trichoderma saturnispoerum* and *Trichoderma ghanense*. Chlamydospores are normal in numerous species. They tend to be globose to ellipsoidal, terminal and intercalary, smooth-walled, yellowish, greenish or lacklustre and 6–15 μm wide in many species.

3.3 *Trichoderma* as Biocontrol Agents

Biocontrol, reducing a pest's population by using the pest's natural enemies, is characterized by the strategy of gainfully utilizing microscopic organisms such as *Trichoderma* spp. as biocontrol specialists against pathogen assault on plants. The biocontrol agent uses a variety of diverse components that act synergistically to achieve natural disease control. Four noteworthy components are reported by which biocontrol agents control other harmful microorganisms: (i) antibiosis (creating antimicrobial metabolites that chemically adjust the rhizosphere so that pathogen cannot develop and/or that kill the pathogen's cells); (ii) mycoparasitism (involving direct physical contact between the pathogen and the biocontrol agent and use of hydrolytic catalysts, anti-infections agents and other harmful compounds produced by the biocontrol agent); (iii) competition (e.g. for space or nutrients); and (iv) fungistasis (e.g. by blocking spore germination). There are a large number of different species of *Trichoderma* some of which are biocontrol agents and they achieve this by using one or more of these methods of control. In addition *Trichoderma* biocontrol agents can bring

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about positive consequences for plants including improving plant development (biofertilization) and boosting the plant's natural immunity. Initiation of every component infers the creation of particular metabolites, for example plant development variables, hydrolytic compounds, siderophores, antimicrobials and permeases.

Bailey and Lumsden (1998) showed that particular strains of *Trichoderma* colonized and infiltrated plant root tissues and launched a progression of morphological and biochemical changes in the plant, which were thought to be part of the plant barrier reaction and in this manner impelled systemic safety. Traditionally, three chief components of activity of *Trichoderma* spp. have been perceived: (i) antibiosis; (ii) mycoparasitism; and (iii) competition for nourishment, space or oxygen. In an average mycoparasitic interaction, the parasitic fungus (e.g. *Trichoderma*) detects chemicals discharged by the host fungus (e.g. *Rhizoctonia solani*) and is chemotropically pulled in towards the host. This is followed by the parasitic fungal hyphae growing in a snake-like fashion coiling around the host hyphae, completely surrounding and covering the host, creating appressoria-like structures and infiltrating the host in order to use the host as a nutrient source, eventually causing lysis of the host (Mukherjee *et al*., 2008).

Successful biocontrol is dependent upon the strain of the biocontrol agent, the organism that it is intended to control and the natural environmental conditions (Papavizas and Lumsden, 1980; Ayers and Adams, 1981; Cook and Baker, 1983).

3.3.1 Type of biocontrol mechanism

As already stated, there are four main components by which biocontrol agents control other harmful microorganisms: (i) antibiosis; (ii) mycoparasitism; (iii) competition; and (iv) fungistasis.

Antibiosis

T. harzianum Rafai is an effective biocontrol agent with a distinct pathosystem that makes a moderate adjustment to the soil and it is not harmful to people, natural life and other valuable microorganisms that help towards plant disease control. It is a powerful biocontrol agent that is used fairly commonly and when used in controlled situations it does not accumulate in the environment and its use is considered to be safe. Other *Trichoderma* strains also deliver metabolites that likewise indicate the incredible reach and prospective application towards controlling pathogenic microorganisms. According to Dennis and Webster (1971), *Trichoderma* strains seem, by all accounts, to be a ceaseless asset as anti-infection agents, producing metabolites that range from the acetaldehydes, gliotoxin and viridian to alpha-pyrone, terpenes, polyketides, isocyanide derivatives, piperazine and complex groups of peptaibols (Ghisalberti and Sivasithamparam, 1991). Mixtures of metabolites such as these may be produced together and act synergistically as well as blending with cell-wall debasing catalysts (Schirmbock *et al*., 1994; Lorito *et al*., 1996) resulting in inhibition of numerous fungal plant pathogens. Vey *et al*. (2001) reported that *Trichoderma* strains create numerous unpredictable and stable compounds including the antibiotic alamethicin, the antifungal agent tricholin, peptaibols, 6-petanyl-α-pyrone, viridian and gliovirin that upset colonization by hostile microorganisms. In a few cases, the biocontrol capacity corresponded with the antimicrobial generation. Likewise Howell (2003) recorded synergism between an endochitinase from *T. harzianum* and gliotoxin and hydrolytic chemicals and peptaibols amid conidial germination of *Botrytis cinerea.*

Peptaibols – a class of straight peptides that for the most part have antimicrobial action against Gram-positive microorganisms – act synergistically with cell-wall corrupting compounds to hinder the development of contagious pathogens and inspire plant imperviousness to pathogens (Wiest *et al*., 2002). In tobacco plants, exogenous applications of peptaibols trigger a resistance reaction and decrease defencelessness to tobacco mosaic infection (Wiest *et al*., 2002). A peptaibol synthetase from *T. virens* has as of late been filtered, and identification of the gene, which has been cloned, will encourage investigations of this compound and its role in biocontrol.

A broad survey of antibiosis and generation of *Trichoderma* auxiliary metabolites is given in Howell (2003). The use of *Trichoderma* species were discovered to be compelling against a few soil-borne and seed-borne plant pathogens, in particular *R. solani*, *Rhizoctonia bataticola*, *Fusarium solani* f. sp. *pisi* (Kumar and Dubey, 2001) and *F. oxysporum* f. sp. *ciceris* (Dubey, 2003;

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Dubey *et al.*, 2007, 2009). More recently, Singh *et al.* (2014) demonstrated the adequacy of *Trichoderma* strains *in vivo* and *in vitro* for control of *Colletotrichum falcatum*, the cause of red rot disease of sugarcane (*Saccharum officinarum*).

Mycoparasitism

Mycoparasitism is an extremely intricate mechanism in which one fungus specifically assaults another fungus, including distinguished pathogens (Chet *et al.*, 1981). The parasitic fungus, on discovering another fungus in the vicinity, grows towards the target host fungus, completely surrounding or covering it and enters the host in order to obtain nutrients from the living tissues. Necrotrophic mycoparasites such as *Trichoderma* kill the host fungus in the process. As a result of this, many diverse types of *Trichoderma* are broadly utilized as farming biopesticides to control plant pathogens, especially those that attack plant roots. *Trichoderma* species are fit for mycoparasitism as they produce various metabolites such as low levels of an extracellular exochitinase enabling them to enter the host fungus. A few strains of *Trichoderma* also produce elicitors and empower safety in plants through colonizing the roots. One of the most noteworthy trademarks of *Trichoderma* is its capacity to parasitize other organisms.

Before contact is made with the target host fungus, dissemination of extracellular exochitinase by *Trichoderma* catalyses the release of cell-wall oligomers from the host fungus, and this in turn prompts the expression of harmful endochitinases (Carsolio *et al.*, 1999), which likewise diffuse and start to attack the target organism (Zeilinger *et al.*, 1999; Viterbo *et al.*, 2007). Once the organisms come into contact, hyphae of *Trichoderma* spp. loop around the host and become attached to it by forming specialized appressoria-like structures on the host surface. Attachment is achieved by sugars in the *Trichoderma* cell wall becoming bound to lectins on the host fungus (Inbar and Chet, 1996). Once in contact, *Trichoderma* produces a few toxic cell-wall degrading enzymes (Chet *et al.*, 1997) and most likely peptaibol antimicrobials too (Schirmbock *et al.*, 1994). The combined activity of these compounds brings about parasitism of the host fungus and disintegration of the cell walls. At the sites of the appressoria, gaps can be made in the host fungus, and *Trichoderma* hyphae can enter into the lumen of the host cell. There are no less than 20–30 genes, proteins and other metabolites that are specifically involved in this interaction indicating the complexity involved in interactions between *Trichoderma* and other organisms.

In the immediate association between *Trichoderma* and the target host fungus there are four stages. These are: (i) chemotropic development – when *Trichoderma* is drawn towards the target fungus chemotropically by chemicals produced by the target fungus; (ii) specific identification between parasite and target host – lectins on the cell surfaces of both pathogen and *Trichoderma* are in charge of this methodology; (iii) coiling of *Trichoderma* hyphae around the target host fungus and attachment to it; and (iv) secretion of lytic proteins. Physical contact is preceded by an early recognition process which leads to the induction of gene expression of hydrolytic enzymes, for example *ech42* which codes for endochitinase in *T. harzianum* (Barbara *et al.*, 2011). Numerous biocontrol genes have been identified, cloned and described, in particular those that code for protease, chitinase, glucanase, tubulins, xylanase, monooxygenase, galacturonase and cell attachment proteins (Muthu and Sharma, 2011). These genes have one kind of capacity in the biocontrol system, for example cell wall degradation, hyphal development or stress resistance. These genes play a vital part in the biocontrol process by managing signals and lead to the emission of chemicals that assist in the debasement of the plant pathogens. The gene *ech42* from *T. harzianum* codes for endochitinase which has altogether stronger action against a wide range of phytopathogenic growths than other chitinolytic compounds (Garcia *et al.*, 1994). The *ech42* endochitinase (42 kDa) hydrolyses *B. cinerea* cell walls *in vitro* and represses spore germination and germ tube elongation in different organisms (Schirmbock *et al.*, 1994). Carsolio *et al.* (1994) demonstrated that *Trichoderma* spp. are potential biocontrol specialists for the control of the fungal diseases. Studies on the subatomic structure and qualities of genes encoding endochitinases (*ech42*) in *Trichoderma* will improve understanding of the connections between the distinctive catalysts involved in biocontrol. Recently, Singh *et al.* (2014) concentrated on the connection between endochitinase

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and the biocontrol action of *Trichoderma* spp. Six *Trichoderma* spp. were utilized for characterization of biocontrol action with the *ech42* gene.

Competition

Effective colonization of given natural surroundings by any life form is subject to its capacity to secure its biological niche and to flourish and develop regardless of competition for nutrients, space and light (Herrera-Estrella and Chet, 2004; Harman, 2006; Vinale *et al*., 2008). In the field a pathogen has to compete with other microorganisms for these resources and this kind of competition provides a basis for disease control. *Trichoderma* species are successful competitors as they are vigorous and develop rapidly in nature and they can survive as chlamydospores when nutrients are scarce, becoming quickly established once conditions improve. A full review of exploitation competition is provided by Lockwood (1981, 1982).

COMPETITION FOR NECROTIC TISSUE. *Trichoderma* spp. that colonize delicate tissues such as leaves are known to control pathogens that attack dead tissues and use this as a base from which they go on into the solid tissues. *Trichoderma* is reported to control *Botrytis* and *Sclerotinia* on strawberry and apple (Tronsmo and Dennis, 1977; Tronsmo and Raa, 1977). Dubos (1987) found that spraying *Trichoderma* spp. on to grape flowers at the time of blossoming resulted in *Trichoderma* colonizing the tissues as they senesced, thereby reducing colonization by *Botrytis* and later disease levels in the fruit. Dubos (1987) explained the importance of administering *Trichoderma* during flowering and that sometimes repeated applications were necessary to ensure its critical spread amid late blossoming.

COMPETITION FOR PLANT EXUDATES. Sometimes, the pathogen's reaction to plant exudates is extremely quick, consequently bringing about disease. As indicated by Nelson (1987) damping-off infection of grains and vegetable seedlings is launched by the fast reaction of the pathogen to the seed exudates. The reaction is rapid to the point that inside a brief time *Pythium* sporangia appear in infected soil and cause disease. As indicated by Ahmad and Baker (1988) seed treated with *Trichoderma* sp. hinders germination of sporangia. Harman and Nelson (1994) expressed that in the above case *Trichoderma* sp. seek germination stimulants. *T. harzianum* (T-35) is reported to control *F. oxysporum* in the rhizosphere of cotton and melon by competition for nutrients (Sivan and Chet, 1989). For this sort of competition, biocontrol agents need to have the capacity to colonize or establish first in the root zone and use root exudates and different nutrients effectively.

COMPETITION ON WOUND SITES. The most defenceless site for pathogen assault is a wound site on a plant. Biocontrol agents when acquainted with such destinations can be extremely useful in controlling a number of pathogens creating infection. Grosclaude *et al*. (1973) and Corke (1974) successfully controlled the pathogen *Chondrostereum purpureum*, the cause of silver leaf in trees, by using *T. viride* in a spray on wound sites created by pruning shears (*T. viride* competing with germinating spores of *C. purpureum* that landed on the freshly exposed sapwood). *Armillaria luteobubalina* (a root pathogen) on freshly cut stumps can be counteracted by *Trichoderma* application (Nelson *et al*., 1995). *T. harzianum* strain T3 is reported to colonize wound sites in cucumber roots and control attack by *Pythium* by competing for nutrients released from the injury.

Wounds resulting from pruning grapevine trunks are one of the important entry points for pathogens causing disease in vascular tissue (Chapuis *et al*., 1998). Grapevine trunk infection, specifically Eutypa dieback, Petri disease, esca, Botryosphaeria canker and Phomopsis dieback instigate moderate grapevine decay, reduce yields, shorten the beneficial life of the vine and increase vineyard administration costs (Creaser and Wicks, 2001). These ailments can conceivably be controlled or anticipated by avoiding contamination or minimizing the impact of the pathogens in the injury tissue. Regular epiphytes are known to likewise colonize grapevine wounds and some restrain wound infection from grapevine trunk pathogens (Munkvold and Marois, 1993). *Trichoderma* species colonize wood tissue and shield grapevine pruning wounds from disease caused by *Eutypa lata* (John *et al*., 2008) and *Phaeomoniella chlamydospora* (Kotze, 2008). Mutawila *et al*. (2011) reported that *T. harzianum* diminished the development of trunk-disease

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pathogens in vaccinated sticks yet did not totally kill them. Be that as it may, it ought to be noted that the pathogen inoculum levels used in this study were much higher (10,000 conidia) than would characteristically be found on a pruning wound in the vineyard. The natural control specialists developed more vigorously than the pathogens in double-vaccinated sticks and there was limited pathogen development which contrasted with single-organism vaccinations. Past studies have reported a reduction in the quantity of shoots contaminated with grapevine trunk-disease pathogens after application of *Trichoderma* species to pruning wounds; however, they did not examine examples of development in the wood (John *et al.*, 2005; Harvey and Hunt, 2006).

COMPETITION FOR NUTRIENTS. Competition for nutrients, principally carbon, nitrogen and iron, may prompt natural control of plant pathogens. In a number of cases, it is observed that nutrients are limited and because of the large number of microorganisms in the soil competing for the nutrients microbial growth is suppressed.

Iron uptake is fundamental for most filamentous growths and during nutrient starvation a large portion of the fungi secrete low subatomic weight, ferric-ion chelating agents called siderophores to assemble natural iron (Eisendle *et al.*, 2004). In *Ustilago maydis* (a fungal pathogen that causes common smut in maize) genes identified with iron uptake have been shown to influence the advancement of plant diseases (McIntyre *et al.*, 2004). *Trichoderma* secretes very productive siderophores that chelate iron and stop the development of other growths (Chet and Inbar, 1994). It is believed that the biocontrol exercised by *Trichoderma* in soil over the viability of *Pythium* is based around accessibility to iron. What is more, *T. harzianum* T-35 controls *F. oxysporum* by both rhizosphere colonization and competing for nutrients, with biocontrol becoming more compelling as the nutrients diminish (Tjamos *et al.*, 1992).

According to Latorre *et al.* (2001) competition has turned out to be especially imperative for the biocontrol of pathogenic organisms, for example *B. cinerea* which is a significant risk causing both pre- and postharvest losses. The higher level of genomic variability of *B. cinerea* makes it possible for new strains to become resistant to essentially any novel chemical fungicide to which it is exposed (Latorre *et al.*, 2001). The advantage of using *Trichoderma* to control *B. cinerea* is the coordination of several mechanisms simultaneously, thus making it practically impossible for resistant strains to appear. *B. cinerea* is particularly sensitive towards nutrition deprivation. Compared with other organisms *Trichoderma* shows a greater capability to mobilize and take up soil nutrients. Efficient use of available nutrients is based on the ability of *Trichoderma* to obtain ATP from the metabolism of different sugars, such as those derived from polymers such as cellulose, glucan and chitin that are widespread in fungal environments, all of them releasing glucose (Chet *et al.*, 1997). The key components of glucose metabolism include assimilation enzymes and permeases, together with proteins involved in membrane and cell wall modifications.

Fungistasis

The majority of soils stifle the germination of organisms to a certain level, by a phenomenon called fungistasis (Dobbs and Hinson, 1953). The power of fungistasis is reliant on the physical and chemical soil properties and in addition soil microbial activity. As indicated by past studies, microorganisms are believed to be the cause of fungistasis, either by limiting the amount of available carbon (nutrient deprivation hypothesis) or by producing antifungal compounds (antibiosis hypothesis). When all is said and done, plant pathogenic growths seem, by all accounts, to be more susceptible than saprophytic organisms (Chalfie *et al.*, 1994; Harman, 2000a). The sensitivity of plant pathogenic fungi to fungistasis is thought to shield them from germination and starting to develop under unfavourable conditions (Harman, 2000a; Lorang *et al.*, 2001). The negative effect of fungistasis on the inoculum density of plant pathogenic fungi has been suggested as a mechanism to explain the commonly found correlation between fungistasis and disease suppressiveness (Hornby, 1983; Larkin *et al.*, 1996). Subsequently, it has been suggested that fungistasis may be manipulatively used as a potential measure to control plant pathogenic growths (Lorang *et al.*, 2001) with the intention of fortifying germination and ensuing drying of the newly developed plant pathogens without a host. A significant number of competitors had

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the capacity to beat the fungistatic impact of soil that results from the vicinity of metabolites created by different species produced under amazing environmental conditions. *Trichoderma* strains become quickly immunized in the soil, in light of the fact that they are commonly impervious to numerous toxic compounds including herbicides, fungicides, insecticides and phenolic compounds (Chet *et al.*, 1997) on the grounds that the strains recuperate quickly after exposure to sub-lethal concentrations of some of these compounds. Imperviousness to lethal compounds may be connected to the ATP-binding cassette (ABC) transport framework in *Trichoderma* strains (Harman *et al.*, 2004). Hence, Vyas and Vyas (1995) recommended replacing fungicides such as captan, methyl bromide or benomyl with preparations of *Trichoderma* strains which were shown to be extremely proficient in controlling a few phytopathogens, for example *R. solani*, *P. ultimum* or *Sclerotium rolfsii*.

3.4 *Trichoderma* as a Source Organism for Useful Genes

Biocontrol microbes must contain a large number of genes that encode products that permit the biocontrol mechanism. A number of genes from *Trichoderma* spp. are cloned and offer great promise as transgenes to produce crops that are resistant to plant diseases. These genes, which are contained in *Trichoderma* spp. and many other beneficial microbes, are the basis of 'natural' organic crop protection and production technology. The processes of mycoparasitism, antibiosis, induced resistance, competition for nutrients and/or space, tolerance to stress through enhanced root or plant development, solubilization and sequestration of inorganic nutrients and inactivation of the pathogen's enzymes have been demonstrated for biocontrol and for enhancement of plant growth. Transgenic expression of high levels of chitinolytic and glucanolytic *Trichoderma* enzymes does not affect plant morphology, development, yield or infection by arbuscular mycorrhizal fungi. Most of these genes have been patented and are commercially available and a number of them are in development to be used in agricultural biotechnology (Lorito, 1998).

3.4.1 Progress in identification of *Trichoderma* genes

Several methods for applying both biocontrol and plant growth promotion exerted by *Trichoderma* strains have recently been demonstrated and it is now clear that hundreds of separate genes and gene products are associated with the processes of competition for nutrients or space, antibiosis, tolerance to stress through enhanced root mycoparasitism, plant development, solubilization, sequestration of inorganic nutrients, inactivation of enzymes produced by pathogens and induced resistance (Monte, 2001). Many of these genes from *Trichoderma* spp. have been identified and cloned (Table 3.2) which offers great potential in the development of genetically transformed *Trichoderma* spp. for traits of importance and will help overcome diseases. However, some of the genes are still unexplored and further work is needed.

Some of the genes that have been identified and isolated are as follows:

- The *tvsp1* gene from *T. virens*, encoding for serine protease which is involved in the degradation of fungal cell walls, was successfully cloned and employed against *R. solani* affecting cotton seedlings and the pathogen was controlled biologically. The gene *tvsp1* was expressed in *Escherichia coli* and cloned in the pET-30 vector (Pozo *et al.*, 2004).
- The trichodiene synthase (*tri5*) gene from *T. harzianum* was found to be responsible for the synthesis of the toxic trichothecene which inhibits protein and DNA synthesis in cells and inhibits the growth of pathogens. Trichothecene shows phytotoxic activity against *Fusarium* species (Gallo *et al.*, 2004).
- The β-tubulin (*β-tub*) gene was isolated and characterized from *T. harzianum*. The structural components of most cells interact with benzimidazole fungicides and play a major role in the biocontrol process. Li and Yang (2007) described the three-dimensional model of the β-tubulin gene as performed by the Swiss-model automated comparative protein modelling server.
- *Sm1* a cysteine-rich protein-coding gene was isolated from *T. virens* (Buensanteai *et al.*, 2010). This gene shows defence activity

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Table 3.2. Progress in identification of *Trichoderma* genes and their activity.

Trichoderma spp.	Biocontrol gene	Function/activity of gene	Effect of biocontrol gene	Reference
Trichoderma harzianum strain IMI206040	Proteinase *prb1* and endochitinase (*ech42* genes)	Parasitic activity against *Sclerotium rolfsii* and *Rhizoctonia solani*	Expression of this gene helps in regulation of hydrolytic enzymes	Cortes *et al*. (1998)
Trichoderma longibrachiatum wild type strain CECT2606	β-1,4-endoglucanase gene, *egl1*	Biocontrol activity against *Pythium ultimum* on cucumber	Shows enhanced biocontrol activity	Migheli *et al*. (1998)
T. harzianum strain P1 74058	*ech*42 gene	Biocontrol activity against *Botrytis cinerea* and *R. solani*	Disruption of this gene affects the biocontrol activity	Woo *et al*. (1999)
Trichoderma atroviride strain P1 (ATCC 74058)	1,3-β-glucosidase gene, *gluc78*	Cell wall degradation of pathogens *Pythium* and *Phytophthora*	Exhibits moderate biocontrol activity	Donzelli *et al*. (2001)
Trichoderma strain SY	Xylanase gene *Xyl*	Helps in breakdown of hemicellulose	Gene isolation	Min *et al*. (2002)
Trichoderma virens strain IMI 304061	*TmkA* mitogen-activated protein kinase (MAPK) gene	Biocontrol activity against pathogens such as *S. rolfsii* and *R. solani*	This gene represses conidial formation in *R. solani*	Mukherjee *et al*. (2003)
T. harzianum strain ATCC 90237	Trichodiene synthase *tri*5 gene	A toxic secondary metabolite responsible for inhibiting DNA or protein synthesis and enhances virulence against *Fusarium* spp.	Increases the virulence against *Fusarium* spp.	Gallo *et al*. (2004)
T. virens strain IMI 304061	*TgaA*, *TgaB* genes	Antagonism against *S. rolfsii* and *R. solani*	Increases virulence in plant pathogenic interactions	Mukherjee *et al*. (2004)
T. virens wild-type strain Gv29-8 and an arginine auxotrophic strain, Tv10.4	*tvsp*1 serine protease-encoding gene	Involved in pathogenesis or biocontrol process of *R. solani*	Exhibits moderate activity against *R. solani*	Pozo *et al*. (2004)
Trichoderma hamatum strain LU593	Chitinase *chit42* and proteinase *prb1* gene	Mycoparasitic activity against *Sclerotinia sclerotiorum*	Exhibits moderate biocontrol activity	Steyaert *et al*. (2004)
T. viride IFO31137	Endo-β-1-6-galactanase gene	A type of arabinogalactan protein that is involved in cell–cell adhesion, expansion and cell death	Expression of gene enhances the production of proteins	Kotake *et al*. (2004)
Trichoderma atroviride strain P1ATCC 74058	*tga1* gene	Chitinase formation and production of antifungal metabolites	Increases the antifungal activity	Reithner *et al*. (2005)
T. virens IMI 304061	*tmkA* gene	Induction of plant systemic resistance and biocontrol activity against *R. solani* (tested under greenhouse conditions)	Shows increased biocontrol activity	Viterbo *et al*. (2005)

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T. harzianum CECT 2413	*ThPTR*2 gene	Di/tripeptide transporter gene, that is involved in mycoparasitic activity against *B. cinerea*	Induces peptide transport that enhances mycoparasitism	Vizcaino *et al*. (2006)
T. harzianum CECT 2413	*erg1* gene	Silencing of the *erg1* gene enhances resistance to terbinafine that shows antifungal activity	Shows enhanced biocontrol activity	Cardoza *et al*. (2006)
T. harzianum Rifai CECT 2413	*qid74* gene	Involved in cell protection and adherence to hydrophobic surfaces that helps in antagonism against *R. solani*	Shows moderate biocontrol activity	Rosado *et al*. (2007)
T. virens Gv29-8	*TvBgn2* and *TvBgn3* genes	These genes help in encoding cell-wall degrading enzymes	Shows enhanced biocontrol activity	Djonovic *et al*. (2007)
T. virens IMI 304061	*tac1*, adenylate cyclase gene	Mycoparasitism against *R. solani*, *S. rolfsii* and *Pythium* spp. and production of secondary metabolites	Shows reduced biocontrol activity	Mukherjee *et al*. (2007)
T. harzianum T88	β-Tubulin gene	Exhibits biocontrol activities like mycoparasitism and antifungal activity	Shows moderate biocontrol activity	Li and Yang (2007)
T. hamatum LU593	Monooxygenase gene	Antagonist activity against *S. sclerotiorum*, *Sclerotinia minor* and *Sclerotinia cepivorum*	Shows enhanced biocontrol activity	Carpenter *et al*. (2008)
T. harzianum E58	*cre1* gene	Production of cellulase and hemicellulase enzymes	Shows enhanced biocontrol activity	Saadia *et al*. (2008)
T. harzianum CECT 2413	T34 *hsp*70	Increases fungal resistance to heat and abiotic stresses	Shows increased biocontrol activity	Montero-Barrientos *et al*. (2008)
T. harzianum (e). (GenBank accession number AJ605116)	*Th-Chit* gene	Antifungal activity in transgenic tobacco	Shows enhanced biocontrol activity	Saiprasad *et al*. (2009)
T. atroviride P1 (ATCC 74058)	*Taabc2* gene	Plays a key role in antagonism against *R. solani*, *P. ultimum* and *B. cinerea*	Shows decreased biocontrol activity	Ruocco *et al*. (2009)
Trichoderma reesei QM9414 (ATCC 26921)	*TrCCD1* gene	Helps in hyphal growth, conidiospore development and carotenoid pigment production	Shows increased biocontrol activity	Zhong *et al*. (2009)
T. harzianum CECT 2413	*Thctf1*transcription factor gene	Antifungal activity against *R. solani*, *Fusarium oxysporum* and *B. cinerea* and production of 6-pentyl-2H-pyran-2-one	Shows enhanced biocontrol activity	Rubio *et al*. (2009)
T. harzianum	Serine protease gene *SL41*	Biocontrol activity against pathogens	Shows enhanced biocontrol activity	Liu *et al*. (2009)

Continued

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Table 3.2. Continued.

Trichoderma spp.	Biocontrol gene	Function/activity of gene	Effect of biocontrol gene	Reference
T. harzianum T34 CECT 2413	Endopolygalacturonase *ThPG1* gene	Secretion of plant cell-wall-degrading enzymes against *R. solani* and *P. ultimum*	Shows enhanced biocontrol activity	Moran-Diez *et al*. (2009)
Trichoderma asperellum (Enzymology Group collection,UFG-ICB)	*tag* 3 gene	Production of cell-wall-degrading enzyme glucanase	Shows significant biocontrol activity	Marcello *et al*. (2010)
T. virens strain TvSMOE38	*Sm1* gene (cysteine-rich protein)	A small cysteine-rich protein that induces defence responses in dicot and monocot plants and in protecting crop diseases	Shows enhanced biocontrol activity	Buensanteai *et al*. (2010)
T. virens (strain IMI 304061)	*TvGST* glutathione transferase gene	Enhances cadmium tolerance in plants	Increases the biocontrol activity	Dixit *et al*. (2011)
Trichoderma brevicompactum IBT40841	*tri*5 gene	Production of trichodermin and antifungal activity against *Candida albicans*, *Candida glabrata* and *Aspergillus fumigatus*	Shows enhanced biocontrol activity	Tijerino *et al*. (2011)
T. harzianum CECT 2413	*Thke11* gene	Expression of this gene in *Arabidopsis thaliana* modulates glucosidase activity and enhances tolerance to salt and osmotic stresses	Enhances the biocontrol activity	Hermosa *et al*. (2011)
T. harzianum CECT 2413	Genes encoding for *N*-acetyl-beta-D-glucosaminidase (*exc*1 and *exc*2), chitinase (*chit42* and *chit33*), protease (*prb1*) and β-glucanase (*bgn13.1*)	Mycoparasitic activity against *F. oxysporum*	Shows enhanced biocontrol activity	Mondejar-Lopez *et al*. (2011)

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against fungal diseases in dicotyledonous and monocotyledonous plants.

- Gene *gluc78*, isolated and characterized from *Trichoderma atroviride*, encodes for an antifungal glucan 1,3-β-glucosidase which plays a significant role in the cell wall degradation of pathogens such as *R. solani* and *P. ultimum* (Donzelli *et al*., 2001).
- A glucose repressor gene *creI* was isolated from *T. harzianum* which causes repression of cellulase and xylanase encoding genes which are the major type of enzymes involved in the cell wall degradation of pathogens (Saadia *et al*., 2008).
- Serine proteases play a key role in fungal biology and are involved in biocontrol activity. A novel serine protease gene named *SL41* from *T. harzianum* was cloned and expressed successfully in *Saccharomyces cerevisiae* (Liu *et al*., 2009).
- A *Trichoderma* strain producing SY xylanase enzyme was isolated from the soil. The *Xyl* gene coding for xylanase was found to be highly expressed in the presence of cellulose as the only source of carbon. The full length cDNA of *Xyl* was amplified and cloned in the pGEM-T vector (Min *et al*., 2002).
- G-protein α subunit genes from *T. virens*, namely *TgaA* and *TgaB*, exhibiting antagonistic activity against pathogens *R. solani* and *S. rolfsii* were cloned and characterized (Mukherjee *et al*., 2004).
- The *T. harzianum*-derived *ThPG1* gene encodes the enzyme endopolygalacturonase which is involved in cell wall degradation of pathogens such as *R. solani* and *P. ultimum* and is also involved in plant beneficial interactions. Moran-Diez *et al*. (2009) performed full length cloning of the *ThPG1* gene using the pSIL-pG1 vector and also examined its phylogenetic relationship by comparing wild-type and mutant strains.
- Galactanase enzymes belong to the family of arabinogalactan proteins that are involved in cell–cell adhesion, cell expansion and cell death. The *Tv6Gal* gene coding for the endo-β-(1→6)-galactanase enzyme from *T. viride* was isolated, cloned and expressed in *E. coli* (Kotake *et al*., 2004).
- Chitinases are one of the cell-wall-degrading proteins that help in antifungal activity. The *Th-Chit* gene isolated from *T. harzianum* confers antifungal activity in transgenic tobacco plants against *Alternaria alternata* (Saiprasad *et al*., 2009).
- *Trichoderma brevicompactum* encodes *tri5*, a trichodiene synthase gene. The overexpression of the *tri5* gene increases the production of trichodermin which shows antifungal activity against *S. cerevisiae*, *Kluyveromyces marxianus*, *Aspergillus fumigatus*, *Candida glabrata*, *Candida albicans* and *Candida tropicalis*. Tijerino *et al*. (2011) reported the cloning of gene *tri5* in pURSPT5 and transformed into *T. brevicompactum*.
- The *erg1* gene from *T. harzianum* encodes the enzyme squalene epoxidase, which helps in the synthesis of ergosterol. Silencing this gene provides resistance to terbinafine, an antifungal compound. The antifungal activity was checked with *S. cerevisiae*. This is the first terpene biosynthesis gene cloned and characterized from the genus *Trichoderma* (Cardoza *et al*., 2006).
- A transcription factor gene *Thctf1* isolated from *T. harzianum* showed antifungal activity against pathogens such as *R. solani*, *B. cinerea*, *F. oxysporum* and *S. rolfsii* and was involved in the production of 6-pentyl-2H-pyran-2-one (Rubio *et al*., 2009).
- The *T. atroviride* G-protein α-subunit gene (*tga1*) is involved in production of chitinases and antifungal metabolites. Chitinases are proteins involved in cell wall degradation. The *tga1* gene was cloned in the pGEM-T vector and characterized. The antifungal activity was examined using a dual culture technique by plating wild-type and mutant Δtga1 strains of *T. atroviride* against plant pathogens *R. solani*, *B. cinerea* and *S. sclerotiorum*. According to Reithner *et al*. (2005) the antifungal activity between the wild-type and mutant strains were affected by altering the *tga1* gene. A mono-oxygenase gene is involved in antifungal activity against *Sclerotinia* spp. Its expression is influenced by physical contact between the two fungi.
- Gene *ech42* from *T. harzianum* encodes for endochitinase. Disruption of the *ech42* gene affects the biocontrol activity of the fungus. Antifungal activity against pathogens *B. cinerea* and *R. solani* with the wild-type and mutant (disrupted) strains was tested (Woo *et al*., 1999).

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- Six genes coding for *N*-acetyl-beta-D-glucosaminidase (*exc1* and *exc2*), chitinase (*chit42* and *chit33*), protease (*prb1*) and β-glucanase (*bgn*) from five different isolates of *T. harzianum* were isolated, cloned and expressed (Mondejar-Lopez *et al.*, 2011). All of these genes play important roles in mycoparasitism activity against pathogens, especially *F. oxysporum*.

According to Montero-Barrientos *et al.* (2008) *Trichoderma* spp. can help plants survive in abiotic stress conditions. An example is a glutathione transferase gene (*TvGST*) from *T. virens* that was cloned, characterized and expressed in a transgenic plant (Dixit *et al.*, 2011). When the transgenic plant expresses this gene against different concentrations of cadmium, it shows tolerance to cadmium accumulation in the plants and thus it acts as a cadmium tolerant gene (Dixit *et al.*, 2011). Another example is the *hsp70* gene from *T. harzianum* T-34 isolate which was cloned, expressed and characterized and it was found to help in increasing the fungal resistance to heat and it also increases salt tolerance, oxidative and osmotic tolerances (Montero-Barrientos *et al.*, 2008). Similarly the *Thkel1* gene from *T. harzianum* encodes for the putative kelch repeat protein which helps in directing the glucosidase action and improves resilience to salt and osmotic stresses in *Arabidopsis thaliana* plants (Hermosa *et al.*, 2011).

3.5 Commercial Utilization

Chemicals produced by *Trichoderma* spp. are generally acknowledged across the board for their various modern commercial applications particularly in the textile industry (Cavaco-Paulo and Gubitz, 2003), the pulp, paper, food and feed industries (Galante *et al.*, 1998) and in agriculture.

3.5.1 *Trichoderma* in the textile industry

Trichoderma reesei is one of the best cellulase-creating filamentous growths for the corruption of crystalline cellulose and is utilized as a modern host to generate heterologous and homologous chemicals (Penttila *et al.*, 2004). In the textile industry cellulase enzymes are widely used for biofinishing cotton and in particular to treat fabrics such as denim to give it an aged 'stone-washed' look. In the past this was achieved using a pumice stone-washing process but this abrasive process has almost entirely been replaced by the enzymatic process of biostoning in which cellulases digest cellulose, the main component of cotton and other natural plant fibres. Biostoning is a more economical and environmentally friendly way to treat denim. The gene for the cellulose enzyme was isolated from *T. reesei* and put into bacteria for mass production. About 10% of textile finishing of cellulose material is estimated to be performed by cellulases and approximately 80% of the 1.8 million pairs of jeans produced annually are finished with cellulases as an alternative to pumice stones (Buchert and Heikinheimo, 1998).

Cellulases may also have potential use in production of synthetic textiles such as viscose, which is made from cellulose that is processed into textile fibres. In the first stage of the process cellulose is changed into an alkali-soluble form using carbon disulfide – a stage that carries environmental risk. Rahkamo *et al.* (1996) found that use of carbon disulfide could be avoided by using purified *T. reesei* cellulases which dissolved the hardwood dissolving pulp (the raw material for the viscose process) increasing its solubility.

3.5.2 *Trichoderma* in the pulp and paper industry

There has been considerable research into potential applications of crude enzyme preparations from *Trichoderma* spp. in the pulp and paper industry. Cellulases have potential use in the pulp and paper industry, for example in de-inking to discharge ink from fibre surfaces, treatment of paper and pulp mill effluent and in biobleaching wastepaper pulp (Suurnakki *et al.*, 2004). Crude preparations of cellulase and xylanase from *T. harzianum* have been shown to have potential use in deinking photocopier waste paper which is needed when such paper is recycled (Pathak *et al.*, 2014). In the timber industry cellulases have been indicated

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to be successful in reducing the energy used for mechanical pulping (Pere *et al.*, 2002).

3.5.3 *Trichoderma* in the food and livestock feed industries

Applications from *Trichoderma* spp. are widely used in the food industry. Enzymes such as pectinases, cellulases and hemicellulases from *Trichoderma* are used for the production of fruit juices, β-glucanases and cellulases are used in brewing and xylanases are used as feed additives for livestock and pet foods (Galante *et al.*, 1998). Cellulases are used together with different hydrolases to degrade anti-nutritional factors such as non-starch polysaccharides to enhance nutritional transformation rates and boost the nutritional value of animal feeds (Galante *et al.*, 1998). The food industry utilizes cellulases together with other plant-cell-wall degrading enzymes in the preparation of fruit and vegetable juices (Urlaub, 2002). Enzymatic hydrolysis of biomass to sugars for ethanol production has also been an area of significant research in recent years.

3.5.4 Use of *Trichoderma* in agriculture and impacts on plants

Trichoderma spp. are found naturally in soils of nearly all types and in other habitats. However, for agricultural purposes (e.g. for biocontrol of pathogens or to promote plant growth) they may be added as a seed treatment or directly to the soil. If applied as a seed treatment the best strains readily colonize the roots of the crop and will persist for up to 18 months after application (Harman, 2000b). Different strains of *Trichoderma* are used as biocontrol agents to control a number of pathogenic fungi, although some strains are more effective than others. Several reputable companies manufacture products which, for example in the USA, are legally registered for control of specific pathogens (Harman, 2000b). *Trichoderma* may also be applied to improve the rate of plant growth, in particular to increase development of a deep root system which can help plants such as maize, turfgrass and ornamentals to tolerate drought (Harman, 2000b).

Biocontrol agents are known to deliver an extensive variety of consequences for plants. *Trichoderma* spp. used as biocontrol agents are known to control infection by pathogens through, for example, mycoparasitism or by the creation of antimicrobial compounds. Notwithstanding this, *Trichoderma* is also known to advance or repress plant development (Wright, 1956; Lindsey and Baker, 1967). Diverse types of *Trichoderma* exist in the rhizosphere and infiltrate plant roots, colonizing the epidermis and a few cells below (Harman *et al.*, 2004). This opportunistic, facultative beneficial interaction between fungus and plant is determined by the capacity of *Trichoderma* to detect sucrose or different nutrients from the plants, in exchange for boosting plant resistance against attacking pathogens and enhancing photosynthetic capacity (Vargas *et al.*, 2011). The proximity of *Trichoderma* spp. in the rhizosphere summons a composed transcriptomic, proteomic and metabolomic reaction in the plant (Shoresh and Harman, 2008). This response by the plant is frequently gainful, enhancing root growth and development, crop yield and resistance to pathogens.

Colonization of the plant root

Colonization infers the capacity to follow and perceive plant roots, enter the plant and withstand lethal metabolites delivered by the plants in response to attack by a remote organic entity. *Trichoderma* strains must colonize plant roots before the plant responds by producing antimicrobial compounds as a defence mechanism against microbial attack (Harman *et al.*, 2004). *Trichoderma* spp. can build enduring colonizations on the surface of plant roots and also enter into the epidermis where plants secrete antimicrobial compounds such as phytoalexins, phenolic compounds, terpenoids, flavonoids and glycones as part of systemic plant reactions against fungal pathogens.

Trichoderma strains have demonstrated greater endurance towards these compounds than other fungi, and their capacity to colonize plant roots unequivocally relies on this ability, which is related to the ABC transport framework in *Trichoderma* spp. Both plant and fungus release

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signal compounds and the interaction between these signals from both partners regulate hormonal signals that encourage root colonization.

Contreras-Cornejo *et al.* (2009) studied the interaction between *T. virens* and *Arabidopsis*. They found that the fungus enhanced biomass production and promoted lateral root growth through an auxin-dependent mechanism. Using a gene knockout technique, Viterbo *et al.* (2010) reported on the part played by ACC (1-aminocyclopropane-1-carboxylate) deaminase in regulation of canola (*Brassica napus*) root growth promotion by *Trichoderma asperellum*. In any case, *Trichoderma* secretes small cysteine-rich hydrophobin-like proteins to encourage connection. Two proteins, TasHyd1 and Qid74, secreted by *T. asperellum* and *T. harzianum*, respectively, encourage connection to the roots (Viterbo and Chet, 2006; Samolski *et al.*, 2012). Entrance into the root is encouraged by secretary expansin-like proteins with cellulose-binding modules and endo-polygalacturonase (Brotman *et al.*, 2008; Moran-Diez *et al.*, 2009). Once infiltration occurs in the root, the fungus begins to grow and develop intercellularly, albeit being restricted to the epidermal layer and a few cells of the cortex immediately below the epidermis. *T. koningii* has been found to suppress phytoalexin production during colonization of *Lotus japonicus* roots (Masunaka *et al.*, 2011).

Trichoderma *as a biofertilizer*

The addition of biofertilizers to soil can improve the retention of nutrients in plants and may encourage soil richness and increased harvest yields. Root colonization by *Trichoderma* strains has a beneficial effect on plants by improving resistance to abiotic stress and uptake of nutrients, as well as increasing root development. Crop productivity in fields can increase up to 300% after the addition of *Trichoderma hamatum* or *T. koningii* as biofertilizer. However, there are very few reports on strains that produce growth factors which have been detected and identified in the laboratory (auxins, cytokinins and ethylene) (Arora *et al.*, 1992). An increase in seed germination was observed when seeds were divided from *Trichoderma* strains by a cellophane film, which demonstrates that *Trichoderma* strains secrete plant-development-advancing elements (Benitez *et al.*, 1998). *Trichoderma* strains deliver cytokinin-like compounds (e.g. zeatin) and gibberellins (e.g. gibberellin A3 (GA3) or a GA3-related compound) which could enhance biofertilization (Osiewacz, 2002).

Trichoderma *advances plant development*

According to Chang *et al.* (1986) improved germination, a fast rate of blooming and increased height and weight of specific plants, namely pepper and chrysanthemums, were observed when soil was treated with conidial suspensions of *T. harzianum*. Shivanna *et al.* (1994, 1996) recorded increased development of wheat and soybean under nursery conditions when treated with *Penicillium* and *Trichoderma*. They further expressed that the reaction varied when the same was attempted in field conditions yet increases in yield were perceived at times. *T. harzianum* strain T-203 enters the roots and acts like a mycorrhizal growth, advancing development (Kleifield and Chet, 1992). *Trichoderma* spp. are known to control minor pathogens such as *Pythium* sp. (Ahmad and Baker, 1988) and in this roundabout way advance development.

Trichoderma *induces plant resistance to pathogens*

The capacity of *Trichoderma* strains to protect plants against attack by root pathogens has long been credited to a hostile impact against the intrusive pathogen (Chet *et al.*, 1997). Colonization of the rhizosphere by *Trichoderma* strains protect plants against various classes of pathogens (viral, bacterial and fungal pathogens) that cause diseases, which indicates the incitement of responses that are similar to plant resistance mechanisms, namely the hypersensitive response (HR), systemic acquired resistance (SAR) and induced systemic resistance (ISR) (Harman *et al.*, 2004).

Activation of plant resistance mechanisms do not necessarily need the presence of the live form of the pathogen as plant genes react to elicitors. *Trichoderma* elicitors might be metabolites that may bring about a combination of phytoalexins, pathogenesis-related (PR) proteins and other compounds produced by the plant which increase protection against a few plant pathogens including microorganisms (Elad *et al.*, 2000; Dana *et al.*, 2001) and resistance to adverse

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abiotic conditions. Yedida *et al.* (2000) showed that inoculation of cucumber roots with *T. harzianum* (strain T-203) resulted in the production of PR proteins, including various hydrolytic chemicals. Plants react promptly to *Trichoderma* intrusion by rapid particle fluxes and an oxidative burst, subsequently demonstrated by the presence of a combination of callose and polyphenols (Shoresh *et al.*, 2010). These events include salicylic acid (SA) and jasmonic acid/ethylene (JA/ET) signalling, resulting in the whole plant gaining varying degrees of resistance to pathogen assault. This reaction is most ordinarily known as JA/ET-mediated ISR and takes after the reaction activated by PGPR. According to recent discoveries (Yoshioka *et al.*, 2012), larger inoculums of *Trichoderma* may enact an SA-mediated SAR reaction, looking like to that summoned by necrotrophic pathogens. The signalling events prompt ISR. An atomic cross-talk between plant (cucumber) and *T. virens* might evidently trigger a downstream guard reaction by ramifications of MAPK (mitogen-activated protein kinase) (Viterbo *et al.*, 2005).

Some newly discovered *Trichoderma* spp., namely *Trichoderma stromaticum*, *Trichoderma amazonicum* and *Trichoderma evansii*, appear to have an endophytic relationship with plants and are not involved with the soil/rhizosphere, and phylogenetic investigation has revealed that these species have recently evolved (Mukherjee *et al.*, 2012). Endophytic *Trichoderma* species are reported to prompt transcriptomic changes in plants and some are known to shield plants from diseases and abiotic stresses (Bailey *et al.*, 2006; Bae *et al.*, 2009). Such endophytes specifically colonize the surface of glandular trichomes and produce appressoria-like structures (Bailey *et al.*, 2009). This is one example where *Trichoderma* utilizes a 'non-root' mode of passage into the plant.

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4 The Role of *Bacillus* Bacterium in Formation of Plant Defence: Mechanism and Reaction

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Abstract
This review analyses data on the physiological and biochemical influence of rhizospheric and endophytic plant growth-promoting rhizobacteria (PGPR) from the genus *Bacillus* on mechanisms of resistance of plants and considers the possibility of their use in agriculture for protecting crops against pathogens and pests. Published results showed that some strains of *Bacillus* spp. elicit significant reduction of incidence or severity of various plant diseases. PGPR-promoted defence of plants develops as a result of their endosymbiotic interrelationships. This mechanism is directly achieved by the bacteria producing peptide antibiotics and hydrolases of chitin and glucan and also because the plants form their own system of induced systemic resistance (ISR), accompanied by changes in the balance of defensive proteins, hormones and pro-/antioxidant status.

4.1 Bacilli Preparations in Plant Growing and Defence

4.1.1 Biofungicides: a short classification and general biological activity

All plants face infections and diseases following attack by a mass of phytopathogens and pests of animal, microbial and viral origin. Plant diseases are responsible for the loss of at least 10% of global food production, representing a threat to food security. For example, potato blight caused by the pathogenic oomycete *Phytophthora infestans* on potato resulted in more than 1 million deaths in Ireland during 'the Great Famine' between 1845 and 1849 (O'Neill, 2009).

The most essential factor for obtaining the highest yield in all agricultural systems is crop protection from pests and diseases. It includes applying economic, selection and agrotechnical measures, application of chemical pesticides and biopreparation. Pest management strategies have evolved from simple empirical procedures to science-based complex events, such as seed treatment or crop spraying. Pesticides are extensively used in intensive agriculture to control pests, diseases, weeds and other crop enemies to reduce yield losses and maintain product quality. Modern preparations created for crop protection can be divided into three groups, which will be discussed in the following three paragraphs.

The first group includes chemicals with a high biocidal effect that kill target 'hazardous' organisms. The efficiency of their use is high. However, they also kill 'beneficial' species of biocenoses, and this is their main disadvantage.

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In addition, they are only partially utilized and their residues accumulate in foods. Although systemic pesticides used against plant diseases are less toxic and they are rapidly utilized in plants, their use entails financial difficulties because they are expensive and pathogens become resistant to them over time. This forces us to search for new chemicals to protect plants permanently (Wisniewski *et al.*, 2007).

Low-molecular substances capable of stimulating the immune potential of plants belong to the second group. By one method of classification they can be divided into the following types: (i) substances that improve the resistance of cell walls to pathogenic attack by storing silicon and lignin in infected tissues; (ii) substances that activate phenolic metabolism; (iii) substances that induce the synthesis of phytoalexins; (iv) substances that provoke plant sensitization (i.e. that prepare plants for pathogenic attack); and (v) substances that boost the susceptibility of fungus cells to external actions, in particular to plant hydrolases.

Biopesticides including living cultures of bacteria or fungi as an active ingredient belong to the third group (Ongena and Jacques, 2008; Saunders and Kohn, 2009; Van der Lelie *et al.*, 2009; Kumar *et al.*, 2012). Their protective effect is based on the ability of microorganisms to produce: (i) antibiotic compounds of various kinds; (ii) siderophores and chelators that promote assimilation of macro- and microelements in plants, such as calcium, iron or, alternatively, isolate heavy metals and toxic substances; (iii) substances that convert insoluble phosphorus into mobile forms and also stimulate the viability of nitrogen-fixing bacteria; (iv) enzymes that can degrade cell walls of pathogens (chitinases, β-1,3-glucanases) or phytotoxins; (v) plant growth regulators and various signalling molecules (auxins, gibberellins, cytokinins, abscisic acid (ABA), salicylates and jasmonates); and (vi) enzymes that stimulate ethylene synthesis in plants. As of early 2013 there were approximately 400 registered active ingredients of biopesticides and more than 1250 biopesticide products (USEPA, 2013).

The principle of the functional action of biopesticides of the second and third groups differs from the action of chemicals because biological agents are aimed at control on the pathogens' level, formation of competitive relations with indigenous pathogenic bacteria and fungi, and induction of systemic resistance. The majority of them act as triggers of the cascade of defence reactions in plants. Many known modern biopesticides combine all the above-mentioned features as a rule. Under the influence of plant growth-promoting rhizobacteria (PGPR) and elicitors, plants activate their own defence mechanisms called induced systemic resistance (ISR) and systemic acquired resistance (SAR) (Van Loon, 2007; Barruiso *et al.*, 2008; Conn *et al.*, 2008).

Even in the early days of research in this area it was shown that some rhizosphere bacterial strains can stimulate plant growth and, at the same time, can protect plants from both biotic and abiotic stresses. These research studies have been expanded into a whole range of work, including reviews dedicated to multifunctional PGPR (Compant *et al.*, 2005; Berg, 2009; Wu *et al.*, 2009; Kumar *et al.*, 2012; Santoyo *et al.*, 2012).

The justifications of using microbial agents as biopesticides is based on: (i) their considerable role within the microbial population of the plant rhizosphere; (ii) their antagonism to many pathogens; (iii) their ability to synthesize physiologically active substances defining their practicality; (iv) their long-term efficiency that remains even over a postharvest period; and (v) the low concentrations required in preparations for direct plant (seed) treatment (Wisniewski *et al.*, 2007; Wang *et al.*, 2010; Maksimov *et al.*, 2011a; Yanez-Mendizabal *et al.*, 2012; Sangwanich *et al.*, 2013). Moreover, it is preferable that biopreparation agents:

- do not affect the growth media;
- are genetically stable;
- are able to survive in unfavourable conditions;
- are prepared in forms that are convenient for their storage and application;
- produce no secondary metabolites that are harmful to humans and animals;
- are stable with regards to the action of chemical pesticides; and
- are non-pathogenic for plants.

Ecological compatibility of PGPR-preparations favours their active introduction into agricultural practice. On the one hand, both producers and customers are interested in green production. This is encouraged both by the population buying food and by state subsidies. On the other

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hand, plant breeders are concerned about significantly reducing the concentration of chemicals used in the environment and in the application of an integrated system of crop protection, in which chemical and biological methods are combined.

Biopesticides have one great disadvantage: they are ineffective in the case of epiphytotics. For effective application of PGPR, it is necessary to take into account the possible economic injury level under which their efficiency becomes inexpedient and at which it is necessary to combine their use with chemical preparation measures. For this reason, it is recommended that they are included as a cultivated preparation only when seeds are lightly infected with root rot agents. In periods of high infection load they should be alternated with a chemical preparation. Weaknesses of microbial biopreparations, compared with chemicals, are: (i) their instability; (ii) their relatively slow onset, which is determined by the need of bacteria to consolidate inside the structure of plant tissues to adapt to new conditions; and (iii) their high specificity both to individual pathogen species and to individual host plants.

Unfortunately, there is still no complete description of the mechanisms of PGPR on the defence system of plants, especially in relation to the endophytic strain of *Bacillus* spp. There are no unique model strains of PGPR capable of defending plants against different forms of pathogens and to serve as a model for studies of plant defence mechanisms. The protective effects of bacteria on plants may exhibit different intensities depending on the bacterial cell concentration. Under adverse environmental conditions, PGPR do not form effective associations with plants. Also the biopreparation may lose efficiency in storage and when used in combination with pesticides. These features narrow the usefulness of the action of PGPR from *Bacillus* spp. to both specific crops and to specific pathogens or pests.

Thus, according to Barruiso *et al.* (2008), no more than 10% of the total number of rhizospheric bacteria species that live in soil have high reproductive activity *in vitro*. This requires being more accurate while creating regulations for production of microbiological specimens, making mixtures out of them, and carrying out selection of strains while taking into consideration the high specificity to this or that culture or even breed. It should be especially emphasized that it is necessary to consider effectors of the defensive system in plants, secreted by microorganisms that can have a negative influence on plant resistance.

4.1.2 Bacilli biofungicides and their activity

PGPR, including *Bacillus* spp., can be divided into three groups (Kumar *et al.*, 2012). The first group includes free-living microorganisms that specifically interact with plants under favourable conditions. The second group is rhizospheric and phylospheric species localized in soil zones adjacent to roots or on the epidermal surface of plant leaves that can hardly exist without a host. The third group is bacteria that are able to form stable associations with certain tissues and organs of plants by penetrating into them through the intercellular space (endophytes). Many representatives of the third PGPR group cannot exist for long without a host and lose their 'useful' properties.

One of the most attractive objects for industrial (commercial) production of biopesticides, including those that are actively used in farming practice, is *Bacillus* spp. strains (Jetiyanon and Kloepper, 2002; Chakraborty *et al.*, 2006; Siddiqui, 2006). Some strains of *Bacillus* spp. are capable of inactivating mycotoxins, have biocide activity against plant bacterial and fungal pathogens, can induce the plant defence system, and are used both as effective probiotics and as an alternative to pesticides. The endophytic property of some *Bacillus* strains allows them to escape from the competitive pressure of other species of saprotrophic rhizobacteria because they occupy the intercellular space of plant tissues. Indeed, several commercial products based on various species of *Bacillus* (e.g. *Bacillus amyloliquefaciens*, *Bacillus licheniformis*, *Bacillus pumilus* and *Bacillus subtilis*) have been marketed as biofungicides (Table 4.1).

Recently *Bacillus* (*Brevibacillus*) *laterosporus* BPM3 with highly insecticidal activity has been shown to exert antagonistic activity towards phytopathogenic fungi, such as *Fusarium oxysporium* f. sp. *cicero*, *Fusarium semitectum*, *Magnaporthe grisea* and *Rhizoctonia oryzae* and Gram-positive

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Table 4.1. Strains of *Bacillus* spp. used as biopesticides for biological control of plant disease.

Biocontrol organism	Product name	Disease	Company
Bacillus subtilis var. *amyloliquefaciens*, FZB-24	FZB-24, RhizoPlus, RhizoVital®42 Taegro	Root rot and wilts (*Alternaria*, *Botrytis cinerea*, *Curvularia radicola*, *Curvularia inaequalis*, *Corynebacterium michiganense*, *Erwinia carotovora*, *Fusarium avenaceum*, *Fusarium culmorum*, *Fusarium oxysporum*, *Gaeumannomyces graminis*, *Gerlachia niveale*, *Phoma chrysanthemi*, *Phomopsis sclerotioides*, *Pyrenochaeta lycopersici*, *Pythium ultimum*, *Rhizoctonia solani*, *Sclerotinia sclerotiorum*, *Stromatinia freesia*, *Verticillium* spp.)	AbiTep GmbH (Germany) Novozyme (Denmark), Earth Biosciences Inc. (USA)
Bacillus subtilis, GB03	Kodiak, GUS 2000	*Rhizoctonia* and *Fusarium* spp.	Gustafson (USA), Bayer CropScience (EEC)
B. subtilis, GB03 and other strains (*B. subtilis*, *Bacillus licheniformis* and *Bacillus megaterium*)	Companion	Root rots (*Aspergillus*, *Golovinomyces cichoracearum*, *F. oxysporum*, *Fusarium nivale*, *Magnaporthe poae*, *Phytophthora*, *Pythium*, *R. solani*, *Sclerospora graminicola*, *Sclerotinia minor*), leaf spot (*Alternaria*, *B. cinerea*, *Colletotrichum orbicular*, *Didymella bryoniae*, *E. carotovora*, *Erwinia tracheiphila*, *Plasmodiophora brassicae*, *Podosphaera xanthi*, *Pseudomonas syringae*, *Xanthomonas campestris*)	Growth Products Ltd (USA)
B. subtilis, GB03 + *B. amyloliquefaciens*, IN937a	BioYield®	Seedling pathogen	Gustafson (USA)
B. subtilis, GB03 and chemical pesticides (metalaxile and PCNB)	System 3	Seedling pathogen	Helena Chemical Co. (USA)
B. subtilis, MBI 600	Subtilex, INTEGRAL, HiStock N/T	*Fusarium* spp., *Rhizoctonia* spp., *Aspergillus*	Becker Underwood (USA)
B. subtilis, 26D	Phytosporin-M	Rots, leaf diseases	BashInkom, (Russia)
B. subtilis, IPM-215	Bactofit	Root rot, *Oidium*, *Septoria*, *Fusarium*, *Pyrenophora*, rust, buckeye rot, bacterial blights	Sibbiopharm (Russia)
B. subtilis, 63-Z	Bacsis	Bare patch, late blight disease, rooteater, phoma rot, early blight, powdery mildew, root rots, bacteriosis, false mildew	Bioformatex (Russia)
B. subtilis, V-10	Alirin_B	Black stem, root rots, fading, late blight disease, powdery mildew, grey rot	Agrobiotechnology (Russia)
B. subtilis, M-22	Gamair		
B. subtilis, CH-13	BisolbiSan	Root rots caused by *Fusarium* and *Helminthosporium*	BisolbiInter (Russia)

Continued

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Table 4.1. Continued.

Biocontrol organism	Product name	Disease	Company
B. subtilis, QST713	Serenade, Serenade Garden Rapsody	Anthracnose (*Colletotrichum* spp.), bacterial leaf spot (*Erwinia*, *Pseudomonas*, *Xanthomonas*), leaf spot (*Cercospora*, *Entomosporium*, *Helminthosporium*, *Myrothecium*, *Septoria*, *Diplocarpon rosea*), grey mould (*B. cinerea*), downy mildew (*Peronospora* spp.), early blight (*Alternaria*), powdery mildew (*Erysiphe*, *Oidium*, *Podosphaera*, *Sphaerotheca*), rust (*Puccinia*), scab (*Venturia inaequalis*), root rot (*R. solani*, *Pythium*, *Fusarium*, *Phytophthora*), dollar spot (*Sclerotinia homeocarpa*), rice blast (*Pyricularia grisea*), soil-borne diseases (*Rhizoctonia*, *Pythium*, *Fusarium* and *Phytophthora*)	AgraQuest Inc. (USA) Bayer CropScience (Germany)
B. subtilis, B246	Avogreen	*Colletotrichum gloeosporioides* and Cercospora spot	Ocean Agriculture (South Africa)
B. subtilis, DB101	Shelter	Botrytis rot, powdery mildew	Dagutat Biolab (South Africa)
B. subtilis, DB102	Artemis	Botrytis rot, powdery mildew	Dagutat Biolab (South Africa)
B. subtilis	Bacillus SPP	*Pseudomonas syringae* pv. *syringae*, *Xanthomonas campestris* pv. *vesicatoria* and *Clavibacter michiganensis* subsp. *michiganensis*	Bio Insumos Nativa Ltd (Chile)
B. subtilis, CFU	Biosubtilin	*Fusarium*, *Verticillium*, *Pythium*, *Cercospora*, *Colletotrichum*, *Alternaria*, *Ascochyta*, *Macrophomina*, *Myrothecium*, *Ramularia*, *Xanthomonas* and *Erysiphe polygoni*	Biotech International Ltd (India)
B. subtilis, QST 713	Cease	*Rhizoctonia*, *Pythium*, *Fusarium*, *Phytophthora*, *Botrytis*, *Erwinia*, *Xanthomonas*	BioWorks Inc. (USA)
B. subtilis, D-747	Ecoshot	*B. cinerea*	Kumiai Chemical Industry (Japan)
B. velezensis, BCRC 14193	Botrybel	*B. cinerea*	Agriclades (Spain)
B. licheniformis, SB3086	EcoGuard	*Rhizoctonia*, *Pythium*, *Fusarium* and *Phytophthora*	Novozyme (Denmark)
B. licheniformis, SB3086	EcoGuard™	Anthracnose (*Colletotrichum graminicola*) and dollar spot (*Sclerotinia homeocarpa*)	Novozymes A/S (Denmark), Novozymes Biologicals (USA)
B. amyloliquefaciens	RhizoVital®42	Soil-borne pathogens	ABiTEP GmbH, (Germany)
Bacillus pumilus, GB34	YieldShield	Soil fungal pathogens	Gustafson, (USA), Bayer CropScience (Germany)

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B. pumilus, QST 2808	Ballad plus Sonata	Root rot (*Rhizoctonia oryzae*), rust (*Puccinia* spp., *Uromyces betae*, *Puccinia sorghi*), rice blast (*Pyricularia oryzae*), powdery mildew (*Peronospora manshurica*, *Erysiphe graminis*, *Erysiphe betae*, *Erysiphe polygoni*), leaf spot (*Cercospora beticola*, *Entyloma*, *Dreschlera*, *Exserohilum turcicum*, *Helminthosporium*, *Bipolaris maydis*, *Cochliobolus heterostrophus*, *Ceratobasidium*, *Ramularia*), bacterial spot (*Xanthomonas* spp.), Asian soybean rust (*Phakopsora pachyrhizi*), brown spot (*Septoria glycines*), white mould (*Sclerotinia sclerotiorum*) pathogens	AgraQuest Inc. (USA) Bayer CropScience (Germany)
Bacillus nigrum, 132	Bactril, SP	Seed mould, Helminthosporium root rot, Fusarium root rot, powdery mildew	Bioformatek (Russia)
B. subtilis, MBI600 and *Bradyrhizobium japonicum*	HiStick N/T, Sublitex, Pro-Mix	Root rot and seed treatments (*Fusarium*, *R. solani*, *Aspergillus*, *Pythium* and *Alternaria*)	Becker Underwood (USA), Premier Horticulture Inc. (Canada)

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bacterium *Staphylococcus aureus*. In greenhouse conditions *B. laterosporus* was used to control rice blast disease, caused by *M. grisea*, and was able to reduce disease incidence by 30–67% (Saikia *et al.*, 2011).

Seventy-six strains of *B. subtilis* were derived from healthy cotton plants (Reva *et al.*, 2002). Some strains decreased the degree of colonization of cotton roots by *Fusarium* fungi and, accordingly, reduced symptoms of wilt on plants (Reva *et al.*, 2002). On the basis of a strain derived from healthy cotton plants strain *B. subtilis* 26D was developed as a commercial preparation named Phytosporin-M (see Table 4.1). It is effective against moulds and decay of various crop seeds, black stem, late blight disease and black spot of potato. From healthy wheat tissues, three *Bacillus* spp. strains and several fungal species were derived (Larran *et al.*, 2002). From leaves and roots of sugarbeet, Chinese investigators derived 221 bacterial isolates, 34 fungal isolates and five actinorhizal isolates (Shi *et al.*, 2009). On the basis of the Australian A-13 line of *B. subtilis*, a new prospective GB03 line was gained (see Table 4.1). It was registered by Uniroyal Agricultural Chemical company (USA) in 1985 and is now used as an important component of biopreparation Kodiak (Gustafson, USA; Bayer CropScience, EEC), Companion (Growth Products Ltd, USA), BioYield® (Gustafson, USA) and System 3 (Helena Chemical Co., USA) (Table 4.1). This line is an effective antagonist of *Rhizoctonia solani* and *F. oxysporium* sp. *vasinfectum* on cotton and groundnut crops. The *B. subtilis* GB07 strain proved effective against *Pythium* spp. pathogens on cotton and the *B. subtilis* FZB-24 strain is highly protective against *F. oxysporum* Schlecht, *Streptomyces scabies* and *Erwinia carotovora* ssp. *atroseptica* on asparagus and potato plants (Kilan *et al.*, 2000). Batsispecin BM made on the basis of *B. subtilis* strain 739 was used to depress the development of root rots and aerogenic infections (stripe rust, brown rust and stem rust) on wheat crops, where its efficiency was no worse than chemical pesticides (Melent'ev, 2007). From the rhizospheric soil of a banana plant the promising strain of *B. subtilis* B106 was isolated. It is used as a biocontrol agent against banana diseases caused by *Pseudocercospora musae* and *Colletotrichum musae* (Fu *et al.*, 2010). The strain *B. amyloliquefaciens* BNM 340 protected soybean plants against damping-off caused by *Pythium ultimum* and was able to increase the seedling emergence rate after inoculation of seeds (Leon *et al.*, 2009). This strain inhibited mycelial growth of *Botrytis cinerea* and *Sclerotinia sclerotorium in vitro* and caused the systemic protection against both of these fungi on leaves from *Brassica napus* seedlings (Simonetti *et al.*, 2012). The strain *B. subtilis* EM7 was evaluated as an effective biocontrol agent for *S. sclerotorium* on oilseed rape (Gao *et al.*, 2014).

Application of a preparation of Bactofit (based on *B. subtilis* IPM-215) decreased the development of root rots on winter wheat by four times and infection with powdery mildew was reduced by ten times (Melent'ev, 2007). Treatment with strains of *Bacillus cereus* AR156 resulted in growth promotion in tomato plants and antagonism against bacterial wilt caused by *Ralstonia solanacearum* and root-knot disease caused by the plant parasitic nematode *Meloidogyne incognita* (Wei *et al.*, 2010). These strains were also successful at reducing the incidence of bacterial speck disease caused by *Pseudomonas syringae* pv. tomato DC3000 (Niu *et al.*, 2012). Treatment of wheat seeds with biopreparations reduced infection with root rot agents by 20.9–51.2% and that with agents of leafy infections by 24.3–63.5%. Safekeeping of stored crops, for example of wheat, tomatoes and apples, increased following treatment by 20–25%; of maize and potato it was up to 20%; of barley, lucerne and cotton it was up to 15%; and of cabbage it was up to 10%. Biological efficiency was 50.5–96.4%. Moreover, treatment of tomato plants with strains of *Bacillus thuringiensis* (Dipel® biopreparation), *Bacillus megaterium* and *B. subtilis* reduced the incidence of root galls and egg masses of *Meloidogyne* spp. by 71.60% and 77.78%, respectively (Khalil, 2013).

There are several reports on the biocontrol of common bunt (*Tilletia caries* and *Tilletia levis*) using some isolates of *Bacillus* spp., resulting in reduction of germination of *T. caries* and *T. levis* teliospores and reduced bunt incidence under field conditions (Kollmorgen and Jones, 1975; Kuz'mina *et al.*, 2003; Khairullin *et al.*, 2009). For example, *B. subtilis* 11RN strains were as effective as tebucanasole fungicide (Khairullin *et al.*, 2009). The protective effect of *B. subtilis* 26D and *Paenibacillus ehimensis* IB-739 against the bunt agent on wheat plants was 60–70% (Kuz'mina *et al.*, 2003).

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4.2 Mechanisms of Plant Disease Resistance, Invoked by PGPR of *Bacillus* spp.

4.2.1 Synthesis by *Bacillus* PGPR antibiotic compounds

Many *Bacillus* spp. such as *B. licheniformis*, *B. amyloliquefaciens*, *B. subtilis*, *B. cereus*, *B. megaterium* and *Bacillus mycoides* are known as very efficient producers of antibiotic molecules. *Bacillus* bacteria can produce up to 167 different antibiotics (Sonenshein *et al*., 2001). These include bacillomycin, mycobacillin, fungistatin, iturin, phengicin, plipastatin, surfactin and bacilizin (Duitman *et al*., 1999; Moyne *et al*., 2004). There are two ways of peptide synthesis (Guder *et al*., 2000; Melent'ev, 2007): (i) non-ribosomal – from precursors with synthetases (non-ribosomal peptide synthetases or NRPSs); and (ii) ribosomal – involving post-translational modification and proteolytic processing of pre-synthesized protein. The structure and synthesis pathway of surfactin, a cyclic lipopeptide of seven amino acid residues associated with the carboxyl group of β-hydroxy acids, is the most studied (Melent'ev, 2007). *B. subtilis* has an average of 4–5% of its genome devoted to antibiotic synthesis and has the potential to produce more than two dozen structurally diverse antimicrobial compounds. All the examined *B. subtilis* strains produce antibiotics but they differ significantly even among closely related strains of the same species. The *Bacillus* bacteria can synthesize lipopeptides with high antibiotic activity (Yazgan *et al*., 2003; Stein, 2005). These oligopeptides inhibit synthesis of pathogens' cell walls, influence membrane structures of cells, and inhibit the formation of the initiation complex on the small ribosomal subunit.

As all the examined *B. subtilis* strains produce different antibiotics, this suggests that the locus determining the kind of antibiotic synthesized is relatively young in evolutionary terms, which is, for example, proved by the high degree of homology of DNA that encodes sublancin in *B. subtilis* (Westers *et al*., 2003). The identity of locuses of genetic clusters for the biosynthesis of subtilin and ericin in *B. subtilis* strains ATCC 6633 and A1/3, as well as interchangeability of genes encoding micosubtilin and fengicin, indicate that they come from a common progenitor (Stein, 2005). With the help of artificial RNA silencing it was shown that iturin plays an important role in anti-pathogenic activity of *B. licheniformis* bacteria, provoked by stability failure in fungal plasmalemma (Hsieh *et al*., 2008; Arrebola *et al*., 2010). It was revealed that some *B. subtilis* strains simultaneously form two or even more antibiotics, such as S499 and RB14 surfactin and iturin synergetically intensifying each other (Guder *et al*., 2000; Tsuge *et al*., 2001). *B. licheniformis* produces lichenizin, which has the same immunochemical reaction as surfactin but differs from it because in the position with one lactonic ring L-glutamic acid is changed into L-glutamine or L-asparaginic acid in the 5 position is changed into L-asparagine.

Among PGPR antibiotics, such post-translation modified peptides as lanthionine and methyllanthionine are singled out. They contain residual thiol bonds (Guder *et al*., 2000; McAuliffe *et al*., 2001). Bacterial self-defence (immunity) from such antibiotics is based on the ATP-binding carrier of proper proteins (LanFEG) that carry lantibiotics out from the cytoplasmic space into the extracellular area (Stein, 2005). *B. subtilis* strains produce a pentacyclic subtilin lantibiotic that contains 32 amino acids (Kawulka *et al*., 2004; Stein, 2005). It is also structurally homologous to nisin (E234) of *Lactococcus lactis* (Ross *et al*., 2002) and has a macrocyclic structure with three inter-residual bonds as bridges between molecules of cysteine and aminoacid α-carbons. It is effective against various Gram-positive bacteria, including those that are potentially pathogenic for humans. *B. subtilis* strain A1/3 produces erycin, which is homologous to subtilin (Stein, 2005). The gene cluster that encodes a precursor of this antibiotic has two structural genes: *eriA* and *eriS*.

The majority of *Bacillus* spp. antibiotics (e.g. polymyxin, circulin and colistin) are active against both pathogenic Gram-positive and Gram-negative bacteria, and pathogenic fungi *Alternaria solani*, *Aspergillus flavus*, *Botryosphaeria ribis*, *Colletotrichum gloeosporioides*, *F. oxysporum*, *Helminthosporium maydis* and *Phomopsis gossypii*. It was found that under the influence of antibiotic substances produced by *B. subtilis*, *Sclerotinia sclerotiorum* hyphae (white rot agent) were swollen. In soil pathogenic fungi *Alternaria alternata*, *Drechslera oryzae* and *Fusarium roseum* this

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process took place under the control of *B. megaterium* and in the cereal rust agent *Puccinia graminis* it took place under the influence of *B. pumilus* (Duffy *et al.*, 2003). Production of fengycin-like lipopeptides by *B. subtilis* CPA-8 promotes biological control of peach brown rot (*Monilinia* spp.) (Yanez-Mendizabal *et al.*, 2012). The phospho-oligopepetide rhizocticin produced by *B. subtilis* ATCC 6633 also displays antifungal and nematicidal activities, but does not retain any bactericidal properties (Borisova *et al.*, 2010). *Bacillus brevis* and *Bacillus polymyxa* produce gramicidin S and polymyxin B peptide antibiotics that strongly inhibited *B. cinerea* spore germination *in vitro* but also exhibited high activity under natural field conditions against Botrytis grey mould caused by this fungus on strawberry. Each family of *Bacillus* peptides displays specific antibiotic activities and may be differentially involved in the antagonism of the various plant pathogens.

It is significant that an active influence of bacterial antibiotics can be involved in the regulation of the defence system of plants. It was revealed that *B. subtilis* surfactin is able to stimulate ISR by activation of lipoxygenases, lipid peroxidases and the formation of reactive oxygen species (ROS) (Ongena *et al.*, 2007; Jourdan *et al.*, 2009). In tobacco surfactin activated phenylalanine ammonia-lyase, medium alkalization and accumulation of hydrogen peroxide (H_2O_2) (Jourdan *et al.*, 2009).

4.2.2 Improvement of phosphoric and nitrogenous nutrition in plants

In ensuring the defence mechanisms of plants against pathogens, the energy component of the cells is important. This depends on plant tissue receiving nitrogen and phosphorus (Selosse *et al.*, 2004; Pérez-García *et al.*, 2011).

Some free-living rhizospheric PGPR, including of some *Bacillus* spp. strains, can fix nitrogen (Ding *et al.*, 2005; Melent'ev, 2007; Kumar *et al.*, 2012). Forchetti *et al.* (2007) identified several endophytic strains of *B. pumilus* isolated from sunflower that are capable of reducing acetylene. This suggests the possibility of nitrogen fixation by these bacteria species. Nitrogen-fixing bacteria species *Bacillus azotofixans*, *Bacillus coagulans*, *B. polymyxa* and *Bacillus macerans* can make up to 18.8% of the total number of spore-forming bacteria in soil (Melent'ev, 2007). It has been proved that *Bacillus* rhizobacteria induce nitrogen fixation of other free-living and associative diazotrophs *Azotobacter*, *Azospirillum*, *Rhizobium* and *Bradirhizobium*. This is more typical for cold climatic zones. The gene *nifH*, encoding the small subunit of nitrogenase, has been detected in some bacilli (Ding *et al.*, 2005). It also confirms the ability of these bacteria to fix nitrogen.

Phosphorus is known to be one of the most important and necessary compounds allowing the functioning of life on the earth. Nevertheless, only 5% of its general volume is relatively available in soil. The plants use bacterial and fungal endophytes to assist in obtaining phosphorus. Some *Bacillus* strains have the best ability to increase the mobility of soluble compounds of phosphorus in the soil through the production of organic acids (Unno *et al.*, 2005; Chen *et al.*, 2010). The most effective organic acids in mobilizing phosphate are considered to be oxalic, tartaric, fumaric, malic, citric and isocitric acids (Ryan *et al.*, 2001). The composition of the acids produced depends on the genotype of the strain (Chen *et al.*, 2006; Girgis *et al.*, 2008; Puente *et al.*, 2009; Hayat *et al.*, 2010, Egorshina *et al.*, 2011; Kumar *et al.*, 2012).

B. subtilis secretes phytases and hydrolysing phytates into the extracellular area – these are salts of the hexaphosphoric ester of inositol (Idriss *et al.*, 2002). It was discovered that there is synergism between the intensity of gluconic acid metabolism in bacteria and their antagonistic activity against pathogens. In our experiments the ability of 23 endophytic *B. subtilis* strains to mobilize sparingly soluble mineral and organic phosphates *in vitro* has been researched (Egorshina *et al.*, 2011). Detection of such activity has been shown to depend on the methods used. When solid medium with insoluble phosphates was used, most of the strains solubilized iron phosphate. In experiments with liquid media the best mobilization of calcium phosphate has been observed but, with the exception of a few strains, bacteria didn't solubilize iron and aluminium phosphates. Wheat grain treated with endophytic phosphate-mobilizing strains has been shown to reduce some parameters of root mycorrhization. From these experiments for

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the first time we advance a hypothesis that phosphate solubilization could be one of the factors reducing the efficiency of endomycorrhizal fungi competition, with phosphate-mobilizing endophytic bacteria colonizing internal plant tissues confirming the authors' hypothesis.

Organic substances may also be a source of phosphorus in the soil if bacteria in the rhizosphere can exhibit phosphatase or phytase activity (Lambers *et al.*, 2008; Plassard and Dell, 2010). The genes encoding phytase (*phy*) or their regulatory activity have been detected in *B. subtilis*, *B. lichenifomis* and *B. amyloliquefaciens* (Idriss *et al.*, 2002; Makarewicz *et al.*, 2006; Jorquera *et al.*, 2011). We have also demonstrated phosphatase activity in both free-living and endophytic strains of *B. subtilis* (Egorshina *et al.*, 2011).

4.2.3 Synthesis of siderophores

Iron is an essential nutrient for all living organisms. Some rhizospheric bacteria, including *Bacillus* strains, have developed iron uptake systems that include siderophores. A siderophore is a compound secreted by microorganisms with a high affinity to bind iron and it is an uptake protein needed to transport iron into the cells (Chen *et al.*, 2010; Shanmugam *et al.*, 2011; Yadav *et al.*, 2011). A lot of research studies dedicated to the investigation of antagonistic PGPR are based on their ability to produce siderophores, such as shizokinen (*B. megaterium*) and bacillibactin (*B. subtilis* and *Bacillus anthracis*), that have high antimicrobial activity and affinity to ions of trivalent iron (Kamnev, 2008; Berg, 2009; Puente *et al.*, 2009; Chen *et al.*, 2010). This deprives pathogens of the element needed for their growth and development, and thus reduces the probability and scale of plant disease. The synthesis of siderophores in bacteria is induced by the low level of Fe^{3+}, and in acid soil. A decrease of siderophore synthesis leads to significant lowering of antifungal activity of the bacterial preparation. In such conditions, the efficiency of iron fixation can be raised by gaining mutant strains capable of synthesizing siderophores. This process is independent of the iron concentration in the soil solution. As a result, Yu *et al.* (2011) found that the siderophore of bacillibactin in liquid culture of *B. subtilis* CAS15 can be involved in the biological control of plant pathogens. The addition of iron to the culture medium of bacteria decreased both the production of siderophores and the antagonistic activity of this strain to the pathogen causing Fusarium wilt in pepper plants. This suggests the involvement of siderophores of *Bacillus* in biological control mechanisms.

Pathogens are thought to be sensitive to suppression by siderophores for several reasons: (i) they produce no siderophores of their own; (ii) they are unable to use siderophores produced by the antagonists or by their immediate environment; (iii) they produce siderophores but too few compared with those produced by the antagonist microbes, and the latter are able to scavenge most of the available iron; and/or (iv) they produce siderophores that can be used by the antagonist, but they are unable to use the antagonist's siderophores (Kumar *et al.*, 2012).

4.2.4 Hydrolases as active components of *Bacilli* bacteria

When the methods of biological control were developing, it was found that some rhizobacteria, for example some strains of *Bacillus* spp., can depress growth and development of mycelial fungi both *in vitro* and *in vivo* (Lee *et al.*, 2007). The ability of bacteria to depress growth and development of root rots and leaf necroses caused, for example, by *Helminthosporium teres* Sacc. or *F. oxysporium* (Chen *et al.*, 2010) was accompanied by lysis of the mycelium. Therefore, bacteria must have other factors beside antibiotics that allow them to depress the growth of pathogenic fungi on plants. Thus, was revealed the ability of *Bacillus* bacteria to secrete chitinases and glucanases into the culture medium (Melent'ev, 2007; Lee *et al.*, 2007; Chen *et al.*, 2010). It is assumed that bacteria producing chitinases provide biological protection of crops from pathogens, especially those that contain chitin and glucans within their cell wall structure.

The major characteristics of chitinases from strains of *Bacillus* spp. and their sources are generalized in reviews by Melent'ev (2007) and Kumar *et al.* (2012). Among them, six isoforms of chitinases of *B. circulans* strain WL-12 were described in detail (Watanabe *et al.*, 1992). Their

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isoelectric point, molecular weight, as well as pH and temperature optimum, were found. In contrast to plant chitinases, bacterial chitinases have a significant advantage because they can destroy chitosan as well. Huang *et al.* (2005) isolated *B. cereus* 28-9, a chitinolytic bacterium, from lily plants in Taiwan where they exhibited biocontrol potential on Botrytis leaf blight of lily, as demonstrated by a detached leaf assay and dual culture assay. It is assumed that applying bacteria producing chitinases to crops can provide biological protection from pathogens, especially those that contain chitin and glucans in their cell wall structure. Moreover, many researchers have shown that chitinase is involved in antifungal activity and can enhance the insecticidal activity of some *Bacillus* spp.

4.2.5 Synthesis of hormone-like compounds and signalling molecules

The ability to actively influence plant growth is a unique feature of PGPR. This feature is thought to be related to the synthesis of various hormonal and signalling metabolites, such as auxins, cytokinins, gibberellins, abscisic acid (ABA), salicylic acid and jasmonic acid (Arkhipova *et al.*, 2006; Sziderics *et al.*, 2007; Forchetti *et al.*, 2007; Berg, 2009; Sgroy *et al.*, 2009; Chen *et al.*, 2010; Merzaeva and Shirokikh, 2010). There are two main ways of stimulating plant growth by PGPR bacteria: (i) the ability to synthesize hormone-like metabolites; and (ii) the effect on the phytohormone balance in plants.

A great deal of information is available about the production of indole-3-acetic acid (IAA) in the culture medium of rhizospheric *Bacillus* spp. strains (Idriss *et al.*, 2007; Sziderics *et al.*, 2007; Chen *et al.*, 2010). *Bacillus* isolates that are IAA producers are *B. megaterium*, *B. brevis*, *B. pumilus*, *B. polymyxa* and *B. mycoides*. *B. pumilus* SE34 secreted high levels of the hormone in tryptophan-amended medium in the stationary phase as determined by chromogenic analysis and high-performance liquid chromatography (Kang *et al.*, 2006). IAA promotes the formation of a better root system in plants, activation of metabolic functions in cells and stimulates not only disease resistance in plants but also allows them to rapidly pass through the stages of early development, when they are most sensitive to pathogens, by increasing water and nutrient uptake (Kilan *et al.*, 2000). Bacterial mutants with low levels of auxins could not influence plant growth (Asgar *et al.*, 2002; Van Loon, 2007; Chen *et al.*, 2010). Idriss *et al.* (2007) demonstrated that growth elongation of maize seedlings was significantly enhanced in the presence of a diluted culture filtrate of *B. subtilis* var. *amyloloquefaciens* FZB42. Moreover, strong curvature obtained after application on maize coleoptiles by bacterial culture filtrates indicated the presence of an IAA-like compound in the supernatant of FZB42. The presence of IAA-like compounds in the culture filtrates of several members of this group, including FZB42, was detected by ELISA tests with IAA-specific antibodies, when those strains were grown at low temperature and low aeration. Significant growth enhancement of lodgepole pine seedlings has been reported due to IAA production by *Bacillus* isolates (Chanway *et al.*, 1991).

It can be assumed that the more auxin produced by a strain the more stimulated the growth of plants. However, this is not correct. For example some *B. subtilis* strains can synthesize indoles causing increased concentrations of exogenous auxin in the medium of pea seedlings, increasing germination yet inhibiting root growth. This fact is proof of the significant role of hormones in the growth-inducing activity of PGPR.

IAA production by PGPR can vary among different species and strains and is influenced by the growth stage of the culture and substrate availability (Idriss *et al.*, 2007). An important source component for IAA synthesis is tryptophan, released into the environment with plant roots (Kamilova *et al.*, 2006; Spaepen *et al.*, 2007). The plant-beneficial Gram-negative bacteria synthesize IAA following different pathways involving indole-3-pyruvic acid, indole-3-acetamide or indole-3-acetonitrile as important intermediates. IAA biosynthesis generally occurs either by involvement of the indole-3-acetamide pathway which is constitutive in nature or by the inducible indole-3-pyruvic acid pathway.

Some strains of *Bacillis* spp. are capable of synthesizing cytokinins and secrete them into the culture medium, for example strain IB-22 of *B. subtilis* (Arkhipova *et al.*, 2006). This strain stimulated the growth of wheat seedlings, while the non-producing strain IS-21 did not have this

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property (Arkhipova *et al.*, 2006). Since the effective agent of BioYield® preparation is a mixture of living strains *B. subtilis* GB03 and *B. amyloliquefaciens* IN937 (Kloepper *et al.*, 2009), it can be assumed that growth activation dependent on photoperiodic lighting is also closely connected with the regulation of chloroplasts' stability by bacterial cytokinins. Increased growth of *Arabidopsis* under the influence of *B. megaterium* was not associated with auxin- or ethylene-dependent mechanisms, and it was necessary for the proper functioning of cytokinin signalling pathways (López-Bucio *et al.*, 2007). Ortíz-Castro *et al.* (2008) have shown that plant growth promotion by *B. megaterium* involves cytokinin signalling and IAA receptors. Cytokinins are likely to serve as an important mediator of relations of compatible endophyte strains of bacteria with plants. This suggests the possibility of developing a method for the selection of such strains on this basis.

Gibberellins may play an important role in the interaction of plants with some strains of *Bacillus* spp. (Joo *et al.*, 2005). Some strains of *Bacillus* bacteria are able to synthesize this hormone, for example Katznelson and Cole (1965) recorded the presence of gibberellins in the culture medium of *B. cereus* and *B. subtilis*. Also gibberellins are found in the culture medium of *B. cereus* MJ-1, *B. pumilus* CJ-69 and *B. macroides* CJ-29 (Joo *et al.*, 2005) and the endophyte *B. subtilis* from giant hogweed (Malfanova *et al.*, 2011) and *B. licheniformis* isolated from the halophyte *Prosopis strombulifera* (Sgroy *et al.*, 2009). Unfortunately, an experimental system of the interaction between bacteria and plants has not revealed accumulation of bacterial gibberellins. However, Gutierrez-Manero *et al.* (2001) confirm the participation of gibberellins produced by strains of *B. pumilus* and *B. licheniformis* with high gibberellin-producing activity in the stimulation of plant growth in alder seedlings.

Some rhizospheric *Bacillus* strains can synthesize ABA (Forchetti *et al.*, 2007; Egorshina *et al.*, 2012). Production of ABA is likely to contribute to the activation of the defence system of host plants. We have found that the inoculation of wheat seedlings by *B. subtilis* 11VM strain led to a rapid increase both of ABA and of IAA levels in shoots and roots (Fig. 4.1) (Egorshina *et al.*, 2012). If the accumulation of ABA in seedlings inoculated with this strain could explain one mechanism of the development of the defence effect in plant cells inoculated with bacteria, the high content of IAA, among other reasons, may be connected with decreased activity of the enzymes involved in the degradation of auxins, for example of IAA oxidase. This is supported by the observation that inoculation of pea seedling roots with strain *B. subtilis* 11VM leads to a decrease of IAA oxidase activity of more than 50%.

Another way of regulation of both plant growth and the defence reaction of plants to stress involving *Bacillus* strains may be the regulation of synthesis of ethylene or activation of enzymes involved in control of its level. Most often discussed is the participation of 1-aminocyclopropane-1-carboxylic acid (ACC) deaminase. This enzyme breaks down ACC – a precursor of ethylene – and lowers the level of ethylene in plant tissues. This effect eliminates the inhibition of growth. The participation of microbial ACC deaminase has been found with the help of analysis of the regulation of ethylene levels in plants by some *Bacillus* strains, for example the endophytic strain *B. subtilis* EZB8 (Sziderics *et al.*, 2007) and the rhizospheric strain *B. subtilis* LDR2 (Barnawal *et al.*, 2013). At the same time the results of López-Bucio *et al.* (2007) suggest that plant growth promotion and root-architecture alterations by *B. megaterium* may involve auxin- and ethylene-independent mechanisms.

It is important that the influence of *Bacillus* strains on the hormonal status is not limited by synthesis of some hormones. Under the influence of both bacteria and hormone-like compounds released by them, a systemic shift of the endogenic hormone balance in plants occurs (Arkhipova *et al.*, 2006). Accordingly, by synthesizing hormones in high concentrations, PGPR exogenically favour the regulation of growth and development processes in plants and form in them the resistance to a whole range of abiotic and biotic factors of the external environment (Saleem *et al.*, 2007; Sziderics *et al.*, 2007).

It has been recently discovered that PGPR are able to synthesize H_2O_2 in bactericidal concentrations and through this they compete with pathogenic microflora for nutrient resources. Nevertheless, it should be mentioned that the H_2O_2-producing activity of PGPR can be connected to the fact that they synthesize some oxidases of organic acids, for example oxalate oxidases (Schoonbeek *et al.*, 2007).

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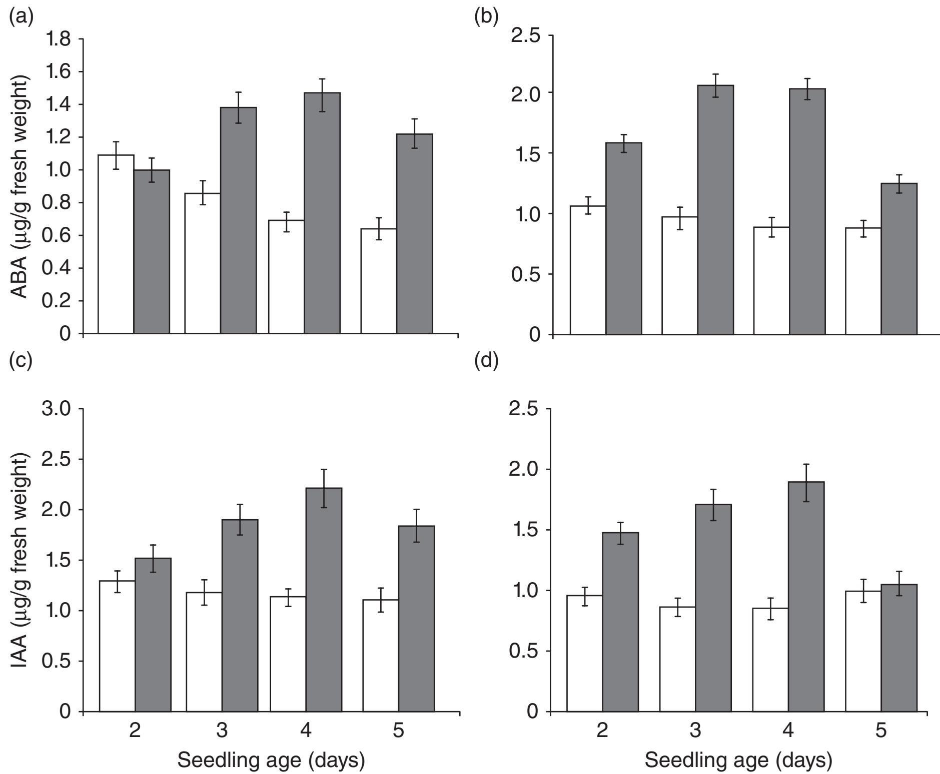

Fig. 4.1. The levels of abscisic acid (ABA) (a, b) and indole-3-acetic acid (IAA) (c, d) in roots (a, c) and shoots (b, d) of wheat seedlings under the influence of *Bacillus subtilis* 11VM strain; control (white bars); seeds treated with bacterial spores (shaded bars). (From Egorshina *et al*., 2012.)

Some strains of *Bacillus* spp. can produce volatile organic compounds (VOCs) (Ryu *et al.*, 2003; Insam and Seewald, 2010) – small organic molecules (molecular mass usually < 300 Da) that characteristically are easily volatilized. For example certain strains of *B. subtilis* and *B. amiloliquefaciens* can synthesize 3-hydroxy-2-butanone (acetoin) and 2,3-butanediol, and *B. megaterium* strain XTBG34 promotes plant growth by producing 2-pentylfuran (Zou *et al.*, 2010). Carbon and nitrogen sources increase VOC production. VOCs can stimulate the growth of *A. thaliana* plants (Zou *et al.*, 2010), have antifungal potential and can act over long distances via diffusion in air and through soil pores (Kai *et al.*, 2009; Insam and Seewald, 2010). With the use of *A. thaliana* mutants it has been shown that a signalling cascade activated by VOCs of *B. subtilis* depends on cytokinin signalling (Ryu *et al.*, 2005). It is established that its effect leads to tissue-specific redistribution of auxins (Zhang *et al.*, 2007), a reduction in the content of ABA in leaves and an increase in the productivity of photosynthesis (Zhang *et al.*, 2008). The VOCs produced by PGPR can elicit tolerance to abiotic stress, including salt and drought in *Arabidopsis* (Cho *et al.*, 2010; Zhang *et al.*, 2008), and ISR against *Erwinia carotovora* but not against *Pseudomonas siringae* pv. *tabaci* in tobacco (Han *et al.*, 2006). 2,3-Butanediol and acetoin from the endophytic *Bacillus* strain IN937a have been shown to confer protection against pathogen infection in *A. thaliana* through the ethylene-dependent signalling pathway (Ryu *et al.*, 2004).

Hydrocyanic acid (HCN) production, as a volatile compound, plays a major role in suppressing the growth of plant pathogens. Its production is highly dependent on the amino acid composition of the culture medium. For example, glycine is a direct precursor of microbial

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cyanide production. After interaction of HCN with the cell wall of the pathogen it is easily degraded (Kumar *et al.*, 2012). That leads to inhibition of spore germination and mycelium growth. As a consequence, cyanide produced by PGPR plays a role in the suppression of different disease-causing agents (Karimi *et al.*, 2012). Hydrogen cyanide effectively blocks the cytochrome oxidase pathway and is highly toxic to all aerobic microorganisms at picomolar concentrations.

4.2.6 Activation of the plant defence system

PGPR, including different strains of *Bacillus* spp., can influence the defence system of many dicotyledonous and monocotyledonous plant species, protecting them against different forms of pathogens (Kumar *et al.*, 2012; Maksimov *et al.*, 2014). This allows plants to activate some genes in their defence system that are involved in SAR or ISR. It is very important to carry out investigations on induction of phytoimmunity under the influence of PGPR to prove the efficiency of their use in farming practice. In 1991, three research teams found out independently that plant resistance to pathogens caused by PGPR is specific and it differs from acquired resistance caused by salicylic acid and elicitors (Alstrom, 1991). Data about the role of some PGPR in plant defence, referred to as ISR, is of a great practical importance (Van Loon, 2007). Investigations of the induction of plant resistance to pathogens under the influence of these symbionts provide a basis for the demonstration of their efficiency and possibility of their use in agriculture (Pieterse *et al.*, 2007). Although many PGPR can induce ISR in plants, activating plant defence mechanisms against pathogen attack, mechanisms of detection and development of the defence reaction with participation of endophytes, exclusively of *B. subtilis*, are not clear. In some plants both SAR and ISR simultaneously develop under the influence of a bacterium, and their interaction can often be observed, demonstrating the high efficiency of the applied biopreparation (Pieterse *et al.*, 2007).

Inoculation by endophytic strains of *Bacillus* spp. increased the resistance of plants to various diseases (Kelemu, 2003) and abiotic stresses (Campanile *et al.*, 2007; Wang *et al.*, 2012). On the basis of 15 plant species, the possibility of ISR development under the influence of rhizobacteria was proved and the major features manifested by this reaction that formed for a long period of time against fungi, bacteria, viruses and sometimes nematodes and insects was defined (Van Loon, 2007).

PGPRs differentially primed genes involved in SAR and ISR during further infection with pathogens (Yang *et al.*, 2009; Valenzuela-Soto *et al.*, 2010). The series of research papers dedicated to the selection of the most sensitive PGPR genes found that quick reaction is typical for approximately 200 plant genes. Some of them decreased their activity and others, on the contrary, greatly increased their activity (there was a 1:1 ratio of genes that were downregulated to those that were upregulated). *B. subtilis* GB03 and *B. amyloliquefaciens* IN937a, as well as strain *Streptomyces* sp. EN28, detected in wheat tissues induced ISR in *Arabidopsis* plants against *E. carotovora* ssp. *carotovora* (Choudhary and Johri, 2009). Strain *B. subtilis* BEB-DN induced the expression of ISR-associated genes in tomato plants and promoted resistance to insects (Valenzuela-Soto *et al.*, 2010). The expression of 70% of genes under the influence of PGPR was connected with ISR. In 13% of genes, it was dependent both on ISR and on SAR. In 17% of genes it was differentially regulated. For example, in the formation of pepper plants' resistance to *Xanthomonas axonopodis* pv. *vesicatoria* bacterial rot under the influence of *B. cereus* strain BS107, the genes of **P**athogenesis **R**elated proteins (PR) were introduced that become activated over the pathogenesis. Some of them, such as PR-1, are induced by salicylic acid, while others (PR-4, PR-10) are induced by jasmonic acid and ethylene or also by H_2O_2 (Yang *et al.*, 2009). The key transcription factor MYC2 was found to be sensitive to jasmonic acid and was included in the rise of sensitivity of tissues to a whole number of pathogens and insects (Pozo *et al.*, 2008). The PGPR strains of *B. subtilis* FB17 and *B. cereus* AR156 induced resistance in *Arabidopsis* plants to the pathogen *P. syringae* pv. tomato DC3000. Furthermore, strain AR156 triggered ISR via simultaneous activation of both the salicylic acid and the jasmonic acid/ethylene-dependent signalling pathways in an NPR1-dependent

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manner that leads to an additive effect on the level of induced protection in *Arabidopsis* (Rudrappa *et al.*, 2010; Niu *et al.*, 2011).

With use of *B. cereus* AR156 it was shown that this bacterium directly induced the expression of defence-related marker genes (*PIN2* and *PR1*) related to both salicylic acid and jasmonic acid dependent pathways (Niu *et al.*, 2012). In a series of investigations undertaken in Van Loon's laboratory (2007), on the basis of using a bacterial mutant for the synthesis of jasmonic acid and ethylene in *Arabidopsis* plants, it was proved that ISR formation under PGPR influence is mediated by ethylene (Pieterse *et al.*, 2007; Sziderics *et al.*, 2007). And whereas the ethylene signal in roots caused local development of defence reactions, the above-ground part experienced systemic priming of ISR genes. The obtained results aroused interest on data on the ability of some *Bacillus* strains to regulate ethylene synthesis by ACC (1-aminocyclopropane-1-carboxylic acid) formation (Sziderics *et al.*, 2007). Ethylene is a stress hormone and it is included in ISR induction together and sequentially with jasmonic acid. Low concentrations of bacterial ACC deaminases can promote active root growth in plants. The defence reaction of plants induced by *Bacillus* bacteria is shown to be associated with ISR and independent of SAR, which is revealed in the absence or even inhibition of transcription activity of genes coupled with it (Kloepper *et al.*, 2004; Pieterse *et al.*, 2007; Liu *et al.*, 2008); early signalling steps of rhizobacteria-induced resistance was controlled by MYB72, a transcription factor of *Arabidopsis* (Van der Ent *et al.*, 2008).

Strain *Bacillus vallismortis* EXTN-1 is capable of stimulating the immune reaction in a broad spectrum of plants, particularly in cucumber (*Cucumis sativus*) where it triggered expression of PR-1 genes (Park *et al.*, 2009). The expression of SAR-associated genes was detected simultaneously with ISR-associated genes in the pathosystem *Capsicum annuum–X. axonopodis* pv. *vesicatoria* under the the influence of *B. cereus* BS107 (Yang *et al.*, 2009). The resistance of *Arabidopsis* to *F. oxysporum* was induced by actinomycete strains *Micromonospora* sp. EN43 and *Streptomyces* sp. EN27 by activation of SAR-associated genes (Conn *et al.*, 2008). The *Streptomyces* sp. EN27 SAR-induced resistance was dependent on NPR1 (non-expresser of PR1 genes), by contrast, *Micromonospora* sp. EN43 promoted NPR1-independent resistance. It means that PGPR have an important role in the priming of plant defence systems (Pieterse *et al.*, 2007). So, there are some facts evidencing the participation of SAR-associated genes in the development of plant resistance induced by endophytes and PGPR despite the conventional belief of the ISR-associated nature of this interaction (Pieterse *et al.*, 2007).

PGPR and other symbionts can influence the signalling systems responsible for the formation of both SAR and ISR. Consequently, the data on their influence on the activity of a key player of these pathways – NPR1 – is very interesting. According to Pieterse *et al.* (2007) inoculation of *npr1* mutants by *Pseudomonas fluorescens* WCS417r did not promote ISR. Influence of *Bacillus* is similar to *Pseudomonas*, but, apparently, strains of *Bacillus* can activate NPR1-independent defence systems resulting in ISR formation (Kloepper *et al.*, 2004).

The influence of PGPR on the functioning of defence systems in resistance of plants is similar to pathogen-induced reactions (Van Loon, 2007). It is stimulated by the ability of endophytes to secrete hormones (Forchetti *et al.*, 2007), oligosaccharides similar to NOD (nodulation) factors of rhizobial compounds, and salicylic and jasmonic acids (Shanmugam and Narayanasamy, 2009; Dodd *et al.*, 2010) into the extracellular area. Some launching components, microbe-associated molecular particles that under the influence of PGPR induce systemic resistance of plants, have been decoded. They are: (i) lipopolysaccharides; (ii) flagellin of bacterial cell walls; (iii) siderophores pseudobacin and pioceolin; (iv) pyocyanin and 2,4-diacyl fluroglucinol antibiotics; and (v) N-acyl homoserine lactones and 2,3-butanediols (Verhagen *et al.*, 2004; Pieterse *et al.*, 2007; Van Loon, 2007).

At last we can assume that PGPR have other classes of molecules that induce ISR which can be specific for each strain, produced and secreted extracellularly: antibiotic peptides and universal signalling molecules such as ethylene, salicylic acid and jasmonic acid. It is important to note that PGPR are able to establish resistance in plants to a range of pathogens and also to sensitize the plant genome to probable pathogenic attack. Unfortunately mechanisms of regulation

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of plant immunity by PGPR and the role of salicylic acid and jasmonic acid are not properly understood.

Plants have receptors that are specific to them that are from Toll-like receptor proteins containing domains rich in leucine. Thus, the reception of *Pseudomonas putida* WCS358 flagellin by plant cells of *Arabidopsis*, tomatoes and beans proceeded through the interaction with membrane-associated kinases FLS2 (flagellin-sensitive 2) (Choudhary and Johri, 2009). Flagellin, which has high elicitor activity, did not always launch a defensive reaction of ISR. Nevertheless, it was quite effective at launching this reaction and was not permanently included in the formation of the endophytic interaction between PGPR and plant tissues (Gomes-Gomes and Boller, 2002).

Accordingly, PGPR must have a different source of molecules that induce ISR in plants. These molecules can be specific and individual for every strain, and may be compounds that are secreted into the extracellular area, for example peptides with antibiotic properties or such universal signalling molecules as ethylene, salicylic and jasmonic acids that have been proved to be secreted by bacteria (Shanmugam and Narayanasamy, 2009). An important role in the induction of ISR by endophytes is played by proteins with properties of hydrolases, acetylases of polysaccharides and oxidases secreted by bacteria and by plants in response to infection. Thus, inside the rhizosphere were found bacteria that secrete enzymes that can destroy oxalate (Schoonbeek *et al.*, 2007). They generate H_2O_2 in an antimicrobial concentration within the zone adjacent to roots and can increase protection of *Arabidopsis* plants from pathogens by 70% (Schoonbeek *et al.*, 2007).

It has been found that *Bacillus* bacteria protected plants from pathogens not only thanks to the high antifungal activity of their antibiotics but also because they mediated the induction of the expression of mainly ISR proteins and accumulation of phenols in the infection zone (Saravanakumara *et al.*, 2007) and enzymes of pro- and antioxidant systems and their functioning products (ROS, phytoalexins, lignin) (Govindappa *et al.*, 2010; White and Torres, 2010; Maksimov *et al.*, 2011b). Inoculation of tomato plants with *B. subtilis* BEB-DN led to the expression of some ISR genes among which the highest activity was shown by PR-4 and PR-6 genes, inhibitors of proteinases and enzymes of lignin synthesis. This made plants resistant to insects (Valenzuela-Soto *et al.*, 2010). Penetration of *B. pumilus* SE34 bacterium into pea roots favoured accumulation of callose, phenol compounds and their polymerization product – xylogen. It is important that PGPR treatment activated in susceptible plants the same isoperoxidases that are induced in resistant plants under the influence of pathogens (Chen *et al.*, 2000; Maksimov *et al.*, 2011b). Lipopeptides of *B. subtilis* strain S499 proved themselves as effective ISR activators in tobacco and bean plants (Ongena *et al.*, 2007). Therefore, endophytic strains of PGPR increase sensitivity of the plant genome over tissue colonization, which allows intensification of plant resistance to infection and prepares the defence system of plants for further response reactions. Meanwhile, it should be noted that such an increase, as a rule, forms ISR aimed at protection of plants from necrotrophs and insects.

Yan *et al.* (2002) observed ISR under the influence of *B. pumilus* SE34 in tomato plants infected with *Phytophthora infestans.* In another study the leaves of *A. thaliana* plants were inoculated with *B. subtilis* FB17, after infection with *P. syringae* pv. tomato (Pst DC3000) and this led to increased expression of the PR-1 gene. After some time it was found that salicylic acid had accumulated in the roots and there was suppression of proliferation of pathogen cells (Rudrappa *et al.*, 2010). It was revealed that the development of plant resistance against the pathogen occurred with the NPR1 protein required for signalling associated with salicylic acid and ethylene-dependent signalling pathways, but not for the jasmonic acid pathway (Rudrappa *et al.*, 2010).

VOCs can act as elicitors of ISR on growing plants under the influence of bacteria of the genus *Bacillus* (Ryu *et al.*, 2003). For example 2,3-butandiol can induce ISR through both the ethylene-dependent and the salicilate- or jasmonate- or *B. subtilis*-independent signal path. Similarly, acetoun (3-hydroxi-2-butanon) produced by strain *B. subtilis* FB17 generated defence in *A. thaliana* plants to infection by *P. syringae* pv. tomato (Pst DC3000) (Rudrappa *et al.*, 2010).

Earlier we are reported that the combination of salicylic acid and jasmonic acid promoted potato resistance to the hemibiotrophic oomycete *P. infestans* (Maksimov *et al.*, 2011b).

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There is considerable evidence in the literature of the jasmonic acid-mediated nature of *B. subtilis*' influence on plant defence reactions. Therefore, in the next step, we conducted an experiment by using a combination of salicylic acid, jasmonic acid and a *B. subtilis* 26D suspension for the investigation of these signalling pathways' interactions, during pathogenesis. Indeed, treatment of potato by *B. subtilis* 26D was an effective method to protect it from potato late blight symptoms (Fig. 4.2). The use of the bacterial suspension with salicylic acid (0.05 M) resulted in reduced symptom development in comparison with plants treated with only *B. subtilis*. It can be assumed that in this case *B. subtilis* initiated jasmonic acid signalling as a substitute of jasmonic acid. The combined influence of exogenic salicylic acid and components of the jasmonic acid pathway induced by the bacterial strain promoted maximum resistance of plants to the potato late blight pathogen. However, it is more difficult to explain the fact that combined treatment of potato by jasmonic acid and *B. subtilis* 26D decreased plant resistance to *P. infestans* significantly in spite of the high defence-stimulating activity of jasmonic acid alone.

The influence of salicylic acid and jasmonic acid in combination with a suspension cells of *B. subtilis* 26D on the transcriptional activity of gene *M21334*, encoding anionic peroxidase during late blight pathogenesis, was investigated (Sorokan *et al*., 2013; Maksimov *et al*., 2014). It was found that infection led to progressive accumulation of *M21334* gene transcripts. In contrast the individual influence of *B. subtilis* 26D resulted in permanent maximal level of transcriptional activity of this gene in infected plants in comparison with non-treated control plants (Fig. 4.3).

Simultaneous treatment of plants by a mix of salicylic acid and *B. subtilis* 26D led to a rather high level of anionic peroxidase gene transcripts (i.e. the influence of *Bacillus* (like jasmonic acid) is not being suppressed by salicylic acid). In infected plants treated with salicylic acid and *B. subtilis* 26D expression of the *M21334* gene was highest among all the variants of the experiment. With the simultaneous use of jasmonic acid and *B. subtilis* 26D, activation of the transcriptional activity of the gene under study was not observed, but we found a 50% decrease of the expression of the anionic peroxidase gene on the first day post-infection. It is to be noted that increasing the transcript's level was in inverse ratio to disease symptom development.

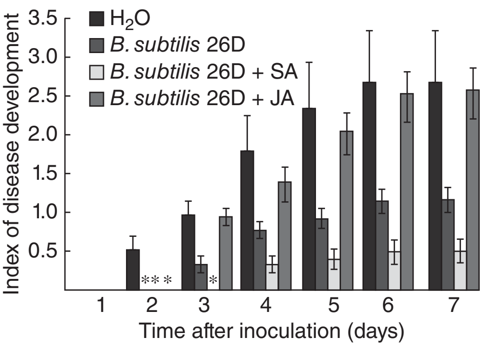

Fig. 4.2. Effects of *Bacillus subtilis* 26D and its composition with salicylic acid (SA) and jasmonic acid (JA) on development of late blight on the leaves of potato plants. *, Disease symptoms were not detected. (Adapted from Sorokan *et al*., 2013.)

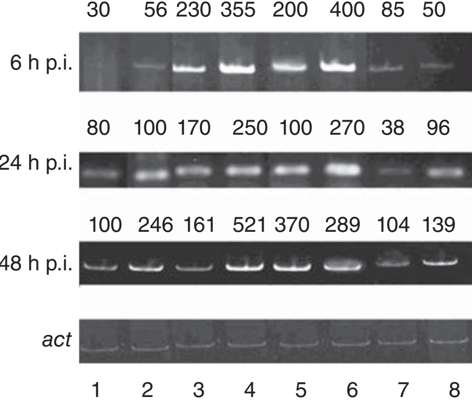

Fig. 4.3. The influence of salicylic acid (SA), jasmonic acid (JA) and *Bacillus subtilis* 26D bacterial cell suspension on transcription of gene *M21334* encoding an anionic peroxidase in potato plants that were uninfected and infected by oomycete *Phytophthora infestans*. Results are normalized against transcription of the gene encoding actin (*act*). Lane 1, Control; lane 2, *P. infestans*; lane 3, *B. subtilis*; lane 4, *B. subtilis* + *P. infestans*; lane 5, SA + *B. subtilis*; lane 6, SA + *B. subtilis* + *P. infestans*; lane 7, JA + *B. subtilis*; lane 8, JA + *B. subtilis* + *P. infestans*. p.i., Post-infection; values shown above each blot are evidence of transcriptional activity after normalization against the actin gene. (From Sorokan *et al*., 2013.)

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According to our results, in potato plants during the development of the defence reactions against the late blight pathogen, the jasmonic acid-mediated signal system prevailed over the salicylic acid-mediated system and was more efficient. Bacterial strain *B. subtilis* 26D displayed rather high defence-stimulating activity similar to that of jasmonic acid. Accordingly, on the basis of the data it can be possible to propose a preliminary hypothesis that ISR triggered by *B. subtilis* 26D is closely related to jasmonic acid-mediated reactions. Another important conclusion based on our investigations is the possibility of using a composite preparation on the basis that salicylic acid and jasmonic acid (or *B. subtilis* 26D) combines growth- and defence-stimulating activities as well as salicylic acid-induced resistance to a range of abiotic factors (Belkadhi *et al*., 2012).

4.2.7 Destruction of mycotoxins and increasing plant resistance against them

Plant pathogens, for example *Fusarium* spp., can produce metabolites that are highly toxic to plants and humans. In these conditions the characteristic of some *Bacillus* spp. strains to increase resistance of plants to toxins through their destruction is exciting (Kutluberdina and Khairullin, 2010). For example, strain *B. subtilis* CE1 can both inhibit the growth of *Fusarium verticillioides*, and decrease fumonisin B1 production *in vitro* (Cavaglieri *et al*., 2005). The reduction in the accumulation of fumonisin B1 was also observed in ears of maize treated with the endophyte *Bacillus mojavensis* (Bacon *et al*., 2008). Some strains of *B. subtilis* can completely destroy zearalenone when cultured in liquid medium (Cho *et al*., 2010; Tinyiro *et al*., 2011). Bacteria of *Bacillus* spp. reduced the level of deoxynivalenol in wheat tissues infected by *Fusarium graminearum* (Palazzini *et al*., 2007). We have shown that an endophytic strain of *B. subtilis* 11RN stimulates the growth of wheat seedlings and reduces the toxic effects of metabolites on plants by 35% (Kutluberdina and Khairullin, 2010).

4.3 Conclusion

Application of endophytic and rhizospheric *Bacillus* spp. strains has great potential. Their advantage is their ability to avoid the competitive pressure of indigenous species, which can affect other PGPR that are artificially included in the agroecosystem. Data on the influence of *Bacillus* bacteria with regards to their being highly antagonistic to pathogens, to their influence on plant growth and productivity and resistance to environmental hostilities has not yet been clarified. Their control of diseases caused by fungi, bacteria and viruses has been demonstrated thoroughly but ISR may also be a successful strategy in management of nematode and insect pests in several crops.

Forcible arguments towards the applied use of PGPR include: (i) their cheapness; (ii) the low energy output during the production process; (iii) the possibility of combining them with other preventive measures; (iv) their inability to provoke infection processes in humans and non-target objects; and (v) their non-pathogenicity towards plants. They have great potential for use as alternatives for crop protection chemicals, even though their use as such alternatives may be limited (Chandrashekhara *et al*., 2007; Melet'ev, 2007; Walters and Fountaine, 2009; Jacometti *et al*., 2010).

Acknowledgements

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5 Biofilm Formation on Plant Surfaces by Rhizobacteria: Impact on Plant Growth and Ecological Significance

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Abstract
Microorganisms have historically been studied as planktonic or free-swimming cells, but most exist as sessile communities attached to surfaces, in multicellular assemblies known as biofilms. Biofilms on plant surfaces are of great importance to plant health. Plant growth-promoting rhizobacteria (PGPR) not only induce plant growth but also provide protection by the process known as biocontrol, whereas other bacteria in the biofilm mode of growth can create a nuisance for plants. Recent advances show that biofilm formation on plant roots is associated with the biological and pathogenic response, but its regulation by the plant is unknown. In this chapter we describe bacterial biofilm processes, the ecological significance and the microbes that form biofilms on plant roots, and the effect of root exudates on biofilms and plant health.

5.1 Introduction

One major challenge for the 21st century will be the production of sufficient food to feed the growing population. The United Nations Population Fund (UNFPA) estimates that the global human population may well reach 10 billion by 2050 (UNFPA 2010). This means increasing agricultural productivity of food crops, as plants form the basis of every food chain. However, agriculture in developed countries already creates a range of serious environmental problems through the use of chemicals, salinization and the depletion of water resources. Furthermore, agricultural production in developing countries cannot be increased without further destroying the forests and turning them into arable land, thus threatening global biodiversity, which is already under stress from human action. There is a serious need to boost global food production in an environmentally sustainable manner (Angus and Hirsch, 2013). Thus, more emphasis has been placed on soil fertility and its maintenance for growing food and fuel crops even as the world around us changes (Morrissey *et al.*, 2004). Similarly, progress in plant biotechnology aims to develop new crop varieties by the introduction of desirable traits, so that these crops: (i) can survive better under conditions of drought or salinity; (ii) have enhanced disease and pest resistance; and (iii) have high nutritional value. However, efforts to understand the microbiome of the rhizosphere and its influence on regulating plant health is not yet fully explored (Morrissey *et al.*, 2004; Berendsen *et al.*, 2012).

Root exudates are well known to exert a significant impact on rhizospheric microorganisms (Bais *et al.*, 2006). Various aspects of the

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interactions between plants and microorganisms have been studied (Ryu *et al.*, 2004; Timmusk *et al.*, 2005; Haggag and Timmusk, 2008). Researchers in the last few decades have established that the majority of the microorganisms often exist in well-organized structures on the surface of plants in biofilms rather than in a planktonic state (Stanley and Lazazzera, 2005; Rudrappa *et al.*, 2007; Beauregard *et al.*, 2013) (Table 5.1). The process of biofilm formation on plant roots involves a complex mechanism which might be understood at physiological and molecular levels (Rudrappa *et al.*, 2007; Vlamakis *et al.*, 2013).

Biofilm formation on both biotic and abiotic surfaces has been the subject of research in the past (O'Toole and Stewart, 2005). The study of bacterial biofilm formation on plant surfaces, especially root surfaces, has not yet been explored fully (Haggag and Timmusk, 2008; Timmusk *et al.*, 2011). Plant growth-promoting rhizobacteria (PGPR) are known to promote the plant growth and yield through both direct and indirect mechanisms which includes phosphate solubilization, siderophore production, nitrogen fixation, indole acetic acid synthesis, production of antibiotic and lytic enzymes, induction of systemic resistance and stress relief (Fujishige *et al.*, 2006; Trivedi *et al.*, 2011).

Bacteria are unicellular organisms that manifest a range of collective behaviours leading to tissue-like functions. Whereas fruiting bodies and swarming are the most spectacular of these, cell aggregation, microcolonies and biofilm formation are the most widespread expression of the collective behaviour of bacteria. Such behaviour provides adaptive strategies during severe environmental stress conditions which can also result in the differentiation of non-specific cells into specialized lines for performing different unique functions not exhibited by single cells (Morris and Monier, 2003; Guttenplan and Kearns, 2013). PGPR are thought to form biofilms. Biofilms are assemblages of cells embedded in a self-produced matrix composed of extracellular polymeric substances (EPS), proteins and sometimes DNA (Beauregard *et al.*, 2013). The formation of microcolonies and biofilms on plant surfaces is associated with the processes of attachment of bacterial cells and production of EPS (Vlamakis *et al.*, 2013).

Bacteria associated with plant surfaces can be found on seeds, leaves and roots. Biofilm formations on abiotic surfaces and bacterial colonization of plant surfaces have some clear similarities, and are controlled by common molecular determinants. Plant-associated communities may usually not be as dense and structured as biofilms formed in other environmental conditions. However, they have an added element of complexity, since the plant surface is not only an inert support for bacterial growth, but also the main source of nutrients for the microorganisms, and an active partner in the system. Plant surface-associated bacteria have a direct role in plant disease and health (Molina *et al.*, 2003; Timmusk *et al.*, 2011; Vlamakis *et al.*, 2013).

In this chapter we review recent progress on plant root-associated biofilms, the impact on the plant–bacteria interaction, the ecological significance, and the influence of root exudates and their potential use for enhancing plant growth and protecting plant health.

5.2 Processes in Biofilm Formation

Biofilm formation is complex, but is generally recognized as consisting of five stages as described by Stoodley *et al.* (2002). These stages are: (i) reversible attachment; (ii) irreversible attachment; (iii) development of microcolonies leading to biofilm establishment; (iv) formation of a mature biofilm with a three-dimensional structure; and (v) dispersion of the biofilm and release of bacterial cells for initiation of new biofilm formation (Fig. 5.1).

5.2.1 Initial attachment

Bacterial cells attach to a surface as a result of a random process, mediated by Brownian motion and gravitational forces. Such attachment can be active or passive (Beloin *et al.*, 2008; Kostakioti *et al.*, 2013). The adhesion of cells during this process depends on the bacterial cell surface characteristics (Kostakioti *et al.*, 2013). The attachment process may be influenced by various factors such as availability of nutrients, pH, temperature and ionic strength. After the initial adherence there is production of extracellular adhesive materials and adhesions.

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Table 5.1. Biofilm formation by beneficial and pathogenic bacteria associated with plant roots. (Partly adapted from Rudrappa *et al*., 2008, and Angus and Hirsh, 2013.)

Bacteria	Nature of association[a]	Plant name	Plant part	References
Acinetobater calcoacetcius P23	PGPR	Duckweed	Root	Yamaga *et al*. (2010)
Azospirillum brasilense	PGPR	Wheat	Root	Kim *et al*. (2005), Shelud'ko *et al*. (2010)
Azorhizobium caulinodans	PGPR	Rice	Root	Van Nieuwenhove *et al*. (2004)
Azotobacter chroococcum	PGPR	Cotton, wheat	Root	Kumar *et al*.(2007)
Bacillus amyloliquefaciens S499	PGPR, biocontrol	*Arabidopsis thaliana*, maize, tomato	Root	Fan *et al*. (2011), Nihorimbere *et al*. (2012)
Bacillus cereus	Under conditions of stress by salt, heat or desiccation	Wild barley	Root	Trivedi *et al*. (2011)
Bacillus pumilis	Under conditions of stress by salt, heat or desiccation	Wild barley	Root	Trivedi *et al*. (2011)
Bacillus polymyxa	PGPR	Cucumber	Root	Yang *et al*. (2004), Nihorimbere *et al*. (2012)
Bacillus subtilis	Biocontrol	*A. thaliana*	Root	Ellis and Cooper (2010), Beauregard *et al*. (2013)
Bacillus megaterium C4	Nitrogen fixation, PGPR	Maize, rice	Root	Liu *et al*. (2006)
Burkholderia cepacia strain Lu10-1	Biocontrol	Mulberry	Root	Ji *et al*. (2010)
Enterobacter agglomerans	Biocontrol	Cotton	Root	Chernin *et al.* (1995)
Enterobacter cloacae	PGPR	Rice	Root	Shankar *et al*. (2011)
Klebsiella pneumoniae	Beneficial	Wheat	Root	Dong *et al*. (2004), Liu *et al*. (2011)
Microsphaeropsis sp.	Biocontrol	Onion	Root	Carisse *et al*. (2001)
Micrococcus sp. NII-0909	PGPR	Cowpea	Root	Dastager *et al*. (2010)
Paenibacillus lentimorbus	Heavy metal tolerance	Chickpea	Root	Khan *et al*. (2012)
Paenibacillus polymyxa	Biocontrol	Peanut	Root	Haggag and Timmusk (2008)
Pantoea agglomerans	PGPR	Chickpea, wheat	Root	Chauhan and Nautiyal (2010)
Pseudomonas aureofaciens	Biocontrol	Wheat	Root	Sigler *et al*. (2001), Zakharchenko *et al*. (2011)
Pseudomonas brassicacearum	Biocontrol	*A. thaliana*	Root	Lalaouna *et al*. (2012)
Pseudomonas chlororaphis	Biocontrol	Wheat	Root	Chin-A-Woeng *et al*. (2000), Shen *et al*. (2012)

Continued

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Table 5.1. Continued.

Bacteria	Nature of association[a]	Plant name	Plant part	References
Pseudomonas fluorescens	Biocontrol	Crop plant	General rhizosphere colonization	Silby and Levy (2004), Barahona *et al*. (2010)
Pseudomonas putida	Draught tolerance, bioremediation	Maize, sunflower, *A. thaliana*	Root	Sandhya *et al*. (2009), Matilla *et al*. (2011), Jakovleva *et al*. (2012)
Pseudomonas aurantiaca SR1	PGPR	Maize, wheat	Root	Rosas *et al*. (2009)
Rhizobium alamii	Heavy metal tolerance	*A. thaliana*, rapeseed	Root	Schue *et al*. (2011)
Rhizobium leguminosarum bv. *viciae* 3841	Nitrogen fixation, PGPR, drought tolerance	Various legumes	Root	Fujishige *et al*. (2006), Williams *et al*. (2008), Janczarek and Skorupska (2011)
R. leguminosarum	Beneficial	Rice	Root	Janczarek (2011)
Rhizobium sp. NGR234	Nitrogen fixation, PGPR,	Legumes (cowpea)	Root	Krysciak *et al*. (2011)
Rhizobium	Symbiosis	Legumes	Root	Fujishige *et al*. (2006), Robledo *et al*. (2012)
Sinorhizobium	Symbiosis	Legumes	Root	Fujishige *et al*. (2006), Khan *et al*. (2012)
Stenotrophomonas maltophilia	Biocontrol, PGPR	Crop plant	Root	Ryan *et al*. (2008)
Shewanella putrefaiens CN-32	Microbial mediated geochemistry	Biofilm on mineral surfaces		Huang *et al*. (2011)
Cyanobacteria spp.	PGPR, biocontrol	Enhanced mixed-species biofilm formation with *Rhizobium*, *Azotobacter*, *Pseudomonas* spp.		Prasanna *et al*. (2011)
Agrobacterium tumefaciens	Pathogenic	Pea	Root	Abarca-Grau *et al*. (2011)
Escherichia coli	Pathogenic	Leafy vegetables	Root	Delaquis *et al*. (2007), Saldaña *et al*. (2011)
Enterococcus faecalis	Pathogenic	*A. thaliana*	Root	Jha *et al*. (2005)

[a]PGPR, Plant growth-promoting rhizobacteria.

At first, the adherent cells, that give rise to biofilm formation, have only a fraction of EPS and a few are able to move freely either by twitching or gliding motility (Guttenplan and Kearns, 2013). The adhesion is reversible at this stage since the attached microorganisms are not yet dedicated to the differentiation process undergoing a series of morphological changes which leads to biofilm development (Hall-Stoodley *et al*., 2004). The surface properties are also important in determining bacterial adhesion. Generally, any surface is susceptible to biofilm development such as plastic, glass, metal, wood and food products. Furthermore, the surfaces that are covered with conditioning film that contains macromolecules, such as organic substances, will enhance the attachment of bacterial cells (Tang *et al*., 2009; Vlamakis *et al*., 2013).

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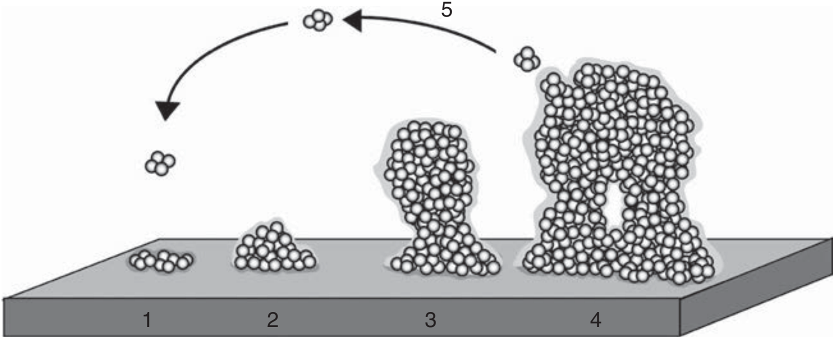

Fig. 5.1. The development of a biofilm, depicted as a five-stage process. Stage 1: attachment of cells to the surface; stage 2: production of extracellular polymeric substances (EPS) matrix; stage 3: development of biofilm architecture; stage 4: maturation; stage 5: dispersion of bacterial cells from the biofilm. (From Lasa, 2006.)

5.2.2 Irreversible attachment

Reversible bacterial cell attachment leads to attachment that is irreversible in nature due to permanent bonding in the presence of EPS (Hall-Stoodley *et al.*, 2004).

In this state of bacterial adherence biofilm becomes tolerant to treatment such as shear force; even chemical treatment with enzymes, detergent and surfactant could not dislodge the bacterial cells (Sinde and Carballo, 2000; Augustin *et al.*, 2004). Vlamakis *et al.* (2013) reported that the extracellular matrix of *Bacillus subtilis* facilitates cell attachment and assists in microcolony formation and biofilm maturation.

5.2.3 Microcolony formation

Microcolony formation results from simultaneous accumulation and growth of microorganisms and is connected with the production of the extracellular matrix (Hall-Stoodley and Stoodley, 2009; Vlamakis *et al.*, 2013) that nourishes the bond between the bacteria and the surface and protects the colony from any environmental stress (Hall-Stoodley *et al.*, 2004; Vlamakis *et al.*, 2013). Lopez *et al.* (2010) found that several species of bacteria in the ecosystem studied showed that accumulation can activate the selection of planktonic cells from the surrounding medium mediated by quorum sensing. Swimming motility was believed to allow the microorganisms to control repulsive forces at the surface water interface and enable them to reach the substratum and form microcolonies (Beauregard *et al.*, 2013). Microcolonies are thought to be beneficial as they allow interspecies substrate exchange and mutual end-product removal to bacteria (Hall-Stoodley *et al.*, 2004; Madsen *et al.*, 2012).

5.2.4 Maturation

The biofilm maturation step results in the development of an organized structure which can be flat or mushroom-shaped depending on the nutrient availability (Vlamakis *et al.*, 2013). Its maturation comprises adhesive processes that link bacteria together during proliferation and disruptive processes that form channels in the biofilm structure (Stoodley *et al.*, 2000). During this phase, surface contact activates responses that lead to gene expression changes, upregulating factors favouring sessility, such as those involved in the formation of the extracellular matrix (Kostakioti *et al.*, 2013). Bacteria grow under sessile form in heterogeneous complex-enclosed microcolonies scattered with open water channels (Stoodley *et al.*, 2000; Vlamakis *et al.*, 2013). Cell division is uncommon in a mature biofilm, and energy is used to produce EPS, which the biofilm cells can use as nutrients. It is not possible to identify general molecular profiles for a given bacterial species because some

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genes are important for biofilm formation under both static and dynamic conditions, whereas others are important only under dynamic biofilm conditions.

5.2.5 Dispersion

Dispersion is the final step in the biofilm formation cycle, and it allows the cells in biofilm mode to return back into their planktonic state (Kaplan, 2010). When the biofilm matures, resource limitation and waste product accumulation activates the dispersion of the biofilm. Hall-Stoodley *et al.* (2004) studied the role of external disturbance such as raised fluid shear stress that is involved in biofilm dispersion. Vlamakis *et al.* (2013) observed that the cells in a mature biofilm of *B. subtilis* released a mixture of D-amino acids (D-tyrosine, D-leucine, D-tryptophan and D-methionine), which help in dissolution and subsequent inhibition of the biofilm. Disseminating bacteria have the capability to restart the process of biofilm formation on availability of a suitable environment. In addition to dissemination of D-amino acids, the ageing *B. subtilis* biofilms possess the polyamine norspermidine, which helps in dispersion of biofilms. The suppressing activity of norspermidine is harmonious with that of D-amino acids, proposing that these molecules act by different mechanisms. It interacts directly and specifically with the EPS. This interaction causes collapse of the EPS, a process that has been visualized by microscopy, and a change in polymer size, as visualized using light scattering.

5.3 Ecological Significance of Biofilm Formation

5.3.1 Defence

A number of benefits to bacterial cells are associated with biofilm formation. A microbial cell in a biofilm mode of growth is well protected due to its EPS. The chemical composition of this self-produced matrix includes mainly EPS, protein and nucleic acid and some other substances (Flemming and Wingender, 2010). EPS provide protection against both physical (e.g. UV radiation, pH shift, osmotic shock and desiccation), chemical and biological stress (Annous *et al.*, 2009). The EPS matrix is also helpful in providing resistance to antimicrobial agents by controlling diffusion of compounds from the surrounding environment into the biofilm (Flemming and Wingender, 2010; Flemming, 2011). Further, EPS was found to seize metals, cations and toxins (Flemming, 2011).

5.3.2 Availability of nutrients to microbes

Biofilm formation can also lead to establishment of nutrient availability and development of syntrophic association between two different bacteria. Such associations have been studied in relation to methanogenic degradation (Schink, 1997). In a study conducted by Yanhong *et al.* (2009) such association was demonstrated in two strains of *Pseudomonas putida* (PCL1444 and PCL1445). *P. putida* PCL1444 effectively utilizes root exudate, degrades naphthalene around the root, protects seeds from being killed by naphthalene and allows the plant to grow normally. Mutants unable to degrade naphthalene do not protect the plant. *P. putida* (PCL1445) was unable to grow on naphthalene in pure culture in the absence of PCL1444, which indicated that the naphthalene degradation product produced by PCL1444 can be utilized by PCL1445 in the rhizosphere resulting in symbiotic relationship.

5.3.3 Colonization

Biofilm formation by such rhizobacteria can provide a mechanism for their establishment and maintenance in favourable environments. Thus, rhizobacteria not only benefit from root exudates but also influence the plant directly or indirectly. *Pseudomonas* is a widely studied genus and members of the genus are fairly widely distributed in plants, soil and water and exhibit different associations in nature (Misas-Villamil *et al.*, 2013). For example, *Pseudomonas syringae* is associated with aerial parts of the host plant while *P. putida* and *Pseudomonas fluorescens* are found in the rhizosphere and help in plant growth promotion and also protect plant health by one or other mechanism (Jakovleva *et al.*, 2012). Similarly *P. putida* was

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found to metabolize toxic aromatic compounds and exhibit rhizosphere colonizing ability and is therefore helpful in rhizoremediation (Kuiper *et al.*, 2004).

5.3.4 Acquisition of new genetic traits

Rhizosphere bacterial populations are hotspots for microbial interaction and biofilm formation. Biofilm modes of bacterial growth provide close proximity for gene transfer through various exchange mechanisms (e.g. conjugation, transformation and transduction) (Merkey *et al.*, 2011). The frequencies of gene transfer are higher in the biofilm mode of growth compared with the planktonic mode. Bacterial plasmids are known to be involved in biofilm formation and biofilms promote plasmid stability and genetic exchange. Plasmids and phages are known to induce the transition to the biofilm mode of growth in their respective hosts through cell–cell interaction (Madsen *et al.*, 2012).

5.4 Biofilms in the Rhizosphere

Bacterial biofilm formation has been extensively studied in the laboratory and in medical systems (Hall-Stoodley *et al.*, 2004; Hoiby *et al.*, 2010). Biofilm-related infections on medical devices and human tissues, such as colonization of cardiac valves and catheters by streptococci, pose serious threats to human health. Interestingly, the mechanisms of biofilm formation within and on the human host are also at work in the plant's environment, especially in the rhizosphere (Angus and Hirsch, 2013). Microscopy-based studies of bacterial colonization in the rhizosphere indicate that bacteria generally form microcolonies or aggregates on root surfaces and that these colonies have a patchy, non-uniform distribution.

Many bacteria are known to form microcolonies during root colonization, these include *P. fluorescens* and other closely related fluorescent pseudomonads that have potential as biocontrol agents (Couillerot *et al.*, 2009), other PGPR such as *B. subtilis* (Vlamakis *et al.*, 2013), free-living nitrogen-fixers such as cyanobacteria (Prasanna *et al.*, 2011) and *Azospirillum* spp. (Shelud'ko *et al.*, 2010).

A distinct exopolymeric matrix covering these microcolonies and aggregates has frequently been encountered (Haggag and Timmusk, 2008; Flemming and Wingender, 2010), particularly when roots were observed with microscopic techniques other than the scanning electron microscope. Most studies of colonization patterns of roots corroborate the notion that bacteria on root surfaces are present primarily as microcolonies at sites of root exudation (Bais *et al.*, 2006). Production of EPS or other exopolymeric material, and consequently the formation of biofilms, may enhance bacterial survival and the potential for colonization of roots. Mutants of *B. subtilis* defective in EPS matrix production showed impaired biofilm formation on the roots of *Arabidopsis thaliana* (Beauregard *et al.*, 2013).

5.4.1 Biofilm formation by PGPR

A number of PGPR form biofilms. The best studied examples of PGPR that form beneficial plant–microbe interactions are of *B. subtilis*, *P. fluorescens* and *Paenibacillus polymyxa* (Table 5.1). Gram-positive microbes, specifically *Bacillus* spp., generally used as effective biocontrol agents, are cosmopolitan and often colonize plants.

Colonization of *A. thaliana* roots by *B. subtilis* requires the production of surfactin. Surfactin, a lipopeptide antimicrobial, is involved in biofilm formation *in vitro*. Surfactin and other lipopeptides produced by *Bacillus* spp. are known to induce systematic resistance in plants and inhibit the growth of phytopathogens. *P. syringae* plant metabolites (malic acid) in root exudates were found to enhance biofilm formation on plant roots by *B. subtilis*. Root exudates from plants infected with *P. syringae* induce matrix gene expression in *B. subtilis*. Malic acid found in tomato root exudates at elevated concentration can influence matrix gene expression and biofilm formation *in vitro* (Vlamakis *et al.*, 2013). Adherent cells can multiply at the site of colonization to form multicellular assemblies. Another root-associated PGPR is *Azospirillum brasilense*, which is commonly associated with cereals (Shelud'ko *et al.*, 2010). Exopolysaccharides, flagellar motility (swimming and swarming) and specific outer membrane proteins are needed for effective

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root colonization, since non-motile and non-chemotactic mutants are among the most impaired in competitive root colonization (Barahona *et al*., 2010). Root hairs and the elongation zone of the root appear to be favoured colonization sites, and dense biofilms may be formed at these positions (Timmusk *et al*., 2005). Burdman *et al.* (1998) reported *Azospirillum* inoculation with nitrogen-fixing rhizobia significantly increased plant growth, and suggested the role of synergism within mixed communities of these microbes. Several *Pseudomonas* spp. and derivatives are effective PGPR, and some are biocontrol agents (Couillerot *et al*., 2009). On wheat roots, a natural population of pseudomonads makes up a significant portion of the microbial community, residing within aggregates and biofilms (Watt *et al*., 2006).

5.4.2 Biofilm formation by phytopathogens

The association of pathogens with roots is identical to that of beneficial bacteria. However, pathogenic pseudomonads have been reported to form thicker, more confluent biofilms on the root surface compared with the more heterogeneous colonization by beneficial pseudomonads (Rudrappa *et al*., 2008). This difference may reflect the interactions that lead to disease, but may also be the consequence of different inoculation strategies, growth conditions and plant hosts. More studies are needed to compare pathogenic and commensal interactions on the same plant and in mixed populations.

The ubiquitous plant disease called crown gall is caused by *Agrobacterium tumefaciens*. Infection occurs at wound sites along roots, and at the crown leads to a horizontal genetic transfer from *A. tumefaciens* to the plant, directing unrestricted growth of the tissue (the gall) and production of nutrients specific for the infecting microbe. The mechanisms of plant attachment have remained elusive, although a two-step model mediated initially by an as-yet-unidentified adhesin and followed by firm attachment via cellulose fibril production has been widely recognized. Once attached to root tissues, *A. tumefaciens* can form thick, anatomically complicated biofilms, abundantly covering the epidermis and root hairs (Matthysse *et al*., 2005). Comparable biofilms formed on abiotic surfaces and several mutants and genetic variants involved in biofilm formation on these surfaces, show similar phenotypes on root tissues (Danhorn *et al*., 2004; Matthysse *et al*., 2005). The role of biofilms during the disease process remains obscure, but may involve proximity to the appropriate infection site, or survival of the basal plant defence response. Oxygen limitation is a common condition in the rhizosphere and also within biofilms (Okinaka *et al*., 2002). An *A. tumefaciens* mutant disrupted for the FNR (fumarate and nitrate reductase regulatory)-type transcription factor SinR develops sparse, patchy biofilms on plant roots and abiotic surfaces (Ramey *et al*., 2004). This regulator is a part of an *A. tumefaciens* oxygen-limitation response pathway, suggesting a link between oxygen levels and biofilm structure. Similarly, limiting phosphorus is common in the rhizosphere due to plant sequestration. Phosphorous limitation enhances biofilm formation by *A. tumefaciens*, compared with the decreased biofilm formation reported for *Pseudomonas aureofaciens* (Danhorn *et al*., 2004).

5.5 Multi-species Biofilms in the Rhizosphere

There has been a significant increase in the knowledge and understanding of microbial biofilms in the last few decades. The majority of studies conducted so far on biofilms are based on monospecies. However, under natural conditions biofilm communities involve different microorganisms or mixed biofilms (Elias and Banin, 2012). Interspecies interactions involve cell–cell communication, via quorum sensing, and metabolic cooperation or competition. The interactions in the mixed biofilms have important ecological and environmental implications. Mixed-species biofilms are certainly the dominant form in the rhizosphere. Mixed biofilm-based inoculants are found to form strong biofilms (Prasanna *et al*., 2011; Seneviratne *et al*., 2011). Prasanna *et al.* (2011) found that cyanobacteria form more robust biofilms when inoculated with *Azotobacter* and *Pseudomonas*.

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5.5.1 Role of bacterial signals in biofilm formation

Successful colonization and biofilm formation depends on initial microbial communication. The role of quorum sensing in biofilm formation is well known (Angus and Hirsch, 2013) and mediates both pathogenic and beneficial plant–microbe interactions. Before biofilm formation, cells in the planktonic mode are involved in chemical signalling. The most prevalent signals involved in bacterial communication are N-acylhomoserine lactones (AHL), autoinducer-2 (Al-2) and 2-heptyl-3hydroxy-4-quinoline (PQS). In *Pseudomonas aeruginosa* the *lasl* gene is engaged in the growth of biofilms. Although, quorum sensing is a unique species-specific communication. Elasri *et al.* (2001) found that the plant-associated bacteria and plant-pathogenic bacteria produce AHL more commonly than soil-borne strains and proposed that these signals play a significant role in biofilm formation. Biofilm formation by *B. subtilis* is influenced by other species mainly by the members of the same genus (Shank *et al.*, 2011).

5.6 Role of Plant Root Exudates on Biofilms

Plant roots ceaselessly create and release a variety of compounds into the rhizosphere in the form of root exudates. The diversity of organic compounds released by plant roots includes various sugars, amino acids, organic acids, fatty acids, sterols, growth factors and vitamins, enzymes, flavonones and purines/nucleotides and several other compounds belonging to different chemical groups (Curl and Truelove, 1986; Uren, 2001; Dakora and Phillips, 2002). Root exudation can be broadly divided into two active processes. The first, root excretion, involves gradient-dependent output of waste materials with unknown functions, whereas the second, secretion, involves exudation of compounds with known functions, such as lubrication and defence (Bais *et al.*, 2006). Roots release compounds via at least two potential mechanisms. Root exudates are transferred across the cellular membrane and discharged into the surrounding rhizosphere. Plant products are also released from root border cells and root border-like cells, which separate from roots as they grow (Vicre *et al.*, 2005). Root exudation clearly represents a significant carbon cost to the plant (Bais *et al.*, 2006), and the magnitude of photosynthates secreted as root exudates differ with the type of soil, age, and physiological state of the plant, and presence of nutrients (Doornbos *et al.*, 2012). Although the functions of most root exudates have not been determined, several compounds present in root exudates play important roles in biological processes (Bais *et al.*, 2006; Doornbos *et al.*, 2012) and are likely to have an effect on bacterial biofilms.

5.7 Biofilms in Relation to Plant Growth and Health Protection

5.7.1 Role of biofilms in biocontrol of plant diseases

The biocontrol ability of bacterial strains is dependent on efficient colonization on the plant surface. Colonization of bacteria on the plant surface involves biofilm formation under natural environments. Bacterial biofilms on the plant root can protect the colonization site and act as a sink for the nutrients, making nutrients in the root exudates unavailable for plant pathogens (Haggag and Timmusk 2008). Plant root-associated beneficial rhizobacteria promote plant growth and yield through improved mineral nutrient uptake, production of hormone(s) and biocontrol activity (Trivedi *et al.*, 2011). For example, *B. subtilis* can protect plants against fungal pathogen attack, and plays a role in the degradation of organic polymers in the soil (Vlamakis *et al.*, 2013). Lugtenberg and Kamilova (2009) demonstrated that *B. subtilis* can be used as a biocontrol agent. Beauregard *et al.* (2013) studied *Arabidopsis* root surfaces inoculated with *B. subtilis* using confocal microscopy to disclose a three-dimensional structure of the *B. subtilis* biofilm. Similarly plant root-associated pseudomonads such as *P. fluorescens* can respond rapidly to the presence of root exudates in soils and at root colonization sites, which results in establishment of strong biofilm networks (Couillerot *et al.*, 2009). Haggag

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and Timmusk (2008) and Chen *et al.* (2012) explored the role of biofilm-forming *Paenibacillus polymyxa* and *B. subtilis* strains in managing *Aspergillus niger* and *Ralstonia solanacearum*, respectively. Therefore to establish effective biocontrol, successful colonization and biofilm formation with the biocontrol agent should be ensured.

5.7.2 Role of biofilms in mitigating stress in the rhizosphere

Survival of agriculturally important microorganisms in the rhizosphere under various stressful conditions is an interesting area of research, directly affecting our food security. PGPR mitigate most effectively the impact of abiotic stresses (drought, low temperature, salinity, metal toxicity and high temperatures) on plants through biofilm formation, which under normal conditions enhance plant growth and under stressful conditions help in better survival (Milošević *et al.*, 2012; Bogino *et al.*, 2013). For example the lipopolysaccharide (LPS) mutant of *Rhizobium leguminosarum* that lacks the biofilm formation property is also unable to tolerate drought conditions (Vanderlinde *et al.*, 2009). Sandhya *et al.* (2009) also found that colonization and biofilm formation by *Pseudomonas putida* strain GAP-P45 will alleviate drought stress effects.

5.8 Conclusion

Bacteria–plant interactions and their associations have been the subject of research for a long time. These interactions may be positive or negative in terms of plant health. In the last few decades it has been established that the biofilm mode of bacterial growth in association with the plant surface provides protection from predation, improved acquisition of nutrients, gene exchange and protection from exposure to toxic chemicals. Biofilms on plant roots also provide a protected environment both for the pathogen and the PGPR. PGPR effective in biofilm formation will be protected from stress conditions and can more effectively help plant growth, and protect plant health through their enhanced survival and metabolic activities. Focused research on the mechanism of biofilm formation and their regulation by plants and the impact of environments needs to be further explored to understand complex microbe–plant root interactions.

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6 Biofilmed Biofertilizers: Application in Agroecosystems

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Abstract

Certain soil microbiota naturally exists as surface-attached microbial communities in a biofilm mode of growth. They have been shown to be more effective at functioning than monocultures or mixed cultures of microbes. Therefore, such beneficial biofilms have been formulated *in vitro* to be used as biofertilizers called biofilmed biofertilizers (BFBFs) in agriculture and plantations. In this chapter we describe the significance of the BFBFs in addressing many issues that affect the sustainability of agroecosystems. In the literature on conventional biofertilizers, it is seen that the importance of surface attachment of microbes and biofilm formation has not been identified, though there are several other reports on the effectiveness of naturally occurring biofilms on soil particles and plant surfaces. However, the density of such biofilms on plant surfaces, particularly on the root system, is too low to have a significant effect on plant growth, as revealed by improved plant growth with BFBF applications to several crops. The BFBFs render numerous biochemical and physiological benefits to plant growth, and improve soil quality, thus leading to a reduction of chemical fertilizer (CF) NPK use by 50% in various crops. This reduction has not been achieved by conventional biofertilizers so far. The role of BFBFs is to reinstate sustainability of degraded agroecosystems through breaking dormancy of the soil microbial seed bank, and in turn restoring microbial diversity and ecosystem functioning. Thus, the concept of BFBFs is not only biofertilization, but also an holistic ecosystem approach. These formulations should therefore be considered as biofilmed microbial ameliorators (BMAs), rather than the BFBFs. If this agronomic practice were adopted in the future, it would lead to a more eco-friendly agriculture with an array of benefits to health, economics and the environment.

6.1 Introduction

Biofertilizers are live formulations of beneficial microorganisms, including nitrogen-fixing bacteria, phosphorus (P) solubilizers, algae, *Azolla* and mycorhizal fungi (Wu *et al.*, 2005). They are capable of performing many tasks such as mobilizing mineral elements from unavailable forms, making atmospheric nitrogen (N_2) available to plants, suppressing pathogens and regulating

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plant growth promotion through biological processes (Tien *et al*., 1979). Conventionally, microbiologists have paid their attention to formulate biofertilizers as monocultures or mixed cultures. The importance of using them as developed microbial communities in surface-attached biofilms was first stressed a decade ago (Seneviratne, 2003; Seneviratne and Jayasinghearachchi, 2003). In early studies, biofilms developed *in vitro* as *Penicillium* mycelium colonized by *Bradyrhizobium elkanii*, called fungal–bacterial biofilms (FBBs), were shown to result in significantly increased biological nitrogen fixation (BNF) over *B. elkanii* alone (Jayasinghearachchi and Seneviratne, 2004). Thereafter, this concept of the FBBs opened a new avenue in biofertilizer research, since subsequent studies showed that biofilms could perform improved biological functions over monocultures and mixed cultures of biofertilizers, for example in P solubilization with enhanced organic acids production (Jayasinghearachchi and Seneviratne, 2006; Seneviratne and Indrasena, 2006) and also in plant growth benefits through increased production of growth hormones (Bandara *et al*., 2006). Later, it was reportedly realized that this was attributable to the biofilms' ability to secrete more stable extracellular substances than mixed cultures (Nadell *et al*., 2009). These biofertilizers have now been named biofilmed biofertilizers (BFBFs) (Seneviratne *et al*., 2008).

The importance of conventional biofertilizers as monocultures or in combination with other beneficial microbes as mixed cultures has been reviewed by Mahdi *et al*. (2010) and Saharan and Nehra (2011). Their survival and function are inconsistent under field conditions due to heterogeneity of biotic and abiotic factors and competition with indigenous organisms. Thus, they have yet to fulfil their promise and potential as commercial inoculants (Nelson, 2004). On the other hand, BFBFs have been tested successfully for their fertilizing potential of many crops, such as maize, rice, a wide range of vegetables and for plantation crops like tea and rubber, under greenhouse and field conditions. Their effectiveness under field conditions has made it possible to reduce the use of chemical fertilizer (CF) NPK by 50%, with several other beneficial functions needed for sustainability of the agroecosystems (Seneviratne *et al*., 2011; Buddhika *et al*., 2012a; Hettiarachchi *et al*., 2012; Weeraratne *et al*., 2012; Seneviratne and Kulasooriya, 2013). To our knowledge, conventional biofertilizers have not been able to achieve this CF reduction so far. The important roles of naturally existing biofilms, when attached to plant surfaces, have been discussed in relation to enhanced plant growth (Rudrappa *et al*., 2008). Natural biofilms and their ecological significance have been reviewed widely (Davey and O'Toole, 2000; Ramey *et al*., 2004; Rudrappa *et al*., 2008), yet incorporation of the biofilm concept into biofertilization has not been assessed adequately, with exception of the study by Malusá *et al*. (2012) who suggest biofilms as an effective biotechnology for inoculating beneficial microbes as biofertilizers. Therefore in this chapter we describe the significance of biofertilizers in biofilm mode in addressing many issues that affect the sustainability of agroecosystems.

6.2 Biofertilizers and the Community Approach of Microbes

The major problem which is faced by current agricultural practices is the creation of undesirable ecological consequences, as many have reported (Choudhury and Kennedy, 2005; Sönmez *et al*., 2007; Seneviratne, 2009; Savci, 2012). These have prompted research into harmless inputs for the sustainability of agroecosystems. Therefore, biofertilizers and organic farming systems in crop cultivation have got attention in safeguarding the soil and producing better quality crop products. The use of biofertilizers has several advantages over conventional chemicals used for agricultural purposes. Generally, biofertilizers are applied either to the seed or to the soil, or both, to accelerate microbial processes in the soil, thereby increasing the nutrient availability in the soil and regulating plant growth through biological processes. Research and applications of biofertilizers have been well documented. For example, *Azospirullum* inoculants were able to reduce nitrogen (N) requirement by 25% in paddy, sorghum and sunflower fields (Varma, 1993). High N levels were observed in plant tissues with biofertilizer application (Bashan *et al*., 2004). Soil inoculation of P-solubilizing bacteria helped to reduce the requirement for phosphate fertilizer as they increased P availability in the soil (Mikanová and Nováková, 2002). Production of organic acids was reported as the major cause in

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P biosolubilization from unavailable nutrient sources. In biofertilizers, microbes belonging to a wide range of genera have been reported to produce plant growth regulators such as indole acetic acid (IAA), gibberellin and cytokinins, thus supporting plant growth and development (Barea *et al*., 1976; Cassan *et al*., 2009). Production of plant growth regulators (e.g. IAA) has been reported not only to be involved in plant growth promotion but also in pathogen suppression (Yu *et al*., 2009). Further, antibiosis and mycoparasitism have been identified as major biological functions of beneficial microbes, which help suppress the growth of pathogens (Badri *et al*., 2008; Bailey *et al*., 2008; Yu *et al*., 2009). Besides, such microbes improve soil properties such as organic matter content (Wu *et al*., 2005) and soil porosity by gluing soil particles together (Czarnes *et al*., 2000), which is also important in soil aggregation and stabilization (Six *et al*., 2004).

Although there have been many reports on microbial monoculture applications as biofertilizers, the importance of biofertilizers as mixed cultures or communities has started to be emphasized lately. It was shown that combined inoculations of nitrogen-fixing and P-solubilizing bacteria were more effective than using single microorganisms for providing more balanced nutrition for crops like rice (Tiwary *et al*., 1998), maize (Pal, 1998) and some other cereals (Afzal *et al*., 2005). Further, Holguin and Bashan (1996) observed that *Azospirillum brasilense* fixed more N_2 when it was grown in a mixed culture with *Staphylococcus* sp. It has been found that large communities of soil microorganisms are effectively involved in detoxification of heavy metals, converting them into non-toxic forms (He *et al*., 2010). The importance of microbial communities rather than monocultures for plant disease suppression has also been revealed (Mazzola, 2007). Metagenomic analysis of disease suppressive soils has claimed that the antagonism is caused by the wide range and high numbers of microbiota existing in the soil (Mendes *et al*., 2011). However, in the above studies, the importance of surface attachment of microbes and biofilm formation has not been identified.

Naturally, soil microbes get attached to soil particles and plant root surfaces and develop into biofilms due to microbial communication through metabolic trading and exchanging signalling molecules (e.g. quorum sensing) (Danhorn and Fuqua, 2007; West *et al*., 2007; Nadell *et al*., 2009). In addition, plant polysaccharides stimulate biofilm formation by providing a substrate for the biofilm exopolysaccharide matrix and also by inducing matrix gene expression (Beauregard *et al*., 2013). Thereby, plants tend to select biofilm-forming microbes to colonize their plant surfaces. However, the density of such naturally formed beneficial biofilms on plant surfaces, particularly on the root system, is too low to have a significant effect on plant growth (Seneviratne *et al*., 2009), as was demonstrated in several studies by increased plant growth via enhanced root colonization, when the developed biofilms were applied (Seneviratne *et al*., 2013). The improved plant growth is attributed to increased biochemical functionality of the BFBFs (Seneviratne and Jayasinghaarachchi, 2003; Seneviratne and Jayasinghaarachchi, 2005). This was illustrated by Herath *et al*. (2013) who demonstrated that biofilms had a wider array of biochemical expressions of exudates compared with the monoculture counterparts of the biofilm. The biochemical functions include hormonal, siderophores and hydrogen cyanide (HCN) production, antifungal activities, nitrogenase activity and biosolubilization of soil inorganic sources (Bandara *et al*., 2006; Herath *et al*., 2013; Triveni *et al*., 2013), which support the fertilizing potential. Regulated metabolism in biofilms through signal exchange optimizes production of plant growth-promoting hormones such as IAA (Bandara *et al*., 2006; West *et al*., 2007; Seneviratne *et al*., 2008; Triveni *et al*., 2013; Buddhika *et al*., 2014). The optimized production of IAA increases root growth, which in turn is important in enhanced nutrient uptake (Appanna, 2007). Thus, metabolism of the BFBFs can support plant growth directly and indirectly.

6.3 Role of BFBFs in Agroecosystems

Application of BFBFs was reported to restore agroecosystems that were depleted due to agronomic practices (e.g. tea cultivation; Seneviratne *et al*., 2011). This was evident from increased soil microbial biomass carbon (MBC), organic carbon, moisture retention and hence drought tolerance, and root-associated nitrogenase activity in the study. In another study, there was a positive

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correlation between leaf area and net photosynthetic rate of tea (Seneviratne *et al.*, 2009). Dense colonization of biofilms on root hairs has been reported to create pseudonodules fixing atmospheric N_2 through BNF, because exopolysaccharides produced in biofilms create an oxygen-restricted environment for triggering BNF (Seneviratne *et al.*, 2008). This was evident from higher root-associated nitrogenase activity in tea with the application of the BFBFs (Seneviratne *et al.*, 2011). The increased N supply in the rhizosphere with the BFBF application was reflected by increased soil NH_4^+ availability (Buddhika *et al.*, 2012b).The BFBF application also reduced NO_3^- availability (Seneviratne *et al.*, 2011), thus increasing N use efficiency, and reducing adverse effects of N on health and the environment. Soil inoculation of BFBF also increased maize root-associated nitrogenase activity approximately fourfold, compared with application of 100% CF alone (Buddhika *et al.*, 2012b). The nitrogenase activity was positively related to leaf chlorophyll content of BFBF-applied plants (Fig. 6.1), possibly due to ample supply of biologically fixed N for chlorophyll synthesis. The nitrogenase activity also extended even up to crop maturation, and was attributed to higher colonization of nitrogen-fixing bacteria on the root surface with the BFBFs than the 100% CF alone. In India, a similarly extended nitrogenase activity in wheat was observed with cyanobacterial BFBFs in a pot experiment (Swarnalakshmi *et al.*, 2013).

The focus of sustainability of an ecosystem is the functional diversity of soil microbes, since it is central to below-ground interactions, including food webs. Soil microbial diversity has a tremendous influence on agriculture as well as on natural ecosystems. It is a well-known fact that conventional agronomic practices, particularly CF application, deplete the diverse microbiome. However, soil application of BFBFs is known to render many beneficial effects that are vital to agricultural sustainability, including increased microbial diversity (Buddhika *et al.*, 2013). The emergence of diverse microbes with BFBF application is caused by breaking dormancy of dormant microbial forms in the soil seed bank as a response to the wide array of biochemicals secreted by the biofilms (Seneviratne and Kulasooriya, 2013).

The increased microbial diversity is considered to be one of the most important indicators of soil quality (Bastidia *et al.*, 2008; Sharma *et al.*, 2011), and is also an important determinant of soil health for increased productive capacity (Fernandes *et al.*, 1997). This is because soil microbial diversity contributes to beneficial functions such as biosolubilization, mineralization (Brookes, 1995; Pankhurst *et al.*, 1995; Yao *et al.*, 2000), rhizoremediation and natural disease suppression (Sharma *et al.*, 2011). Higher disease suppression of *Rhizoctonia solani* in potato was found in plots with the highest soil microbial diversity (Garbeva *et al.*, 2004). Similarly, there were significantly lower counts of shot hole borer in the BFBF-treated plants compared with the 100% CF-treated plants in tea cultivation (Fig. 6.2). As such, it appears that natural pest or pathogen suppression is caused by the improved soil microbial diversity, which can be achieved by BFBF application. In this way, use of biocontrol agents, some of which have been reported as unsafe (e.g. Simberloff, 2012), are not required when BFBFs are applied in agroecosystems. A natural weed-control process has also been observed recently in tea-growing soils applied with BFBFs for some time (A.P.D.A. Jayasekara, 2014, unpublished data). Cyanobacterial diversity

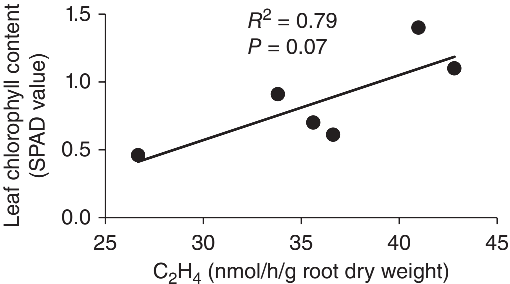

Fig. 6.1. Relationship between root-associated nitrogenase activity as evaluated by acetylene reduction to ethylene, and leaf chlorophyll content of maize plants applied with biofilmed biofertilizers (BFBFs) in a field experiment in Sri Lanka.

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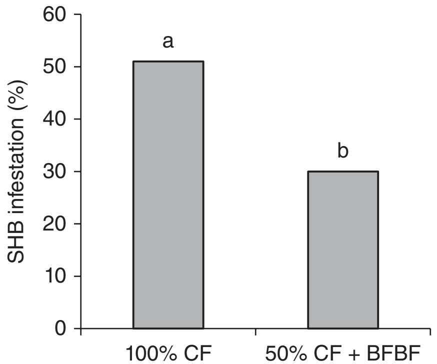

Fig. 6.2. Percentage shot hole borer (SHB) infestation of young tea plants treated with recommended chemical fertilizer (100% CF) and 50% CF + biofilmed biofertilizer (BFBF) on a tea estate at the Tea Research Institute at Talawakelle, Sri Lanka. Bars marked with different lower case letters indicate a significant difference at 5% probability level.

loss in agricultural soils has been reversed by applying cyanobacteria as monocultures (Prasanna *et al*., 2009) and/or as biofilms (Prasanna *et al*., 2011; Swarnalakshmi *et al*., 2013). These workers suggested that the utilization of a broader spectrum of biological functions of the developed cyanobacterial biofilms is the key to develop effective inocula in sustainable agriculture. However, sole application of developed FBBs as BFBFs, even in the absence of applied cyanobacterial monocultures or biofilms, has demonstrated an increased cyanobacterial diversity in a cropland soil (Buddhika *et al*., 2013) because of dormancy breaking of the soil microbial seed bank (Seneviratne and Kulasooriya, 2013). Thus, it is expected that BFBFs would increase demand for biofertilizers in the future, because they replenish the largely depleted microbiome in conventional agriculture, leading to sustainability of the agroecosystems.

BFBFs have been observed to play a vital role in agriculture starting from seed germination. Enhanced seed germination and improved seedling vigour with BFBFs compared with monoculture inoculation has been reported in several crop plants (Buddhika *et al*., 2012a; Herath *et al*., 2013; Triveni *et al*., 2013). Maize seeds tested with BFBFs for seed germination and growth showed improved performances due to regulated IAA production by the BFBFs, compared with their monoculture bacteria (Buddhika *et al*., 2014). This regulated IAA production was attributed to interactions of microbes in the biofilms, and such interactions have been reported to play amazing functions in biofilms (West *et al*., 2007). FBBs with a higher number of bacterial species, generally called higher order biofilms, were observed to pose an enhanced effect on plant growth (Seneviratne *et al*., 2009) due to their effective establishment in the soil–plant system (Swarnalakshmi *et al*., 2013). Further, collective behaviour of multiple bacterial species in biofilms has been observed to be involved in coordination, interactions and communication among the species for many ecologically important biological processes (Davey and O'Toole, 2000; West *et al*., 2007).

6.4 Fertilizing Potential of BFBFs

Different biofilms have been developed by using rhizosphere fungi and nitrogen-fixing bacteria from a wide range of genera, in order to be used as biofertilizers in agriculture and plantations (Jayasinghearachichi and Seneviratne, 2004; Seneviratne *et al*., 2011; Triveni *et al*., 2013). Application of BFBFs was first tested for soybean as a fungal–rhizobial biofilm, with increased N_2 fixation (by *ca*.30%), shoot and root growth, nodulation and soil N accumulation over the application of the rhizobium alone (Jayasinghearachchi and Seneviratne, 2004). Subsequently, developed biofilms were started to be tested extensively as biofertilizers for non-leguminous crops in several agroclimatic regions of Sri Lanka (Seneviratne *et al*., 2009). Either soil or seed inoculation, or both at the same time, supplemented with 50% of the recommended CF (i.e. 50% CF + BFBF) was compared with the full dose (100%) of CF as the positive control. The 50% CF + BFBF was used here because it was confirmed from initial studies that 50% CF was the optimum level to be coupled with the BFBFs for maximizing yields in diverse soils (Seneviratne *et al*., 2009). Generally, application of BFBFs alone is not recommended, since they are fungal-bacterial biofertilizers which may incorporate a considerable fraction of plant-available soil nutrients to the fungal biomass, thus reducing plant growth. So far, the BFBFs have been tested for 12 different crops in agricultural research centres as well as farmers' fields at 25 locations covering 12 districts in the country

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(Fig. 6.3). Results revealed that crop yields with 50% CF + BFBF were not significantly different ($P > 0.05$), and hence comparable to, yields with 100% CF (Table 6.1). This clearly shows the potential of BFBFs in reducing CF use by 50% with numerous health, economic and environmental benefits to agriculture and plantations. Widely varying soil and climatic conditions at the different locations tended to produce high variability in the yields of the same crop with the same treatment. It was reported recently that growth of the crops treated with BFBFs was limited by low levels of P in the soil (Buddhika and Seneviratne, 2014). BFBFs applied to rubber plants in the nursery also illustrated their potential in reducing CF use by 50% (Hettiarachchi *et al.*, 2012). In India, applications of cyanobacteria and plant growth-promoting rhizobacteria (PGPR)-based BFBFs were observed to increase plant growth and yields of mung bean and soybean (Prasanna *et al.*, 2014), and improve micronutrient biofortification in wheat (Rana *et al.*, 2012a, b).

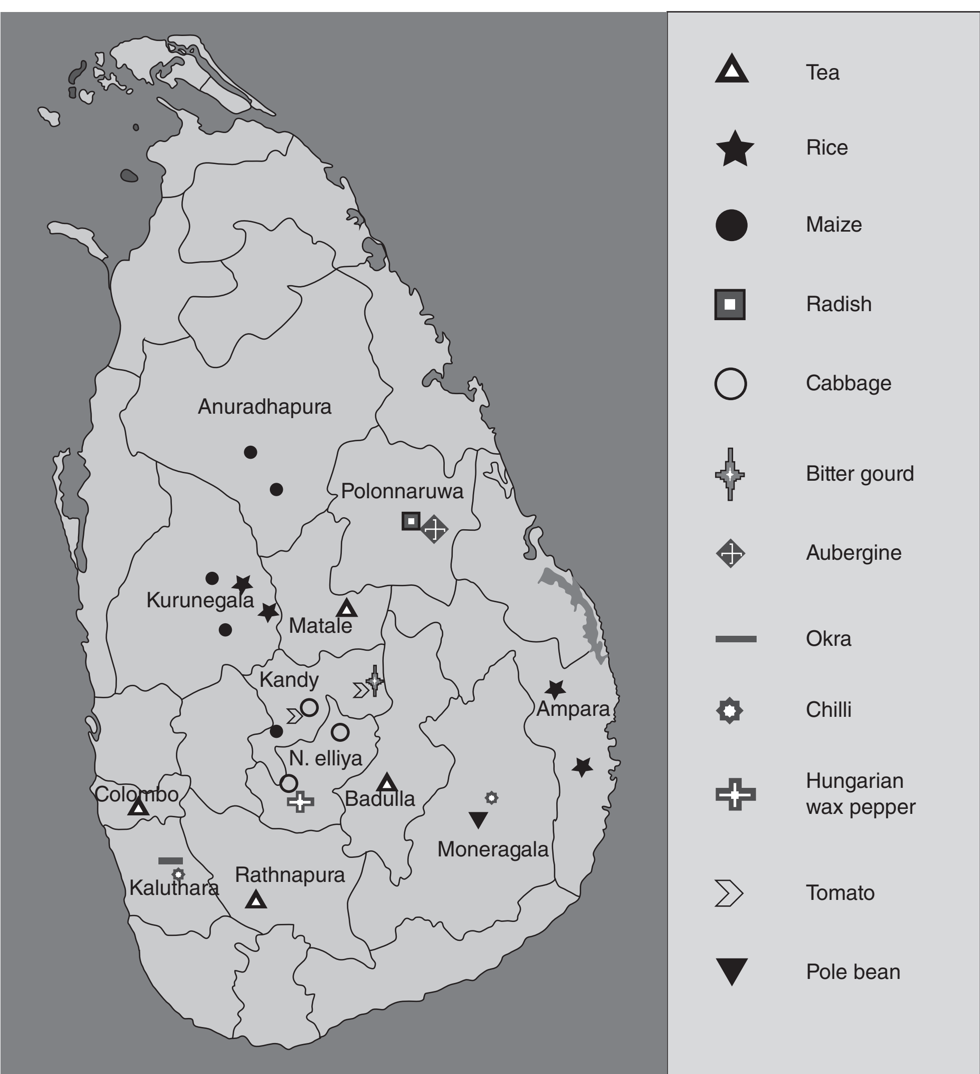

Fig. 6.3. Locations and crops of field experiments conducted with biofilmed biofertlizers (BFBFs) in different districts of Sri Lanka.

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Table 6.1. Mean crop yields following application of biofilmed biofertilizer (BFBF) combined with 50% of the recommended rate of chemical fertilizer (50% CF) compared with application of the recommended rate of chemical fertilizer (100% CF) in field experiments conducted in different agroecological regions of Sri Lanka.

Crop[a]	Mean ± SE crop yield (kg/ha)		Number of sites
	50% CF + BFBF	100% CF	
Tea	4300 ± 606	4100 ± 678	4
Rice	4420 ± 715	3580 ± 1295	5
Maize	2681 ± 322	2502 ± 338	3
Radish	1192 ± 251	992 ± 188	4
Cabbage	1302 ± 342	980 ± 249	4
Bitter gourd	1547 ± 445	1563 ± 440	4
Aubergine	748 ± 175	678 ± 260	4
Okra	3107 ± 1719	1739 ± 710	3
Chilli	3478 ± 1754	2350 ± 919	3
Hungarian wax pepper	238 ± 50	152 ± 39	3
Tomato	335 ± 86	397 ± 131	3
Pole bean	2762 ± 886	2396 ± 753	3

[a]Rice and maize field experiments were conducted during one or two seasons. Field experiments for vegetables were carried out during two consecutive dry and wet seasons. In the case of tea, the yields are annual averages over 4 years. In the same crop, mean yields of the two treatments were not significantly different at 5% probability level, according to Student's *t*-test.

6.5 Conclusion

The action of BFBFs differs from that of conventional biofertilizers which influence a limited set of functions such as BNF, mineral solubilization and plant growth hormone production. BFBFs show a wider range of more stable biochemical expressions and regulated metabolism for maximal effect, which are important in numerous functions of agroecosystems. BFBFs reinstate sustainability of degraded agroecosystems through breaking dormancy in the soil microbial seed bank, and in turn restoring microbial diversity and ecosystem functioning. Thus, the concept of BFBFs is an holistic ecosystem approach. BFBFs show not only enhanced biofertilization traits, but also biocontrol and other health and environment-related features. These formulations should therefore be considered as biofilmed microbial ameliorators (BMAs), rather than the BFBFs. Extensive studies conducted in various agroecosystems in the country clearly show the potential of the BMAs in reducing CF use by 50% without lowering current yields of numerous agricultural and plantation crops. If this agronomic practice was adopted in the future, it would lead to a more eco-friendly agriculture with an array of benefits to health, economy and the environment.

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7 Microbial Nanoformulation: Exploring Potential for Coherent Nano-farming

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Abstract

Recently nanotechnology has emerged as the sixth revolutionary technology after the green revolution of the 1960s and the biotechnology revolution of the 1990s. Today when agricultural scientists are facing major challenges such as reduced crop production, nutrient deficiency and climate change, nanotechnology has offered promising applications for precision farming. This innovative technology embraces wide applications such as plant disease control, enhanced nutrient uptake, improved plant growth and sustained release of agrochemicals. Interestingly, a nanoparticle (NP)-based strategy has gained momentum and become increasingly popular in the agricultural sector as a result of its unique properties compared with biopesticides. NPs are highly stable and could be synthesized using microbes which offer a non-toxic, cost-effective and eco-friendly means of synthesis over chemical synthesis. This green synthesis approach has a major advantage over the chemical method which is associated with ecotoxicity. Therefore, here we highlight the exploitation of agriculturally important microbes for NP synthesis and their further future role in agriculture.

7.1 Introduction

The emergence of nanotechnology has revolutionized the scientific world because of its novelty, high-growth and pertinent broad impacts. Nanotechnology is the study of nanoscale (1–100 nm) materials known as nanoparticles (NPs), exhibiting unique and novel physical, chemical and biological properties (Li *et al.*, 2001). These NPs have flexible physical properties with a large surface area to volume ratio and a strong affinity for proteins (Kumar *et al.*, 2010). Therefore, this new field has evolved as a fascinating breakthrough technology with broad and noteworthy applications in various fields such as those of medicine, electronics, cosmetics and in environmental remediation (Feiner, 2006; Patolsky *et al.*, 2006; Caruthers *et al.*, 2007; Hu and Chen, 2007; Bakshi *et al.*, 2014).

The successful application of nanotechnology in diverse fields has attracted considerable attention from agricultural scientists in order to modernize the agricultural sector towards agri-nanotechnology (Nair *et al.*, 2010). With respect to plant health, this innovative technology has a wide range of applications such as plant disease control, enhanced nutrient uptake, improved plant growth and sustained release of

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agrochemicals (Salamanca-Buentello *et al.*, 2005; Mishra *et al.*, 2014a; Mishra and Singh, 2015a, b). In this chapter we highlight the innovations and opportunities that nanotechnology offers in the agricultural sector.

7.2 Applications of Nanotechnology in Agriculture: Bridging the Gap

There is an obvious need to increase crop production through the use of traditional and advanced technologies while simultaneously maintaining sustainability in agroecosystems. Biotic and abiotic stresses are the major challenges faced by the agricultural sector and these hinder total agricultural production. Hence, development of agricultural technology becomes necessary to cope with these challenges. Over the past decade use of nanotechnology in the agricultural field has gained momentum by delivering robust applications (Tarafdar, 2012; Kah and Hofmann, 2014). These include nanotechnological applications in plant disease resistance and plant growth that have overwhelmingly remoulded the agricultural sector (Ghormade *et al.*, 2011; Chowdappa and Gowda, 2013).

During the 1970s, the use of chemical pesticides for plant disease management came into existence after the promotion of the green revolution and this changed the overall picture of the agricultural sector. Application of chemical pesticides results in successful control over plant diseases, but at the same time it leads to many environmental and health hazards including a reduction in soil fertility, development of pesticide-resistant pathogens and accumulation of pesticide residues in the food chain (Tilman *et al.*, 2002). To control pathogens and pests, around ~2 t of chemical pesticides are used annually around the globe (Stephenson, 2003).

There has been an increasing demand for biopesticides in the last few decades due to increasing awareness of the health and environmental hazards caused by chemical pesticides. Biopesticides are a beneficial, environmentally friendly means of controlling plant diseases. Currently, the major global market share is occupied by chemical pesticides while biopesticides are only in their infancy (Lehr, 2010; Glare *et al.*, 2012). Recently, the pioneering approach of harnessing nanotechnology and its applications in agriculture is gaining remarkable importance and it is recognized that this technology may be able to tackle some of the major concerns related to use of biopesticides (e.g. shelf life, on-field stability, coverage area and required dose) (Ghormade *et al.*, 2011; Singh, 2014; Mishra *et al.*, 2014b; Mishra and Singh, 2015a).

NPs in the size range of 100 nm or less, produced through nanotechnology, possess exclusive physical, biological and chemical characteristics which contribute to promising applications in agriculture. These NPs have a large surface area, are highly stable and provide size-dependent qualities (Nair *et al.*, 2010). Synthesis of NPs is performed through physical and chemical methods and so far many inorganic and organic materials are generated using these methods. However, the materials generated through these methods are unstable, expensive and environmentally hazardous. Therefore, currently synthesis of NPs through biological means, either of microbial or plant origin, is gaining popularity as they are non-toxic, low cost and eco-friendly (Gardea-Torresdey *et al.*, 2002; Rajesh *et al.*, 2009; Mishra *et al.*, 2014a).

It is evident from earlier reports that biosynthesized silver nanoparticles (AgNPs) act as a strong fungicide against various phytopathogens and have successfully controlled plant diseases caused by them (Jaidev and Narasimha, 2010; Mala *et al.*, 2012; Gopinath and Velusamy, 2013; Mishra *et al.*, 2014a). Additionally, they are also reported to deliver promising applications for fruit and vegetable preservation (Fayaz *et al.*, 2009). Moreover, application of biosynthesized zinc oxide (ZnO) NPs improves Zn nutritional status and also helps in secretion of phosphorus-mobilizing enzymes that would ultimately lead to uptake of phosphorus as a nutrient (Raliya and Tarafdar, 2013). These reports clearly suggest the robust applicability of NPs in agriculture but since the ongoing research studies are based in the laboratory, further research needs to be done to strengthen the findings. Some roles of biosynthesized NPs in agriculture are listed in Table 7.1.

Major applications of nanotechnology in agriculture include:

- improved seed germination of rainfed crops mediated by carbon nanotubes (CNTs);
- formulation of nano-fertilizers for balanced crop nutrition;

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Table 7.1. Multifarious role of biosynthesized nanoparticles (NPs) in agriculture.

Biological sources	NPs	Applications	Reference
Serratia sp.	Silver (Ag) NPs	Antifungal activity towards *Bipolaris sorokiniana*	Mishra *et al*. (2014a)
Brassica rapa	AgNPs	Antifungal activity against wood-rotting pathogens	Narayanan and Park (2014)
Calotropis procera	AgNPs	Antimicrobial activity	Mohamed *et al*. (2014)
Bacillus sp.	AgNPs	Antifungal activity towards *Fusarium oxysporum*	Gopinath and Velusamy (2013)
Aspergillus niger	AgNPs	Antifungal and antibacterial activity	Jaidev and Narasimha (2010)
Trichoderma viride	AgNPs	Vegetable and fruit preservation	Fayaz *et al*. (2009)
Spirulina platensis	AgNPs	Bactericidal activity against phytopathogens	Mala *et al*. (2012)
Cow's milk	AgNPs	Antifungal activity against phytopathogens	Lee *et al*. (2013)
Aspergillus fumigatus	Zinc oxide (ZnO) NPs	Enhanced native phosphorus-mobilizing enzymes and gum production in cluster bean	Raliya and Tarafdar (2013)
Brassica juncea	Copper (Cu) NPs	Antifungal activity against phytopathogens	Umer *et al.* (2012)

- development of efficient nanoformulations comprising mainly AgNPs for plant disease control;
- development of nano-herbicides for weed control;
- management of postharvest diseases using an NP-based strategy;
- diagnostic devices based on nano-sensors for monitoring agroecosystems; and
- improved agricultural engineering using nanotechnology in the field of agricultural machinery.

7.3 Role of Various Microbes in the Synthesis of NPs

Microbes in general are known to have a remarkable ability to form exquisite inorganic structures often of nanodimensions. Microorganisms are 6–10 nm in size, reproduce fast and as they are generators of NPs are usually referred as 'nanofactories'. This ability of living creatures has captured the attention of material scientists who wish to learn about these biological systems and improve their skills for precise fabrication of nanomaterials at ambient conditions. Although physical and chemical methods of synthesis of NPs are more popular, the use of toxic chemicals to a great extent limits their applicability. The biosynthesis of NPs using biological means has received increasing attention due to a growing need and demand to develop environmentally safe, reliable and non-toxic technologies in material synthesis (Kalishwaralal *et al*., 2008). The origin of the idea for biogenic synthesis of NPs using microbes germinated from the experiments on biosorption of metals with Gram-negative and Gram-positive bacteria. With an enzymatic process, the use of expensive chemicals is removed and the 'green route' synthesis is further supported by the fact that most of the bacterial species inhabit ambient conditions of varying temperature, pH and pressure. The particles thus generated by these processes have higher catalytic reactivity, greater specific surface area, and better contact between the enzyme and metal salt due to the bacterial carrier matrix (Bhattacharya and Mukherjee, 2008). The first evidence of synthesizing AgNPs was established using the microorganism *Pseudomonas stutzeri* AG259, a bacterial strain that was originally isolated from a silver mine in 1984 (Haefeli *et al*., 1984). Biological synthesis of NPs by the various microbes requires a unique trait of resistance of the organism to silver ions themselves,

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as many reports prove that where on the one hand the lower concentration of silver nitrate triggers synthesis of AgNPs, on the other hand higher concentrations can kill the organism within minutes (Kalimuthu *et al.*, 2008; Pandian *et al.*, 2010). To date a number of microorganisms have been successfully used for the synthesis of various kinds of NPs. Some of them are mentioned in Table 7.2. Further, the microbes have also been reported to synthesize various kinds of NPs which have been used for multifarious roles (Fig. 7.1).

In general the production of various NPs via biological methods includes metallic NPs, other metal NPs, oxide NPs, sulfide NPs, and other miscellaneous NPs.

Table 7.2. Some microorganisms used for synthesis of nanoparticles (NPs).

Microorganisms	Reference
Lactobacillus strains	Nair and Pradeep (2002)
Bacillus megaterium	Fu *et al*. (1999)
Escherichia coli	Gurunathan *et al*. (2009)
Staphylococcus aureus	Nanda and Saravanan (2009)
Geobacter sulfurreducens	Law *et al*. (2008)
Morganella sp.	Parikh *et al*. (2008)
Proteus mirabilis	Samadi *et al*. (2009)
Trichoderma asperellum	Mukherjee *et al*. (2008)
Penicillium fellutanum	Kathiresan *et al*. (2009)
Fusarium semitectum	Basavaraja *et al*. (2008)
Serratia BHU-S4	Mishra *et al*. (2014a)
Rhodococcus species	Ahmad *et al*. (2003)
Stenotrophomonas maltophilia	Oves *et al*. (2013)
Pseudomonas aeruginosa	Jeyaraja *et al*. (2013)
Cladosporium cladosporioides	Balaji *et al*. (2009)
Plectonema boryanum	Lengke *et al*. (2006)
Candida utilis	Gericke and Pinches (2006)
Verticillium sp.	Ahmad *et al*. (2004)
Neurospora crassa	Castro-Longoria *et al*. (2011)
Yeast	Zheng *et al*. (2010)

7.3.1 Metallic NPs

A number of methods have been published pertaining to chemical synthesis of metallic NPs. Synthesis of nanomaterials via microorganisms is an alternative and is comparatively less explored. However, in nature, some nanomaterials such as intracellular magnetite or greigite nanocrystallites are synthesized by magnetotactic bacteria as a result of biological processes occurring inside the cells (Blakemore, 1982; Mann *et al.*, 1990). Microorganisms are gifted with the ability of adsorbing and accumulating metals along with the secretion of large amounts of enzymes involved in the enzymatic reduction of metals ions (Rai and Durn, 2011; Zhang *et al.*, 2011). Metallic NPs generally include gold, silver, alloys and other metal NPs. Synthesis of gold nanoparticles (AuNPs) can be traced back to ancient Roman times where they were used to stain glasses for decorative purposes. Microbial synthesis of AuNPs was first reported in *Bacillus subtilis* 168 which showed the presence of 5–25 nm octahedral NPs inside the cell wall (Beveridge and Murray, 1980). In recent years the synthesis of AuNPs has been an area of great interest among researchers because of their emerging and immense application in a number of areas such as bioimaging, biosensors, biolabels and biomedicines. Among microorganisms, prokaryotes have grabbed most of the attention in the area of AuNP synthesis while fungi appear to be more promising for large-scale production of NPs because of their ability to grow both in the laboratory and on an industrial scale, along with the gifted trait of secretion of a large amount of proteins. In this context, extracellular synthesis of AuNPs by the fungus *Fusarium oxysporum* and by the actinomycete *Thermomonospora* sp. has been reported by Gurunathan *et al.* (2003). Certain yeasts like *Pichia jadinii* and *Yerrowia lipolytica* have also shown their potential for synthesis of AuNPs (Gericke and Pinches, 2006; Agnihotri *et al.*, 2009).

Among the other metal NPs, AgNPs have been largely explored because of the inhibitory and bactericidal effects. In this context, various microbes are known to reduce silver ions to form AgNPs, most of which are spherical in shape. Many reports have confirmed synthesis of AgNPs in the form of a film, production in solution or accumulation on the cell surface when fungi such as

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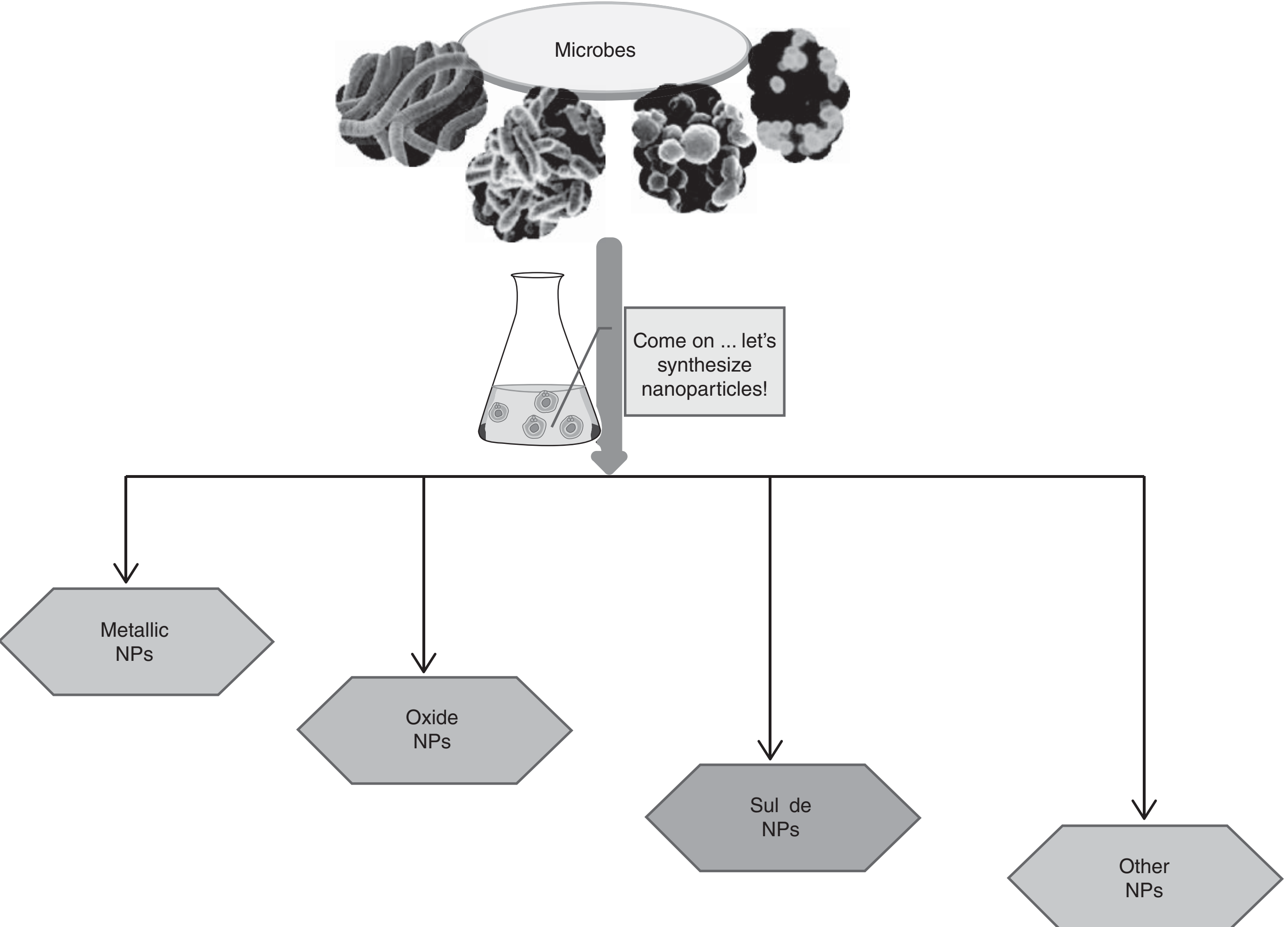

Fig. 7.1. Various kinds of nanoparticles (NPs) reported to be synthesized from microbes.

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Verticillium, *F. oxysporum* or *Aspergillus flavus* were employed for NP synthesis (Vigneshwaran *et al.*, 2006; Pandian *et al.*, 2009; Castro-Longoria *et al.*, 2011).

In addition to the AuNPs and AgNPs, alloy NPs have gained the attention of researchers due to their applications in catalysis, electronics, and as optical materials and coatings. In a 2005 study it was found that exposure of a mixture of 1 mm $HAuCl_4$ and 1 mm $AgNO_3$ solutions to different amounts of fungal biomass (*F. oxysporum*) resulted in the formation of highly stable Au–Ag alloy NPs (Senapati *et al.*, 2005). Similarly, Sawle *et al.* (2008) demonstrated the synthesis of core-shell Au–Ag alloy NPs from fungal strains of *Fusarium semitectum* which were quite stable for many weeks. The possible mechanism of synthesis of AgNPs using a bacterial culture is explained in Fig. 7.2.

7.3.2 Oxide NPs

Oxide NPs are an important category of compound NP synthesized by microbes. Formation of oxide NPs occurs at higher redox potentials in oxic and anoxic conditions. Similar to the metallic NPs, phylogenetically dissimilar bacteria such as *Rhodobacter*, *Klebsiella*, *Lactobacillus* and sulfate-reducing bacteria have been reported to synthesize compound NPs. Oxide NPs have been mainly categorized into two groups, namely: (i) magnetic oxide NPs; and (ii) non-magnetic oxide NPs. Many magnetotactic bacteria are reported to synthesize intracellular magnetic particles comprising iron oxide, iron sulfides, or both (Bazylinski *et al.*, 1994, 1995). The magnetotactic NPs are aligned in chains within the bacterium which enable the bacterium to migrate along oxygen gradients in aquatic environments, under the control of the earth's geomagnetic field. In a 2008 study, bacterium *Actinobacter* sp. was shown to be able to extracellularly synthesize iron-based magnetic NPs, namely maghemite (gamma-Fe_2O_3) and greigite (Fe_3S_4) depending on the nature of precursors used under ambient conditions (Bharde *et al.*, 2008). Apart from magnetic oxide NPs, many workers have studied other oxide NPs including TiO_2, Sb_2O_3, SiO_2, $BaTiO_3$ and ZrO_2 NPs (Bansal *et al.*, 2005; Jha and Prasad, 2009).

7.3.3 Sulfide NPs

Among nanocrystalline semiconductors, metal sulfides are of great significance due to their optical, electronic and magnetic properties. Sulfide NPs such as cadmium sulfide (CdS), zinc sulfide (ZnS) and lead sulfide (PbS) have been greatly explored and find applications as fluorescent biological labels, optoelectronic photocatalysts, sensors, photoelectric and thermoelectric materials, and use in photoimaging and photodetection (Li *et al.*, 2011). CdS nanocrystal is a typical type of sulfide NP which has been worked on a great deal. In a 2012 study, rapid and low-cost biosynthesis of CdS using culture supernatants of *Escherichia coli* ATCC 8739, *Bacillus subtilis* ATCC 6633 and *Lactobacillus acidophilus* DSMZ 20079T was reported (Abd El-Raheem *et al.*, 2012). Recently, an eco-friendly synthesis of CdS NPs using the marine bacterium *Enterococcus* sp. has been reported (Rajeshkumar *et al.*, 2014). Apart from CdS NPs, ZnS and PbS NPs have also been successfully synthesized by microorganisms like *Rhodobacter sphaeroides* and members of the family *Desulfobacteraceae* (Labrenz *et al.*, 2000; Bai *et al.*, 2006).

7.3.4 Other NPs

In nature, a large array of organisms form organic/inorganic composites with ordered structures with the help of biopolymers like protein and microbial cells. In addition to the NPs already mentioned, $PbCO_3$, $CdCO_3$, $SrCO_3$, PHB, $Zn_3(PO_4)_2$ and CdSe NPs are other ones which are reported to be synthesized by microbes (Li *et al.*, 2011). In a study, zinc phosphate nanopowders were synthesized using yeasts as biotemplates (Gurunathan and Kumar, 2009).

7.4 Possible Mechanisms for Antimicrobial Action of NPs Against Plant Pathogens

With the increase in microbial organisms that are resistant to multiple antibiotics and pesticides, many researchers have looked to nanotechnology for developing new, effective antimicrobial

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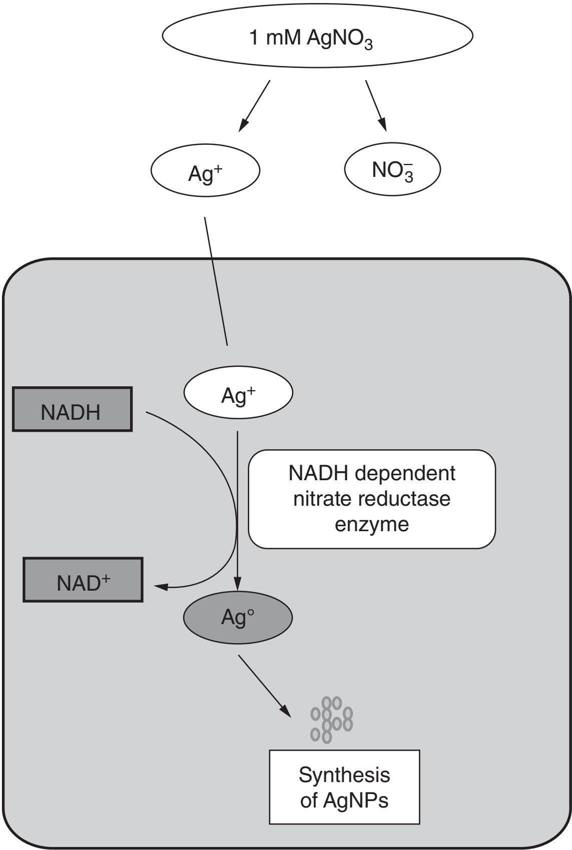

Fig. 7.2. The possible mechanism of synthesis of silver nanoparticles (AgNPs) by bacteria. NAD, Nicotinamide adenine dinucleotide; NADH, nicotinamide adenine dinucleotide – hydrogen (reduced).

reagents free of resistance and cost. Among the different kind of NPs, AgNPs have been most worked upon. Silver and its compounds have been reported to show strong inhibitory and antimicrobial activities against bacteria, fungi and viruses and when compared with other metals, silver is reported to exhibit higher toxicity to microorganisms than to mammalian cells. The exact mechanism which NPs make use of to kill a microbe is not clearly known and to date this is a topic of debate. There are, however, various theories which are in the public domain to explain the most plausible mode of action adopted by NPs. The antimicrobial modes of action of Ag^+ ions have been revealed in some detail (Fig. 7.3). According to the first theory, AgNPs have the capability of attaching to the bacterial cell wall and breaking through it, thereby causing structural changes in the cell membrane affecting the permeability of the cell membrane and death of the cell. This is followed by formation of 'pits' on the cell surface where NPs accumulate in large numbers on the cell surface (Sondi and Salopek-Sondi, 2004). The second theory considers formation of free radicals by the silver to be the mechanism responsible for cell death (Kim *et al*., 2007). According to the third theory, release of silver ions by the NPs can result in interaction of the ions with the thiol groups of many vital enzymes thereby inactivating the enzymes (Matsumura *et al*., 2003). The fourth theory suggests that reaction between NPs and sulfur/phosphorus or both leads to problems in DNA replication of the bacteria and thus kills the microbes (Hatchett and Henry, 1996). According to another group of workers, dephosphorylation of the peptide substrates on tyrosine

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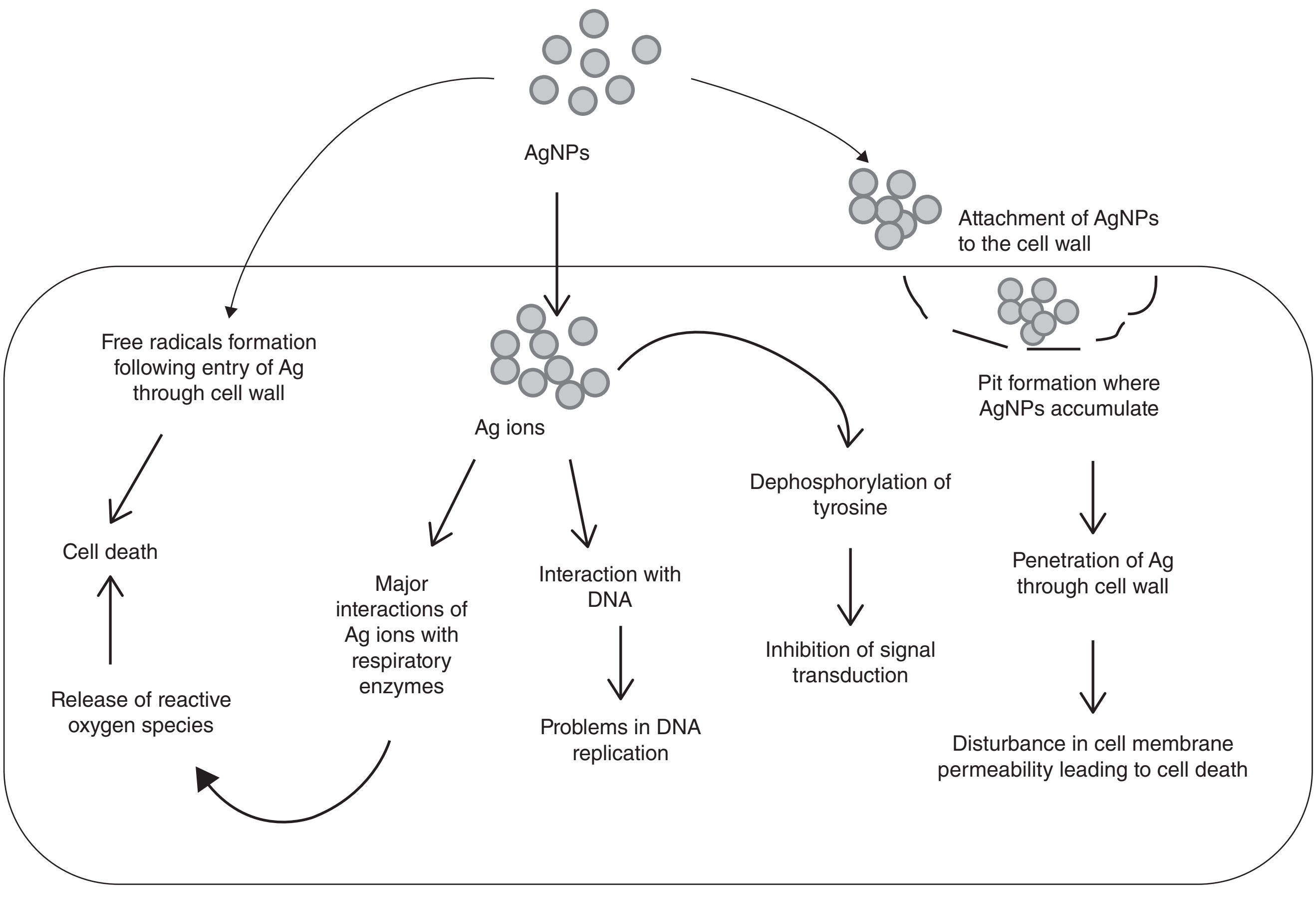

Fig. 7.3. Possible mechanisms of antimicrobial action of silver nanoparticles (AgNPs).

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residues by NPs can lead to signal transduction inhibition, thereby stopping the growth of the microorganism. Recently, a group of workers through proteomic analyses identified accumulation of envelope protein precursors indicating that AgNPs might have targeted the bacterial membrane, leading to a dissipation of the proton motive force (Lok *et al.*, 2006). Since most of the mechanisms have been studied on pathogens causing human diseases it is uncertain whether these mechanisms can also explain the antimicrobial action against plant pathogens. However, since the cell machinery is almost the same, it is expected that these theories could be of use for exploring the mechanism adopted by NPs in curbing plant pathogens.

7.5 Issues Related to Environmental Biosafety of Metal NPs

The potential application of nanoscience and nanotechnology in agricultural, food and pharmaceutical sectors, with their apparent modest implications for human and environmental health, are particularly attractive especially to countries concerned with sustainable development. However, caution is observed in such promising yet uncertain scientific endeavours as not enough is known of the effects of the use of nanomaterials. Further, while nanotechnology has high prospects of offering key solutions to threatening environmental problems at the same time it itself has been criticized for posing magnified environmental threats in order to promote its use (Kandlikar *et al.*, 2007; Grieger *et al.*, 2009).

Forecasting severe threats

Current emphasis on conventional approaches to assess the environmental impact of nanomaterials cannot be realized from the knowledge of the bulkier compounds from which they are synthesized (SCENIHR, 2006). The imperceptible myopic view on various potential nano-ecotoxicological factors could possibly have an uncertain and profound impact in the future (Balbus *et al.*, 2007; Kandlikar *et al.*, 2007). Therefore, it is vitally important that these nanomaterials be treated as novel and distinct materials and be individually assessed through rigorous international regulatory mechanisms.

The rising concern of environmentalists and the scientific community concerning the use of nanomaterials is based on growing evidence which strongly suggests serious harm in the long run. The potential threats include:

- unrestricted and uncontrollable movement of metal NPs in the food chain;
- cross reaction of these free nanomaterials with other environmental pollutants which may enhance their toxicity to local biodiversity (Baun *et al.*, 2008); and
- their potential to freely trespass trans-organ boundaries, which is one of their most beneficial properties but is also one of the most threatening dimensions if misused.

Regarding the last point, there is evidence that demonstrates that these nanomaterials can easily enter all vital organs, including the reproductive system casting doom on the existence of humans by unrestricted trans-generational movement (Lockman *et al.*, 2004; SCENIHR, 2009).

7.6 Regulations for Nanotechnology

Environmental regulation should be amended to effectively handle nano-scale products and applications in order to preclude environmental problems. Nanotechnology and its applications can be regulated both directly and indirectly. Direct regulations focus on strict management of specific aspects of nanotechnology and its use while indirect regulations deal with the overall nano-scale product or processes of nanotechnology in a more general sense.

7.6.1 European Union's approach to regulating nanotechnology

The regulatory framework for nanotechnology in Europe is heavily influenced by the precautionary principle, with a high priority for social safety. The precautionary principle is a regulatory strategy which can be beneficially adopted if a suspected nanotechnology's application has insufficient biosafety data and lacks extensive

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knowledge on risk assessment and may cause potential harm to human or environmental health. On the other hand, the adoption of the precautionary principle as a basis for regulating nanotechnology processes may hinder future developments in nanotechnology due to regulation of exploratory research in nanoscience and technology.

7.6.2 International standards for nanotechnology-based research

Framing of international policies and standards to ensure safety of human and environmental health are governed by the Organization for Economic Co-operation and Development (OECD) and the International Organization for Standardization (ISO). The working party of the OECD (www.oecd.org/sti/nano) maintains databases on environmental safety and human health research. Research methods used are regularly examined and the OECD working party is actively involved in developing consistent test strategies to analyse the safety of nanomaterials research. There is an obvious need for developing global standards for nanoscience and technology research which can be adapted to new approaches and methods while at the same time framing of codes of conduct (Mantovani *et al.*, 2010). The European Commission has released a code of conduct for nanoscience and technology research (http://ec.europa.eu/nanotechnology/pdf/nanocode-rec_pe0894c_en.pdf). The core components of this code include principles of precaution, sustainability and accountability.

7.6.3 International scenario on biosafety of nanoproducts

A 2003 report by the Better Regulation Commission, formed by the UK government, recommended that the public should be provided with relevant information to enable them to consider the risks of nano-products for themselves and that their decisions would be taken into consideration in the making of government policy regarding regulations for managing risk (Better Regulation Task Force, 2003). This commission acted as a frontrunner in managing matters of risk. According to a 2004 report of the UK's Royal Society it was proposed that the potential exposure risks of nanomaterials by manufacturers should be assessed and their results should be properly published and made available to regulatory groups on nanotechnology (Royal Society and Royal Academy of Engineering, 2004). In 2007, US president George W. Bush outlined that tagging NPs (i.e. labelling of consumer products containing NPs) would not be required and regulations were relaxed. However, critics condemned the government for lack of sufficient labelling of nanomaterials. On 10 December 2008 the US National Research Council published a report summoning additional regulations to ensure the biosafety of nanomaterials. The European Union has commissioned the Scientific Committee on Emerging and Newly Identified Health Risks to analyse the consequences of nanotechnology and has created an inventory of hazards posed by NPs.

7.7 Conclusions and Recommendations

The environmentally friendly supremacy of promising technologies confronts a major challenge of the remarkable balance needed between exploitation of advantages while avoiding unidentified harms. As such, a life cycle perspective should be taken with regards to the use of nanomaterials, considering the environmental benefits they may bring but also assessing the long-term impacts of their manufacture, use and disposal. Extensive research on nano-ecotoxicity should be executed along with evaluation of nanomaterials in complex systems in terms of their performance for biosafety.

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8 *Bacillus thuringiensis*: a Natural Tool in Insect Pest Control

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Abstract

Bacillus thuringiensis is a Gram-positive, ubiquitous, spore-forming bacterium that produces large amounts of proteins that crystallize inside the cell during the sporulation stage known as *B. thuriniensis* delta-endotoxins or Cry proteins. The insecticidal Cry proteins produced by *B. thuringiensis* have provided a particular, secure and effective tool for the control of a wide diversity of insect pests around the world for over 70 years. The Cry proteins are lethal to insect larvae in the orders Lepidoptera, Diptera and Coleoptera. More recently, isolates have been identified with activity against the organisms in the orders Hymenoptera, Homoptera, Orthoptera and Mallophaga and also nematodes, mites, lice and protozoa. Furthermore, in the global biopesticide market *B. thuringiensis* represents nearly US$600 million/year. At least 900 different Cry toxin sequences have been found and classified into 73 family groups (by 2014). Biotechnology and genetic manipulation of *cry* genes present in *B. thuringiensis* can potentially improve the efficacy and cost-effectiveness of *B. thuringiensis*-based commercial products. The combination of genes from different *B. thuringiensis* strains to enhance their activity, extend their host range and improve the spectrum of insecticidal activity has been achieved with recombinant technologies. These genetically modified *B. thuringiensis* products are currently commercially available. It has been recently established that Cry hybrid proteins of *B. thuringiensis*, gained by domain swapping, resulted in enhanced toxicities when compared with wild-type proteins. Nowadays, *B. thuringiensis* insecticidal genes have been included in several of the most important crop plants where they provide a model for biotechnology in agriculture. The *B. thuringiensis* transgenic crop has received more attention in cases where *cry* genes have been brought together by a mixture of mutagenesis and oligonucleotide synthesis to produce synthetic genes. In this chapter, manipulation of Cry proteins from the soil bacterium *B. thuringiensis* and its biotechnological applications are described. The future prospects are also discussed.

8.1 Short Story of *Bacillus thuringiensis*

Interest in microbial insecticides dates from the middle of the nineteenth century. Research into disease of the silkworm *Bombyx mori* by Pasteur in 1849 focused the attention of bacteriologists on insects and the discovery of a number of microbial diseases of insects (Sanchis, 2010).

Bacillus thuringiensis is an aerobic Gram-positive endospore-forming bacterium which is a part of the family *Bacillaceae* and it is widely used in agriculture as a biological pesticide (Aronson *et al*., 1986; Höfte and Whiteley, 1989; Feitelson *et al*., 1992). *B. thuringiensis* was isolated from

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infected silkworms (*B. mori*) by the Japanese Ishiwata (1901). Later, in 1911, the bacterium was found by Berliner, who separated it from contaminated chrysalids of *Ephestia kuehniella*, collected in the province of Thuringe (Germany). Berliner named it *Bacillus thuringiensis* Berliner and reported the occurrence of a crystal within a *B. thuringiensis* strain. The insecticidal activity of this crystal was discovered later. Soon after that, *B. thuringiensis* was used as an entomopathogenic organism because small amounts of this bacterium killed insect larvae, and *B. thuringiensis* began to be used as an important tool for biological control of pest insects (Sanchis, 2010).

B. thuringiensis was used for the first time as an insect control in the late 1920s against *Lymantria dispar* larvae in the USA and against *Ostrinia nubilalis* larvae in Hungary. In 1938 the first *B. thuringiensis* commercial product, 'Sporéine', was produced in France and since then *B. thuringiensis* has been used in pest insect management for more than 70 years (Sanchis, 2010). The importance of *B. thuringiensis* is that it can produce an insecticidal crystal protein which is toxic to pest insects. The crystal inclusion, named Cry, consists of a union of polypeptides with a molecular mass of between 27 kDa and 140 kDa (Höfte and Whiteley, 1989). These crystals represent 20–30% of the total protein after the processes of cellular lysis and spore liberation. Cry proteins are the active ingredient for commercial preparations (Sanchis, 2010).

The crystals, after being ingested by susceptible insect larvae, cause cell death by toxaemia. Angus (1954) found that a suspension of crystals injected directly into the haemocoel was highly toxic, and he proposed the hypothesis that states that crystals, or their constituents, were protoxins that when being processed by enzymes in the gut of the insect would release a toxic compound. Lecadet and Martouret (1962), working with *Pieris brassicae* larvae, showed that after swallowing crystals the larvae liberates soluble toxic substances. Later studies allowed us to prove Angus' hypothesis.

The advantages of the use of bio-based *B. thuringiensis* are primarily derived from its high specificity against target pest insects and its safety for the environment, beneficial insects and other living organisms, including humans. Some disadvantages of using commercial bioinsecticides based on *B. thuringiensis* currently on the market are: (i) its relatively high cost; (ii) loss of viability of the bacteria over long periods of storage under unfavourable conditions; and (iii) fast denaturation of the toxin in the environment. The effectiveness of *B. thuringiensis* sprayed in the field is also affected by the coverage of the application, formulation type and susceptibility to washing by rainwater. Another problem is the appearance of resistant insects (Hernández-Fernández and López-Pazos, 2011).

In the 1970s it was commonly acknowledged that moths and butterflies (lepidopteran insects) were the only susceptible insect larvae of *B. thuringiensis*. Goldberg and Margalit (1977) reported that a new *B. thuringiensis* subspecies named *israelensis* killed mosquito and black fly larvae (dipteran insects). This was the first *B. thuringiensis* strain killing an insect other than a caterpillar (Goldberg and Margalit, 1977). *B. thuringiensis* subsp. *israelensis* was used for disease control, providing both medical and environmental benefits. Krieg *et al*. (1983) isolated a new subspecies of *B. thuringiensis* called subsp. *morrisoni* var. *tenebrionis*; this strain had tested activity against the larvae of beetles' species (coleopteran insects) and therefore this led to more commercial development of the bioinsecticide. Also, *B. thuringiensis* crystal proteins have activity against nematode pests that infect animals and plants. Furthermore, some Cry toxins have been reported to have biological activity against the larvae of Hymenoptera, Mallophaga and Acari and other invertebrates such Platyhelminthes (Schnepf *et al*., 1998). *B. thuringiensis* has shown activity against cancer cells, leukaemia and against protozoa of medical importance such as *Giardia lamblia* and *Plasmodium berghei* (Mizuki *et al*., 1996; Wei *et al*., 2003). The search for new *cry* genes, which encode toxins with new and/or better specificities, is one of the main objectives of the research programmes in *B. thuringiensis* due to the emergence and resistance of new insect pests (Schnepf *et al*., 1998; Cerda and Wright, 2002). A small number of toxins have a broader spectrum of activity that can control two or three orders of insects. For instance, Cry1Ba toxin is active against larvae of moths, flies and beetles (van Frankenhuyzen, 2009). The modern international market for pesticides (herbicides, insecticides, fungicides, nematicides and fumigants) is prized at US$25.3 billion. Biopesticides correspond to only 2.5% of

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this market but their shares were projected to increase to about 4.2%, or over US$1 billion, in 2010 (Ibrahim *et al*., 2010).

Heimpel (1967) proposed a permanent and official terminology for the different toxins produced by *B. thuringiensis* and introduced the term delta-endotoxins or insecticidal crystal proteins (ICPs). The classification of different isolates of *B. thuringiensis* was performed on the basis of serotyping according to the flagellar antigen H given by de Barjac and Franchon (1990). This classification was accepted and is now the basis for the classification of different strains of *B. thuringiensis*, although there is no strict relationship between strain serotype and crystaliferous protein type, and therefore its insecticidal activity. Periodically, new strains have been identified and to date, up to 92 serotypes and 84 serovars have been described and defined as subspecies (International Entomopathogenic Bacillus Centre, Pasteur Institute, Paris, France, 2010) (Table 8.1).

The genes encoding delta-endotoxin, called *cry* genes, have been cloned and sequenced. The study of these has permitted the organization of this information to give rise to a new classification based on amino acid sequence homology and specific pathotype. Hofte and Whiteley (1989) presented a classification with the sequence of 14 *cry* genes encoding delta-endotoxin of *B. thuringiensis*, into four major classes:

- CryI (lepidoptera-active);
- CryII (diptera-lepidoptera-active);
- CryIII (coleoptera-active); and
- CryIV (diptera-active).

In addition there is an unrelated class of Cyt proteins with cytolytic and mosquitocidal activity.

Efforts to identify new strains with more effective toxins represent an option for the control

Table 8.1. Characterization of *Bacillus thuringiensis* serovars according to the H antigen. (From de Barjac and Franchon, 1990; International Entomopathogenic Bacillus Centre, 2010.)

H antigen	Serovar	H antigen	Serovar	H antigen	Serovar
1	*thuringiensis*	19	*tochigiensis*	44	*higo*
2	*finitimus*	20a20b	*yunnanensis*	45	*roskidiensis*
3a3c	*alesti*	20a20c	*pondicheriensis*	46	*champaisis*
3a3b3c	*kurstaki*	21	*colmeri*	47	*wratislaviensis*
3a3d	*sumiyoshiensis*	22	*shandongiensis*	48	*balearica*
3a3d3e	*fukuokaensis*	23	*japonensis*	49	*muju*
4a4b	*sotto*	24a24b	*neoleonensis*	50	*navarrensis*
4a4c	*kenyae*	24a24c	*novosibirsk*	51	*xianguangiensis*
5a5b	*galleriae*	25	*coreanensis*	52	*kim*
5a5c	*canadiensis*	26	*silo*	53	*asturiensis*
6	*entomocidus*	27	*mexicanensis*	54	*poloniensis*
7	*aizawaï*	28a28b	*monterrey*	55	*palmanyolensis*
8a8b	*morrisoni*	28a28c	*jegathesan*	56	*rongseni*
8a8c	*ostriniae*	29	*amagiensis*	57	*pirenaica*
8b8d	*nigeriensis*	30	*medellin*	58	*argentinensis*
9	*tolworthi*	31	*toguchi*	59	*iberica*
10a10b	*darmstadiensis*	32	*cameroun*	60	*pingluonsis*
10a10c	*londrina*	33	*leesis*	61	*sylvestriensis*
11a11b	*toumanoffi*	34	*konkutian*	62	*zhaodongensis*
11a11c	*kyushuensis*	35	*seoulensis*	63	*bolivia*
12	*thompsoni*	36	*malaysiensis*	64	*azorensis*
13	*pakistani*	37	*andaluciensis*	65	*pulsiensis*
14	*israelensis*	38	*ozwaldocruzi*	66	*graciosensis*
15	*dakota*	39	*brasiliensis*	67	*vazensis*
16	*indiana*	40	*huazhongensis*	68	*thailandensis*
17	*tohokuensis*	41	*sooncheon*	69	*pahangi*
18a18b	*kumamotoensis*	42	*jinhongiensis*	70	*sinensis*
18a18c	*yosoo*	43	*guiyangiensis*	71	*jordanica*

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of insects. This is how the list of 14 dissimilar *cry* genes sequences (Hofte and Whiteley, 1989) has increased dramatically from the 29 published in March 1992 (Feitelson *et al.*, 1992) to 51 presented in 1998 (Schnepf *et al.*, 1998). The discovery of 29 new genes encoding toxic proteins of *B. thuringiensis* added the CryV and CryVI classes (nematode active) to the classification (Feitelson *et al.*, 1992).

With the same 51 sequences of *cry* genes, Crickmore *et al.* (2014) presented a new nomenclature based on amino acid sequence homology of the *cry* genes. Comparing the sequences, 18 different groups were determined. Nowadays, there are families from Cry1 to Cry73 and a Cyt3 group of proteins, and these are separated into different subgroups (A, B, C, etc.). This information is constantly being updated and is available at the following URL: http://www.lifesci.sussex.ac.uk/Home/Neil_Crickmore/Bt/.

8.2 Biodiversity of Toxin Proteins

The variety of known Cry proteins is the result of a continuing effort in many countries to isolate and characterize new strains of *B. thuringiensis* with the goal of finding novel toxins which can control the pests with significant agronomical and medical implications (Hernández-Fernández and López-Pazos, 2011). The strains have been isolated from different environments such as soil (DeLucca *et al.*, 1981; Martin and Travers, 1989; Hossain *et al.*, 1997; Hernández-Fernández *et al.*, 2011), stored grain dusts (DeLucca *et al.*, 1984), mills (Meadows *et al.*, 1992), foods (Rosenquist *et al.*, 2005; Frederiksen *et al.*, 2006), insect cadavers (Hansen *et al.*, 1998; Eilenberg *et al.*, 2000), freshwater (Espinasse *et al.*, 2003), phylloplanes of conifer and deciduous leaves (Collier *et al.*, 2005; Bizzarri and Bishop, 2007), rhizosphere (Manonmani *et al.*, 1991), annelids (Hendriksen and Hansen, 2002), crustaceans (Swiecicka and Mahillon, 2006), insectivorous mammals (Swiecicka *et al.*, 2002) and the mangrove ecosystem (Bolívar and Hernández-Fernández, 2012). Characterization of native strains of *B. thuringiensis*, globally, has allowed recognition of multiple gene combinations that are more common than the others. The amazing variety of Cry toxins is supposed to be due to a high degree of genetic plasticity (Schnepf *et al.*, 1998). *B. thuringiensis* is typifed by the occurrence of a parasporal body, a crystal that is responsible for the toxic action of the bacteria. This crystal consists of one or more proteinaceous toxins, commonly called delta-endotoxins or Cry proteins, which are well known for their insecticidal properties. The Cry proteins are composed of several polypeptides bound together; the polypeptides have molecular masses ranging from 27 kDa to 140 kDa (Hofte and Whiteley, 1989) and vary between 369 (Cry34) and 1344 amino acids (Cry43) in length (Adang *et al.*, 2014). These crystals make up from 20% to 30% of the total protein after cell lysis and the release of the spore and the crystals. Conditional on the structure of the insecticidal proteins, the crystals occur in different shapes, such as bipyramidal, cuboidal, flat rhomboid or a compound with two or more crystal types. The genes that codify for the delta-endotoxins are situated on plasmids, which are replicating extra-chromosomal DNA and may be moved by conjugation between dissimilar strains of *B. thuringiensis*. Additionally, the plasmids are moderately large and may have one-quarter of the genetic coding capacity of the bacterial chromosome (Klier *et al.*, 1982; Carlton and Gonzalez, 1985).

A wide biodiversity of Cry toxins have been classified according to their primary amino acid sequences (981 different Cry proteins) into 73 groups (Cry1–Cry73) (Table 8.2) (Crickmore *et al.*, 2014). These Cry protein sequences have been classified into four phylogentically non-related protein families that may have dissimilar modes of action: (i) three domain Cry toxins (3D); (ii) the mosquitocidal Cry toxins (Mtx); (iii) the binary-like Cry toxins (Bin); and (iv) the Cyt family toxins (Table 8.3) (Bravo *et al.*, 2013). Cry1 toxins are part of the three domain family. These toxins are the most diverse group of Cry proteins. Cry1 proteins are synthesized in protoxin form and must be processed by proteases to produce an active toxin, including protoxins of 70–130 KDa (Roh *et al.*, 2007). To date, ten three-dimensional structures of toxins of *B. thuringiensis* have been described by X-ray crystallography: Cry1Aa (Grochulski *et al.*, 1995), Cry2Aa (Morse *et al.*, 2001), Cry3Aa (Li *et al.*, 1991), Cry3Bb (Galitsky *et al.*, 2001), Cry4Aa (Boonserm *et al.*, 2006), Cry4Ba (Boonserm *et al.*, 2005), Cyt2Ba (Cohen *et al.*, 2008), Cyt2Aa (Li *et al.*, 1996), Cry46Aa/parasporin-2 (Akiba *et al.*, 2009) and Cry8Ea (Guo *et al.*, 2009).

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Table 8.2. *B. thuringiensis* Cry protein groups and their diversity. (From Crickmore *et al*., 2014.)

Families of *Bt* proteins	Sequences reported	Families of *Bt* proteins	Sequences reported	Families of *Bt* proteins	Sequences reported
Cry1	297	Cry25	1	Cry49	5
Cry2	112	Cry26	1	Cry50	3
Cry3	100	Cry27	1	Cry51	2
Cry4	48	Cry28	2	Cry52	2
Cry5	39	Cry29	2	Cry53	2
Cry6	26	Cry30	13	Cry54	5
Cry7	42	Cry31	11	Cry55	3
Cry8	57	Cry32	28	Cry56	4
Cry9	36	Cry33	1	Cry57	2
Cry10	5	Cry34	12	Cry58	1
Cry11	8	Cry35	11	Cry59	2
Cry12	1	Cry36	1	Cry60	6
Cry13	1	Cry37	1	Cry61	3
Cry14	2	Cry38	1	Cry62	1
Cry15	1	Cry39	1	Cry63	1
Cry16	1	Cry40	4	Cry64	1
Cry17	1	Cry41	4	Cry65	2
Cry18	3	Cry42	1	Cry66	2
Cry19	3	Cry43	7	Cry67	2
Cry20	4	Cry44	1	Cry68	1
Cry21	10	Cry45	1	Cry69	3
Cry22	7	Cry46	3	Cry70	3
Cry23	1	Cry47	1	Cry71	1
Cry24	3	Cry48	5	Cry72	1
				Cry73	1

Some strains make extra insecticidal toxins, called Vip (vegetative insecticidal proteins) that are secreted during the vegetative phase of growth (Estruch *et al*., 1996) and have wide toxicity against lepidopteran species. Vip proteins were first described in the mid-1990s, and the rate of discovery of related toxins has increased in a similar fashion to that seen with the Cry toxins a decade before. Genetically engineered products expressing Vip3A are being analysed in cotton and maize plants. Even though it has related properties to the delta-endotoxins, the Vip3A toxin has not been classified as a delta-endotoxin. Today, over 120 Vip proteins have been reported and these are represented by four families of well-structured proteins (Table 8.3) (Crickmore *et al*., 2014).

Other members include those of the Cyt family (cytolytic), a group of dipteran proteins (Table 8.3) (Crickmore *et al*., 2014). Although the families' proteins are unrelated to each other, the Cyt and Cry proteins interact to potentiate their effects, in a synergistic manner, against certain species of mosquitoes and black flies (Federici, 1999; Butko, 2003; Soberón *et al*., 2007), for example Cry1Ac1 and Cyt1A1 toxicity to *Trichoplusia ni* both *in vitro* and *in vivo*.

Even though the most comprehensively studied members of the Cry family are insecticidal, nematocidal toxins (Wei *et al*., 2003) and other Cry proteins known as parasporins are specifically active against certain cancer cells (Ohba *et al*., 2009) (Table 8.3).

B. thuringiensis produce other insecticidal molecules called beta-exotoxin or thuringiensin (Lambert and Peferoen, 1992). These molecules are toxic against non-target organisms as well as mice, some aquatic insects and fish (Beegle and Yamamoto, 1992). Beta-exotoxin contributes to the insecticidal toxicity against lepidopteran, dipteran and coleopteran insects (Crickmore *et al*., 2005) and as it is toxic to almost all other forms of life its occurrence is prohibited in *B. thuringiensis* microbial products (US EPA, 2007).

The Cry toxin has three domains from the amino (N) terminus to the carboxyl (C) terminus:

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Table 8.3. List of toxins and their classification based on homology groups of *B. thuringiensis*. (From Krishnan, 2013; Crickmore *et al*., 2014.)

Cry1Aa	Cry1Ab	Cry1Ac	Cry1Ad	Cry1Ae	Cry1Af	Cry1Ag	Cry1Ah	Cry1Ai	Cry1Ba	Cry1Bb				
Cry1Bc	Cry1Bd	Cry1Be	Cry1Bf	Cry1Bg	Cry1Bh	Cry1Bi	Cry1Ca	Cry1Cb	Cry1Da	Cry1Db	Cry1Dc	Cry1Ea		
Cry1Eb	Cry1Fa	Cry1Fb	Cry1Ga	Cry1Gb	Cry1Gc	Cry1Ha	Cry1Hb	Cry1Ia	Cry1Ib	Cry1Ic				
Cry1Id	Cry1Ie	Cry1If	Cry1Ig	Cry1Ja	Cry1Jb	Cry1Jc	Cry1Jd	Cry1Ka	Cry1La	Cry1Ma	Cry1Na	Cry1Nb		
Cry2Aa	Cry2Ab	Cry2Ac	Cry2Ad	Cry2Ae	Cry2Af	Cry2Ag	Cry2Ah	Cry2Ai	Cry2Aj	Cry2Ak	Cry2Ba			
Cry3Aa	Cry3Ba	Cry3Bb	Cry3Ca	Cry4Aa	Cry4Ba	Cry4Ca	Cry4Cb	Cry4Cc						
Cry5Aa	Cry5Ab	Cry5Ac	Cry5Ad	Cry5Ba	Cry5Ca	Cry5Da	Cry5Ea	Cry6Aa	Cry6Ba	Cry7Aa	Cry7Ab	Cry7Ba	Cry7Bb	
Cry7Aa	Cry7Ca	Cry7Cb	Cry7Da	Cry7Ea	Cry7Fa	Cry7Fb	Cry7Ga	Cry7Gb	Cry7Gc	Cry7Gd	Cry7Ha	Cry7Ia	Cry7Ja	Cry7Ka
Cry8Aa	Cry8Ab	Cry8Ac	Cry8Ad	Cry8Ba	Cry8Bb	Cry8Bc	Cry8Ca	Cry8Da	Cry8Db	Cry8Ea	Cry8Fa	Cry8Ga		
Cry8Ha	Cry8Ia	Cry8Ib	Cry8Ja	Cry8Ka	Cry8Kb	Cry8La	Cry8Ma	Cry8Na	Cry8Pa	Cry8Qa	Cry8Ra	Cry8Sa	Cry8Ta	
Cry9Aa	Cry9Ba	Cry9Bb	Cry9Ca	Cry9Da	Cry9Db	Cry9Dc	Cry9Ea	Cry9Eb	Cry9Ec	Cry9Ed	Cry9Ee	Cry9Fa	Cry9Ga	
Cry10Aa	Cry11Aa	Cry11Ba	Cry11Bb	Cry12Aa	Cry13Aa	Cry14Aa	Cry14Ab	Cry15Aa	Cry16Aa	Cry17Aa	Cry18Aa	Cry18Ba	Cry18Ca	
Cry19Aa	Cry19Ba	Cry20Aa	Cry20Ba	Cry21Aa	Cry21Ba	Cry21Ca	Cry21Da	Cry22Aa	Cry22Ab	Cry22Ba	Cry22Bb	Cry23Aa	Cry24Aa	
Cry24Ba	Cry24Ca	Cry25Aa	Cry26Aa	Cry27Aa	Cry28Aa	Cry29Aa	Cry30Aa	Cry30Ba	Cry30Ca	Cry30Da	Cry30Db	Cry30Ea	Cry30Fa	Cry30Ga
Cry31Aa	Cry31Ab	Cry31Ac	Cry31Ad	Cry32Aa	Cry32Ab	Cry32Ba	Cry32Ca	Cry32Cb	Cry32Da	Cry32Ea	Cry32Eb	Cry32Fa	Cry32Ga	
Cry32Ha	Cry32Hb	Cry32Ia	Cry32Ja	Cry32Ka	Cry32La	Cry32Ma	Cry32Mb	Cry32Na	Cry32Oa	Cry32Pa	Cry32Qa	Cry32Ra	Cry32Sa	Cry32Ta
Cry32Ua	Cry33Aa	Cry34Aa	Cry34Ab	Cry34Ac	Cry34Ba	Cry35Aa	Cry35Ab	Cry35Ac	Cry35Ba	Cry36Aa	Cry37Aa	Cry38Aa	Cry39Aa	Cry40Aa
Cry40Ba	Cry40Ca	Cry40Da	Cry41Aa	Cry41Ab	Cry41Ba	Cry42Aa	Cry43Aa	Cry43Ba	Cry43Ca	Cry43Cb	Cry43Cc	Cry44Aa	Cry45Aa	
Cry46Aa	Cry46Ab	Cry47Aa	Cry48Aa	Cry48Ab	Cry49Aa	Cry49Ab	Cry50Aa	Cry50Ba	Cry51Aa	Cry52Aa				
Cry53Aa	Cry53Ab	Cry54Aa	Cry54Ab	Cry54Ba	Cry55Aa	Cry56Aa	Cry57Aa	Cry58Aa	Cry59Aa	Cry59Ba				
Cry60Aa	Cry60Ba	Cry61Aa	Cry62Aa	Cry63Aa	Cry64Aa	Cry65Aa	Cry66Aa	Cry67Aa	Cry68Aa	Cry69Aa	Cry62Ab			
Cry70Aa	Cry70Ba	Cry70Bb	Cry71Aa	Cry72Aa	Cry73Aa									
Cyt1Ab	Cyt1Aa	Cyt1Ba	Cyt1Ca	Cyt1Da	Cyt2Aa	Cyt2Ba	Cyt2Bb	Cyt2Bc	Cyt2Ca	Cyt3Aa				
Vip1Ab	Vip1Aa	Vip1Ac	Vip1Ba	Vip1Bb	Vip1Ca	Vip1Da			Sip1					
Vip2Ab	Vip2Aa	Vip2Ac	Vip2Ad	Vip2Ae	Vip2Af	Vip2Ba	Vip2Bb							
Vip3Ab	Vip3Aa	Vip3Ac	Vip3Ad	Vip3Ae	Vip3Af	Vip3Ag	Vip3Ah	Vip3Ba	Vip3Bb	Vip3Ca	Vip4Aa			

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(i) a seven helix bundle (Domain I); (ii) a triple anti-parallel β-sheet (Domain II); and (iii) a β-sheet sandwich of two anti-parallel β-sheets (Domain III). Domain I is implicated in membrane inclusion, toxin oligomerization and pore formation. Domain II is implicated in receptor recognition and Domain III is implicated in insect specificity by mediating specific exchanges with different insect gut proteins (Bravo *et al.*, 2011). A feature of all the *B. thuringiensis* Cry toxins is that they are built from five highly conserved sequence blocks, which indicates that all the proteins in this *cry* gene family adopted the same general fold. The protoxin and the active toxin sequences are mainly different due to their large C-terminal end which is highly conserved among some of the protoxin sequences (Hernández-Fernández and López-Pazos, 2011). The possible function of this long C-terminal segment is to help Cry proteins as they form an ordered structured crystalline strain (Schnepf *et al.*, 1998).

8.3 Mode of Action of *Bacillus thuringiensis* Cry Proteins

The most important point about Cry toxins in terms of their mode of action is their insect specificity. This characteristic is established by the specific binding of Cry toxins to surface proteins that are found in the microvilli of larvae midgut cells (Bravo *et al.*, 2011).

The mode of action of Cry proteins has been studied mainly using Cry1 proteins. Based on these studies, a model of the insecticidal activity was proposed (Gill *et al.*, 1992; Knowles and Dow, 1993; Pigott and Ellar, 2007; Bravo *et al.*, 2011; Adang *et al.*, 2014). The binding proteins in the model have been recognized as cadherin-like proteins, glycosylphophatidylinositol (GPI)-anchored aminopeptidase-N (APN), GPI-anchored alkaline phosphatase (ALP), a 270 kDa glycoconjugate and a 250 kDa protein named P252 (Pigott and Ellar, 2007). The process occurs as follows:

1. Ingestion of crystals by susceptible insects.
2. Dissolution of the crystals and liberation of the protoxin in the insect gut. Gut conditions must be alkaline in order to realize dissolution, where they break the disulfide bonds. This factor helps to determine potential toxicity and the host range of *B. thuringiensis*.
3. Proteolytic activation of the protoxin, producing a toxic fragment. The protoxin is activated through proteolytic cleavage by the insect midgut proteases, so that the mature toxin consists of the N-terminal part of the protoxin. In Cry1A protoxins the cleavage is done by chymotrypsin-like or trypsin-like proteases. The molecular weight of the protein decreases from about 130–140 kDa to 55–65 kDa (Sanchis, 2010).
4. This fragment 'toxin', through the peritrophic membrane, joins to specific receptors on the apical brush border of the midgut microvilliae in susceptible insects. The toxin interaction with the cadherin-like glycoprotein receptor (Zhang *et al.*, 2005) or/and aminopeptidase N (APN) (Yaoi *et al.*, 1999) induces the formation of the oligomer, which is responsible for membrane insertion and pore formation. In 2007, 38 different amino-peptidases were reported for 12 different lepidopteran insects (Pigott and Ellar, 2007).
5. The pore allows the passage of ions and water into the cells, as well as passage of monovalent cations into columnar cells, causing depolarization of the cell membrane until cell lysis and eventual death of the insect larvae (Sanchis, 2010). The lysis of the epithelial cells leads to paralysis of the insect's digestive system and it stops eating. The death of the insect occurs 1–3 days after ingestion of the crystals. The insects also ingest *B. thuringiensis* spores, which produces septicaemia due to the germination of the spores in the insect's haemocoel. This activity optimizes the toxic effect of the *B. thuringiensis* toxins (Pigott and Ellar, 2007; Bravo *et al.*, 2011; Adang *et al.*, 2014).

8.4 *B. thuringiensis* Cry toxins as Bioinsecticide Products

Commercial formulations of *B. thuringiensis* have been used for the last few decades in the control of lepidopteran insects and, in more recent times, beetles and flies. Many of these products are based on spore-crystal preparations derived from a small number of wild-type strains. The potency of these bioinsecticides is adjusted according to different patterns, such as the amount required to remove a larva of a species

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in 1 cm^2 of artificial diet; the rates are established by biological activity assays and the bioinsecticide concentration is expressed in international units (IU) (Lüthy, 1986). Some commercial preparations (e.g. Sandox and Javellin) take *Spodoptera* spp. as the reference insect, specifying the quantity of product to be applied per hectare. One milligram of a dry preparation of *B. thuringiensis* contains about 16,000 IU. When comparing the potency of these biopesticides with synthetic pyrethroids and organophosphates, we see that while synthetic pyrethroids and organophosphates are used at a rate of 3×10^{24} and 8×10^{26} molecules/ha, respectively, *B. thuringiensis*' products are applied in a quantity of 10^{22} molecules/ha. This means the biopesticide is between 300 and 80,000 times more potent than the two kinds of chemicals (Feitelson *et al.*, 1992).

On the other hand, there are some disadvantages to the use of *B. thuringiensis*-based biopesticides, including: (i) they have a delayed action; (ii) they have a reduced host range; (iii) they have narrow persistence in the field (mainly as a result of the impact of solar radiation and temperature); and (iv) they do not reach insects that attack roots or internal parts of the plant.

With the purpose of resolving some of these issues, some recombinant DNA techniques have been used to produce transgenic bacteria. Among soil bacteria which have been transformed with the genes of *B. thuringiensis* are *Pseudomonas cepacia* (Stock *et al.*, 1990), *Pseudomonas fluorescens* (Waalwijk *et al.*, 1991), *Agrobacterium radiobacter* (Obukowicz *et al.*, 1986), *Bradyrhizobium* sp. (Nambiar *et al.*, 1990) and the endophyte *Clavibacter xyli* subsp. *cynodontis* (Turner *et al.*, 1991). Using these transgenic bacteria has extended the distribution range of Cry proteins to occupy other ecological niches, for example *Clavibacter* is an endophytic bacterium that grows in the vascular tissue of maize and other plants, and *Pseudomonas* or *Agrobacterium* grow in the rhizosphere.

Andy Barrero and Frank Gaertner of the Mycogen Corporation developed a transgenic bacterium, *Pseudomonas*, transformed with a *cry* gene of *B. thuringiensis*. This bacterium produces insecticidal crystal fermentation. *Pseudomonas* does not sporulate hence the crystals are not released as strains of *B. thuringiensis*. Finally, for the fermentation a fixative is added to kill the cells which contain the Cry proteins. This product has been approved for marketing in the USA (the product is CellCap™ of the Mycogen Corporation).

A diverse number of strains of *B. thuringiensis* have been identified that contain different *cry* genes and two subspecies have been developed into biopesticide products (*kurstaki* and *aizawaï*) to control lepidopteran pests. The commercial products arising from these strains include Dipel®, Javelin®, Thuricide®, Worm Attack®, Caterpillar Killer® and Bactospeine®, but many small companies sell similar products under a variety of trade names (Table 8.4).

Bacillus thuringiensis var. *israelensis* (Bti) is highly toxic to mosquito and blackfly larvae, both vectors of malaria, onchocercosis and dengue fever. For this reason, Bti is used for the control of mosquitoes and blackfly. Many commercial *B. thuringiensis* products that utilize Bti are also available; among them VectoBac®, TeKnar®, Bactimos® and Skeetal® (Table 8.4) (Sanchis, 2010).

8.5 Development of Transgenic *B. thuringiensis* Crops

In the 1980s, several scientists demonstrated that plants can be genetically engineered and this led to the advance of transgenic *B. thuringiensis* plants. The method used to introduce foreign DNA into plants was a transformation technique based on the transfer of the Ti plasmid from *Agrobacterium tumefaciens* (Sanchis and Bourguet, 2008). The Plant Genetic Systems Company developed a genetically engineered plant expressing a *cry1Ab* gene to produce plants that were resistant to insect attack; however, it did not produce sufficient expression of the insecticidal proteins (Vaeck *et al.*, 1987). Later, other scientists transformed *B. thuringiensis* genes into tomato and cotton plants. The transformation with *A. tumefaciens* was restricted to dicotyledonous plants. The advance of innovative methods of transformation, such as electroporation or particle bombardment, made it possible to transform monocotyledonous plants (Koziel *et al.*, 1993). Several advances have occurred since (Sanchis and Bourguet, 2008), among them the most important are:

1. The *cry1A* toxin gene was used with the cauliflower mosaic virus (CaMV) 35S promoter.

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Table 8.4. *B. thuringiensis*-based bioinsecticides toxic to lepidopteran, coleopteran and dipteran insects. (Adapted from Sauka and Benintende, 2008, and Sansinenea, 2012.)

Commercial name	Company	Serovar from *B. thuringiensis*	Order of insect	Cry protein[a]
Dipel, BiobitXL, Foray	Abbott Labs	*kurstaki*	Lepidoptera	1Aa, 1Ab, 1Ac, 2Aa, 2Ab
Florbac, Xentari		*aizawaï*	Lepidoptera	1Aa, 1Ab, 1Ac, 2Aa, 2Ab
Futura		*kurstaki*	Lepidoptera	1Aa, 1Ab, 1Ac, 2Aa, 2Ab
Bernan Bt	Bactec	*kurstaki*	Lepidoptera	1Aa, 1Ab, 1Ac, 2Aa, 2Ab
Bathurin	Chamapol-Biokrna	*thuringiensis*	Lepidoptera	NI
Bactis	Compagnia di Recerca chim. (CRC)	*kurstaki*	Lepidoptera	1Aa, 1Ab, 1Ac, 2Aa, 2Ab
Biospor	Farbwerbe-Hoechst	*kurstaki*	Lepidoptera	1Aa, 1Ab, 1Ac, 2Aa, 2Ab
Cutlass	Fermenta ASC Co.	*kurstaki*	Lepidoptera	1Aa, 1Ab, 1Ac, 2Aa, 2Ab
Muscabac	Farmos	*thuringiensis*	Lepidoptera	β-exotoxina
DendroBacillin	Glavmikro-bioprom	*dendrolimus*	Lepidoptera	NI
EndoVacterin		*galleriae*	Lepidoptera	1Cb
Insektin		*thuringiensis*	Lepidoptera	1Ba
Mianfeng pesticide	Huazhong Agricultural University	YBT-1520	Lepidoptera	NI
Shuangdu preparation		*chinesensis* Ct-43	Lepidoptera and Coleoptera	NI
Bitayon	Jewin-Joffe Industry Limited	ND	Lepidoptera	NI
Larvo-Bt	Knoll Bioproducts	*kurstaki*	Lepidoptera	1Aa, 1Ab, 1Ac, 2Aa, 2Ab
Bt	Korea Explosives Kyowa-Hakko Kogyo Co.	*kurstaki*	Lepidoptera	1Aa, 1Ab, 1Ac, 2Aa, 2Ab
Selectgyn		*aizawaï*	Lepidoptera	1Aa, 1Ab, 1Ba, 1Ca, 1Da
Sporoine	LIBEC	*kurstaki*	Lepidoptera	1Aa, 1Ab, 1Ac, 2Aa, 2Ab
M-Peril	Mycogen	*kurstaki*	Lepidoptera	1Aa, 1Ab, 1Ac, 2Aa, 2Ab
Biobit Foray	Novo Nordisk	*kurstaki*	Lepidoptera	1Aa, 1Ab, 1Ac, 2Aa, 2Ab
SOK	Nor-Am Chemical	*kurstaki*	Lepidoptera	1Aa, 1Ab, 1Ac, 2Aa, 2Ab
Bactospeine	Philips	*kurstaki*	Lepidoptera	1Aa, 1Ab, 1Ac, 2Aa, 2Ab
Plantibac	Procida	*kurstaki*	Lepidoptera	1Aa, 1Ab, 1Ac, 2Aa, 2Ab
Baturad	Radonja	*kurstaki*	Lepidoptera	1Aa, 1Ab, 1Ac, 2Aa, 2Ab
Nubilacid		*kurstaki*	Lepidoptera	1Aa, 1Ab, 1Ac, 2Aa, 2Ab
Bt 8010, Rijan	Scientific & Technology Developing	ND	Lepidoptera	NI

Continued

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Table 8.4. Continued.

Commercial name	Company	Serovar from *B. thuringiensis*	Order of insect	Cry protein[a]
Delfin, Thuricide	SDS Biotech KK	ND	Lepidoptera	NI
Able	Thermo, Trilogy Coporation	*kurstaki*	Lepidoptera	NI
Thuricide		*kurstaki*	Lepidoptera	1Aa, 1Ab, 1Ac, 2Aa, 2Ab
Javelin, Delfin		*kurstaki*	Lepidoptera	1Aa, 1Ab, 1Ac, 2Aa, 2Ab
CoStar		*kurstaki*	Lepidoptera	1Aa, 1Ab, 1Ac, 2Aa, 2Ab
Steward		*kurstaki*	Lepidoptera	1Aa, 1Ab, 1Ac, 2Aa, 2Ab
Vault		*kurstaki*	Lepidoptera	1Aa, 1Ab, 1Ac, 2Aa, 2Ab
Basillex	Shionogi Co.	*thuringiensis*	Lepidoptera	β-exotoxina
Bactur	Thomsoni Hayward Co.	*kurstaki*	Lepidoptera	1Aa, 1Ab, 1Ac, 2Aa, 2Ab
Toaro, Toaro-Ct	Towagosei Chem	*kurstaki*	Lepidoptera	1Aa, 1Ab, 1Ac, 2Aa, 2Ab
Spicturin	Tuticorin Alcali Chemicals and Fertilisers Limited	*galleriae*	Lepidoptera	1Cb
Tobaggi	Dongbu Hannong Chemicals	*aizawaï*	Lepidoptera	NI
Solbichae	Gree Biotech Co.	*aizawaï*	Lepidoptera	NI
Selectgyn	Kyowa-Hakko Kogyo Co.	*aizawaï*	Lepidoptera	NI
Gnatrol	Valent Bioscience Co.	*israelensis*	Diptera	NI
Skeetal	Novo Nordisk	*israelensis*	Diptera	NI
Moskitur	JZD Slusovice	*israelensis*	Diptera	NI
Baktokulicid	VPO Biopreparat	*israelensis*	Diptera	NI
Bactimos, VectoBac, TeKnar	Valent Bioscience Co.	*israelensis*	Diptera	NI
M-One, M-Trak	Mycogen	*morrisoni* or *tenebrionis*	Coleoptera	NI
Trident, Trident II	Sandoz Agro Inc.	*morrisoni* or *tenebrionis*	Coleoptera	NI
Di Terra	Valent Bioscience Co.	*morrisoni* or *tenebrionis*	Coleoptera	NI
Novodor	Novo Nordisk	*morrisoni* or *tenebrionis*	Coleoptera	NI
Foil	Ecogen Inc.	*morrisoni* or *tenebrionis*	Coleoptera and Lepidoptera	NI

[a]NI, No information.

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However, low levels of expression of the Cry toxin protein and therefore reduced protection against insect damage were reported (Barton *et al*., 1987).
2. The toxic N-terminal element of the protein was expressed in plants for improvement of expression Cry proteins (Fischhoff *et al*., 1987). However, the protein level was low.
3. High adenine and thymine content of *cry* genes led to aberrations in mRNA processing and translation (Ely, 1993). To enhance the expression of *cry* genes in plants, DNA sequence modifications were executed to remove polyadenylation signal sites without changing the encoded amino acid sequence of the toxin.
4. Site-directed mutagenesis improved toxin protein production in tobacco from 0.02% or the minimal insecticidal level (Perlak *et al*., 1991). These levels were insufficient for consistent control of pests in transgenic crops produced in the field.
5. DNA resynthesis of *cry* genes enabled enough levels of expression of the toxin protein to stabilize the control of insect pests in field conditions to be obtained.
6. Levels of expression were between 0.2% and 1% of soluble protein. More successful plants, that used synthetic genes designed to be more compatible with plant expression, were introduced a few years later (Koziel *et al*., 1993).
7. Higher levels of expression were reached by transforming tobacco chloroplasts (McBride *et al*., 1995).
8. The US Environmental Protection Agency (EPA) accepted the first registration of *B. thuringiensis* potato, maize and cotton products expressing toxin genes at potentially commercially reliable levels (produced by Syngenta Seeds and Mycogen Seeds in 1995). This included two transgenic maize hybrids expressing a *cry1Ab* toxin gene active against *Ostrinia nubilalis* (Hbn.). These varieties were rapidly discontinued.

After these events, insertion of *cry* genes of *B. thuringiensis* into crop plants became more common. For example two maize varieties expressing the Cry1Ab1 toxin (in 1996 by Northrup King and Monsanto) were released with the product names Agrisure and YieldGard, respectively. The first transgenic cotton expressing a modified Cry1Ac toxin was also released (in 1995 by Monsanto), named Bollgard and Ingard. Later, novel transgenic maize expressing the Cry1F protein was developed (in 2001 by Pioneer Hi-Bred and Dow Agro-Sciences) and marketed with the name Herculex I. The insect pests controlled by these products are: *Agrotis ipsilon* (Hufn.), *Spodoptera frugiperda* (Smith) and *O. nubilalis* (Hbn.).

B. thuringiensis cotton expressed two toxins, Cry1AC and Cry2Ab, active against different lepidopteran pests of cotton (released in 2002 by Monsanto). Another maize variety (also named YieldGard) resistant to *Diabrotica virgifera virgifera* (Lec.) was released in 2003 by Monsanto. It expressed a synthetic version of the wild-type Cry3Bb1 toxin gene active against Coleoptera. Promptly, a YieldGard Plus was developed (in 2003 by Monsanto) which contained both Cry-1Ab1 and Cry3Bb1 toxins, for control of lepidopteran and coleopteran insects.

In 2005, Dow AgroSciences and Pioneer Hi-Bred released Hercule RW, a maize variety expressing the binary toxins of 14 kDa and 44 kDa proteins, Cry34Ab1/Cry35Ab1, respectively, active against insect pests of the Chrysomelidae family.

Estruch *et al*. (1996) discovered *B. thuringiensis* Vip3A, in the vegetative insecticidal protein class, which has toxicity against lepidopteran insects. Then, cotton and maize plants expressing Vip3A were evaluated.

Since 1996 the use of these *B. thuringiensis* insect-resistant crops has led to a considerable reduction in pesticide application and cost savings for growers and the confidence in the remuneration of *B. thuringiensis* crops has rapidly increased across the world, except in Europe (Sanchis, 2010).

The global number of hectares used for biotech crops has increased more than 100-fold from 1.7 million ha in 1996 to over 175 million ha in 2013 – demonstrating the rapid adoption of biotech crops that talks for itself of the benefits it produces for farmers and clients (James, 2013).

In the 18-year period, 1996–2013, millions of farmers in ~30 countries worldwide, have grown more than 1.6 billion ha of transgenic crops. This is a region comparable to > 150% the size of the total land mass of the USA or China which is an enormous area (James, 2013). There is one principal and crushing motive that underpins the confidence of risk-averse

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farmers to biotechnology – biotech crops distribute substantial, and sustainable, socio-economic and environmental benefits (James, 2013). A comprehensive USA study carried out in Europe in 2011, confirmed that biotech crops are sustainable (James, 2013). Biotech crops are contributing to sustainability by:

- contributing to food, feed and fibre security and self-sufficiency, including more affordable food, by increasing productivity and economic benefits sustainably at the farmer level;
- conserving biodiversity;
- reducing agriculture's environmental footprint;
- by increasing efficiency of water usage which will have a major impact on conservation and availability of water globally; and
- helping mitigate climate change and reduce greenhouse gases (Brookes and Barfoot, 2012; James, 2013).

In 2013, 18 million farmers, compared with 17.3 million in 2012, cultivated transgenic plants (James, 2013). Latin American, Asian and African farmers collectively grew 94 million ha or 54% of the global 175 million biotech ha (52% in 2012). This is the opposite of the situation predicted by critics who declared that biotech crops were for industrial countries and would never be established and adopted by developing countries, and principally small poor farmers (James, 2013). Brazil is now second only to the USA with regards to cultivation of transgenic crops.

From 1996 to 2012, biotech crops have contributed to food safety, sustainability and climate change by:

- increasing crop production by an estimated US$116.9 billion;
- providing a superior environment, by saving 497 million kg of pesticides;
- reducing CO_2 emissions by 26.7 billion kg in 2012 alone;
- conserving biodiversity in the period 1996–2012 by saving 123 million ha of land; and
- helping alleviate poverty by assisting > 16.5 million small farmers and their families (a total of > 65 million people), who are some of the poorest people in the world (James, 2013).

8.6 Protein Engineering in *B. thuringiensis* Toxins

Mutagenesis of Cry toxins of *B. thuringiensis* has been used to produce new recombinant toxins (Bravo *et al*., 2007). Joining domains from different Cry proteins may perhaps generate active toxins with original specificities (Gatehouse, 2008). For example a fusion Cry protein, with domains I and III of Cry1Ba and domain II of Cry1Ia (proteins with activity against lepidopteran insects only) conferred resistance against both lepidopteran and coleopteran insect pests *Phthorimaea operculella* and *Leptinotarsa decemlineata*, respectively, in transgenic potato plants (Naimov *et al*., 2003). The domain III swapping along with different Cry toxins domains has resulted in novel recombinant hybrid toxins with enhanced toxicities against insects (Bosch *et al*., 1994). Assembly of a hybrid toxin containing domains I and II of Cry1Ab toxin and domain III of Cry1C conferred a sixfold higher toxicity against *Spodoptera exigua* (de Maagd *et al*., 2000). Another case was the combining of domains I and II of Cry3Aa and domain III from Cry1Ab (eCry3.1Ab) which presented as being toxic to *Diabrotica virgifera*, in contrast with the two toxins that showed no toxicity to this insect (Walters *et al*., 2010). These results suggest that domain III swapping may perhaps be an interesting approach to improve toxicity of Cry toxins or to make novel hybrid toxins with toxicity against pests that show no susceptibility to the parental Cry toxins (Bravo *et al*., 2013).

An alternative to the 'domain swap' approach has been the use of *B. thuringiensis* toxins modified by site-directed mutagenesis to increase toxicity to target pests (Gatehouse, 2008). Domain II in three-domain Cry proteins has been used for such modifications, involving mutation of amino acid residues in the loop regions of this domain (Gatehouse, 2008). Mutation of Cry1Ab augmented its toxicity to larvae of *Lymantria dispar* by up to 40-fold (Rajamohan *et al*., 1996).

The enhancement of the insecticidal activity by site-directed mutagenesis of the binding epitopes found in domains II and III has a lot of potential for selection of Cry toxins with superior activity against diverse insect pests (Bravo *et al*., 2013).

Phage display technology for producing toxins with enhanced binding to a receptor, has been developed (Azzazy and Highsmith, 2002; Mullen *et al*., 2006; Ishikawa *et al*., 2007). The distinct

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protein DNA sequence is combined with a coat protein gene allowing the fusion protein to be displayed on the surface of the phage that can interact with ligands, a method known as biopanning (Bravo *et al.*, 2013). This technique maintains a connection between the shown protein and the encoding gene and enables more mutagenesis and choice by *in vitro* high throughput molecular evolution of proteins (Droge *et al.*, 2003).

A transgenic maize variety with resistance to *Diabrotica virgifera* expresses a modified adaptation of the Bt Cry3Bb1 toxin (Vaughn *et al.*, 2005). A large number of variants of the native Cry3Bb, with specific mutations, were directed to improve the channel-forming capacity of the toxin (English *et al.*, 2003). These mutations were perfomed to: (i) enhance the hydrophobicity of the protein; (ii) augment the mobility of the channel-forming; (iii) add to the mobility and plasticity of loop regions in domain I; (iv) modify ion pair interactions and metal binding sites; and (v) decrease or remove binding to carbohydrates in the insect gut. The toxicity of the protein to *D. virgifera* was augmented approximately eightfold (Gatehouse, 2008).

Hybrid toxins which include substitutions of domain III have produced novel toxins with a wider spectrum or higher toxicity against new targets of insect, when compared with the parental toxins. Several Cry1 toxins (Cry1Ab, Cry1Ac, Cry1Ba and Cry1Ea) with low specificity against *Spodoptera exigua* become active when their domain III is substituted by that of Cry1Ca (Pardo-López *et al.*, 2006). The Cry1Ab protein is not toxic to *S. exigua* but the hybrid toxin containing domain III of Cry1C showed ten times higher toxicity against *S. exigua* than the parental Cry1C toxin (Pardo-López *et al.*, 2006). In another work, replacing domain III of Cry1Aa with that of Cry1Ac produced 300-fold more toxicity against *Heliothis virescens* (Caramori *et al.*, 1991). It has been suggested that domain III may have occurred in nature as an evolutionary mechanism to generate toxins with new specificities (Bravo, 1997; Pardo-López *et al.*, 2006).

8.7 Conclusion and Future Prospects

The Cry toxins are friendly to the environment and are an ideal insecticide against different target pests, besides, the development of resistance is low. However, some pest insects are not controlled effectively by the Cry toxins discovered to date. It is necessary to continue studies and research to isolate and characterize novel proteins, or use recombinant DNA and engineering of proteins (site directed mutagenesis methods) to change the sequences of *B. thuringiensis cry* genes to develop proteins, that have new and improved activity against agricultural and pests.

Colombia has made several collections of native strains of *B. thuringiensis*. Among these Jorge Tadeo Lozano University, CORPOICA (the Colombian Corporation for Agricultural Research), the National University of Colombia and the Corporation for Biological Research have been conducting research since 1992 to find novel *B. thuringiensis* strains that have a capacity to produce delta-endotoxins with a wider action spectrum.

Hernández-Fernández *et al.* (2011), at Jorge Tadeo Lozano University's Molecular Biology Laboratories, collected soil samples from various regions and different ecosystems, in five departments of Colombia. They isolated about 200 strains of *B. thuringiensis* and characterized them at the microscopic, biochemical, molecular and biological level. The main goal of this investigation was to identify novel native *B. thuringiensis* strains that contained *cry1* genes in order to control *Tuta absoluta* larvae. The characterized strains had amorphous, bi-pyramidal, square, round and triangular crystal forms. Eighteen morphological groups with different crystal combinations were established, showing high biodiversity. The Colombian native strains revealed protein bands ranging from 28 kDa to 150 kDa, and between one and five protein bands were observed for individual native strains (Hernández-Fernández *et al.*, 2011).

Molecular characterizations permit the identification of between one and five specific *cry1* genes in each of the native *B. thuringiensis* strains, producing 13 different profiles. The characterization of novel native *B. thuringiensis* strains from Colombia contributes to an appreciation of the high *B. thuringiensis* biodiversity in Colombian agricultural areas. Regarding toxicity, ten native *B. thuringiensis* strains were tested against second instar larvae of *T. absoluta*. The native *B. thuringiensis* strain ZCUJTL11 showed the highest potential to develop a microbiological control method

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against *T. absoluta*. This strain showed threefold more toxicity than the reference strain *B. thuringiensis* subspecies *kurstaki* (Hernández-Fernández *et al*., 2011).

Eco-friendly strategies with environmental benefits used for controlling insects, based on the *B. thuringiensis* Cry proteins, should increase in the future, in particular with the broad implementation of transgenic crops. The detection of new toxins and new ways of presenting the toxin to susceptible insects, which includes the development of recombinant microorganisms and proteomic technology, may perhaps be modified by the investigation of *B. thuringiensis* Cry proteins. Furthermore, interaction studies between Cry toxins and pest insects connecting modes of action and resistance methods should be carried out. Such research studies are essential in order to permit the progress of existing *B. thuringiensis* application strategies and the capacity to design the most well-organized options.

B. thuringiensis Cry toxins represent an important tool for insect control and the increase of transgenic plants that are self-resistant to insect attack. The use of this knowledge has been successful because it has reduced the use of chemical insecticides that pollute the environment and agricultural products. Identification of new and more powerful Cry proteins can help in the development of new biopesticides and genetically engineered plants and also provide a reservoir of Cry toxins that can be used in the event of emergence of resistance.

Future important work in this field includes deeper analysis using the latest technologies such as genomics, proteomics, transcriptomic and metabolomics, and the valuable tools of modern bioinformatics to develop *in vitro* designs before realization of the full application *in vivo*. We have to further study the molecular basis of toxin action and study the role of receptor molecules in toxicity. This knowledge will provide new tools for a rational design of Cry toxins to control insect pests that cause considerable losses in agriculture or harm to human health.

Subsequent generations of *B. thuringiensis* crops should occur with the expression of a number of toxins that minimize the risk of development of resistance of insects, while controlling insect species in different orders, not only coleopteran, lepidopteran and dipteran insects.

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9 *Pleurotus* as an Exclusive Eco-Friendly Modular Biotool

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Abstract

The basidiomycete genus *Pleurotus*, commonly known as the oyster mushroom, is a well estabilished and recognized model biotool for many biochemical and biotechnological activities. Members of this genus are naturally widespread in temperate and subtropical environments throughout the world as decomposers of wood. *Pleurotus* is capable of growing on a wide range of lignocellulosic substrates, including various agricultural and forest wastes. *Pleurotus* spp. possess a cassette of genes producing important metabolites including lignocellulolytic enzymes which enables this group of fungi to be used as an 'eco-friendly biotool' for biodegradation, bioremediation, production of multipurpose enzymes, food derivatives, medicinal products, animal fodder, compost and an agent of biobleaching. This chapter compiles comprehensive and detailed accounts of several of the aforementioned activities. As a result of its remarkable characteristics, this fungus has now become an important biotool and the choice of biotechnologists for use as a model employed in innovative research studies, industries and various fields from the laboratory bench to large-scale activities.

9.1 Introduction

Pleurotus is a genus of saprophytic mushrooms taxonomically placed in the phylum Basidomycotina, order Agricales and family Pleurotaceae (Liu *et al*., 2013). In general *Pleurotus* is referred to as the 'oyster mushroom' in many parts of the world while it is known as the 'abalone mushroom' in China and 'dhingri' in India (Shukla and Jaitly, 2011). There are more than 40 biological species in the genus *Pleurotus* (Jose and Janardhanan, 2000; Skrobiszewski *et al*., 2013). These are commonly found in nature under different climates, in both tropical and temperate zones. *Pleurotus* spp. are one of the best choice of edible mushrooms which can be cultivated in the tropics. They have gained significant importance during last decade and are now cultivated worldwide in subtropical and temperate zones.

Pleurotus is one of the second most cultivated mushrooms because of the presence of nutritional and taste components including carbohydrates, free amino acids and 50 flavour nucleotides (Yang *et al*., 2001). Mushrooms have medicinal as well as nutritive value and have been used extensively as human food since time immemorial. The fruiting bodies or mycelia have been used as food and food-flavouring material for centuries due to their nutritional and medicinal values and the diversity of their bioactive components.

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Members of the genus *Pleurotus* may be considered as model organisms, and include some species that are capable of growing on a variety of lignocellulosic agricultural wastes and can degrade dyes and aromatic compounds (Parenti *et al.*, 2013). These fungi are useful for decontaminating wastes with a high content of several toxic compounds (Faraco *et al.*, 2009). *Pleurotus ostreatus* produces a large number of extracellular enzymes and is used as a source of lignin-degrading enzymes for the biotreatment of wastes and effluents (Ruiz-Duenas *et al.*, 2009), as well as for bio-pretreatment of lignocellulose for the production of second-generation biofuels (Talebnia *et al.*, 2009). The extract of *Pleurotus* has been reported to exhibit an anti-ageing effect, antitumour activity (Hawang *et al.*, 2003), blood-glucose-lowering property (Kang *et al.*, 2001), inhibitory effects on angiogenesis-related enzymes (Kang *et al.*, 2003), antioxidant activity (Hui *et al.*, 2002) and immune regulatory activity (Kang *et al.*, 2004). *Pleurotus* spp. have been extensively used for their medicinal value (Wasser and Weis, 1999) and different types of mushroom of the genus *Pleurotus* are highly valued as a source of nutrition, namely *P. ostreatus* (Maity *et al.*, 2011), *Pleurotus sajor-caju* (Pramanik *et al.*, 2007), *Pleurotus florida* (Rout *et al.*, 2008) and *Pleurotus citrinopileatus.* These are reported as commonly available edible mushrooms having similar immune-enhancing polysaccharides, while different polysaccharides from *P. florida* and its varieties were reported by several workers (Dey *et al.*, 2010).

Pleurotus has been studied by several authors with the most varied objectives, including the production of: (i) liquid inoculum (Rosado *et al.*, 2002); (ii) extracellular enzymes (Naraian *et al.*, 2010); (iii) flavouring agents (Martin, 1992); (iv) antimicrobials (Wisbeck *et al.*, 2002); and (v) multivitamins (Solomko and Eliseeva, 1988).

In this chapter we provide a detailed account of *Pleurotus* spp. employed in a variety of activities and discuss how *Pleurotus* is recognized as an exclusive eco-friendly modular biotool of the successful microbiologist/biotechnologist.

9.2 Historic Details of *Pleurotus*

According to the general account of *Pleurotus*, this fungus grows naturally on dead wood in logs and tree stems. The first successful attempt to grow *P. ostreatus* under artificial conditions for human consumption was conducted by a German worker Falck (1917). Bench-level cultivation in the laboratory of diverse varieties of oyster mushroom began in India during the period of the early 1960s, however, its commercial cultivation began during the mid-1970s. Bano and Srivastava (1962) started cultivation of *Pleurotus* mushroom on paddy straw. Furthermore, various researchers worldwide were drawn to cultivate various species of *Pleurotus* which has broad adaptability and can flourish under diverse agroclimatic conditions.

Several species of *Pleurotus* are cheaper and easier to grow than most cultivated edible mushrooms. Cultivation does not need difficult substrate preparation and these species can be grown on ordinary lignocellulosic agroindustrial residues containing lignin, cellulose and hemicelluloses. Thus, the cultivation of this mushroom is very simple and economical and is liked by people living in rural areas where raw materials are easily available.

In terms of production oyster mushrooms are the second largest cultivated mushrooms worldwide due to its easy cultivation technology. China, the world leader in production of oyster mushrooms, contributes nearly 85% of the total world production of about 1 million t. Presently oyster mushrooms are being cultivated almost worldwide, producing a large amount of fleshy edible fruiting bodies. Based on current knowledge the present production of the oyster mushroom in India is around 1500 t due to its strange morphology and low domestic demand.

9.3 Biological Species of *Pleurotus*

The oyster mushroom, commonly called 'dhingri' in India, was primarily described in 1775 by the Dutch naturalist Nikolaus Joseph and nominated as *Agaricus ostreatus*. Later in 1871 the German mycologist Paul Kummer shifted this to a separate, newly defined genus *Pleurotus* (Kummer, 1871), which is now a well-established genus of macrofungi. The generic term *Pleurotus* in Latin means 'side ear' (i.e. lateral attachment of the stem); however, *ostreatus* refers to oysters, due to the shape of the fruit bodies resembling the shape of oyster shells.

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Based on studies of mating compatibility this basidiomycete genus includes more than 40 biological species (Jose and Janardhanan, 2000) commonly referred as 'oyster mushrooms'. Species of *Pleurotus* that have been identified are: *P. populinus*, *P. ostreatus*, *P. florida*, *P. pulmonarius*, *P. colombinus*, *P. sapidus*, *P. populinus*, *P. eryngii* (king oyster mushroom), *P. ferulae*, *P. fossulatus*, *P. nebrodensis*, *P. abieticola*, *P. albidus*, *P. djamor-cornucopiae*, *P. eryngii*, *P. cornucopiae* (branched oyster mushroom), *P. citrinopileatus* (golden oyster mushroom), *P. euosmus*, *P. djamor* (pink oyster mushroom), *P. flabellatus*, *P. salmoneo-stramineus*, *P. salmonicolor*, *P. opuntiae*, *P. calyptratus*, *P. cystidiosus* (abalone mushroom), *P. abalones*, *P. fuscosquamulosus*, *P. smithii*, *P. dryinus*, *P. levis*, *P. tuber-regium* (king tuber mushroom), *P. australis* (brown oyster mushroom), *P. purpureo-olivaceus*, *P. rattenburyi*, *P. gardneri*, *P. parsonsii*, *P. velatus*, *P. sajor-caju*, *P. nidiformis*, *P. incarnatus*, *P. fuscus* var. *ferulae*. These species are distributed both in temperate and tropical climates, and are the second most cultivated mushrooms in the world (Arora *et al*., 1991).

9.4 *Pleurotus* as a Modular Biotool

Over several decades a huge number of different microorganisms including bacteria, yeasts and fungi have been successfully employed due to their potential for various important activities concerning environment protection and human welfare. However, several biological species of the basidiomycete genus *Pleurotus* are strongly preferred on account of their capacity to secrete lignocellulolytic enzymes, nutraceuticals and compounds of therapeutic value. Thus *Pleurotus* is a well-estabilished genus and its members are used as biotool for conducting multiple activites including biodegradation, bioremediation, production of multipurpose enzymes, food derivatives, medicinal products, animal feed and fodder, compost and as an agent of biobleaching. Detailed accounts of these activities are discussed in the following subsections.

9.4.1 Biotool for degradation and bioremediation

Bioremediation is a process employed for removal of pollutants from the contaminated environment using microorganisms. It uses naturally occurring bacteria and fungi or plants to degrade substances hazardous to human health and the environment (Vidali, 2001). Under this process wastes are biologically converted to harmless compounds to a level below concentration limits (Cohen *et al*., 2002). Various species of *Pleurotus* are able to efficiently mineralize a variety of harmful polymeric compounds including agricultural wastes (lignocelluloses), polycyclic aromatic hydrocarbons (PAHs), industrial effluents from textile dyes and waste pulp from paper mills, herbicides, pesticides, metals and explosives; most of them have carcinogenic properties (Fig. 9.1).

Degradation of lignocelluloses

Lignocelluloses are one of the most abundant and major agricultural by-products. They are used as fodder or as fuels. However, often surplus mass material is negligently burnt or thrown away in the surrounding environment which creates post-disposal problems in the environment. As a result mineralization of these agricultural wastes has become necessary for a healthy environment.

Pleurotus is a versatile genus of white-rot basidiomycete fungi well known for the complexity of their enzymatic system and prominent lignocellulolytic property. Members of this genus can colonize a wide range of natural lignocellulosic wastes (Naraian *et al*., 2010). The use of *Pleurotus* spp. is one of the most important aspects for the biodegradation of organo-pollutants, xenobiotics and industrial contaminants (Cohen *et al*., 2002). *Pleurotus* spp. have a massive capability to decompose lignocellulose directly, thus a huge mass of lignocellulosic wastes can be recycled. The most common examples of the agricultural wastes studied as substrates for *Pleurotus* spp. are coffee pulp, flax shives, maize cobs, sugarcane bagasse and rice hulls (Cohen *et al*., 2002).

The degradation of biomass or lignocellulosic materials to simpler sugars is a process that requires the cooperative action of enzymes that act synergistically to degrade: (i) cellulose (endoglucanases, cellobiohydrolases, β-glucosidase); (ii) hemicellulose (xylanases, mannanases, β-xylosidases); (iii) acetyl xylan esterase, feruloyl esterases, ρ-coumaryl esterases and α-glucuronidase; and (iv) lignin (manganese peroxidase (MnP),

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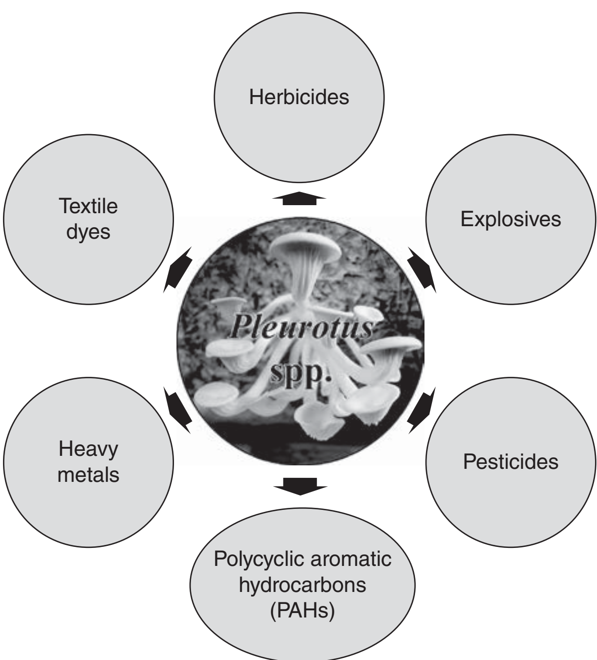

Fig. 9.1. Environmental pollutants for which *Pleurotus* spp. are used for biodegradation and bioremediation.

lignin peroxidise (LiP) and laccases) (Martinez *et al.*, 2005). The reproducible bioconversion of the agricultural lignocellulosic residues on an industrial scale by using enzymatic preparations may have many practical applications like degradation of lignocellulosic and organo-pollutants (Cohen *et al.*, 2002).

Decolorization of textile dyes

Various white-rot fungal cultures have been extensively studied to develop bioprocesses for the mineralization of dyes. *Pleurotus* spp. are one of the most widely studied white-rot fungi employed for decolorization of different dyes. Several studies from different laboratories have tested different species of *Pleurotus* for the purpose of dye decolorization and have got significant results. *P. ostreatus* is an excellent producer of the industrially important enzyme laccase. The anthraquinone dye SN4R can be effectively decolorized (66%) by the crude laccase (Hongman *et al.*, 2004). Also *P. ostreatus* produced an extracellular peroxidase that can decolorize Remazol Brilliant Blue R reported by Shin *et al.* (1997). Shin and Kim (1998) found that eight different dyes, including triphenylmethane, heterocyclic, azo and polymeric dyes, were decolorized by this peroxidase to a certain limit after 5 min. Laccase and MnP activities of *P. ostreatus* also play a big role in decolorization of Reactive B 185, Acid Black 194, Orisol Blue BH and Orisol Turquoise JL (Rodriguez *et al.*, 1999). Several other workers also studied different species of *Pleurotus* spp. for the decolorization of various dyes and a few reports are summarized in Table 9.1.

Bioremediation of heavy metals

Contamination of soils with heavy metals originating from agricultural or industrial activities is a major environmental problem. Metal pollution has become a serious environmental problem worldwide because of the widespread use of heavy metals, their distribution and particularly their toxicity to humans and the biosphere (Alkorta *et al.*, 2004). To reduce the toxicity of heavy metals, several physicochemical methods are used worldwide but these are inherently problematic in their application to metal-bearing waste streams (Javaid e*t al.*, 2011).

Mycoremediation is the technology which offers an eco-friendly and low-cost technique using fungi; it is a natural process and usually does

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Table 9.1. Different *Pleurotus* species and their enzymatic system that degrades textile dyes that can create environmental problems.

Pleurotus species	Dyes	Enzymatic system	References
Pleurotus florida	Black CA, Black B133, Corazol Violet SR	Laccase (enzymatic oxidation)	Moorthi *et al*. (2007)
P. florida, *Pleurotus citrinopileatus*, *Pleurotus eryngii*	Direct Blue 14	Laccase, peroxidase and immobilized crude enzyme extract	Singh *et al*. (2012)
P. florida	Reactive Green, Yellow, Blue	Laccase (crude enzyme extract), tyrosinase	Shanmugam *et al*. (2005)
P. florida	Bromophenol Blue, Brilliant Green, Methyl Red	Laccase (crude enzyme extract)	Radhika *et al*. (2014)
P. florida, *Pleurotus sajor-caju*	Synazol Red HF6BN	Laccase, phenol-oxidizing, bioremediation	Ilyas *et al*. (2012)
Pleurotus pulmonarius	Remozol Brilliant Blue R, Methyl Violet, Ethyl Violet, Brilliant Cresyl Blue	Laccase, phenol-oxidizing enzyme	Zilly *et al*. (2002)
Pleurotus ostreatus	Blue HFRT	Laccase fermentation	Devi *et al*. (2012)
P. florida	Crystal Violet, Orange, Malachite Green	Laccase, phenol-oxidizing enzyme	Krishnaveni and Kowsalya (2011)

not produce toxic by-products. It also provides a permanent solution for the contaminants in the environment (Perelo, 2010). Mycelia of the phoenix oyster mushroom were reported to eliminate up to 97% of the mercury from contaminated water (Arica *et al*., 2003). As observed by Humer *et al.* (2004), mushrooms significantly removed copper and chromium in treated woods. Adsorption of metals is an excellent alternative to the physicochemical methods mentioned above (Javaid *et al*., 2011).

Pleurotus belongs to a group of white-rot fungi well known for their capacity for heavy metal adsorption and excellent metal uptake (Purvis and Halls, 1996). Several workers have studied the uptake of heavy metals by various mushroom fruiting bodies collected adjacent to heavy metal smelters, landfills of sewage sludge and emission areas (Svoboda and Kalac, 2003; Svoboda *et al*., 2006; Antonijevic and Maric, 2008). Danis (2010) studied the removal of copper from aqueous solutions through biosorption by *Pleurotus cornucopiae.* In another study *P. ostreatus* successfully removed Cr, Cu, Ni and Zn ions from tannery wastewater (Javaid *et al*., 2011).

Degradation of pesticides

Pesticides are substances which mitigate the damage done by pests and protect various agricultural crops. Use of chemical pesticides has become common to protect agricultural crops. The widespread use of these chemicals creates serious environmental problems which ultimately need remediation. *Pleurotus* spp. are a major fungal biotool with the capability of degrading various kinds of chemical pesticide into non-harmful metabolites. During the last five decades, valuable efforts have been spent by some investigators studying the degradation by *Pleurotus* spp. of some recalcitrant organopollutants. Using *P. sajor-caju* Arisoy and Kolankaya (1997) determined that the degradation of lindane started to increase after 10 days of incubation and reached the maximum level in 20 days. The ability of spent mushroom waste of *P. ostreatus* to degrade 1,1,1-trichloro-2,2-bis (4-chlorophenyl) ethane (DDT) was also evaluated and resulted in a reduction of up to 48% DDT following 28 days incubation and 51% of DDT following 56 days (Purnomo *et al*., 2010).

Degradation of herbicides

Atrazine (2-chloro-4-ethylamine-6-isopropylamine-s-triazine) is a herbicide that is regularly used for weed control in sugarcane, maize and soybean crops (ANVISA, 2011). The chemical structure of this herbicide, possessing a triazine ring substituted with chlorine, ethylamine and isopropylamine,

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makes it more recalcitrant to biological degradation in the agricultural environment (Colla *et al*., 2008). However, commonly used triazine herbicides are efficiently degraded by a number of species of white-rot fungi (Bending *et al*., 2002; Nwachukwu and Osuji, 2007), for example atrazine can be degraded by *Pleurotus pulmonarius*.

In a recent study of optimized atrazine degradation by *P. ostreatus* it was found that after 15 days of incubation with the fungus 94.5% of the atrazine was degraded. This observation demonstrated the efficiency of *P. ostreatus* for atrazine degradation, and verified the potential of this fungus as a bioremediation agent (Pereira *et al*., 2013).

Degradation of explosives

Pollutant explosives include nitro-compounds such as 2, 4, 6-trinitrotoluene (TNT) and RDX (cyclotrimethylenetrinitramine), HMX (cyclotetramethylenetetranitramine), PETN (pentaerythritol tetranitrate) and EGDN (ethylene glycol dinitrate). Several attempts have been made to remove and dispose of these environmentally persistent compounds. Fungi were able to degrade TNT as a recalcitrant pollutant at abandoned military areas and performed complete degradation of meta and parachlorophenol within 15 days (Perez *et al*., 1997). Surprisingly it took only 15 days to degrade 2, 4-DCP (2, 4-dichlorophenol) to 2% of the original level using *P. sajor-caju* (Rodriguez *et al*., 2004). In a similar study *P. ostreatus* was able to grow successfully on a high concentration of TNT, and caused its extensive degradation as it was adsorbed by the mycelia after only 4 h (Axtell *et al*., 2000).

Degradation of polycyclic aromatic hydrocarbons (PAH)

PAH are of high interest, since a lot of them are potent carcinogens (Dipple, 1976). PAH containing four or more condensed rings cannot be degraded easily by microorganisms (Bezalel *et al*., 1996b; Tabak *et al*., 1981). Several recent studies have reported that the *P. ostreatus* is able to degrade a variety of PAH (Sack and Gunther, 1993; Bezalel *et al*., 1996a, b). It has been proved that *Pleurotus* spp. and related fungal strains are reasonably capable of mineralizing [^{14}C] pyrene (Martens and Zadrazil, 1997).

In a study Wolter *et al.* (1997) investigated degradation of eight unlabelled highly condensed PAH and the mineralization of three ^{14}C-labelled PAH by the white-rot fungus *P. florida*. Based on their findings, Wolter *et al*. (1997) reported disappearance of the unlabelled four- to six-ring PAH: pyrene, benzo[a]anthracene, chrysene, benzo[b]uoranthene, benzo[k]fluoranthene, benzo[a] pyrene, dibenz[a,h]anthracene and benzo[ghi]perylene. After 15 weeks of incubation, less than 25% of the initial amounts of 50 g were recovered (compared with the controls where above 85% were recovered).

9.4.2 Biotool for production of multipurpose enzymes

Enzyme production is an important field of bioprocessing in microbial biotechnology. Most enzyme manufacturers produce enzymes by submerged fermentation techniques. However, in the last decades there has been an increasing trend towards the use of a solid-state fermentation technique to produce several enzymes (Hashim, 2012).

The genus *Pleurotus* includes species that belong to group of white-rot fungi which have a unique ability to produce extracellular lignocellulolytic enzymes including laccase and MnP (Stajic *et al*., 2006), xylanase (Elisashvili *et al*., 2008), CMCase (carboxymethyl cellulase), β-glucosidase and β-xylosidase (Naraian *et al*., 2010) (Fig. 9.2). These enzymes have shown enormous potential as they can be widely used for lignocellulose degradation (Ren and Diller, 2007) and detoxification of agroindustrial residues with high phenolic content (Mata *et al*., 2005).

Different applications for these enzymes include upgrade of animal feed (Akin *et al*., 1993), pulp and paper production (Breen and Singleton, 1999), textile dye bleaching, bioremediation and effluent detoxification, as a component in washing powder, for removal of phenolics from wines and transformation of antibiotics and steroids (Mayer and Staples, 2002; Lazzè *et al*., 2004). These potential applications in industrial processes should be put into practice as *Pleurotus* spp. are an inexpensive source of these enzymes.

Production of protease

Protease is an enzyme that breaks the peptide bonds of protein to produce free amino acids

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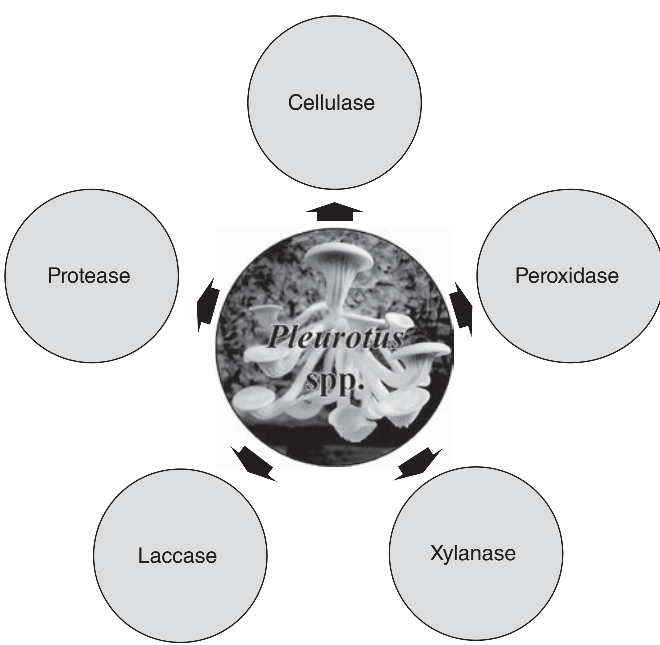

Fig. 9.2. Multipurpose enzymes generally produced by the several species of *Pleurotus* spp.

and other smaller peptides (Mitchell *et al*., 2007). This is one of the largest groups of hydrolytic enzymes having a 60% share in the world enzyme market. Proteases are widely used in various industries, for example that of food, leather, detergent, pharmaceutical, diagnostics, waste management, and several newer applications are being explored (Nirmal *et al*., 2011). Despite the fact that the production of extracellular proteolytic enzymes is a common feature among fungi, relatively few proteases secreted from lignin-degrading fungi have been characterized on a molecular level (Datta, 1992; Mellon and Cotty, 1996). Palmieri *et al.* (2001) purified and characterized a novel extracellular protease from *P. ostreatus.* It has been reported (Bockle *et al*., 1999) that proteases in liquid culture of *P. pulmonarius* are not responsible for the decrease in peroxidase activity. In a recent study protease was isolated from the medicinal mushroom *P. sajor-caju*, and the protease has been partially purified and characterized (Ravikumar *et al*., 2012).

Production of cellulase

The enzyme that catalyses the hydrolysis of cellulose is known as cellulase. Unlike most of the enzymes cellulase is a complex of enzymes that work synergistically to attack the native cellulose polymer. Cellulase is a family of at least three groups of enzymes: (i) endoglucanases which act randomly on soluble and insoluble cellulose chains; (ii) exoglucanases that act to liberate cellobiose from the reducing and non-reducing ends of cellulose chains; and (iii) β-glucosidases which liberate glucose from cellobiose (Deswal *et al*., 2001).

Cellulases have many potential applications, for example formulation of washing powder, in animal feed production (Han and He, 2010), in the textile industry, the pulp and paper industry, starch processing, grain alcohol fermentation, malting and brewing, and in the extraction of fruit and vegetable juices (Bhat, 2000; Deswal *et al*., 2011). Recently Naraian *et al*. (2010) detected cellulase activity from three different species, namely *P. sajor-caju*, *P. florida* and *P. eryngii.*

Production of xylanase

Xylanase is an enzyme that catalyses the hydrolysis of the 1, 4-β-D-xylosidic linkages in xylans which are constituents of hemicellulose, a structural component of plant cell walls. Xylanases

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are produced by marine algae, protozoans, crustaceans, insects, snails and seeds of land plants (Sunna and Antranikian, 1997). Among microbial sources, filamentous fungi which secrete xylanases are especially interesting as they have been found to secrete these enzymes into the medium at higher levels than yeasts and bacteria (Polizeli *et al*., 2005). In recent years, the various biotechnological uses of xylanases has grown remarkably (Techapun *et al*., 2003). Xylanase began to be used in the 1980s, initially in the preparation of animal feed and later in the food, textile and paper industries (Polizeli *et al*., 2005). Currently, xylanases are used in animal feed, the manufacture of bread, food and drinks, and textiles. The ability of *P. eryngii* and *Flamulina velutipes* to produce xylanlytic enzymes in submerged fermentation was checked for the first time in 2010 (Simair *et al*., 2010). Recently Singh *et al.* (2012) studied the production of extracellular xylanase by three species of *Pleurotus* including *P. florida*, *P. flabellatus* and *P. sajor-caju*.

Production of peroxidases

In the last few decades more emphasis has been placed on analysing MnP, because this enzyme is produced by most white-rot fungi, including many species which lack LiP (Lobos *et al*., 1994; Kerem and Hadar, 1995). Martinez *et al*. (1996) studied the catalytic properties of *P. eryngii* MnP isoenzymes from liquid peptone medium. *P. eryngii* peroxidase efficiently oxidized substituted phenols which could not be oxidized by the *Phanerochaete chrysosporium* peroxidases (Martinez *et al*., 1996). Naraian *et al*. (2010) studied the expression of MnP in three different species of *Pleurotus* including *P. florida*, *P. sajor-caju* and *P. eryngii* and found it was dependent on the substrate and its chemical composition.

Production of laccase

Laccases, enzymes with *p*-diphenol oxidase activity, are members of a group of proteins collectively known as multicopper or blue copper oxidases. Laccases catalyse the oxidation of a great variety of phenolic compounds and aromatic amines using molecular oxygen as the electron acceptor (Yaropolov *et al*., 1994).

In recent years laccases have attracted the attention of researchers due to their ability to oxidize both phenolic and non-phenolic compounds and even highly recalcitrant environmental pollutants, which makes them very useful for their applications in biotechnological processes. Fungal laccases may also play a role in physiological processes, such as sporulation, pigment production, plant pathogenesis, delignification, humification and morphogenesis (Saparrat *et al*., 2007).

The growth as well as laccase production by *Pleurotus* spp. has been extensively studied. The ability of four local fungal isolates of *P. ostreatus* for extracellular laccase production was positively demonstrated with humidified sawdust as the substrate in a mineral salt medium (Hashim, 2012). Several other investigators also reported high levels of laccase production, for example Gupte *et al*. (2007) examined *P. ostreatus* laccase production on wheat straw and Souza *et al*. (2002) round *P. pulmonarius* showed high laccase activity when examining different inoculum sizes used for laccase production in solid state fermentation. Naraian *et al.* (2010) observed production of laccase during *in vitro* fermentation of maize cobs using *P. florida*, *P. sajor-caju* and *P. eryngii*.

Laccases have wide commercial applications within the food industry, pulp and paper industries and textile industry, and also for synthetic chemistry, soil bioremediation and removal of endocrine disruptors (Cuoto *et al*., 2006).

9.4.3 Biotool for production of food and food derivatives

Oyster mushroom (*Pleurotus*) has been used for centuries worldwide both as a food and a traditional medicine. It contains low amounts of fat and digestible carbohydrates, but has higher protein content than most ordinary vegetables (Kalyoncu *et al*., 2010). Thus it is considered as a healthy food, as it is both low in calories and fat, but rich in proteins, minerals and dietary fibre (Manzi and Pizzoferrato, 2000) (Fig. 9.3).

Biotool producing single cell protein (SCP)

SCP is protein derived from the culture of microorganisms grown on abundantly available agricultural and industrial wastes. SCP is produced from many species of microorganisms including the white-rot fungus *Pleurotus*. It is very convenient to use fungi for production of SCP particularly when they can be grown on inexpensive

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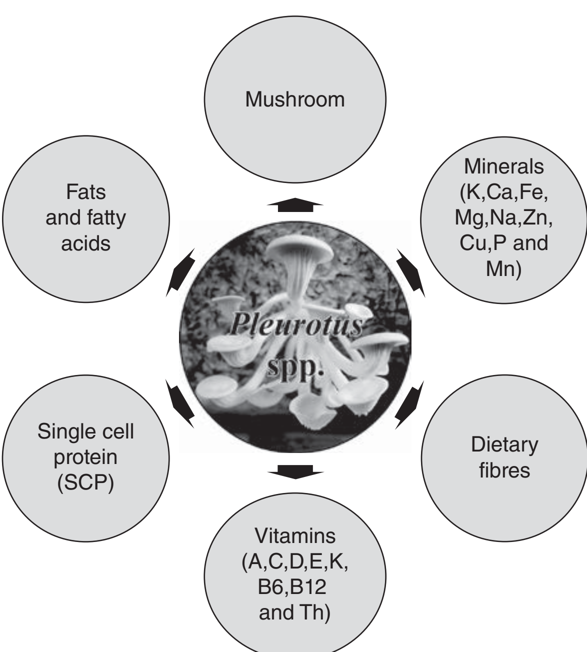

Fig. 9.3. Nutritional and food derivatives produced by *Pleurotus* spp. and found in their fruit bodies and culture extracts.

waste material. Their rapid growth and high protein content have made them prime candidates for use as sources of SCP (Anupama and Ravindra, 2000).

Biotool producing mushrooms

Mushrooms have been extensively used as a source of nutrition since ancient times. The consumption of mushrooms these days is growing fast due to their balanced nutritient composition (Lee *et al*., 2011). Oyster mushroom (*Pleurotus* spp.) is considered a good dietary option because of its nutritional value, as it is rich in protein, fibre, carbohydrates, vitamins and minerals and it has few calories, and is low in fat and sodium (Cohen *et al*., 2002). Oyster mushroom cultivation and production has increased tremendously throughout the world over the last few decades. Among wild species in the genus *Pleurotus*, the economic importance of *P. eryngii* is well established (Cohen *et al*., 2002). Oyster mushroom, *P. ostreatus*, is one of the most commonly cultivated species of genus *Pleurotus*, however, other frequently cultivated species include grey oyster mushroom (*P. sajor-caju*), abalone mushroom (*P. cystidiosus*), golden oyster mushroom (*P. citrinopileatus* Sing.), pink oyster mushroom (*P. flabellatus*), black oyster mushroom (*P. sapidus*), *P. eryngii*, *P. djamor* and *P. tuber-regium*. Recently Naraian *et al*. (2009) successfully cultivated *P. florida* using maize cobs as the basal substrate and different nitrogen-rich oilseed cakes as supplements.

Mushrooms with their delicate flavour and texture are recognized as a nutritious food and an important source of biologically active compounds. Generally, mushrooms are low in energy and high in dietary fibre and are an excellent source of antioxidants as they accumulate a variety of secondary metabolites, including phenolic compounds (Cheung *et al*., 2003; Yim *et al*., 2011).

Biotool producing multivitamins

Oyster mushrooms are consumed worldwide by humans for their availability of multiple vitamins. The edible fruit bodies of *Pleurotus* spp. generally contain high levels of different vitamins including vitamins B1, B2, B5 (Raganathan *et al*., 1996; Olivieri *et al*., 2006), B6 and B7 (Solomko and Eliseeva, 1988). *Pleurotus* mushroom are also rich in vitamins B, D and K, and sometimes vitamins A and C, making it

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suitable for low-calorie diets (Kurtzman, 1997; Mattila *et al.*, 2001). Surprisingly Kumari and Achal (2008) reported that vitamins A, C and E were present in both fresh fruit bodies and powdered samples of *P. ostreatus.*

Biotool producing minerals

Wild as well as artificially cultivated oyster mushrooms, like all living things in the nature, contain several minerals. Potassium (K) seems to be the most abundant in mushrooms (Kurtzman, 1993). It is believed that these mushrooms may also be a good nutrient source of micro- and macroelements, such as K, Mg, P, Zn, Fe, Cu and Ca (Stamets, 1993; Isiloglu *et al.*, 2001). This has been also reported that *P. ostreatus* and are naturally rich in selenium (Mattila *et al.*, 2001; Cocchi *et al.*, 2006; Falandysz *et al.*, 2008).

Biotool producing dietary fibres

Fungi such as *Pleurotus* are an excellent source of dietary fibre because their cell wall is composed mainly of chitin, hemicellulose, mannans and β-glucans (Manzi and Pizzoferrato, 2000). It is strongly believed that the fruit body of *Pleurotus* in particular contains a surplus amount of dietery fibre which is required for a healthy diet. The sclerotium, a solid mass of mushroom hyphae, contains high levels of dietary fibre (Cheung and Lee, 2000). Besides, the fruit body and sclerotium of *P. tuber-regium* are also useful for food, such as soup and sausage (Akobundu and Eluchie, 1992). Oyster mushroom should be a preferred healthy food as it can assist intestinal motility and increase stool bulk, decreasing absorption of harmful toxic, carcinogenic substances and leading to lower incidence of colorectal cancer (Manzi and Pizzoferrato, 2000).

9.4.4 Biotool for production of medicinal products

Mushrooms are used not only for consumption but they also have a medicinal use. There are over 14,000 varities of mushrooms in the world, of which about 3000 are edible, about 700 have known medicinal properties and around 1400 have been recognized as poisonous (Choudhury *et al.*, 2011).

Mushrooms are suitable for people with hypertension, obesity and diabetes as they have a low sodium: potassium ratio, and are low in starch, fat and calorific value. Alkaline ash and high fibre content makes them suitable for consumption to those having hyperacidity and constipation. A polycyclic aromatic compound pleurotin has been isolated from *Pleurotus griseus* which possesses antibiotic properties. The folic acid present in oyster mushrooms helps to cure anaemia. The cell walls of edible macrofungi also contain chitin, hemicelluloses, glucans, mannans, and especially, branched non-cellulosic β-glucans, which have beneficial health properties (Chen and Seviour, 2007).

Biotool producing antioxidants

Humans and other organisms possess antioxidant defence and repair systems that have evolved to protect them against oxidative damage, however, these systems are insufficient to totally prevent damage (Simic, 1988). The antioxidants in human diets are of great interest as possible protective agents to help the human body reduce oxidative damage (Mau *et al.*, 2002).

Fruiting bodies of *Pleurotus* possess a higher concentration of antioxidants than other commercial mushrooms (Lo, 2005; Patel *et al.*, 2012). This activity is mainly due to the presence of the polysaccharide pleuran (β-glucan) that has been isolated from *P. ostreatus* and shows a positive effect on rat colon with precancerous lesions (Bobek and Galbavy, 2001). *P. ostreatus* increased the activities of important antioxidant enzymes (namely superoxide dismutase, catalase and peroxidase), thereby reducing oxidative damage in humans (Yang *et al.*, 2002). Methanolic extract of *Pleurotus eous* significantly enhanced the activity of antioxidant enzymes (Ramkumar *et al.*, 2010).

Several phenolic compounds commonly found in the fruiting bodies of mushrooms are antioxidants, with redox properties that act as reducing agents, hydrogen donors, free radical scavengers and singlet oxygen quenchers (Barros *et al.*, 2007). Recently extracts of oyster mushrooms (*Pleurotus pulmonarius*, *P. ostreatus*, *P. djamor* var. *djamor* and *P. djamor* var. *roseus*) and the split gill mushroom (*Schizophyllum commune*) were successfully investigated for their antioxidant properties (Arbaayah and Kalsom, 2013).

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Biotool producing polysaccharides

Oyster mushrooms contain numerous biologically active polysaccharides in the fruiting bodies, cultured mycelium and broth culture extracts. Polysaccharides have a broad range of important bioactivities, including anticoagulant, antioxidant, antiproliferative, antitumour, anticomplement, anti-inflammatory, antiviral, antipeptic and anti-adhesive activities (Rocha *et al*., 2005; Costa *et al*., 2010). Polysaccharides belong to a structurally diverse class of macromolecules and are polymers of monosaccharide residues joined to each other by glycosidic linkages (Sharon and Lis, 1993). In a study it was found that mushroom polysaccharides are present mostly as glucans with different types of glycosidic linkages, such as (1-3), (1-6)-β-glucans and (1-3)-α-glucans, but some are true heteroglycans. Among the polysaccharides produced by *Pleurotus* spp., β-1, 3 glucans play an important role as biological response modifiers (Bohn and BeMiller, 1995).

Pleurotus spp. are appreciated not only for their sensory characteristics, but also for their nutritional value and functional properties (Alexopoulos *et al*., 1996). In a comparative investigation data on mushroom polysaccharides were collected from more than 600 species of 182 genera of higher hetero- and homobasidiomycetes (Wasser, 2002). These polysaccharides differed in their chemical composition, but most belonged to the group of β-glucans.

Fruit bodies of the oyster mushroom *P. ostreatus* contain polysaccharides (Yoshioka *et al*., 1972; Karacsonyi and Kuniak, 1994), as does the extract from the cultured mycelia of different *Pleurotus* spp., including *P. pulmonarius* (Zhuang *et al*., 1993; Gutierrez *et al*., 1996), *P. citrinopileatus* (Zhang *et al*., 1994) and *P. sajor-caju* (Zhuang *et al*., 1993). Mycelial growth and exopolysaccharide production by the submerged fermentation of *P. tuber-regium* have been reported (Zhang and Cheung, 2011).

9.4.5 Biotool for production of animal feed and fodder

The idea of using white-rot fungi to improve the digestibility of lignocellulosic material for ruminants was first developed almost a century ago as was the suggested use of the fungi for the improvement of lignocellulosic wastes (Cohen *et al*., 2002). Biological delignification of straw using white-rot fungi seems to be the most promising way of improving its digestibility (Kamra and Zadrazil, 1986; Zadrazil and Reinger, 1988). Several authors have examined this possibility, using mainly wheat straw and *Pleurotus* spp. under different conditions and substrate pretreatments (Zadrazil, 1980; Streeter *et al*., 1982; Kamra and Zadrazil, 1986). The role of fungi in agricultural waste conversion by different fungal species was recently reviewed (Cohen and Hadar, 2001).

Several studies using *Pleurotus* spp. for upgrading different substrates into valuable animal feed products have been conducted. Hadar *et al.* (1992) studied the utilization of lignocellulosic waste by *P. ostreatus* for increased digestibility, using cotton stalks as the substrate. Many workers made efforts to improve *in-vitro* digestibility of wheat straw for improved ruminant consumption using different species of *Pleurotus*, namely *P. ostreatus* (Lindenfelser *et al*., 1979; Zadrazil, 1985; Jalc *et al*., 1996), *P. eryngii*, *Pleurotus serotinus* and *P. sajor-caju* (Kamra and Zadrazil, 1986; Martinez *et al*., 1994). Malik *et al*. (1999) worked on *P. ostreatus* for cardboard protein enrichment, and similarly Hutterman *et al*. (2000) reported increased digestibility of rice straw and cotton stalks and substrate improvement using *Pleurotus* spp. The mushroom industry has been considering problems with spent mushroom substrates from an environmental standpoint concerning its effective disposal and recycling. Previous studies have shown the feasibility of using these kinds of waste to produce animal feed (Zhang *et al*., 1995; Adamovic *et al*., 1998; Bae *et al*., 2006).

9.4.6 Biotool for production of compost

The culture wastes of *Pleurotus* produced by mushroom farms are mostly discarded but are partly used for making compost. If we can reutilize the culture wastes for producing fruit bodies of *Lyophyllum decastes*, it can reduce the cost of mushroom production and achieve effective use of wood resources (Ohga *et al*., 1993; Nakaya *et al*., 1997). Mushroom growing produces a great deal of waste, however, spent mushroom compost (SMC) can provide the physico-chemical

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environment suitable for use as the basis of seedling growth media for different plant species, especially vegetables. Peksen and Uzun (2008) studied this and concluded that SMC + P or SMC can be used as vegetable seedling media for both kale and broccoli.

9.4.7 Biotool for biobleaching

Biotool of pulp bleaching

Wood pulp bleaching is the chemical process carried out on various types of wood pulp to decolorize the pulp, to make it whiter. This is usually done by chlorine bleaching but this process is not eco-friendly. However, the enzyme produced by a group of white-rot fungi can break down hemicellulose and lignin and provides an environmentally friendly alternative to chlorine bleaching, and this is called biobleaching. Non-chlorine bleaching of pulp with laccase was first patented in 1994 using an enzyme treatment to obtain a brighter pulp with low lignin content (Luisa *et al*., 1996). In spite of this new method, the pretreatments of wood pulp with laccase can provide milder and cleaner strategies of delignification that also respect the integrity of cellulose (Barreca *et al*., 2003; Xu *et al*., 2006). Laccases are able to delignify pulp when they are used together with mediators (Bourbonnais *et al*., 1997).

Once the potential of the laccase mediator systems for delignifying and bleaching paper pulps has been demonstrated, a crucial aspect for their industrial implementation is to optimize the integration of the enzymatic delignification systems into 'elemental chlorine free' and 'total chlorine free' sequences. Fungal laccases, from *P. ostreatus* IMI 39554571 have been used to treat pulp.

Biotool of skin bleaching

According to Nawamura *et al*. (2007) *P. nebrodensis* has antioxidative ability and a suppressive effect on melanin generation. *P. abalones* extract has the ability to inhibit platelet aggregation and improve rough skin as reported in the Japanese unpublished patent application no. 2002-348247 while Nawamura *et al*. (2007) reported that when this extract is applied to the skin there are improvements regarding skin ageing or skin diseases. *P. nebrodensis* and *P. abalones* are reported to be whitening agents (Nawamura *et al*., 2007).

9.5 Conclusion

White-rot fungi in the genus *Pleurotus* have powerful and complex enzymatic machinery with a cassette of structural genes coding for important metabolites that enables them to be used for a variety of biological activities. Now with the current advancement of knowledge this offers significant opportunities for elucidating roles and interactions that may be exploited in numerous eco-friendly processes that are of benefit to society. As a result of its remarkable characteristics, this fungus has now become an important biotool and the choice of biotechnologists for use as a model employed in innovative research studies, industries and fields from the laboratory bench to large-scale activities.

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10 Use of Biotechnology in Promoting Novel Food and Agriculturally Important Microorganisms

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Abstract
This chapter reviews the various applications of microbes in agriculture, characterization of novel microbial genes and enzymes useful in food and agriculture, and biotechnological tools used to augment the activity of microbes and their products. Microorganisms have many associations with plant communities such as commensalism, synergism, mutualism and antagonism. Some of these associations result in beneficial interactions between plants and microorganisms for various purposes, such as promotion of plant growth, guarding the plant from other harmful interactions and production of safer foods which are free from inorganic chemicals. In addition, microorganisms have been studied for their vital roles in various food production and fermentation industries. The utility of microorganisms demonstrated so far in the field of food and agriculture necessitates further characterization and identification of novel microbes and the untapped potential use of their enzymes and gene products. The availability of various traditional and classical approaches aid in the identification and characterization of microbes to some extent, however, conspicuously the advent of biotechnology has opened up many more techniques that can be used to characterize and promote agriculturally important beneficial microorganisms.

10.1 Introduction

A broad range of microorganisms are found in various natural and agricultural ecosystems that play an integrated role in plant vigour, crop productivity, recycling of nutrients, fixing atmospheric nitrogen so it is in a useable form for plants, allowing plant roots to access soil nutrients, and conservation of ecosystem functions at various levels. Microorganisms are found almost everywhere and comprise about 60% of the earth's biomass (Singh *et al*., 2009). For aquatic ecosystems, such as the oceans, the number of microbial cells has been estimated to be approximately 1.2×10^{29} microbial cells, while in terrestrial environments the soil could support as many as 4×10^{30} microbial cells. The estimated number of prokaryotic cells in this planet's soil is 2.6×10^{29}, providing an enormous capacity for genetic diversity and a great potential for exploitation in the field of agriculture, food and industrial microbiology (Singh *et al*., 2009). As a result of several challenges such as increase in global population, changes in climate and increase in greenhouse gas emissions, it has become necessary to understand the ecology and dynamics of plant-associated microbes, as well as their implications for plant

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physiology and improvement. Of the different microbial communities, beneficial fungi, yeasts and bacteria tend to play important roles in food and agriculture by means of enhanced nutrient use and protection of crops against biotic and abiotic stresses (Saravanakumar and Samiyappan, 2007; Saravanakumar *et al.*, 2007a, b). In addition, the microbes have greater potential in the food industry in terms of serving as the source of the industrial enzymes and enhancing the shelf life of horticultural commodities after harvest. Thereby considering the current scenario and the potential role of beneficial microorganisms in the field of food and agriculture, biotechnology can be used to provide various insights on the improvement of microbes that could result in greater use of potential microbes to enhance food production all over the world. In this context, the current chapter focuses on:

- major uses of microorganisms in food and agriculture, particularly as biofertilizers and biocontrol agents;
- biotechnology-mediated approaches for the detection and identification of potential microbes; and
- molecular approaches for genetic recombination of beneficial microorganisms and genes in order to enhance the activity of a particular microorganism.

10.2 Role of Microbes in Agriculture and the Food Industry

Agriculture has been transformed fundamentally in almost all developed and developing countries by the use of high-yielding varieties through hybridization programmes and intensive application of synthetic fertilizers to supplement nutrition to plants and pesticides for the management of pests and diseases. The intensive application of synthetic fertilizers has resulted in reduced efficiency of uptake of nutrients by plants, besides reducing the quality of soil properties. Further, the indiscriminate use of synthetic pesticides for the control of pests and diseases has led to several concerns, including high cost, exposure risks, fungicide residues and other health and environmental hazards. In addition to these hazards, increases in per capita income and the purchasing power of people in developed countries have led to increased demand for the production of quality food products that are free from toxic residues. All these issues in modern-day agriculture necessitate alternative strategies for crop management and crop protection which mainly include the conservation of microbial biodiversity and introduction of beneficial microbes in the plant niche to improve plant growth and alleviate biotic and abiotic stresses, and thereby increase the crop yield significantly.

10.2.1 Biofertilizers and plant growth promoters

Plant growth depends primarily on the use of nutrients. Although soil contains enormous amounts of nutrients, the utilization of nutrients by plants requires the mediation of microbes. It is evident from several research studies that microbes play an important role in fixing and solubilizing plant growth nutrients and thus increasing their availability to the plants. Considering the close association of microbes with plants and their role as mediators for nutrient uptake by plants needed for their growth and development, the plant growth-associated microbes have been designated as biofertilizers. According to Vessey (2003), biofertilizers are substances containing living microorganisms applied to seeds, plant surfaces or soils to colonize the rhizosphere or any parts of the plants internally or externally and promote growth by increasing the supply or availability of primary nutrients to the host. Most commonly, bacteria such as *Azospirillum*, *Herbaspirrilum*, *Acetobacter*, *Azotobacter*, *Azoarocus* and *Rhizobium* are used as biofertilizers due to their property to fix atmospheric nitrogen and they are mostly associated with leguminous crops. Interestingly some of the bacterial species of *Achromobacter*, *Acetobacter*, *Alcaligenes*, *Arthrobacter*, *Azomonas*, *Bacillus*, *Beijerinckia*, *Campylobacter*, *Clostridium*, *Corynebacterium*, *Desulfovibrio*, *Derxia*, *Enterobacter*, *Erwinia*, *Herbaspirillum*, *Lignobacter*, *Klebsiella*, *Methylosinus*, *Mycobacterium*, *Rhodospirillum*, *Rhodo-pseudomonas* and *Xanthobacter* have been reported to associate with non-legume crops in the fixation of atmospheric nitrogen. Similarly,

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the bio-inoculants have specific and non-specific interactions with the plants. While *Azolla*, blue green algae and *Rhizobium* are known to be plant specific, the bioformulations from *Azospirillum*, *Azotobacter*, phosphorus-solubilizing bacteria and vesicular arbuscular mycorrhizal (VAM) fungi have greater adaptability and are able to colonize a wide range of crops. These are considered as biofertilizers due to their broad-spectrum activity. Although several nitrogen-fixing bacteria have been isolated from the rhizosphere of various plants, species of *Azospirillum* and *Azotobacter* are commercially applied to enhance the yield in cereals and legumes under field conditions. In addition to nitrogen fixing by various microbes, *Bacillus megaterium*, *Bacillus circulans*, *Bacillus subtilis*, *Alcaligenes*, *Acinetobacter*, *Arthrobacter*, *Azospirillum*, *Burkholderia*, *Enterobacter*, *Erwinia*, *Flavobacterium*, *Pseudomonas*, *Rhizobium* and *Serratia* have been involved in the solubilization of mineral phosphates. Similarly VAM fungi enable plants to obtain nutrients and minerals from the soil by solubilization and guarding the rhizosphere of crop plants from harmful phytopathogens (Mohammadi and Sohrabi, 2012). The application and potential utility of fungi- and bacteria-based biofertilizers in sustainable crop production has been critically reviewed by several authors (Vessey, 2003; Kaewchai *et al.*, 2009; Das *et al.*, 2013).

10.2.2 Microbes as biotic stress managers

Further to the nutrient association of microbes with plants, microbes play a major role in the mitigation of abiotic and biotic stresses in plants by displaying different mechanisms of action. Pests and diseases cause significant damage and economic losses in agricultural and horticultural crops every year. Global losses caused by pests and diseases have been estimated to be 30% of the potential crop production (Oerke, 2006) despite the continuous release of new pesticides and resistant cultivars. Furthermore, pests are continuously adapting and becoming resistant to existing pest-resistant varieties and pesticides and a few of the pesticides are being withdrawn from the market for environmental reasons. Thus the use of antagonistic microbes including fungi, bacteria and bacteriophages has gained significance in the management of pests and diseases in sustainable agriculture.

The biocontrol properties of microbes are attributed to more than one character and thus it is imperative to study the genes involved in the different mechanisms of action. Extensive work on biological control has defined the following modes of action by biocontrol agents: (i) effective colonization and competition for nutrients against phytopathogens around the rhizosphere and secretion of growth-promoting hormones such as auxins and cytokinin (Saravanakumar *et al.*, 2008a, b); (ii) production of antibiotics and lytic enzymes, namely chitinases and β-1, 3-glucanases (Haas and Defago, 2005; Saravanakumar *et al.*, 2009); and (iii) induction of defence-related genes in plants involved in the control of pests and diseases (Saravanakumar *et al.*, 2008a, b). Various modes of action, either individually or in combination, determine the success of biological control of plant pests. Hence, an in-depth study on the mechanism of action could offer a prelude for tapping potential genes for further improvement of biocontrol strains. Of several microbes, the bacteria isolated from the rhizosphere region designated as plant growth-promoting rhizobacteria (PGPR) have been found to exert beneficial effects on plant growth besides controlling pests and diseases in culture or in a protected environment (Kloepper *et al.*, 2004; Saravanakumar *et al.*, 2007a, b; Prabhukarthikeyan *et al.*, 2014). In addition to bacteria, fungi such as *Trichoderma*, *Glomus*, non-pathogenic *Fusarium*, *Coniothyrium* and *Ampelomyces* are well known for their antagonism against several soil-borne plant pathogens (Rojo *et al.*, 2007). It has also been demonstrated that biocontrol fungi like VAM and *Trichoderma* promoted plant growth besides reducing the disease severity of soil-borne pathogens (Sallam *et al.*, 2009; John *et al.*, 2010). Similarly, some yeast strains such as *Candida*, *Metschnikowia*, *Pichia*, *Rhodotorula* and *Aureobasidium* are studied for their involvement in the control of postharvest diseases besides enhancing the shelf life of fruits after harvest (Spadaro and Gullino, 2004; Saravanakumar *et al.*, 2008a; Zhang *et al.*, 2010). In addition to biotic stress managers, microbes have also been studied for their potential to alleviate abiotic stresses imposed in all stages of plant growth in agriculture.

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10.2.3 Microbes as abiotic stress alleviators

Abiotic stresses like soil salinity, drought, flooding and high and low temperature are considered to be worldwide problems for agriculture as these stresses limit the cultivation of plants in tropical, subtropical and temperate regions. Abiotic stresses are reported to cause increased production of ethylene in most of the plants (Feng and Barker, 1992). In many instances, removing or blocking the effect of stress-induced ethylene production alleviates the stress effect (Glick *et al.*, 2007). In these circumstances, the function of reducing ethylene production could be a viable tool to manage the plant stand against abiotic stresses.

It is well known that microbes have a mechanism to mitigate the higher synthesis of ethylene in plants during stress-related conditions. When plants are exposed to these conditions, ethylene is synthesized from a precursor of 1-aminocyclopropane-1-carboxylic acid (ACC) resulting in senescence and retardation of root growth in plants. In such conditions, the introduction of a fungus or bacterium which has the enzyme ACC deaminase could stop the higher synthesis of stress ethylene by converting the precursor ACC into ammonia and α-ketobutyrate. Several researchers have demonstrated an enhanced survival fitness of plants against various types of abiotic stress, such as salinity (Mayak *et al.*, 2004a; Saravanakumar and Samiyappan, 2007), water stress (Mayak *et al.*, 2004b; Saravanakumar *et al.*, 2011) and flooding (Grichko and Glick, 2001), through the enzymatic action of ACC deaminase. The microbes have also been reported to degrade heavy metals, herbicides and pesticides which have the huge potential to pollute the soil in various environments including agricultural ecosystems (Hernández *et al.*, 2008; Chen *et al.*, 2012).

10.2.4 Microbes in food processing

Microorganisms, namely bacteria, yeasts and moulds, are considered to be a vital part of the food processing system. Efforts have been made to explore new habitats to look for microbes which are capable of making novel products and processes. The microbial traits that are considered to be most important for commercial food processing are: (i) sensory quality; (ii) bacteriophage resistance; (iii) the ability to secrete antimicrobial compounds for inhibiting the growth of undesirable microorganisms; (iv) the ability to produce enzymes of industrial value; and (v) degradation or inactivation of toxins. Since the application of microbes in the food industry is a large topic to deal with, this chapter focuses on the microbes that are involved in degradation of natural toxins, anti-nutritional factors, mycotoxins and specific biotechnology-relevant enzymes in the food industry.

Degradation of natural toxins

Toxigenic compounds are natural compounds found in some agricultural products such as cyanogens in cassava, sorghum, linseed and in livestock feeds. The natural toxins need to be removed from the food products as they cause health hazards to humans and livestock. Strains of *Lactobacillus plantarum*, *Leuconostoc mesenteroides* and *Candida tropicalis* producing the enzyme β-glucosidase showed higher potential than the non-producers to degrade the cyanogenic glycosides in fermented food products of cassava and linseed (Lei *et al.*, 1999). Similarly, bacterial strains of *Bacillus licheniformis*, *Rhodococcus*, *Klebsiella* and *Alcaligenes* (Ramarethinam and Rajalakshmi, 2004; Mohapatra *et al.*, 2006) and fungal isolates of *Penicillium*, *Aspergillus* and *Rhizopus* (Brand *et al.*, 2000) were efficient in detoxification of caffeine during the processing of coffee and tea through different pathways by producing N-demethylases and caffeine oxidases. They also served as potential sources for biodecaffeination as caffeine has lots of negative impacts on the environment causing pollution and soil infertility (Dash and Gummadi, 2006). The identification and characterization of genes responsible for caffeine catabolism enzymes like N-demethylases and caffeine oxidases could provide the scope for genetic manipulation in a single microorganism to increase degradation activity.

Biotransformation of mycotoxins

In addition to the detoxification of natural toxins and alkaloids from plant products and beverages, microbes have greater application in the

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biotransformation of mycotoxins. Mycotoxins are produced by a specific group of fungi in cereals, oilseeds, livestock feeds, dried and processed fruits, spices, fruit juices and wines. For instance, the aflatoxin B1 produced by filamentous fungi *Aspergillus flavus* and *Aspergillus parasiticus* have been considered to be highly mutagenic, toxic, carcinogenic and teratogenic to humans and animals. Cell free cultures of bacterial strains *Mycobacterium fluoranthenivorans* DSM 44556T and *Rhodococcus erythropolis* DSM 14303 have degraded more than 90% of aflatoxin B1 (Teniola *et al.*, 2005). The genes responsible for detoxification of several polyaromatic compounds were encoded by the *bphC* gene of *R. erythropolis* (Takeda *et al.*, 2004) suggesting the expression of multiple *bphC* dioxygenases will have an added advantage in the degradation of polyaromatic compounds like aflatoxin B1 in food and feed processing.

Molnar *et al.* (2004) have isolated a novel yeast strain, *Trichosporon mycotoxinivorans*, which has the ability to degrade ochratoxin A and zearalenone. Similarly, the yeast strains *Rhodosporidium kratochvilovae* LS11 and *Sporobolomyces* IAM 13481 have effectively degraded the patulin accumulation in pome fruits and their derived products (Castoria *et al.*, 2011). The genome sequencing of yeast strain *Sporobolomyces* sp. IAM 13481 has revealed the genes responsible for patulin degradation (Ianiri *et al.*, 2013). The identification of patulin degradation genes has opened up the possibility of producing apples incorporating patulin degradation genes or the industrial use of the yeast strain *Sporobolomyces* sp. IAM 13481 in contaminated apple juices.

Novel microbial enzymes with wider adoptability and utility

It is also possible to generate enzymes from microbes with modified structures that confer novel desired properties, such as improved activity, thermostability, or the ability to work on a new substrate or at a higher pH. For example a novel β-galactosidase gene *BGalH* from the microbe *Halomonas* sp. S62 (identified through whole genome sequencing) has been shown to completely hydrolyse lactose in milk, and it exhibited high activity at low temperature and also stability over a wide range of pH. These properties were found to be suitable for the removal of lactose from dairy products at low temperatures (Wang *et al.*, 2013). Ko *et al.* (2013) characterized a novel steviol-producing β-glucosidase enzyme from *Penicillium decumbens* and optimized the conditions for production of the steviol which has use as a sweetener for seasonings, pickles and salted foods. Similarly, the purification and characterization of a thermostable novel enzyme, glucoamylase, from *Fusarium solani* may have application in the food industry for saccharification of starch, as resistance to thermal inactivation has become a desirable property in many industrial applications (Bhatti *et al.*, 2007). Likewise, the bacterium *Oenococcus oeni* was responsible for malolactic fermentation through the action of β-glycosidases, a secondary fermentation that takes place in many wines which imparts positive sensory properties (Borneman *et al.*, 2010, 2012). This enzyme liberates the volatile aroma compounds from non-volatile sugar molecules in wines.

With these wider applications of microbes and their products in the field of food and agriculture, this chapter now considers how biotechnology could be employed to identify, characterize and improve the microorganisms for their enhanced activity and increased use in food and agriculture.

10.3 Biotechnology for Characterization of Agricultural Microbes

10.3.1 Identification of microbes using 16S rRNA analysis

Biotechnology is the broad term used for the characterization of genes of interest (in part or full), gene manipulation, transfer and expression of genes in different systems and use of molecular markers, in this case in respect of agricultural microbes. By characterization of the genes, either the novel microbes or the particular trait of the identified potential microbes could be improved, with the ultimate goal of enhancing agricultural production and/or their use in the food industry. With the advent of PCR, it has become handy to identify organisms with an amplification and sequencing of the conserved

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DNA regions. With respect to the culturable microbes associated with plant growth and development, the use of universal primers from the 16S rDNA region helps to sequence the nucleotides and identify the microbial taxonomy of the organisms. The availability of genomic databases such as the National Center for Biotechnology Information (NCBI), the European Molecular Biology Laboratory (EMBL) and the DNA Databank of Japan (DDBJ) have further widened the opportunities and possibilities for identification of microorganisms of regional importance. For instance Hernández *et al*. (2008) observed that *Stenotrophomonas* strain P51 and *Arthrobacter* strain P52 isolated from long-term herbicide-treated agricultural soils in central Chile were capable of degrading *s*-triazine herbicides. Using 16S rRNA gene sequencing they were able to characterize these strains and identify more populations of these novel microbes in the contaminated soils thereby degrading the herbicides. Further characterization of microbial genes responsible for *s*-triazine degradation could be useful as biocatalysts for the bioremediation of herbicides in agricultural soils.

10.3.2 Characterization of microbial genes useful in agriculture

Plant–microbe interactions take place on the basis of biochemical and genomic interactions between plants and their associated microbes. It is essential to characterize the genes involved in orchestrating the beneficial associations via different signals and sensing of root metabolites that promote the uptake of nutrients through fixing and solubilization of soil nutrients and keeping phytopathogens away from the plants. For instance, *nif* genes are responsible for nodulation and fixing nitrogen by *Rhizobium* in leguminous plants. Similarly, for many years several microbiological and biochemical studies have been focused on mechanisms involved in the biocontrol of pests and diseases (Elad, 2000; Ramjegathesh *et al*., 2013).

The recent advancement in molecular techniques has been greatly attributed to the development of new tools and approaches for shedding light on the various mechanisms of biological control and for improving insights provided by biochemical and microbiological studies. From the genes involved in biocontrol properties, the genetic basis of mechanisms of action can be understood. Of several strategies available, targeted gene sequence from a known database, differential display techniques and whole genome sequencing of an organism may provide better insights into mechanisms of action (Saveetha *et al*., 2010). Besides these techniques, gene inactivation and overexpression studies through insertion mutagenesis, PCR tilling and RNA silencing studies could be employed to provide knowledge on the transcription and regulation of these genes and to confirm the biocontrol potential (Massart and Jijakli, 2007).

An example of a microbial gene that is useful in agriculture is the gene *acdS* which codes for the enzyme ACC deaminase in rhizobacteria; this enzyme can mitigate the higher synthesis of ethylene in plants under stress-related conditions (as explained earlier in section 10.2.3). Genetically engineering the ACC deaminase gene *acdS* into a plant-associated bacterium that otherwise does not possess this gene could enhance the beneficial effect of the bacterium on plant growth under stressful environmental conditions.

Escherichia coli and strains of *Pseudomonas* that are deficient in ACC deaminase activity have been transformed with the *acdS* gene from *Pseudomonas* so as to enable root elongation in canola plants (Shah *et al*., 1998). Similarly, the biocontrol efficacy of pseudomonads has been significantly improved with the transformation of the *acdS* gene (Wang *et al*., 2000). More importantly, researchers have ensured that the complex transcriptional regulatory system controlling the expression of *acdS* genes works in all transformants. This was clearly demonstrated in two different systems. In the first system, ACC deaminase activity was not expressed in the *Azospirillum* transformants when a *Pseudomonas acdS* gene was transformed under the control of regulatory gene *acdR*. In the second system, ACC deaminase activity was highly expressed in *Azospirillum* transformants and promoted plant growth when the regulatory portion of the *acdS* gene was placed under the control of *E. coli*'s *lac* promoter or *tet* promoter (Holguin and Glick, 2001).

Similarly, the transfer of the *acdS* gene from *Rhizobium leguminosarum* bv. *viciae* 128C53K to *Sinorhizobium meliloti* enabled higher nodulation

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in lucerne plants and stimulated plant growth by 35–40% more than the non-transformed strain (Ma *et al.*, 2004). These studies clearly indicated the possibility of improving the efficacy of different rhizobacterial strains with genetic engineering of the ACC deaminase gene. It was also demonstrated that application of ACC deaminase-secreting *Pseudomonas putida* UW4 with *Gigaspora rosea* BEG9 resulted in synergistic interactions between the two microbial systems and in turn enhanced growth in cucumber plants (Gamalero *et al.*, 2009). ACC deaminase-mediated stress resistance with its potential for improving agriculture and use of this gene in biotechnology are well reviewed by Glick *et al.* (2007) and Saravanakumar (2012). To provide an insight to the microbial genes useful in the field of agriculture, the candidate genes identified from different microbes using molecular techniques have been presented in Table 10.1.

10.3.3 Targeted gene sequence

The targeted gene strategy requires prior selection of one or a few genes. This selection can be based either on pre-existing data or on extrapolation of an existing model developed in a study of other beneficial microbes in the field of agriculture, for instance it could be a biocontrol agent. Whatever the selection process, the first step is to design degenerate primers amplifying part of the gene sequence. The degenerate primers could be picked up from the amino acid sequence of the characterized proteins, or from the sequence alignment of similar proteins from other microbes, and/or from the primers used previously for other microbes. After PCR amplification with the degenerate primers, the amplified DNA product could be isolated, cloned and sequenced. The sequence data obtained could be further used to track the full sequence of the gene including *cis*- and *trans*-regions either by employing a modified cRACE protocol or by hybridizing the cloned product with a genomic DNA library (Raguchander *et al.*, 2011). Yet, this strategy focuses only on one or a few genes, whereas in the case of biological control, the properties often depend on the regulation and the mutual interaction of numerous genes.

10.3.4 Differential expression studies

The genes involved in plant–microbe interactions could be identified by assessing the differential gene expression under varied exposure conditions. The molecular techniques, cDNA amplified fragment length polymorphism analysis (cDNA-AFLP), differential display and subtractive hybridization are normally used for differential expression studies. For example the assessment of gene expression by biocontrol agents in the presence and absence of pathogenic microorganisms may provide information on the overexpression or repression of novel genes. Those genes can be isolated, cloned and sequenced for further characterization and property studies. Nevertheless, the relevance of the genes identified by such methods with regards to their putative involvement in biocontrol properties depends heavily on the selected comparison model (Saveetha *et al.*, 2010).

10.3.5 Genome sequencing

In recent years, functional genomics, a relatively new area of research, has been developed. It aims to determine the patterns of gene expression and interaction in the genome, based on the knowledge of an extensive part of or the complete genomic sequence of an organism. It can provide an understanding of how microorganisms respond to environmental influences at the genetic level (i.e. by expressing specific genes in different situations or ecologies) and should therefore allow adaptation of conditions to improve technological processes. In each microbial genome that has been sequenced, 40–50% of the putative genes encode proteins of unknown function and 20–30% encode unknown proteins apparently unique to that species. Genomic analysis also suggests that less than 1% of the microbes on earth have been cultured and studied in the laboratory. With the advent of genome sequencing, it is now possible in many organisms to observe the expression of many genes simultaneously, even those with unknown biological functions, as they are switched on and off during normal development or while an organism attempts to cope with pathogens or the changing environmental conditions.

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Table 10.1. Genes characterized from plant-associated microbes for potential use in food and agriculture.

Source microbe	Gene/plasmid/cosmid	Use in agriculture	References
Nitrogen-fixing/phosphate-solubilization genes			
Pseudomonas stutzeri A1501	X940 cosmid encoding *nif* genes	Biocontrol bacterium *Pseudomonas protegens* Pf-5 transformed with *nif* genes improved plant growth in *Arabidopsis*, lucerne, tall fescue and maize under nitrogen-deficient conditions	Setten *et al*. (2013)
Klebsiella pneumonia	*nifD*, *nif K*, *nifN*, *nifV*, *nifB*, *nifQ*, *nifE*, *nifX*, *nifU*, *nifS*	Encodes the enzymes involved in nitrogen fixation; 24 kb of DNA contains 20 genes	Swain and Abhijita (2013)
Rhizobium leguminosarum	*acdS* gene	Plant growth promotion during stress conditions	Ma *et al*. (2004)
Serratia marcescens	pKG3791	Solubilization of mineral phosphorus in the soil and helps in plant nutrient uptake	Krishnaraj and Goldstein (2001)
Pseudomonas fluorescens	More than 20 *rhi* genes	Nutrient acquisition and stress response	Rainey (1999)
Genes associated with root colonization			
P. fluorescens strain WCS365	*sss*	Rhizosphere colonization and enhanced biocontrol activity against *Fusarium oxysporum* f.sp. *radicis-lycopersici*	Dekkers *et al*. (2000)
Genes for antibiotic production			
Trichoderma brevicompactum IBT40841	*tri5*	Involved in trichodermin antibiotic production for the biocontrol of plant diseases	Tijerino *et al*. (2011)
Bacillus amyloliquefaciens FZB42	*srfAA*, *bacA*, *bmyB* and *fend*	Antifungal, antibacterial and nematicidal activity involved in biological control	Koumoutsi *et al*. (2004)
Bacillus strain QST713	*bacA*, *srfAA*, *bmyB*, *fenD* and *ituC*	Commercial biofungicide for the management of plant diseases	Joshi and McSpadden-Gardener (2006)
P. fluorescens 2-79	*phz A*, *B*, *C*, *D*, *E*, *F*, *G*	Involved in the synthesis of phenazine carboxylic acid	Mavrodi *et al*. (1998)
P. fluorescens Q2-87	*phl A*, *C*, *B*, *D*	Synthesis of 2,4-diacetylphloro glucinol (2,4-DAPG) which has biocontrol activity against damping-off, root rot, wilt and take all diseases	Mavrodi *et al*. (2001)
P. fluorescens Pf-5	*plt F*, *A*, *B*, *C*, *D*, *E*, *F*, *G*	Synthesis of pyoluteorin involved in the control of oomycetes (chromista) pathogens	Kraus and Loper (1995)
Genes encoding lytic enzymes			
Trichoderma harzianum, *Trichoderma virens*	*ech42*	Biocontrol of soil-borne pathogens	Garcia *et al*. (1994), Baek *et al*. (1999)
Clonostachys rosea strain IK726	*cr-ech58*, *cr-ech42*, *cr-ech37*	Chitinase gene in biocontrol	Mamarabadi *et al*. (2008)
Aeromonas hydrophila HS4, *Aeromonas punctata* HS6	Chitinases	Biocontrol of phytopathogenic fungi and harmful insects	Kuddus and Ahmad (2013)

Continued

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Table 10.1. Continued.

Source microbe	Gene/plasmid/cosmid	Use in agriculture	References
Abiotic stress tolerance			
Aspergillus niger	Glucose oxidase encoding gene (*goxA*)	*Trichoderma atroviride* SJ3-4 strain with glucose oxidase encoding gene (*goxA*) from *A. niger* under a homologous chitinase (*nag1*) promoter has significantly induced systemic resistance in plants	Brunner *et al*. (2005)
Pseudomonas putida, *P. fluorescens*, *Enterobacter*	*accD*	Reduces the accumulation of stress ethylene. Broad-spectrum activity against water stress, saline stress and flooding	Mayak *et al*. (2004a, b), Glick *et al*. (2007), Saravanakumar and Samiyappan (2007)
Streptoccocus thermophilus	*Clp L*	Heat shock and cold tolerance in agriculture	Varcamonti *et al*. (2006)
Pantoea dispersa strain 1A, *Serratia marcescens* strain SRM	–	Cold-tolerant plant growth-promoting bacterial strain	Selvakumar *et al* (2008a, b)
Bioremediation genes			
Stenotrophomonas, *Arthrobacter*	*atzA*, *atzB*, *atzC*, *atzD*, *atzE* and *atzF*	*s*-Triazine herbicide degradation in the agricultural field	Hernández *et al*. (2008)
Candida pelliculosa	–	Bifenthrin pesticide degradation in the agricultural field	Chen *et al*. (2012)
Food processing related genes/enzymes			
Sporobolomyces sp. IAM 13481, *Rhodosporidium kratochvilovae*	–	Patulin degradation	Ianiri *et al*. (2013)
Trichosporon mycotoxinivorans	–	Aflatoxin B1 detoxification	Molnar *et al*. (2004)
C. rosea	*zhd101* (lactonohydrolase)	Zearalenone detoxification	Takahashi-Ando *et al*. (2005)
Fusarium sporotrichioides, *Fusarium graminearum*	*Tri101* (trichothecene 3-O-acetyltrans-ferase)	Trichothecene detoxification	Garvey *et al*. (2008)
Halomonas sp. S62	*BGalH*	Greater specificity to lactose, high activity at low temperature and stability over a wide range of pH	Wang *et al*. (2013)
Fusarium solani	Glucoamylase	Thermostable enzyme for saccharification of starch	Bhatti *et al*. (2007)

The genome sequences of many plant-associated microorganisms have been completed using the whole shotgun sequence method and large numbers of microbial genome sequencing projects are also in progress. The whole genomic sequencing of several important agriculturally beneficial microorganisms such as *Pseudomonas fluorescens* Pf-5, *Bacillus amyloliquefaciens* subsp. *plantarum* UCMB5033, *Rhizobium leguminosarum*, *Serratia plymuthica* strain AS9, *Trichoderma virens* Gv29-8 and *Trichoderma atroviride* IMI 206040 opens up a new avenue to advance the knowledge on beneficial interactions through a genomics approach (Paulsen *et al*., 2005; MacLean

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et al., 2007; Kubicek *et al.*, 2011; Neupane *et al.*, 2012; Niazi *et al.*, 2013). Full genome sequencing of the antagonistic bacterium *P. fluorescens* Pf-5 revealed that 6% of the full genome (7.07 Mb) sequence has been attributed to the biosynthesis of secondary metabolites such as antibiotics and siderophores (involved in sequestering iron compounds to starve and inhibit the phytopathogens). In this study, Paulsen *et al.* (2005) used a new approach consisting of a combination of genome sequence analysis and isotope guided fractionation (genomisotopic approach) to identify the bioactive compounds (orfamide A) involved in biological control. Similarly, comparison of the whole genome sequence of two biocontrol mycoparasitic organisms such as *T. virens* and *T. atroviride* revealed that the genome of *T. virens* contains 28 non-ribosomal peptide synthetases, including the gene for the strongly antifungal compound gliotoxin, compared with 16 non-ribosomal peptide synthetases for *T. atroviride* (Kubicek *et al.*, 2011).

10.3.6 Metagenomics for new beneficial inoculants

Traditional isolation and culturing methods have so far targeted a small group of copiotrophic microorganisms as candidates for use in agriculture. However, culture-independent studies have shown the existence of significant populations of unexplored soil microorganisms in agricultural settings which have not been characterized and identified in the isolation and culturing methods using nutrient-based media. In such circumstances, genomic sequencing and analysis of uncultured soil microroganisms uses the technique known as metagenomics. This technique has been applied to explicit novel microbial genes which are responsible for antibiotic production and bioremediation. Further, the cloning of large fragments of DNA isolated directly from microbes in natural environments provides a method to access the soil metagenomic DNA and to study the properties of microbes in various soil conditions.

Metagenomics analysis can be accomplished in two ways: (i) sequence-driven analysis; and (ii) functional-driven analysis. In the case of sequence-driven analysis, the conserved DNA sequences have greater utility in designing PCR primers or hybridizaton probes to screen and analyse the metagenomic libraries. The sequencing of clones carrying phylogenetic regions, namely the internal transcribed spacer (ITS) and the 16S rRNA gene, could provide taxonomical information about the microorganisms from which the clones were obtained. The functional-driven analysis mainly consists of identification of cloned DNA fragments expressing a desired trait and characterization of these clones by biochemical and sequence analysis. This particular metagenomics approach has greater potential in agriculture as it could help in identifying useful metabolites, genes and proteins in agriculture from the uncultured microorganisms. The major limitation of this approach could be the expression of a desirable trait in the target cell and orchestrating all the genes involved in the functional expression (Schloss and Handelsman, 2003). Chhabra *et al.* (2013) utilized the functional metagenomic approach to identify novel uncultivatable microbes which had greater capacity to solubilize mineral phosphate compounds from a barley rhizosphere soil. The application of metagenomics in tracking novel compounds from soil microbes has been extensively reviewed by Ghosh (2012). In conclusion, metagenomics could provide significant information about the novel genes responsible for production of antibiotics and metabolic enzymes and competition for its microbial fitness in the ecological niche (Handelsman, 2004). However, the various strategies so far developed to identify genes by researchers have their own advantages and drawbacks. Hence, the selection and use of the specific technique relies heavily on the information to be derived.

10.4 Biotechnology to Enhance the Activity of Microorganisms Useful to Agriculture

The application of biotechnology in enhancing microbes is not only limited to the characterization of genes and genetic elucidation of functional genes but also involves the genetic transfer and manipulation of single or multiple genes involved in promoting plant growth, alleviation of biotic and abiotic stresses and degradation of toxins in food. Also it is possible to design the

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superior microbe with multiple genes encoding for different properties such as the capability of acting as a biofertilizer, biocontrol agent and the ability to degrade natural toxicants in plants.

In general, protoplast fusion, transposon mutagenesis and transformation have been used for genetic manipulation of fungi. Whole fungal cells could be transformed using the lithium acetate method by electroporation, or by particle bombardment and/or by *Agrobacterium*-mediated transformation. Bacterial transformation is mainly performed using electroporation, or osmotic shock or by means of *E. coli*-mediated conjugation. Similarly, the genes encoding for undesirable characters that reduce the efficacy of biocontrol agents could also be deleted or suppressed using molecular techniques like transposon mutagenesis.

10.4.1 Protoplast fusion

Protoplast fusion is considered as a quick and easy method to develop improved hybrid strains via genetic recombination in filamentous fungi (Lalithakumari and Mathivanan, 2003) and antagonistic bacteria (Abdel-Salam *et al.*, 2007). This technique is mainly applied to bring out the most desirable attributes in one strain from two distinguished elite strains. Most frequently, the introduction of exogenous DNA through protoplast fusion is done in the presence of polyethylene glycol (PEG) and calcium chloride. This technique in particular has been effectively used in improving many *Trichoderma* strains for their biocontrol potential against various plant diseases (Lalithakumari and Mathivanan, 2003). An interspecific protoplast fusion has been effective in enhancing the cellulase production in *Trichoderma reesei* (Ogawa *et al.*, 1989). Similarly, self-fusion of protoplasts from *Trichoderma harzianum* strain PTh18 showed a twofold increase in chitinase and biocontrol activity against *Rhizoctonia solani* when compared with the parent strain (Prabavathy *et al.*, 2006). Recent studies have indicated the successful fusion of protoplasts between two antagonistic bacterial strains. Protoplast fusion between two antibiotic-producing *Pseudomonas aeruginosa* and *P. fluorescens* strains demonstrated improved efficacy against *Fusarium oxysporum*. The superiority of the fusant in reducing the pathogen could be because they have the killing system from both parental strains and more gene copies of an antagonistic system (Abdel-Salam *et al.*, 2007).

10.4.2 Genetic recombination

Genetically modified microorganisms (GMMs) based on recombinant DNA techniques have been constructed since the 1970s. Genetic recombination is an advanced biotechnology which results in microorganisms with enriched superior characteristics of interest. It offers the opportunity to create artificial combinations of genes that do not exist together in nature. The most frequently used techniques include engineering with single genes or operons, pathway construction and alterations of the sequences of existing genes (Dale and Park, 2007).

It has also been proposed that chromosomal insertion of the genes is one of the techniques to overcome the horizontal transfer of introduced genes within the rhizosphere (Rodríguez *et al.*, 2007). Bacteria, fungi and viruses are the major candidates of choice for genetic manipulation. However, considering genetic manipulation, ease of commercial production and application of potential GMM in agriculture, bacterial organisms have been found to be most suitable when compared to fungi and viruses (Vidaver *et al.*, 2012).

The first step in the genetic recombination of microbes is the selection of a suitable gene. Next, the DNA fragment to be cloned is inserted into a vector and introduced into host cells. The modified bacteria are called recombinant cells. The following step is production of multiple gene copies and selection of cells containing recombinant DNA. The final step includes screening for clones with the desired DNA inserts and biological properties (Wasilkowski *et al.*, 2012). Thus, the isolation of DNA, modification of DNA via deletion or insertion of nucleotides, transfer and expression of the desired gene into the host cell, selectable marker genes, knowledge of nucleotide sequences which control gene expression, and selection and subsequent propagation have assumed great significance in successful gene transformation in microorganisms. In some cases, undesirable characters have to be deleted from the microorganisms so as to obtain the beneficial activity, especially in the field of the food industry.

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For instance, frost damage is a major agricultural problem affecting many annual crops and subtropical plants. Frost damage is initiated on the surface of plant cells by a membrane protein of ice-nucleating bacteria, namely *Pseudomonas*, *Xanthomonas* and *Erwinia*. Researchers formulated the idea of developing non-ice-nucleating bacteria to outcompete ice-nucleating bacteria. The non-ice-nucleating bacteria were isolated by treating the ice-nucleating bacteria with chemical mutagens and this has been seen as possibility of introducing multiple mutations that could adversely impact their genetic stability and ecological fitness. To avoid multiple mutations, ice-nucleation-deficient mutants of *Pseudomonas syringae* were constructed by deleting the genes conferring ice nucleation. These genetically engineered mutants were able to compete successfully with ice-nucleating *P. syringae* for the colonization of plant leaf surfaces. Field tests showed that plants treated with ice-nucleation-deficient *P. syringae* suffered significantly less frost damage than the untreated control plants (Lindow and Panopoulos, 1988). Similarly, it was reported that *P. putida* GR12-2 secretes antifreeze proteins into the surrounding medium when the bacterium is grown at low temperatures (Sun *et al*., 1995).

In the case of biological control, the development of a superior biocontrol agent is necessary for the successful management of plant diseases after isolation and characterization of genes involved in biocontrol properties. This could involve the construction of elite strains that produce increased levels of lytic enzymes and antibiotics. In contrast, the suppression or deletion of a gene from the biocontrol strain could also enhance the sustained biocontrol activity in some of the strains. This was clearly demonstrated in a modified strain of *Agrobacterium radiobacter* strain K84 that controlled crown gall disease caused by *Agrobacterium tumefaciens*. Agrocin 84 antibiotic produced by *A. radiobacter* is toxic to agrobacteria possessing a nopaline and agrocinopine A type Ti plasmid (McClure *et al*., 1994). So the pathogen *A. tumefaciens* can develop agrocin 84 resistance if the plasmid carrying the genes responsible for the biosynthesis of agrocin 84 could accidentally be transferred from *A. radiobacter*. To avoid this possibility, the DNA region responsible for plasmid transfer was axed from the agrocin 84 plasmid. In this way the mutant strain of *A. radiobacter* was developed so that it lost its property of transferring the modified agrocin plasmid to pathogenic agrobacteria and retained the potential to act as an effective biocontrol agent. Similarly, Smith and Saddler (2001) reported the biocontrol activity of avirulent mutants of *Ralstonia solanacearum* against bacterial wilt in potato. These strains containing transposon-induced insertions in the *hrp* gene were able to invade the plant, survive and multiply within the plant excluding pathogenic strains.

Rodríguez *et al*. (2007) have recently reviewed the advantages of developing genetically modified rhizobacteria over developing transgenic plants for improving plant performance, expressing the view that it is easier to modify a bacterium than complex higher organisms. The same authors have stated that plant growth-promoting traits could be combined in a single organism instead of engineering crop by crop. This suggested the possibility of constructing a superior plant-beneficial strain with multiple mechanisms of action. In line with this statement, the biocontrol rhizobacterium *Pseudomonas protegens* Pf-5, which has greater colonization ability and adaptability to a wide variety of plant species, has been genetically engineered with nitrogenase-encoding genes transferred from *Pseudomonas stutzeri* A1501 via the X940 cosmid in order to have the ability to fix atmospheric nitrogen (Setten *et al*., 2013).

In the detoxification of mycotoxins, the most popular fermentation yeast *Saccharomyces cerevisiae* and strains of the probiotic food bacteria *Lactobacillus* have been used as vector systems to carry detoxification genes. Takahashi-Ando *et al*. (2002) isolated and cloned a zearalenone detoxifying gene *zhd101* (lactonohydrolase) from *Clonostachys rosea*. The same authors have introduced the gene into *S. cerevisiae* and evaluated the detoxification activity against zearalenone toxin (Takahashi-Ando *et al*., 2005). The transformation system in the yeast strain showed greater detoxification when compared with the gene *zhd101* expressed in *Lactobacillus* strains (El-Nezami *et al*., 2002). Similarly, trichothecene detoxification gene *Tri101* encoding trichothecene 3-O-acetyltransferase was isolated from *Fusarium sporotrichioides* and *Fusarium graminearum*. The gene was cloned and transiently expressed in *S. cerevisiae*, wheat,

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barley and tobacco to study its expression in different systems and its ability to detoxify the mycotoxin (Garvey *et al.*, 2008) produced by various species of *Fusarium*. The authors observed the differential expression of the *Tri101* gene in various transformation systems. This indicated that the expression of genes in different transient systems partly or fully relies on the use of suitable vectors and regulator systems.

10.4.3 Use of regulators for expression of microbial genes

One of the most critical factors that determine the success of transgenesis in microbial strains is the availability of suitable promoters to bring out the expression of exogenous or endogenous novel transgenes.

To understand this process, Margolles-Clark *et al.* (1996) used the cellulase promoter *cbh1* from *T. reesei* to express the endochitinase gene (*ThEn-42*) of *T. harzianum* in the transformation system. The transformants showed a tenfold increase in chitinase activity. Yet, it was difficult to predict the functional system of the transformants under natural conditions, as the higher production of chitinase was triggered by the cellulose-inducing components because of the *chb1* promoter. Also no elicitation was observed in the presence of chitin which is actually considered to be a substrate specific for the overproduction of a chitinase enzyme. The activity of 42 kDa endochitinase was increased to fivefold when the same gene was duplicated into the host cell along with the 5′ and 3′ regulatory sequences to the full length of 3.5 kb genomic DNA. This particular research has supported the homologous transformation of chitinase genes in the biocontrol *Trichoderma* species. It was also concluded in many of the studies that several species of *Trichoderma* have the 42 kDa endochitinase gene as a conserved region (Fekete *et al.*, 1996). In that case, the specific triggering of the conserved endochitinase gene could be a useful trait in improving the biocontrol fungi. Lorito and co-researchers have shown that specific triggering of chitinase genes occurred when a mycoparasitic protein complex recognized the promoter sequences and disconnected the association of catabolite repressor proteins (Lorito *et al.*, 1996). Similarly, the introduction of glucose oxidase encoding gene (*goxA*) from *Aspergillus niger* into *T. atroviride* SJ3-4 and expression under the control of a homologous chitinase (*nag1*) promoter has increased the biocontrol potential of fungal transformant *T. atroviride* SJ3-4 against *R. solani*, *Botrytis cinerea* and *Pythium ultimum*. The expression of gene *goxA* and secretion of the enzyme was observed immediately when it recognized the pathogen. The timely secretion of the glucose oxidase enzyme was held responsible for the effective mycoparasitism and lysis of the plant pathogens, *R. solani* and *P. ultimum*. This clearly demonstrated that use of pathogen-inducible promoters in an expression of heterologous genes could enhance the biocontrol potential and systemic resistance-inducing properties of biocontrol agents. Further, these microbes could be more useful in providing defence molecules such as glucose oxidase to plants so that the plants can recognize the pathogen and enhance their defences to pathogens (Brunner *et al.*, 2005).

Nowadays research is focused on the construction of adaptive and suitable vectors so that the vector could carry and express the desired traits under varied environmental conditions which are very common in the field of agriculture. In line with this statement, the endochitinase *ChiA* gene from *Serratia marcescens* B2 was introduced into an indigenous epiphytic bacterium (*Erwinia ananas* NR1) that colonizes rice leaves. The genes were placed under the control of homologous promoters derived from the same bacterium (i.e. *E. ananas* NR1). The evaluation of the transformed strain NR1 has demonstrated its greater efficacy in controlling rice blast disease. The advantage of the use of the indigenous strains for the transformation is claimed to be that it would have greater adaptability to the local conditions and might not be affected by abiotic or biotic factors. Therefore an introduction of disease suppressive genes under the control of promoters derived from the recipient itself has offered a novel technique for development of the new biocontrol agents (Someya and Akutsu, 2006).

In some studies, it has been shown that the production of various antifungal metabolites could be controlled by a global regulator. In that case the antibiotic production of a particular

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microorganism could be very well enhanced by modifying these global regulators. It was reported in *P. fluorescens* CHA0 that when it encodes housekeeping sigma factor ~70, it also enhanced the production of antibiotics and improved the protection against damping-off of cucumber caused by *P. ultimum* (Schnider *et al*., 1995). Similarly, another strain of *P. fluorescens*, an antagonist of *R. solani*, has been genetically modified for increased production of the antibiotic pyrrolnitrin (Ligon *et al*., 1999) by modifying or introducing an extra copy of the wild-type global regulator gene *gacA*. The use of the GacS and GacA two-component system in *Pseudomonas* spp. had a positive influence on the production of antifungal metabolites required for the control of plant diseases (Haas and Keel, 2003). Further, some of the specific transcriptional activators and repressors could also regulate the transcription of biosynthetic genes encoding for the production of antibiotics. For instance the PhlF protein expressed from the phloroglucinol (Phl) locus of *P. fluorescens* repressed the transcriptional proteins (PhlA–D) that guide the synthesis of phloroglucinol (Schnider *et al*., 2000). In this case the mutation of genes encoding the PhlF protein had subsequently increased the production of phloroglucinol. Similarly, the overexpression of *phlA–D* resulted in overproduction of phloroglucinol and ultimately it improved the biocontrol potential against *P. ultimum* (Delany *et al*., 2000). Thus it is necessary to understand the expression of homologous and heterologous genes and promoters for the successful genetic recombination of microorganisms.

10.5 Concerns About the Use of GMMs

There is worldwide debate over the production and consumption of food items produced from genetically modified micro-organisms. With respect to the use of GMMs in the field of agriculture and food processing there are lots of concerns about possible horizontal gene transfer, viability and the effects on the biodiversity of native organisms. Further, it warrants selective markers and tools to monitor the introduced organisms in the soil and environment. In these circumstances, the symbiotic colonization behaviour of the GMMs may be engineered in addition to the actual trait so as to make the organism bind to the rhizosphere region of a particular crop and so it will not be introduced into the surrounding soil. This was demonstrated in the case of the rhizoremediation of polychlorinated biphenyls by *P. fluorescens*, in which biphenyl degradation is regulated using a system that responds to signals from lucerne roots (Villacieros *et al*., 2005). Another promising approach could be the use of endophytic bacteria as recipients for the introduced novel genes, so that the genetically modified organisms would be confined within the plant. In the case of food-related GMMs, there are only a few examples of authorized use of GMMs. Hence it is necessary to apply the functional genomics and bioinformatics tools for risk analysis of GMMs. Further, the safety assessment of GMMs is important in addressing public concern about food safety and security.

10.6 Future Work

Microbes have many applications in the field of food and agriculture. The prevalence of inconsistent performance by microorganisms could be a significant drawback encountered in the use of microbes. In these circumstances, it is mandatory to develop the technologies to retain the constant performance of microbes and their products under various environmental conditions, including for example different substrates, temperatures and pH. Nevertheless this planet has a huge variety of microorganisms and their enzymes are at present left untapped for their potential use in various fields. Most of the research studies have been confined to *in vitro* studies and the useful traits have to be tailored to suit the field and commercial-level application. It is also understood from the publications arising from microbial research that biotechnological applications have greater scope in promoting novel microbes in various agricultural and allied industries than is currently being realized. In conclusion, a greater understanding of microbes can improve the livelihood of humand kind in ensuring food safety and security.

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11 Endophytes: an Emerging Microbial Tool for Plant Disease Management

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Abstract

In 1884, de Barry first used the term 'endophyte' to define bacteria or fungi that occur within asymptomatic plant tissues. Endophytes include bacteria, fungi and actinomycetes. These microorganisms live inside plant tissues for part of their life cycle or for their entire lifespan without showing their presence as the plants do not express any visible symptoms. They offer great-untapped potential, which can be exploited to maintain healthy crops. They exist inside living plant tissues in different relationships, such as symbiotic relationships where both the partners get benefit or as antagonistic relationships in which the growth of one partner is suppressed while the growth of the other is favoured. Endophytes have been reported as potential antagonists against several plant pathogens. The potential to colonize internal host tissues for the benefit of crop growth and disease suppression have made endophytes an important component to improve crop performance. Novel endophytes are also directly associated with the production of secondary natural products or with the processes involved. Therefore identification, understanding interactions and utilization of novel endophytes or their products to enhance crop productivity and for management of plant diseases are an integral part of sustainable agricultural production.

11.1 Introduction

The term 'endophyte' consists of two Greek words, 'endo' meaning within and 'phyte' meaning plant. In nature, every plant species harbours endophytic microorganisms inside it and they live asymptomatically inside the tissues (Saikkonen *et al.*, 1998). Endophytes are most often isolated from symptomless plants of various species (McInroy and Kloepper, 1995) as they inhabit the tissues of roots, stems, leaves and seeds of various symptomless and healthy plants (Johri, 2006). They are also considered as plant mutualists. They are common in tissues of the aboveground parts of plants but are only occasionally observed in roots and are distinguished from mycorrhiza as they lack external hyphae or mantels (Saikkonen *et al.*, 1998). Endophytic microorganisms cannot be considered as saprophytes due to their association with the living hosts (tissues) and also due to their contribution to better plant health (Haggag, 2010). They offer great untapped potential that can be exploited to maintain healthy crops. They contribute towards better plant health by producing a plethora of substances which protect the plants

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and aid survival. These compounds, upon isolation and characterization, may have use in medicine, industry and agriculture. It has been observed that the presence of a mutualistic endophyte may act as a 'biological trigger' to activate the system of stress response more strongly and rapidly than in non-mutualistic plants (Redman *et al*., 2002). True endophytes never produce external fructifications on the host or on any substrate but may produce mycelium or spores externally on the host surface affecting flower and seed production (Siegel *et al*., 1987).

Transmission of endophytes may be either vertical (directly from parent plant to offspring) or horizontal (from individual to unrelated individual). Vertical transmission is usually observed in endophytes which have asexual cycles and are transmitted by penetrating the host's seed. These organisms are mutualistic as their reproductive fitness is tied to that of their host plant. Conversely, endophytes that are transmitted horizontally have sexual cycles and are transmitted via spores carried by the wind and/or insect vectors. Since they spread in a similar way to pathogens, horizontally transmitted endophytes are closely related to pathogenic organisms because of the similar mode of dissemination, although they are not pathogenic to hosts.

11.1.1 Advantages of endophytes in plant disease management

Advantages of endophytes in plant disease management include:

- Biological control of plant diseases by exploitation of endophytes is considered as an alternative to pesticides and reduces the use of harmful chemicals in crop production.
- Endophyte–plant interactions can be exploited to promote plant health and may play a significant role in sustainable low-input agriculture for both non-food and food crops.
- Use of specific endophytes is more preferable than the use of non-specific chemical pesticides or fertilizers due to their effectiveness, low cost and contributions to sustainable agricultural production.
- Biocontrol agents provide control consistently well throughout crop cycles, whereas chemicals remain active only for very short period as far as suppression of plant pathogens are concerned. If biocontrol agents (endophytes) are used as one of the components of integrated pest management (IPM) of seed production, this reduces costs and pollution levels as compared with use of chemicals.
- In nature endophytes are easily available and specific as they suppress or kill only the target pathogens or organisms.

In addition to these advantages there are some limitations to the use of endophytes. Some endophytic strains have been associated with increased susceptibility to diseases, for example turfgrasses when infected with endophytes become more susceptible to *Pythium* blight (Funk *et al*., 1994).

11.1.2 Biodiversity of endophytes

Temperate and tropical rainforests are considered to be the most biologically diverse terrestrial ecosystems for endophytes on earth. These spots only cover 1.44% of the land's surface, but they harbour more than 60% of the world's terrestrial biodiversity (Mittermeier *et al*., 1999). Areas having high plant endemism may also possess endophytes, which are specific and may have evolved with the endemic plant species. Ultimately, biological diversity indicates chemical diversity as a result of the constant chemical innovation which exists in ecosystems and the survival of evolutionary races is very active. Tropical rainforests in particular are a remarkable example of such types of environment. Resources are limited, competition is very high and selection pressure is at its peak. This indicates that the rainforests are probably the source of novel molecules and biologically active compounds (Redell *et al*., 2000). The metabolic distinction between tropical and temperate endophytes was described by Bills *et al*. (2002) through statistical data by comparing the number of bioactive natural products from endophytes of temperate origin with the number of such products from endophytes of tropical regions. The study revealed that tropical endophytes provide more active natural products than temperate endophytes. It was also observed that tropical endophytes produced a significantly greater number of active secondary metabolites than fungi living on other tropical substrata. This study suggested that the host plant may also influence the general metabolism of endophytes.

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11.2 Major Endophytic Microorganisms

Three main organisms are considered as endophytes: fungi, bacteria and actinomycetes.

11.2.1 Fungal endophytes

Most endophytic fungal species belong to the phylum Ascomycota (Schradl and Philips, 1997), which produce ascospores as the sexual spore and ascocarps as the sexual fruiting structure. Most of them are closely related to fungi known to cause diseases, either through active penetration into healthy tissue or as secondary invaders of wounded tissues (Schardl and Philips, 1997). Fungal endophytes live internally, either intracellularly or intercellularly, and asymptomatically, causing no apparent damage to the host (Saikkonen *et al.*, 1998). Like many fungal pathogens fungal endophytes also possess exoenzymes that are required to colonize their host and they usually grow in the apoplastic washing fluid of the hosts. Fungal endophyte–host interactions are characterized by an equilibrium between plant defence and fungal virulence and such endophytes are beneficial to plants as they promote plant growth (Dai *et al.*, 2008), protect plants from pests and diseases (Wilkinson *et al.*, 2000; Tanaka *et al.*, 2005; Vega *et al.*, 2008) and enhance resistance to various kinds of stress (Lewis, 2004; Malinowski *et al.*, 2004).

Some examples of the fungal endophytes are:

- *Neotyphodium* sp.;
- *Epichloe* sp.;
- *Trichoderma* sp.;
- *Phomopsis cassia*;
- *Gliocladium* sp.;
- *Cryptoscoriopsis quercina*; and
- *Aspergillus* sp.

11.2.2 Bacterial endophytes

Like endophytic fungi, endophytic bacteria have also been found colonizing internal tissues of virtually every plant studied, where they form a range of different types of relationships including commensalistic, mutualistic, symbiotic and trophobiotic. The origins of the majority of such endophytes appear to be either from the rhizosphere or from the phyllosphere; however, some endophytes seem to be transmitted through the seed. Bacterial endophytes can also promote plant growth and yield, and can act as antagonists. Endophytes can be beneficial to their host by producing a range of natural products and these natural products/metabolites could be harnessed for effective use in agriculture, medicine or in industry.

In addition to the benefits already mentioned, bacterial endophytes have the potential to indirectly remove soil contaminants through enhancement of phytoremediation and also they may play a significant role in soil fertility status through nitrogen fixation and phosphate solubilization. At the present time there is increased interest in developing the potential of modified endophytic microorganisms through biotechnological approaches for the sustainable production of non-food crops for biofuel and biomass production.

Some examples of bacterial endophytes are:

- *Pseudomonas fluorescens*;
- *Bacillus subtilis*;
- *Serracia marcescens*;
- *Curtobacterium luteum*; and
- *Methylobacterium extorquens*.

11.2.3 Actinomycetes

Actinomycetes are prokaryotic organisms with branched mycelia that produce spores; as a result actinomycetes are similar to fungi in character. Hence these Gram-positive bacteria are also known as filamentous fungi. As well as being Gram-positive, they are aerobic and constitute a large part of the rhizosphere microbiota. Their isolation is an important step for screening for new bioactive compounds. Excluding the coryneform bacteria, actinomycetes are also found inside plant tissues, and such associations may play an important role in plant growth and development. There are several reports that refer to actinomycetes acting as potential antagonists protecting plants

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against pathogens and also concerning the effects of their metabolic products on plant growth and physiology (Katznelson and Cole, 1965; Gupta *et al*., 1995).

Some examples of the endophytic actinomycetes are:

- *Frankia* sp.;
- *Streptomyces* sp.;
- *Nocardia* sp.;
- *Micromonospora* sp.;
- *Streptosporangium* sp.;
- *Microbispora* sp.; and
- *Streptoverticillium* sp.

Antimicrobial activity of endophytic actinomycetes

A number of actinomyctes have been found to be capable of suppressing fungal pathogens *in vitro* and *in planta* in wheat, including *Pythium* spp., *Gaeumannomyces graminis* var. *tritici* and *Rhizoctonia solani*, which indicates their potential as biocontrol agents (Coombs and Franco, 2003). They are also effective against *Bacillus subtilis*, *Staphylococcus aureus*, *Micrococcus luteus* and the yeast *Candida albicans*.

11.3 Endophyte–Host Relationship

Endophyte–host interactions are variable, and range from mutualistic to antagonistic (Saikkonen *et al*., 1998). Endophytes help the host plants by suppressing pathogens from colonizing inside them. Extensive multiplication and colonization of plant tissues by endophytes results in a 'barrier effect', where the existing endophytes compete with the pathogenic microorganisms and prevent them from taking hold. Chemicals produced by endophytes may also inhibit the growth of pathogens. Higher rates of water loss in leaves have been observed in plants with fungal endophytes in the internal tissues. All fungi establishing themselves in plant foliage have an asymptomatic period in their life, which varies from a short period as in pathogens to a lifetime as observed in the case of *Neotyphodium* endophytes in grasses. Grass–endophyte associations are generally considered to be mutualistic associations, although host–endophyte interactions and associations are based on mutual exploitation.

11.4 Bioactivity of Endophytes

Endophytes have been found to be bioactive in the following ways:

- Metabolites produced by endophytes have been exploited to combat pathogens and cancers even in human and animals.
- Endophytes are being tested for their roles in biofuel production and in agriculture.
- Activities of endophytic organisms have been recorded to be either due to the induction of host resistance or as a result of production of secondary metabolites, which indirectly protect the plants from harmful pathogens.
- Grass–endophyte interactions produce more metabolites, which are necessary for crop protection, than endophyte–woody plant interactions (Saikkonen *et al*., 2004).
- Tolerance to abiotic stresses is also enhanced due to endophytic associations (West, 1994).
- Plant roots infected by endophytic fungi have been recorded to produce phenolics and this elicits a greater plant defence reaction (Schulz *et al*., 1999).
- Phytoremediation of volatile organics and herbicides can be enhanced by endophytes.

11.5 Mechanisms of Action of Endophytes

Recently, endophytes have been studied and evaluated by many researchers for their possible use as biocontrol agents to protect plants against pathogens and insects. Based on such reports, it has been found that there are various mechanisms of endophytic inhibition of pathogens including direct and indirect parasitism.

11.5.1 Direct parasitism

In direct parasitism, endophytes directly suppress pathogens by the production of antibiotics and through secretion of lytic enzymes.

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Antibiosis

Antibiosis is the inhibition or death of an organism as a result of the toxic action of metabolites produced by another organism. Secondary metabolites produced by many fungal endophytes are antifungal and antibacterial in nature, which strongly suppresses the growth of other microorganisms or plant pathogens (Gunatilaka, 2006). Several endophytic bioagents are capable of producing single or multiple types of antibiotics including alkaloids, terpenoids, polypeptides and aromatic compounds, and plant pathogens are sensitive to those antibiotics (Table 11.1). It has been found that bacterial endophytes adhere to the fungal mycelium of pathogens such as in the case of the charcoal rot fungus, *Rhizoctonia bataticola*, causing deformation and cell wall lysis. Hyphal tip swelling, bursting and loss of structural integrity, were also observed (Senthilkumar *et al*., 2007).

Production of lytic enzymes

Lytic enzymes produced by many microorganisms can hydrolyse a variety of polymeric compounds (Gao *et al*., 2010), including proteins, cellulose, hemicellulose, chitin and DNA (Tripathi *et al*., 2008). During colonization of the plant (host) surface, endophytes produce enzymes and hydrolyse plant cell walls (Table 11.2). Therefore, these enzymes are effective at suppressing pathogenic activities directly and have the ability to degrade the cell walls of fungi (chitin as the cell wall component) and oomycetes (cellulose as the cell wall material). These enzymes include cellulases, β-1, 3-glucanases and chitinases. The enzymes may not be the sole factor for antagonism but they may contribute as part of a combination of mechanisms.

Table 11.1. Antibiotics produced by endophytes.

Endophytic microorganisms	Antibiotics	Pathogens	Disease	Reference
Phomopsis cassiae	Cadinane sesquiterpenes 3,11,12 trihydroxycadalen	*Cladosporium sphaerospermum*	–	Silva *et al*. (2006)
Alternaria spp.	Altersetin	Gram-positive bacteria	–	Hellwig *et al*. (2002)
Paenibacillus sp.	Peptide antifungal	*Rhizoctonia bataticola*	Charcoal rot (soybean)	Senthilkumar *et al*. (2007)
Colletotrichum magna	Phenylalanine ammonia-lyase	*Colletotrichum orbiculare* and *Fusarium oxysporum*	–	Redman *et al*. (1999)
Acremonium zeae	Pyrrocidines A, B	*Aspergillus flavus*	–	Wicklow *et al*. (2005)
Verticillium sp.	Massariphenoe	*Pyricularia grisea*	Rice blast	You *et al*. (2009)
Cryptoscoriopsis quercina	Cryptocin	*P. grisea*	Rice blast	Li *et al*. (2000)
Pseudomonas	Pseudomycins	*Mycosphaerella fifiensis*	Black sigatoka of banana	Harrison *et al*. (1991)
Xylaria sp.	Griseofulvin	*P. grisea*, *Puccinia recondita*	Rice blast and yellow rust	Park *et al*. (2005)
Colletotrichum gloeosporioides	Colletotric acid	*Helminthosporium sativum*	–	Zhou *et al*. (2000)
Epichloe typhina	Sesquiterpene chokols A-G(15-21)	*Cladosporium phlei*	Purple rot disease	Koshino *et al*. (1989)
Ampelomyces sp.	Altersolanol A	*Staphylococcus aureus*	–	Li *et al*. (2008)

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Table 11.2. Production of various enzymes by endophytes.

Endophytic microorganism	Enzymes	Pathogens	Reference
Lysobacter enzymogenes	1,3-glucanase	*Pythium*	Palumbo *et al*. (2005)
Serratia marcescens	Chitinase	*Sclerotium rolfsii*	Ordentlich *et al*. (1988)
Pseudomonas stutzeri	Chitinase and laminarinase	*Fusarium solani*	Lim *et al*. (1991)
Paenibacillus sp. and *Streptomyces*	β-1,3-glucanase	*Fusarium oxysporum* f.sp. *cucumerinum*	Singh *et al*. (1999)
Pseudomonas cepacia	β-1,3-glucanase	*Rhizoctonia solani*, *S. rolfsii* and *Pythium ultimum*	Fridlender *et al*. (1993)

Detoxification and degradation of virulence factors

Detoxification of pathogen virulence factors is another mechanism of biological control. Certain biocontrol agents are capable of detoxifying albicidin toxin of *Xanthomonas albilineans* (Zhang and Birch, 1997). Detoxification includes synthesis of a protein that binds reversibly to the toxin in case of *Klebsiella oxytoca* (Walker *et al*., 1988) and *Alcaligenes denitrificans* (Basnayake *et al*., 1995), whereas irreversible detoxification of albicidin (mediated by an esterase) occurs in *Pantoea dispersa*.

As a mechanism of self-defence against biocontrol agents, often pathogen toxins show a broad-spectrum of activity against not only the specific host plant but also microbial competitors (including endophytes), or the toxin can detoxify antibiotics produced by some antagonists (Duffy *et al*., 2003).

Hyperparasites and predation

Hyperparasitism is the phenomenon where the endophytic antagonist or biocontrol agent either directly kills the pathogen or its propagules (Tripathi *et al*., 2008). Hyperparasitism in fungi is evidenced by the antagonist colonizing the pathogen's hyphae by twisting around them or by the antagonist penetrating the pathogen's hyphae initially and later secreting lytic enzymes that decompose the pathogen's cell wall. This mode of action is followed by the potential fungal antagonist *Trichoderma* when it parasitizes plant pathogens such as *Rhizoctonia solani* and also applies to many other situations of pathogen antagonism by *Trichoderma* spp. (Grosch *et al*., 2006). In contrast to this mechanism of hyperparasitism, microbial predation is a general means of suppressing pathogens by endophytes under nutrient-limited conditions. For example *Trichoderma* spp. exhibit predatory behaviour by producing a range of enzymes which are utilized to degrade the cell walls of pathogenic fungi and then to degrade the remaining fragments of the pathogen (Benhamou and Chet, 1997).

11.5.2 Indirect effects

Plants have a series of mechanisms to resist abiotic stresses (e.g. cold, drought or salt stress) and biotic stresses (including plant pathogens). During stress conditions plants rapidly express different morphological and biochemical changes, which include the hypersensitive response or cellular necrosis, and production of phytoalexins in response to pathogen attack and abiotic stresses. In terms of long-term host defence mechanisms developed to defend against pathogen attack, innate resistance may be specific or non-specific (general) (Király *et al*., 2007). The non-specific or general resistance is effective against a wide range of pathogenic species, while specific resistance can confer protection against one or a few pathogenic strains. Actually, the defence mechanism of plants associated with endophytes is triggered through induced resistance enhancement and by the production of secondary metabolites.

Induction of plant resistance

Over the past two decades researchers have mostly focused on host–pathogen interactions and expression of resistance by the host under different circumstances. Two well-studied major types of resistance mechanisms are: (i) induced

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systemic resistance (ISR); and (ii) systemic acquired resistance (SAR). ISR is a non-pathogenic (beneficial) rhizobacteria-induced resistance mechanism and is regulated by the jasmonic acid or ethylene pathway. ISR is not associated with the accumulation of pathogenesis related (PR) proteins (Vallad and Goodman, 2004; Tripathi *et al.*, 2008). SAR is induced as a result of host–pathogen interaction and is mediated by the salicylic acid pathway. SAR is associated with PR protein accumulation. Non-pathogenic strains of *Fusarium solani* isolated from tomato root tissues elicited ISR against *Septoria lycopersici*, the tomato foliar pathogen, which triggered PR genes and expression of PR5 and PR7 observed in roots (Kavroulakis *et al.*, 2007). Besides SAR and ISR, when endophytes colonize inside plant tissues, plants acquire the ability to mount a rapid defence response. High levels of lignin deposition, phenylalanine ammonia-lyase activity and peroxidise activity, as a part of post-infectional defence mechanism, was observed when *Citrullus lanatus* and *Cucumis sativus* were exposed to infection by a non-pathogenic mutant of *Colletotrichum magna* and it provided resistance against plant-pathogenic *Colletotrichum orbiculare* and *Fusarium oxysporum* (Redman *et al.*, 1999).

Stimulation of plant secondary metabolites

Secondary metabolites in plants are that group of chemical compounds which are not essential for basic life functions in plants, but play a major role in the adaptation of plants to different types of environment (Bourgaud *et al.*, 2001). Among plant-based secondary metabolites, phytoalexins are most important low-molecular-weight antimicrobial molecules (Smith, 1996), containing multiple substances including flavonoids and terpenoids. These are mainly produced as a result of host–pathogen interactions; otherwise neither the hosts nor the organisms alone can produce such substances. Phytoalexin production has also been reported under various non-biological stress conditions including heavy metal ions or salt stress and UV light. The discovery of phytoalexins dates back to the work of French botanist Noel Bernard (Stoessl and Arditti, 1984) in *Orchis morio* and *Loroglossum hircinum* as a response to fungal attack. Plant secondary metabolite production can be enhanced by coculturing with endophytic elicitors, which in turn increase plant resistance. Elicitors are pathogen-derived signal molecules that later induce a defence response in the plants. Endophytic elicitors, which induced plant secondary metabolite production, also follow similar mechanisms to stimulate plant resistance. Multiplication and colonization of fungal endophytes inside plant tissues results in the secretion of the enzyme hydrolase from the plant cell, and this enzyme limits the growth of fungal pathogens, thus endophyte fragments, acting as elicitors, are produced by hydrolyzation.

Different elicitors such as polysaccharides, lipopolysaccharides and glycoprotein stimulate the plant defence mechanism and trigger production of plant secondary metabolites, which successfully help in suppression of pathogen attack.

Promotion of plant growth and physiology

Endophytes, as discussed above, contribute towards protection of their host plant against phytopathogens through control of plant physiology (Giménez *et al.*, 2007). Endophytes have the ability to enhance plant resistance to a variety of abiotic and biotic stresses, thus increasing plant health which is reflected in plant growth and vigour or persistence (Kuldau and Bacon, 2008). Researchers across the world have demonstrated the growth promotion activity, resistance to drought stress (Swarthout *et al.*, 2009) and tolerance to unsuitable soil conditions (Belesky and Fedders, 1995; Malinowski *et al.*, 2004) observed in the plant hosts colonized by endophytes (Barka *et al.*, 2002). Plant growth promotion may be enhanced due to the influence of phytohormones produced by fungal endophytes. Indole acetic acid (IAA) is produced by *Colletotrichum* sp., an endophytic fungus in *Artimesia annua*, and IAA regulates plant processes (Lu *et al.*, 2000). Similarly Dai *et al.* (2008) argued that host growth stimulated by fungal endophytes might be attributed to the effect of phytohormones produced by the endophytic fungi.

Increased demands for energy, carbon skeletons and reducing equivalents associated with defence responses are provided by primary metabolic pathways (Bolton, 2009). It has been established that enhancement of plant growth promotion induced by fungal endophytes increase protection against pathogens indirectly.

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Competition

Competition between endophytes and pathogens mainly occurs for their nutrition (e.g. sugars, carbohydrates, growth factors, etc.) and/or space (host surface for colonization). As a result of their rapid colonization, endophytes exhaust the limited available substrates and thereby prevent the entry of the pathogens into the host.

11.6 Endophytes: an Emerging Tool as Biocontrol Agents

11.6.1 Potential endophytic antagonists

Potential endophytic antagonists could be used directly for seed treatments or application to the transplants. Precautions should be taken to limit the side effects of biotic and abiotic factors on the biocontrol agent by immediate protection of them within plant tissues. Mutualistic endophytes are in protected environments in the endosphere and this provides a competitive advantage over rhizospheric and phyllosphere organisms in respect of the constant nutrient flow, moisture, pH, as well as protection from competitors which remain in high numbers and densities. It seems that the organisms dwelling in the endosphere are not there accidentally but probably have been selected by the plant for this niche, because of the beneficial effects they offer to their host and their abilities to resist biochemical plant defence products. The energy cost for the host plant in the production of endophytic biomass is adequately compensated for by the beneficial effects of the mutualistic microorganisms towards plant health (Backman and Sikora, 2008). Finally, the amount of inoculum required per hectare to protect the host is minimized through precise targeting of endophyte treatments. The entire approach must be attractive to the biotechnology industry looking for some alternatives to replace traditional pesticides, as targeting the pathozone of plant pathogen, pests and nematode infections assures improved efficacy. Integrated use of biocontrol agents (endophytes) in combination with commercial pesticides when applied to seed or seedlings could lead to synergistic effects and can act on one or multiple pathogens. The chemicals could provide immediate suppression of pathogenic organisms, while the effect of the biological agent is sustained and could provide protection to the plants throughout the crop cycle. IPM on the seed reduces costs and can protect the environment, while allowing the biocontrol agent to build up momentum in the soil. Fungal and bacterial endophytes that are effective against plant pathogens are listed in Tables 11.3 and 11.4, respectively.

Table 11.3. Fungal endophytes effective against plant pathogens.

Endophytic fungus	Pathogens	Host	Reference
Epichloe typhina	*Cladosporium phlei*	Timothy plants	Shimanuki (1987)
Colletotrichum gloeosporioides	*Helminthosporium sativum*	–	Zhou *et al*. (2000)
Epichloe sp., *Neotyphodium* sp.	*Cryptonectria parasitica*	Chestnut	Yue *et al*. (2000)
Trichophyton sp., *Chrysosporium* sp., *Candida pseudotropicalis*, *Candida tropicalis*	*Sclerotinia sclerotiorum*	Bean	Rocha *et al*. (2011)
Neotyphodium spp.	*Rhizoctonia zea*	–	Gwinn and Gavin (1992)
Gliocladium spp.	*Pythium ultimum*, *Verticillum dahliae*	–	Stinson *et al*. (2003)
Aspergillus terreus	*Botrytis cinerea*	–	Cazar *et al*. (2005)
Verticillium sp.	*Pyricularia grisea*	Rice	You *et al*. (2009)
Cryptoscoriopsis quercina	*Pyricularia grisea*	Rice	Li *et al*. (2000)
Acremonium zeae	*Aspergillus flavus*	–	Wicklow *et al*. (2005)
Phomopsis cassiae	*Cladosporium sphaerospermum*	–	Silva *et al*. (2006)
Colletotrichum magna	*Colletotrichum orbiculare*, *Fusarium oxysporum*	–	Redman *et al*. (1999)
Alternaria spp.	Gram-positive bacteria	–	Hellwig *et al*. (2002)

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Table 11.4. Bacterial endophytes effective against plant pathogens.

Endophytic bacteria	Pathogens	Host and disease	Reference
Pseudomonas sp., *Curtobacterium luteum*, *Pantoea agglomerans*	*Erwinia caratovora*	Potato (soft rot)	Sturz *et al*. (1999)
Paenibacillus sp. (HKA-15), *Bacillus* sp. (HKA-121)	*Rhizoctonia bataticola*	Soybean	Senthilkumar *et al*. (2009)
Burkholderia sp.	*Phytophthora capsici*, *Fusarium graminearum*, *Sclerotinia libertiana*	*Huperzia serrata*	Wang *et al*. (2010)
Pseudomonas fluorescens	*Phytophthora capsici*	Black pepper	Paul *et al*. (2005)
Pseudomonas fluorescens, *Bacillus subtilis*	*Xanthomonas campestris* pv. *campestris*	Cauliflower (black rot)	Singh *et al*. (2010)
Paenibacillus polymyxa	*Leptosphaeria maculans*	Canola (black leg disease)	Beatty and Jensen (2002)
Serratia plymuthica	*Verticillium dahliae*, *Rhizoctonia solani*	–	Berg *et al*. (2005)
Fluorescent pseudomonads	*Macrophomina phaseolina*, *Sclerotium sclerotiorum*	–	Gupta *et al*. (2001)
Streptomyces, *Streptoverticillium* sp., *Streptosporangium* sp.	*Fusarium oxysporium* f.sp. *cubense*	Banana (Fusarium wilt)	Cao *et al*. (2005)
Streptomyces sp., *Microbiospora* sp., *Micromonospora* sp., *Nocardioides* sp.	*R. solani*, *Pythium* sp., *Gaeumannomyces graminis* var. *tritici*	–	Coombs and Franco (2003)
Serratia marcescens	*Sclerotium rolfsii*	–	Ordentlich *et al*. (1988)
Pseudomonas stutzeri	*Fusarium solani*	–	Lim *et al*. (1991)

11.6.2 Endophytes' metabolites active against insects and nematodes

In addition to their potential use against plant pathogens, endophytes may also be used for the management of insect and nematode pests.

Insects

Endophytes are believed to play a role in the reduction in weight gain by insect larvae and also reduce the leaf area consumed by insects. For example Hardy *et al*. (1985) studying fall armyworm (*Spodoptera frugiperda*) found survival of the insect pest and their weight gain was negatively affected by the fungal endophyte of perennial ryegrass. Later Breen (1994) found association with endophytes enhanced host plant resistance to insects. Fungal endophytes produce alkaloids that are active against insects and some of these are listed in Table 11.5.

Nematodes

Endophytes also help to bring down the nematode population density in infected in crops mostly by inducing resistance in the host plant. For example endophytic *Pseudomonads* and *Arthrobacter* spp. are effective against the root knot nematode in black pepper (*Piper nigrum* L.) (Aravind *et al*., 2009).

11.6.3 Protocol for isolation of endophytic microorganisms

In order to work on endophytes and establish, for example, if a particular species has a

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Table 11.5. Alkaloids produced by fungal endophytes against insects.

Endophytic fungus	Alkaloid	Effectiveness	Reference
Balansiae cyperi	Agroclavine	*Spodoptera frugiperda*	Clay and Cheplik (1989)
Neotyphodium coenophialium	Pyrrolopyrazine	Argentine stem weevil	Schardl and Philips (1997)
Acremonium coenophialum, *Epichloe typhina*	Loline, peramine	Aphids	Siegel *et al*. (1990)
Nodulisporium sp.	Nodulisporic acid A	*Aedes* mosquito, blowfly	Ondeyka *et al*. (1999)

role to play in biocontrol, the endophytic organism needs to be isolated. Isolation of endophytes from their associated hosts is not simple, unlike isolation of other disease-causing organisms. The protocol for isolating endophytic microorganisms is as follows:

- The plant material (root, stem, leaf) is washed carefully under running tap water.
- The sample is examined macroscopically and areas with lesions and discolorations are discarded so that only healthy parts are used.
- The material is surface sterilized for 1 min in ethanol (70%), 4.5 min sodium hypochlorite (5% active chlorine) followed by three washing steps for at least 5 min with sterile water.
- After surface sterilization the samples are dried on sterile filter paper and imprinted on biomalt agar 50 g/l Biomalt (Villa Natura Gesundprodukte GmbH, Germany) + 20 g/l Bacto™ Agar (Becton Dickinson and Company, France), pH 5.6 with the antibiotics penicillin G Na (60 mg/l), streptomycin sulfate (80 mg/l) and (oxy) tetracycline HCl (50 mg/l) as a sterility check. These cultures serve as control plates to check the surface sterilization procedure.
- The samples are cut into small pieces and mixed together.
- For the isolation of endophytes the samples are incubated on biomalt agar.
- The culture plates are monitored for up to 6 weeks for outgrowth of mycelium, which will then be transferred immediately to fresh biomalt medium.

11.7 Conclusion

Endophytes represent a wide group of microorganisms that are abundant in natural ecosystems and are a dependable source of bioactive and novel chemical compounds that have considerable potential to be exploited in the medical, agricultural and industrial sectors. The mechanisms that enable an endophytic existence, including the response to their surroundings, needs be studied to enable a better understanding of their association with higher plants. Studies that include the isolation of endophytes from a wide range of plant host species may lead to the discovery of new products. Endophytes have been established as potential antagonists for protecting plants against diseases. They are also potential candidates for the enhancement of nutrient cycling and bioremediation. The major challenge today facing the study of endophyte biology and the discovery of natural products is the rapid loss of rainforests, the greatest reservoir for acquiring novel microorganisms and their products, due to deforestation (Strobel and Daisy, 2003). The study of endophytes as potential antagonists or biocontrol agents against plant diseases has been conducted across the world but few endophytic species have been studied sufficiently well to enable them to be exploited as alternatives to toxic chemicals for crop protection. However, countries need to generate data on the endophytic species isolated and to establish information bases of their biodiversity. Nationwide collection of the endophytic species in different geographic areas of the world needs to begin to provide a resource for researchers. In the near future endophytes will emerge as a microbial tool that can be used by the agricultural sector for crop protection and will also contribute towards nutrient cycling and bioremediation.

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12 Role of *Listeria monocytogenes* in Human Health: Disadvantages and Advantages

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Abstract

The genus *Listeria* consists of eight species: *Listeria monocytogenes*, *Listeria innocua*, *Listeria ivanovii*, *Listeria seeligeri*, *Listeria welshimeri*, *Listeria grayi*, *Listeria rocourtii* and *Listeria marthii*. Among *Listeria* species only *L. monocytogenes* and *L. ivanovii* are pathogenic to humans and animals. Unlike *L. monocytogenes* which causes listeriosis in humans and animals *L. ivanovii* is solely an animal pathogen. The bacterium may be rod shaped, coccoid or filamentous depending on nutrients, environmental and cultural conditions. Among pathogenic bacteria *L. monocytogenes* is atypical as it has the ability to survive and grow in a broad temperature range (1–45°C), a wide pH range (4.1–9.6) and it can tolerate sodium chloride concentrations of 5–25% and a variety of other toxic chemicals such as tellurite, acridine dyes, lithium chloride, nalidixic acid and cycloheximide. The bacterium has been found widely in the environment and in humans and animals. *L. monocytogenes* has emerged as an important food-borne pathogen in human beings, especially in the developed world. Many sporadic cases and outbreaks of listeriosis have been reported linked with the consumption of various plant and animal food products contaminated with *L. monocytogenes*. Listeriosis can be clinically defined as the presence of the organism in bloodstream, cerebrospinal fluid or in a normally sterile body site. Predominant clinical manifestations of human listeriosis are diarrhoea, meningitis, encephalitis, cerebritis and septicaemia. Besides these manifestations, endocarditis, pericarditis and pyogenic abscesses have also been documented. Most human infections involve the central nervous system whereas encephalitis, characterized by multiple abscesses in the brain stem, rarely occurs. Immunocompromised patients, neonates and pregnant women are more susceptible to listeriosis. However, the disease can also develop in apparently healthy individuals. *L. monocytogenes* has evolved an exclusive array of mechanisms that allow it to exploit the host: attaching and invading host cells, escaping from the host cell vacuole, spreading from cell to cell, crossing barriers and evading the host's humoral immune system. Knowledge of the cell biology of the organism has made it possible to use the bacterium as a vector in vaccine development, in the treatment of cancer and infectious diseases. Therefore it can be concluded that the bacterium is playing a vital role in human life. In this chapter we discuss various concerns about *L. monocytogenes* including their harmful and beneficial aspects on human life.

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12.1 Introduction

Listeria monocytogenes is a Gram-positive, rod-shaped, non-spore-forming, non-acid fast bacterium that is a facultative anaerobe. The organism exhibits a characteristic tumbling motility at temperatures of 20–25°C. The genus *Listeria* includes eight species: *Listeria monocytogenes*, *Listeria innocua*, *Listeria ivanovii*, *Listeria seeligeri*, *Listeria welshimeri*, *Listeria grayi*, *Listeria rocourtii* and *Listeria marthii*, the latter two being included in the genus in 2010 (Sauders *et al*., 2012). *Listeria* species can be differentiated using some biochemical assays such as haemolytic activity and sugar fermentation (Table 12.1). Generally, all members of the genus *Listeria* are aerobes or facultative anaerobes, are catalase positive and oxidase negative. Among all species of the genus, *L. monocytogenes* exhibits a positive reaction in the Christie Atkins Munch-Peterson (CAMP) assay and shows an increased zone of haemolysis on sheep blood agar plates (Fig. 12.1). At temperatures lower than 30°C the bacterium is motile in nature with the help of peritrichous flagella. It is widely distributed throughout the environment. The bacterium also has the ability to grow in a wide range of temperatures (1–45°C), pH (4.1–9.6) and osmotic pressures. These properties enable these bacteria to survive under adverse conditions for a long time. Therefore they are sometimes considered as resilient bacteria (Adzitey and Huda, 2010).

L. monocytogenes causes several illnesses in human beings and animals. Annual incidence of human listeriosis is reported as between 1.6 and 6 cases per million people (Rocourt *et al*., 2000). Listeriosis can be clinically defined as the presence of the organism in the bloodstream, cerebrospinal fluid or in a normally sterile body site. The principal clinical symptoms of human listeriosis include diarrhoea, meningitis, encephalitis, cerebritis and septicaemia. A hospitalization rate as high as 91% is reported for untreated cases. Among patients receiving therapy there is a mortality rate of up to 30%. In addition, endocarditis, pericarditis and pyogenic abscesses (local inflammatory reactions in various organs) have been documented. Most human infections involve the central nervous system (CNS) whereas it is rare that encephalitis, characterized by multiple abscesses on the brain stem, occurs. Immunocompromised patients, neonates and pregnant

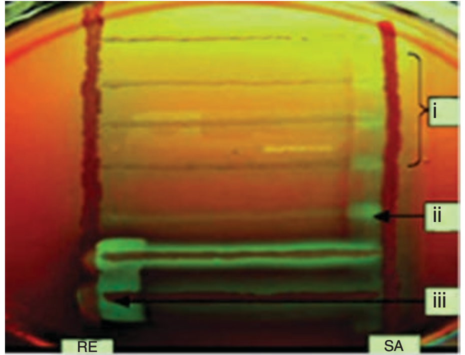

Fig. 12.1. Christie Atkins Munch-Petersen (CAMP) assay. (i) and (ii) *Listeria monocytogenes* showing synergistic haemolytic activity with *Staphylococcus aureus*. (iii) Standard *Listeria ivanovii* showing synergistic haemolytic activity with *Rhodococcus equi*. RE, *R. equi*; SA, *S. aureus*.

Table 12.1. Biochemical differentiation[a] of *Listeria* species. (From Hitchens, 1998.)

	Biochemical characters				
			Acid production from		
Listeria spp.	β-Haemolysis	CAMP[b]	D-Xylose	L-Rhamnose	Mannitol
L. monocytogenes	+	+	–	+	–
L. ivanovii	+	–	+	–	–
L. innocua	–	–	–	V	–
L. seeligeri	+	+/–	+	–	–
L. welshimeri	–	–	+	V	–
L. grayi	–	–	–	V	+

[a]+, Positive; +/–, weakly positive; –, negative; V, variable.
[b]CAMP, Christie Atkins Munch-Peterson assay.

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women are more susceptible to listeriosis. However, the disease can also develop in apparently healthy individuals (Sim *et al.*, 2002; Aureli *et al.*, 2003; Carrique-Mas *et al.*, 2003; Dumen *et al.*, 2008; El-Malek *et al.*, 2010; Hoelzer *et al.*, 2012; CDC, 2014).

L. monocytogenes has been found to be pathogenic to both animals and humans without showing any significant host specificity. The infection includes four consecutive steps: (i) bacteria enter the host; (ii) phagosomal vacuole lysis; (iii) multiplication of the bacteria in the cytosol; and (iv) cell-to-cell spread using actin-based motility. It is observed that each step needs expression of specific virulence factors. Studies have suggested that the major virulence genes are positioned in a cluster of genes at two different DNA loci. These loci are mainly influenced by a protein called positive regulatory factor A or prfA. Several groups of virulence factors (e.g. internalines, listeriolysin O, phosphatidylinositol-specific phospholipase C (PI-PLC), actin, lecithinase and fibronectin) have been characterized that play an important role in the pathogenicity of *L. monocytogenes*. Among these the internalines, encoded and regulated with different internaline genes (*inl*), participate in the invasion of epithelial cells and are also responsible for the tissue tropism of *L. monocytogenes*. Listeriolysin O and PI-PLC are encoded by the genes *hlyA* and *plcA*, respectively, and are involved in lysis of the phagosomes of the host cell. Therefore these factors are responsible for the intracellular growth of *Listeria* in cells. The actin protein encoded by the gene *actA* has been found to be involved in motility whereas fibronectin-binding protein, FbpA, has recently been considered as a novel multifunctional protein. Moreover, a few enzymes such as lecithinase, zinc metal protease and serine protease also participate in virulence of the bacterium (Vazquez-Boland *et al.*, 2001; Den Bakker *et al.*, 2010).

12.2 Disadvantages and Advantages of the Bacterium

Microorganisms play a significant role in human life and affect life in many ways. Sometimes they are harmful whereas they may also be beneficial. For example a variety of bacterial species are causative agents of various infectious diseases whereas several bacteria provide a variety of products, such as several antibiotics, enzymes, vitamins and vaccines, that are required for the improved quality of life of human beings. In this section a few roles of the bacterium *L. monocytogenes* including harmful and beneficial aspects are discussed.

12.2.1 Disadvantages

L. monocytogenes has emerged as an important food-borne pathogen in human beings, especially in the developed world. The bacterium is responsible for about 98% of human cases and 85% animal cases of listeriosis (Das *et al.*, 2013). Therefore, it has gained considerable attention from researchers and medical practitioners. Several diseases and their symptoms associated with infection by the bacterium, including food poisoning, abortion, meningitis, will now be described.

Food poisioning

The bacterium *L. monocytogenes* has been isolated from various food products of plant and animal origin associated with many listeriosis outbreaks. Therefore, contaminated foods are believed to be the primary source of transmission of listeria infection in sporadic cases as well as outbreaks (Adzitey and Huda, 2010). Major outbreaks of human listeriosis are increasing globally. The first investigated outbreak occurred in 1981 in the Maritime provinces of Canada where seven adults and 34 prenatal cases of listeriosis resulted due to consumption of contaminated coleslaw with a case fatality rate of 44% (18 deaths). Later on, many food-borne outbreaks (involving pasteurized whole milk and milk products) of listeriosis have been reported (Amagliani *et al.*, 2004; Lawley, 2013). A case control study (Olsen *et al.*, 2005) established in a multistate outbreak of *L. monocytogenes* that infection was linked to delicatessen Turkey meat. Cases of infection caused by these isolates were associated with four deaths and three miscarriages. In 2007 an outbreak of *L. monocytogenes* infections occurred associated with pasteurized milk from a local dairy, in Massachusetts (MMWR, 2008). This outbreak illustrated the potential for contamination of liquid milk products after

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pasteurization and the difficulty in detecting outbreaks of *L. monocytogenes* infections. Similarly, due to consumption of 'Quargel' cheese a multinational outbreak of listeriosis due to *L. monocytogenes* was reported in Austria and Germany. The outbreak comprised 14 cases (including five fatalities) infected by a serotype 1/2a *L. monocytogenes*, with onset of illness from June 2009 to January 2010. A second strain of *L. monocytogenes* serotype 1/2a spread by this product could be linked to further 13 cases in Austria (two fatal), six in Germany (one fatal) and one case in the Czech Republic, with onset of disease from December 2009 to the end of February 2010 (Fretz *et al.*, 2010). In the USA, the Centre for Disease Control and Prevention (CDC) monitors various diseases including food-borne outbreaks and illness. In the past few years an increase in outbreaks and illness due to *L. monocytogenes* has been observed. A comprehensive list of various outbreaks due to *L. monocytogenes* in the USA is shown in Table 12.2. A 5 year summary of confirmed cases of *L. monocytogenes* infection in Europe is listed in Table 12.3.

Abortion

L. monocytogenes can cause abortion in animals as well as humans. Pregnant women are more susceptible to listeria infection: the risk of infection by the bacterium in pregnant women has been reported to be 17 times higher than that of the normal population. In humans and other mammals, the bacterium is able to cross the intestinal barrier. The haematogenous dissemination of the organism that occurs may cause invasive disease that typically leads to infection of the placento-foetal unit, or meningitis in immunocompromised individuals. During pregnancy the organism can lead to intrauterine infection that results in severe complications such as preterm labour, spontaneous abortion, stillbirth or neonatal infection notably granulomatosis infantiseptica (Jamshidi *et al.*, 2009).

A number of cases of abortion in women have been observed from all over the world including India (Kaur *et al.*, 2007). According to a report published by Mylonakis *et al.* (2002), infections caused by *L. monocytogenes* were reported

Table 12.2. Summary of *Listeria monocytogenes*-associated food-borne outbreaks in the USA from 2000 to 2014. (From Marler Clark Network, 2014.)

Study period	Total number of cases	Number of hospitalizations	Number of deaths	*L. monocytogenes* serotype	Food vehicle
2000	13	13	0	4b	Mexican style cheese
	30	29	4	1/2a	Deli meat
2001	28	0	0	1/2a	Deli meat
2002	54	NA[a]	8	4b	Deli meat
2003	3	NA	NA	4b	Unknown
	12	12	1	4b	Mexican style cheese
2005	6	6	0	4b	Unknown
	3	3	0	1/2b	Grilled chicken
	13	13	1	1/2a	Deli meat
	12	12	0	1/2b	Mexican style cheese
2006	2	1	1	4b	Unknown
	2	0	0	1/2b	Tacho or nacho salad
	3	2	1	4b	Cheese
2007	5	5	3	4b	Milk
2008	5	5	3	1/2a	Tuna salad
	20	2	0	1/2a	Sprouts
	8	4	0	1/2a	Mexican style cheese
2011[a]	147	147	33	1/2a, 1/2b	Cantaloupes
2012[a]	22	20	4	NA	Ricotta salata cheese
2013[a]	6	6	1	NA	Soft ripened cheese
2014[a]	8	7	1	NA	Soft or semisoft Hispanic cheese

[a]NA, Not available.

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Table 12.3. Summary of confirmed human listeriosis cases from 2008 to 2012 in Europe. (From EFSA, 2014.)

	Number of confirmed cases				
Country	2008	2009	2010	2011	2012
Austria	31	46	34	26	36
Belgium	64	58	40	70	83
Bulgaria	5	5	4	4	10
Cyprus	0	0	1	2	1
Czech Republic	37	32	26	35	32
Denmark	51	97	62	49	50
Estonia	8	3	5	3	3
Finland	40	34	71	43	61
France	276	328	312	282	348
Germany	306	394	377	330	412
Greece	1	4	10	10	11
Hungary	19	16	20	11	13
Ireland	13	10	10	7	11
Italy	118	109	137	100	36
Latvia	5	4	7	7	6
Lithuania	7	5	5	6	8
Luxembourg	1	3	0	2	2
Malta	0	0	1	2	1
The Netherlands	45	44	72	87	73
Poland	33	32	59	62	54
Portugal[a]	–	–	–	–	–
Romania	0	6	6	1	11
Slovakia	8	10	5	31	11
Slovenia	3	6	11	5	7
Spain[b]	88	121	129	91	107
Sweden	60	73	63	56	72
UK	206	235	176	164	183
Norway	34	31	22	21	30
Switzerland	43	41	67	47	39
Total	1502	1747	1732	1554	1711

[a]No surveillance system exists so no reports available.
[b]Sentinel surveillance; notification rates calculated on estimated coverage of 25%.

in a total of 7.4 per million pregnant women, accounting for about 30% of all cases in 2000. An outbreak in 2011 in the USA was found to be linked to cantaloupes that affected 147 people. A total of 33 people died and one pregnant woman experienced a miscarriage among the affected 147 persons. The pregnant females (third trimester of pregnancy) infected with *L. monocytogenes* were treated with antibiotics. However, routine optimal antibiotic treatment for listeriosis in early pregnancy has not yet been determined. Despite this, in December 2011, a 28-year-old previously healthy woman was admitted to hospital in Boston, Massachusetts, with fever, headache and neck stiffness. The woman was 12 weeks pregnant and her blood was found to be positive for *L. monocytogenes*. She was successfully treated with intravenous ampicillin for 2 weeks (2 g every 4 h) and gentamycin (100 mg every 8 h) followed by ampicillin alone for 2 weeks (Chan *et al.*, 2013).

It has been described that *L. monocytogenes* affects 30 species of mammals and birds. Outbreaks of abortion due to *L. monocytogenes* have been observed in various animals, namely cattle and sheep (McDonald, 1967; Shoukat *et al.*, 2013). In cattle, abortion occurs approximately 1 week after exposure most commonly during the last trimester of pregnancy. However, it may occur as early as the fourth month of gestation. The aborted foetus is often autolysed. The cows may show clinical signs of disease as well,

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although once returned to health they appear to resist reinfection (Hovingh, 2009).The bacterium may be the most common bacterial cause of multiple abortions in herds.

Meningitis

Meningitis causes swelling of the brain and spinal cord linings. The symptoms of the disease include stiff neck, fever, headache, vomiting, trouble staying awake and seizures. It is usually caused by a virus or bacteria (e.g. *Streptococcus pneumoniae* and *Neisseria meningitides*) (Karriem Norwood, 2014). *L. monocytogenes* is the third most frequent cause of bacterial meningitis after *S. pneumoniae* and *N. meningitides* (Laguna-Del Estal *et al*., 2013). *Listeria* meningitis occurs most often in newborns, older adults and people with long-term illnesses or weak immune systems. It can be a serious illness, causing death in some cases. In non-pregnant adults, infections of *L. monocytogenes* affect the CNS in 55–70% of cases (Vazquez-Boland *et al*., 2001). These infections normally develop as a meningo-encephalitis accompanied by severe changes in consciousness and movement disorders. In some cases, paralysis of the cranial nerves has also been observed. The encephalitic form is common in animals, however, it is rare in humans. The bacterial organism can be isolated with difficulty from the cerebrospinal fluid in this form. The first phase of the disease is known as the 'subfebrile phase'. The symptoms of this phase may include headache, vomiting, visual disorders and general malaise which are followed by a second phase showing severe signs of rhombencephalitis. The mortality rate for CNS infection is around 20–60% (Vazquez-Boland *et al*., 2001). It is also estimated that *L. monocytogenes* accounts for 10% of community-acquired bacterial meningitis (Amaya Villar *et al*., 2010). The study conducted by Brouwer *et al*. (2006) in The Netherlands found 30 episodes of *L. monocytogenes* meningitis. All patients were immunocompromised or were less than 50 years old. In 19 (63%) of 30 patients, symptoms were present within 24 h whereas in eight patients (27%) symptoms were present less than or at the fourth day. In a review study on meningitis incidence, 103 episodes of acute bacterial meningitis in adults hospitalized from 1985 to 1996 were reviewed in two of the largest hospitals of Edmonton. Most cases were found to be community acquired (87%) and *L. monocytogenes* was the second most common organism found in 12.5% positive cases. The overall mortality was 18%, whereas the fatality rate for specific *L. monocytogenes* cases was 40%. In certain high-risk groups, such as cancer patients, it is also observed that *L. monocytogenes* is one of the most common bacteria that cause bacterial meningitis (Vazquez-Boland *et al*., 2001).

Early administration of sufficient antibiotic therapy is the basis of successful treatment of meningitis patients. Meningitis caused by *L. monocytogenes* is still a serious disease, however, a wide variety of antimicrobial agents has been developed. The choice of treatment against *L. monocytogenes* infections includes β-lactam antibiotics, normally ampicillin, alone or in combination with an aminoglycoside (classically gentamycin). The second line treatment includes trimethoprim or sulfamethoxazole, erythromycin, vancomycin and fluoroquinolones. The resistance among clinical isolates of *L. monocytogenes* to these antibiotics has been observed to be lower than to other antibiotics (Mateus *et al*., 2013). In a study conducted by Calder (1997), ampicillin with or without an aminoglycoside was reported to be the best treatment for meningitis caused by *L. monocytogenes*. Ampicillin and penicillin are considered to be equally effective against *L. monocytogenes*, both administered in daily doses of 6 g or more. However, chloramphenicol alone or in combination has found to be less effective against *Listeria* meningitis. Among penicillin-allergic patients, co-trimoxazole has been observed to be the best alternative antibiotic therapy. Erythromycin has been used successfully to treat listeria septicaemia. However, cephalosporins which are commonly used as a first-line treatment for non-*Listeria* meningitis was not found to be effective to treat *Listeria* meningitis due to inherent resistance against *L. monocytogenes* (Jones *et al*., 1997). Intravenous ampicillin and aminoglycosides have been used in various cases for treating meningitis caused by *L. monocytogenes* (Amaya-Villar *et al*., 2010).

Other infections

Another frequent form of listeriosis is bacteraemia or septicaemia which is reported in 15–50% of all listeriosis cases. The mortality rate is very high (up to 70%) if it is associated

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with severe underlying devastating conditions. In terms of serious infections septicaemia is reported more frequently than CNS infection. Some other atypical clinical forms of listeriosis such as endocarditis (the third most frequent form), myocarditis, arteritis, pneumonia, pleuritis, hepatitis, colecystitis, peritonitis, localized abscesses (e.g. brain abscess that accounts for about 10% of CNS infections caused by *Listeria* spp.), arthritis, osteomyelitis, sinusitis, otitis, conjunctivitis and ophthalmitis have been reported in humans (Vazquez-Boland *et al*., 2001; Sahu *et al*., 2012).

12.2.2 Advantages

In the last few years, research on behaviour and the mode of pathogenicity of *L. monocytogenes* has revealed some important aspects of the bacterium. A number of biological attributes of *L. monocytogenes* allow a promising role for the bacterium for use as a vector in vaccine development in the treatment of several cancer and infectious diseases. A few very important applications of the organism are discussed here.

L. monocytogenes *as a live bacterial vaccine vector*

Several viral and bacterial species have been utilized as live antigen delivery vectors, including vaccinia virus, *Salmonella*, *Shigella*, *Legionella*, *Lactococcus* and *Mycobacterium* (BCG (Bacillus Calmette-Guérin) vaccine). However, several unique features have made *L. monocytogenes* an attractive choice to be used as a vaccine vector (Paterson and Maciag, 2005; Singh and Paterson, 2006). Various *in vitro* and *in vivo* studies have confirmed the ability of *L. monocytogenes* to be used as a vector for intracellular gene or protein delivery. The bacterium has also displayed safety and efficacy during clinical trials. Therefore, this intracellular pathogen has shown to be a promising therapeutic vector for the delivery of DNA, RNA or protein to prime immune responses against specific antigens (Paterson and Johnson, 2004). The bacterium triggers strong innate and adaptive immunity in the infected host which are required for clearance of the organism. *L. monocytogenes* has the ability to live in both the phagosome and the cytoplasm of host cells. This characteristic feature in combination with its inherent immunostimulatory capacities enhances antigen presentation and stimulation of effector and memory T-cells. Due to the efficiency of these cell-mediated immune responses, efforts are being made to develop this bacterium as a recombinant antigen delivery vector for inducing protective cellular immunity against various infections or cancers (Tangney and Gahan, 2010). The antigen representation by *Listeria* includes two pathways: (i) exogenous; and (ii) endogenous. In the exogenous pathway, the phagocytosed bacterial cells are degraded in phagolysosomal compartments and the resulting protein fragments are directed into major histocompatibility complex (MHC) class II-containing vesicles for loading and presentation to $CD4^+$ T-cells. The endogenous pathway is used when the bacterium has escaped into the cytoplasm and secretes proteins which are processed by the cytosolic proteosomal machinery (Bruhn *et al*., 2007). The resulting peptides are transported into the endoplasmic reticulum (ER) for loading on to MHC class I molecules and presentation to $CD8^+$ T-cells (Fig. 12.2).

L. monocytogenes *in anti-cancer therapies*

The many research studies on *L. monocytogenes* have led to exciting developments in cancer therapies. Two key approaches have been explored recently. One is the use of *Listeria*-mediated bactofection of tumour cells (transfer of DNA to tumour cells by bacteria) and vaccination using *L. monocytogenes* bacterium expressing cancer-related antigens. The second approach has exploited the ability of *L. monocytogenes* to stimulate a strong $CD8^+$ T-cell response. This feature of *L. monocytogenes* infections offers the possibility of introducing heterologous antigens and triggering a $CD8^+$ T-cell mediated immune response (Tangney and Gahan, 2010).

John Rothman has described how *L. monocytogenes* invokes autoimmune responses in cancer patients. Rothman and his colleagues worked on Lovaxin C which is a *Listeria*-based vaccine. They have tested the vaccines on 15 women from 2005 to 2007. These women were suffering with progressive, recurrent or advanced cervical cancer and earlier these patients had not been treated successfully with chemotherapy, radiotherapy or surgery. The researcher observed that the patients generally tolerated this recombinant

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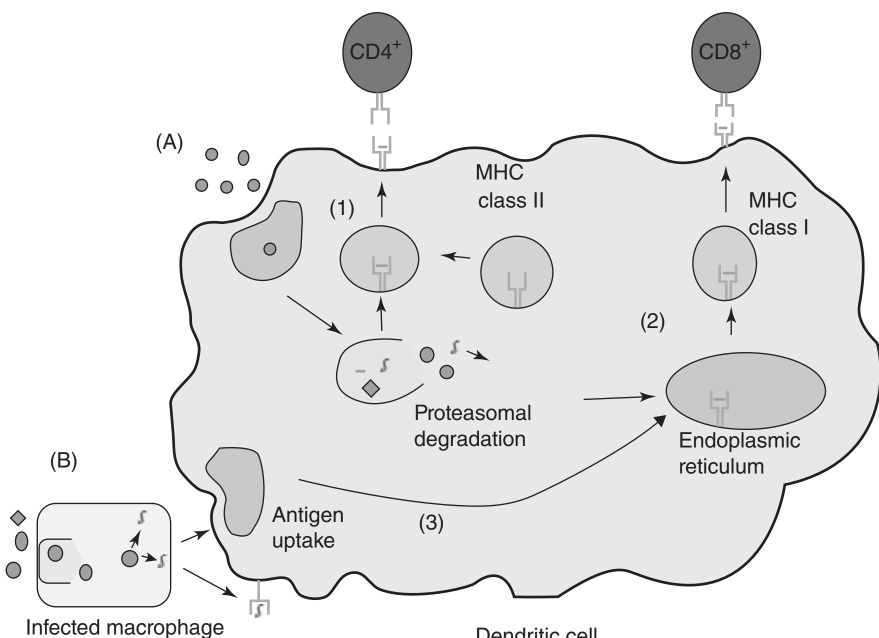

Fig. 12.2. Antigen presentation pathways for listerial antigens. (A) *Listeria* bacteria (classical pathway); (B) macrophage infected with *Listeria* (cross-presentation pathway). (1) Exogenous pathway (phagocytosed bacterial cell is degraded by phagolysosomal compartment and resulting protein fragments are directed to MHC class II-containing vesicles for loading and presentation to CD4+ T-cells). (2) Endogenous pathway (bacterium is subject to proteasomal degradation in the cytoplasm and the resulting peptides are taken up by the endoplasmic reticulum for loading on to MHC class I molecules and presentation to CD8+ T-cells). (3) Cross presentation of the two pathways.

vaccine (Greenemeier, 2008). The human papilloma virus (HPV) is a sexually transmitted virus which can lead to invasive cervical cancer if not diagnosed properly and left untreated. Lovaxin C (vaccine) was also tested for treatment against the HPV (Greenemeier, 2008).

Bruhn *et al*. (2007) reviewed various aspects of cancer immunotherapy using *L. monocytogenes*. They described the protection of tumours associated with HPV antigens. The HPV-16 early protein E7 is highly expressed by virally transformed cancer cells. Several preclinical studies have demonstrated that a recombinant *Listeria* strain expressing HPV-16 E7 can show both prophylactic and therapeutic efficacy against E7-expressing tumours. The review also described the application of recombinant *L. monocytogenes* against several cancers such as melanoma, breast, lung, pancreatic and ovarian cancer and tumours such as those in the gastrointestinal tract in animal models.

A recent review has described various pivotal events in the development of *L. monocytogenes*-based vaccines, including their part in clinical trials for the treatment of different type of cancers (Wood and Paterson, 2014). According to the review, *L. monocytogenes* was used initially as a vector for foreign antigens in 1992. Then in 1994 the first *L. monocytogenes*-based vaccine with the fusion of listeriolysin O was developed. After successive progress at various stages of vaccine development researchers were able to suppress tumour growth and kill tumour cells in 2009. The first clinical trial was performed in the same year and success of a phase II clinical trial of the vaccine was reported in 2012. In 2013 the synergistic efficacy of a *L. monocytogenes*-based vaccine and a common inhibitory receptor such as programmed death 1 (PD1) was evaluated.

Treatment of infectious diseases

In an *in vivo* study using a mouse model, recombinant *Listeria* carrying a number of different antigens has been shown to provide protective immunity against various infectious organisms. Strains of the

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bacterium have been engineered to express a number of human immunodeficiency virus (HIV)/simian immunodeficiency virus (SIV) antigens. Strong cell-mediated immunity was noticed after oral and parenteral immunization in mouse whereas oral immunization showed similar results in rhesus macaque using recombinant *L. monocytogenes* (Gunn *et al.*, 2001; Paterson and Johnson, 2004). Therefore the bacterium *L. monocytogenes* has been used against both neoplastic diseases and several infectious diseases such as HIV, HPV and *Mycobacterium* infections (Wieskirch and Paterson, 1997; Curtiss, 2002; Yin *et al.*, 2013).

12.3 Conclusion and Future Prospects

Overall, the bacterium *L. monocytogenes* plays a significant role in the lives of both humans and animals. The increased health risk associated with *L. monocytogenes* infection has drawn the attention of various health agencies. The bacterium has been reported to be associated with a wide variety of foods, however, the pathobiology of the bacterium is such that it is impractical to totally eliminate *L. monocytogenes* from food. From a food safety point of view, it is important to understand how the bacterium *L. monocytogenes* is able to adapt its cellular physiology successfully to overcome various forms of environmental stress. In particular *L. monocytogenes* cold stress adaptation is one of the fundamental attributes enabling its dissemination in food environments. Therefore, introduction and identification of methodology for use in the food industry, clinical laboratories and governmental laboratories and more accurate survey techniques for the presence of this pathogen in foods, including locally produced foods, can improve food safety and protection from the organism.

Although the bacterium has been found linked with a wide variety of health problems simultaneously it has been highlighted as an important tool for the immunologist and cell biologist. The bacterium *L. monocytogenes* has been employed as a model organism for studying host–pathogen interactions and immune responses against intracellular pathogens. As a result, several studies have provided significant insight about the *L. monocytogenes* interaction with host mucosal surfaces of the human gastrointestinal tract and the immune system that triggers the antibacterial immune responses. Understanding the cell biology of this organism has made it possible to use the bacterium as a vector in vaccine development, in the treatment of cancer and infectious diseases. The recombinant *Listeria* strains have shown a number of advantages as live vaccine delivery vectors. Proinflammatory cytokine cascades developed from multiple immune responses due to *L. monocytogenes* lead to both $CD4^+$ and $CD8^+$ effector and memory T-cell populations. However, past immunity to *Listeria* does not appear to prevent effective use of the vector. The preclinical results accumulated until now argue for the continued development of *Listeria* vectors for therapeutic use in humans. Assessment of vaccine efficacy and safety is still largely empirical because the literature has shown that most of the studies are conducted in animal models, such as mice and guinea pigs, and there is still a need for comparative studies for quick and efficient evaluation of the best candidates among developed strains for human trials. Therefore, the bacterium *L. monocytogenes* plays an important role in the life of human beings as both 'friend' and 'foe'.

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13 Natural Weapons against Cancer from Bacteria

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Abstract
Cancer is an enduring disease responsible for numerous deaths worldwide. Currently resistance towards anticancer drugs and the side effects of chemotherapy are a major problem in the treatment of cancer, so there is an increased requirement for new effective antitumour agents that are active against fatal tumours but with fewer side effects. It has been known for more than two decades that there are a large number of natural compounds secreted by bacteria that exhibit anticancer activity. These alternative microbial agents can be exploited as antitumour agents. Antitumour compounds isolated from marine bacteria and the anticancer activity of lactic acid bacteria are particularly promising. This chapter presents an overview of the major compounds produced by bacteria that have potential as therapeutic agents in the treatment of cancer.

13.1 Introduction

Cancer is fundamentally the uncontrolled growth of cells in the body. Initially cancer cells invade tissues locally and later spread or metastasize throughout the body. Hanahan and Weinberg (2000) have reported six unique characters defining traits of cancer. The vital characters of cancer cells are as follows: (i) they are self-sufficient in producing their own growth signals therefore reducing dependency; (ii) they are insensitive to antigrowth signals; (iii) they evade apoptosis meaning they are resistant to cellular suicide mechanisms; (iv) they have limitless replicative capacity; (v) they have angiogenic ability, enabling the growth of new blood vessels for tumour growth; and (vi) they are capable of tissue invasion and metastasis.

According to the World Health Organization (WHO), cancer is one of the major causes of death worldwide and because of this awful disease about 8.2 million deaths occurred in 2012 (WHO, 2012).

Generally chemotherapy is used for the treatment of cancer. The drawback of this therapy is that lots of normal cells are also killed at the process, and the reason behind this is mostly anticancer drugs are not able to differentiate between cancer cells and non-cancer cells. High toxicity and adverse side effects are generally associated with cancer chemotherapy drugs (Gatti and Zunino, 2005; You *et al.*, 2010). Nowadays resistance towards anticancer drugs and the side effects of chemotherapy are a major problem in the treatment of cancer, so there is an increased requirement for new effective antitumour drugs that are active against fatal tumours but with fewer side effects.

In the late 1990s the pharmaceutical industry was more dependent on monoclonal antibodies

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and synthetic protein kinase inhibitors, but in 2007, when three drugs that were naturally derived were approved (e.g. alvespimycin, salinosporamide), attention was drawn to the importance of natural compounds that originated from bacteria (Bailly, 2009). Several important anticancerous commercialized drugs with fewer side effects have been derived by structural modification of natural compounds. Actinomycetes are a major source of natural anticancerous compounds (Berdy, 2005).

This chapter presents an overview of natural compounds of bacterial origin. Several compounds with antitumour activity have been isolated from marine bacteria. Antimicrobial peptides (bacteriocins) from bacteria and lactic acid bacteria (LAB) have also shown anticancer activity.

13.2 Anticancer Compounds from Marine Bacteria

The living conditions of marine bacteria are quite different from those of terrestrial bacteria and only a few studies have been done on the compounds produced by marine bacteria compared with the many studies on products of terrestrial bacteria. As marine bacteria live in an unusual environment, they have the capacity to produce specific and effective anticancer compounds. In view of the above there should be much more attention in the investigation of the anticancer compounds from marine bacteria. Polyketides, isoprenoids, butenolide, indolocarbazole and non-ribosomal peptides produced by marine bacteria are the important natural compounds that have been found to exhibit antitumour activities. Examples of these compounds are listed in Table 13.1.

Matsuda *et al*. (2003) have isolated and purified a sulfated polysaccharide, B-1, from the culture filtrate of marine *Pseudomonas* sp. B-1 was evaluated for anticancer activity against a panel of 39 cell lines. Among the cancer cell lines tested, high sensitivities to B-1 were observed in central nervous system cancer and lung cancer cell lines.

Matsuda *et al*. (1999) have also reported the production of a galactose, glucose and sulfate composed extracellular polysaccharide from marine *Pseudomonas* sp. This polysaccharide showed cytotoxicity to cultured human cancer cells such as MT-4.

Four new 3-methyl-4-ethylideneproline-containing peptides, lucentamycins A–D, have been isolated by Cho *et al*. (2007) from the marine-derived actinomycete *Nocardiopsis lucentensis* (strain CNR-712). Out of the four compounds, lucentamycins A and B showed significant *in vitro* cytotoxicity against HCT-116 human colon carcinoma cells.

Asolkar *et al*. (2009) have evaluated the cytotoxic activity of three new cyclohexadepsipeptides, arenamides A–C, against the human colon carcinoma cell line HCT-116. Arenamides A and B showed moderate cytotoxicity with IC_{50} (half maximal inhibitory concentration) values

Table 13.1. Anticancer compounds from marine bacteria.

Compounds	Organism	References
Saliniketal A, saliniketal B (polyketide)	*Salinispora arenicola*	Jensen *et al*. (2007), William *et al*. (2007)
Chartreusin (polyketide)	*Streptomyces* sp. QD518	Wu *et al*. (2006)
Actinofuranones A and B (polyketide)	*Streptomyces* sp. CNQ766	Cho *et al*. (2006)
Marinomycins (polyketide)	*Marinispora* sp. CNQ-140	Kwon *et al*. (2006)
Piperazimycins (peptide)	*Streptomyces* sp. CNQ-593	Miller *et al*. (2007)
Urukthapelstatin (peptide)	*Mechercharimyces asporophorigenes* YM11-542	Matsuo *et al*. (2007)
Mechercharmycins (peptide)	*Thermoactinomyces* sp.	Kanoh *et al*. (2005)
Altemicidin (isoprenoid)	*Streptomyces sioyaensis* SA-1758	Takahashi *et al*. (1989a, 1989b)
Butenolide	*Streptoverticillium luteoverticillatum*	Li *et al*. (2006)
K252c (indolocarbazole)	Actinomycete strain Z2039-2	Liu *et al*. (2007)

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of 13.2 μg/ml and 19.2 μg/ml, respectively. These compounds were isolated from the marine bacterial strain identified as *Salinispora arenicola.*

13.3 Anticancer Activities of Lactic Acid Bacteria (LAB)

Marteau *et al*. (2002) defined LAB as 'the microbial preparations or components of microbial cells that have a beneficial effect on health and well-being'. Selected LAB such as *Lactobacillus* spp., *Bifidobacterium* spp., *Lactococcus* spp. and *Enterococcus* spp. are referred to as probiotics. These bacteria have properties that enable them to survive in the gut environment and have been proved to be beneficial for the host by boosting its intestinal microbial balance (Salminen *et al*., 1998; Vimala and Dileep, 2006). These beneficial bacteria can be ingested in fermented milk products or as a supplement.

Recently, LAB have captured growing research attention because of their role in maintaining gut health and this is supported by extensive reports in the literature focusing on the health benefits of consuming LAB (Fuller, 1989; Gilliland, 1990; Aso *et al*., 1995). LAB have shown a positive effect in preventing cancer in the colon. The exact mechanism of control of colon cancer by LAB is currently unknown although there are several possible mechanisms (Rafter, 2002). According to Hirayama and Rafter (1999) and Rafter (2002) these include:

- modifications of the metabolic activities of the intestinal microflora;
- modification in the physico-chemical conditions in the colon;
- the ability to bind and degrade potential carcinogens;
- quantitative and/or qualitative alterations in the intestinal microflora;
- production of antitumourigenic or antimutagenic compounds;
- enhancing the host's immune response; and/or
- effects on the physiology of the host.

Kim *et al*. (2002) investigated whole cells, cytoplasm and peptidoglycans of ten different LAB against human cancer cell lines using the 3H-thymidine incorporation assay. They concluded that the cytoplasm fractions of *Bifidobacterium longum* and *Lactococcus lactis* ssp. *lactis* inhibited proliferation of two cancer cell lines by 50% at 33 μg/ml and 23 μg/ml for SNUC2A (a human colon adenocarcinoma cell line) and 17 μg/ml and 11 μg/ml for SNU-1 (a human gastric cancer cell line), respectively.

Baldwin *et al*. (2010) reported that a probiotic *Lactobacillus acidophilus* and *Lactobacillus casei* mix can enhance the apoptosis of a colorectal cancer cell line in the presence of 5-fluorouracil. They hypothesized that LAB or probiotics could be used as an adjuvant treatment during anticancer chemotherapy. *B. longum* has also inhibited colon cancer and modulates the intermediate biomarkers of colon carcinogenesis (Singh *et al*., 1997).

The probiotic strains *Enterococcus faecium* RM11 and *Lactobacillus fermentum* RM28 also triggered antiproliferation of colon cancer cells at the rates of 21–29% and 22–29%, respectively. Hence both strains could be used as potential probiotics for colon cancer biological products (Thirabunyanon *et al*., 2009).

The effects of LAB have been recorded not only on colon cancer but also on other cancers. LAB such as *Lactobacillus rhamnosus* (Seow *et al*., 2002) and *L. casei* (Ohashi *et al*., 2002) have shown reduction in bladder cancer. LAB such as *Bifidobacterium infantis*, *Bifidobacterium bifidum*, *Bifidobacterium animalis*, *Lactobacillus acidophilus* and *Lactobacillus paracasei* have inhibited the growth of the MCF7 breast cancer cell line (Biffi, *et al*., 1997). Ohashi *et al*. (2002) reported that regular intake of *L. casei* strain Shirota reduced the risk of bladder cancer. So from the above it is shown that LAB have considerable potential to combat fatal diseases such as cancer.

13.4 Anticancer Activities of Bacteriocins/Antimicrobial Peptides Isolated from Bacteria

Antimicrobial peptides isolated from bacteria, also called as bacteriocins, are extremely effective candidates as therapeutic agents owing to a wide range of antimicrobial activity. These molecules have also shown potential benefits in the treatment of cancer (Meyer and Nes, 1997; Tanaka, 2001). Bacteriocins help in the survival

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of individual bacterial cells by killing other bacteria present in the same environment that might compete for space and nutrients. Bacteriocin does not harm the host bacterium due to post-translational modification and/or a specific immunity mechanism. Antimicrobial peptides contribute to the defence mechanisms of bacteria (Keymanesh *et al.*, 2009).

The mechanism of the killing of prokaryotic and eukaryotic cells by antimicrobial peptides has been conceptualized by thorough research. Two mechanisms have been suggested: (i) pore formation in the plasma membrane; and (ii) mitochondrial membrane disruption that induces apoptosis (Papo and Shai, 2005).

Antimicrobial peptides are different from small molecular antibiotics. Various antimicrobial peptides, including both cationic and neutral peptides, are secreted by both Gram-positive and Gram-negative bacteria (Jack *et al.*, 1995). As cancer cells have developed resistance towards some anticancer drugs this has lead to interest in developing anticancer agents with novel mechanisms of killing cancerous cells.

Although a number of bacteriocins have been isolated there are very few examples of bacteriocins showing toxic effects on eukaryotic cells (Lagos *et al.*, 2009). However, pyocin, colicin, pediocin and microcin are a handful of bacteriocins reported to have antineoplastic activities (Gilbert *et al.*, 2008). Colicins are produced by *Escherichia coli* and other members of the *Enterobacteriaceae.* Colicins inhibit proliferation of tumour cell lines in a colicin-type and cell-line dependent fashion and are more toxic to tumour cells than to normal cells within the body (Lancaster *et al.*, 2007). The effect of four purified colicins (A, E1, E3, U) have been examined for their inhibitory effect on 11 human cell lines and one standard fibroblast cell line by Chumchalova and Smarda (2003). Results showed that the colicins showed a cytotoxic effect and the authors concluded that in many cases tumour cells were more sensitive to colicins than normal cells (Chumchalova and Smarda, 2003). Cytotoxic effects of a colicin on the mouse neoplastic L60T line have also been reported by Saito and Watanabe (1979).

Ali *et al.* (2004) reported the inhibitory effect of pyocin S2 on human tumour cell lines HepG2 and Im9 and no inhibitory effects were observed on the normal cell line HFFF (human foetal foreskin fibroblast). Pyocin S2 has been isolated from *Pseudomonas aeruginosa*.

Microcins are secreted by Gram-negative bacteria and are usually low molecular weight bacteriocins (Lagos *et al.*, 2009). Microcin E492 produced by *Klebsiella pneumonia* RYC492 is one of the important antimicrobial peptides that shows potential inhibitory action on cancer cell lines (Hetz *et al.*, 2002).

Katayama *et al.* (2005) concluded that parasporin-1 isolated from *Bacillus thuriengiensis* strain BMG1.7 is a novel cytotoxic protein that is toxic to human cancer cells. The mode of action of parasporin-1 differs from that of other cytotoxic proteins from *B. thuringiensis* including Cyt protein and other bacterial pore-forming toxins. Approximately 13 parasporin proteins have been isolated from 11 strains of *B. thuringiensis* (Ohba *et al.*, 2009).

As explained above, there are relatively few documented examples of a bacteriocin possessing an inhibitory effect on cancer cell lines. However, if more bacteriocins were tested then the likelihood of finding more compounds with potential for cancer therapy would significantly increase.

13.5 Conclusion

Undoubtedly as resistance is increasing against conventional chemotherapy drugs there is increasing interest in natural compounds produced by bacteria that have anticancer activity. Further, as bacteria are natural biofactories for the production of these antineoplastic compounds they could be an economical alternative.

Bacterial anticancerous compounds are now in a big demand because of their advantages over conventional compounds. LAB are excellent candidates for cancer therapy as they are probiotics and can colonize tumour cells as a continuous source of anticancer compound. Microcin E492 has been shown to have a very pivotal role in the treatment of cancer. Marine bacteria have also gained considerable attention as they produce a large number of compounds showing potential anticancer activity.

Although natural compounds derived from bacteria have proved that they are potential candidates for the treatment of cancer, some questions are still unanswered, for example: (i) Do the bacteria initiate, promote or just attract the tumour

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cell?; and (ii) Can the natural compounds secreted by bacteria be used safely and effectively as therapeutic agents for cancer treatment? (Mager, 2006). Until these questions are answered, research will continue for effective treatment of this terrible disease.

So these antitumour compounds from bacteria appear to have great potential because of their possible therapeutic applications, however, it is clear that more work is needed to identify more compounds and establish a greater understanding of their mode of action.

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14 *Giardia* and Giardiasis: an Overview of Recent Developments

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Abstract

Giardia lamblia is one of the most common protozoan enteric pathogens that inhabits the upper small intestine of humans and several other vertebrates and causes giardiasis. Global prevalence of giardiasis has been estimated to be 300 million cases annually. To adapt in environments both inside and outside the small intestine of the host, this protozoan parasite undergoes significant developmental changes during its life cycle. It has been confirmed that *G. lamblia* has become drug resistant and biochemical studies have been undertaken to investigate the cause of resistance. This chapter focuses on the most current findings regarding the important advances in understanding the molecular mechanisms that regulate the antigen switching process, including oxidative stress and expressional modifications in *Giardia*, and potential drug targets for the treatment of giardiasis are discussed.

14.1 Introduction

The micro-aerotolerant *Giardia lamblia*, a unicellular, gastrointestinal flagellated protozoan causes one of the most frequent parasitic infections worldwide (Adam, 1991). It lacks conventional mitochondria, Golgi body and peroxisomes. In 2002 an estimated 280 million symptomatic human infections were reported every year (Lane and Lloyd, 2002) but more recently this has risen to 300 million cases annually (Morrison *et al*., 2007). The symptoms of giardiasis are watery diarrhoea, abdominal pain, irritable bowel syndrome, nausea, vomiting, weight loss and the symptoms appear 6–15 days after infection (Farthing, 1997). The disease symptoms have been observed to be more profound in malnourished children and in immunodeficient individuals. Metronidazole or other nitroimidazoles are the common treatment options. *Giardia* species cannot invade the gut and it secretes no well-known toxin but recent data suggests that *Giardia* increases intestinal permeability by augmenting apoptosis of the inner cell lining of the intestine (Singer and Nash, 2000; Scott *et al*., 2002). Due to its potential as a zoonotic pathogen, farm animals get infected hampering the economic yield (O'Handley *et al*., 2001). Even though this infection is chronic nearly half of cases are asymptomatic and infection subsides without drug treatment (Farthing, 1997). It has been put forward that certain gastrointestinal disorders like irritable bowel syndrome can be related to a previous *Giardia* infection (Hanevik *et al*., 2009). Therefore, the symptomatology differs from person to person.

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The protist genus *Giardia* is a member of the diplomonads, which is a group of binucleated flagellates that are now classified as part of the super group Excavata (Simpson, 2003). Being one of the most divergent eukaryotes examined to date, it presents us with unique opportunities to have a better understanding into the key pathways that characterize eukaryotic cells as well as new molecular mechanisms within them. *G. lamblia* acts as a good model system due to its unique and unusual ultrastructure, simple *in vitro* differentiation, sequenced genome and a metabolism resembling that of a bacterium (Lauwaet *et al.*, 2007). Reductive processes in *Giardia* combined with its parasitic lifestyle play a vital role in its survivability and at the same time highlight its evolution. *Giardia* is of widespread biological interest because of its early evolutionary position and also because of its striking adaptation to two very different and hostile environments. The flagellated trophozoite form specifically colonizes the small intestinal lumen of humans, and the dormant cyst form survives in cold fresh water (Gillin *et al.*, 1996).

G. lamblia is a micro-aerophilic organism, which does not usually tolerate elevated oxygen pressure. In the upper intestinal cell lining, where this organism generally resides, the oxygen (O_2) concentration there has been measured at 60 μM (Raj *et al.*, 2014). In addition to this, the amitochondriate parasite lacks some of the conventional enzymes of detoxifying reactive oxygen species (ROS), such as superoxide dismutase (SOD), catalase, peroxidase and glutathione reductase. A recent finding suggests that *Giardia*, which is highly susceptible to oxygen tension, prefers to reside in the proximal small intestine than the colon as the latter has a higher redox buffering capacity (Mastronicola *et al.*, 2011). The detailed mechanism by which the parasite could aid the detoxification of ROS produced during oxidative stress is as yet unknown. Cysteine is neither synthesized *de novo* nor from cystine and is thought to be imported inside the cell by passive diffusion, although active transport accounts for some of the attainment of cysteine (Lujan *et al.*, 1994). The toxicity of thiol-blocking agents unable to penetrate intact cells has demonstrated the significance of free thiol groups on the surfaces of trophozoites (Gillin *et al.*, 1984).

Giardia is a micro-aerophilic parasite infecting all vertebrates that lacks mitochondria but contains mitosomes (apparent relic mitochondria). This organism is a eukaryote and possesses many typical characteristics such as a distinct nucleus and nuclear membrane, endomembrane system and cytoskeleton; however, other aspects of the cell such as small-subunit (SSU) ribosomal RNA (rRNA) and some key metabolic enzymes are prokaryotic-like (Svärd *et al.*, 2003). Although controversial, the majority of investigators think *Giardia*, which has diverged during transition of mitochondrial acquisition, is an important organism to understand the evolution in eukaryotic cells (Thompson *et al.*, 2004). The *Giardia* genome project has greatly enriched our knowledge about this intriguing organism.

14.2 Historical Background of *Giardia*

G. lamblia, the intestinal protozoan with a long and venerable history of about 300 years, remains a biological enigma even today. The great Dutch microscopist Antonie van Leeuwenhoek, who recovered it from his own stool in 1681, is credited for its discovery (Dobell, 1920). The first reports on the morphology of *Giardia* are those of Lambl (1859, 1860) and Cunningham (1881) (all as cited in Erlandsen and Meyer, 1984). The genus name *Giardia* was established by Kunstler (1882); however, Blanchard (1888) suggested that the genus be named *lamblia* to commemorate Lambl (1859) and it was Stiles (1915) who named this parasite *Giardia lamblia* (all as cited in Erlandsen and Meyer, 1984).

14.3 Systemic Classification of *Giardia*

Conventionally, all living organisms have been categorized as eukaryotes or prokaryotes, and some are still a matter of debate for retaining characters of both. However, the most extensively established classification now utilizes three major divisions, Archaea (archaebacteria), Bacteria (eubacteria) and Eukarya (eukaryotes) (Woese *et al.*, 1990), which can then be divided into kingdoms. *G. lamblia* is a distinct eukaryotic organism and has been placed in the subkingdom of Protozoa, the more 'animal-like'

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of the unicellular eukaryotes. Traditionally, according to their morphology protozoa had been differentiated into ciliates, sporozoa, flagellates and amoeba (rhizopods). Therefore, it shared the same family of flagellated protozoans comprised of the kinetoplastids (e.g. *Leishmania* spp. and *Trypanosoma* spp.), parabasalids (e.g. *Trichomonas vaginalis*) and *Dientamoeba* (e.g. *Dientamoeba fragilis*). *Giardia* has been positioned in the order Diplomonadida and the family Hexamitidae. The phylogenetic tree of a gene, namely *cpn 60*, which is considered to be of mitochondrial origin, also proposes that *Giardia* is an early branching eukaryote (Germot *et al.*, 1996). Iron-dependent hydrogenases of *G. lamblia* provide evidence for lateral gene transfer (Nixon *et al.*, 2003). Recently, the triose phosphate isomerase gene has proved to be a very useful genetic marker for the population genetic study of *G. lamblia*. Thus, all the prevalent reports suggest that *Giardia* and other diplomonads lie in accordance with existing eukaryotes.

14.4 Epidemiology

Giardia is a genus of intestinal flagellates that infect a wide variety of vertebrate hosts. The genus currently comprises six species, namely *Giardia agilis*, *Giardia ardeae*, *Giardia psittaci*, *Giardia microti*, *Giardia muris* and *Giardia duodenalis* (syn. *intestinalis*) infecting amphibians, birds, rodents and mammals (Adam, 1991). These species can be distinguished on the basis of their trophozoites' morphology and ultrastructure. Among them only *G. lamblia* (= *duodenalis*) causes giardiasis in humans, although it can also infect other vertebrates (Thompson *et al.*, 2004). According to previous reports, the members of species *G. lamblia* have negligible morphological variations but great genetic diversity. Therefore, they are considered as a species complex (Monis *et al.*, 2003) with eight distinct genetic groups or assemblages (A–H) (Lasek-Nesselquist *et al.*, 2010). Assemblage A and B can infect and multiply in humans and other mammals whereas the rest show a much restricted host range. Likewise C and D mostly infect the canids, whereas E, F, G and H infect livestock, cats, rodents and marine vertebrates, respectively (Cacciò and Ryan, 2008).

Among the enormous list of waterborne diarrhoeal diseases, giardiasis is a major disease that comes second to respiratory infection as causes of mortality and morbidity worldwide (Wolfe, 1992). The tropics and subtropics are regions which are highly prone to this infection, especially among socio-economically underprivileged individuals (Yassin *et al.*, 1999). Poor hygienic conditions favour its spread, particularly where there is scarcity of clean drinking water and effective sewage disposal systems, resulting in faecal contamination of the sewage. Though the disease is endemic in developing countries, it is also responsible for many localized outbreaks in the USA caused by contaminated drinking water. In India intestinal parasites are a major health problem (Saha *et al.*, 1996). Direct faecal–oral transmission is the other major means by which *G. lamblia* is spread both in adults and in children (Sethi *et al.*, 1999). Food-borne transmission is less common but well documented (Osterholm *et al.*, 1981). These outbreaks have most probably occurred through contamination of freshly prepared food by infected food handlers.

Every year the number of immunosuppressed individuals is increasing worldwide in part due to the continuous spread of human immunodeficiency virus (HIV), chemotherapy and implementation of immunosuppressive drugs. Immunosuppressed individuals are at a higher risk of getting infected by opportunistic parasites (Stark *et al.*, 2009). *Giardia* is not listed among the opportunistic parasites because it does not cause prolonged symptoms and therapy is independent of the patient's immune status. However, the observed prevalence of *Giardia* infection of immunosuppressed individuals varies between 1.5% and 17.7% in the few reports published (Stark *et al.*, 2009). HIV-infected individuals with giardiasis exhibit symptoms which are similar to that of symptoms shown by HIV-negative individuals with giardiasis, with asymptomatic infection prevalent in the presence of HIV (Faubert, 2000). However, when $CD4^+$ counts are reduced and cause progressive immunosuppression, the chance of symptomatic *Giardia* infection increases, with a tendency towards chronic diarrhoea (Dwivedi *et al.*, 2007).

It is rather obvious that the distinction between mixed infections and true recombinants is crucial. This will require single cell analysis (i.e. single cysts) to be formally undisputedly identified. Since genotyping of single cysts is technically feasible (Miller and Sterling, 2007), and

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assemblage-specific PCR-based assays are available (Geurden *et al.*, 2009), research in this direction will be of paramount importance. A prerequisite for inter- and intra-assemblage recombination is that mixed *Giardia* infections occur in an individual host. This seems to be the case, especially in humans and dogs (Sprong *et al.*, 2009), where in multilocus genotyping analysis between ~20% and ~30% of the isolates were inter-assemblage mixtures. Multiple genotypes of *Giardia* have profound significance towards the etiology of giardiasis as it cannot be clearly stated as to how and when vertebrates get affected with more than one genotype. Either infection with different *Giardia* genotypes occurs simultaneously, due to environmental contamination, through water for example, or asymptomatic individuals affected by one *Giardia* assemblage may become symptomatic on being infected by another *Giardia* assemblage. This hypothesis is sustained by studies conducted on asymptomatic subjects. Mixed infections are important for molecular typing of *Giardia*. Assignment of a single marker can correctly identify isolates of specific sub-assemblages as different markers give different results. For example one marker can classify isolates as potentially zoonotic whereas another marker can identify the same set of isolates as host adapted. Therefore, use of multiple markers for typing gives reliable results.

14.5 Life Cycle

The life cycle of *G. lamblia* has two stages, the vegetative trophozoites stage and the infective cyst stage. Infection normally follows ingestion of cysts in faecally unhygienic water or less commonly via contaminated foods (Adam, 1991). Excystation is activated by the low pH of the gastric acid in the stomach and stimulated by the slightly alkaline pH and proteolytic action of the duodenum fluid (Rice and Schaefer, 1981). This key step in infection is dependent upon a cysteine protease stored in peripheral vesicles of the trophozoites and released into the space between the trophozoites and the cell wall during excystation (Ward *et al.*, 1997). After excystation, the emerging parasite quickly segregates into two identical binucleate trophozoites that attach to and specifically inhabit the small intestine of humans. They swim (Shant *et al.*, 2004) in the lumenal fluid with four pairs of flagella and also adhere to the mucus strand *in vivo* and *in vitro* (Adam, 1991). They also penetrate the mucus layer and adhere to the intestinal epithelial cells via their unique ventral adhesive disc (Nemanic *et al.*, 1979). In this position they feed on the host's gut contents not by phagocytosing large particles such as bacteria but through micropinocytotic vesicles. Here they grow in number by binary fission. When the trophozoites are moved downstream by the flow of the intestinal fluid they encyst, as they could not survive outside the host. The elevated pH and intestinal bile concentration of the intestinal lumen induces the encystations and cyst antigen synthesis (Gillin *et al.*, 1996). The most visible overall change during encystation is that the trophozoites gradually round up and detach, lose mobility and become refractive.

14.5.1 Trophozoite structure

Pear-shaped trophozoites of *G. lamblia* are around 12–15 μm long and 8–10 μm wide. The cytoskeleton comprises of a median body, four pairs of flagella (anterior, posterior, caudal and ventral), and a ventral disk. Trophozoites have two nuclei without nucleoli that are positioned anteriorly and are symmetric with reference to the long axis. Lysosomal vacuoles, as well as ribosomal and glycogen granules, are found in the cytoplasm. Golgi complexes become observable in encysting trophozoites but have not been confirmed to be present in vegetative trophozoites (Gillin *et al.*, 1996). However, stacked membranes suggestive of Golgi complexes have been demonstrated (Lanfredi-Rangel *et al.*, 1999).

Attachment of the trophozoites to the intestinal wall is brought about by the ventral adhesive disc from where they gather the necessary nutrients and the disc holds them in place preventing them from travelling beyond the jejunum. The disc is composed of the contractile protein actinin, α-actinin, tropomyosin and myosin and they all form the biochemical basis on which adherence takes place (Feely *et al.*, 1982). Attachment is supported by active metabolism and is hampered at temperatures below 37°C, high oxygen levels or reduced cysteine concentrations (Gillin and Reiner, 1982).

The trophozoites possess four pairs of flagella arising from two sets of basal bodies located

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near the midline and anteroventral position of the nucleus. The two ventral flagella are extrapolated at one side by paraflagellar rods. Recent studies have proposed that trophozoites have a specialized membrane with an electron transport chain which agrees with the fact that *Giardia* might not be primitive after all, but has originated from an aerobic mitochondria-containing flagellate (Lloyd *et al*., 2002; Tovar *et al*., 2003).

14.5.2 Mitosome

The discovery of nuclear genes of putative mitochondrial ancestry in *Giardia* (Silberman *et al*., 2002) and the current discovery of mitochondrial remnant organelles in amitochondrial protists such as *Entamoeba histolytica* (Anderson *et al*., 2005) and *Trachipleistophora hominis* (Gribaldo and Philippe, 2002) put forward the theory that the eukaryotic amitochondrial state is not a primitive condition but is rather the consequence of reductive evolution. Two mitochondrial marker proteins involved in iron-sulfur cluster biosynthesis – IscS and IscU – have been identified using an *in vitro* protein reconstitution assay and specific antibodies. This demonstration shows the mitochondrial remnant organelles (mitosomes) are present in *Giardia*, which functions in iron–sulfur protein maturation. This evidence shows that *Giardia* is not amitochondrial and that it has a functional organelle retained from the original mitochondrial endosymbiont (Tovar *et al*., 2003).

14.5.3 Cyst structure

Cysts are composed of an outer filamentous layer and an inner membranous layer. The dimensions of the oval-shaped cyst are 7–10 μm in width and 10–20 μm in length enclosed by a wall that is 0.3–0.5 μm thick. Proteins and a novel *N*-acetylgalactosamine (GalNAc) polysaccharide form the cyst wall. GalNAc is initially not present in the trophozoites, but arises during encystation via an inducible pathway involving enzymes that produce UDP-GalNAc from fructose-6-phosphate (Lopez *et al*., 2003).

14.6 Molecular Biology

14.6.1 Transfection

A transient and stable transfection system contributes considerably to the understanding of the genetics of *G. lamblia*. A transfection system is constituted of a luciferase flanked at the 5′ end by a short region of the *gdh* gene and at the 3′ end by a putative polyadenylation signal. DNA is introduced by electroporation in such transfection procedures. The *pac* gene which confers resistance to the antibiotic puromycin or the *neo* gene that confers resistance to neomycin are used as selectable markers for stable episomal transfection. The transfection vectors contain sequences of bacterial plasmids that act as origins of replication in *G. lamblia*.

14.6.2 Transcription and translation

Transcription in *G. lamblia* is particularly eukaryotic in nature; none the less, it has a number of features that are more characteristic of prokaryotes such as amanitin-resistant transcription using RNA polymerase II (Seshadri *et al*., 2003). As in all eukaryotes, the transcript is formed in the nucleus and transported to the cytoplasm for translation.

14.6.3 Tranposons

Mobile genetic elements, by virtue of their ability to move towards new chromosomal locations, are considered important in determining the evolutionary course of the genome. They occur widely in the biological kingdom. Among protozoan parasites several types of transposable elements are encountered. The largest variety is seen in the trypanosomatids. Three families of transposon elements were detected in the WB isolate of *G. lamblia* currently being used for the genome sequencing project. Two of these are subtelomeric in location while the other one is chromosome-internal and dead (Arkhipova and Morrison, 2001). Nucleotide sequence analysis of all the elements shows that they are all retrotransposons, and all of them are non-long-terminal-repeat (LTR) retrotransposons (Bhattacharya *et al*., 2002).

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These elements are clearly distinct from all other previously described non-LTR lineages.

14.7 The RNA Interference (RNAi) Pathway in *Giardia*

Considered one of the most outstanding discoveries in recent years, the RNAi process has become one of the most useful tools used to study expression of genes, leading to the understanding of specific functions in organisms where genetic approaches do not work. Like other organisms, *Giardia* presents RNAi machinery, although it is very simple. Recently, several researchers revealed exciting insights about the significance of small RNAs in this parasite (Kolev and Ullu, 2009).

14.8 Oxidative Stress

Oxidative stress triggers a series of physiological, pathological and adaptive responses in cells either due to cellular damage or through specific signalling molecules. Transcriptional outputs influencing cell survival and disease processes are modulated by these responses. Over the past two decades, various transcription factors and signalling pathways have been recognized to play vital roles in critical transcriptional responses to oxidative stress. How changes in the intracellular ROS concentration brings about distinct and functional alterations in gene expression are demonstrated effectively by these examples.

To elucidate this problem, a *G. lamblia* DNA library containing more than 10,000 clones has been used to recognize the differentially regulated genes in the oxidative stressed cells compared with the control set (Raj *et al.*, 2014). The differentially regulated genes fall into five groups of functionally related proteins. These functional categories are: (i) metabolic enzymes; (ii) structural proteins; (iii) kinases and phosphatases; (iv) cell cycle and proliferation controllers; and (v) cell death regulators.

The study has depicted a view on genes which are differentially expressed during oxidative stress and taking important roles in *Giardia* stress management. NADH ferredoxin oxidoreductase, NADH oxidase, thioredoxin reductase, nitroreductase, pyruvate ferredoxin oxidoreductase, arginine deiminase and alcohol dehydrogenase are some of the enzymes that play a pivotal role in oxidative stress management. Presence of these enzymes is an indication of several pathways, as they represent some major biochemical pathways in eukaryotes. In addition to this, a good number of hypothetical proteins are present that indicates their significant role in stress regulation. Some other proteins such as Hsp90, β-giardin and cysteine-rich variant-specific surface proteins (VSPs), calmodulin (CaM) kinase and serine threonine protein phosphatase also play some important roles in *Giardia* survival. Cell death regulation has been studied along with the cell survival strategy under oxidative stress management (Ghosh *et al.*, 2009). Investigation of the cell cycle has revealed that increased oxygen tension affects the cell cycle progression and during death levels of G_1, as assessed by flow cytometry, were below normal. Further studies proved that the parasite cells are not dying through necrosis but via a programmed cell death (PCD) mechanism.

14.9 Mode of Cell Death

PCD is a well-regulated cellular process which is widely recognized in multicellular organisms. It has been observed in a cumulative number of unicellular eukaryotes as well, including the trypanosomatids, *Dictyostelium*, *Plasmodium*, *T. vaginalis*, *E. histolytica* and *Blastocystis* (Bruchhaus *et al.*, 2007). *Giardia* possesses two forms of cell death – apoptosis and autophagy (Corrêa *et al.*, 2009). Interestingly, Corrêa *et al.* proposed the presence of caspases and a caspase-dependent mechanism using CaspaTag and fluorescence microscopy. This method, although frequently used, has been shown by several authors to have questionable specificity (Darzynkiewicz and Pozarowski, 2007). The lack of caspase in unicellular eukaryotes and the possession of an apoptotic pathway independent of caspases have been well documented. In recent years there has been various reports highlighting the involvement of metacaspases in an apoptotic-like PCD in unicellular organisms. Metacaspases have shown a well-established functional activity in yeast (Mazzoni and Falcone, 2008), but work in *Trypanosoma brucei* (Helms *et al.*, 2006) and *Plasmodium berghei* (Le Chat *et al.*, 2007) has

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been inconclusive. Caspase-independent apoptosis does occur; however, this typically requires functional mitochondria and therefore this pathway is unlikely in *Giardia* because this organism lacks mitochondria. *Giardia* does, however, have a relic structure termed the mitosome but this organelle is not capable of generating energy and no clear metabolic or signalling role has been related to it (Rosa Ide *et al*., 2008). Analysis of the *Giardia* genome for apoptotic-related genes including caspases and metacaspases has been unsuccessful as none of the components could be identified (Raj *et al*., 2014).

14.10 Immunology

Over the last decade, considerable light has been shed on the role played by the host's immune system in determining the outcome of *Giardia* infection. Spontaneous resolution of infection in humans has been demonstrated and in an experimental animal and this strongly suggests the existence of a protective insusceptible response to *Giardia*. Increased prevalence of giardiasis in patients with hypogammaglobulinemia emphasizes the importance of antibody in the immunity against this parasite. Several giardiasis outbreaks show that individuals repeatedly exposed to *G. lamblia* have a lower incidence of infection suggesting that prior exposure imparts partial resistance to reinfection. In addition a high prevalence of infection by *Giardia* in homosexuals suggests the role of cellular immunity in *Giardia* infection since many of the homosexuals have acquired a T-cell defect. It has also been found that removal of *G. muris* is impaired in hypothalamic mice, and in mice deficient in B-cells and mast cells. Macrophages have also been implicated for immunity to this parasite. More than one immunological mechanism seems to be involved in providing protection against *Giardia*.

14.10.1 Humoral response

Giardia infection induces a local as well as a systemic response. Serum antibodies to *G. lamblia* were first demonstrated by Ridley and Ridley (1976) in patients of giardiasis with malabsorption, using the indirect immunofluorescence antibody test. These antibodies were IgG, IgA and IgM which were directed against surface antigens. The antibody-mediated killing may or may not require complement (Nash and Aggarwal, 1986). The increase in the titre value of antibodies in some patients with recurrent infection suggests that antibodies alone are not protective. The cell-mediated response may also be essential for the defensive immune response (Eckmann, 2003). Thus a combination of immunological events ultimately clears the infection and memory develops in the majority of affected human hosts. But how the secretary antibody or cellular components react with *Giardia* is completely unknown.

14.10.2 Cell-mediated response

Cell-mediated immune response in giardiasis is poorly understood although a role for T-lymphocytes has been well established. The available evidence suggests that macrophages do not spontaneously eliminate *Giardia* species but may act as antigen-presenting cells for CD4 lymphocytes and/or participate in antibody-mediated killing of the trophozoites. Theoretically a protective effect of anti-trophozoite antibodies might result in inhibition of trophozoite attachment to the intestinal epithelial cells or opsonization of trophozoites for killing or ingestion by phagocytes. Of these possibilities there is evidence that trophozoite attachment to intestinal epithelium is inhibited by antibodies. The non-immune protection system includes the intestinal mucosal layer, intestinal motility and human breast milk for infants (Tellez *et al*., 2003). A T-cell dependent mechanism is also involved for controlling acute *Giardia* infection and this mechanism is devoid of B-cell activation (Singer and Nash, 2000). Interleukin 6 (IL-6) has been revealed to play a key role in controlling acute giardiasis (Bienz *et al*., 2003).

14.11 Antigenic Variation

14.11.1 Occurrence of antigenic variation

Antigenic variation is brought about by the cysteine-rich surface antigens in *G. lamblia* trophozoites (Nash *et al*., 1990). It was found that the complementary monoclonal antibody

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to a 170 kDa surface antigen (initially called CRP170 but now called VSPA6) from the WB isolate showed cytotoxicity towards the WB trophozoites but not towards isolates carrying different surface antigens. Consequent reports have proved that only one VSP is expressed by an individual organism at a time; however, subpopulations of trophozoites exhibiting various VSPs have resulted in studies where multiple surface-labelled bands were detected. Variation occurs approximately once in every six to 12 generations with a frequency of 10^{-3} to 10^{-4} (Nash *et al.*, 1990). Variable surface proteins including ornithine carbamoyl transferase, fructose-1,6-bisphosphate aldolase, α-giardins and arginine deiminase act as immunoreactive agents during acute giardiasis and they may be used for diagnostic purposes depending on the diagnostic procedure used. Taglin, a surface antigen, moves as a doublet of 28 kDa and 30 kDa on Western blots (Ward *et al.*, 1987) and shows protease-induced lectin activity (Lev *et al.*, 1986). It binds to mannose-6-phosphate on being treated by trypsin. Consistent expression of this antigen occurs throughout excystation and encystation. Another protein, α1-giardin, which is a highly immunoreactive glycosaminoglycan (GAG)-binding protein, plays a vital role in the host–parasite interaction (Weiland *et al.*, 2003). A 49 kDa antigen (GP49) is also attached to the membrane surface of *G. lamblia* trophozoites by a glycosylphosphatidylinositol (GPI) anchor (Das *et al.*, 1991). Incorporation of exogenous phosphatidyl inositol occurs during synthesis of the GPI-anchor but myoinositol is converted to phosphatidyl inositol prior to incorporation (Subramanian *et al.*, 2000).

14.11.2 Molecular mechanism in the control of antigenic variation

The VSPs form a major subject of study to describe the cellular and humoral immune responses in several animal models as they are the only boundary between the parasite and the host (Pimenta *et al.*, 1991). GS/H7 isolate of *G. lamblia* is the only known isolate which has the potential of infecting both humans and mice (Byrd *et al.*, 1994), making it a most sought-after strain for immunological analysis (Von Allmen *et al.*, 2004). Work on this strain has indicated that regulation of VSP expression is brought about by a post-transcriptional gene silencing mechanism (Prucca *et al.*, 2008). Nuclear run-on experiments have been used to show the simultaneous transcription of several *vsps*, although only one mRNA corresponding to the VSP that was expressed at the trophozoite plasma membrane accumulated in the cytoplasm. Antisense VSP transcripts were identified and were shown to be generated in the nuclei of the parasite by a relaxed mechanism controlling transcription (Elmendorf *et al.*, 2001). Moreover, direct proof of the involvement of an RNAi-based mechanism for the regulation of VSP expression has come from the expression of Dicer or RNA-dependent RNA polymerase knock-down experiments. In the non-existence of these enzymatic activities, multiple VSPs were simultaneously expressed on the surface of trophozoites, as detected by immunofluorescence assays with specific monoclonal antibodies.

14.12 Pathology and Pathogenesis

Up until now it has been unclear as to how *Giardia* causes the disease. There is mounting evidence that the characteristics of both the parasite and the host have a hand in the outcome of the infection. In symptomatic cases of *Giardia*, trophozoites attach to the microvilli brush border by virtue of their ventral disc and produce a barrier effect or damage the brush border. This may result in decreased brush border enzymes, which ultimately leads to malabsorption of fat, vitamin B12, lactose and protein. The pathological changes are more marked in patients with hypogammaglobulinemia and may account for increased frequency of overt giardiasis in these cases (Lebwohl *et al.*, 2003). Two antigenically distinct isolates were used to infect gerbils and human volunteers and a marked difference in pathogenicity was shown between these two isolates. In subsequent studies it has been found that the same isolate expressed antigen differently in different hosts. In one group of human volunteers it produced a 72 kDa surface antigen and caused successful infection, whereas in the other group it expressed 200 kDa surface antigens and remained avirulent. Though the data available was not conclusive, this attribute of the parasite might be important

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(Nash *et al.*, 1990). A number of predisposing factors in the host may also take part in causing the disease including: (i) age; (ii) nutritional status; and (iii) genetic predisposition, as suggested by frequency of infection in blood group A and various states of immunodeficiency. Adult reactive arthritis is also associated with *G. lamblia* infection. In conclusion it can be said that variable characteristics of the infecting strains resulting in varying degrees of virulence in combination with host factors determine the susceptibility to *Giardia* infection.

14.12.1 Clinical features

The clinical manifestation of giardiasis varies from asymptomatic infection to chronic diarrhoea with malabsorption. Patients with symptomatic giardiasis have diarrhoea with loose foul-smelling stools that is slimy, foamy or bulky. Other common gastrointestinal symptoms include abdominal cramps and bloating, nausea and decreased appetite. Malaise and weight loss is seen in the majority of patients and fever is occasionally present, especially in the early part of the infection. On the other hand about half of the infected people are asymptomatic, and the infection commonly resolves spontaneously (Adam, 1991).

14.12.2 Diagnosis

Diagnosis of *G. lamblia* can be done in several ways (Kapoor *et al.*, 2001):

- microscopic examination of stools;
- examination of duodenal contents;
- small bowel biopsy;
- gastrointestinal radiology;
- immunodiagnosis; and
- using a DNA probe.

Microscopic stool examination

Microscopic examination of stools for cysts and trophozoites is usually the first diagnostic test performed in patients with suspected giardiasis. *Giardia* cysts are found in stools of most patients with giardiasis whereas trophozoites are found less commonly, but correlates with symptomatic infection (Goka *et al.*, 1990). Stool specimens are examined by light microscopy either fresh or fixed with polyvinyl alcohol or formalin and then stained with trichrome or iron haematoxylin. The accuracy of daily examinations of a single stool specimen is approximately 50–70% (Goka *et al.*, 1990). Two or three specimens collected on different days should be analysed as symptoms of the illness begin approximately 1–7 days before excretion of cysts start. Sedimentation and immunomagnetic separation of *G. lamblia* cysts followed by microscopic examination has shown better sensitivity and specificity (Massanet-Nicolau, 2003).

Examination of intestinal fluid

In patients where repeated stool examinations are negative, diagnosis can be done by examination of the duodenal contents. The duodenal fluid can be obtained with a duodenal tube or with the entero-test (HEDECO Company, Mountain View, California). In the entero-test the patient swallows a gelatin capsule on a string. After several hours the capsule is removed from the intestine and examined microscopically. Examination of the duodenal content is more sensitive than examination of stool samples (Hopper *et al.*, 2006). The inclusion of routine duodenal biopsies as part of upper endoscopy in paediatric patients yields additional pathological findings that otherwise could have been missed (Kori *et al.*, 2003).

Small bowel biopsy

Small intestinal biopsies may yield better diagnosis of *Giardia* infection where other methods fail. Such specimens may be examined after sectioning and Giemsa staining. The parasite is usually found to attach to the microvillus border, particularly in the crypts. Though both sensitivity and specificity are high for small bowel biopsy and the duodenal fluid test, these tests are not used often for diagnosis as they are invasive (Brown *et al.*, 1996).

Gastrointestinal radiology

Giardiasis may result in non-specific intestinal changes that may be useful for disease diagnosis in about 20% of giardiasis patients. These abnormalities have been detected by barium examination.

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Immunodiagnosis

Higher sensitivity and specificity are offered by immunoassay procedures compared with conventional staining methods. For screening a large number of patients, especially during an epidemic or for patients with negligible symptoms, these techniques are quite effective. They depend on detection of: (i) specific antibody in the patient's serum; or (ii) *Giardia* antigen in a stool sample and duodenal fluid. Different immunoassay kits based on direct immunofluorescent-antibody assay and ELISA are available in the market. Comparative studies of these kits using microscopic and other direct methods (Aziz *et al.*, 2001) have shown high specificity (100%) for these techniques while kits vary in sensitivity from 94% to 99%.

DNA probe

Over the last few years, scientists have devoted themselves to prepare more sensitive, sophisticated diagnostic systems based on nucleic acid detection. Butcher and Farthing (1989) reported on a DNA probe to diagnose *Giardia* infection while Lewis *et al.* (1990) reported on a total genomic DNA probe to detect *G. lamblia*. The latter probe can detect as much as 10 μg of *G. lamblia* DNA, 10^4 trophozoites and 10^4 cysts but it shows cross reactivity with a high amount (5 μg) of *Trypanosoma cruzi* DNA. Different DNA probes have been designed to detect *Giardia* in stool samples. The non-transcribed spacer region within the rRNA gene of *G. lamblia* has been successfully used to differentiate between *G. lamblia* and other enteric pathogens. In 1991 Ronald M. Atlas reported the utility of PCR in detection of *G. lamblia*. In this detection system the giardin gene has been targeted for amplification. Since then a lot of PCR-based detection systems have been proposed. Real time PCR (Verweij *et al.*, 2003) and colorimetric detection of PCR product have also been reported. PCR detection has the advantage over other detection systems with regard to both sensitivity and specificity.

Molecular diagnosis – nucleic acid detection methods

PCR-based methods show higher sensitivity than other conventional methods and immunological techniques for detecting *G. lamblia* in faeces. Special molecular techniques are necessary to determine *G. lamblia* assemblages and sub-assemblages. Identifying *G. lamblia* assemblages infecting humans (A and B) is necessary as it helps to determine the epidemiology of the disease and likely transmission routes. As DNA sequence-based *Giardia* surveys have also found assemblages A and B in non-human hosts, the zoonotic potential of some assemblage A and B isolates must be borne in mind (Cacciò and Ryan, 2008; Ortega-Pierres *et al.*, 2009). Molecular sub-typing methods are less developed for *G. lamblia* than for other protozoan pathogens, but loci on the following genes have been targeted: (i) small subunit ribosomal RNA (*ssu-rRNA*); (ii) β giardin (*bg*); (iii) glutamate dehydrogenase (*gdh*); (iv) elongation factor 1-alpha (*ef-1*); (v) triose phosphate isomerase (*tpi*); (vi) GLORF-C4 (*C4*); and (vii) the inter-genomic rRNA spacer region (IGS) (Cacciò and Ryan, 2008). It is highlighted that there are potential typing and sub-typing complications caused by:

- intra-isolate sequence heterogeneity (the presence of mixed templates that influence the identification of subtypes within each assemblage); and
- the unreliable assignment of isolates to *G. lamblia* assemblages generated by different genetic markers, especially when single genetic markers are used.

14.13 Treatment of Giardiasis

During the last decade significant achievements have been obtained in treatment of giardiasis (Petri, 2003). A number of attempts have been made to determine the susceptibility of *G. lamblia* to different drugs *in vitro.* To date the following drugs have been used for treatment of giardiasis: albendazole, mebendazole, ornidazole, tinidazole, metronidazole, mepacrine, furazolidone, secnidazole, hemezole, nifuroxazide, etofamide, nalidixic acid, quinfamide and nitazoxanide (Minenoa and Avery, 2003).

14.13.1 *G. lamblia* susceptibility towards aminoglycosides

The aminoglycosides are a large and diverse group of antibiotics, a significant number of them target the 3′ end of single-stranded (ss) RNA. The 3′

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end of ss rRNA plays a vital function in protein synthesis (binding mRNA and tRNA) and has been characterized as the site of action of several aminoglycoside antibiotics. Kasugamycin, the first antibiotic for which an rRNA target has been implicated, acts on the two methyl groups added to nucleotides A-1518 and A-1519 in bacteria. *G. lamblia* rRNAs like rRNAs of all organisms includes the AA dinucleotide, although there are no indications of the methylation status. *G. lamblia* has been found to be partially susceptible to this drug. Hygromycin acts on the U-1495 and also interacts with G-1494. These nucleotides are present in *G. lamblia* also within the highly conserved 1492–1506 sequences. *G. lamblia* WB strain is highly susceptible to this drug at an ID_{50} (the infective dose of microorganism that will cause 50% of exposed individuals to become infected) of 50 µg/ml. But this is of no clinical utility because of the lack of selective toxicity displayed by this aminoglycoside. Paramomycin interacts at two sites that are separated with respect to sequence but are at the same location with respect to secondary structure. Apparently the 1409–1491 base pair and not a specific sequence confer susceptibility in *Tetrahymena*. This base pair is present in *G. lamblia* and the parasite has been observed to be highly susceptible to this drug. However, the drug is rarely used for treatment for its high ID_{50}. However, since paramomycin like aminoglycosides is poorly absorbed from the gut, reducing the chances of systemic toxicity, this drug has been recommended in pregnancy. Kanamycin and apramycin interact with A-1408 in bacteria. In *G. lamblia* this nucleotide is substituted by G, and so these drugs have no effect on this parasite. Gentamycin interacts with G-1405 of bacteria ss rRNA. Though this is present in *G. lamblia* the parasite is highly resistant to this aminoglycoside. So probably there are other requirements for gentamicin activity. Other aminoglycosides like neomycin, sisomicin, ribostamycin, butirosin and tobramycin are ineffective against this parasite (Edlind, 1989).

14.13.2 Susceptibility to nitroheterocyclic drugs

Giardia infection is presently very effectively controlled by drugs of the 5′-nitroimidazole family, and metronidazole and tinidazole in particular. They exhibit a wide range of activity against pathogens and anaerobic bacteria. Metronidazole is widely used for treatment of giardiasis and is more than effective when given as a 5-day course (Adam, 1991). The mechanism of action of this drug is the reduction of the nitro group to toxic nitro anion radical metabolites via reduced ferredoxin (Edwards *et al.*, 1993) and is this is brought about by the enzyme pyruvate ferredoxin oxidoreductase (PFOR) (Townson *et al.*, 1996). The toxic radicals are thought to bind to DNA disabling the whole cell (Edwards *et al.*, 1993), but there is no evidence of it in nucleated organisms like *Giardia*. There are also indications that the radicals may have toxic effects on the enzymes of the respiratory chain of the parasite (Adam, 1991). The decrease in PFOR activity results in drug resistance. The drug acts as a mutagen in bacteria and if used in high doses for prolonged periods it is carcinogenic to mice. In humans, nausea and general malaise are common when undergoing treatment. A disulfirum-like interaction with ethanol is seen but acute complications are rare. Tinidazole is effective when introduced in single doses and is well accepted by the human system. Consequently this is the drug of choice; although there is evidence of genetic damage induced by tinidazole in human lymphocytes (López Nigro *et al.*, 2001). Furazolidone, another member of the nitroheterocyclic drugs, is also reduced to cytotoxic products in a similar manner to the 5′-nitroimidazoles but not via ferredoxin. In *Giardia* the enzyme NADH oxidase activates furazolidone (Brown *et al.*, 1996) but does not seem to interfere with mechanisms of furazolidone resistance. Furazolidone resistance is thought to be brought about by membrane changes that prevent access of furazolidone to its site of activation within the parasite.

Mode of action of tinidazole

After oral administration, tinidazole is rapidly and completely absorbed. Tinidazole is distributed into virtually all tissues and body fluids and also crosses the blood–brain barrier. Tinidazole is significantly metabolized in humans prior to excretion. The plasma half-life of tinidazole is approximately 12–14 h.

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14.13.3 Susceptibility to benzimidazoles

Mebendazole, a benzimidazole, is a broad-spectrum anthelmintic agent that shows giardicidal activity through its interaction with β-tubulin. Mebendazole causes degenerative alterations in the tegument and intestinal cells of parasitic worms by binding to the colchicine-sensitive site of tubulin, thus inhibiting its polymerization or assembly into microtubules. The loss of the cytoplasmic microtubules leads to impaired uptake of glucose by the larval and adult stages of the susceptible parasites, and depletes their glycogen stores. Degenerative changes in the endoplasmic reticulum, the mitochondria of the germinal layer, and the subsequent release of lysosomes result in decreased production of ATP, which is the energy required for the survival of the helminth. Due to diminished energy production, the parasite is immobilized and eventually dies.

Albendazole, another benzimidazole, is capable of acting against *G. lamblia* even outside a host body (Meloni *et al*., 1990). Two different methods were used to measure and compare the action of metronidazole and albendazole against *Giardia* trophozoites. These methods were: (i) Meloni's method, based on the loss of adherence of parasites to surfaces; and (ii) the Hill method, based on the loss of the parasite's capacity to divide. It was found that Meloni's method is more sensitive than Hill's method under low and moderate inhibitory albendazole concentrations. However, under high metronidazole concentrations, the Hill's method proved to be more sensitive. Both methods seem to be effective to determine IC_{50} values. However, the inhibition of adherence method had even better performance for the benzimidazole-like drugs (Cruz *et al*., 2003). Metronidazole- and albendazole-resistant giardiasis can be successfully treated with nitrazoxanide in acquired immunodeficiency syndrome patients (Abboud *et al*., 2001). Albendazole and mebendazole analogues are as active as antiprotozoal agents as metronidazole against *G. lamblia* (Navarrete-Vázquez *et al*., 2003).

Secnidazole, another benzimidazole derivative, can be used for treatment of giardiasis to effectively eliminate chronic *Giardia* infection (Escobedo *et al*., 2003). Fenbendazole is also a very effective treatment for giardiasis, eliminating *Giardia* trophozoites from the small intestine (Keith *et al*., 2003). Quinacrine is a flavoantagonist and probably suppresses the oxidation of NADPH in *G. lamblia*. Though it is highly effective, gastrointestinal side effects are however common (Upcroft *et al*., 1996).

14.13.4 Other agents

Other agents with *in vitro* activity include chloroquine, pyrimethamine, mefloquine, rifampin, azithromycin and doxycycline. Ciprofloxacin exhibits cytotoxic effects in *G. lamblia* trophozoites and is used as an alternative drug in treatment of giardiasis (Sousa *et al*., 2001). Patients who fail to respond to treatment usually respond to a second course of treatment with the original or another agent. Combined treatment with quinacrine and metronidazole has been found very effective in the treatment of giardiasis.

14.14 Potential Drug Target Against *Giardia*

Giardiasis could be self-limiting in some cases, but because of the potential for chronic or sporadic symptoms, treatment is recommended. As already explained there are several drugs with different mechanisms that are available to treat giardiasis. However, new drugs need to be developed that take into account the undesirable side effects of the current therapeutics. In addition, reappearance of symptoms after treatment and reinfection are significant reasons for the progression of newer treatments.

The absence of the arginine deiminase (ADI) gene in the human genome, together with its important function in both pathogenic protozoa and bacteria, makes this enzyme an attractive therapeutic drug target for treatment of bacterial and parasite infections. Because ADI is involved in an important energy-producing pathway in *Giardia* and antigenic variation, it is a desirable target for further drug design (Knodler *et al*., 1998). The next challenge is to design inhibitors, with the ADI catalytic cysteine being an obvious target for an active site-directed electrophile or a suicide substrate.

In parasitic infections, some models are currently exploiting the use of amino acids as

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therapeutic agents. For severe malaria, human volunteers were treated with arginine intravenously and the dosing regimen was found to be safe, with no clinically significant adverse effects (Weinberg *et al*., 2008).

14.15 Prevention of Giardiasis

The best way to avoid *G. lamblia* infection is by prevention by adhering to the following guidelines:

- Practice good hygiene in day care centres, retirement homes and at home to prevent the spread of infection.
- Wash hands frequently with soap and water for at least 30 s.
- Avoid contact with the faeces of an infected person.
- When travelling in regions where giardiasis is common, avoid infection by using packaged drinking water and avoiding consumption of raw fruits and vegetables.
- Do not use untreated water from susceptible water bodies where the parasite might be present, such as lakes, rivers and streams. If water from such sources has to be used then it should be boiled for at least 5 min before use.
- Public swimming pools that are not properly treated and maintained are another potential source of contamination. Avoid swallowing water in swimming pools and spas. Do not swim when sick with diarrhoea.

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15 Power of Bifidobacteria in Food Applications for Health Promotion

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Abstract
Bifidobacteria are the predominant bacteria in the normal intestinal biota of healthy breast-fed newborns (more than 95% of the total bacterial population), and represent approximately 20% of the microbiota in the gastrointestinal tract of adults. Nowadays, these bacteria are the second most commonly used microorganisms in the production of probiotic food products after members of the genus *Lactobacillus*. Bifidobacteria have been intensively studied for the prevention and treatment of human and/or animal gastrointestinal disorders, such as colonic transit disorders, intestinal infections, colonic adenomas and cancer. The market of probiotic foods (an important segment of functional foods) is estimated to increase annually by about 6.8% from 2013 to 2018 and has an estimated total sales value of US$44.9 billion in 2018. This chapter focuses on the general characteristics and health effects of bifidobacteria as well as on potential applications of these bacteria in the production of different food products.

15.1 Introduction

Nowadays, even in developed countries, the role of diet is not limited to the provision of enough nutrients; it may also modulate various physiological functions and may play detrimental or beneficial roles in some diseases (Koletzko *et al*., 1998). The concept of the use of foods to promote a state of well-being, improving health and reducing the risk of diseases has become a new frontier in the nutrition sciences and related fields (Granato *et al*., 2010). Furthermore, this concept is particularly important in the steady increase in life expectancy and the desire of elderly people for an improved quality of life as well as the deceleration of the increase in cost of health care (Roberfroid, 2007). Moreover, in many countries the emphasis has moved from the dogma of medication to that of prevention. In this context, development and contribution of functional foods – prebiotics, probiotics and synbiotics – should be key pillars of the health care system. Probiotics, according to the most recent definition of the Food and Agriculture Organization of the United Nations/World Health Organization (FAO/WHO), are live microorganisms that benefically affect the health of the host (FAO/WHO, 2002). Bifidobacteria, naturally present in the dominant colonic microbiota (up to 25% of the cultivable faecal bacteria in adults and 80% in infants), are the second most commonly used bacteria in the production of probiotic products after members of the genus *Lactobacillus* (Heller, 2001; Soccol *et al*., 2010). Based on clinical studies, many beneficial health effects are due to bifidobacteria such as: (i) the inhibition of

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pathogens and harmful bacteria that colonize and/or infect the gut mucosa; (ii) the regulation of intestinal microbial homeostasis; (iii) the repression of pro-carcinogenic enzymatic activities within the microbiota; (iv) the modulation of local and systemic immune responses; (v) the production of vitamins; (vi) the bioconversion of a number of dietary compounds into bioactive molecules; and (vii) the prevention of cancer (Saavedra *et al.*, 1994; Gill *et al.*, 2001; Xiao *et al.*, 2003; Wang *et al.*, 2004a, b; Furrie *et al.*, 2005; Leu *et al.*, 2010). The global market for probiotic ingredients, supplements and foods was worth US$27.9 billion in 2011 and is expected to reach US$44.9 billion in 2018, growing at a compound annual growth rate of 6.8% from 2013 to 2018 (Transparency Market Research, 2013). In 2008, the value of this market was reported to be only about US$16 billion (Granato *et al.*, 2010). This segment of the market still has potential to grow dynamically. Hence this chapter focuses on the general characteristics and health effects of bifidobacteria as well discussing potential applications of these bacteria in the production of different food products.

15.2 The *Bifidobacterium*

15.2.1 Taxonomy and general features

Officially, the genus *Bifidobacterium* was classified in 1973 with 23 species and belongs to the family *Bifidobacteriaceae*, order *Bifidobacteriales*, subclass *Actinobacteridae*, class *Actinobacteria* and phylum *Actinobacteria*. Later, Scardovi (1986) updated the number of bifidobacterial species to 24. Recently, with the description of two new species *Bifidobacterium bombi* (Killer *et al.*, 2009) and *Bifidobacterium crudilactis* (Delcenserie *et al.*, 2007), the number of species in this genus has reached 31 taxa (Table 15.1) (Ventura *et al.*, 2007). The history of this genus started in 1889, when Henry Tissier – a French paediatrician – observed that bacteria cells with Y-shaped morphology (bifid form) were dominant in the gut microbiota of healthy babies and were missing or at reduced numbers in the gastrointestinal tract of infants who had diarrhoea. At the same time he had successfully isolated this bacterium from stool samples of breast-fed infants, and named the organism *Bacillus bifidus communis* (Tissier, 1900). Thereafter different designations have been employed such as *Bacteroides bifidus* and *Lactobacillus bifidus*. In 1924, Orla-Jensen suggested a separate genus, namely *Bifidobacterium* (Orla-Jensen, 1924), but this suggestion was only accepted officially 50 years later (in 1973). The name of the genus is derived from the fact that members typically appear as bifid, branched or V- and Y-shaped rods (Fig. 15.1). Bifidobacteria are Gram-positive, non-motile, non-sporulating, catalase-negative, heterofermentative, anaerobic or microaerophylic, and have a high G+C content ranging from 45% to 67% (Biavati and Mattarelli, 2001; Ventura *et al.*, 2007). The G+C content of *Bifidobacterium inopinatum* was reported to be the lowest (45%), whereas *Bifidobacterium choerinum* has G+C content about 66.3% (Table 15.1). The G+C contents of *Bifidobacterium adolescentis*, *Bifidobacterium lactis*, *Bifidobacterium longum* and *Bifidobacterium bifidum* are 59%, 60%, 60% and 63%, respectively. Numerous bifidobacterial genomic sequencing projects are running at different institutes (Ventura and Turroni, 2012). The public genomic databases to date contain 21 complete bifidobacterial genomes (National Center for Biotechnology Information, 2015) that vary in size from 1.9 Mb to 2.8 Mb. Genomic lengths of the *Bifidobacterium dentium* Bd1, *B. adolescentis* ATCC 15703, *Bifidobacterium infantis* ATCC 15697, *B. longum* DJO10A, *B. lactis* B4-01 (Barrangou *et al.*, 2009) and *B. bifidum* ATCC 29521 strains are 2.636 Mb, 2.090 Mb, 2.833 Mb, 2.375 Mb, 1.939 Mb and 2.211 Mb, respectively (Table 15.1).

Bifidobacteria are one of the important microorganisms in the microbiota of warm-blooded animals. The main habitat of them is the faeces (Reuter, 1963; Scardovi and Crociani, 1974), but they have also been reported as being isolated from other parts of the digestion system such as the rumen, or the vagina (Orla-Jensen, 1924; Reuter, 1963; Biavati and Mattarelli, 1991). *Bifidobacterium breve* and *B. infantis* are only present in the intestinal tracts of infants, while *B. bifidum*, *Bifidobacterium catenulatum*, *B. longum* and *Bifidobacterium pseudocatenulatum* can be found in both infants and adults, whereas *B. adolescentis* was isolated only from the intestinal tracts of adults (Reuter, 1963; Matsuki *et al.*, 1999; Mayer *et al.*, 2003). Some other *Bifidobacterium* species are not of human origin, but were proved to have probiotic properties such as *B. lactis*, which was isolated from fermented

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Table 15.1. Habitats and some genomic properties of the genus *Bifidobacterium*. (Adapted from Bifid Project, 2014.)

Bifidobacterium species	Source	G+C content (%)[a]	Genomic size (bp)	References
Human origin				
B. adolescentis	Adult faeces	59.2 (ATCC 15703)	2,089,645	Reuter (1963)
B. angulatum	Adult faeces	59.4 (DSM 20098)	2,007,108	Scardovi and Crociani (1974)
B. bifidum	Child and adult faeces, vagina	62.68 (ATCC 29521)	2,198,551	Orla-Jensen (1924)
B. breve synonym *B. parvolulorum*	Child faeces, vagina	58 (DSM20213)	2,297,799	Reuter (1963)
B. catenulatum	Child and adult faeces, vagina	56 (DSM16992)	2,058,429	Scardovi and Crociani (1974)
B. denticolens	Buccal cavity	55		Crociani *et al*. (1996)
B. dentium	Buccal cavity, adult faeces	58.54 (Bd1)	2,636,368	Scardovi and Crociani, (1974), Ventura *et al*. (2009)
B. gallicum	Adult faeces	57 (DSM20093)	2,016,380	Lauer (1990)
B. infantis synonym *B. liberorum* and *B. lactentis*	Child faeces, vagina	60.5	2,828,958	Reuter (1963), Fukuda *et al*. (2011)
B. inopinatum	Buccal cavity	45		Crociani *et al*. (1996)
B. longum	Child and adult faeces and vagina	60 (DJO10A)	2,375,792	Reuter (1963)
B. pseudocatenulatum	Child faeces	56 (DSM20438)	2,304,808	Scardovi *et al*. (1979)
Environmental and food origins				
B. lactis (subjective synonym of *B. animalis*)	Fermented milk	60 (DSM10140)	1,938,483	Meile *et al*. (1997), Cay *et al*. (2000)
B. minimum	Sewage			Scardovi and Trovatelli (1974)
B. subtile	Sewage			Scardovi and Trovatelli (1974)
B. thermacidophilum	Anaerobic digester			Dong *et al*. (2000)
Animals				
B. animalis ssp. *lactis*	Rat, chicken, rabbit and calf faeces	60.6 (ATCC 25527)	1,932,693	Mitsuoka (1969)
B. asteroides	Bees	60.05 (PRL2011)	2,167,304	Orla-Jensen (1924)
B. boum	Rumen, piglet faeces			Scardovi *et al*. (1979)
B. choerinum	Piglet faeces	66.3		Scardovi *et al*. (1979)
B. coryneforme	Bees			Scardovi and Trovatelli (1969)
B. cuniculi	Rabbit faeces			Scardovi *et al*. (1979)
B. gallinarum	Chicken faeces			Watabe *et al*. (1983)
B. indicum	Bees			Scardovi and Trovatelli (1969)
B. magnum	Rabbit faeces			Scardovi and Zani (1974)
B. merycicum	Rumen			Biavati *et al*. (1991)

Continued

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Table 15.1. Continued.

Bifidobacterium species	Source	G+C content (%)[a]	Genomic size (bp)	References
B. pseudolongum ssp. *pseudolongum*	Piglet, chicken, calf and rat faeces, rumen			Mitsuoka (1969)
B. pseudolongum ssp. *globosum*	Piglet, chicken, calf and rat faeces, rumen			Scardovi *et al*. (1969)
B. pullorum	Chicken faeces			Trovatelli *et al*. (1974)
B. ruminantium	Rumen			Biavati and Mattarelli (1991)
B. saeculare	Rabbit faeces			Biavati *et al*. (1991)
B. suis	Piglet faeces			Matteuzzi *et al*. (1971)
B. thermophilum synonym *B. ruminale*	Piglet, chicken and calf faeces, rumen			Mitsuoka (1969)
B. biavatii	Faeces of common marmoset, red-handed tamarin			Endo *et al*. (2012)

[a]Number of the type species is shown in parentheses. ATTC, American Type Culture Collection; DSM, Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH.

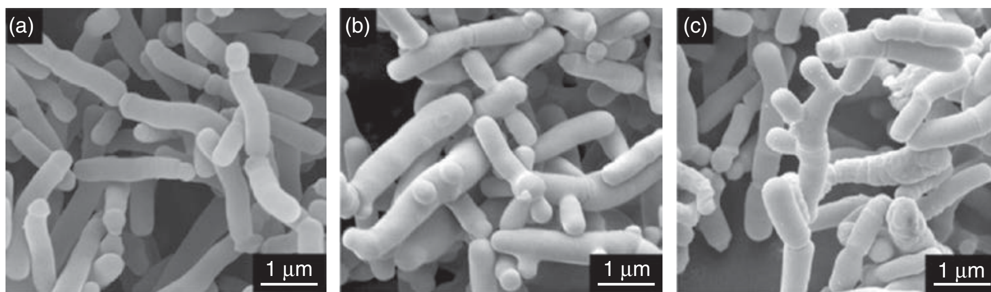

Fig. 15.1. Scanning electron micrographs of *Bifidobacterium longum* NCC2705 cells grown anaerobically in MRS (de Man, Rogosa and Sharpe) medium. Typical rod shapes are observed in exponential phase (a), while branched-shaped forms are observed in transition (b) and stationary (c) phase. (From Klijn *et al*., 2005, with permission.)

milk, and *Bifidobacterium animalis* from faeces of rat, chicken, rabbit and calf. *B. adolescentis*, *B. bifidum*, *B. breve* and *B. longum* are predominant in the vagina, whereas the *B. dentium* is the common species of microbiota of the oral cavity. *B. dentium* is responsible for approximately 8% of the bacteria cultured from dental caries (Scardovi and Crociani, 1974; Ventura *et al*., 2009). Unfortunately, *B. dentium*, *B. inopinatum* and *Bifidobacterium denticolens* are considered to be pathogens because they cause dental caries or ulcerative colitis. In 2012 researchers at Baylor College of Medicine and Texas Children's Hospital identified the first commensal bacteria strain – *B. dentium* – in the human intestine that is capable of secreting large amounts of

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gamma-aminobutyric acid (GABA). This molecule is a major inhibitory neurotransmitter in the central and enteric nervous systems and thus may play a role in preventing or treating inflammatory bowel diseases such as Crohn's disease (Medical News Today, 2012). *Bifidobacterium minimum* and *Bifidobacterium subtile* are only present in sewage and they can be used as indicator microorganisms in determination of faecal contamination of wastewater (O'Sullivan and Kullen, 1998; Biavati *et al.*, 2000; Ventura *et al.*, 2004). In 2002, Jian and Dong did the phylogenetic analysis of both 16S rRNA and HSP60 sequences as well as the DNA base compositions and some phenotypic characteristics. Based on the results obtained, they proposed that *B. inopinatum* DSM 10107T and *B. denticolens* DSM 10105T should taxonomically be classified as *Scardovia inopinata* and *Parascardovia denticolens*, respectively (Jian and Dong, 2002).

In the genus *Bifidobacterium*, several species are associated in groups that have been named according to the oldest name included in the group. These groups are:

- *B. adolescentis* group (*B. angulatum*, *B. pseudocatenulatum*, *B. catenulatum*, *B. dentium*, *B. adolescentis*, *B. merycicum* and *B. ruminantium*);
- *Bifidobacterium pullorum* group (*B. gallinarum*, *B. pullorum* and *B. saeculare*);
- *Bifidobacterium asteroides* group (*B. asteroides*, *B. coryneforme*, *Bifidobacterium indicum*);
- *Bifidobacterium boum* group (*B. boum*, *Bifidobacterium thermacidophilum*, *Bifidobacterium thermophilum*); and
- *Bifidobacterium pseudolongum* group (*B. animalis*, *B. cuniculi*, *B. choerinum*, *B. gallicum* and *B. pseudolongum*).

The species *B. breve* and *B. longum* form a couple according to different phylogenetic analyses, as well as *B. minimum* and *B. psychroaerophilum* (less supported). *B. bifidum*, *B. magnum*, *B. scardovii* and *B. subtile* form distinct branches (Felis and Dellaglio, 2007).

15.2.2 Structure of the cell wall

Bifidobacteria have a typical Gram-positive cell wall structure (Gomes and Malcata, 1999) that consists of numerous layers (generally, three layers in a Gram-positive and one layer in a Gram-negative bacterium) of peptidoglycan (murein), teichoic acids, lipoteichoic acids, wall-associated proteins, polysaccharides and cytoplasmatic membrane. Structurally, the backbone of the murein is build up by alternating molecules of *N*-acetylglucosamine (NAG) and *N*-acetylmuramic acid (NAM) linked by a β-1,4-glycoside bonds. The 3-carbon of NAM is substituted with a lactyl ether group derived from pyruvate that connects a peptide side chain that contains L-alanine, D-glutamate, L-ornithine and D-alanine (Todar, 2014) to the glycan backbone, but in some cases L-ornithine is substituted by L-lysine. The glycan backbone thus can be cross-linked by inter-peptide bridges of amino acids that connect a free amino group on L-ornithine or L-lysine to a free carboxy group on D-alanine of a nearby tetrapeptide side chain. Assembly of the inter-bridge is inhibited by β-lactam antibiotics. Moreover, due to lack of the outer membrane, bifidobacteria (Gram-positive) are more sensitive to penicillin than Gram-negative bacteria. The presence of L-ornithine or L-alanine as well as the exact composition of the individual inter-peptide bridge and number of these bridges in the murein differ among the various *Bifidobacterium* species, and even vary from strain to strain. Therefore, this characteristic can be used for identification of strains (Leahy *et al.*, 2005). The polysaccharides consist of glucose and galactose, and frequently rhamnose (Biavati *et al.*, 2000). Of course in this case, the quality and quantity of individual sugars also varies from species to species, and even from strain to strain, and depends on culture conditions. Chemically, teichoic acids are linear polymers with the main chain consisting of polyglycerol or polyribitol substituted with phosphates and a few amino acids and sugars. These polymers are apparently directed outwards at right angles to the layers of peptidoglycan and are occasionally anchored to the plasma membrane. To date, the functions of teichoic acid are still not known clearly, but they play an essential role in the viability of Gram-positive bacteria in the wild. Moreover, the lipotecichoic acids and surface proteins play an important role in the formation of the hydrophobic character of the surface of the cell-wall (Arunachalam, 1999; Biavati *et al.*, 2000). Two proposed functions of teichoic acids are: (i) they can act as a transporter by providing a channel of regularly oriented negative

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charges for threading substances with positive charge through the complicated peptidoglycan layer; and (ii) they improve the adherence of the bacteria to tissue surfaces by the regulation and assembly of muramic acid subunits on the outside of the plasma membrane.

15.2.3 Oxygen tolerance

Even though bifidobacteria are known as strictly anaerobes, the degree of tolerance to oxygen in the case of individual species can vary (Meile *et al.*, 1997; Beerens *et al.*, 2000; Simpson *et al.*, 2005; Kawasaki *et al.*, 2006) depending on the species itself, its morphology and the culture medium (Boylston *et al.*, 2004; Rodrigues *et al.*, 2011). *B. infantis*, *B. breve* and *B. longum* are able to grow under partial aeration (low oxygen concentration), whereas *B. adolescentis* is very intolerant to even a very low concentration of oxygen. Moreover, *B. boum* and *B. thermophilum* were shown to grow well even at 20% O_2 concentration (Kawasaki *et al.*, 2006; Ninomiya *et al.*, 2009). Unlike an aerobic prokaryote, bifidobacteria, except *B. indicum* and *B. asteroides*, are unable to reduce hydrogen peroxide to water by catalase. However, the harmful effect of hydrogen peroxide, that is well known to cause oxidative damage, can be inactivated with fructose-6-phosphate-phosphoketolase (Scardovi, 1981), which is a key enzyme in the pentose phosphate pathway. In bifidobacteria during metabolism of glucose, nicotinamide adenine dinucleotide (NAD) is reduced to NADH. In the presence of oxygen molecules the enzyme NADH oxidase (oxidoreductase) reduces oxygen to hydrogen peroxide. Then the hydrogen peroxide is transported outside of the cell where it is converted by the enzyme NADH peroxidase (the heterolytic cleavage of the peroxide bond to catalyse the two-electron reduction of hydrogen peroxide to water) (Crane *et al.*, 2000). Thus, the degree of tolerance to oxygen should be reciprocally proportional with the activity of NADH oxidase and peroxidase enzymes.

15.2.4 Temperature and pH

The optimum temperature for the growth of bifidobacteria is 37–41°C, the minimum is 25–28°C and the maximum is 43–45°C (Scardovi, 1981; Shah, 2007). Most of the bifidobacteria are unable to grow at temperatures lower than 20°C or higher than 46°C, except *B. thermacidophilum* that grows well at 49.5°C (Dong *et al.*, 2000) and *Bifidobacterium psychraerophilum* that can grow even at 4°C (Simpson *et al.*, 2004). Gavini and co-workers (1991) proposed a growth temperature of 45°C to distinguish the origins of bifidobacteria, because the ones of human origin cannot grow at temperatures higher than this.

The optimum pH for the growth of bifidobacteria is 6.5–7, and they are unable to grow below pH 4.5 or above pH 8.5 (Scardovi, 1981; Shah, 2007). Matsumoto and co-workers (2004) reported growth of *B. lactis* and *B. animalis* even at pH 3.5. In a liquid complex media (like fermented dairy products) negligible losses of bifidobacteria occurred at pH 5, but if the pH dropped to pH 4, cell numbers decreased from 0.1 to 7.6 log orders (Vinderola *et al.*, 2002a). The *B. longum* strain 1941 and the *B. pseudolongum* strain 20097 exhibited more tolerance to the acidic conditions than the seven other strains of bifidobacteria under strong acidic conditions (pH 1.5–3.0). Following a 3 h incubation period, the cell counts of these two acid-tolerant strains decreased less than 1.0 log, while the cell counts of the other bifidobacteria strains evaluated decreased up to 7.0 log (Lankaputhra and Shah, 1995).

15.2.5 Metabolism of carbohydrates

It is well known that the gut microbiota is capable of degrading complex carbohydrates in the host to monosaccharides and then converting it to bioenergy and cell structures. These complex carbohydrates can be dietary compounds (e.g. resistant starches, cellulose, hemicellulose, glycogen, galactan, xylan, pullulan, pectins and gums), host-derived compounds (e.g. mucin, glycosphingolipids, chondroitin sulfate, hyaluronic acid and heparin) (Hooper *et al.*, 2002) or carbon sources produced by other members of the gastrointestinal tract microbial community (Korakli *et al.*, 2002). Analysis of the genome map of *B. breve* (a typical member of the natural microbiota of an infant intestine) shows that this bacterium encodes various carbohydrate-modifying enzymes that degrade, modify or create glycosidic bonds (Pokusaeva *et al.*, 2011). Most

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of them are cytoplasmic enzymes while only a small number of these glycosidases are found extracellularly.

Bifidobacteria are saccharolytic organisms, and all species are able to metabolize glucose, galactose and fructose (Leahy *et al.*, 2005). Hexose metabolism of bifidobacteria is unique. Due to lack of aldolase and glucose-6-phosphate-dehyrdogenase, bifidobacteria are unable to metabolize hexose through either glycolysis or the hexose-monophosphate pathway. Instead, bifidobacteria degrade hexose sugars through a particular metabolic pathway, the so-called 'bifidus shunt', where the enzyme fructose-6-phosphate-phosphoketolase (EC 4.1.2.2) plays a key role (Fig. 15.2) (de Vries and Stouthamer, 1967). This enzyme is considered to be a taxonomic marker for the family of *Bifidobacteriaceae* (Felis and Dellaglio, 2007). Initially, the hexokinase together with ATP converts glucose to glucose-6-phosphate and then it is converted to fructose-6-phosphate with the glucose-6-phosphate isomerase. From this point, fructose-6-phosphate phosphoketolase splits the fructose-6-phosphate into two molecules: (i) erythrose-4-phosphate; and (ii) acetyl phosphate. The acetyl phosphate is then converted to ethanol via acetyl-CoA or to acetic acid directly. The erythrose-4-phosphate and another molecule of fructose-6-phosphate react with each other to yield glyceraldehyde-3-phosphate and acetyl phosphate. The glyceraldehyde-3-phosphate is converted to lactic acid through the pyruvic pathway. Therefore, theoretically, through the 'bifidus pathway', two molecules of lactic acid and three molecules of acetic acid are generated from the fermentation of two molecules of glucose (de Vries and Stouthamer, 1967; Kun *et al.*, 2008). In contrast, the homofermentative group of lactic acid bacteria (LAB) produces two molecules of ATP and two molecules of lactic acid from one molecule of glucose, whereas heterofermentative LAB produce one molecule each of lactic acid, ethanol and ATP per one molecule of fermented glucose (Salminen *et al.*, 2004). The ratio of lactate to acetate formed by bifidobacteria may vary depending on the carbon source utilized and also on the species examined (Palframan *et al.*,

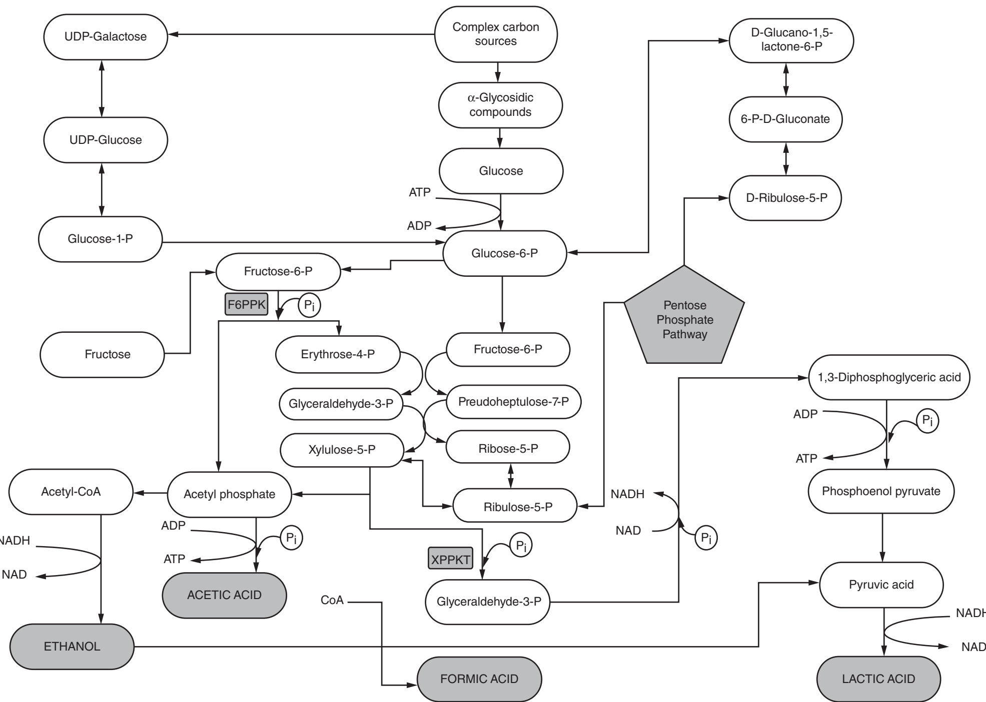

Fig. 15.2. Schematic metabolism of carbohydrates by bifidobacteria (the bifidus shunt or bifidus pathway). Abbreviations: F6PPK, fructose-6-phosphate phosphoketolase; XPPKT, xylulose-5-phosphate/fructose-6-phosphate phosphoketolase; Pi, phosphate.

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2003). In some cases, a small amount of succinic acid is detectable, whereas no butyric or propionic acids are produced (Scardovi, 1981; Degnan and Macfarlane, 1994; Biavati *et al.*, 2000). Carbon dioxide is released only in the case of degradation of gluconic acid. Furthermore, the bifidus shunt yields five molecules of ATP for every two molecules of glucose. However, during the metabolism of lactose, initially it is split into glucose and galactose by β-galactosidase and then galactose is converted to fructose-6-P through the Leloir pathway for feeding into the major pathway of glucose metabolism (Vedamuthu, 2006). The β-galactosidase also exhibits a galactosyltransferase to synthetize different galacto-oligosaccharides (Rabiu *et al.*, 2001; Ventura *et al.*, 2004). It is also suggested that the proportions of the final fermentation products vary considerably from one strain to another, and also depend on the carbon source used (de Vries and Stouthamer, 1968). However, many of the characterized strains can utilize ribose, galactose, fructose, glucose, sucrose, maltose, melibiose and raffinose, but generally cannot ferment L-arabinose, rhamnose, NAG, sorbitol, melezitose, trehalose, glycerol, xylitol and inulin (Pokusaeva *et al.*, 2011).

15.2.6 Sensitivity to antibiotics

Generally, species belonging to the genus *Bifidobacterium* are resistant to various antibiotics such as kanamycin, neomycin, streptomycin, polymyxin, gentamicin, nalidixin and metronidazole, but their growth and activity are inhibited by oleandomycin, lincomycin, clindamycin, vancomycin, penicillin G, ampicillin, erythromycin, bacitracin, chloramphenicol and nitrofurantoin (Kheadr *et al.*, 2004; Moubareck *et al.*, 2005). Kanamycin and neomycin are widely applied in preparation of selective media for isolation of bifidobacteria (Temmerman *et al.*, 2002). Charteris and co-workers (1998) investigated antibiotic susceptibility of 16 *Bifidobacterium* isolates to 44 antibiotics and found that most of the isolates were resistant to amikacin (30 μg), aztreonam (30 μg), cefoxitin (30 μg), colistin sulfate (10 μg), fusidic acid (10 μg), gentamycin (10 μg), kanamycin (30 μg), metronidazole (5 μg), nalidixic acid (30 μg), norfloxacin (10 μg), polymyxin B (300 μg), trimethoprim (5 μg), streptomycin (10 μg) and vancomycin (30 μg). They were resistant or moderately susceptible to ceftazidime (30 μg), ciprofloxacin (5 μg), cotrimoxazole (25 μg), netilmicin (10 μg) and sulfamethoxazole (100 μg). Tetracycline (30 μg) moderately affected the *Bifidobacterium* isolates. Susceptibilities of most of the isolates to penicillin-type antibiotics (i.e. bacitracin (10 μg), cephalothin (30 μg), cefuroxime (30 μg), cefaclor (30 μg), ceftizoxime (30 μg), cefotaxime (30 μg), chloramphenicol (30 μg), clindamycin (2 μg), erythromycin (15 μg), nitrofurantoin (300 μg) and rifampicin (5 μg)) were high, and to cephradine (30 μg), cephazolin (30 μg), cefoperazone (75 μg), ceftriaxone (30 μg), furazolidone (15 μg) and ofloxacin (5 μg) were variable. All *B. bifidum* isolates were susceptible to cefixime (5 μg). Based on the results of β-lactamase activity of four microorganism–drug combinations these workers suggested that cell wall impermeability was responsible for cephalosporin resistance among bifidobacteria. The *B. bifidum* PRL2010 strain did not show MIC (minimum inhibitory concentration) values higher than those indicated by the European Food Safety Authority as critical values except for an atypically high level of resistance to streptomycin (Serafini *et al.*, 2013). However, a high level of resistance to this antibiotic has been noticed for several other bifidobacterial strains (Kiwaki and Sato, 2009).

No doubt the antibiotic susceptibility of *Bifidobacterium* varies depending on the origin of the strain. Kheadr and co-workers (2004) found that strains originating from infants were much more sensitive to chloramphenicol, novobiocin and tetracycline than commercial ones. In contrast, the resistance to pirlimycin of commercial strains was higher than that of strains from infants. Different results are available in the literature about susceptibility of bifidobacteria to vancomycin that is a natural glycopeptide and able to block synthesis of peptidoglycan by formation of a complex with D-alanine. Charteris and co-workers (1998) as well as Kheadr and co-workers (2004) reported the resistance of bifidobacteria to vancomycin, whereas others (Tynkkynen *et al.*, 1998; Moubareck *et al.*, 2005; Zhou *et al.*, 2005) found that it inhibited growth of various *Bifidobacterium* strains. Zhou

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and co-workers (2005) detected the resistance of only one strain, namely *B. lactis* Bb-12.

15.3 Microbial Ecosystem and Health Effects

15.3.1 Gut microbiota

The human gastrointestinal tract is a very complex ecosystem, the microbial composition of which has yet to be fully determined even if it is generally accepted that representatives of four bacterial phyla (*Bacteroidetes*, *Firmicutes*, *Actinobacteria* and *Proteobacteria*) are the main residents of this ecosystem (Eckburg *et al.*, 2005; Turroni *et al.*, 2008). At the very beginning (before the birth) the gastrointestinal tract of the infant is sterile. Colonization begins immediately after birth and is influenced by the mode of delivery, infant diet, hygiene levels and medication (Grönlund *et al.*, 1999). Bifidobacteria are estimated to represent 40–50% and 60–90% of total bacteria in formula-fed and breast-fed infants, respectively (Harmsen *et al.*, 2000), but the population of bifidobacteria decreases after the weaning period and they were shown to represent 3% of the faecal flora in adults (Vaughan *et al.*, 2002) until an advanced age when the population of bifidobacteria appear to decline (Leahy *et al.*, 2005). The digestive microbiota in the gastrointestinal tract develops during life and its composition changes based on the maturation of the intestinal mucosa and dietary diversification (Galdeano *et al.*, 2010). Generally, the gastrointestinal tract of a healthy 18-year-old man houses over 10^{14} microbial cells with over 1000 diverse bacterial types, mostly in the colon (Wallace *et al.*, 2011). This constitutes approximately tenfold more bacterial cells than human cells in the body, and represents 1–2 kg of bacteria within the adult gut (Shanahan, 2002; O'Hara and Shanahan, 2006). The majority of bacteria in the adult gut are non-sporing anaerobes, the most numerically predominant of which include *Bacteroides* spp. and *Bifidobacterium* spp., *Eubacterium* spp., *Clostridium* spp., *Lactobacillus* spp., *Fusobacterium* spp. and various Gram-positive cocci (O'Hara and Shanahan, 2006). The interaction between gut bacteria and the host is a symbiotic relationship, where the host provides a nutrient-rich habitat and the bacteria can bring important benefits to the host's health (Shanahan, 2002), thus the gut microbiota has important and specific functions in both human health and disease (Guarner and Malagelada, 2003).

15.3.2 Diarrhoea

Acute diarrhoea is a serious cause of infant morbidity and mortality. Supplementation of food or oral administration with probiotic strains benefits antibiotic-associated or virus-associated diarrhoea (Table 15.2). Hotta and co-workers (1987) investigated the effects of administration of *Bifidobacterium* preparations and commercially available bifidus yoghurt on infantile intractable diarrhoea. Fifteen patients were treated and received antibiotic therapy. In most cases, an abnormal microbiota was observed: *Candida* or *Enterococcus* often predominated with a marked decrease of anaerobes and aerobes in the stool biota. In all patients, the stool frequency and appearance were dramatically improved within 3–7 days after oral administration of bifidus yoghurt enriched with bifidobacteria. The intestinal microflora of all subjects also became normal with a predominance of the resident *Bifidobacterium* or administered *B. breve*. Corrêa and co-workers (2005) also reported a significant reduction in the risk of gastrointestinal infections in the *B. lactis* and *Streptococcus thermophilus*-supplemented formula group compared with the control formula group. The supplementation of infant formula with *S. thermophilus* mixed with a strain of *B. bifidum* can reduce to one-fourth the incidence of acute diarrhoea and rotavirus shedding in infants admitted to hospital. Saavedra and co-workers (1994) conducted a double-blind, placebo-controlled trial, in which infants aged 5–24 months were randomized to receive a standard infant formula or the same formula supplemented with *B. bifidum* and *S. thermophilus.* These infants were admitted to a chronic medical care hospital. They reported that during the course of the study, 31% of control patients (who received the control formula) and 7% (who received the supplemented formula) developed diarrhoea. Moreover, meanwhile 39% of the control group shed rotavirus at some

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Table 15.2. Beneficial health effects of bifidobacteria.

Strains	Health benefits	References
Bifidobacterium breve	Immune modulation and stimulation, reduced symptoms of irritable bowel disease, reduced faecal butyric levels	Brigidi *et al*. (2001), Matsumoto *et al*. (2001), Hoarau *et al*. (2006), Wang *et al*. (2007), Latvala *et al*. (2008), Okada *et al*. (2009)
Bifidobacterium animalis	Increased IgA secretion	Bakker-Zierikzee *et al*. (2006)
Bifidobacterium longum BB536	Treatment of allergy, Crohn's disease	Takahashi *et al*. (2006), Isolauri and Salminen (2008), Steed *et al*. (2010)
Bifidobacterium lactis Bb12	Shortening the frequency of rotavirus and travellers' diarrhoea, inhibitory effect against *Helicobacter pylori*, reducing the numbers of enterobacteria and clostridia, increased total faecal IgA and anti-poliovirus IgA	Saavedra *et al*. (1994), Fukushima *et al*. (1998), Fukushima *et al*. (1999), Wang *et al*. (2004a), Mohan *et al*. (2006)
Bifidobacterium bifidum and *B. breve*	Ulcerative colitis	Ishikawa *et al*. (2003)
Bifidobacterium infantis 35624	Caused a significant reduction in a composite irritable bowel syndrome score	O'Mahony *et al*. (2005)
B. longum	Treatment of ulcerative colitis	Furrie *et al*. (2005), Fujimori *et al*. (2009)
B. bifidum BGN4 and *B. lactis* AD011 (probiotic mix with *Lactobacillus acidophilus*)	Prevents the development of eczema in infants at high risk of allergy during the first year of life	Kim *et al*. (2010)
B. bifidum	Role in toxin neutralization, thus reducing the incidence of antibiotic-associated diarrhoea	Plummer *et al*. (2004)
B. longum BB536	Inhibits 2-amino-3-methylimidazo[4,5-f] quinoline-induced intestinal, liver and mammary carcinogenesis	Reddy and Rivenson (1993)
B. longum	Inhibition of azoxymethane (AOM)-induced colon carcinogenesis, decrease in colonic mucosal cell proliferation, colonic mucosal and tumour ornithine decarboxylase activity and ras-p21 activities	Reddy (1998)

time during the study, whereas only 10% of those who received the supplemented formula shed rotavirus.

15.3.3 Inflammatory bowel diseases

Irritable bowel syndrome (IBS) is a functional bowel disorder characterized by abdominal pain, bloating and disordered defaecation, which may take the form of constipation, diarrhoea or a mixture of the two. The probiotic preparation VSL-3 containing *B. infantis* Y1 and *B. breve* Y8 strains were administered in a clinical trial with ten patients who are suffering from IBS or functional diarrhoea. Changes in the composition and biochemistry of faecal microbiota were detected in the case of the administration of VSL-3, thus it significantly improved the clinical picture of patients. The probiotic

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treatment resulted in a significant increase in lactobacilli, bifidobacteria and *S. thermophilus*, while the cell count of *Bacteroides*, coliforms, enterococci and *Clostridium perfringens* did not change significantly. In addition, faecal β-galactosidase increased and urease activities decreased as a result of changes in the intestinal microbiota induced by VSL-3 administration (Brigidi *et al.*, 2001). The fermented dairy product containing probiotic *B. lactis* DN-173 010 together with two classical yoghurt starters resulted in improvements in objectively measured abdominal girth and gastrointestinal transit, as well as reduced symptomatology (Agrawal *et al.*, 2008). Fifty patients with diarrhoea-dominant IBS were randomized into placebo or probiotic mixture (*Lactobacillus acidophilus*, *Lactobacillus plantarum*, *Lactobacillus rhamnosus*, *B. breve*, *B. lactis*, *B. longum* and *S. thermophilus*) groups. The results showed that the proportion of adequate relief was consistently higher in the probiotics group than in the placebo group throughout the 10-week period ($P < 0.05$). The proportion of responders was significantly higher in the probiotics group than in the placebo group (48% versus 12%, $P = 0.01$). Stool consistency improved significantly in the probiotics group compared with the placebo group. Percentage changes in individual symptom scores were similar in the two groups, but IBS quality of life improvement tended to be higher in the probiotics group. The therapeutic effect of probiotics is associated with the stabilization of intestinal microbiota (Ki *et al.*, 2012).

Treatment with *B. infantis* 35624 was shown to cause a significant reduction in a composite IBS score, which included abdominal pain and bloating and difficulties with bowel movements. An immune-modulating role of this organism is suggested based on the normalization of the ratio of an anti-inflammatory to a proinflammatory cytokine (interleukin (IL)-10 to IL-12) (O'Mahony *et al.*, 2005).

15.3.4 Allergenic diseases

The prevention of asthma in infants is possible by modulating gut microbiota with probiotics and prebiotics. Generally, infants with atopic dermatitis have a high risk of developing asthma. Taniuchi and co-workers (2005) investigated 17 infants with cow's milk hypersensitivity with atopic dermatitis, who had less than 30% *Bifidobacterium* in their intestinal microbiota. The proportion of *Bifidobacterium* in the faecal microbiota increased while the proportion of aerobic bacteria decreased after 3 months of the administration, and was associated with a significant improvement of allergic symptoms compared with the beginning of the study. In the control group, there were no significant changes to the overall faecal microbiota and total allergic score during the entire study period. Van der Aa and co-workers (2011) suggested that a synbiotic mixture, a combination of probiotics and prebiotics, prevents asthma-like symptoms in infants with atopic dermatitis. They investigated the effect of early intervention with synbiotics on the prevalence of it. The applied synbiotic contained a combination of *B. breve* M-16V and a specific mixture of 90% short-chain galacto-oligosaccharides and 10% long-chain fructo-oligosaccharides (Immunofortis®). In a double-blind, placebo-controlled multicentre trial 90 infants with atopic dermatitis, age < 7 months were randomized during 12 weeks. They found that significantly fewer children in the synbiotic than in the placebo group had started to use asthma medication after baseline (5.6% versus 25.6%, absolute risk reduction 20.1% with 95% confidence intervals 35.7% to 4.5%). Total IgE levels did not differ between the two groups. Earlier Kukkonen and co-workers (2007) tested the effect of a mixture of four probiotic bacterial strains (*L. rhamnosus* GG (ATCC 53103), *L. rhamnosus* LC705 (DSM 7061), *B. breve* Bb99 (DSM 13692) and *Propionibacterium freudenreichii* ssp. *shermanii* JS (DSM 7076)) along with prebiotic galacto-oligosaccharides in preventing allergic diseases. They found that no effect on the incidence of allergic diseases by age 2 years was detected in the case of probiotic treatment. The only tendancy was a reduction in IgE-associated (atopic) diseases and asthma, especially atopic asthma, was prevented. The same trial was used by Wickens *et al.* and the Probiotic Study Group (2008) and they found that supplementation with *L. rhamnosus*, but not *B. animalis* ssp. *lactis*, substantially reduced the cumulative prevalence of eczema, but not atopy, by 2 years.

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The mechanism of improvement of the allergic symptoms by the administration of *Bifidobacterium* is thought to be as follows:

1. Lipoteichoic acid in the cell wall of Gram-positive bacteria such as *Bifidobacterium* binds to Toll-like receptor 2 on the antigen-presenting cells, which produce IL-12 to activate the type 1 $CD4^+$ lymphocytes (Th1) (Cleveland *et al.*, 1996).
2. *Bifidobacterium* may regulate the Th1/Th2 balance through regulatory T-cells.
3. Administration of probiotics is likely to suppress the increase in the gut permeability induced by milk allergy (Isolauri *et al.*, 1993).

15.3.5 Cancer prevention

The potential ability of bifidobacteria to prevent cancers of the gut – that are prevalent and believed to have both genetic and dietary causes – has been studied by some groups. Reddy and co-workers (1997) reported that the feeding of lyophilized cultures of *B. longum* significantly inhibited the formation of the aberrant crypt foci (ACF) (53%) and crypt multiplicity in the colon of rats. Combined administration of the *Bifidobacterium* and inulin resulted in more potent inhibition of colonic ACF than administration of the two separately, achieving 80% inhibition of small ACF (Rowland *et al.*, 1998). The ACF would be an early preneoplastic marker of malignant potential in the process of colon carcinogenesis (Kulkarni and Reddy, 1994). In addition, the pH-lowering effect of bifidobacteria in the colon subsequently inhibited the growth of *Escherichia coli* and clostridia. A decrease in growth of such pathogenic microorganisms may also produce the modulation of bacterial enzymes such as β-glucuronidase that can convert procarcinogens to proximate carcinogens (Liong, 2008).

Chiang and co-workers (2000) carried out a double-blind, three-stage before-and-after intervention trial with 50 Taiwanese citizens in order to define the cellular basis for immune enhancement by a probiotic bacteria strain (*B. lactis* HN019) as well as to determine the effects of oligosaccharide-enriched low-fat milk on immune enhancement. No significant effects on immune responses were showed in group 1 (who received only low-fat milk). The intervention with probiotic bacteria (group 2) significantly enhanced polymorphonuclear neutrophil (PMN) cell phagocytosis and natural killer (NK) cell tumour killing activity. In the group 3, who consumed *B. lactis* in an oligosaccharide-rich substrate, lactose-hydrolysed low-fat milk, increases in PMN and NK cell activity were greatest (Chiang *et al.*, 2000). The effects of prolonged ingestion of *Bifidobacterium* sp. fermented milk (BFM) with or without inulin on faecal bifidobacteria and some bacterial enzymatic activities were investigated in healthy humans (Bouhnik *et al.*, 1996). Twelve volunteers were randomly divided into two groups. During the ingestion period, they received BFM in association with either 18 g/day inulin or placebo for 12 days. The administration of BFM with placebo led to an increase in total bifidobacteria (indigenous and exogenous) and exogenous bifidobacteria and a decrease in β-glucuronidase activity. Simultaneous administration of BFM and inulin led to an increase in total bifidobacteria and exogenous bifidobacteria, but had no effect on β-glucuronidase activity. Rowland and co-workers (1998), however, found that consumption of diets containing *B. longum* and inulin were also associated with decreases in β-glucuronidase activity and ammonia concentration in the caecal contents. Both these factors have been associated with carcinogenesis of the colon in experimental animal models. Administrated alone or with inulin, BFM did not change faecal total anaerobe counts, pH, nitrate reductase, nitroreductase and azoreductase activities (Bouhnik *et al.*, 1996). The synbiotic combination of resistant starch and *B. lactis* significantly protected against the development of colorectal cancer in the rat-azoxymethane model beyond the benefit of either agent alone (Leu *et al.*, 2010). Unfortunately, most of the studies that have suggested a reduction of carcinogen-induced cancerous cells are carried out in mice, thus human trials are needed to provide evidence of a protective effect of this type of supplementation (Lee and O'Sullivan, 2010).

15.3.6 *Helicobacter pylori* infection

Probiotic bifidobacteria have the potential to inhibit the growth of both antibiotic-sensitive and antibiotic-resistant *H. pylori* strains through the synthesis of antimicrobial peptides. Collado and co-workers (2005) found that six of the

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24 different *Bifidobacterium* strains they tested were shown to inhibit *H. pylori*. These antagonistic effects were due to compouds that are sensitive to protease and resistant to heating at 100°C for 10 min.

15.3.7 Lactose intolerance

Lactose intolerance is the inability to digest lactose, a sugar found in milk and to a lesser extent in milk-derived dairy products. Chemically, lactose is a disaccharide consisting of one glucose and one galactose connected to each other through a β-galactosidic bond. Hydrolysis of lactose in the digestive system of the human requires lactase activity and there may be insufficient levels of this enzyme in lactose-intolerant individuals. In this group, after consuming significant amounts of lactose some symptoms arise including abdominal bloating and cramps, flatulence, diarrhoea and nausea. Some studies have produced evidence that milk consumption by lactose-intolerant individuals causes inflammatory bowel disease (Joachim, 1999; Vesa *et al.*, 2000). Thus, the key factor to prevent these diseases should be colonic metabolism of lactose. The milk can be treated by exogenous lactase to remove the lactose or it can be fermented by yoghurt cultures in combination with probiotic bacteria that have endogenous lactase activity. He and co-workers (2008) studied the effects of supplementation of *B. longum* and a yoghurt-enriched with *B. animalis* on the composition and metabolic activities of the colonic microbiota as well as the ability to alleviate symptoms in lactose-intolerant individuals. It was worth noting that *B. animalis* was not present in the colon of the investigated group. He *et al.*, (2008) found that supplementation modified the composition and metabolic activities of the colonic microbiota, but this was definitely not due to the endogenous small-intestinal lactase activity. The lactase activity of probiotic bifidobacteria can be induced separately by growth in medium containing lactose. Jiang and co-workers (1996) observed that higher lactase activity was assayed in milk containing *Bifidobacterium longum* B6 cultured with lactose than lactose and glucose. They also found that consumption of milk containing B6 grown with lactose resulted in significantly less hydrogen production and flatulence than control samples. Thus, milk containing *B. longum* B6 might reduce symptoms from lactose malabsorption.

15.4 Bifidobacteria in Food Products

15.4.1 Dairy-based matrices

Dairy foods have been the first commercialized probiotic food products and these products are still consumed in large quantities throughout the world. Nutritionally, milk and milk products are highly beneficial because of their composition. It is well known that dairy foods in both fermented and non-fermented forms have played important roles in the diet of humans worldwide for thousands of years, even before the discovery of microorganisms and LAB. It is reported that written records of using fermented milk for various gastrointestinal infections goes back to 76 BC (Jankovic *et al.*, 2010). Moreover, dairy foods have proved to be very good carriers to deliver probiotic strains to the host due to their inherent properties and storage temperature (Gardiner *et al.*, 1999; Ross *et al.*, 2002; Phillips *et al.*, 2006; Saarela *et al.*, 2006). By storage at refrigerated temperatures, dairy food matrices have many technical advantages over others. Currently hundreds of probiotic dairy products are manufactured and consumed around the world include sour and fresh milk, yoghurt and cheese (Awaisheh *et al.*, 2005; Kailasapathy and Phillips, 2008; Cruz *et al.*, 2009; Awaisheh, 2011). In retail stores these products are presented on the shelves with different brand names such as Acidophilus milk, Acidophilus buttermilk, Actimel, Bifidus milk, Sweet acidophilus milk, Nu-Trish A/B, Yakult, Procult drink, Gaio and ProViva (Özer and Kirmaci, 2010). Due to technological and functional characteristics, *Lactobacillus* and *Bifidobacterium* strains have been used predominantly as commercial probiotics in the food and pharmaceutical industry. Some commercial dairy probiotics are summarized in Table 15.3.

Milk

Generally, bifidobacteria are unable to grow (or grow very slowly) in pure milk (Klaver *et al.*,

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Table 15.3. Some probiotic products in the market containing *Bifidobacterium.*

Product	Strains	Manufacturer	Category	References
Bio yoghurt	*Lactobacillus acidophilus*, *Lactobacillus casei*, *Lactobacillus gasseri*, *Lactobacillus rhamnosus*, *Lactobacillus reuteri*, *Bifidobacterium bifidum*, *Bifidobacterium animalis*, *Bifidobacterium infantis*, *Bifidobacterium longum*		Yoghurt	Bio-Foods Ltd (2014)
Bifidus milk	*B. longum*, *B. bifidum*		Milk	Arunachalam (1999), Gürakan *et al*. (2009)
AB milk, *Acidophilus bifidus* milk	*L. acidophilus*, *B. bifidum*		Milk	Heller, (2001), Özer and Kirmaci (2009)
AB yoghurt	*L. acidophilus*, a species of *Bifidobacterium*, *Streptococcus thermophilus*, *Lactobacillus bulgaricus*		Yoghurt	Lourens-Hattingh and Viljoen (2001)
Bifidus yoghurt	*B. bifidum*, *B. longum*, *S. thermophilus*, *Lactobacillus delbrueckii* ssp. *bulgaricus*, *L. acidophilus*	Morinaga Milk Industry Company	Yoghurt	Boylston *et al*. (2004)
Bifighurt	*B. longum*, *S. thermophilus*			Gürakan *et al*. (2009), Özer and Kirmaci (2009)
Activia	*B. animalis* DN173010	Danone	Yoghurt	Danone (2014)
Parmalat Len6	*L. acidophilus*, *L. casei*, *B. animalis* ssp. *lactis*	Parmalat	Yoghurt	Parmalat (2014)
BioBest	*L. acidophilus* NCFM, *Bifidobacterium lactis* Bi-07	Parmalat	Yoghurt	Parmalat (2014)
Activia (stirred)	*B. animalis* DN173010	Danone	Yoghurt	Danone (2014)
Activia (drinkable)	*B. animalis* DN173010	Danone	Yoghurt	Danone (2014)
Lective (stirred)	*B. animalis* ssp. *lactis*	Vigor	Yoghurt	Vigor (2014)
Lective (drinkable)	*B. animalis* ssp. *lactis*	Vigor	Yoghurt	Vigor (2014)
Biofibras (stirred)	*B. animalis* ssp. *lactis*, *L. acidophilus*	Batavo	Yoghurt	Batavo (2014)
Biofibras (drinkable)	*B. animalis* ssp. *lactis*, *L. acidophilus*	Batavo	Yoghurt	Batavo (2014)
Nesvita (stirred)	*B. animalis* ssp. *lactis*	Nestlé	Yoghurt	Nestlé (2014)
Nesvita (drinkable)	*B. animalis* ssp. *lactis*	Nestlé	Yoghurt	Nestlé (2014)
Cheddar-like cheese	*B. infantis*		Cheese	Daigle *et al*. (1999)
Ice cream	*L. acidophilus* LA-5, *B. animalis* ssp. *lactis* BB-12, *Propionibacterium jensenii* 702		Ice cream	Ranadheera *et al*. (2013)
Cheese	*B. bifidum* (or *B. longum*), *S. thermophilus*, *L. delbrueckii* ssp. *bulgaricus*		Cheese	Corbo *et al*. (2001)

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1993) compared with other LAB used frequently in fermented dairy products (Beerens *et al.*, 2000). This may be due to the low concentration of free amino acids and small peptides in milk. *Bifidobacterium* spp. are known to lack proteolytic activity and grow insufficiently on this type of substrate, thus production of bifidus milk has to face this technological problem. However, some manufacturers have used different techniques to overcome it, such as the use of a higher amount of inoculum of bifidobacteria (Yakult Milk may apply this for production of bifidus milk) or the addition of growth-promoting factors as a nitrogen source (Gomes *et al.*, 1995; Beerens *et al.*, 2000). Bifidus milk was one of the first milk products containing probiotic bifidobacteria produced by the Morinaga Milk Industry Company in Japan in 1977 (Stanton *et al.*, 2003; Gürakan *et al.*, 2009). This product has a slightly acidic flavour because the ratio of lactic acid to acetic acid is about 2:3. The production protocol is quite simple. Briefly, the cow's milk is first concentrated to dry matter of about 150–200 g/kg and then treated by heat at 80–120°C for 5–30 min. After cooling to 37°C, the milk is inoculated with a mixed culture of *B. bifidum* and *B. longum* at a level of 10% and kept to ferment until a pH of approximately 4.5 is obtained. By the end of fermentation a bacterial cell concentration of about 10^8–10^9 c.f.u./ml is reached (Özer and Kirmaci, 2009). The bifidus milk is obtained by cooling to 10°C and packaged (Gürakan *et al.*, 2009). Another milk product containing bifidobacteria is AB milk (acidophilus bifidus milk). Two bacteria (*L. acidophilus* and *B. bifidum*) are applied for production of this product, thus it has better flavour characteristics due to presence of LAB. Synergistic and growth-promoting reactions between *L. acidophilus* and *B. bifidum* in milk are known to occur, providing the necessary growth stimulants for bifidobacteria (Hansen, 1985). The technology of production is similar to that of bifidus milk described above. This milk product is unfermented and also contains about 10^8–10^9 c.f.u./ml (Özer and Kirmaci, 2009).

Yoghurt

One of the popular fermented milk products are yoghurt types, including yoghurt and kefir that have been known for thousands of years (since AD 800). As it is rich in valuable nutrient compounds (e.g. protein, vitamins, riboflavins and calcium) yoghurt is considered to be a better food product than milk. For the last few decades, yoghurt was widely selected as the matrix to carry probiotic bacteria (Prajapati and Nair, 2008), thus many probiotic yoghurt products can be found on the shelves of food stores.

Dairy yoghurt is produced using *Lactobacillus delbrueckii* ssp. *bulgaricus* and *S. thermophilus* bacteria that are able to ferment lactose in milk to produce lactic acid. On the one hand, the lactic acid acts on the milk protein to provide good characteristics in the form of texture and taste for yoghurt products; on the other hand, it makes the pH of yoghurts drop into a range between 3.7 and 4.3 (Hamann and Marth, 1983). Meanwhile this pH value is good for probiotic *Lactobacillus* spp., whereas it is one of the major barriers for the incorporation of bifidobacteria into yoghurt products. Generally, bifidobacteria grow well at a pH between 6.5 and 7.0 and they are inhibited at pH values below 5.0 and sensitive to pH values below 4.6 (Scardovi, 1986; Lourens-Hattingh and Viljoen, 2001). For industrial application, the pH value of yoghurt should be maintained above 4.6 when incorporating bifidobacteria (Tamime and Robinson, 1988; Laroia and Martin, 1990; Modler *et al.*, 1990; Vinderola *et al.*, 2002a). Recently, *B. bifidum*, *B. breve*, *B. longum* and *B. animalis* are commonly used for the production of probiotic yoghurt in combination with other LAB. *B. animalis* ssp. *lactis* Bb-12 has been found to have remarkable technological properties such as surviving passage through the gastrointestinal tract and the capability of adhering extraordinarily to enterocytes (Haschke *et al.*, 1998). Moreover, it does not have adverse effects on the flavour, appearance or taste of the foods and the organism remains viable in high concentrations until the probiotic product is consumed (Möller and de Vrese, 2004).

In 1979, bifidus yoghurt was launched by the Morinaga Milk Industry Company in Japan (Stanton *et al.*, 2003). It was produced by a starter culture of *B. bifidum* or *B. longum* in combination with yoghurt cultures (*S. thermophilus* and *L. delbrueckii* ssp. *bulgaricus*) with or without *L. acidophilus*. For yoghurt manufacture, the solids content of the milk is increased to 16%, with 1–5% being fat and 11–14% being non-fat solids. After the solids composition is adjusted, the

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milk goes for pasteurization by heating. Then it is cooled to 41–46°C and inoculated with yoghurt cultures of *S. thermophilus* and *L. bulgaricus* combined with probiotic bacteria (LAB and/or bifidobacteria) at a concentration of about 2–4%. The fermentation process runs at 40–44°C for 4–5 h. During fermentation, lactic acid is produced from lactose by the yoghurt bacteria, the population of which increases from 100-fold to 10,000-fold to a final concentration of approximately 10^9 c.f.u./ml. The reduction in pH, due to the production of lactic acid, causes a destabilization of the micellar casein at a pH of 5.1–5.2, with complete coagulation occurring around pH 4.6. At the desired final pH, the coagulated milk is cooled quickly to 4–10°C to slow down the fermentation process. In the case of Bifighurt a mixed starter culture consisting of *B. longum* CKL 1969 or DSM2054 (a slime producer) and *S. thermophilus* (Arunachalam, 1999) is applied. The final product has a mild acidic flavour and contains L-(+)-lactic acid at a level of 95% as well as a slimy texture (Gürakan *et al.*, 2009).

Strains of *L. acidophilus* and species of *Bifidobacterium* (known as AB cultures) in combination with conventional yoghurt organisms (*S. thermophilus* and *L. bulgaricus*) are frequently used to produce bio-yoghurt that is quite a popular food product worldwide. The production process looks like the traditional one for yoghurt, where the pasteurized milk is firstly inoculated with the conventional starter culture under certain conditions. After the fermentation and cooling down to 4°C, the probiotic cultures can be added into the product, then it is ready for packing (Lourens-Hattingh and Viljoen, 2001).

Recently, fruit-added fermented milk products share almost half of the yoghurt-like products market (Santo *et al.*, 2011). However, due to antimicrobial activities of fruits, the addition of them into yoghurt may cause a decline of probiotic bacteria.

Cheese

Compared with milk or yoghurts, cheeses have numerous markedly suitable characteristics to support the growth and viability of bifidobacteria, thus they should be ideal alternative carriers for delivering probiotics to the host system. The processing steps and conditions used in cheese making vary from type to type. Generally, the processing of cheese starts with the addition of rennet and LAB to milk to facilitate the precipitation of the casein molecules and concentration of milk solids (Wikipedia, 2015). Then the precipitated curds are moulded, pressed and salted. The next step is ripening that plays an important role in the formation of the characteristics of cheese (flavours and texture) through the lipolytic and proteolytic activity of the bacteria. Thus the microorganisms used, as well as the specific processing treatments, are key factors in making the different types of cheese. By the end, the pH of cheese ranges from 4.8 to 5.6 and is higher than the pH of fermented milks or yoghurt (pH 3.7–4.3). This pH is suitable for the long-term survival even of the acid-sensitive bifidobacteria (Boylston *et al.*, 2004). Additionally, the LAB used in cheese making can support the bifidobacteria through altering the pH, growth promoters and inhibitors and the oxygen content of the cheese (Okonogi *et al.*, 1984; Tempel *et al.*, 2002). Furthermore, due to a relatively high fat content, the cheese matrix can act as a protector for the probiotic bifidobacteria and help them to pass through the gastrointestinal tract (Gardiner *et al.*, 1998; Corbo *et al.*, 2001; Vinderola *et al.*, 2002b).

Some *Bifidobacterium* strains have been successfully blended into different cheeses (Boylston *et al.*, 2004; Escobar *et al.*, 2012; Lollo *et al.*, 2012; Albenzio *et al.*, 2013). Probiotic Cheddar-like cheeses were produced with cream enriched with native phosphocaseinate retentate and fermented by *Lactobacillus lactis* ssp. *lactis* and/or *L. lactis* ssp. *cremoris* in combination with *B. infantis* (Daigle *et al.*, 1999). The viability of bifidobacteria was quite good (above 3×10^6 c.f.u./g of cheese remained after keeping at 4°C for 84 days in vacuum-sealed bags). There were no significant differences between cheeses produced with or without bifidobacteria in terms of fat, protein, moisture, salt, ash and pH. Dinakar and Mistry (1994) studied the growth and viability of *B. bifidum* (ATCC 15696) in Cheddar cheese and reported that treatments did not affect cheese composition and bifidobacteria remained viable and increased in numbers in the cheese during 24 weeks of storage, but did not affect the flavour, flavour intensity, texture or appearance of the cheese compared with that of the control. Typical Iranian white-brined cheese was made

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and studied by Ghoddusio and Robinson (1996) using full-cream pasteurized milk and inoculated with *B. bifidum* or *B. adolescentis* (1%) and 1% of either a mixture of yoghurt cultures (*S. thermophilus* and *L. delbruekii* ssp. *bulgaricus*) or a mixture of cheese cultures (*L. lactis* ssp. *lactis* and *L. lactis* ssp. *cremoris*). *B. adolescentis* showed poor survival in the presence of both yoghurt and cheese cultures. The cheese made with yoghurt culture and *B. bifidum* showed acceptable survival of bifidobacteria up to 60 days after the manufacture. Moreover, *B. bifidum* was more tolerant of the salt and acidity associated with the white-brined cheese than *B. adolescentis*. Argentinian fresco cheese was produced with bifidobacteria (five *Bifidobacterium* strains including two strains of *B. bifidum* and two strains of *B. longum*) and *L. acidophilus* (two strains) and/or *Lactobacillus casei* (Vinderola *et al*., 2000) and investigated as well as gouda which was manufactured with bifidobacteria and *L. acidophilus* (Gomes *et al*., 1995). Murad and co-workers (1998) reported that more than 10^8 c.f.u./g bifidobacterial cell concentration was detected in the bifidus kariesh cheese after a 10-day ripening period. Furthermore, it received higher sensory scores than the control cheese preparations, meaning the introduction of bifidobacteria resulted in cheeses with new dietetic and probiotic characteristics and improved organoleptic properties. *B. bifidum* or *B. longum* was successfully incorporated into Canestrato Pugliese hard cheese (Corbo *et al*., 2001). Recently, Albenzio and co-workers (2013) investigated Scamorza ewe's milk cheese produced by incorporating probiotic strains (a mix of *B. longum* and *B. lactis* or *L. acidophilus*) into the cheese matrix. Probiotic cell recovery in the cheese was 7.55 ± 0.07 log c.f.u./g and 9.09 ± 0.04 log c.f.u./g in S-LA (*L. acidophilus*) and S-BB (bifidobacteria) cheese, respectively. The matured Scamorza cheese containing the mix of *B. longum* and *B. lactis* was characterized by significantly higher levels of Gln, Ser, Arg, Ile and Leu, whereas cheese containing *L. acidophilus* was characterized by higher levels of Tyr and Met.

Overall, the cheese matrix has potential to be a proper carrier for delivery of probiotic bifidobacteria to the host system. The success of the incorporation of bifidobacteria into cheeses depends on many factors, but two strains, *B. bifidum* and *B. longum*, demonstrated good viability during the processing and storage of the cheese, thus they could be preferred ones for the production of cheese dairy products.

Ice cream

Ice cream is one of the most popular dairy products, enjoyed by consumers of all ages. Although it is regarded as a summer treat more and more people like ice cream's refreshing flavour throughout the year. Technically, the ice cream mixture is made up of liquid milk (approximately 3.0% fat), milk cream (approximately 40% fat), about 11% non-fat milk solids, sweeteners, stabilizer/emulsifier and flavouring agents (Prasanna *et al*., 2014). Products such as some puddings, fruit and nut ice creams, plain ice cream, frozen yoghurt and reduced-fat, low-fat and non-fat ice creams should be classified in this category (Marshall and Arbuckle, 1996).

Production of ice cream includes the following steps. The liquid milk is initially heated to 40–45°C and mixed with the solid ingredients. Then, the mixture is heated and homogenized at around 75°C. After homogenization the ice cream is pasteurized at 85°C for 30 min and cooled to 2°C. The mix is then aged for at least 4 h but usually overnight. This allows time for the fat to cool down and crystallize, and for the proteins and polysaccharides to fully hydrate. Flavouring is added after ageing and before the freezing process. The temperature inside freezers is generally kept at −40°C. Before storage and shipping, the ice cream must be hardened to a temperature of −23°C (Marshall *et al*., 2003).

The final products have relatively high pH values (from 5.5 up to 6.5) and generally the lower acidity (high content of lactic acid) increases consumer acceptance of such products, especially by those who prefer mild products (Cruz *et al*., 2009). However, these relatively high pH values could also favour increased survival of probiotic cultures during storage. Ice cream has many other advanced properties that mean that it may be an ideal matrix for carrying probiotic bacteria (bifidobacteria), for example it is kept at low temperature because it is a frozen product, it has a low milk protein and fat content, and it may be low or lactose free (Cruz *et al*., 2009). As a general rule, the addition of probiotic strains into a food matrix implies the need to assure the viability of the probiotic culture at

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high levels during the storage period (Stanton *et al.*, 2003) as well as ensuring it does not dramatically change the product's overall quality, such as the melting rate and the sensory features (Cruz *et al.*, 2009).

In evaluation of the processing steps, probiotic bacteria may be incorporated into ice creams in two ways: (i) direct addition to the product during its manufacture; or (ii) production of probiotic/bifidus yoghurt (as described in the subsection 'Yoghurt') and then freezing. In the first case classical ice cream is made and the *Bifidobacterium* culture or fermented milk products are added to the mixture after the ageing step (Magarinos *et al.*, 2007). Ice cream containing probiotic bacteria was produced by mixing fortified milk fermented with probiotic strains *L. acidophilus* LA-14 and *B. lactis* BL-01 by Akin and co-workers (2007); they found that the addition of inulin stimulated the growth of probiotic bacteria and improved the viability of these organisms. In another example, *L. acidophilus* LA-5, *B. animalis* ssp. *lactis* Bb-12 and a novel probiotic *Propionibacterium jensenii* 702A were successfully used for production of chocolate-flavoured probiotic ice cream based on goat's milk (Ranadheera *et al.*, 2013).

The incorporation of bifidobacteria into ice cream, in particular, may face some intrinsic technological problems such as the beating step and storage under freezing temperatures. Not all probiotic bacteria prefer freezing temperatures during the storage period. In addition, flavouring ingredients such as fruit pulp/juice could also improve the final quality of the product (Cruz *et al.*, 2009).

15.4.2 Non-dairy based matrices

Fruits and vegetables

For historical and technological reasons most probiotic foods are based on dairy products. Unfortunately, this may cause inconvenience for some consumers who do not tolerate lactose, or who are allergic to milk proteins or are simply vegetarian (Heenan *et al.*, 2004). According to the National Institute of Diabetes and Digestive and Kidney Diseases of the US National Institutes of Health, about 75% of the world population is lactose intolerant, whereas approximately 2.5% of children younger than 3 years of age are allergic to milk (mainly cow's milk) (Brill, 2008). The prevalence of milk allergy in adults is between 0.1% and 0.5% (Høst, 2002). Since fruits and vegetables already contain beneficial nutrients such as minerals, vitamins, dietary fibres and antioxidants (Yoon *et al.*, 2004) while lacking the dairy allergens, they may serve as ideal food matrices for carrying probiotic bacteria. Furthermore, fruit juices have pleasing taste profiles to all age groups and they are perceived as being healthy and refreshing. Thus, new non-dairy probiotic food products have recently been developed (Table 15.4), to meet the consumer's expectancy for health (Luckow and Delahunty, 2004).

Many studies have been carried out to develop novel probiotic fruit or vegetable products containing bifidobacteria (Lee *et al.*, 1999; Savard *et al.*, 2003; Wang *et al.*, 2009). The suitability of carrot juice as a raw material for the production of probiotic food with *Bifidobacterium* strains was investigated by Kun and co-workers (2008). They found that bifidobacteria were capable of growing and could perform biochemical activities in carrot juice without any nutrient supplementation, but a 15–45% decrease in the carotenoid contents was experienced after fermentation (Kun *et al.*, 2008). Wang and co-workers (2009) also studied the probiotic potential of noni juice fermented with LAB (*L. plantarum* and *L. casei*) and bifidobacteria (*B. longum*). These bacterial cultures grew well in noni juice at 30°C, and the viable cell count was nearly 10^9 c.f.u./ml after 48 h of fermentation. Both *B. longum* and *L. plantarum* retained viability at low pH and under highly acidic conditions in fermented noni juice during cold storage at 4°C (Wang *et al.*, 2009). Saarela and co-workers (2006) studied whether different substrates affect the cultivable stability of freeze-dried *B. animalis* ssp. *lactis* preparations during storage in milk and fruit juice. It was reported that cells produced in different ways had comparable stability in milk, whereas in juice, sucrose-protected cells survived better than reconstituted skimmed milk-protected cells (Saarela *et al.*, 2006). The *B. longum* JK-2 strain showed the highest viability maintaining a population of 10^6 c.f.u./ml after 1 week at 4°C (Lee *et al.*, 1999). Additionally, carrots and red beet were also evaluated as potential substrates for the production of probiotic juices by some species of LAB and bifidobacteria (Buruleanu *et al.*, 2009).

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Table 15.4. Some non-dairy probiotic products recently developed.

Category	Product	Species	Reference
Fruit and vegetable based	Peanut milk	*Bifidobacterium pseudocatenulatum G4*	Barka *et al*. (2009)
	Orange juice, pineapple juice, cranberry juice	*Bifidobacterium animalis* ssp. *lactis* Bb-12	Sheehan *et al*. (2007)
	Orange, grape and passion fruit mix	*B. animalis* ssp. *lactis* Bb-12	Saarela *et al*. (2006)
	Noni juice	*Bifidobacterium longum*	Wang *et al*. (2009)
	Naked juice	*Bifidobacterium*	Naked Juice Company (2015)
Soy based	Soymilk	*Bifidobacterium infantis* CCRC 14633	Chou and Hou (2000)
	Soymilk	*Bifidobacterium breve* Yakult strain	Shimakawa *et al*. (2003)
	Non-fermented, frozen soy dessert	*Bifidobacterium lactis* BBDB2, *B. lactis* BB-12	Heenan *et al*. (2005)
	Soymilk	*B. lactis* LAFTI® B94, *B. longum* Bl536	Donkor *et al*. (2007)
Cereal based	Traditional fermented mahewu, non-alcoholic cereal-based beverages maize (corn), millet and sorghum	*B. lactis* DSM 10140	McMaster *et al*. (2005)
	Milk- and water-based puddings containing both maize and rice flour	*B. animalis* Bb12	Helland *et al*. (2005)
	Oat-based product	*Bifidobacterium bifidum* DSM 20456	Martensson *et al*. (2002)

In Asia, soybean-based food products are very popular due to their availability and the fact that they are rich in high quality proteins, essential amino acids, calcium, phosphorus, iron, vitamins (especially A and B) and vegetable oil. In the last few decades soymilk has caught the attention of researchers and innovators in order to produce probiotics (Champagne and Gardner, 2008) because of some health benefits including prevention of chronic diseases, menopausal disorders, cancer, atherosclerosis and osteoporosis (Shimakawa *et al*., 2003). However, consumption of soymilk is hindered because of the presence of unpleasant off-flavours carried over from soybean as well as various oligosaccharides (soy-oligosaccharides) including raffinose and stachyose that may cause consumers to suffer a gastrointestinal discomfort known as flatulence. These matters can be addressed and eliminated by treatment using fermentation by probiotic bacteria (Song *et al*., 2012). Soymilk itself has been reported to support the growth of bifidobacteria, but it is not as good as reconstituted skimmed milk (Farnworth *et al*., 2007). Fermented soy yoghurt with a good sensory acceptance (82.5%) by potential consumers was produced and investigated by Marinho and co-workers (1994). Other products that also presented high acceptance indices include: (i) iron-fortified soy yoghurt (Umbelino *et al*., 2001); (ii) fermented water-soluble soy extract with bifidobacteria (Shimakawa *et al*., 2003); and (iii) a soy yoghurt product supplemented with oligofructose and inulin (Hauly *et al*., 2005).

Cereals

Due to the presence of non-digestible components, cereals themselves may have prebiotic potential

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and they are also good substrates for the growth of probiotic strains (Charalampopoulos *et al.*, 2002; Salovaara and Gänzle, 2011). Most of the commercially available cereal-based probiotic products are fermented with LAB often in combination with yeasts or moulds (Blandino *et al.*, 2003; Champagne, 2009), which is not favourable for strictly anaerobic bifidobacteria. However, some studies have been carried out to overcome this problem. Rozada-Sánchez and co-workers (2008) studied the growth and metabolic activity of four different *Bifidobacterium* spp. (*B. adolescentis* NCIMB 702204, *B. infantis* NCIMB 702205, *B. breve* NCIMB 702257 and *B. longum* NCIMB 702259) in a malt hydrolisate for the production of a potentially probiotic malt-based beverage. They reported that malt hydrolysate was a good growth substrate for all the investigated *Bifidobacterium* strains and higher molarities of lactic acid over acetic acid were detected in the cases of *B. breve*, *B. infantis* and *B. adolescentis* (good organoleptical properties). Appropriate growth of *Lactobacillus reuteri*, *L. acidophilus* and *B. bifidum* in oat-based substrates was also studied (Martensson *et al.*, 2002). Blandino and co-workers (2003) reported that Yosa, a new oat-based fermented (LAB and bifidobacteria) food had a flavour similar to yoghurt or porridge. Therefore, it is considered a healthy food. Another idea to incorporate bifidobacteria into cereal-based food matrices is a special direct liquid inoculation system in that the probiotic bacteria can be directly added to the finished food product. Use of this technique results in a higher number of viable microorganisms, and thus increases its functionality (Prado *et al.*, 2008).

15.5 Concluding Remarks

Bifidobacteria are believed to play an important role in gut homeostasis and normal development, and thus enhance colon health and prevent diseases. Hence in the past few decades, intensive research into probiotic foods, especially with those containing LAB and bifidobacteria, has been carried out worldwide. Some scientific and technological fields still need much more attention from researchers and developers to explore and realize their potential, including the following:

- More clinical evidence is needed to demonstrate the beneficial effects of probiotics as in the last few years there has been increasing sceptism in society about functional foods, doubting these beneficial effects.
- More scientific data is required on the mechanisms of probiotic action and understanding how the human body's immune system can differentiate between beneficial and pathogenic organisms.
- New species and strains of bifidobacteria with different technological and specific advantages (e.g. oxygen and acid tolerance to tolerate gastrointestinal transit, production of bacteriocin and exo-polysaccharides) need to be isolated and identified for future developments.
- There needs to be more research into fermentation using mixed probiotic cultures (LAB and bifidobacteria) in different combinations with other additive compounds in order to design symbiotic food products with organoleptic characteristics that are acceptable to consumers. Such mixed cultures and additives eliminate the problem caused by the metabolism of carbohydrates by bifidobacteria producing acetic acid and lactic acid in a ratio of 3:2 which results in the presence of too much acetic acid in the food matrix causing an odour that consumers do not like.

Hence there are considerable technological challenges that need to be addressed for the future design and application of new synbiotic food products.

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16 Probiotics and Dental Caries: a Recent Outlook on Conventional Therapy

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Abstract

In the oral cavity several hundred species of microbe reside harmoniously. However, sometimes microorganisms that can cause inflammation and infection and induce demineralization of dental enamel can flourish. Such infection can destroy the dentin and the connective tissue of the tooth. Remedies include restoration, endodontic therapy and removal of the damaged tooth. These methods are not conclusively proved to be the best methods for preventing new primary caries or for treating secondary caries. Use of probiotic *Lactobacillus* spp. and *Bifidobacterium* spp. in treating or preventing dental caries and other related problems can change the conventional therapy of drilling, filling and billing. The mechanism of action of probiotic bacteria in the oral cavity may be direct, for example by replacing the pathogenic flora by competing for the adhesion site, nutrients and growth requirements and producing antimicrobial agents, thereby inhibiting growth of the pathogen. Some indirect mechanisms of action include: (i) modulating the systemic immune function; (ii) the effect on local immunity; (iii) the effect on non-immunologic/non-specific defence mechanisms; (iv) prevention of plaque formation by neutralizing free electrons and production of antioxidants; and (v) modifying the surrounding environment.

16.1 Introduction

Dental disease remains a 'silent epidemic' in the world that threatens children and adults alike. In India it affects about 80% of the population (Sidhu, 2008). Caries and periodontal diseases are major public health problems that affect all countries across the globe (Caglar *et al.*, 2005). Fermentation of sugars by bacteria cause local damage to tooth enamel and dentin. Commonly known as 'dental plaque', oral microbial communities are one of the important complex bacterial floras related to the human body. So far, more than 700 different bacteria have been identified from the oral cavity of humans, and the majority of them are related to dental plaque (Aas *et al.*, 2005; Paster *et al.*, 2006). *Streptococcus mutans* is one of the most important enamel-degrading bacteria which is found in the mouth cavity and is responsible for caries (Marsh and Martin, 2000). These bacteria along with the polymers (glucans and levans) form a biofilm/dental plaque. *S. mutans* is considered to be part of the normal oral flora of humans (Lehner, 1992). Over the last few years several studies have suggested that probiotic bacteria can maintain gut health, and they may be helpful for oral health. The aim of this chapter is to examine

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possible potential mechanisms of probiotic bacteria in the prevention and healing of dental caries and to summarize the observed effects of probiotics with respect to oral health.

16.2 Dental Caries

Streptococci, enterococci and actinomycetes are found in abundance in the human mouth and are supposed to be the etiological agents of caries. They are carbohydrate users, but are not strictly acid tolerant. Severe changes in the mouth cavity may result in growth of numerous bacteria including *Streptococcus sorbrinus*, *S. mutans* and *Porphyromonas gingivalis* which are the primary cause of dental caries. *S. mutans* is the important cause of childhood dental caries (Becker *et al.*, 2002). *Enterococcus faecalis* is an opportunistic pathogen responsible for oral infection (Patidar *et al.*, 2011). In 10–20% of cases, *S. sobrinus* is to blame for caries development (Aas *et al.*, 2008). Dental caries or tooth decay is due to bacterial processes that damage the hard tooth structure. Bacterial metabolic activities cause prolonged plaque acidification which leads to permanent establishment of a cariogenic microflora and demineralization of the tooth. It is an irreversible process, as once damage is done it cannot be repaired.

Today caries are widely spread throughout the world (Dwivedi *et al.*, 2011). They are found universally, in all age groups, both sexes, irrespective of caste, creed or geographical location and with multifactorial causes. The prevalence of dental caries in the population in India has reached up to 80% in the last two decades (Sidhu, 2008). Three factors are solely responsible for caries: (i) susceptible host teeth; (ii) microorganisms in the dental plaque; and (iii) the substrate composition (e.g. composition of the diet). However, human defence mechanisms such as saliva, lysozyme, lactoferrin and secretory immunoglobulin A (SIgA) antibodies hinder the formation of plaque (Damle, 2000). The composition of the diet is an important factor in the aetiology of caries. A sugar-rich diet increases the possibility of caries. Minerals (e.g. calcium, phosphorus and fluorine), vitamins (A, D, K and B) and fats have an inhibitory action on plaque formation (Kennedy *et al.*, 1993).

There is a need to develop a potent topical antimicrobial agent that targets the prevention of microbes of teeth. Treatment of dental caries comprises removal of the decay by using operative procedures and restoration with fillings such as silver and gold, composite resin, glass ionomer cement, full metal or porcelain crowns (James *et al.*, 2001). In the advanced stages, when the pulp is damaged, endodontic treatment (or root canal treatment) is a must (Hargreaves, 2006). If there is extensive destruction, extraction of the tooth and replacement with an artificial prosthesis is an option (Smales and Gao, 2000).

Caries can be prevented by consuming milk which contains fluoride, water and salts. Toothpaste containing an adequate quantity of fluoride and intake of less sugary food can be used as prophylactic measures for caries. Probiotics are 'live microorganisms or their products which when administered in sufficient amount confer health benefits on the host'. Probiotics have been known to improve gastrointestinal health for quite some time and their popularity has prompted increased interest in the maintenance of oral health also. Most probiotics include lactobacilli or bifidobacteria and they are used in milk fermentation. Mechanisms by which probiotics benefit the host are unknown, but they modify pH, show antagonistic activity by producing antimicrobial compounds, compete for pathogen-binding and receptor sites, stimulate immunomodulatory cells and produce lactase (Bhushan and Chandra, 2010).

Acid is harmful to teeth, so care must be taken to avoid selecting strains with high fermentation capacity. Most probiotics in dairy form contain high levels of calcium, which reduces demineralization of teeth. It is possible that probiotics act as a biofilm to keep pathogens away and occupy a space that might otherwise to be occupied by a pathogen. Saraf *et al.* (2010) observed that probiotics stick to dental tissues to establish a cariostatic effect and thus form a biofilm to fight against cariogenic bacteria. Data on understanding the possible mechanisms of probiotic action in the oral cavity is still insufficient.

16.3 Potential of Probiotics for Prevention or Treatment of Dental Caries

The concept of probiotics as therapeutics was put forward by Lily and Stillwell in 1965 (Lilly

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and Stillwell, 1965). Probiotics (meaning 'for life') are microbes that include *Lactobacillus casei*, *Lactobacillus acidophilus*, *Lactobacillus sporogenes*, *Lactobacillus rhamnosus*, *Lactobacillus delbrukii*, *Lactobacillus plantarum* and *Bifidobacterium bifidum*, *Bifidobacterium infantis* and *Bifidobacterium longum*. Probiotics are reported to be involved in immunomodulation (Dietrich *et al.*, 2003), have anti-cancerous properties (Rafter, 2003), be antimicrobial (Jain *et al.*, 2009; Singh *et al.*, 2009) and have antitoxin (Chen *et al.*, 2006) and anti-allergic (Winkler *et al.*, 2007) properties. Probiotics maintain gut homeostasis (Bhardwaj, 2010), may be used as an oral vaccine (Amdekar *et al.*, 2010) and are modulators of Toll-like receptor (TLR)2 of the urinary tract (Amdekar *et al.*, 2011).

Lactobacillus spp. prevent microbial infections and cancer because of their immunomodulatory properties (Manisha *et al.*, 2001; Meurman, 2005). These bacteria can inhabit the biofilm and defend oral tissues from disease. They have cariostatic activity which helps in preventing *Candida* colonization and act as antioxidants (Patil and Reddy, 2006).

16.3.1 Using probiotics to replace the pathogenic flora

Despite over 50 years of antibiotic use, infectious diseases remain a major health problem. Instead of using chemicals, one microorganism can be countered with another microorganism, as the old Indian proverb goes: 'use a thorn to remove a thorn'. This is the concept behind replacement therapy or probiotic therapy. Probiotics as replacement therapy for dental caries is a new and fascinating concept and it has taken over from the old conventional therapy. As a result of their cariogenic properties lactobacilli have been the focus of great interest in dentistry for several years. Table 16.1 provides a brief overview of some studies showing the effect of probiotics on dental caries.

16.4 Direct and Indirect Mechanisms of Probiotic Action

Probiotics have been used for maintaining gut health for some time, and over the last few decades major studies have been designed to examine the use of probiotics for immunomodulation to avoid diseases of the urinary and respiratory tract, and improve allergic and atopic diseases in infants (Parvez *et al.*, 2006). However, very few studies have been conducted on probiotics and their effect on oral health. The mechanism of action of probiotic bacteria in the oral cavity may be direct, for example by replacing the pathogenic flora by competing for the adhesion site, nutrients and growth requirements and producing antimicrobial agents, thereby inhibiting growth of the pathogen (Boirivant and Strober, 2007; Cosseau *et al.*, 2008; Haukioja *et al.*, 2008; Sliepen *et al.*, 2009; Twetman *et al.*, 2009). These are similar to the role played by probiotics in the gastrointestinal tract. However, probiotics may need some extra properties when they are to be used in the oral cavity because oral probiotic bacteria must adhere and colonize the periodontal tissue, including hard non-shedding surfaces, and form a biofilm but they shouldn't ferment sugars as this is harmful for teeth and leads to caries (Meurman, 2005).

Some indirect mechanisms of action include: (i) modulating the systemic immune function; (ii) the effect on local immunity; (iii) the effect on non-immunologic/non-specific defence mechanisms; (iv) prevention of plaque formation by neutralizing free electrons and production of antioxidants; and (v) modifying the surrounding environment. These will be discussed below in more detail.

Direct and indirect mechanisms of probiotic action in the oral cavity are shown in Fig. 16.1.

16.4.1 Modulation of systemic immune system

Gut probiotics may elicit a beneficial systemic immune response and thus positively influence oral health, however, this idea is highly controversial. Probiotics improve both mucosal and systemic immunity (Ríos-Olivares *et al.*, 2006). Probiotic bacteria release pro-inflammatory cytokines such as tumour necrosis factor-α and interleukin-6, thus reflecting stimulation of non-specific immunity. *Bifidobacterium* and *Lactobacillus* strains have been found to activate macrophages and phagocytosis in the murine model (Zhu *et al.*, 2011). Macrophages are key mediators of the innate immune response. They

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Table 16.1. A brief overview on some studies showing the effect of probiotics on dental caries.

Study design	Number of participants	Probiotics	Duration	Effect	Reference
Double blind, randomized, controlled placebo trial	78	*Lactobacillus paracasei*	14 days	Probiotic effect	Chuang *et al*. (2011)
Cross-sectional survey	400	*Lactobacillus*	–	*Streptococcus mutans* was observed in 87.37% and *Lactobacillus* in 36.71% of samples	Hedge *et al*. (2005)
Double blind placebo trial	24	*Lactobacillus acidophilus* DSM 20079	–	*L. acidophilus* DSM 20079 has good adherence properties	Tahmourespour and Kermanshahi (2011)
Randomized controlled trial	276	*Lactobacillus rhamnosus* GG	16 weeks	Prevalence of *Candida* infection decreased in probiotic group	Hatakka *et al*. (2007)
Double blind placebo trial	45	Probiotic mouth rinse	4 weeks	Probiotic and chlorhexidine groups lacked plaque accumu-lation	Harini and Anegundi (2010)
Double blind placebo trial	245	*Lactobacillus* spp.	16 weeks	Reduction in the incidence rate of dental caries in probiotic group compared with control	Gonzalez *et al*. (1990)
Cluster randomized study	248	*L. rhamnosus* LB21	21 months	Daily consumption of milk containing probiotic bacteria and fluoride reduced caries	Stecksén-Blicks *et al*. (2009)
Clinical controlled study	28	Probiotic milk drink	4 weeks	Probiotic milk drink reduced the effects of plaque induced by gingival inflammation	Slawik *et al*. (2011)
Pilot study	50	*Lactobacillus plantarum* 299	–	No difference between the effect of *L. plantarum* 299 and chlorhexidine used in oral care procedure	Klarin *et al*. (2008)

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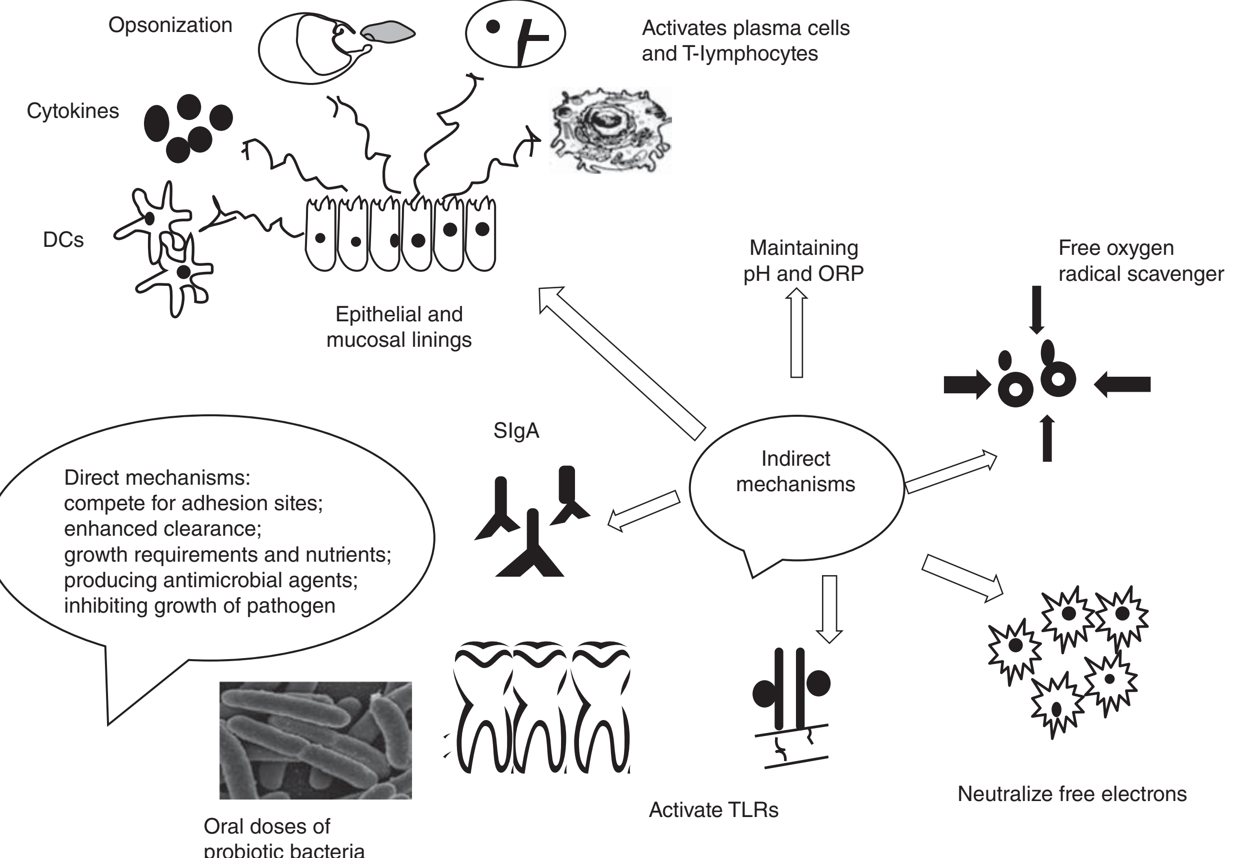

Fig. 16.1. Direct and indirect mechanisms of probiotic action in the oral cavity. Direct mechanisms of action include competition for adherence sites, growth requirements and nutrients, and secretion of antimicrobial compounds thereby inhibiting the growth of the pathogen. Indirect mechanisms of action include: secretary IgA (SIgA) synthesis in the oral cavity; activation of Toll-like receptors (TLR)2 and TLR4; neutralizing free electrons; free oxygen scavenging; maintaining pH and oxidation/reduction potential (ORP) and systemic non-specific immunomodulation which activates phagocytes and plasma cells to secrete cytokines, activates dendritic cells (DCs) and T-lymphocytes.

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function as antigen-presenting cells in initiating adaptive immune responses. Phagocytosis should be activated before antibody production. These phagocytes release toxic components, like reactive oxygen species (ROS) and lytic enzymes, which cause recruitment of immunocompetent cells and the generation of the inflammatory response. Oral administration of *Lactobacillus* and *Bifidobacterium* have been shown to have a time- and dose-dependent effect in rotavirus-induced diarrhoea (Szajewska *et al.*, 2001), pouchitis (Vanderhoof, 2001), irritable bowel syndrome, autoimmune disorders such as rheumatoid arthritis or lupus, and infections caused by enteropathogens (Salminen *et al.*, 2009; Turroni *et al.*, 2009; Lopez *et al.*, 2010; Amdekar *et al.*, 2011).

Other workers have also found that probiotic bacteria influence the inflammatory response elicited by pathogens through specific signalling pathways, for example Yurong *et al.* (2005) and Chichlowski *et al.* (2007) found they activated lymphocytes and produced antibodies. The immunomodulatory property of probiotics is due to increased production of T-lymphocytes, CD^+ cells and antibody-secreting plasma cells, pro- and anti-inflammatory cytokines, interleukins, interferon, natural killer cells, respiratory burst of macrophages, opsonization and delayed hypersensitivity reactions (Panda *et al.*, 2003; Ng *et al.*, 2009; Ohashi and Ushida, 2009). These phenomena also occur in the oral cavity, however, to date no studies have examined this.

16.4.2 Effect on local immunity

The oral immune system is an extensive and compartmentalized mucosa-associated system (Czerkinsky *et al.*, 1999). As the oral epithelium is part of the immune system it produces cytokines. Studies have suggested that bacteria can be a stimulus for the epithelial cells to produce cytokines (Walker, 2004). The association of probiotic bacteria with oral epithelium is sufficient to trigger signalling cascades that ultimately activate the underlying immune cells in the lamina propria. They might also secrete some soluble factors that cause immunomodulation (Hoarau *et al.*, 2006). Adherence to the epithelium is not the sole criterion for probiotic action in the oral cavity; rather these probiotics activate immune cells. The epithelium can be activated by TLRs. TLRs identify conserved pathogen-associated molecular patterns (PAMPs) which are found in many microorganisms (Sugawara *et al.*, 2002). These act as a receptor for common oral pathogens (Netea *et al.*, 2002). Thus TLRs stimulate epithelial cells which in turn mediate chemokines secretion, regulate intercellular adhesion molecule-1 expression and promote lymphocyte recruitment leading to cytokine secretion. TLRs may protect the host by sequestrating PAMPs (Dutz, 2009). Based on the specificity of ligand recognition, TLR2, TLR4 and CD14 form a set of receptors that can recognize most oral microorganisms associated with caries and other dental problems.

16.4.3 Effect on non-immunologic/ non-specific defence mechanisms

Non-immunologic defence mechanisms also operate to protect the oral cavity and teeth. Saliva is one of the primary defences against bacteria and other infectious agents. Saliva contains some antibiotic agents and is an important natural defence mechanism of the body (Dar-Odeh and Al-Kayed, 2002). The salivary bicarbonate and carbon dioxide buffer system and calcium and phosphate ions play an important role in protecting teeth from caries (Speirs, 1984). A group of salivary proteins, lysozyme, lactoferrin and peroxidase act in conjunction with other components of saliva to limit the growth of bacteria or kill them directly. Also SIgA found in saliva (Tomasi *et al.*, 1965) is known to protect the teeth from caries. *L. casei* and *L. acidophilus* have been shown to increase the SIgA in intestinal fluids from mice challenged with *Salmonella enteritidis* (Jain *et al.*, 2008). Similar results were also observed when *Lactobacillus* GG and *Bifidobacterium lactis* Bb 12 were given to infants. This treatment increased the maturation of SIgA (Rautava *et al.*, 2006). In another study, two probiotic strains *Bifidobacterium animalis* ssp. *lactis*, BB-12® and *Lactobacillus paracasei* ssp. *paracasei*, *L. casei* 431® increased the SIgA level in plasma and saliva samples (Rizzardini *et al.*, 2012). *Lactobacillus pentosus* strain b240 increased the salivary SIgA level in elderly (human) individuals (Kotani *et al.*, 2010). Hence it appears that probiotics may increase the SIgA level which

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would contribute to maintaining oral health and protecting teeth and other tissues of the oral cavity. It is possible that some factors secreted by probiotic bacteria activate plasma cells to secrete IgA or enhance the maturation of the IgA response in the oral cavity. However, more studies are needed in order to find out the reason for increasing SIgA levels in the oral cavity following treatment with probiotics.

16.4.5 Prevention of plaque formation by neutralizing free electrons and production of antioxidants

Free radical formation leads to arterial damage and plaque formation which harm the oral cavity. They are highly reactive species of short life span and have free/unpaired electrons. Free radicals are useful in small quantities but in excess they may lead to severe damage (Krasse *et al.*, 2006). The most dangerous free radicals of the oral cavity are: (i) fluorine gas; (ii) free radicals generated by antibacterial agents; (iii) tap water; and (iv) food particles. Rotten food particles contain oxidants. These free radicals may lead to gingivitis and periodontal disease. Oxidants cause stains and dental plaque formation. Rust forms in the oral cavity due to the oxidation of minerals with saliva. When calcium oxidizes in saliva, the mineral deposits on the teeth and forms plaque. Other inorganic minerals also oxidize and form stains on the teeth. There is a new theory that postulates that dental plaque and stains are formed by an electromagnetic field around protein particles, plaque-forming bacteria attracting minerals by magnetism caused by free electrons. This theory is scientifically based but is as yet unproven.

Antioxidants diminish the harmful effects of free radicals. Antioxidants have become the focus of many scientific studies because oxidative stress is associated with many diseases (Osuntoki and Korie, 2010). Antioxidants are believed to protect the oral cavity by healing periodontal disease, reducing oral cancers, reducing mucosal inflammatory reactions in the oral cavity and metal-based restoration reactions (Sakagami *et al.*, 1999). Natural antioxidants are present in some foods but vitamin supplements and probiotic bacteria that produce antioxidants are the best source of antioxidants. Antioxidant toothpowder and probiotic mouthwashes are new approaches to taking antioxidants for treating gingivitis and periodontal disease; probiotics breakdown putrescent odours efficiently by fixing the toxic gases and changing them into harmless gases that can be metabolized. The probiotic *Lactobacillus reuteri* has been shown to be efficacious in curing gingival infection and dental plaque (Krasse *et al.*, 2006).

16.4.6 Probiotics change the environment

Certain probiotic bacteria have been found to affect and maintain conditions in the nearby environment by modulating the pH and the oxidation/reduction potential (ORP), which may inhibit the ability of pathogens to become established.

Probiotic bacteria lower the pH making it more acidic, hence plaque bacteria cannot form dental plaque and calculus. The ability of probiotic bacteria to lower the pH is due to the secretion of acids. In the vaginal ecosystem these acids include mainly lactic acid, acetic acid, hydrogen peroxides and bacteriocins. These maintain the pH at less than 4.5, repressing other (potentially pathogenic) bacteria residing inside the vagina (Alla *et al.*, 2001). Such secretions are also observed in the gastrointestinal tract and oral cavity, also decreasing the pH (Sartor, 2004; Quigley and Quera, 2006; Sheikh *et al.*, 2011). The exact mode of action of lactic acid is not known, however, it functions as a permeabilizer of the Gram-negative bacterial cell outer membrane (Alakomi *et al.*, 2000). Organic acids, in an undissociated form, diffuse across the cell membrane towards the more alkaline cytosol and cause damage, interfering with essential metabolic functions. Lactic and acetic acid reduce the intracellular pH and cause dissipation of the membrane potential (Lorca and de Valdez, 2009). In addition, lactic acid works as a chelating molecule, which binds with some other ions like iron (Fe) and act as a nutrient for probiotic bacteria (Oelschlaeger, 2010).

Another mechanism by which probiotic bacteria can change the surroundings is by affecting the ORP. *Lactobacilli* usually alter the ORP by producing metabolites which make the environment

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less conducive for organisms requiring oxygen, thus inhibiting oral pathogens from surviving in the oral cavity (Olanrewaju, 2007).

16.5 Concluding Remarks and Future Directions

Probiotics have the potential to change the traditional 'drill', 'fill' and 'bill' therapy for treatment of dental caries and management of other dental diseases. However, the mechanisms of action of probiotics in the oral cavity are not yet fully understood, although it is known that probiotic bacteria affect both bacteria residing in the oral cavity and immune responses. As bacteria in food or foodstuffs can affect the stability of the oral microbiota, there is a need for further study into possible mechanisms of action of probiotics, including a detailed molecular understanding of the processes involved, and *in vivo* studies of bacterial interactions leading to identification of naturally occurring strains for probiotic-mediated rather than probiotic-assisted therapy. In addition, long-term clinical trials are required to prove that probiotics can be used in preventing/treating oral diseases.

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17 Human Microbiota for Human Health

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Abstract

Microorganisms are considered to be absent in the human foetus during early development in the mother's womb, but later after delivery the numbers of microbes exceeds the number of total human cells. This human microbiota has tremendous influence on human physiology, development, nutrition, immunity and resistance to pathogens and is also known to be associated with normal development and function of the mucosal and gut immune system. In health the microorganisms contribute to effective metabolism by producing enzymes such as 'carbohydrate active enzymes' (CAZymes) and also help to develop a mucosal barrier, innate and adaptive immune responses and effectively suppress establishment of pathogens. This chapter discusses the distribution of microorganisms and their benefits to human beings, along with future developments such as engineering these microbes to produce stimulants, antidepressants and satiety-inducing drugs and even delivery of drugs, therapeutic peptides or vaccine antigens by microbes at mucosal surfaces which hold tremendous promise for the treatment of diverse diseases and also leads to generation of wealth.

17.1 Introduction

Health is defined as the state of being free from illness or injury and the word 'wealth' is derived from Middle English 'welthe', from well or weal, on the pattern of health. Wealth can also be defined as the state of being rich, having material prosperity and plentiful supplies of a particular resource/desirable things and well-being. Even though wealth directly means an abundance of valuable possessions or money, in the present situation health is considered to be the real wealth.

The World Health Organization (WHO) defined health in its broader sense in 1946 as 'a state of complete physical, mental, and social well-being and not merely the absence of disease or infirmity' (Grad, 2002). There is no doubt that an individual's health is heavily dependent on the microbial ecosystems associated with them. Many perplexing ailments such as asthma, allergies, obesity, diabetes, autoimmune diseases, autism and even degenerative diseases may be due to the breakdown of homeostasis caused by differential diet, misuse of antibiotics and over-emphasis on cleanliness. It has recently been suggested that caesarean deliveries may disrupt the excellent relationship between the human foetus and human microbiota in which the microbes help maintain human health.

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Microbes inhabited this planet approximately 4 billion years before the existence of man, which suggests a significant role for them in human life. However, despite decades of microbiological research very little is known as to how many microbes affect the health of humans and how the microbiology of the human host changes as human's age.

Microorganisms evolved earlier than human beings and their association started during the early stages of human evolution influencing the development of humans. Invertebrate models have proved that bacteria may influence a specific host's development; this finding will have some relevance to both animal and human development. A good example in this regard is the association of the bioluminescent, marine bacteria *Vibrio fischeri* with the seawater squid *Euprymna scolopes*. The relationship between these organisms develops within a few hours of the squid hatching from its egg. *V. fischeri* releases diffusible signals that are involved in morphological changes needed for the development of a special light organ in the growing squid.

Evidence is available from the research that microbes involved in relationships with mammalian cells may also have influence at the molecular level on development. For example, in work carried out on germ-free (GF) mice infected with *Bacteroides thetaiotaomicron*, the bacteria were reported to have influence on the various host genes related to the uptake of nutrients, its metabolism, functioning of the mucosal barrier, angiogenesis and development of the nervous system (Xu and Gordon, 2003).

In view of the possible influence of microbes on human beings, a lot of research has been done to demonstrate the association of a huge number of microbes with humans from the early ages of development until death. Microorganisms that are part of the normal microflora of human beings are estimated to be present in larger numbers than human cells (Goodman and Gordon, 2010): the healthy human is estimated to contain approximately ten times more bacteria than healthy cells (Tlaskalová-Hogenová *et al.*, 2004).

These microorganisms are reported to play a major role in human physiology and several homeostatic responses such as immunity and resistance including nutrition uptake and development (Backhed *et al.*, 2004; Ordovas and Mooser 2006; Belda-Ferre *et al.*, 2011; Kau *et al.*, 2011; Mirmonsef *et al.*, 2011). A microbiome is also known to be associated with mucosal functioning and its normal development and immunity of the gut (Weinstein and Cebra, 1991; Cebra, 1999; Shanahan, 2002; Backhed *et al.*, 2004; Rakoff-Nahoum *et al.*, 2004; Ley *et al.*, 2005; Mazmanian *et al.*, 2005).

17.2 Distribution of Microbes in the Human Body

It has been estimated that there are over 10^{14} microbes in the human body and the human gut itself is known to possess about 7000 different microbial strains (Pollan, 2013). Every part of the human body is inhabited by different microorganisms, but these numbers will vary considerably from one location to another and the human intestine is estimated to have a higher microbial load than any other part of the body (Fig. 17.1). The population size of the microorganisms on the skin is $10–10^6$/cm^2, saliva is 10^8/ml, dental plaque 10^{12}/g, ilieal contents 10^8/ml, colon (faeces) 10^{10}/g and vagina 10^8/ml (Backhed *et al.*, 2005).

Even though there are 2000 different bacterial phylotypes available within the human body, only eight of 55 main divisions of bacteria colonize the human body, indicating the possible role of host selection. These selected groups of microbes constitute the 'microbiome' (Blaser, 2005) and they use the human as a host in order to carry out fermentation through which they can enhance their numbers (Nicholson *et al.*, 2005). In general bacteria from the following families are the major inhabitants of the human body: *Actinomycetaceae*, *Bacilaceae*, *Bacteroidaceae*, *Bifidobacteriaceae*, *Clostridiaceae*, *Coriobacteriaceae*, *Corynebacteriaceae*, *Desulfoviroionaceae*, *Enaterobacteriaceae*, *Erysipelotrichaceae*, *Eubacteriaceae*, *Fusobacteriaceae*, *Helicobacteriaceae*, *Lachnospiraceae*, *Lactobacillaceae*, *Mycobacteriaceae*, *Moraxellaceae*, *Neisseriaceae*, *Pasteurellaceae*, *Ruminococcaceae*, *Staphylococcaceae*, *Streptococcaceae* and *Veillonellaceae*.

Among several groups of microorganisms distributed in the human body, *Staphylococcus* sp. *Acinetobacter* sp., *Malassazia furfur*, *Micrococcus* sp. *Corynebacterium* sp. and *Propionibacterium* sp. are

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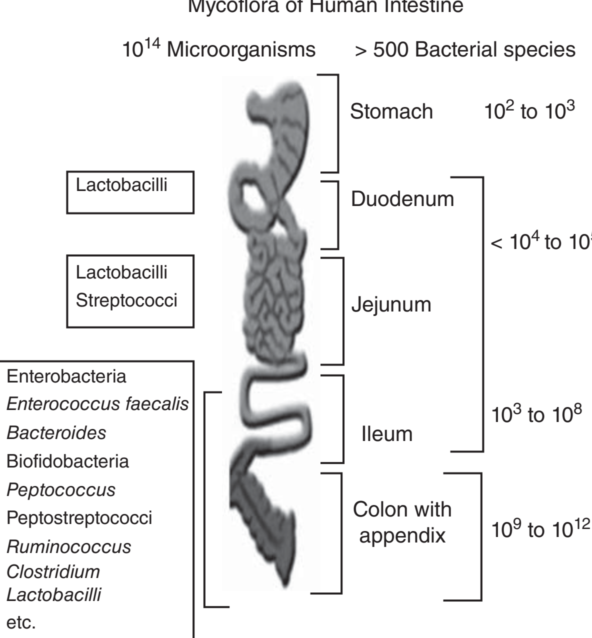

Fig. 17.1. Normal flora of the human intestine.

considered to be inhabitants of normal healthy skin. The number of bacteria detected on the skin surface is about 10^{12}, which is equivalent to $10^4/cm^2$, which equates to about one bacterium per 100 mm^2. This indicates that the bacteria are not evenly distributed but exist in microcolonies on the skin surface often growing as biofilms.

The following species are commonly observed in the oral cavity: *Actinomyces* sp., *Prevotella* sp. *Porphyromonas* sp., *Lactobacillus* sp., *Fusobacterium* sp., *Bifidobacterium* sp., *Streptococcus* sp., *Campylobacter* sp., *Rothia dentocariosa*, *Capnocytophaga* sp., *Propionibacterium acne*, *Eubacterium* sp., *Veillonella* sp., *Peptostreptococcus* sp., *Neisseria* sp., *Haemophilus parainfluenzae*, *Selenomonas* sp., *Actinobacillus* sp., *Capnocytophaga*, *Bacteroides* sp. and *Wolinella* sp. The load of bacterial species is very large in the human gastrointestinal tract (including the stomach, jejunum, ileum and colon), and in the upper and lower respiratory tract. As already mentioned, the human gut is considered to be an important microbial niche, which depends on the human regular diet for nutrition. These microbes are critically important as they influence the health condition of an individual.

In order to increase knowledge and understanding of the microbial communities of the normal human adult and their role in health and disease the Human Microbiome Project (HMP) was initiated. Whole genome shotgun sequence information on microbial communities isolated from five major body sites, including the gut, airways, oral, urogenital and skin surfaces, were analysed (The HMP Consortium, 2012). This has helped not only increase understanding of the microbial communities and their role in maintaining health but has also provided a critical baseline for further metagenomic studies from these sites. The study, conducted on samples collected up to three times from 15–18 body sites of 242 healthy adults, has generated 5177 microbial taxonomic profiles from 16S ribosomal RNA genes and another 3.5 terabases of metagenomic sequence resources (The HMP Consortium, 2012).

17.3 Microbial Flora Acquired During Development

During early development of the foetus in the mother's womb until its birth, microbes are not

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generally observed. Recent observations have revealed that the first microbes were obtained during delivery and even after delivery. The microbes acquired during delivery are considered to be companions of the human for life, but their numbers vary during ageing and according to the health of the individual, being altered by disease conditions. These companion microbes maintain a mutualistic relationship with humans and are referred to as the normal microflora. Historically the human–microbe relationship is considered to be one of commensalism. However, it would appear that the human–microbe relationship is not one of commensalism, because each partner has the ability to influence the other. Therefore, the commensal microflora of the body is often better described as the ‘indigenous microbiota’, as suggested by Dubos *et al*. (1965).

The capacity of the indigenous microbiota to cause harm and disease to the host is severely limited by the host’s immune system which helps to maintain a microbe–host homeostasis, which in turn helps to prevent the generation of significant microbial disturbances. However, if disturbances to the indigenous microbiota do occur, such as after surgical procedures or with chronic wounds, a person’s host defences are often compromised predisposing them to changes in their microflora and initiation of infection and diseases.

17.4 Human Microbiota and Health

In health the indigenous microbiota of the human contributes to effective metabolism by producing enzymes and also helps to develop a mucosal barrier, innate and adaptive immune responses and effectively suppress establishment of pathogens (Tlaskalová-Hogenová *et al*., 2011). It is important to understand the microbes involved in the indigenous microbiota of the healthy individual and how this varies in disease states from pre-clinical conditions and disease onset through progressive states of disease.

In disease, with a breach of the mucosal barrier, commensal bacteria can become a chronic inflammatory stimulus to adjacent tissues (Tanner *et al*., 1998; Kumar *et al*., 2006) as well as a source of immune perturbation in conditions such as atherosclerosis, type 2 diabetes, non-alcoholic fatty liver disease, obesity and inflammatory bowel disease (Ott *et al*., 2006; Creely *et al*., 2007; Pussinen *et al*., 2007; Al-Attas *et al*., 2009; Ley, 2010). In the absence of pathogenic microorganisms, the commensal gut microflora is known to trigger immune processes leading to a relapsing/remitting autoimmune disease driven by myelin-specific CD^{4+} T-cells (Berer *et al*., 2011).

17.5 Variation in the Human Microbiota

As mentioned earlier, the microbiota varies from one body site to another and also during the developmental stages of human beings. Among the indigenous microbiota of children *Bifidobacterium* sp. are found to be predominant, whereas in adult guts which are exposed to different food habits *Bacteroides* sp. are the normal inhabitant. In older people who suffer from senescence-related complications such as a decline in health and nutritional conditions, variations in the usual microbiota are observed including: (i) lower numbers of *Veillonella* sp. and bifidobacteria and instead they show increased numbers of *Clostridium* sp., enterobacteria and lactobacilli; (ii) an increase in organisms that cause urinary tract infections; (iii) increased colonization in the oropharynx by *Candida albicans* and *Klebsiella* sp.; (iv) increased levels of enterobacteria and streptococci on the skin; and (v) increased levels of Gram-negative bacteria in the eye and oral cavity (Wilson, 2004).

The gut microbiota of the elderly varies in composition according to diet and health status. Claesson *et al*. (2012) proved this by examining the composition of gut microorganisms in faeces of elderly people selected from different locations. They recorded variation in the microbiota and showed that it was significantly correlated with an individual’s health status and even the environment with which they are associated. For example it was observed that the microbiota of people who lived in long-stay care homes harboured less diverse microbiota than people living in the community. A community-associated microbiota was found to be directly correlated with effective metabolism and its absence may lead to increased weakness.

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Collectively, these findings indicate that the microbiota plays an important role in deriving energy and nutrition from the diet which is linked to health status and on the other hand, a change in diet also influences the variation in the microbiota of individuals. This variation is reported to be associated with increased health risk upon ageing and may be linked to obesity and inflammatory diseases. Yatsunenko *et al.* (2012) examined the gut microbiota in faecal samples of 531 individuals of different ages and geographical regions and they examined the gene content of 110 healthy children and adults looking at the possible influence of variation in the gut microbiome on human genetics leading to metabolic diversity. They observed differences in assemblages of bacteria among the people even during infancy, as well as adulthood, and even people belonging to different countries showed different functional gene repertoires. This study highlights the need to understand an individual's microbiome for their development, variation in physiology due to nutritional diversity and even the impact of westernization.

Devkota *et al.* (2012) reported that dietary fats, by promoting changes in host bile acid composition, can markedly alter conditions for gut microbial assemblages, resulting in dysbiosis that can perturb immune homeostasis. These workers also provided data on the influence of Western-type diets which are rich in saturated fats and the effect on complex immune-mediated diseases such as inflammatory bowel disease in genetically susceptible hosts (Devkota *et al.*, 2012).

As mentioned in the Introduction (section 17.1) incessant and indiscriminate use of antibiotics can disturb the microbial ecology of individuals. Cho *et al.* (2012) demonstrated that administration of antibiotics in the early life of young mice can disturb metabolic homeostasis. They showed that it had a direct influence on adiposity in the mice, increased hormone levels related to metabolism and substantial taxonomic changes were observed in the microbiome, as well as changes in copies of key genes involved in the metabolism of carbohydrates to short-chain fatty acids (SCFAs), increases in colonic SCFA levels, and alterations in the regulation of hepatic metabolism of lipids and cholesterol. The spatial and temporal aspects of the composition of the intestinal microbiota are depicted in Fig. 17.2 (Sekirov *et al.*, 2010).

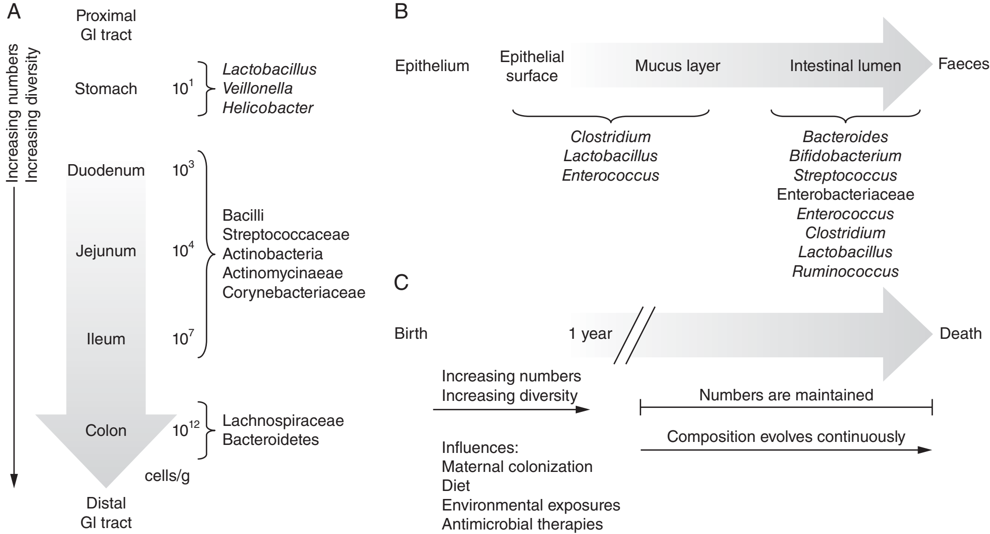

Fig. 17.2. Spatial and temporal aspects of the composition of the intestinal microbiota. A, Variations in microbial numbers and composition across the length of the gastrointestinal (GI) tract; B, longitudinal variations in microbial composition in the intestine; C, temporal aspects of microbiota establishment and maintenance of factors influencing microbial composition. (From Sekirov *et al.*, 2010.)

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17.6 Gut Microflora and Human Metabolism

The plant cell wall is a good nutrient source but it is highly variable (Carpita, 1996; Gorshkova *et al.*, 1996). Plant carbohydrates are not easily digestible by enzymatic breakdown as they are both chemically complex, due to the composition of the polysaccharides, and structurally complex, due to their arrangement in a three-dimensional network (Gilbert, 2010). In particular cellulose microfibrils are associated with a wide range of homo- and heteropolysaccharides such as hemicelluloses and pectins with different monosaccharides. Ester substituents or non-carbohydrate polymers such as lignin, proteins, cutin and suberin add further difficulty for enzymatic degradation by increasing structural complexity. However, these carbohydrates are a source of energy not only for the host but also for the microorganisms comprising the microbiome and hence the nutrient environment serves as a mediator to maintain complex relationships between both (Hooper *et al.*, 2002).

By contrast, analysis of the human genome has revealed that it codes for less than 20 enzymes required for carbohydrate breakdown, indicating the need of other sources as supplements for enzymes required for total sugar breakdown. Human gut microbiota that colonize the digestive tract are known to be effective degraders of several complex plant carbohydrates and hence support human beings in this regard (Backhed *et al.*, 2005). These microorganisms have to respond rapidly to these carbohydrates with release of specific enzymes which are necessary to metabolize the plant cell wall structures in every meal.

Microbes have an extraordinary capacity to respond to the changing environment quickly with their simple gene regulation strategies. They can also produce different enzymes in response to different specific substrates. Hence the human distal gut harbours different microorganisms which enable the host to increase its metabolic capabilities by deriving energy even from indigestible dietary polysaccharides. Studies of a few unrelated, healthy adults have revealed substantial diversity in their gut communities, as measured by sequencing 16S rRNA genes, yet how this diversity relates to function and to the rest of the genes in the collective genomes of the microbiota (the gut microbiome) remains obscure. The role of the microbiome in digestion is evident from studies conducted on lean and obese mice gut microbiota which has been proved to have influence on energy balance by increasing calorie harvest from a meal, its usage and storage (Turnbaugh *et al.*, 2006).

Recent studies also support the view that the human gut microbiota contributes enzymes required for degradation/digestion of almost all polysaccharides of the plant cell wall including cellulose. Turnbaugh *et al.* (2010) reported that dockerin-containing cellulolytic enzymes required for breakdown of cellulose are coded by the distal gut microbiota of humans. These are multienzyme complexes assembled on large scaffolding proteins generally called cellulosomes (Bayer *et al.*, 2004; Fontes and Gilbert 2010). The HMP analysis added information on the distribution of such proteins in the vagina, oral and nasal samples and even in the other non-digestive sites of the human body (Cantarel *et al.*, 2012).

The three important enzymes involved in catalysing the breakdown of complex carbohydrates are: (i) glycosyltransferases (GTs); (ii) glycoside hydrolases (GHs); and (iii) polysaccharide lyases (PLs). These enzymes together are generally called 'carbohydrate active enzymes' (CAZymes) and they are classified in a number of sequence-based families on the CAZy database. The CAZy database of the human genome reveals that humans have the ability to synthesize 97 GHs and, as already mentioned, 20 enzymes to utilize carbohydrates as nutrients. It is surprising that out of these, several are yet to be fully characterized. It was also observed that, even though the total genome size is larger in humans, the numbers of GHs are comparatively few when compared with some of the gut bacteria such as *B. thetaiotaomicron*, which is known to encode over 260 GHs. The complex web of gut microbiota contributing to host physiology is represented in Fig. 17.3 (Sekirov *et al.*, 2010).

Analysis of 520 metagenomic samples obtained from five major body sites revealed that even when the microbial community composition varies, the CAZyme profiles were very similar within a body site (Cantarel *et al.*, 2012). It was also noted that the functional profile and microbial colonization in a particular part of the body appear to have adapted to the available

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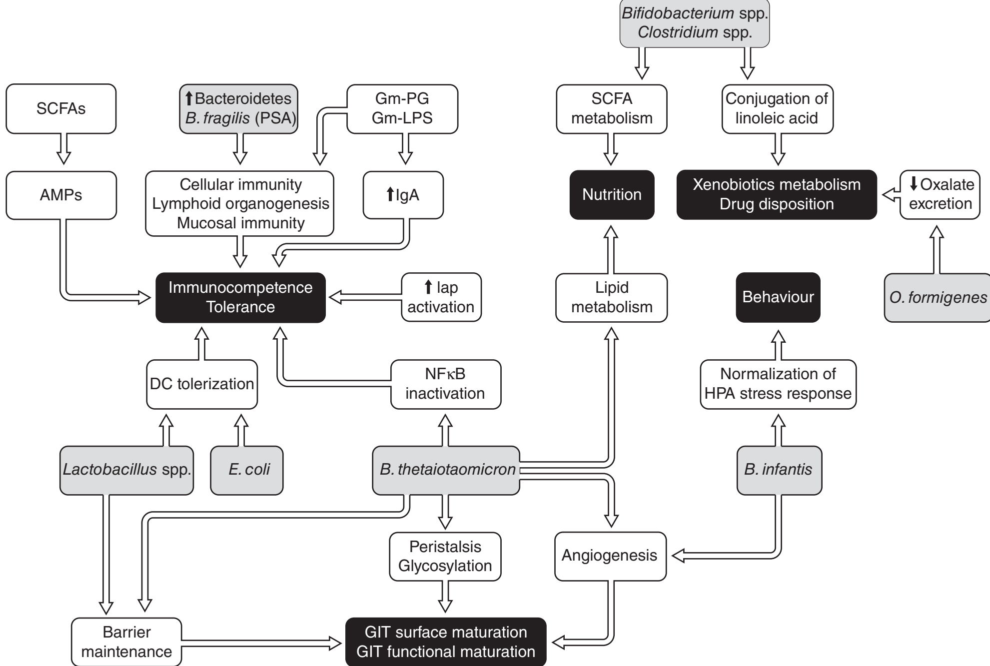

Fig. 17.3. The complex web of gut microbiota contributions to host physiology. Different gut microbiota components can affect many aspects of normal host development, while the microbiota as a whole often exhibits functional redundancy. Members of the microbiota (e.g. *Bacteroides fragilis*, *Bacteroides thetaiotaomicron*, *Bifidobacterium infantis*, *Clostridium* spp., *Escherichia coli*, *Lactobacillus* spp. and *Oxalobacter formigenes*) are shown in grey, with their components or products of their metabolism. Their effects on the host at the cellular or organ level are shown in white. Only some examples of microbial members/components contributing to any given phenotype are shown. AMP, Antimicrobial peptides; DC, dendritic cells; GIT, gastrointestinal tract; Gm-, Gram negative; HPA, hypothalamus-pituitary-adrenal; Iap, intestinal alkaline phosphatase; LPS, lipopolysaccharide; NFκB, nuclear factor-κB; PG, peptidoglycan; PSA, polysaccharide A; SCFA, short-chain fatty acid. (From Sekirov *et al.*, 2010.)

carbohydrate and its composition. Cantarel *et al.* (2012) observed that among the five different body sites examined the gastrointestinal tract possessed the maximum capacity to degrade sugars completely, whereas dextran degradation was found to be highly effective in the mouth and peptidoglycan degradation in the vagina. These findings suggest that the composition of different carbohydrates influences the microbiome composition of the particular body site and also serves as a major driving force for the distribution of microbiota in different parts of the body and even the CAZyme profile of these microbes.

There is also substantial research carried out to address how the diversity of enzymes involved in carbohydrate breakdown has evolved in gut microflora even by acquiring new genes from microbes living outside the gut. The recent outcome of research by Hehemann *et al.* (2010) showed that novel CAZymes have been added to the gut microflora as a result of flexible microbial interactions. They reported that genes coding for porphyranases, agarases and associated proteins have been acquired by the gut bacterium *Bacteroides plebeius*, which is a normal part of the gut microflora of Japanese individuals. They showed that these genes are initially observed in a marine Bacteroidetes, *Zobellia galactanivorans*, which are active against the sulfated polysaccharide porphyran generally found in marine red algae of the genus *Porphyra*. This clearly shows that gut microflora might have acquired these genes through molecular cross talk with marine

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bacteria associated with seaweeds, which are important in the daily diet for the Japanese.

Comparative metagenome analysis of Japanese and North American individuals conducted by these authors revealed that porphyranases and agarases are more commonly observed in the Japanese than North Americans. This difference is ascribed to the Japanese individuals consuming non-sterile food which will contribute to CAZyme diversity in human gut microbes, because these will have continuous interaction with other food-specific microflora. These interactions will contribute to an increased number of genes added to the gut which are required to breakdown complex plant cell wall constituents. The absence of GH6 cellulases, which are commonly observed in soil bacteria and fungi in the distal gut microflora, involved in anaerobic degradation of carbohydrates reveals the possible selection force of the genes encoding enzymes required for carbohydrate metabolism.

Due to this variation in functional adaptations among gut microflora, the distal portion of the gastrointestinal tract receives a large amount of undigested dietary carbohydrates such as cellulose, xylan and pectin (mostly from plant cell walls), and undigested starch along with glycans (mucins and glycosphingolipids) derived from the host. They pass into the distal regions of the small intestine (ileum) and colon where they are degraded by resident microbes. SCFAs derived by further bacterial fermentation of the resultant monosaccharide are absorbed and utilized by the host. Acetate, propionate and butyrate, which are three major SCFAs produced by gut microflora, are the preferred energy substrate of the colonic epithelial cells (Roediger, 1982). It has been estimated that in humans 50–60 g of carbohydrate is typically fermented per day, yielding 0.5–0.6 mol of SCFA, with a total energy value of 140–180 kcal. However, the amount and type of SCFA produced varies according to the dietary factors such as the fibre content. In addition to their nutritional value, SCFAs have important effects on gut physiology, with butyrate, in particular, affecting epithelial proliferation and differentiation (O'Keefe, 2008). *B. thetaiotaomicron* has an extraordinary capacity for acquiring and degrading polysaccharides and also affects the metabolic machinery of host cells, leading to more efficient nutrient uptake and utilization.

Unlike humans, many species of bacteria can synthesize folate (Klipstein and Samloff, 1966; Crittenden *et al.*, 2003) and their contribution to the folate content of the large intestine in human infants is sufficiently great to potentially affect the folate status of the host (Kim *et al.*, 2004). The gut bacteria are also a significant source of a range of vitamins, particularly those of the B group and vitamin K. It has been shown that germ-free (GF) mice require vitamin K and higher amounts of B vitamins (B12, biotin, folic acid and pantothetane) in their diets, in contrast to those that are colonized with a conventional microbiota. In addition, the gut bacteria aid in the absorption of minerals such as calcium, magnesium and iron (O'Hara and Shanahan, 2006).

Studies conducted using adult male GF Wistar rats revealed that conventionally reared rats required 30% less caloric intake to maintain their body weight compared with GF animals. This indicates the importance of the microbiota in assisting the host to obtain the maximum nutritional value (i.e. energy) from the diet. Additional comparative studies involving GF and colonized animals have confirmed that the intestinal microbiota affects the host machinery and plays an important role in the control of energy storage in the host. GF mice showed 40% less total body fat compared with conventionally raised animals even though the latter consume less per day. One proposed mechanism for this phenomenon is that the microbiota promotes storage of calories harvested from the diet into fat, acting through the intestinally derived protein FIAF (fasting-induced adipocyte factor), which is involved in coordinating increased hepatic lipogenesis with increasing lipoprotein lipase activity in adipocytes (Backhed *et al.*, 2004).

Individuals with low levels of the enzyme lactase have limited ability to digest lactose, which can result in intestinal distress, or lactose intolerance. People with lactose intolerance suffer from bloating, flatulence, abdominal pain and diarrhoea. Typically, they will restrict their intake of dairy products, which puts them at risk of deficiencies in several nutrients, most notably calcium (Montalto *et al.*, 2006). *Streptococcus thermophilus*, a species belonging to the *Streptococcus salivarius* group of microorganisms, has been used as a probiotic because of its ability to ferment lactose. The contribution of lactase by

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bacterial cultures is thought to improve the digestion of lactose in lactose-intolerant individuals (Sanders, 2000). The inability of adults to digest lactose is widespread, although generally these patients tolerate lactose better from yogurt than from milk (Suarez *et al.*, 1995). The yogurt starter cultures (*S. thermophilus* and *Lactobacillus delbrueckii* subsp. *bulgaricus*) present at levels normally seen in yogurt effectively improve the digestion of lactose in lactose maldigesters.

Probiotics also play an important role in bringing down elevated levels of certain blood lipids which are considered to be a risk factor for cardiovascular disease. A proposed mechanism is based on the ability of certain probiotic lactobacilli and bifidobacteria to deconjugate bile acids enzymatically, increasing their rates of excretion (De Smet *et al.*, 1994). Cholesterol is a precursor of bile acids, and the loss of bile acids through excretion is replaced by the conversion of cholesterol molecules to bile acids. Probiotic bacteria also ferment food-derived indigestible carbohydrates to produce SCFAs in the gut. This can cause a decrease in the systemic levels of blood lipids by inhibiting hepatic cholesterol synthesis and/or redistributing cholesterol from plasma to the liver. Several immunomodulatory functions of probiotic microflora of human intestine are summarized in Table 17.1.

Recently work done by Wang *et al.* (2011) on GF mice proved the critical role of gut microflora and dietary choline on production of trimethylamine N-oxide (TMAO), foam cell formation and accumulation of cholesterol by augmented macrophages. They also observed that dietary choline-enhanced atherosclerosis is inhibited by suppressing the intestinal microflora. This indicates the important relationship between gut microflora-induced metabolism and its impact on different pathogenesis including atherosclerotic heart disease. This will invite more insights during development of treatment strategies and diagnostic tests for such diseases (Wang *et al.*, 2011).

17.7 Horizontal Gene Transfer (HGT) Favours Bacterial Efficiency

HGT has played a vital role in bacterial evolution and is considered to be highly efficient in bacteria for rapid access to genetic innovations, allowing traits such as virulence, antibiotic

Table 17.1. Immunomodulatory effects enhanced by probiotic bacteria. (From Sanders, 1999.)

Test product	Effect on immune system
Fermented milk (*Streptococcus thermophilus*) with *Lactobacillus johnsonii*	Phagocytic activity + respiratory burst of peripheral blood leukocytes
Lactobacillus GG capsules	Increased serum immunoglobulin (Ig)A response to *Salmonella typhi* lipopolysaccharide vaccine
Lactobacillus GG powder capsules	Increased IgM secreting cells against rotavirus infection
Fermented milk with *Lactobacillus casei* Shirota	No effect on natural killer cell activity, phagocytosis or cytokine production
Fermented milk with *L. johnsonii* La1 (7×10^{10}) or *Bifidobacterium bifidum* Bb12 (10^{10})	No effect on lymphocyte subsets but increased levels of phagocytosis of *Escherichia coli* compared with pre-feeding levels
Lactobacillus brevis subsp. *coagulans* (Labre) tablet, live and heat killed	Increased levels of interferon (IFN)-α
Fermented milk with 4×10^9/day *L. johnsonii* La1 and *Bifidobacterium*	Increased levels of serum IgA response to *S. typhi* vaccine
Yogurt with 3×10^{12}/day *S. thermophilus* and *Lactobacillus delbrueckii* subsp. *bulgaricus*	Increased levels of serum IFN-γ, B-lymphocytes and natural killer cell subsets
Lactobacillus GG ($2 \times 10^{10-11}$)	Increased levels of rotavirus-specific IgA antibody secreting cells in rotavirus-infected infants
2×10^{10} c.f.u./day dried *Lactobacillus* GG	Increased levels of IgA-secreting cells and no change in clinical status of Crohn's disease patients
Bifidobacterium lactis formula	Increased levels of IgA-secreting cells

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resistance and xenobiotic metabolism to spread through the human microbiome. Within the human microbiome this ecological architecture continues across multiple spatial scales, functional classes and ecological niches with transfer further enriched among bacteria that inhabit the same body site, have the same oxygen tolerance or have the same ability to cause disease. This structure offers a window into the molecular traits that define ecological niches, insight that can be used to uncover sources of antibiotic resistance and identify genes associated with the pathology of meningitis and other diseases (Smillie *et al.*, 2011).

To what extent do host genetics control the composition of the gut microbiome? Studies comparing the gut microbiota in human twins and across inbred mouse lines have yielded inconsistent answers to this question. However, candidate gene approaches, in which one gene is deleted or added to a model host organism, showed that a single host gene can have a tremendous effect on the diversity and population structure of the gut microbiota. Now, quantitative genetics is emerging as a highly promising approach that can be used to better understand the overall architecture of host genetic influence on the microbiota, and to discover additional host genes controlling microbial diversity in the gut (Spor *et al.*, 2011).

17.8 Microbiota and Disease

Various factors (e.g. diet, antibiotic treatment, stress, age) may 'shift' the balance of the gut microflora away from potentially beneficial or health-promoting bacteria (e.g. lactobacilli and bifidobacteria) towards a predominance of potentially harmful or pathogenic bacteria such as *Clostridium* spp., sulfate-reducers and certain *Bacteroides* spp. (Fooks *et al.*, 1999).

Predominance of these latter populations are known to predispose an individual to a number of clinical conditions such as cancer and inflammatory disorders while making the host more susceptible to infections by transient enteropathogens such as *Salmonella* sp., *Escherichia coli* and *Listeria* sp. (Sanders, 2000). Consequently, people are seeking healthier lifestyles, and evidence is mounting that supports the idea that our health can be affected by the daily consumption of specific bacteria or 'probiotics'. The word 'probiotic' is derived from Greek and means 'for life'. A fuller definition of probiotics is: 'live microbial feed supplements which beneficially affect the host by improving the intestinal microbial balance' (Fuller, 1989). However, a later definition proposed by the Food and Agricultural Organization of the United Nations and the WHO describe probiotics as 'live microorganisms which when administered in adequate amounts confer a health benefit on the host' (McFarland and Elmer, 1995).

Constipation is a major digestive complaint and is traditionally defined in terms of bowel movement varying from between three times a week to once a week or less. Current evidence suggests that probiotics, specifically lactic acid bacteria, may improve intestinal mobility and relieve constipation, possibly through a reduction in gut pH. One study showed that the consumption of bifidus milk improved intestinal mobility and bowel behaviour in a group of constipated elderly people. However, a review of the literature does not substantiate this claim, and the causes of constipation may actually relate to physical inactivity, low-fibre diet, insufficient liquid intake and certain drugs.

17.9 Biotherapeutic Agents from Human Milk

Some of the important criteria generally recommended for human probiotics are: (i) they should be of human origin; (ii) they should have a history of prolonged use by sensitive populations such as neonates and infants; and (iii) they should have the enzymes to digest mucosal disaccharides and the ability to adapt to a varied diet (of carbohydrates). Probiotics isolated from human milk satisfy all these recommendations and hence nowadays are considered to be an important source of these bacteria (Collins *et al.*, 1998).

To understand the importance of these microbes, recently the first microbiome study focused on human milk was published by Hunt *et al.* (2011). Recent understanding of the microflora of human milk has disproved the traditional concept of human milk being sterile; rather it has been shown to represent a continuous supply of commensal, mutualistic and/or potentially

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probiotic bacteria to the infant gut. Culture-dependent techniques and other studies conducted on human milk have shown the presence of staphylococci, streptococci, lactic acid bacteria and bifidobacteria, which later become a component of the infant gut, contributing to maturation of the immune system, protecting the infant against diarrhoeal and respiratory diseases and reducing the risk of developing other diseases such as diabetes or obesity (Hunt *et al.*, 2011; Sanz, 2011). A few strains of lactic acid bacteria isolated from human breastmilk are reported to inhibit human immunodeficiency virus (HIV) type 1 (Martin *et al.*, 2010). It is also quite interesting to observe that the maternal gut microbiota is subject to variation during late pregnancy and during lactation to support the transfer of beneficial microflora from the gut to breastmilk through gut monocytes (Fernándeza *et al.*, 2013).

Lactobacillus gasseri, *Lactobacillus salivarius*, *Lactobacillus reuteri*, *Lactobacillus fermentum* and *Bifidobacterium breve* isolated from human milk enjoy high probiotic potential, because they naturally qualify for the QPS (Qualified Presumption of Safety) status set by the European Food Safety Authority. It was proved that exclusive breastfeeding during the first month of life can safeguard the baby from asthma (Gdalevich *et al.*, 2001a) and especially lactic acid bacteria present in the milk have been found to be highly effective against atopic dermatitis (Gdalevich *et al.*, 2001b; Kalliomäki *et al.*, 2001). Further, these bacteria are also known to enhance gut-associated lymphoid tissue and anti-allergic processes. Hence, healthy human milk is considered to be a potential source of effective probiotics which will impart a biotherapeutic effect to infants against many diseases.

17.10 Microbiota and its Future in Wealth Generation

Microbes are considered to play an important role in the catabolism of cholesterol in the gut, production of ultraviolet light-absorbing molecules on the skin, and secretion of pathogen-killing antimicrobials in the urogenital or respiratory tracts. Along with these, it may be possible to engineer our microbiota to produce diffusible small molecules that enter our bloodstream, cross the blood–brain barrier and exert neurological activities. In future, microbiota may be engineered to produce stimulants, antidepressants and satiety-inducing drugs and, if designed thoughtfully, microbial production could be modulated by dietary inputs dictated by the host or by microbial sensing of host biochemistry. The delivery of drugs, therapeutic peptides or vaccine antigens by microbes at mucosal surfaces holds tremendous promise for the treatment of diverse diseases.

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18 Biotechnological Production of Polyunsaturated Fatty Acids

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Abstract
The market for omega-3 fatty acids or polyunsaturated fatty acids (PUFAs) for biodiesel production is continuously increasing. The main source of omega-3 fatty acids is from the extraction of oil from cold-water fish that usually inhabit deep-water environments, whereas biodiesel is produced from vegetable oils. Microbial oil can be an interesting alternative for the production of PUFAs. This chapter reviews the production, extraction, chemical characterization and future trends on microbial oil production for applications in the food, pharmaceutical and chemical industries. From the analysis of several studies available in the literature, it has been shown that the production of lipids by the biotechnological route can be an effective way to complement the production of fatty acids for biodiesel production or to substitute the traditional extraction of fish oil for production of PUFAs. However, more technologies should be developed to enable the industrial production of microbial lipids.

18.1 Introduction

Fungi are eukaryotic organisms that are among the major components of the tree of life and their current classification includes heterotrophs that feed by absorption with branched development and reproduce by spores (Kendrick, 2000). Yeasts and fungi are considered as potential sources of oil, since they can accumulate large amounts of lipids, and yields of methyl esters are very satisfactory (Vicente *et al.*, 2010). According to Ratledge (1996), yeasts and fungi are the major classes of microorganisms used for the production of lipids and they are considered to be oleaginous microorganisms, since up to 40% of their biomass can be lipids. The substrate and its constituents determine the efficiency of oil production. The most frequently used substrates are glycerol and glucose. Generally the production of lipids is greater where there is a high ratio of carbon to nitrogen (C:N). The operational conditions in terms of agitation and temperature can determine the amount of each unsaturated fatty acid that is produced. The oxygenation, temperature, pH and nutrients available in the medium determine the characteristics of the lipids produced.

Fatty acids are constituents of cells, exerting structural, energy and signalling functions, among others. The polyunsaturated fatty acid (PUFA) families include omega-6 and omega-3 fatty acids, such as linoleic acid and α-linolenic acid, respectively. These acids are essential because they play an important role in the nervous system activity and development of the organism (Clemente *et al.*, 2007). Regarding the size of

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the carbon chain, PUFAs with more than 16 carbon atoms are called, by some authors, the long-chain fatty acids, whereas PUFAs with greater than 22 carbon chain atoms are called very long-chain fatty acids. In the literature it is reported that 84% of cardiovascular diseases are associated with low rates of omega-3, especially eicosapentaenoic acid (EPA), docosahexaenoic acid (DHA) and docosapentanoic acid (DPA). The consumption of omega-3 fatty acids is scientifically proven to help in the prevention of myocardial infarction, arrhythmia, atherosclerosis and hypertension. Among the omega-3 fatty acids, there is special interest in EPA, due to its contribution to the control of coronary diseases listed above, in addition to its beneficial effects on vasodilation and platelet activation (Jackowski *et al.*, 2012).

Considering that omega-3 fatty acids are important compounds for a variety of nutraceuticals and pharmaceuticals, the market for these fatty acids is continuously increasing. At the moment, the main commercial sources of omega-3 fatty acids are from the extraction of oil from cold-water fish that usually inhabit deep-water environments. The accumulation of omega-3 fatty acids in marine fish is actually due to the ingestion of seaweed (Cao *et al.*, 2012). In the long term, marine fish oil will not be able to meet the growing demand for these fatty acids, given that the fish population could shrink dramatically or even disappear due to overfishing. Furthermore, the production of omega-3 fatty acids from fish oil has some disadvantages, such as unattractive odour, contamination, high cost and instability following purification (Wen and Chen, 2001). Concerns about the quality and availability of the supply of fish oil have generated interest in the production of omega-3 fatty acids by an alternative, sustainable and low-cost source, for example its production by the biotechnological route.

The PUFAs also play an important role as a raw material for biodiesel production. However, the chemical structure and the contents of each of the substances present in the raw material alter the physicochemical properties of the biofuel. Brazil stands out as the leading country in the production of biodiesel due to its ability to produce biodiesel from vegetable oil extracted from a variety of oleaginous grains. In Brazil, biodiesel is blended with diesel at a proportion of 5% and it is called B5. To supply the actual demand, the installed capacity of production is approximately 5.1 million m^3 of biodiesel (Poli, 2014). As a consequence, large areas of land are used for cultivating oilseeds for biodiesel production, generating much discussion because of the competition between biodiesel and food production. An alternative is the use of the biotechnological route for the production of PUFAs, which, in this case, are the raw material for biofuel production (Ratledge, 2004). The microbial oil has many advantages compared with cultivating oilseeds for biodiesel production such as: (i) the raw materials are available in large quantities and at low cost; (ii) there is less influence on production from the environment, seasons and climate; and (iii) the ability to increase production (Poli, 2014). However, biodiesel production from microbial oil is dependent of the fatty acids that are dependent on the microorganism employed for oil production.

Based on the aspects presented above, the production of microbial oil is seen as an interesting alternative for PUFAs for food and pharmaceutical applications as well as production of microbial oil as an alternative source of raw material for the biodiesel industry. This chapter addresses some of the important aspects relating to the production of microbial oil using yeast and fungi.

18.2 Microbial Metabolism for PUFA Production

It is important to know how the production of PUFAs by microorganisms occurs because in this way conditions can be optimized to obtain better yields. The lipids can be produced in two ways: (i) by *de novo* synthesis, involving a metabolic pathway using precursors of fatty acids such as acetyl-coenzyme A (CoA) and malonyl-CoA to produce PUFAs; and (ii) by accumulation of intracellular lipids, which involves the uptake of fatty acids, oil and triacylglycerols from the culture medium and their accumulation in the cell where they may be modified (Beopoulos *et al.*, 2009).

Liang and Jiang (2013) showed that the *de novo* synthesis occurs when the oleaginous microorganisms use glucose as the carbon source,

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with conversion of glucose to pyruvate in the cytoplasm. By moving to the mitochondria, pyruvate is converted to acetyl-CoA, which condenses with oxaloacetate, thereby forming the citrate. When the amount of mitochondrial citrate is too high, it comes out of the organelle and moves to the cytoplasm where it is cleaved to form acetyl-CoA and oxaloacetate.

The accumulation of lipids by the microorganisms depends of many factors, but the ratio of carbon:nitrogen (C:N) is particularly critical. Excess carbon and nitrogen in limiting conditions favours the process because the microorganism rapidly depletes the nitrogen source, but continues to assimilate carbon, resulting in the accumulation of triglycerides. For lipid production to occur it is essential that there is a supply of intracellular nicotinamide adenine dinucleotide phosphate (NADPH) and acetyl-CoA. The species of oleaginous microorganisms, unlike other microorganisms, possess the enzyme ATP-citrate lyase, which catalyses the reaction between citrate and CoA, forming acetyl-CoA and oxaloacetate. Initially, there is an increase in the activity of adenosine monophosphate (AMP) deaminase, decreasing the amount of AMP in the cell. Thus inhibition of the enzyme isocitrate dehydrogenase, which is dependent on AMP, occurs in oleaginous microorganisms. Then, isocitrate is not metabolized and is balanced with the action of citrate via aconitase, with the accumulation of citrate in the mitochondria. Mitochondrial citrate goes to the cytoplasm and is used by ATP-citrate lyase in the formation of oxaloacetate from acetyl-CoA, which is used in the synthesis of fatty acids (Ratledge, 2004).

18.3 Oleaginous Microorganisms

Oleaginous microorganisms are species with an oil content greater than 20% in the whole biomass (Meng *et al.*, 2009). Microbial oil is produced by certain microorganisms, such as yeasts, fungi, bacteria and algae. The best known oleaginous yeasts are of the genus *Candida*, *Cryptococcus*, *Rhodotorula*, *Trichosporon* and *Yarrowia*. These yeasts are of great relevance considering that an average value for the accumulation of lipids corresponds to 40% of their biomass, and in optimal conditions this accumulation may reach 70% of their biomass (Beopoulos *et al.*, 2009). There are also species of fungi, such as *Aspergillus terreus*, *Claviceps purpurea*, *Tolyposporium*, *Mortierella alpina*, *Mortierella isabellina* and *Rhizopus oryzae* which accumulate lipids. Table 18.1 shows some species of yeasts and fungi that produce lipids, the substrate used and lipid content.

It can be seen from Table 18.1 that the highest microbial oil yield for yeast were for the species *Rhodosporidium toruloides* and *Rhodotorula glacialis* DBVPG 4785, with 67.5% and 68% of their dry weight biomass, respectively. It is important to mention that the substrate used is glucose which facilitates the production of PUFA, because there are no intermediate reactions in the process, since the biochemical pathway of most microorganisms begins with the conversion of glucose to pyruvate. The composition of the oil obtained is similar for both microorganisms, with the exception of the palmitic acid and α-linolenic acid produced by *R. toruloides*, which were not produced by *R. glacialis* DBVPG 4785. Regarding the microbial oil-producing fungi, *Mortierella isabellina* provided the best results. In the studies by Economou *et al.* (2011) and Gao *et al.* (2013) the microbial oil yields were around 64% with *M. isabellina*, using different substrates. Evaluating the chemical composition of the oil produced showed an identical profile of PUFAs in both studies.

18.4 Production, Extraction and Chemical Characterization of Microbial Oil

18.4.1 Production of Microbial Oil

Analysing Table 18.1, it is seen that the microbial oil yields obtained are high in some studies, but this does not guarantee the success of the process from an industrial viewpoint. In some cases, the procedure for oil extraction is too expensive to apply for production at scale. For this reason, the viability of production of microbial oil is dependent of high conversion of substrate to intracellular lipids combined with high extraction efficiency at low energy consumption. Often there are cases in which the yield is satisfactory,

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Table 18.1. Lipid production by yeasts and fungi.

Microorganism	Substrate	Lipid (% of dry weight biomass)	Chemical composition of oil	Reference
Yeast				
Rhodosporidium toruloides	Biodiesel waste (industrial glycerol)	41.0	Oleic, stearic, linoleic and palmitic acids	Kiran *et al.* (2013)
R. toruloides and *Rhodotorula glutinis*	Levoglucosan	44.2	Oleic, stearic, linoleic and palmitic acids	Lian *et al.* (2013)
R. toruloides	Glucose	67.5	Palmitic, palmitoleic, stearic, oleic, linoleic and α-linoleic acids	Koutinas *et al.* (2014)
Rhodotorula glacialis DBVPG 4785	Glucose	68.0	Oleic, stearic, linoleic and palmitoleic acids	Amaretti *et al.* (2010)
Torulaspora globosa YU5/2	Glucose	45.7	Total lipid	Leesing and Baojungharn (2011)
Trichosporon cutaneum	Maize cob	45.4	Oleic, stearic, linoleic and palmitic acids	Chen *et al.* (2013)
Yarrowia lipolytica QU21	Glucose and glycerol	30.1	Oleic and linoleic acids	Poli *et al.* (2014)
Fungi				
Aspergillus terreus IBB M1	Glucose	54.0	Oleic, palmitic and stearic acids	Khot *et al*. (2012)
Cryptococcus curvatus	Glycerol	52.0	Oleic, palmitic, stearic and linoleic acids	Liang *et al.* (2010)
Mortierella isabellina	Rice hull hydrolysate	64.3	Oleic, palmitic and linoleic acids	Economou *et al.* (2011)
M. isabellina	Xylose	64.5	Oleic, palmitic and linoleic acids	Gao *et al.* (2013)
Mortierella sp.	Glucose, fructose, sucrose and lactose	43.5	Oleic, palmitic, stearic and linoleic acids	Kumar *et al.* (2011)
Sporobolomyces carnicolor	Hexose, pentose other polysaccharides	50.0	Oleic and linoleic acids	Matsui *et al.* (2012)
Trichosporon capitatum	Sugarcane molasses	43.1	Oleic acids	Wu *et al*. (2011)

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but there are high costs for the extraction and purification of the final product of interest. So it is important to know the processes for the production of PUFAs and the steps required for extracting them. Table 18.2 shows some processes used in the production of PUFAs reported in the literature.

Some studies reported in Table 18.2 deserve more attention and are discussed in a little more detail here. Liang *et al.* (2010) employed a 2 l fed-batch reactor with a paddle stirrer, obtaining a lipid yield of 52%. In this work, the authors carried our two fed-batch fermentations using glycerol as the substrate. For the first fermentation in the fed-batch process, the initial feed concentration of glycerol was 25.8 g/l and the substrate and a nitrogen source (NH_4Cl) were fed intermittently at different times. For the second fermentation, the glycerol concentration was 30 g/l and the substrate and the nitrogen source (NH_4Cl) were added discontinuously in the process medium (day 6). Both fermentations occurred in a time interval of 12 days.

Kiran *et al.* (2013) also used the fed-batch process to obtain microbial oil and obtained a yield of 41%. The solid state fermentation was supplemented with glycerol at the start of the process and every 24 h to keep the C:N ratio in the range of 70–90. The nitrogen source was free amino nitrogen (FAN) and the fermentation occurred over 3 days. Amaretti *et al.* (2010) obtained a lipid yield of 68% using different substrate concentrations and temperatures in a 2 l batch bioreactor to optimize the production of PUFAs. The glucose concentration used was 120 g/l and a temperature of 20°C was considered to be the optimum for the growth of microorganisms. Other work that is important to highlight is that published by Santamauro *et al.* (2014), because they used a bioreactor of

Table 18.2. Processes used to produce PUFAs with substrates, microorganisms and yields obtained.

Microorganism	Substrate	Processes	Lipid (% of dry weight biomass)	Reference
Aspergillus terreus	Sugarcane bagasse	Batch with 100 ml	19.0	Kamat *et al*. (2013)
A. terreus IBB M1	Glucose	Batch with 50 ml	54.0	Khot *et al*. (2012)
Colletotrichum sp. and *Alternaria* sp.	Glucose, sucrose, fructose, lactose and xylose	Batch with 250 ml	50.0	Dey *et al*. (2011)
Cryptococcus curvatus	Glycerol	Fed-batch with 2 l	52.0	Liang *et al*. (2010)
Metschnikowia pulcherrima	Lignocellulose	Batch with 500 l	40.0	Santamauro *et al*. (2014)
Mortierella isabellina	Rice hull hydrolysate	Batch with 50 ml	64.3	Economou *et al*. (2011)
M. isabellina	Whey	Batch with 100 ml	21.3	Demir *et al*. (2013)
M. isabellina	Wheat bran	Batch with 50 ml	53.0	Zeng *et al*. (2013)
Rhodosporidium toruloides	Biodiesel waste (industrial glycerol)	Fed-batch with 1 l	41.0	Kiran *et al*. (2013)
Rhodotorula glacialis DBVPG 4785	Glucose	Batch with 2 l	68.0	Amaretti *et al*. (2010)
Thamnidium elegans and *Zygorhynchus moelleri*	Olive mill wastewater	Batch with 1.5 l	60.0	Bellou *et al*. (2014)
Torulaspora globosa YU5/2	Glucose	Batch with 50 ml	45.7	Leesing and Baojungharn (2011)
Trichosporon cutaneum	Maize cob	Batch with 50 ml	45.4	Chen *et al*. (2013)
Trichosporonoides spathulata and *Kodamaea ohmeri*	Crude glycerol	Batch with 100 ml	53.3	Kitcha and Cheirsilp (2011)
Yarrowia lipolytica	Glucose	Batch with 25 ml	30.0	Xue *et al*. (2013)

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500 l, exploiting the accumulation of lipids using *Metschnikowia pulcherrima* in a large-scale process. The culture medium was optimized with a reduced content of glycerol to establish the cultures in two open-air bioreactors with a capacity of 500 l, located in a heated oven. The bioreactor was inoculated with a 500 ml culture of *M. pulcherrima* and the medium was stirred by well-fitted blades rotating at 10 rpm and aerated through two nozzles on opposite sides of the pond. The process took place for 28 days.

18.4.2 Extraction of Microbial Oil

Regarding the methods used for the extraction of PUFAs from microbial cells, Table 18.3 presents some processes/procedures reported in the literature. It is important to note that most methods that have been employed for extraction are for a laboratory-scale process, without the preoccupation of technical and economical feasibility of extraction on an industrial scale. In addition, it is important to clarify that many

Table 18.3. Methods used for the extraction of polyunsaturated fatty acids (PUFAs).

Methodology	Solvents	Reference
The extraction was performed using a mixture of methanol and chloroform, which was centrifuged and afterwards evaporation of solvent was carried out (Bligh-Dyer method)	Methanol: chloroform	Matsui *et al.* (2012)
Extracted by the method according to Folch *et al*. (1957) using chloroform and methanol 2:1 (v/v) determined gravimetrically after evaporation of the solvent *in vacuo*	Methanol: chloroform	Bellou *et al.* (2014)
The mycelia was separated by centrifugation then washed with water and lyophilized. The lipids were extracted from the biomass using chloroform, methanol and water	Methanol: chloroform	Xia *et al.* (2011)
Lipid extraction from the dry matter was performed using hexane and after extraction the solvent was removed by evaporation and the oil was weighed	Hexane	Economou *et al.* (2011)
The extraction was performed with chloroform: methanol (2:1) using a Soxhlet extractor according to the method used by Bligh and Dyer (1959)	Methanol:chloroform	Kumar *et al.* (2011)
The lipids were extracted from lyophilized matter in accordance with the procedure of Bligh and Dyer. After the mixture was extracted with chloroform: methanol (2:1, v/v) for 1 h, the mixture was centrifuged and then the solvent evaporated *in vacuo*	Methanol: chloroform	Wu *et al.* (2011)
The extraction was performed with chloroform: methanol (1:1) using a Soxhlet extractor. The extraction time was 2 h at 140°C	Methanol: chloroform	Kiran *et al.* (2013)
The lipid extraction of the dried biomass was in accordance with the procedure of Folch *et al*. (1957) using hexanes with a mixture of chloroform:methanol (2:1, v/v). The mixture was centrifuged and then the solvent evaporated	Methanol: chloroform	Chen *et al.* (2013)
Dried microbial mass was mixed in a tank with 25% hexane (w/w) for extraction. Afterwards the microbial cells were disrupted in a homogenizer	Hexane	Koutinas *et al.* (2014)
Lipid was extracted by an adaptation of a method reported by Bligh and Dyer. The samples were stirred in a mixture of chloroform and methanol (2:1 w/v) over 48 h and the biomass was filtered and washed with additional chloroform	Methanol: chloroform	Santamauro *et al.* (2014)
Total cellular lipid was extracted from the dried mycelia with a mixture of chloroform/methanol 2: 1 (v/v) and weighed after evaporation of the solvent in a rotary evaporator	Methanol: chloroform	Zikou *et al*. (2013)
The lipids were extracted from the dry matter of pelleted cells using the extraction method of Bligh and Dyer with methanol and chloroform	Methanol: chloroform	Schneider *et al.* (2013)

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researchers use a particular method of extraction as a sample preparation step for future determinations of the composition. If the extraction is carried out with the aim of sample preparation, the greatest concern is to minimize possible interference in the recovery of the desired products, without any cost–benefit analysis of the final product.

Generally the conventional processes of extraction use hexane or a mixture of organic solvents, which present some disadvantages. These include: (i) there is degradation of some constituents of microbial oil produced due to the high temperatures needed during extraction and solvent removal; and (ii) these organic solvents solubilize other components of the cell wall of the microorganism (Bertholet *et al*., 2004). In an attempt to minimize the negative effects of the use of organic solvents, different extraction methods have been reported (Bertholet *et al.*, 2004; Ranjan *et al.*, 2010; Halim *et al.*, 2011; Mercer and Armenta, 2011; Pieber *et al.*, 2012). Among the proposed technologies has been the use of supercritical carbon dioxide (CO_2) (Sahena *et al.*, 2009; Walker *et al.*, 2009; Catchpole *et al.*, 2009; Nisha *et al.*, 2012), supercritical CO_2 combined with using a co-solvent (Rodríguez-Meizoso *et al.*, 2008; Pieber *et al.*, 2012), microwave (Camel, 2001) and ultrasound (Adam *et al.*, 2012).

18.4.3 Chemical Characterization of Microbial Oil

Regarding the analytical methods reported in the literature for chemical characterization of PUFA, gas chromatography (GC) predominates. Some of the methods and procedures will be presented here to give readers an idea of the range of methods that may be adopted for chemical characterization of microbial oil.

Qiao *et al.* (2013) evaluated the chemical composition of PUFAs obtained from *Penicillium chrysogenum* by liquid chromatography–electrospray ionization and gas chromatography–mass spectrometry (GC–MS) and identified compounds such as linoleic acid and hexadienoic acid. Singh *et al.* (2013) evaluated the chemical composition of PUFAs by gas chromatography–flame ionization detector (GC–FID) obtaining a varied chemical composition of oleic, palmitic, palmitoleic and stearic acids. The chemical composition of microbial oil from a fungus (*Pythium irregulare*) was determined by GC–FID by the authors Lio and Wang (2013), and they reported compounds such as linoleic and and α-linoleic acids.

Shahnaz and Shameel (2009) found palmitic and oleic acids in the chemical composition analysed by GC–MS, whereas Zhang *et al*. (2006) used column chromatography for purification of arachidonic acid (ARA) extracted from fungal cells. Cipak *et al*. (2006) characterized microbial oil from yeast by GC–MS and found compounds such as hexadienoic and linoleic acids. It can be seen that most of the fatty acids reported in the studies consists of linoleic acid and its derivatives, determined by GC coupled with a mass detector or GC–FID.

18.5 Microbial Oil's Applications

The application of microbial oil will depend on the profile of fatty acids produced, which is dependent on the microorganism and culture conditions. Yeasts, unlike many filamentous fungi, tend to produce a limited amount of PUFAs, and some strains may have relatively high levels of stearic acid (Ratledge, 2005). Some species of filamentous fungi are exploited for their ability to produce special lipids, such as DHA, γ-linolenic acid (GLA), ARA and EPA (Liang and Jiang, 2013). In recent years, attention has been focused on the use of microorganisms as alternatives for industrial and food applications (Garay *et al.*, 2014). Microbial PUFAs may be used as functional foods, and as a raw material for biodiesel production (Zhao *et al*., 2014).

PUFAs also have various physiological functions and are therefore widely used in food, medicine and cosmetics (Papanikolaou *et al.*, 2002). According to Gołębiowski *et al*. (2014), PUFAs play a variety of roles including having significant antifungal activity. PUFAs are also important in skin care, promoting hair growth, and such natural fats and oils may be added to cosmetics although the nutritional protective effect of ARA and GLA to skin has not been shown (Zhao *et al*., 2014). Biotechnological techniques based on solid state fermentation and genetic engineering have been used to prepare functional cereals naturally enriched with PUFAs (Certík *et al*., 2013). According to Dong and Walker (2008)

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PUFAs and additives when used in pharmaceutical products have proved beneficial to the heart and for prevention of circulatory diseases, cancer and inflammatory diseases. Wongsakul *et al*. (2003) similarly reported on the health benefits of PUFAs including reducing the risk of coronary heart disease, preventing certain cancers and improved immune function. Indeed a number of authors have recorded the health benefits of these fatty acids including: (i) that they have a significant effect on lowering blood pressure (Engler and Engler, 1998); (ii) that they may reduce the risk of colon cancer as shown by several epidemiological studies (Fan *et al*., 2003); and (iii) they can be used for the prevention of cardiovascular and metabolic diseases (Sealls *et al*., 2008).

The importance of omega fatty acids in health is mainly due to their role in affecting inflammatory manifestations in the body. The interactions between pro-inflammatory molecules derived from omega-6 and anti-inflammatory actions of molecules derived from omega-3 underlie the significant cardiovascular benefits attributed to increased consumption of omega-3 and at the same time decreasing consumption of omega-6 (Zhao *et al*., 2014).

In the petrochemical industry biodiesel can be produced by esterification of fatty acids from microbial oil. The composition of most microbial oil is similar to that of vegetable oils in general, including C16 and C18 fatty acids such as oleic acid, palmitic acid, stearic acid and linoleic acid, so vegetable oil used in the production of biodiesel can be substituted by microbial oil for both technical and economic reasons (Zhao *et al*., 2010).

18.6 Future Trends

When assessing the future prospects of technology deployment is important to carefully evaluate what is being deposited as industrial patents. Apt *et al*. (2014) have a patent for the production of microbial oil using yeast strains including *Pseudozyma aphdis*, *Pseudozyma rugulosa*, *Sporidiobolus pararoseus* and *Rhodotorula ingeniosa* and a large range of other microorganisms which produce at least 20–75% of their dry cell weight as oil. Jackson *et al*. (2012) also have a patent for the production of PUFAs in oleaginous yeasts, producing omega-3 and/or omega-6 fatty acids from yeast grown on a fermentable carbon source consisting of inverted sucrose, glucose, fructose and combinations thereof. This invention provides a method for producing PUFAs by transgenic oleaginous yeast, producing at least 25% of its dry cell weight as oil. The preferred oleaginous yeast is selected from a group consisting of *Yarrowia*, *Candida*, *Rhodotorula*, *Rhodosporidium*, *Cryptococcus*, *Trichosporon* and *Lipomyces*. The most preferred species is *Yarrowia lipolytica*. Another patent deposited by Akimoto *et al*. (2011) reported on a way to artificially treat microorganisms that have an ability to produce and intracellularly accumulate lipid vesicles containing unsaturated fatty acids. Further research is required to identify other processes to produce PUFAs with high yields in order to improve both the technical and the economic viability.

18.7 Concluding Remarks

This chapter has reviewed the production, extraction, chemical characterization and future trends on microbial oil production for applications in the food, pharmaceutical and chemical industries. More specific fatty acids with properties that are useful for the food and pharmaceutical industries are produced mainly by fungi, whereas microbial oil from yeast can be used for biodiesel production. The yield of microbial oil is dependent on the microorganism, the cultivation conditions and the extraction process used. In general, extraction using organic solvents is employed, but there is a growing interest in the use of green procedures for obtaining solvent-free oil with less expenditure of energy. This chapter has shown that the production of lipids by the biotechnological route can be an effective way to complement the production of fatty acids for biodiesel production or to substitute the traditional extraction of fish oil for production of PUFAs. However, more technologies should be developed to enable the industrial production of microbial lipids.

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19 Functional Enzymes for Animal Feed Applications

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Abstract
The great demand for animal feed additives is directly proportional to the increased world human population. Such increase in demand has inspired scientists to look for alternatives to traditional feed additives in order to cope with the increased requirements in animal feeds which ultimately enter the human food chain. Antibiotics have been used traditionally as animal feed additives for their various beneficial effects. However, their application has been recently banned after discovering several potential side effects, which will ultimately enter the human food chain, resulting in many health problems. In addition to probiotics and prebiotics, functional enzymes are considered to be one of the major sources for non-traditional feed additives, and they have been intensively investigated in the last two decades. This chapter highlights the application of different functional enzymes as animal feed additives. Moreover, the different groups of phytases and xylanases will be discussed in detail for their role as feed additives, including their characterization as well as their microbial production and application.

19.1 Introduction

The current world human population reached 7.3 billion in 2015 and is expected to reach 9.7 billion in 2050 as reported in the United Nations 2015 Revision (UN DESA, 2015). In addition, the world is expected to experience food shortages due to environmental climate changes, which are the main factors contributing to food shortages in many countries such as India, China, the Horn of Africa and South Asian countries. Therefore, healthy food requirements should be achieved to cover the production cost and the increasing demand for food as well as animal products (Barekatain *et al*., 2013). Antibiotics have been traditionally applied as feed additives to cover the low quality of animal products and to enhance the growth and development of such animals. However, the use of antibiotic growth promoters are understood to create a lot of problems for animal health such as increasing resistance of pathogens while at the same time affecting the beneficial microbial flora normally present in the animal intestine. Such problems will affect the general health of animals, their growth, development and products, which will be eventually reflected on the end users (i.e. humans), who will have to suffer

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from the side effects of the materials added as feed. Alternatively, different feed additives have been applied as antibiotic replacements in animal feeding, including functional enzymes, probiotics, prebiotics, minerals, herbs and mushrooms to increase animal production and thus generate additional cost savings on animal farms (Gao *et al.*, 2013). Such feed additives are used to increase the health, fertility and performance of animals through the improvement of the feed conversion ratio mainly by regulating feed intake. Moreover, they are used to cover the needs for essential nutrients such as phosphate from the hydrolysis of phytate in plant fibres through the addition of exogenous phytase enzymes. Additionally, they increase the digestibility, the absorption of nutrients and the energy uptake, thus contributing to increasing the production of animal products (Cowieson *et al.*, 2004). Nowadays, it is well established that the addition of probiotics in animal feeds may increase the colonization of microorganisms in the animal intestine and therefore increase feed digestion and absorption (Liu *et al.*, 2011). Moreover, better nutrient availability can be achieved by supplying highly available forms of nutrients or by applying special feeds designed according to the daily animal needs.

In most countries, the use of feed additives in animal production is strictly subject to government regulations. Wenk (2002) lists different substances which have been approved by the European Union (EU) for their application as animal feed additives as follows:

- substances with antioxidative effects;
- flavours and substances affecting food intake;
- coccidiostats;
- emulsifiers, stabilizers (e.g. organic acids);
- colouring substances;
- preservatives;
- vitamins, pro-vitamins;
- trace elements;
- binding substances;
- probiotics; and
- enzyme mixtures.

Since 1995, the EU has banned the use of growth and performance promoters such as antibiotics (Ravindran and Son, 2011), human growth hormones and metabolic modifiers such as β-agonists (Wenk, 2002). Therefore, functional enzymes have been applied in order to replace antibiotics and other undesirable additives in animal feeding (Bedford and Schulze, 1998).

Nowadays, exogenous functional enzymes are widely used in animal feeds to improve nutrient availability, feed intake, digestibility, nutrient absorption and the colonization of microorganisms in the animal intestine (Beauchemin *et al.*, 2004). The application of exogenous enzymes is not only limited to ruminants but is also used for monogastric species for the reduction of viscosity and transit time in the lower digestive tract in order to increase nutrient availability and absorption. In addition, natural medicinal products originating from herbs or fungi such as mushrooms have been used as supplements in ethno-veterinary medicine (Guo *et al.*, 2003, 2004). These bioactive compounds, mainly poly and oligosaccharides as glycosides, alkaloids, volatile oils and organic acids have been found to exert their potential effects by acting as prebiotic substances for growth promotion and immune enhancement (Verstegen and Schaafsma, 1999; Guo *et al.*, 2004).

19.2 Application of Functional Enzymes in Animal Feeding

In the last two decades, many investigations have proven that the addition of functional enzymes such as fibrolytic enzymes, pectinases and amylases to animal feed can increase milk production and animal weight. This was attributed to the better digestibility and absorption of such nutrients (Beauchemin *et al.*, 2004; Tricarico *et al.*, 2008; van de Vyver and Useni, 2012). Different enzyme preparations have been generally used to improve the intestinal health of the animal. However, their application should be based on the required effect and the animal needs (i.e. to increase the availability of essential nutrients, feed intake or the growth performance) (Wenk, 2002). Table 19.1 summarizes some of the functional enzymes applied as animal feed additives together with the microorganisms that produce them and their functions.

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Table 19.1. Enzymes that are currently used in animal feed. (Adapted from Pariza and Cook, 2010.)

Trivial name	Classification	Production organism	Function
α-Amylase	Carbohydrase	*Aspergillus* sp., *Bacillus* sp., *Rhizopus* sp., animal pancreatic tissue, barley malt	Hydrolyses *Rhizopus* starch
Maltogenic α-amylase	Carbohydrase	*Bacillus subtilis*, *Bacillus stearothermophilus*	Hydrolyses starch with production of maltose
β-Amylase	Carbohydrase	Barley malt	Hydrolyses starch with production of maltose
Cellulase	Carbohydrase	*Aspergillus niger*, *Humicola insolens*, *Trichoderma reesei*	Breaks down cellulose
α-Galactosidase	Carbohydrase	*A. niger*, *Mortierella vinaceae* var. *raffinoseutilizer*, *Saccharomyces* sp.	Hydrolyses oligosaccharides
β-Glucanase	Carbohydrase	*Aspergillus* sp., *Bacillus* sp., *H. insolens*, *Penicillium funiculosum*, *T. reesei*	Hydrolyses β-glucans
β-Glucosidase	Carbohydrase	*A. niger*	Hydrolyses cellulose degradation products to glucose
Glucoamylase (amylo-glucosidase)	Carbohydrase	*Aspergillus* sp., *Rhizopus* sp.	Hydrolyses starch with production of glucose
Hemicellulase	Carbohydrase	*Aspergillus* sp., *Bacillus* sp., *H. insolens*, *T. reesei*	Breaks down hemicellulose
Invertase	Carbohydrase	*A. niger*, *Saccharomyces* sp.	Hydrolyses sucrose to glucose and fructose
Lactase	Carbohydrase	*Aspergillus* sp., *Candida pseudotropicalis*, *Kluyveromyces marxianis* var. *lactis*	Hydrolyses lactose to glucose and galactose
β-Mannanase	Carbohydrase	*A. niger*, *Bacillus lentus*, *T. reesei*	Hydrolyses β-mannans
Pectinase	Carbohydrase	*Aspergillus aculeatus*, *A. niger*, *Rhizopus oryzae*	Breaks down pectin
Pullulanase	Carbohydrase	*Bacillus* sp.	Hydrolyses starch
Xylanase	Carbohydrase	*Aspergillus* sp., *Thermomyces lanuginosus*, *H. insolens*, *P. funiculosum*, *T. reesei*	Hydrolyses xylans
Lipase	Lipase	*Aspergillus* sp., *Candida rugosa*, *Rhizomucor miehei*, *Rhizopus oryzae*, animal pancreatic tissue, animal forestomach	Hydrolyse triglycerides, diglycerides and glycerol monoesters
Ficain	Protease	Fig (*Ficus glabrata*)	Hydrolyses proteins
Keratinase	Protease	*Bacillus licheniformis*	Hydrolyses proteins
Papain	Protease	Papaya (*Carica papaya*)	Hydrolyses proteins
Pepsin	Protease	Animal stomachs	Hydrolyses proteins
Protease	Protease	*Aspergillus* sp., *Bacillus* sp.	Hydrolyses proteins
Trypsin	Protease	Animal pancreas	Hydrolyses proteins
Catalase	Oxido-reductase	*A. niger*, *Micrococcus lysodeikticus*	Produces water and oxygen from hydrogen peroxide
Glucose oxidase	Oxido-reductase	*A. niger*	Degrades glucose to hydrogen peroxide and gluconic acid
Phytase	Phosphatase	*Aspergillus* sp., *P. funiculosum*, *Schizosaccharomyces pombe*	Hydrolyses phytate

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19.3 Phytases (Chemical Abstracts Service (CAS) # 37288-11-2)

Phytase (myo-inositol hexakisphosphate-phosphohydrolase) is a phosphatase enzyme which catalyses the hydrolysis of phytic acid with the release of organic phosphorus found in grains and seeds making it available for animals (Mullaney *et al.*, 2000, 2002). Phytase enzymes have been found in animals, plants, fungi and bacteria, however, the fungal phytase is the most studied and characterized (Mullaney and Ullah, 2003). According to the International Union of Biochemistry and Molecular Biology (IUBMB), phytase is classified based on the location of the first phosphate within the inositol ring that is the first phosphate group hydrolysed during synthesis (Oh *et al.*, 2006). The Enzyme Nomenclature Committee of the IUBMB recognizes different types of phytase enzymes based on the position of the first phosphate hydrolysed, and these are 3-phytase (EC 3.1.3.8), 4- or 6-phytase (EC 3.1.3.26) and 5-phytase (EC 3.1.3.72). Phytase is active over a wide range of pH, from 4.5 to 7.0, based on the producing microorganism. Bacterial and fungal phytases have the phosphate in phytic acid at position 3. Unusually, plant phytases release the first phosphate at position 6 of the inositol ring before releasing the remaining phosphate groups (Greiner *et al.*, 2002).

When phytic acid is not properly digested in non-ruminant digestive tracts, it acts as a chelating agent for some of the divalent cations such as Zn, Fe, Ca and Mg. This will affect the bioavailability of these cations in our body. The deficiency of these minerals can cause health problems since they are important for bone and teeth structure. Furthermore, mineral deficiency may disturb energy and metabolic processes, the building of protein, the formation of blood and the digestibility of proteins, starch and lipids under neutral, acidic and alkaline conditions (Lei and Porres, 2003). It has been found that supplementation of sodium phytate to glucose-based diets in broiler feeds increases the endogenous losses of amino acids and minerals (Cowieson *et al.*, 2004). Therefore, most animal feeds are supplemented with inorganic phosphate. However, the excessive supplementation of inorganic phosphate and its low absorption in the gastrointestinal tracts of monogastric or even in ruminant animals may result in the excretion of excessive phosphorus in the effluent of animals, especially in the case of pigs and poultry (Chesson, 1993). This causes ecological problems such as eutrophication across the globe, resulting from pollution of water by phosphorus from human and animal wastes (Naqvi *et al.*, 2000; Vats *et al.*, 2005). Therefore, phytase is an important additive in animal feeds in order to hydrolyse the non-digestable phytic acid in feeds.

19.3.1 Characterization and production of phytase

Phytase activity is usually measured by the amount of inorganic phosphate released per minute from a selected substrate under optimum pH and temperature conditions. For commercial applications, most of the isolated phytase should have a relatively high specific activity at a wide range of pH, between 2.0 and 6.0, in order to tolerate the digestive tract environment of the animals. This is very important for monogastric animals which have an acidic stomach (pH 2.0–3.0) and an alkaline small intestine (pH 4.0–7.0). Therefore fungal phytases have been used because they can withstand the acidic conditions of the upper intestinal tract. Most of the commercialized phytases present in the market are produced by soil fungal strains such as *Aspergillus niger* or *Aspergillus ficuum*. However, instead of giving higher enzymatic activity in acidic conditions, they have higher specific activities, almost 100 IU/mg of protein, and they are thermostable at high processing temperatures (van Gorcom *et al.*, 1995). Table 19.2 summarizes different molecular and biochemical characteristics for different phytase enzymes. It can be observed that fungal phytases are acidophilic in nature and are also known as histidine acid phosphatases (HAPs). They have an optimum pH range from 2.5 to 5.0, and exhibit minor activities above pH 5.5, which is compatible with the internal environment of the digestive tract of monogastric animals (Ullah and Gibson, 1987; van Gorcom *et al.*, 1995; Oh *et al.*, 2004). Currently, Natuphos®, a phytase commercialized by BASF (Ludwigshafen, Germany), contains the fermentation extract of *A. niger*, and has an optimal activity at pH 5.5

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Table 19.2. Molecular and biochemical characteristics of histidine acid phosphatases (HAPs) and alkaline phytases. (From Oh *et al*., 2004.)

	HAPs			Alkaline phytases
Characteristics	PhyA	PhyB	PhyC	PhyD
Molecular mass (kDa)	62–128	270	42–45	38–45
Glycosylation	Yes	Yes	No	No
Optimum pH	2.5–5.0	2.5	5.0–6.0	7.0–8.0
Optimum temperature (°C)	55–60	55–60	40–60	55–70
Thermal stability	Low (60°C)	Low (60°C)	Low (60°C)	High (85–95°C)
Effect of Ca^{2+}	Inhibition	Inhibition	Inhibition	Stimulation
Effect of EDTA[a]	Stimulation	Stimulation	Stimulation	Inhibition
Substrate specificity	Broad	Broad	Broad	Specific
Nature of phytate	Metal-free phytate	Metal-free phytate	Metal-free phytate	Ca-phytate
Position specificity	D-3 position of phytate	D-3 position of phytate	D-6 position of phytate	D-3 position of phytate
Final product[b]	$IP_1 + 5P_i$	$IP_1 + 5P_i$	$IP_1 + 5P_i$	$IP_3 + 3P_i$
Active site	(+) Charged amino acid	(+) Charged amino acid	(+) Charged amino acid	(-) Charged amino acid
Crystal structure	A large α/β and a small α-domain	A large α/β and a small α-domain	A large α/β and a small α-domain	Six-bladed β-propeller
Source	*Aspergillus niger*, *Aspergillus awanmori*, *Aspergillus fumigatus*, *Aspergillus terrus*, *Emericella nidulans*, *Myceliophthora thermophila*, *Talaromyces thermophilus*	*A. niger*, *Saccharomyces cerevisiae*, *Schizo-saccharomyces pombe*	*Escherichia coli*, lysosomal and prostatic acid phosphatases from rat and human	*Bacillus* and some plants, such as *Typha latifolia*, pollen and some legume seeds

[a]EDTA, Ethylenediaminetetraacetic acid.
[b]IP_1, Myo-inositol monophosphate; IP_3, myo-inositol trisphosphate; P_i, inorganic phosphate.

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after 30–60 min. On the other hand, bacterial phytases liberate inorganic phosphate at a pH range between 6.0 and 7.0, such as phytases from *Bacillus subtilis* and *Bacillus amyloliquefaciens* (Powar and Jagannathan, 1982; Shimizu, 1992). However, for optimum phytase activity, the appropriate pH range is not the only requirement; the reaction temperature should also be optimized to allow the liberation of phosphorus from phytic acid. It has been found that alkaline phytases naturally excreted by *B. amyloliquefaciens* can withstand temperatures up to 90°C with an optimal activity at temperatures ranging from 55°C to 70°C (Oh *et al.*, 2004). For commercial applications, the enzyme is generally used in pellet form, which is recommended to ease application and to reduce the risk of enzymatic activity loss. Since the feed pelleting process usually involves treatments at higher temperature (between 60°C and 80°C) and steam, all feed enzymes should be heat stable in order to avoid substantial activity loss during manufacturing. One of the disadvantages of the enzymes having their optimum activity at high temperatures, usually more than 40°C, is their low specific activities at ambient temperatures. This is associated with the great rigidity and thus decreased flexibility of the protein. These relatively high optimal temperatures preclude full activity of phytase at the stomach temperature of pigs or poultry (37–40°C) and result in even poorer performance of phytase in fish (Bosch *et al.*, 1998). In addition, the low enzymatic activity or substrate specificity usually limits the application of such enzymes.

Most microbial and plant phytases are classified as HAPs which react with the metal-free phytate as the substrate at a pH range between 2.5 and 5.0 (Oh *et al.*, 2006). Based on the crystal structures of phytase produced by *Escherichia coli*, they are categorized under the HAPs groups. It has been found that the positively charged active site of this phytic acid class favours metal-free phytase to initiate the hydrolysis of organic phosphate from phytase (Lim *et al.*, 2000a). However, there is no hydrolytic activity when a metal-free phytase complex is initiated. Therefore, the presence of mineral chelators such as ethylenediaminetetraacetic acid (EDTA) and phthalate esters can activate the dephosphorylation of phytic acid by discharging the divalent metal captions in the complexes (Shimizu, 1993). The hydrolysis of phytate initiated by HAPs on the phosphate group, either at position 3 or 6 of the phytin molecule, resulted in free inositol as the final product (Mullaney *et al.*, 2002; Oh *et al.*, 2006). Furthermore, the HAP class is sub-classified into different sub-groups known as Phy A, Phy B and Phy C with molecular mass of 62–128, 270 and 42–45 kDa, respectively (Liu *et al.*, 1998; Oh *et al.*, 2006).

Alkaline phytases, Phy D, represent another group of phytases with molecular weights ranging from 38 kDa to 45 kDa. They are produced by bacterial species, mainly *Bacillus* sp., and they initiate the dephosphorylation of the phosphate group in the calcium–phytate complex. Alkaline phytases require the calcium ion for their enzymatic catalysis (Oh *et al.*, 2006). The optimal hydrolytic activity is achieved at a pH range from 7.0 to 8.0 (Powar and Jagannathan, 1982; Shimizu, 1992; Kim *et al.*, 1998; Idriss *et al.*, 2000; Choi *et al.*, 2001). Alkaline phytases are notably unique for their formation of the calcium–phytate complex (Oh *et al.*, 2004), however, they do not exert any activity on the other phosphoester groups. This is characterized by the formation of phosphate bridges between Ca^{2+} and the two oxianions from the phosphate groups of phytate (Oh *et al.*, 2006). The hydrolysis of the calcium–phytate complex proceeds preferentially at C_3 and the dephosphorylation produces an intermediate known as D-myo-inositol-(1,2,4,5,6)-penthakisphosphate as a leading derivative, which finally produces myo-inositol trisphosphate (Idriss *et al.*, 2000; Kerovuo *et al.*, 2000). However, research carried out by Bohn *et al.* (2008) has revealed that this enzyme can initiate the hydrolysis of the calcium–phytate complex at C_5.

Currently, the world commercial supply of phytase is provided from preparations containing microbial enzymes, which have been produced either by solid state or submerged fermentation of microorganisms including bacteria, yeast and fungi. The selection of the production process for phytase production depends on the origin of the strain and enzyme purity required. Fungi produce extracellular phytases (Howson and Davis, 1983), whereas most bacteria produce intracellular phytases. In the latter case, cell disruption during downstream processing is required to release the produced enzymes, except for *B. subtilis* (Powar and Jagannathan, 1982)

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and *Enterobacter* (Yoon *et al*., 1996) which can secrete the enzyme in the fermentation broth during submerged cultivation. However, both fermentation techniques were not able to sustain the availability of phytase in the market. It is well established that commercial application has not been feasible for many years, due to the low activity yield and the anticipated high cost of the conventional phytase fermentation systems. Therefore, the development of genetic engineering protocols for heterologus protein expression from microbial systems will provide phytase enzymes to the animal feed market in the required amounts at relatively low costs. Theoretically, phytase preparation should be catalytically efficient, proteolysis-resistant, thermostable, and cheap (Lei and Stahl, 2001). Nowadays, single or multiple traits of phytase enzymes have been successfully improved by genetic manipulation. Genetic engineering techniques are improved by site-directed mutagenesis on the phytase gene. Crystal structure determination in phytase for biochemical investigations has been used to improve the enzyme specific activity at a wide range of pH in the heat-stable enzymes such as *Aspergillus fumigatus* phytase, *AppA* phytase gene from *E. coli* (Rodriguez *et al*., 2000a, b) and the *PhyA* phytase gene from *A. niger* (Mullaney *et al*., 2000; Rodriguez *et al*., 2000b).

19.3.2 Enzymatic hydrolysis of phytic acid

The phytic acid molecule, having six phosphate groups, can be hydrolysed by phytase at different rates and in different orders. Most phytases are able to cleave five of the six phosphate groups in phytic acid (Konietzny and Greiner, 2002). Phytases have been grouped based on the first phosphate position of phytic acid being hydrolysed. Hydrolysis of the first phosphate group at position 3 may result in the removal of one inorganic phosphate and the presumptive formation of inositol-(1,2,4,5,6)P_5 intermediates. Hydrolysis of the second phosphate will release inorganic phosphate at position 1 (IP-1). However, if the IP-1 is removed first in the dephosphorylation step, IP-3 will be then removed in the second dephosphorylation step, resulting in the removal of two inorganic phosphates. Concomitantly, inositol-(2,4,5,6)P_4 will accumulate and will be then further hydrolysed with the accumulation of inositol triphosphate (IP_3) < inositol diphosphate (IP_2) < inositol monophosphate (IP_1), and the release of up to five or six inorganic phosphates. Phytase also tends to remove the phosphate either at the IP-6 or the IP-4 position. However, intermediates resulting from the hydrolysis at IP-4 or IP-6 do not accumulate, suggesting that they are tightly bound to the enzymes. Finally, the removal of the second phosphate results in the accumulation of inositol tetraphosphate (IP_4) intermediates, (1,2,3,5)P_4 with further dephosphorylating steps resulting in the accumulation of IP_3 and the release of phosphates.

19.3.3 Application of phytases in animal feed

Phytases are considered to be very useful and important additives in animal feeds for the liberation of organic phosphate and intermediates such as inositol, which can be easily absorbed in the intestine and provide different nutritional benefits to the animals. Phytases can be formulated into powder and added to raw materials rich in phytate, for example cereal-, legume- and seed-based feed. The addition of phytases has been reported to reduce the excretion of inorganic phosphates to about 30–50% (Jorquera *et al*., 2008). Additionally, these authors concluded that the amount of phytase incorporated during feed preparation depends on the amount of phytate present in the raw materials and its relevant processing techniques. Phytic acid, phytate or phytin, a chemical component known as inositol-hexakisphosphate (IP_6), can be found in most plant tissues. Generally, higher percentages can be found mainly in the bran and aleurone, as the main storage centre of phosphorus in plants. Phytic acid plays an important role in energy storage in seeds and grains and inositol acts as a cation source (Anderson, 2005). The degradation of phytic acid is catalysed by phytases, which successively dephosphorylate phytic acid. This hydrolysis results in the release of inorganic phosphate and the myo-inositol group, which in turn increases the absorption and reduces nutrient constraints in the digestive tract (Lei and Porres,

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2003). Technically, plant phytates contain organic phosphate that is not available, and its intestinal absorption is normally limited in monogastric animals. However, this is not the case with ruminant animals since they can depend on phytate-degrading enzymes produced by microorganisms naturally living in their digestive systems (Morse *et al.*, 1992). Moreover, Clark *et al.* (1986) reported that the optimum hydrolysis of phytic acid was achieved when dairy cattle were fed with 50% grain and 50% maize silage. However, in ruminants, the organic phosphorus is still not enough to meet their requirements.

The amount of phytase required to be added in animal feed is significantly correlated with the type of the diet, efficacy of phytase and different techniques managing the animal diets (Rosen, 2003; Selle and Ravindran, 2007). The use of plant by-products such as rice bran and wheat bran, which contain more than 79% of phytate, is not recommended unless an appropriate level of phytase is supplemented in the feed (Cabahug *et al.*, 1999). In addition, phytase supplementation has been shown to generate a good growth performance response in ruminants, including bone mineralization, energy and nitrogen utilization. Ruminants digest phytate through the action of phytase produced by the microbial flora in their digestive system. The anaerobic gut microflora is responsible for the colonization and digestion of plant materials based on their ability to release the appropriate enzymes within the rumen. The liberation of inorganic phosphate from phytate by microbial phytases is not only beneficial to the host but also to the microflora in the rumen.

However, in the case of monogastric animals, the situation is different because of the absence of gastrointestinal microbial phytase producers. Therefore, monogastric animals such as pigs, poultry and fish are unable to metabolize phytic acid. Accordingly, inorganic phosphate is usually added to their feed in order to cope with the phosphate requirements (Bosch *et al.*, 1998). Since phytic acid is found within the hulls of nuts, seeds and grains of the feed, which are normally fed to monogastric animals, most of the phytate ingested will be excreted in the faeces. Thus, supplementation of feed with exogenous phytases will reduce the excretion of phytic acid in the faeces almost by 30–50%, especially when the enzyme is supplemented in a dose ranging from 500 IU/kg to 1000 IU/kg of the diet (Kemme *et al.* 1997; Liu *et al.*, 1997). Interestingly, Selle *et al.* (1999) evaluated sorghum-based broiler diets supplemented with 600 IU/kg and they reported a significant increase in the weight gain of the broilers after 7–25 days of up to 7.6% and an improvement in the feed efficiency of up to 4.7%, compared with the control diets. This improvement was obtained with a specific reduction in the excreted phosphorus, Ca^{2+} and protein/amino acids. Another study evaluated the addition of phytase at levels up to 1500 IU/kg to animal diets containing 4.5 g/kg total phytate (Simons *et al.*, 1990). Their results gave consistent enhancement of growth performance. However, Woyengo *et al.* (2008), who investigated three levels of phytase (0, 250 and 500 IU/kg) in wheat-based diet for broilers, reported no increment in the apparent ileal digestibility of phosphorus, Ca^{2+} and protein/amino acids. This was attributed to the fact that a wheat-based diet has a low non-phytate phosphorus content. Another important factor, which should be considered when supplementing the animal diet with the appropriate level of phytase, is the viscosity of the feed in the digestive tract. The viscosity of the digesta of the basal diets increased from 3.22 cP to 3.38 cP upon increasing the phytase level from 0.0 IU/kg to 250 IU/kg. However, further increase in the phytase level up to 500 IU/kg decreased the viscosity of the digesta to 2.73 cP (Woyengo *et al.*, 2008). This may contribute to the increased digestibility of phosphorus in the poultry diets. Furthermore, increasing the phytase content in the diets of laying hens from 500 IU/kg to 5000 IU/kg increased the ileal phosphorus digestibility, and hence increased the laying performance, egg quality and bone mineralization. These results were comparable to those obtained with diets containing a novel transgenic maize-derived phytase (Gao *et al.*, 2013). Generally, most of the researchers recommend that broiler diets should have a low non-phytate level in order to maximize the growth of the broilers (Lim *et al.*, 2000b; Singh and Khatta, 2003; Singh *et al.*, 2003).

19.4 Xylanases (CAS # 9025-57-4)

Xylanases are glycosidases catalysing the endohydrolysis of 1,4-β-D-xylosidic linkages in xylan

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of hemicellulose present in the plant cell wall and convert it into utilizable monomer sugar. Xylan hydrolysis can be mediated by many microorganisms such as bacteria, actinomycetes, yeasts and fungi by synthesizing different types of xylanases. In 1961, the IUBMB assigned xylanase the enzyme code EC 3.2.1.8 and also recognized it as an endo-1,4-β-xylanase. However, there are other commonly used synonyms such as xylanase, endoxylanase, 1,4-β-D-xylan-xylanohydrolase, endo-1,4-β-D-xylanase, β-1,4-xylanase and β-xylanase. Many microbial species are able to hydrolyse xylan during their growth through the synthesis of β-1,4-endoxylanase and β-xylosidase enzymes. These are a widespread group of enzymes involving endoxylanases (EC 3.2.1.8) and β-xylosidases (EC 3.2.1.37), which are mostly produced by fungi such as *A. niger* and *Trichoderma viride*. Most of these xylanase producers are reported to be plant pathogens as they can attack and degrade the plant tissues by enzymatic hydrolysis (Esteban *et al.*, 1982). Microbial xylanases have a wide range of industrial applications with many future potential uses, such as in the pulp and paper industry and in the food and feed industries. Many research groups have diverted their attention to these microbial species to enhance the production of xylanases. It has been reported that highly thermophilic and halophilic xylanases can be produced from extremophilic microbial species (Balakrishnan *et al*., 2000). In addition, some bacterial species such as *Bacillus stearothermophilus* are able to produce different types of thermostable xylanases (Beg *et al*., 2001).

Xylans are the major constituents of hemicelluloses which are heterogeneous polysaccharides found in plant cell walls (De Vries and Visser, 2001). These are complex structures when compared with cellulose due to a number of sugar monomeric side chains (usually up to 500–3000 side chains). The classification of hemicelluloses is based on the type of sugar monomers attached. The most common sugar monomers involved are D-xylose, D-mannose, D-galactose and L-arabinose (Bastawde, 1992). Xylans, as derivatives of hemicelluloses, are heteropolysaccharides composed of simpler subunits that have acetyl, 4-O-methyl-D-glucuronosyl and α-arabinofuranosyl residues. These residues are linked to the β-1,4-linked xylopyranose backbone either by covalent or non-covalent bonding (Shoham *et al*., 1992). In plants, xylans are found in between the cellulosic fibre sheets and lignin with a complex binding relationship with lignin and cellulose through hydrogen bonding. Xylan forms a thick wall over cellulose to provide protection from degradation by different cellulose-degrading enzymes (Joseleau *et al*., 1992). Xylans are also known as non-starch polysaccharides (NSPs) having linear or branched alkaline soluble structures. They are easily precipitated by alcohol from their aqueous solution and hydrolysed by mineral acids (Wilkie, 1983; Uffen, 1997). Xylans and their derivatives have found many industrial applications over the past few decades. They were classified according to the common substituent found on their backbone into: (i) linear homo-xylan; (ii) arabino-xylan; (iii) glucurono-xylan; and (iv) glucuronoarabinoxylan (Voragen *et al*., 1992; Sunna and Antranikian, 1997).

19.4.1 Classification and production

The classification of xylanases is based on their physiochemical properties including isoelectric point (pI) and molecular weight. Xylanases are also classified based on their protein sequences alignment, that is family 10 (F10), family 11 (G11) and β-xylosidases (Bajpai, 2009). The higher molecular weight endoxylanases with low isoelectric point (pI value) have been grouped in F10, while the low molecular weight endoxylanases with high pI value were grouped in G11 (Collins *et al*., 2005). Another family, known as β-xylosidases (collectively xylanases), is divided into five families of glycoside hydrolases: 3, 39, 43, 52 and 54. Enzymes from the families 3, 39, 52 and 54 catalyse the hydrolysis of xylooligomers via the retaining mechanism, while the enzymes from the family 43 carry out the hydrolysis through the inversion of the anomeric configuration (Juturu and Wu, 2012). The endoxylanases belonging to F10 are high molecular weight enzymes (molecular mass > 40 kDa), and are structurally composed of a cellulose-binding domain and a catalytic domain connected by a linker peptide with a $(\beta/\alpha)_8$ fold TIM (triose phosphate isomerise) barrel (Biely *et al.*, 1997; Bajpai, 2009). On the other hand, family G11 endoxylanases contain low molecular weight

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enzymes which have relatively small sizes. These endoxylanases can pass through the pores of the hemicellulose network, which will result in efficient hydrolysis (molecular mass less than 20 kDa). Based on their isoelectric points, this group is further sub-divided into two subgroups, alkaline and acidic, and are known to have a β-jelly roll structure (Törrönen and Rouvinen, 1997).

Since the xylan structure is heterogeneous and complex, its complete hydrolysis into its main pentose backbone sugar (D-xylose) requires a variety of multifunctional xylanolytic enzymes (Beg *et al.*, 2001; Subramaniyan and Prema, 2002; Juturu and Wu, 2012). The rate of xylan hydrolysis depends on the chain length of the oligomeric substrate, the nature of the bonds present in between the D-xylose monomers and the side chain complexity (Lee and Forsberg, 1987). The hydrolysis of xylan involves the action of many hydrolysing enzymes (Li *et al.*, 2000). Endo-1,4-β-D-xylanases (EC 3.2.1.8) randomly cleave the xylan backbone, β-D-xylosidases (EC 3.2.1.37) cleave xylose monomers from the non-reducing end of xylooligosaccharides and xylobiose, while the removal of the side groups is catalysed by α-L-arabinofuranosidases (EC3.2.1.55), α-D-glucuronidases (EC 3.2.1.139), acetylxylanesterases (EC 3.1.1.72) and a group of phenolic enzymes, ferulic acid esterases (EC 3.1.1.73) and ρ-coumaric acid esterases (EC 3.1.1.-) (Beg *et al.*, 2001; Collins *et al.*, 2005).

For industrial applications, thermostable enzymes that can withstand processing temperatures above 50°C are required. Fungal and bacterial xylanases have been extensively studied more than enzymes from other sources. In general, they are stable enzymes working at high temperatures and at a pH range between 5.0 and 9.0 (Table 19.3). Fungal xylanases, produced mainly by *Aspergillus* sp., are generally the better choice for the hydrolysis of substrates at pH values around 5.0 and 6.0 (Beg *et al.*, 2001). However, the enzyme has high stability even in acidic or alkaline environments and exhibits catalytic activity between pH 2.0 and 9.0 (Bajpai, 2009). On the other hand, most of the xylanases produced by *Bacillus* sp. are slightly more thermostable compared with fungal xylanases. Normally, the molecular weight of xylanases lies between 20 kDa and 60 kDa. On the other hand, some strains can produce high molecular weight xylanases up to 180 kDa such as strains of *Thermo anaerobacterium* sp. and *Thermotoga maritime* (Winterhalter and Liebel, 1995). Low molecular weight xylanases of 20 kDa with basic isoelectric points (pI 8–9.5) show homology to the family G11 of glycosyl hydrolases (Beg *et al.*, 2001). Other high molecular weight xylanases (molecular mass more than 40 kDa) and lower pI values belong to the other identified endoxylanase family, F10.

19.4.2 Application in animal feed

Xylanases are widely used in the industrial manufacture of animal feeds. They are usually applied combined with other hydrolytic enzymes such as pectinases, proteases, celluloses, lipases, glucanases, alginases and phytases. These enzymes are generally used to reduce the viscosity of raw materials used in animal feeds. The complex arabinoxylan present on the cell wall of most grains may reduce the effective digestion and absorption of nutrients in animal digestive tracts. Hence it is necessary to make the feed easily absorbable by hydrolysing the arabinoxylan, which is achieved by xylanase enzymes. Studies in this field have reported that the xylanase-treated feed has better digestibility and nutrient absorption rate when compared with raw feeds (Bedford and Classen, 1992). Xylanases have been widely used to reduce the anti-nutritional effects of NSPs and to improve the nutritional values of energy and protein in wheat-based diets (Selle *et al.*, 2009). Nowadays, xylanases are used increasingly in maize-based diets (Beg *et al.*, 2001), sometimes in combination with other fibrolytic enzymes.

Xylanase-based enzyme products are now widely used throughout the world as supplements for pig and poultry diets, which are based on wheat, triticale and rye. Additionally, substantial amounts of barley, wheat, rye and sunflower can only be used in broiler diets if they are supplemented with enzymes. The addition of enzymes ensures the maximum utilization of nutrients trapped inside the plant cells, as well as reducing the viscosity created by NSPs in the

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Table 19.3. Characterization of thermostable xylanases for industrial application.[a] (From Beg *et al*., 2001.)

Microorganisms	Molecular weight (kDa)	Optimal		Stability		pI[b]	K_m (mg/ml)[c]	V_{max}[d]	Reference
		pH	°C	pH	°C				
Bacillus circulans WL-12	15	5.5–7.0	–	–	–	9.1	4	–	Esteban *et al*. (1982)
Bacillus stearothermophilus T6	43	6.5	55	6.5–10	70	7–9	1.63	288	Khasin *et al*. (1993)
Bacillus sp. strain BP-23	32	5.5	50	9.5–11	55	9.3	–	–	Blanco *et al*. (1995)
Bacillus sp. strain BP-7	22–120	6.0	55	8.0–9.0	65	7–9	–	–	Lopez *et al*. (1998)
Bacillus polymyxa CECT 153	61	6.5	50	–	–	4.7	17.1	112	Morales *et al*. (1995)
Bacillus sp. strain K-1	23	5.5	60	5.0–12.0	50–60	–	–	–	Ratankhanokchai *et al*. (1999)
Bacillus sp. NG-27	–	7.0, 8.4	70	6.0–11.0	40–90	–	–	–	Gupta *et al*. (1992)
Bacillus sp. SPS-0	–	6.0	75	6.0–9.0	85	–	–	–	Bataillon *et al*. (1998)
Bacillus sp. strain AR-009	23, 48	9.0–10.0	60–75	8.0–9.0	60–65	–	–	–	Gessesse (1998)
Bacillus sp. NCIM 59	15.8, 35	6.0	50–60	7.0	50	4, 8	1.58, 3.50	0.017, 0.742	Dey *et al*. (1992)
Staphylococcus sp. SG-13	60	7.5, 9.2	50	7.5–9.5	50	–	4	90	Ximenes *et al*. (1999)
Thermoanaerobacterium sp. JW/SL-YS 485	24–180	6.2	80	–	–	4.37	3	–	Winterhalter and Liebel (1995)
Thermotoga maritima MSB8	40, 120	5.4, 6.2	92–105	–	–	5.6	1.1, 0.29	374, 4760	
Acrophialophora nainiana	17	6.0	50	5.0	50	–	0.731, 0.343	–	Ximenes *et al*. (1999)
Aspergillus niger	13.5–14.0	5.5	45	5.0–6.0	60	9	–	–	Frederick *et al*.(1985)
Aspergillus kawachii IFO 4308	26–35	2.0–5.5	50–60	1.0–10.0	30–60	3.5–6.7	–	–	Ito *et al*. (1992)
Aspergillus nidulans	22–34	5.4	55	5.4	24–40	–	–	–	Fernandez-Epsinar *et al*. (1992)
Aspergillus fischeri Fxn1	31	6.0	60	5.0–9.5	55	–	4.88	5.88	Raj and Chandra (1996)
Aspergillus sojae	32.7, 35.5	5, 5.5	60, 50	5–8, 5–9	50, 35	3.5, 3.75	–	–	Kimura *et al*. (1995)

[a]Where two values are shown this is because the same strain produces two different types of thermostable xylanase with different characteristics.
[b]pI, Isoelectric point.
[c]K_m, Michaelis constant which is the substrate concentration when the velocity of the reaction reaches one-half of V_{max}.
[d] V_{max}, The maximal velocity, or rate of enzymatic reaction, at saturating substrate concentrations.

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bird's digestive tract. This was confirmed by the study of Barekatain *et al.* (2013), where the addition of xylanase reduced the concentration of insoluble NSPs in the ileum. Moreover, the fed birds noticeably responded to xylanase supplementation in terms of better feed conversion ratio and body weight gain, as reflected in the concentrations of arabinose, xylose and total insoluble NSP (Selle *et al.*, 2009). Wu and Ravindran (2004) carried out another investigation on wheat-based diets fed to broiler chickens. They found that feeding the broiler chicken with whole wheat increased the feed efficiency in comparison to broilers fed with ground wheat (Plavnik *et al.*, 2002; Wu *et al.*, 2003). However, the supplementation of both ground wheat and whole wheat diets with xylanases showed an improvement in the feed efficiency due to the increase in the grinding activity of the larger gizzard and the enhanced mixing of the substrate with the supplemented xylanases (Bedford and Schulze, 1998; Preston *et al.*, 2000; Wu and Ravindran, 2004).

Nowadays, by-products from biorefinery activities (e.g the ethanol industry) such as corn (maize) distillers' dried grains with solubles (cDDGS), which contain high concentrations of maize distillers' dried grains, are used in animal feeds. There are two to three times the levels of NSP, protein and fat in cDDGS in comparison with maize grains (Cromwell *et al.*, 1993; Belyea *et al.*, 2004). cDDGS is widely used as feed in poultry diets with accepted levels up to 60 g/kg in starter broiler diets, and levels of 120–150 g/kg in grower-finisher broiler diets (Lumpkins *et al.*, 2004; Świątkiewicz and Koreleski, 2007). However, increasing the amount of cDDGS in maize-based diets of starter broilers to 180 g/kg reduced body weight gain by about 27 g per chick and caused a deterioration in the feed conversion ratio, and increasing the amount of cDDGS up to 250 g/kg for 35–49-day-old broilers significantly decreased body weight gain, even though there was no effect on the feed intake (Lumpkins *et al.*, 2004). Therefore, as xylanases have been widely used to reduce the anti-nutritional effects of NSP and to improve the nutritional values of energy and protein in wheat-based diets (Selle *et al.*, 2009; Barekatain *et al.*, 2013) xylanases may be similarly used on maize-based diets. Indeed, the inclusion of xylanases to broiler diets containing cDDGS can improve growth performance and digestibility of dry matter hemicelluloses by 20% and energy by 620 kJ/kg (Lazaro *et al.*, 2003; Świątkiewicz and Koreleski, 2007). Liu *et al.* (2011) reported that the appropriate dose of enzymes and the NSP levels of the maize-based diets are very important factors to ensure adequate digestibility and functionality of the diets, and hence to improve the growth performance and animal productivity.

The fibrolytic activity of xylanases and other enzymes can increase the quality of silage fed to ruminants, which will in turn increase the rate of digestion of xylan sugars in plant cell walls and enable further breakdown by various other enzymes in the animal's abdomen. At the same time, not only the host but also the growth performance of the microbiota in the animal rumen can benefit. For ruminants, the addition of β-glucanase and xylanase to the animal feed altered the milk fat content and improved the productivity of beef cattle (Meale *et al.*, 2014). Moreover, Phakachoed *et al.* (2012) investigated the effects of xylanase supplementation on ruminal disappearance and rumen fermentation of a rice-straw-based diet in fistulated non-lactating dairy cows. The enzyme supplementation did not change the dry matter intake, ruminal pH and NH_3-N concentrations.

19.5 Future Trends

In addition to the traditional use of microbial cells being used as biofactories for production of functional enzymes for feed additives, in future the animal feed industry may be able to use transgenic plants with heterologous expression of thermostable phytases and xylanases with a wide range of pH activity. Such enzyme production in transgenic plant cells would be cost-effective and in addition there would be fewer handling steps in the production process for generating functional feed additives compared with the traditional use of adding exogenous microbial enzymes during animal feed manufacturing.

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20 Microbial Xylanases: Production, Applications and Challenges

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Abstract
Xylanases (enzyme code EC 3.2.1.8) are a class of enzymes catalysing the degradation of the linear polysaccharide β-1,4-xylan into xylose, thus breaking down hemicellulose, a major constituent of the plant cell wall. Xylanase production has been reported in a wide spectrum of microorganisms, including bacteria, actinomycetes, yeasts and filamentous fungi. In the past few years, xylanases have drawn significant attention of the scientific fraternity due to their widespread biotechnological applications such as: (i) in pretreatment of lignocellulosic waste to simple sugars; (ii) production of biobutenol; (iii) animal feed processing; (iv) improvement of bread quality; (v) biobleaching of fabrics; (vi) pulp bleaching; (vii) silage production; and (viii) treatment of organic waste. The main bottleneck in commercial applications of xylanase-based enzymatic processes is the bulk production of xylanases at an economically viable rate. Therefore, it is exigent to work on cost-effective strategies for large-scale production of xylanases by microbes.

20.1 Introduction

Xylanases are a class of enzymes known to degrade the linear polysaccharide β-1,4-xylan into xylose, thereby breaking down hemicellulose, a major constituent of the cell wall of plants (Vimalashanmugam and Viruthagiri, 2013). Xylanases were reported for the first time in 1955 (Whistler and Masek, 1955) and named as pentosanases. Xylanases were recognized in 1961 by the International Union of Biochemistry and Molecular Biology (IUBMB) and were assigned the enzyme code EC 3.2.1.8. Officially xylanases are called endo-1,4-β-xylanase, but other synonyms include xylanase, endoxylanase, β-1,4-D-xylan-xylanohydrolase, endo-1,4-β-D-xylanase, β-1,4-xylanase and β-xylanase (Chundawat *et al.*, 2011).

The plant cell wall is primarily comprised of cellulose (20–50% on a dry weight basis), hemicelluloses (15–35%) and lignin (10–30%) (Chundawat *et al.*, 2011). Xylan is the principal constituent of hemicelluloses (Ning *et al.*, 2008). Conversion of xylan into useful end products requires deconstruction of the hemicelluloses to their constituent sugars (Dodd *et al.*, 2009). In this respect endoxylanases play a significant role owing to their ability to hydrolyse xylan into xylose, xylobiose and xylooligomers.

Release of fermentable sugars from cellulosic materials, especially agricultural wastes, is receiving increasing interest for the production of biofuel and bio-based chemicals (Adsul *et al.*, 2005; Dhiman *et al.*, 2008; Zhou *et al.*, 2008; Zheng *et al.*, 2010).

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Synergistic action of several enzymes is required for biodegradation of xylan. β-1,4-Endoxylanase, β-xylosidase, α-L-arabinofuranosidase, α-glucuronidase, acetyl xylan esterase and phenolic acid (ferulic and ρ-coumaric acid) esterase altogether represent a typical xylan-degrading enzyme system (Coughlan and Hazlewood, 1993).

Xylanase production has been documented in a wide spectrum of microorganisms, including bacteria, actinomycetes, yeasts and filamentous fungi (Nascimento *et al.*, 2003; Bakri *et al.*, 2008). Xylanases have drawn significant attention in the past few years because of their diverse applications in bleaching and pulping processes employing cellulose-free combinations, in the food processing industry, textile processes and enzymatic pretreatment of lingo-cellulosic materials and treatment of organic waste (Mechaly *et al.*, 1997; Wong *et al.*, 1998). Such enzymes not only offer eco-incentive alternatives for bleaching of paper pulp discouraging the use of toxic chlorine compounds but also improve the physical and optical properties of the paper pulp. The large-scale production of xylanases at a cost-effective rate is a key hurdle in the commercial application of xylanase-based enzymatic processes. Therefore, it is obligatory to explore strategies paving the way for cost-effective bulk production of xylanases for industrial applications.

The best strategy to meet the goal of increased production of xylanases at low cost is the exploration of xylanase hypersecretary microbial strains capable of metabolizing available waste material. To this end, cryptic information of molecular details of xylanases is imperative along with the successful expression of xylanase genes in appropriate expression vectors. In the light of the aforesaid context, the present chapter encompasses the manifold aspects of xylanases, their production under different cultivation strategies, possible applications and challenges in their commercial applications.

20.2 Xylan: Composition and Structure

As already mentioned, the plant cell wall consists primarily of cellulose (20–50% on a dry weight basis), hemicelluloses (15–35%) and lignin (10–30%), (Chundawat *et al.*, 2011). Major heteropolymers of hemicellulose are mannan, xylan, galactan and arabinan. Classification of these heteropolymers is based on the peculiar sugar moieties linked to the heteropolymers. In the majority of hemicelluloses D-xylose, D-mannose, D-galactose and L-arabinose are found as the principal monomers. In cell walls of angiosperms (flowering plants) xylan is the most common hemicellulosic polysaccharide constituting up to 30–35% of the total dry weight (Joseleau *et al.*, 1992). Xylan is a complex polysaccharide consisting of a rachis of xylose residues connected by β-1,4-glycosidic bonds (Fig. 20.1) (Sunna and Antranikian, 1997). The backbone of xylan consists of β-xylopyranose residues (Whistler and Richards, 1970). The majority of xylans are found as heteropolysaccharides, bearing different substituent groups (e.g. acetyl, arabinosyl and glucuronysyl residues) (Whistler and Richards, 1970) in the rachis chain and in the side chain (Biely, 1985). Xylan is a complex and extensively branched heteropolysaccharide. The variation in structure of xylan in angiosperms is because of the homopolymeric rachis of 1,4-linked β-D-xylopyranosyl which can be substituted with α-L-arabinofuranosyl, 4-*O*-methyl-D-glucuronopyranosyl, glucuronopyranosyl, acetyl and ρ-coumaroyl side-chain groups (Fig. 20.1) (Kulkarni *et al.*, 1999; Li *et al.*, 2000).

Xylans can further be categorized as linear homoxylan, arabinoxylan, glucuronoxylan or glucuronoarabinoxylan depending on the common substituent present on the rachis. Homoxylans are exclusively comprised of xylosyl moieties and are not frequently found in plants; they have been documented only in a few cases such as esparto grass, tobacco stalks and guar seed husks (Sunna and Antranikian, 1997). On the basis of its substituent, a broad differentiation may therefore be made among xylans, in which the complexity widens from linear to extensively substituted xylans. Generally, four families of xylans can be distinguished (Voragen *et al.*, 1992):

- arabinoxylans, containing side chains of single distal units of α-L-arabinofuranosyl;
- glucuronoxylans, with α-D-glucuronic acid or its 4-O-methyl ether as sole substituent derivative;
- glucuronoarabinoxylan, with both α-D-glucuronic and α-L-arabinose; and

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Fig. 20.1. Structure of xylan and the xylanolytic enzymes involved in its degradation. Ac, Acetyl group; α-Araf, α-arabinofuranose; α-4-*O*-Me-GlcUA, α-4-*O*-methylglucuronic acid. (From Sunna and Antranikian, 1997.)

- galactoglucuronoarabinoxylans, with terminal β-D-galactopyranosyl moieties on complex oligosaccharide side chains of xylans.

20.3 Xylanases: Classification and Characteristics

Endoxylanase (endo-1,4-β-xylanase, EC 3.2.1.8), β-xylosidase (xylan 1,4-β-xylosidase, EC 3.2.1.37), α-glucuronidase (α-glucosiduronase, EC 3.2.1.139), α-arabinofuranosidase (α-L-arabinofuranosidase, EC 3.2.1.55) and acetylxylan esterase (EC 3.1.1.72) constitute a typical xylan-degrading enzyme system (Choi *et al.*, 2000). Endo-β-1,4-xylanases bring about the catalytic hydrolysis of the rachis of xylan-producing xylooligosaccharides, which in turn can be converted to xylose by β-xylosidase (Zhang *et al.*, 2007).

Depending on the amino acid sequence homologies and hydrophobic cluster analysis, xylanases can be classified chiefly into two families of glycosyl hydrolases (GH): (i) family F or GH10; and (ii) family G or GH11 (Jeffries, 1996; Zhou *et al.*, 2008). However, other glycosyl hydrolase families, 5, 7, 8 and 43, have also been documented to have different catalytic divisions with a proven endo-1,4-β-xylanase activity (Collins *et al.*, 2005).

Xylanases belonging to family G are of low molecular mass with isoelectric point (pI) values of 8–9.5 as compared with those in family F which are of high molecular mass with lower pI values (Buchert *et al.*, 1994). The favourable pH for xylan hydrolysis has been reported to be around pH 5 for most fungal xylanases (Yu *et al.*, 1987), whereas pH optima of bacterial xylanases has been found to be slightly higher (Table 20.1). The xylanases and cellulases together with pectinases share about 20% of the global enzyme market (Polizeli *et al.*, 2005).

20.3.1 Endo-1,4-β-xylanases

Endo-1,4-β-xylanase (1,4-β-D-xylan xylanohydrolase; EC 3.2.1.8) results in the cleavage of the glycosidic bonds in the xylan rachis and retards the degree of polymerization of starch (Li *et al.*, 2000). Endoxylanases can be demarcated based on the final products released from the hydrolysis of the xylan rachis (e.g. xylose, xylobiose and xylotriose, arabinose). The optimum activity of endoxylanases occurs at temperatures between 40°C and 80°C, and between pH 4.0 and 6.5. Endoxylanases from bacteria and fungi are single subunit proteins with molecular weights from 8.5 kDa to 85 kDa and pI values between 4.0 and 10.3 and the majority of them are glycosylated (Polizeli *et al.*, 2005).

20.3.2 β-Xylosidases

β-D-Xylosidases (1,4-β-D-xylan xylohydrolase; EC 3.2.1.37) have been classified on the basis

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Table 20.1. Characteristics of xylanases from different microorganisms.

		Optimum			
Microorganisms	pI = isoelectric point	Molecular weight (kDa)	pH	Temperature (°C)	Reference
Bacteria					
Acidobacterium capsulatum	7.3	41	5	65	Inagaki *et al.* (1998)
Bacillus circulans WL-12	9.1	15	5.5–7	–	Joshi *et al*. (2008)
Bacillus stearothermophilus	7.9	43	6.5	55	Khasin *et al*. (1993)
Bacillus polymyxa CECT 153	4.7	61	6.5	50	Morales *et al.* (1995)
Bacillus sp. strain K-1	–	23	5.5	60	Ratanakhanokchai *et al.* (1999)
Cellulomonas fimi	4.5–8.5	14–150	5–6.5	40–45	Khanna (1993)
Staphylococcus sp. SG-13	–	60	7.5, 9.2	50	Gupta *et al*. (2000)
Thermotoga maritima MSB8	5.6	40, 120	5.4, 6.2	92–105	Winterhalter and Liebl (1995)
Fungi					
Aspergillus niger ANL-301	9	13.5–14.0	5.5	45	Okafor *et al*. (2010)
Cephalosporium sp.	–	30, 70	8	40	Bansod *et al.* (1993)
Fusarium oxysporum	–	20.8, 23.5	6	60, 55	Christakopoulos *et al.* (1996)
Penicillium purpurogenum	8.6, 5.9	33, 23	7, 3.5	60, 50	Belancic *et al*. (1995)
Trichoderma harzianum	–	20	5	50	Ahmed *et al*. (2011)
Malbranchea flava	9.0	30	9	60	Sharma *et al.* (2010)
Yeast					
Cryptococcus albidus	–	48	5	25	Morosoli *et al.* (1986)
Trichosporon cutaneum SL409	–	–	6.5	50	Liu *et al.* (1998)
Pichia pinus	–	16	4.5	30	Ravindra *et al.* (2007)
Thermomonospora curvata	4.2–8.4	15–36	6.8–7.8	75	Stutzenberger and Bodine (2008)

of their relative affinities for xylobiose and high molecular weight xylooligosaccharides. β-D-Xylosidases may be monomeric, dimeric or tetrameric with molecular weights ranging from 26 kDa to 360 kDa. They are secreted by different types of bacteria and fungi (Corral and Villasenor-Ortega, 2006). Purified β-xylosidases generally do not hydrolyse xylan, their best substrate is xylobiose and their affinity for xylooligosaccharides is inversely proportional to its degree of polymerization. They are, however, capable of cleaving synthetic substrates such as p-nitrophenyl- and o-nitrophenyl-β-D-xylopyranoside (Polizeli *et al*., 2005). The optimum temperature for the enzyme activity can differ from 40°C to 80°C, but the majority of β-xylosidases give optimum activity at 60°C. The thermostability of β-xylosidases greatly differs in microorganisms (Rizzatti *et al*., 2001).

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20.3.3 α-Arabinofuranosidases

There are two types of α-arabinofuranosidases based on their mode of action:

- exo-α-L-arabinofuranosidase (EC 3.2.1.55), degrading p-nitrophenyl-α-L-arabinofuranosides and arabinans (Fig. 20.1); and
- endo-1,5-α-L-arabinase (EC 3.2.1.99) hydrolysing linear arabinans (De Vries *et al.*, 2000).

Subsequently after release of the arabinose, there is no degradation in the xylan backbone as there will be production of xylooligosaccharides.

20.3.4 Acetylxylan esterase

Acetylxylan esterase (EC 3.1.1.6) is involved in removal of *O*-acetyl substituents at positions 2 and 3 of xylose moieties. The majority of xylans are deacetylated subsequently after alkaline extraction (Sunna and Antranikian, 1997). Acetylxylan plays a significant role in the hydrolysis of xylan because the acetyl side groups, by steric hindrance, prevent or retard interactions with the rachis of xylose residues, so enzymes such as acetylxylan esterase that cleave them from the rachis, resulting in their removal, thereby facilitate endoxylanases activity (Corral and Villasenor-Ortega, 2006).

20.3.5 α-Glucuronidases

α-Glucuronidase (EC 3.2.1.131) results in hydrolysis of the α-1,2 bonds connecting glucuronic acid moieties and β-D-xylopyranosyl units in glucuronoxylan. The substrate specificity of α-glucuronidases has been documented to vary with the microbial source of the enzyme (Tenkanen and Siika-aho, 2000). It has also been observed that acetyl functional groups near to glucuronosyl substituents alter the α-glucuronidase activity to some extent.

20.4 Production of Xylanases

Xylanases are abundantly distributed in nature and are produced by microorganisms. Higher eukaryotes such as algae, protozoa, crustaceans, arthropods (insects), gastropods (snails) and germinating plant seeds also produce xylanases (Bastawde, 1992; Polizeli *et al.*, 2005). Complete xylanolytic enzyme systems are widely distributed among different types of microorganisms (Table 20.2) such as bacteria (Sunna and Antranikian, 1997; Gilbert and Hazlewood, 1999), fungi (Sunna and Antranikian, 1997), actinomycetes (Bastawde, 1992) and yeasts (Gilbert and Hazlewood, 1999; Sharma *et al.*, 2010), which share diverse and widespread ecological niches.

20.4.1 Fungi

Production of xylanases and other xylan-degrading enzymes has been extensively reported in filamentous fungi. They secrete the enzymes extracellularly. The rate of xylanase production in filamentous fungi has been reported to be higher than that of yeasts and bacteria, and in some fungal genera such as *Aspergillus*, *Fusarium* and *Trichoderma* production of xylanases is particularly high (Adsul *et al.*, 2005). Basidiomycetes also have been reported to produce extracellular xylanases which act on lignocellulosic materials (Ball and McCarthy, 1999) and produce metabolites of interest to the pharmaceutical, cosmetic and food industries (Hrmova *et al.*, 1984). White-rot basidiomycetes are known to produce high titres of xylanases. For example *Phanerochaete chrysosporium* produces high levels of α-glucuronidase (Buswell and Chang, 1994) and *Coriolus versicolor* produces a complex of xylanolytic enzymes (Qinnghe *et al.*, 2004). *Cuninghamella subvermispora* also produces xylanase when growing on plant cell-wall polysaccharides or on wood chips (Castanares *et al.*, 1995).

20.4.2 Bacteria

Production of xylanases has also been documented in some non-plant pathogenic species of bacterial genera such as *Bacillus* (Abd El-Nasser *et al.*, 1997). The production of α-L-arabinofuranosidase has been reported in the extreme thermophile *Rhodothermus marinus* (De Souza-Cruz *et al.*, 2004). The production of

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Table 20.2. Xylanase-producing microorganisms.

Microorganisms	Xylanases	Cultivation conditions	Media	Reference
Penicillium canescens	18,895 IU/g	pH 7.0, 30°C	Soya oil cake and casein peptone	Verma and Satyanarayana (2012)
Bacillus pumilus	5 IU/g	pH 7.0 and at 60°C	Wheat bran and olive oil	Yoon *et al.* (2004)
Streptomyces sp. P12–137	27.8 IU/ml	pH 7.2, 28°C	Wheat bran and KNO_3	Assamoi *et al*. (2010)
Penicillium fellutanum	39.7 IU/ml	30°C	Oat spelt xylan, urea, peptone and yeast extract	Battan *et al*. (2012)
Penicillium clerotiorum	7.5 IU/ml	pH 6.5, 30°C	Wheat bran	Coman and Bahrim (2011)
Aspergillus niger PPI	16.0 IU/ml	pH 5.0, 28°C	Oat and urea	Palaniswamy *et al*. (2008)
Aspergillus oryzae	2830.7 IU/g	28°C	Wheat bran	Knob and Carmona (2008)
Cochliobolus sativus Cs6	1,469.4 IU/g	pH 4.5, 30°C	Wheat straw and $NaNO_3$	Pandey and Pandey (2002)
Bacillus circulans D1	8.4 IU/ml	pH 9.0, 45°C	Bagasse hydrolysates	Pirota *et al*. (2013)
Paecilomyces themophila J18	18,580.0 IU/g	pH 6.9, 50°C	Wheat straw and yeast extract	Arabi *et al*. (2011)
Bacillus mojavensis	7.45 IU/ml	pH 8.0, 50°C	Barley bran	Bocchini *et al*. (2005)
Kluyvera sp.	5.12 IU/ml	pH 5.0–9.0, 50–70°C	Palm oil fibre	Yang *et al*. (2006)

α-arabinofuranosidases by *Bacillus polymyxa* has been very well demonstrated at the gene level (Joshi *et al.*, 2008). Bacteria that produce xylanases have attracted researchers because of their alkaline-thermostable xylanase-producing character (Esteban *et al.*, 1982). The optimum pH for the production of xylanases has been reported to be higher in bacteria than fungi, the latter preferring low pH for optimum production of xylanases (Inagaki *et al.*, 1998), making bacterial xylanase production appropriate for diverse biotechnological applications. Thermostable xylanases have been reported in *Bacillus firmus*, *Actinomadura* sp. strain Cpt20, *Geobacillus thermoleovorans* and *Saccharopolyspora pathunthaniensis* S582. All these strains produce xylanases that are active at temperatures ranging from 65°C to 90°C (Gomes *et al*., 2000). One xylanase, reported from *Thermotoga* sp. (Subramaniyan and Prema, 2002), has been found to be optimally active even at temperatures between 100°C and 105°C.

20.5 Production of Xylanases Under Solid State Fermentation (SSF) and Submerged Fermentation (SmF)

Xylanase biosynthesis can be achieved by both solid state fermentation (SSF) and submerged fermentation (SmF) (Cai *et al.*, 1998; Haddara *et al.*, 2012; Xin and He, 2013).

20.5.1 Xylanase production by SmF

SmF is comparatively beneficial compared with other techniques because more nutrients are available, there is sufficient oxygen supply and less time is required for the fermentation (Gwande and Kamat, 1999). About 80–90% of all xylanases are produced in submerged culture (Buchert *et al.*, 1994). In order to reduce the cost of the xylanases, natural lignocellulosic substrates such as wheat bran, sugarcane bagasse,

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rice straw and maize (corn) cobs may be used (Kansoh and Gammal, 2001) for the enzyme production (Table 20.3). Moreover, SmF offers better control of the culture conditions influencing the fermentation (Gouda, 2000).

20.5.2 Production of xylanase by SSF

SSF is generally a fermentation process occurring in the absence or near-absence of free water, employing a natural substrate or an inert substrate as solid support. During recent years, SSF has shown much progression in the manufacturing of enumerable fermentation products. SSF offers a medium deprived of free-flowing water molecules, wherein water is present either in an absorbed or in a complex form with the solid matrix and the substrate. SSF offers an attractive alternative for the production of enzymes at commercial scale due to the low capital investment needed and the low operating cost, along with many other characteristic properties. SSF provides a nature-identical environment for the microorganisms and therefore it facilitates the microbial growth for increased production of enzymes and metabolites that otherwise could not be produced or could be produced only in low yields under SmF (Farrel *et al.*, 1996). In SSF, xylanase can be obtained as a secondary metabolite from fungi cultivated on the lignocellulosic substrate. The most commonly used substrate among lignocellulosic waste is wheat bran. It not only contains sufficient nutrients but it is also able to remain loose under moist conditions, thereby providing a large surface area (Freixo *et al.*, 2008). Wheat bran contains appreciable amounts of soluble sugars such as

Table 20.3. Production of xylanases in submerged fermentation (SmF).

Organism	Substrate	Cultivation conditions	Xylanase activity (IU/ml)	References
Jonesia denitrificans BN13	Birchwood xylan, 7 g/l	4 l Fermentation, 37°C, pH 7, 2 days	10.80	Oliveira *et al*. (2006)
Streptomyces sp. strain AMT-3	Birchwood xylan, 10 g/l	Shake flask, 30°C, pH 7.0, 10 days	10.30	Nascimento *et al*. (2003)
Pichia pinus	1% Oat spelt xylan	Shake flask, 30°C, pH 4.5, 7 days	545	Morosoli *et al*. (1986)
Streptomyces sp. Ab106	Xylan, 10 g/l	4 l Fermentation, 55°C, pH 7.5, 5 days	8	Frost and Moss (1987)
Streptomyces actuosus	Rice bran, 50 g/l	37°C, pH 5, 4 days	11.60	Nawel *et al*. (2011)
Streptomyces malaysiensis	Birchwood xylan, 10 g/l	Shake flask, 30°C, pH 7, 6 days	11.90	Techapun *et al*. (2002)
Bacillus coagulans	Birchwood xylan, 10 g/l	Shake flask, 45°C, pH 7, 24 h	24.20	Viet *et al*. (1991)
Bacillus subtilis	Birchwood xylan, 10 g/l	3 l Laboratory fermenter, 55°C, pH 9, 2 days	128	Nascimento *et al*. (2003)
Bacillus pumilus	Wheat bran 10 g/l	Shake flask, 35°C, pH 9, 3 days	430	Chaudhury *et al*. (2006)
Thermobifida fusca	Wheat bran 20 g/l	Shake flask 50°C, pH 9.0, 3 days	6.68	Annamalai *et al*. (2009)
Aspergillus foetidus MTCC 4898	Birchwood xylan, 10 g/l	Shake flask, 30°C, pH 5, 3 days	210	Prema and Poorna (2006)
Fusarium solani F7	Wheat straw 30 g/l	Shake flask, pH 5.5, 30°C, 10 days	78.32	Liu *et al*. (2007)
Penicillium kloeckeri NRRL 1017	Birchwood xylan, 10 g/l	Shake flask, 26–28°C, 5 days	12.20	Shah and Madamwar (2005)
Coriolus versicolor	Tomato pomace	Shake flask, 25°C, 14 days	2.56	Gupta *et al*. (2009)

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glucose (42.5% dry weight), xylose (15.4% dry weight), arabinose (3.1% dry weight) and galactose (2.7% dry weight), which is a requisite for the initiation of growth and replication of microorganisms (Jecu, 2000). It also contains hemicelluloses (45%), which may play the role of inducers, and organic nitrogen sources (23%) that are essential for protein synthesis (Archana and Satyanarayana, 1997).

Critical parameters affecting SSF include the initial moisture content and inoculum size. At the lowest and highest water content the rate of decomposition of the total organic matter decreases which in turn affects enzyme production (Lequart *et al.*, 1999). The inoculum size also plays a crucial role in the production of metabolites under SSF. A low level of inoculum may not be sufficient enough to initiate growth and enzyme synthesis on different substrates. On the other hand, if the inoculum size increases beyond a certain limit the enzyme production decreases, which may be attributed to depletion of nutrients which result in decrease in metabolic activity. Hence, a balance between biomass and nutrients is required for maximum enzyme yield (Babu and Satyanarayana, 1996).

20.6 Biotechnological Applications

In the past few years, the biotechnological applications of xylans and xylanases have grown from strength to strength because of diverse applications in many industrial processes (Pandey, 1994; Sabu *et al.*, 2005). Use of xylanase started in the 1980s in the making of animal feed and in the food, textile and paper industries. Xylanase and cellulase, together with pectinases, constitute up to 20% of the global enzyme market (Buchert *et al.*, 1994; Khandeparker and Numan, 2008). Some important applications at commercial scale are described as follows.

20.6.1 Bioethanol production

Ethanol production from lignocellulosic waste involves the removal of recalcitrant compounds like lignin from the pulp and hydrolysis of cellulose and hemicellulose to monosaccharides (Techapun et *al.*, 2002). The acid hydrolysis at high temperature requires input of a significant amount of energy and acid-resisting equipment which makes the process expensive. However, acid-based pretreatment of lignocellulosic waste can be replaced with microbial enzymes in general and with xylanases in particular, offering an inexpensive option.

The complex structure of lignocellulosic waste demands the synergistic action of several enzymes, namely endoglucanases (EC 3.2.1.4), β-glucosidases (EC 3.2.1.21), endo-1,4-β-xylanases (EC 3.2.1.8) and β-xylosidases (EC 3.2.1.37), for complete hydrolysis (Topakas *et al.*, 2013). In some cases, endo-1,4-β-xylanase has been proved to be a bi-functional enzyme with endo-1,4-β-xylanase as well as cellulase activity. Bi-functionality of endo-1,4-β-xylanase has led to more efficient and cheaper saccharification of agricultural waste, and municipal and industrial wastes used as a feedstock for bioethanol production, as it can degrade both cellulose and xylan residues. Fermentation of both hexose and pentose sugars present in lignocellulosic waste into methanol has also been reported (Beg *et al.*, 2001).

20.6.2 Animal feedstocks

It has been documented that incorporation of endo-1,4-β-xylanase in animal feed not only increases animal growth rates by improving digestibility but also improves the quality of animal litter (Fuller *et al.*, 1995; Sunna and Antranikian, 1997). The endosperm cell walls of cereals are generally rich in polysaccharides which are present in the form of arabinoxylans mixed with linked β-glucans, constituting a major proportion, along with celluloses, mannans and galactans (Longland *et al.*, 1995). For example arabinoxylans are abundant in wheat, triticale and rye (Bonnin *et al.*, 1998), whereas oats and barley contain more β-glucans (Cui *et al.*, 2000). Generally, the viscous properties of the polysaccharides make them difficult to be digested by ruminants. Hence, incorporation of xylanase in wheat- or rye-based diets or β-glucanase in barley-based diets could be an important strategy to facilitate the availability of such polysaccharides (Bedford and Classen, 1993). Endo-1,4-β-xylanase and 1,3-1,4-β-glucanase catalyse the cleavage of the internal β-1,4-linkages of the 1,4-β-D-xylan backbone (Jeffries, 1996) and cleavage of the

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1,4-β-D-glucosidic bonds adjacent to the β-1,3-linkages in the mixed-linked-β-glucans, respectively (Planas, 2000). Consequently, endo-1,4-β-xylanase not only thins out the gut contents, promoting increased nutrient absorption, but also induces increased diffusion of the pancreatic enzymes.

20.6.3 Xylanases in the pulp and paper industry

In developed and developing nations, the pulp and paper industry has emerged as one of the major water-polluting industries (Battan *et al.*, 2007). In the past few years, several environmental protection groups have taken stern action to restrict the release of toxic pollutants into water bodies by these industries. With a view to protect the environment, it is thus obligatory to explore environmentally cleaner technologies. Various trials have been conducted by employing enumerable enzymes (Dhiman *et al.*, 2008), redox-mediators (Suurnakki *et al.*, 2010) and microorganisms (Yadav *et al.*, 2010) for use in the pulp and paper industry. For the production of paper, it is necessary to retain cellulose with the simultaneous removal of lignin from the paper pulp. Use of chlorine for bleaching of the paper pulp is a traditional approach in this respect.

Endo-1,4-β-xylanase has boundless potential to split hemicellulose chains which binds lignin tightly to the cellulose fibres. Therefore, endo-1,4-β-xylanase treatment of the pulp is considered more appropriate than lignin-degrading systems (Garg *et al.*, 2010). Moreover, applications of xylanases result in lowering the chlorine dosage, the cost of chemicals, and the release of chloro-organic pollutants in pulp and effluent (Khandeparker and Numan, 2008).

Pulp fibre morphology

A comparison between softwood sulfate pulp, before and after xylanase pre-bleaching and alkali extraction (Pekarovicova *et al.*, 1992), indicated no significant alterations in the shape of the fibres, however, flattening of the fibre was seen after extraction (alkaline) indicating that the lignin extraction from the cell wall leads to its collapse. In an another report, it was documented that xylanase-based pretreatment of sulfite pulp bagasse results in the formation of 'peels' and 'cracks' on fibre surfaces (Agnihotri *et al.*, 2012).

Biobleaching of pulp

During the pulping processes, the processed pulp appears brown in colour because of the presence of residual lignin and its derivatives. Therefore, with a view to obtain white and brighter pulp for the manufacturing of good quality paper, it becomes obligatory to bleach the pulp to eliminate lignin and its degradation products (Lundgren *et al.*, 1994). Traditionally chlorine has been used as the bleaching agent in the pulp and paper industry but due to environmental concerns biobleaching is considered to be a better alternative. It has been documented that biobleaching of pulp is comparatively more effective with microbial xylanases instead of other lignin-degrading enzymes. This is due to lignin being cross-linked to the hemicelluloses which is more readily depolymerized than lignin (Khandeparker and Numan, 2008).

20.6.4 Xylanases in the baking industry

Enzymes are known to play an important role in baking technology. The applications of xylanases have been reported in the making of bread (Senn and Pieper, 2001) where they are involved in enzymatic hydrolysis of non-starch polysaccharides and improve the rheological properties of dough, crumb firmness and bread specific volume (Martinez-Anaya and Jimenez, 1997). Endo-1,4-β-xylanase (xylanase) randomly acts on the arabinoxylan rachis which decreases the degree of polymerization, thereby leaving a strong impact on the structural integrity of arabinoxylan and its function (Courtin and Delcour, 2002). At an optimum dose, xylanases have been demonstrated to improve dough machinability, crumb structure, loaf volume, oven spring, dough stability and shelf life (Poutanen, 1997). The hydrolytic action of xylanase liberates free sugars such as pentoses which might be used by microbes for fermentation.

In the baking industry, the elasticity of the gluten network is increased with the help of xylanases which are utilized as additives. Xylanases enhance the handling as well as the stability of the dough. Subsequently, the enzyme gets denatured and inactivated during the

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bread-baking process. Addition of xylanases to wheat flour for bread making alters the arabinoxylans present in the flour and result in approximately a 10% more voluminous loaf (Garg *et al*., 2010). Several xylanases from bacterial and fungal sources are used in the baking industry (Pariza and Johnson, 2001). Xylanase action also improves crumb softness following storage. Here it is noteworthy that owing to dissimilarities in substrate specificities, interactions with inhibitors, kinetics capabilities and action patterns, all xylanases are not effective in baking (Pariza and Johnson, 2001). A comparison of the efficiencies of the different xylanases from *Aspergillus oryzae*, *Humicola insolens* and *Trichoderma reesei* revealed that the xylanase from *A. oryzae* was the most effective in improving the quality of bread made from wheat flour (Basinskiene *et al.*, 2006). The specific volume of the bread was found to increase by 8–13% and crumb firmness was decreased by 15–24% when compared to bread without xylanase. However, maximum anti-staling effect was recorded with xylanase from *T. reesei.* Jiang *et al.* (2005) reported an improvement in the specific volume of wheat bread using endo-1,4-β-xylanase B from *Thermotoga maritima.*

20.6.5 Fruit juice and beer clarification

Endo-1,4-β-xylanase has been reported to help in increasing juice yield from fruits or vegetables and also in the maceration process. Xylanases also lower the viscosity of the fruit juice thereby improving its filterability (Biely, 1985). Addition of endo-1,4-β-xylanase facilitates the release of more fermentable sugars from barley and is therefore useful in making beer, as well as being useful in processing the spent barley for animal feed and lowering the viscosity of the brewing liquid (Biely, 1985).

20.6.6 Improving silage

Endo-1,4-β-xylanase and cellulase pretreatment of forages results in production of better quality silage and improves plant-cell-wall digestion by ruminants. A considerable amount of sugar sequestered in the xylan of plant biomass gets released during endo-1,4-β-xylanase treatment, thereby increasing nutritive sugar, useful for digestion of lignocellulose in cows and other ruminants. It has been observed that endo-1, 4-β-xylanase also produces compounds which are the source of the nutrients for many ruminal microflora (Garg *et al.*, 2010).

20.6.7 Lignocellulosic bioconversions

Pretreatment of lignocellulosic materials using xylanase liberates sugars, which could be utilized for producing xylooligosaccharides, ethanol, organic acids, single cell protein and biohydrogen (Barnard *et al*., 2010; Lo *et al*., 2010; Li and Wang, 2011). Xylooligosaccarides have been proved to have their applications as prebiotics and preventing dental caries.

20.6.8 Xylanases in textile processing

In the textile industry, the pretreatment of cotton fabric is necessary for subsequent wet-processing treatments such as dyeing, printing or finishing. Such pretreatment practices for cotton preparations include desizing and scouring to make it hydrophilic and allow bleaching to reach a standard level of whiteness (Karmakar, 1999; Rouette, 2001). Desizing requires removal of the adhesive substance, known as size, from the warp threads which is pre-coated to prevent the thread breaking during weaving and the process involves treatment of the fabric with chemicals such as acids, alkali or oxidizing agents. In scouring, several non-cellulosic components such as fats, waxes, proteins, pectins, natural colourants, minerals, non-cellulosic polysaccharides and water-soluble compounds largely found in the primary cell wall are either completely or partially removed from native cotton. Scouring imparts the fabric with high and even wettability, so that it can be bleached and dyed successfully. Generally, highly alkaline chemicals like sodium hydroxide are used for scouring in the textile industry. Scouring not only removes the non-cellulosic impurities from the cotton, but also attacks the cellulose which leads to heavy strength loss and weight loss in the fabric. Moreover, the use of these hazardous chemicals results in high COD (chemical oxygen demand), BOD (biological

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oxygen demand) and TDS (total dissolved salts) values in the waste water. Replacement of the chemical-based scouring agents with xylanase could lead to hydrolysis of the tiny fibres that attached the seedcoat fragments to the fabric, eventually making the seedcoat fragments more accessible to chemicals, thereby reducing the consumption of hydrogen peroxide in the consecutive chemical bleaching step (Csiszar *et al.*, 2001).

20.6.9 Bioenergy

Chiranjeevi *et al.* (2012) optimized the physicochemical parameters influencing the production of holocellulases (a mixture of cellulases and xylanases) from *Cladosporium cladosporioides* under SmF. They found that a cocktail of holocellulases has a pivotal role in the commercialization of textile, biorefinery and paper manufacturing industries and detergent formulation. Song *et al.* (2012) reported that wheat straw is a primary source of lignocellulosic biomass for second generation biorefining. They engineered GH11 xylanase with an objective to improve its biomass-degrading ability. However, they also suggested that enzyme engineering alone cannot overcome the limitations imposed due to the complex organization of the plant cell wall and the lignin barrier. Cavka *et al.* (2011) demonstrated the potential of converting waste fibre sludge from pulp mills into liquid biofuel with xylanase enzymes as co-products in lignocelluloses-based biorefineries. A thermal- and acid-stable xylanase called as 'Xtreme' xylanase has been discovered in the bacterium, *Alicyclobacillus acidocaldarius* isolated from Nymph Creek in Yellowstone National Park's Norris Geyser basin (Idaho National Laboratory, 2015). The enzyme is both stable as well as active at temperatures from hot tap water to nearly boiling, and under acidic conditions ranging from battery acid to acid rain. The enzyme can efficiently convert hemicellulose and cellulose components of biomass into fermentable sugars which can be used to make fuels and high value chemicals.

20.7 Xylanases: Challenges

The pulp and paper industry is considered to be one of the fastest growing sectors, which is looking forward for thermostable xylanases in order to meet processing conditions. The proven stability of xylanases at high temperatures is the primary issue associated with their use, because thermostable xylanases have the capacity to increase the residence time when required, making the process more efficient. Kraft cooking, a process associated with pulping of wood chips, is performed at a very high temperature (170°C) in the presence of sodium hydroxide and sulfides. Although the temperature drops down to 90°C during the bleaching step, most of the existing xylanases are not able to withstand this temperature. Xylanases from *T. maritima* and *G. thermoleovorans* have been reported to have temperature optima above 80°C, but alkali-stability seems to be the main hurdle associated with these enzymes (Yoon *et al.*, 2004: Sharma *et al.*, 2007). Thermostable xylanases are also required in the animal feed industry especially during pellet making. Although, most of the xylanases exhibit activity at higher temperatures (50–90°C) they are optimally active at either acidic or neutral pH (Sunna and Bergquist, 2003; Wang *et al.*, 2010). Xylanases from different strains of *Bacillus halodurans* and *G. thermoleovoans* are optimally active in alkaline conditions (Sunna and Bergquist, 2003; Song *et al.*, 2012), however, their stability at higher temperatures is not adequate for the process conditions in the paper industry. Xylanases should be preferably of low molecular weight so that they can diffuse easily into the pulp fibres in order to facilitate the catalytic reaction. They must also be cellulase-free so as to protect the cellulosic content of the pulp. To this end, the bacterial xylanases of the family GH11 has emerged as the most suitable, being both cellulase-free and of low molecular mass (Mamo *et al.*, 2006: Wang *et al.*, 2010).

Recombinant DNA technology has eventually resulted in the selection of xylanolytic enzymes that are more suitable for industrial applications (Techapun *et al.*, 2002). The specific challenges for recombinant DNA technology recognized in xylan bioconversion are: (i) production of cellulose-free xylanolytic systems; and (ii) an improvement of the fermentation characteristics of the associated organisms by introducing genes for xylanase and xylosidase (Sunna and Antranikian, 1997).

Large-scale production of xylanases requires reduction in their costs for diverse industrial

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applications which is still very challenging. Low molecular weight endoxylanases have already been expressed successfully in various hosts albeit their expression levels were not similar in all the cases but ranged from 2 mg/l to 148 mg/l, which was far from the anticipated level for commercial applications.

Multimeric enzymes when expressed in the secretory mode cannot easily pass through the cell wall barrier and accumulate within the periplasmic space instead of being secreted into the fermentation media (Hermann *et al.*, 2008). To meet this challenge application of *gas1* gene knockout yeast strains for recombinant protein expressions could be an alternative option. Gas1 null mutation affects yeast cell wall synthesis thereby generating progeny with a leaky cell wall, which facilitates the release of multimeric proteins extracellularly into the fermentation media (Marx *et al*., 2006). Multimeric protein expression may result in an improperly or partially folded protein. Nascent polypeptide chains produced during overexpression of proteins either saturate or overload the cellular endoplasmic reticulum (ER) machinery leading to improperly or partially folded proteins.

Co-expression of the protein disulfide isomerase chaperone and/or homologues of ER resident chaperones at the time of recombinant protein expression could assist in the proper folding of recombinant proteins, eventually promoting functional expression of proteins. The structures of β-xylosidases have not yet been well elucidated due to the difficulty in obtaining their full crystallographic information. Moreover, overexpression of other hemicellulase enzymes, such as arabinofuranosidases, mannanases and ferulic acid esterases, involved in the hydrolysis of xylan side chains has not received much attention as yet (Moreira and Filho, 2008). Complete hydrolysis of hemicellulose to monomer sugars requires a synergistic action of all hemicellulase enzymes; hence, their optimal ratio is also an important factor which affects both the hydrolysis efficiency and the enzyme dosage. Unfortunately, this subject seems to be hardly addressed in the existing literature. Compared with other hydrolytic enzymes the nucleotide entries of hemicellulases are relatively limited and mining new genes from nature could be a good way to find out new and powerful xylanases as well as other hemicellulase enzymes to meet the industrial requirements, such as operations at high temperature, high or low pH, and high salt concentration or in the presence of toxic chemicals. Rational engineering and directed evolution of known genes are also powerful tools to get tailor-made hemicellulase enzymes suitable for various industrial applications.

20.8 Conclusion and Future Prospects

For complete hydrolysis of xylan into fermentable sugars, the joint action of different hemicellulases is required. Endo-1,4-β-xylanase is the dominant enzyme of this system which breaks the glycosidic linkage between xylosides, resulting in formation of small xylooligosaccharides. Most of the explored xylanases have been place within the GH10 or GH11 families, whereas the xylanases belonging to the families 5, 7, 8 and 43 are still being researched.

The conversion of xylan into useful products is part of the strategies being made to consolidate the entire economics of the pretreatment of lignocellulosic material, and to this end xylanases have been identified as key enzymes with diverse industrial applications. Therefore, in future, there is a need to develop easier and cost-effective production of xylanases to cater for the needs of various industries. In this connection, instead of application of existing lignocellulosic biomass from agriculture for the production of xylanases either by SmF or SSF, there is also a need to investigate revolutionary molecular techniques in addition to those that are being used to improve the enzyme's characteristics and increase its expression rates.

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21 Microbial Chitinase: Production and Potential Applications

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Abstract
Chitin, a biopolymer of *N*-acetyl-D-glucosamine, is extensively found in marine and terrestrial environments. The chitinase enzyme is able to hydrolyse insoluble chitin into its oligo and monomeric components and has received increased attention because of the broad range of biotechnological and industrial applications including: (i) pharmaceutically significant chito-oligosaccharides; (ii) single-cell proteins; (iii) protoplast isolation; (iv) control of pathogenic fungi; and (v) treatment of chitinous waste. The enzyme chitinase is found in various organisms such as bacteria, fungi, crustaceans, insects, invertebrates and higher plants. The commercial application of microbial chitinase is attractive as by comparison to other sources of chitinase it lends itself to large-scale production to fulfill the demands of the current world. This chapter covers microbial sources of chitinase, its structure, its production and applications in industrial and biotechnological sectors.

21.1 Introduction

Chitinase, also known as chitinolytic enzymes, is a group of enzymes which are able to degrade chitin directly into low molecular weight products. It is found in various organisms such as bacteria, fungi, crustaceans, insects, invertebrates, higher plants and in some vertebrates (Yong *et al*., 2005). In 1911, chitinase was first time described by Bernard who found a thermo-sensitive antifungal factor in orchid bulbs (Gokul *et al*., 2000). Chitinases are classified into endochitinases and exochitinases based on their mode of action on chitin or chito-oligomers. Endochitinases have the capacity to cleave chitin at internal sites to generate multimers of *N*-acetyl-D-glucosamine (GlcNAc or NAG). However, exochitinases catalyse chitin progressively to produce GlcNAc, chitobiose or chitotriose. Chitinases belong to two major families of carbohydrate enzymes (families 18 and 19) depending on their sequence similarities, structure and function. Family 18 comprises chitinases found in bacteria, fungi, viruses and animals, and some classes of plant chitinases (class III or V). Family 19 includes class I, II and IV chitinases of plant origin only, with exception of chitinase C isolated from *Streptomyces griseus* HUT 6037 (Ohno *et al*., 1996) and chitinases F and G from *Streptomyces coelicolor* (Saito *et al*., 1999). It is well known that chitinolytic enzymes have a range of industrial and biotechnological applications including preparation of single-cell protein, pharmaceutically important chito-oligosaccharides and GlcNAc (Pichyangkura *et al*., 2002; Gohel *et al*.,

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2007). It is also applied for control of pathogenic fungi and malaria transmission, treatment of chitinous waste and isolation of protoplasts (Dahiya *et al.*, 2006). The production of inexpensive chitinases is important in the use of chitin-containing waste, particularly in the seafood industry, where it can not only solve an environmental problem but also add value in certain cases (Wang *et al.*, 1995; Suginta *et al.*, 2000). The enzymatic hydrolysis of chitin results into chito-oligomers which are used in diverse fields such as in medical, agricultural and industrial applications. The chito-oligomers are used as antibacterial, antifungal, antihypertensive and hypocholesterolaemic agents, and as a food quality enhancer (Bhattacharya *et al.*, 2007). In recent years, efforts have been made throughout the world to enhance the production and isolation of gene(s) encoding for the chitinase enzyme to be utilized as candidate gene(s) to combat with fungal pathogens (Tsujibo *et al.*, 2000; Viterbo *et al.*, 2001). In nature, bacterial and fungal chitinases play an important role in maintaining the balance between carbon and nitrogen trapped in the large biomass as insoluble chitin (Aronson *et al.*, 2003; Li, 2006).

21.2 General Structure and Properties of Chitin

Chitin is a polymer of GlcNAc connected to each other by β-1,4 glycosidic linkages (Majeti and Kumar, 2000). It is an essential structural polymer present in the fungal cell wall, the exoskeleton of arthropods and in the microfilarial sheath of nematodes, acting as a protective layer against harsh conditions (Tharanathan and Kittur, 2003). It is a colourless to off-white, hard, inelastic, nitrogenous polysaccharide. The molecular weight of chitin ranges from 1.03×10^6 Da to 2.5×10^6 Da. The literature includes numerous reports on its multidimensional properties in biomedical and other industrial applications. Chitin is biocompatible and biodegradable and has antibacterial and wound-healing capabilities (Jolle and Muzzarelli, 1999). It has a highly ordered crystalline structure and is insoluble in water as suggested by X-ray diffraction studies (Roberts, 1992). One of its many significant features is its ability to be easily processed into different forms such as fibres, cotton, powder, hydrogels, beads, sponges, films, flakes and membranes (Mano and Omori, 2007). The properties and polymer chain arrangement of chitin depends on its origin (Rinaudo, 2006). It is analogous to cellulose in both chemical structure and in biological function with some exceptions (Fig. 21.1).

Chitin is a white, amorphous, semi-transparent mass that is insoluble in common solvents such as water, acid, alkali, ethanol and other organic solvents but soluble in concentrated HCl, H_2SO_4, acetic acid and 78–97% phosphoric acid. The basic units of this substance are linked together by condensation reactions to make up long chains. Hydrogen bonds link the chains together and help to make chitin rigid and strong. The solubility of chitin is enhanced by partial deacetylation under mild conditions that do not degrade the polymer, thus increasing the polarity and electrostatic repulsion of the amino groups. The literature suggests that chitins with a degree of acetylation display good solubility in aqueous media (Cho *et al.*, 2000).

There are three different crystalline polymorphic forms of chitin, namely α, β and γ (Jolle and Muzzarelli, 1999; Khoushab and Yamabhai, 2010).

Fig. 21.1. Chemical structure of (a) chitin (b) cellulose.

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21.2.1 α-Chitin

α-Chitin consists of alternating parallel and anti-parallel chains (Fig. 21.2). It is stable and tightly compacted due to its crystalline structure and it is the most abundant isomorphous form occurring mainly in crustaceans, insects and fungi in nature. α-Chitin is insoluble and does not swell in common solvents.

21.2.2 β-Chitin

β-Chitin consists of only parallel chains and occurs only in marine organisms (Fig. 21.2). It is soluble in formic acid, forms a series of crystalline hydrate structures and can be swollen in water by intercalating between the stacks of chains (Kurita, 2001). By treatment with acid (cold 6 N hydrochloric acid or in a solution of formic acid) and 45% fuming nitric acid, β-chitin can be irreversibly converted into α-chitin (Lotmar and Picken, 1950; Blackwell, 1988).

21.2.3 γ-Chitin

γ-Chitin is a mixture of both α- and β-chitins (Atkins, 1985) (Fig. 21.2).

21.3 General Structure, Properties and Classification of Chitinase

Chitinases (EC 3.2.1.14) are glycosyl hydrolases, which catalyse chitin hydrolysis between the C1 and C4 of two consecutive GlcNAc by either an endolytic or an exolytic mechanism (Parani *et al.*, 2011) and produce GlcNAc and *N*-acetyl chito-oligosaccharides, and chitinase has been applied as a biologically functional material in many biotechnological areas (Han *et al.*, 2008). Chitinases are generally found in plants, animals, insects, bacteria, fungi, viruses and actinomycetes (Khoushab and Yamabhai, 2010). Mostly, chitin-containing organisms contain chitinases which are required for morphogenesis of cell walls and exoskeletons. However, some organisms do not contain chitin but produce chitinases, for example soil bacteria (Gooday, 1977). The enzyme is involved in cell division and cell separation such as in *S. cerevisiae* (Kuranda and Robbins, 1991). The yeast cells are unable to separate normally if they lack chitinase activity in the logarithmic growth phase, and instead they aggregate at the septum regions and remain as clusters (Kuranda and Robbins, 1991). The functions of chitinases in various organisms are diverse. In plants chitinase secretion is induced by infections of chitin-containing microbes (fungi, insects) or other injuries (Pegg and Young, 1982; Boller *et al.*, 1983). Chitinase activity is induced by fungal, bacterial and viral infections and as such chitinases are considered as pathogenesis-related proteins (Neuhaus, 1999). In fungi, chitinase activity plays a physiological role in apical growth and morphogenesis of fungal hyphae (Gooday *et al.*, 1986). In bacteria, chitinases play a role in nutrition and parasitism. In insects and crustaceans, chitinases are required for the partial degradation of old cuticle (Dahiya *et al.*, 2006), while in humans, chitinases can be found in gastric juices and they are involved in catabolic activity (Paoletti *et al.*, 2007). In addition to the above, chitinolytic enzymes have been widely used in various applications including agricultural, biological, biotechnological and environmental fields (Chuan, 2006). These enzymes can be directly applied in the biological

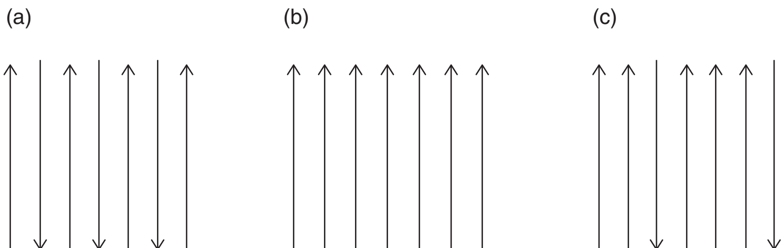

Fig. 21.2. Three polymorphic configurations of chitin. (a) α-Chitin, (b) β-chitin and (c) γ-chitin.

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control of microorganisms, or indirectly through genetic manipulation of the purified enzyme (Oppenheim and Chet, 1992; Ahmadian *et al*., 2007). In recent years, bacterial chitinase genes from *Alteromonas* (Tsujibo *et al*., 1993), *Bacillus circulans* (Watanabe *et al*., 1994), *Vibrio harveyi* (Soto-Gil and Zyskind, 1984) and *Vibrio vulnifucus* (Chang *et al*., 2003) have been cloned and characterized. Due to its antifungal activity chitinases have huge biotechnological potential in food and seed preservation (Dempsey *et al*., 1998).

Chitinases belong to a group of complex hydrolytic enzymes that catalyse the depolymerization of chitin and are divided into two main categories on the basis of their mode of action (Fig. 21.3). The two main categories are:

- Endochitinases (EC 3.2.1.14) or poly β-(1,4)-2-acetamide-2-D glucoside glycanohydrolase which randomly cleaves the chitin chain at internal sites, generating multimers of GlcNAc.
- Exochitinases which are further classified into two subcategories: (i) chitobiosidases (EC 3.2.1.29); and (ii) β-(1,4)-*N*-acetyl glucosaminidases (EC 3.2.1.30). Chitobiosidases catalyse the gradual discharge of diacetylchitobiose starting from the non-reducing end of chitin. However, β-(1,4)-*N*-acetyl glucosaminidases cleave the oligomeric products of endochitinases and chitobiosidases, resulting into formation of GlcNAc monomers (Sahai and Manocha, 1993).

As already mentioned chitinases are classified into two families which further divide into five known classes of chitinase (Henrissat and Bairoch, 1993). Both families of chitinases do not share sequence similarity and have a different three-dimensional structure, and it is assumed that they have developed from a different ancestor (Davies and Henrissat, 1995; Hamel *et al*., 1997). They also differ in several of their biochemical properties.

Family18 consists of class III and class V chitinases found in bacteria, plants, animals, yeast, viruses and fungi. Family 18 chitinases play an important role in pathogens and pests control. Family 18 chitinases use a retention mechanism, keeping the catalysis product in the same configuration as the substrate (i.e. β-anomeric form). In addition, they hydrolyse GlcNAc–GlcNAc or GlcNAc–GlcN linkages. Finally, family 18 chitinases are likely to function according to a substrate-assisted catalysis model (Brameld *et al*., 1998). Crystallographic analysis of family 18 chitinases isolated from both bacteria (Perrakis *et al*., 1994) and plants (Terwisscha van Scheltinga *et al*., 1994, 1996) revealed a common structural motif within two short homologous sequences. Thus, the family 18 chitinases share a similar active site, located at the carboxy terminus and comprising a $(\beta/\alpha)_8$-barrel domain. Hydrolysis has been reported to proceed with retention of the anomeric configuration (Armand *et al*., 1994), consistent with the double displacement hydrolysis mechanism. Family 18 chitinases broadly occur in five kingdoms of life, for example *Thermococcus kodakarensis* in Archaea (Fukui *et al*., 2005), *Serratia marcescens* in Bacteria (Brurberg *et al*., 1994), *Coccidioides immitis* in Fungi (Bortone *et al*., 2002), tobacco in Plantae (Melchers *et al*., 1994) and the sandfly and

N-acetyl-β-glucosaminidase Chitobiosidase Endochitinase and lysozyme

Chitin

Polymer of β-(1-4)-*N*-acetyl-D-glucosamine units

Fig. 21.3. Cleavage sites on chitin by exo- and endochitinases.

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human in Animalia (Fusetti *et al.*, 2002; Ramalho-Ortigao and Traub-Cseko, 2003).

Family 19 consists of class I, II and IV chitinases, which are exclusively present in plants. However, a single family 19 chitinase was reported in *S. griseus* (Ohno *et al.*, 1996; Watanabe *et al.*, 1999). Members of family 19 use an inversion mechanism converting the product into the α-anomeric form (Brameld and Goddard, 1998) and it acts on GlcNAc–GlcNAc or GlcN–GlcNAc linkages (Ohno *et al.*, 1996).

Chitinases isolated from bacteria belong to both family 18 and family 19. Generally the structure and function of bacterial chitinases have been studied by using enzymes from *Serratia marcescens* (Vaaje-Kolstad *et al.*, 2004), *B. circulans* (Hardt and Laine, 2004) and *Vibrio* sp. (Songsiriritthigul *et al.*, 2008). The crystal structure of a native bacterial chitinase A from *S. marcescens* has been reported by Perrakis *et al.*, (1994) (Fig. 21.4). The enzyme comprises three domains: (i) an all-β-strand amino-terminal domain; (ii) a catalytic α/β-barrel domain; and (iii) a small α+β-fold domain. There are several residues with unusual geometries in the structure. The reaction mechanism seems to be similar to that of lysozyme and most other glycosyl hydrolases (Perrakis *et al.*, 1994).

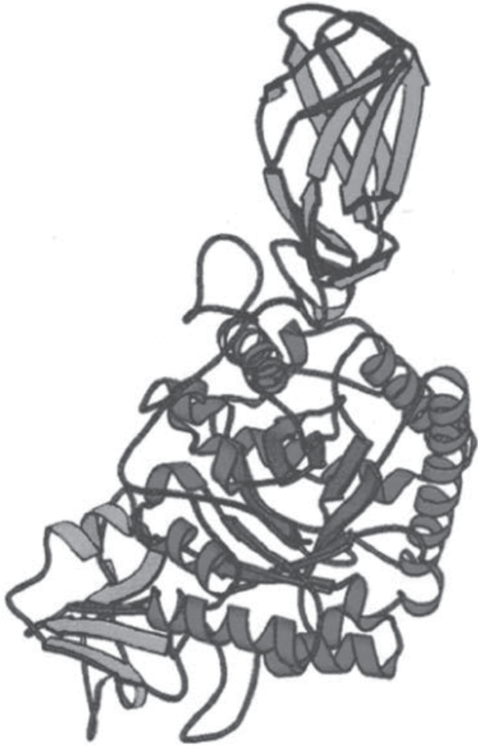

Fig. 21.4. Crystal structure of a bacterial chitinase (License number 3525290565441) showing the three domains (see text). (From Perrakis *et al.*, 1994.)

21.4 Source of Microbial Chitinase

Chitinase-producing microorganisms occur in nature and are an ideal source for production of chitinase because of their low production cost and easy availability of raw materials in comparison to plant, human and insect chitinases (Table 21.1).

21.4.1 Bacterial chitinase

Some bacteria including *Stenotrophomonas maltophilia*, *S. marcescens* and *Xanthomonas maltophilia* are recognized as effective chitin-degrading bacterial agents (Kobayashi *et al.*, 1995; Zhang and Yuen, 2000; Jung *et al.*, 2003). Chitinase from other bacteria such as *Aeromonas* sp. PTCC1691 (Al-Ahmadi *et al.*, 2011), *Bacillus* sp. (Thamthiankul *et al.*, 2001) and *Serratia* sp. (Haynes *et al.*, 1999) are reported to produce GlcNAc and chito-oligosaccharides by the biodegradation of chitin. The marine bacterium *Vibrio furnissii* (Keyhani *et al.*, 2000), *Clostridium* sp. (Konagaya *et al.*, 2006) and *Microbispora* sp. (Nawani *et al.*, 2002) can degrade chitin to chito-oligosaccharides, which can be metabolized and further used by other microorganisms as a sole source of nitrogen and carbon. Thermophilic chitinase of *Aeromonas* sp. DYU-T007 was purified and used for chitobiose production. Moreover, thermophilic organisms *Bacillus licheniformis* (Takayanagi *et al.*, 1991), *Bacillus* sp. BG-11 (Bharat and Hoondal, 1998) and *Strteptomyces thermoviolaceus* OPC-520 (Tsujibo *et al.*, 1995) are reported as a major sources of chitinases.

21.4.2 Fungal chitinase

Myrothecium verrucavia (Govindsamy *et al.*, 1998) and *Trichoderma* sp. (Howell, 2003) are suggested as major sources of chitinase among fungi. *Trichoderma harzianum* and *Fusarium chlamydosporum* are used as biocontrol agents.

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Table 21.1. Source of chitinase-producing microorganisms (published from 2000 onwards).

Microorganisms	Source of isolation	Reference
Serratia marcescens	Soil of fish market	Rebecca *et al*. (2013)
Bacillus amyloliquefaciens	Beaches	Das *et al*. (2012)
Kurthia gibsonii	Coastal environments	Paul *et al*. (2012)
Bacillus thuringiensis	Soil including chitin wastes	Kuzu *et al*. (2012)
Bacillus subtilis (JN032305)	Chilli rhizosphere soil	Narasimhan and Shivakumar (2012)
Streptomyces sp. SJKP9	Shore soil sample	Jagadeeswari and Selvam (2012)
Enterobacter sp. KB3	Coastal soils	Velusamy and Kim (2011)
S. marcescens XJ-01	Fishing field	Xia *et al*. (2011)
B. subtilis	Soil sample of agricultural field	Karunya *et al*. (2011)
S. marcescens	Fresh vegetables	Zeki and Muslim (2010)
Massilia timonae	Soil sample	Faramarzi *et al*. (2009)
Bacillus licheniformis	Red palm weavil gut	Khiyami and Masmali (2008)
B. licheniformis	Rhizosphere soil samples	Shanmugaiah *et al*. (2008)
Aeromonas sp. DYUT007	Beach sand	Lien *et al*. (2007)
B. licheniformis	Plant rhizospheric soil	Kamil *et al*. (2007)
B. licheniformis MB-2	Geothermal springs	Toharisman *et al*. (2005)
Bacillus cereus YQ 308	Soil samples	Chang *et al*. (2003)
Streptomyces sp. M-20	Mongolian soil	Kim *et al*. (2003)
Vibrio sp. 98CJ11027	Coastal area	Park *et al*. (2000)

Chitinases from *Trichoderma* sp. and *Aspergillus* sp. are used in the production of GlcNAc (Rattanakit *et al*., 2003; Abd-Aziz *et al*., 2008). Several antifungal chitinases of actinomycetes, especially from *Streptomyces* sp. have been produced and purified. Examples are *Streptomyces halstedii* (Joo, 2005), *Streptomyces hygroscopicus* (Haggag and Abdallh, 2012), *Streptomyces aureofaciens* (Taechowisan *et al*., 2003), *S. griseus* (Tarentino and Maley, 1974), *Streptomyces plicatus* (Robbins *et al*., 1988) and *Streptomyces lividans* (Miyashita *et al*., 1991). Chitinase from other fungi such as *Penicillium janthinellum* (Giambattista *et al*., 2001), *Acremonium obclavatum* (Kawachi *et al*., 2001), *Candida albicans* (Mellor *et al*., 1994), *Mucor fragilis* (Yamamoto *et al*., 1986), *Piromyces communis* (Sakurada *et al*., 1997) and *Acremonium obclavatum* (Gunaratna and Balasubramanian, 1994) have been reported.

21.5 Production of Chitinase

Fermentation technology plays an extremely significant role in the field of biotechnology not only for the production of various enzymes but it also reduces the cost of enzyme production. Microorganisms are considered as an ideal source for chitinase production because: (i) they have a short generation time; (ii) they are capable of high product yield; (iii) they are highly adaptable to environmental conditions; and (iv) the simplicity of genetic manipulation.

21.5.1 Submerged fermentation (SmF) system

Chitinases are enzymes which degrade chitin into its monomers and are of great importance to the biotechnological sector. Submerged fermentation (SmF) is the process by which microorganisms are cultivated in a liquid medium that contains soluble carbon along with other nutrients. In order to obtain the maximum yield of chitinase using SmF various parameters may be altered, such as using different substrates, carbon sources, nitrogen sources, metal ions, pH and fermentation times. Using the SmF process, lots of microbial strains have been used for chitinase production including bacteria, fungi, yeasts and algae. A list of chitinase-producing microbes and their production parameters are summarized in Table 21.2.

Carbon sources

In the growth of microorganisms, carbon is an essential nutrient along with others. In many studies it has been found that chitin itself is an

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Table 21.2. Optimum fermentation conditions for chitinase production (published from 2000 onwards).

Microorganisms	Incubation period (h)	Optimum temperature (°C)	Optimum pH	Substrate[a]	Reference
Vibrio alginolyticus JN863235	72	30	6.5	0.3% CC	Ravikumar and Meignanalakshmi (2013)
Aeromonas sp. ZD-05	72	30	7	1% CC	Dehdari *et al*. (2012)
Serratia marcescens CBC-5	72	30	7	1% CC	Chakraborty *et al*. (2012)
S. marcescens DSM 30121	144	30	6	2% CC	Lamine *et al*. (2012)
Streptomyces sp.	144	30	7	0.4% CC	Chandrasekaran *et al*. (2012)
S. marcescens XJ-01	32	32	8	0.75% CC	Xia *et al*. (2011)
Bacillius subtilis	96	35	7	0.3% CC	Karunya *et al*. (2011)
S. marcescens SMG	92	30	NM	1.75% chitin	Das (2011)
Bacillus cereus 1.21	36	55	7	NM	Mubarik *et al*. (2010)
Micrococcus sp. AG84	42	35	8	NM	Annamalai *et al*. (2010)
S. marcescens B4A	NM	30	7.9	1% chitin	Zarei *et al*. (2011)
Paenibacillius sp. D1	NM	30	7	NM	Singh (2010)
Streptomyces sp. ANU 6277	60	35	6	1% chitin	Narayana and Vijayalakshmi (2009)
Streptomyces	72	30–35	7	1.6% CC	Saadoun *et al*. (2009)
Streptococcus canus	120	40–60	8	1.2% CC	Mane and Deshmukh (2009)
Streptomyces pseudogriseolus	132	40–50	8	1% CC	Mane and Deshmukh (2009)
Micromonospora brevicatiana	132	40–60	8	1.2% CC	Mane and Deshmukh (2009)
Aeromonas sp. JK1	48	30	8	0.75% CC	Al-Ahmadi *et al*. (2011)
Bacillus laterosprous MML2270	NM	35	8	0.3% CC	Shanmugaiah *et al*. (2008)
Streptomyces venezuelae P10	96	30	NM	0.6% CC	Mukherjee and Sen (2006)
Streptomyces aureofaciens	NM	30–40	6.5–7	1% CC	Taechowisan *et al*. (2003)
Alcaligenes xylosoxidans	72	NM	8	NM	Vaidya *et al*. (2001)

[a]CC, Colloidal chitin; NM, not mentioned.

essential inducer for chitinase production, and no additional sugars are required. It has also been seen that the effect of the same carbon source differs among species, for example Miyashita *et al*. (1991) found addition of glucose to the chitin medium repressed chitinase production while Bhushan (1998) reported that the presence of glucose enhanced chitinase production. Some of the best carbon sources for chitinase production are given in Table 21.3.

Nitrogen sources

In industrial microbiology, regulation of nitrogen concentration plays an important role since it affects the synthesis of enzymes involved in both primary and secondary metabolism. Many reports are available on the effects of the supplementation of nitrogen sources on production of chitinase (Table 21.4).

Metal ions

Growth and chitinase production by microorganisms are greatly affected by the chemical constituents of the production medium. The influence of metal ion supplements on the production of chitinase by microorganisms has also received much attention and many reports are available (Table 21.5).

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Table 21.3. Carbon sources for microbial chitinase production (published from 2000 onwards).

Bacterial species	Best carbon source	Reference
Brevibacillus laterosporus	Colloidal chitin	Prasanna *et al*. (2013)
Vibrio alginolyticus JN863235	Glucose	Ravikumar and Meignanalakshmi (2013)
Bacillus licheniformis	Shrimp shell	Abirami *et al*. (2012)
Trichoderma viride	Colloidal chitin	Sharaf *et al*. (2012)
Streptomyces sp.	Sucrose	Sowmya *et al*. (2012)
Streptomyces sp. SJKP9	Sucrose	Jagadeeswari and Selvam (2012)
Serratia marcescens CBC-5	Colloidal chitin	Chakraborty *et al*. (2012)
S. marcescens XJ-01	Colloidal chitin	Xia *et al*. (2011)
Paenibacillus sp. DI	Crab shell chitin	Singh (2010)
S. marcescens	Chitin	Natarajan and Murthy (2010)
Metarhizium anisopliae	Colloidal chitin and dextrose	Dhar and Kaur (2009)
Streptomyces sp. ANU 6277	Starch	Narayana and Vijayalakshmi (2009)
Aeromonas sp. JK1	Chitin	Al-Ahmadi *et al*. (2011)
Streptomyces halstedii	Glucose with chitin	Joo (2005)
Bacillus cereus YQ 308	Shrimp and crab shell powder	Chang *et al*. (2003)
Alcaligenes xylosoxidans	Arabinose	Vaidya *et al*. (2001)

Table 21.4. Nitrogen sources for microbial chitinase production (published from 2000 onwards).

Bacterial species	Best nitrogen source	Reference
Vibrio alginolyticus JN863235	Yeast extract	Ravikumar and Meignanalakshmi (2013)
Trichoderma viride	Peptone	Sharaf *et al*. (2012)
Streptomyces sp. SJKP9	Yeast extract	Jagadeeswari and Selvam (2012)
Serratia marcescens CBC-5	Yeast extract	Chakraborty *et al*. (2012)
S. marcescens XJ-01	$(NH_4)_2SO_4$	Xia *et al*. (2011)
S. marcescens	Peptone	Natarajan and Murthy (2010)
Bacillus subtilis MTCC 2387, *Pseudomonas auregenosa* MTCC 4676, *S. marscencens* MTCC 4822	Yeast extract	Jaganmohan *et al*. (2010)
Streptomyces sp. ANU 6277	Yeast extract	Narayana and Vijayalakshmi (2009)
Metarhizium anisopliae	Yeast extract	Dhar and Kaur (2009)
Aeromonas sp. JK1	$(NH_4)_2SO_4$ and peptone	Ahmadian *et al*. (2007)
Trichoderma harzianum	Peptone and tryptone	Sandhya *et al*. (2004)
Aspergillius sp. SI 13	$(NH_4)_2SO_4$	Rattanakit *et al*. (2002)
Myrothecium verrucaria	Peptone and urea	Vaidya *et al*. (2001)
Alcaligenes xylosoxidans	Yeast extract	Vaidya *et al*. (2003)

21.5.2 Solid state fermentation (SSF) system

Solid-state fermentation (SSF) is the process of fermentation in which microorganisms grow on a solid fermentation substance in the absence of free water. In the process of SSF natural raw materials are used as the carbon and energy source and an inert material may also be used as a solid matrix for supporting the nutrient solution. The commonly used substrates for SSF processes are agrocrops and and agro-industrial residues. The most important agrocrops are barley and cassava and agro-industrial residues include wheat bran, rice bran, bagasse of sugarcane and cassava, and oil cakes of coconut, soybean and groundnut. Fruit pulps (e.g. apple), seeds (e.g. jack fruit), maize (corn) cobs, sawdust, coffee husk and pulp, shrimp shells as well as insect cuticles are also utilized as substrates for SSF (Rattanakit *et al*., 2003; Rustiguel *et al*., 2012). A list of chitinase-producing microorganisms that use

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different agro-industrial waste as the substrate for SSF is presented in Table 21.6.

The commercialization of the chitinase enzyme and its industrial applications are still limited by the production cost. Therefore, there is a great deal of interest in reducing the production cost of chitinase and utilization of agro-industrial wastes may be one of the ways to achieve this.

21.5.3 Statistical optimization of chitinase production

Several statistical and non-statistical methods are available for optimization of production medium constituents (Montgomery, 2002). Plackett-Burman and response surface methodology are the most widely used statistical approaches. Although optimizing the parameters by statistical methods reduces the time and expense, selection of media components for use in the Plackett-Burman design is either decided by replication or by random selection. The conventional method for medium optimization involves changing one parameter at a time while keeping all the others constant. This method may be very expensive and time consuming. In addition, it fails to determine the combined effect of different factors. A number of statistical experimental designs have been used to address these problems. A list of chitinase-producing microorganisms used for chitinase production by statistical optimization methods is presented in Table 21.7.

Table 21.5. Metal ions for microbial chitinase production (published from 2000 onwards).

Bacterial species	Best metal ion	Reference
Trichoderma viride	Fe and Zn	Sharaf *et al*. (2012)
Panibacillus sp.D1	Fe	Singh (2010)
Aeromonas sp. JK1	Mg and Mn	Al-Ahmadi *et al*. (2011)
Panibacillus sabina JD2	Ca	Patel *et al*. (2007)
Pentoea dispersa	Ca	Gohel *et al*. (2006)

21.5.4 Purification of chitinase and its characterization

Purification of chitinase is important in order to understand enzyme structure and the structure–function relationships of proteins (Aires-Barros *et al*., 1994; Saxena *et al*., 2003). For industrial purposes, the employed purification strategies should be inexpensive, rapid, high yielding and amenable to large-scale operations. Various processes are used to purify chitinases and these are highlighted in Fig. 21.5. The significance of purified chitinase is widely accepted in numerous applications, but the homogeneous preparation is not required for all commercial and industrial applications.

The properties of purified chitinase along with its specific catalytic activity are important

Table 21.6. Agro-industrial waste used for chitinase production (published from 2000 onwards).

Microorganism	Agro-substrate	Reference
Metarhizium anisopliae	Silkworm chrysalis	Rustiguel *et al*. (2012)
Trichoderma harzianum	Sugarcane bagasse	Sudhakar and Nagarajan (2011b)
Oerskovia xanthineolytica	Wheat bran with colloidal chitin as base	Waghmare *et al*. (2011)
Serratia marcescens	Rice bran and sugarcane bagasse	Sudhakar and Nagarajan (2011a)
Penicillium chrysogenum	Wheat bran and chitin	Patidar *et al*. (2005)
T. harzianum	Wheat bran	Sandhya *et al*. (2004)
Trichoderma longibrachiatum ATCC 36838	Wheat bran and crude chitin	Kovacs *et al*. (2004)
T. harzianum	Wheat bran, chitin and yeast extract	Nampoothiri *et al*. (2004)
Aspergillus sp. SI-13	Crab shell	Rattanakit *et al*. (2003)

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Table 21.7. Microbes used for statistical optimization of chitinase production (published from 2000 onwards).

Microorganism	Source of microbes[a]	Reference
Bacillus subtilis	Rhizosphere soil	Narasimhan and Shivakumar (2012)
Chitiolyticbacter meiyuanensis	Soil	Hao *et al*. (2012)
Bacillus pumilus	NM	Tasharrofi *et al*. (2011)
Alternaria alternate	Fishery polluted soil	Ghanem *et al*. (2011)
Beauveria bassiana SFB-205	Seoul National University, Korea	Kim *et al*. (2011)
Aspergillus terreus	Fishery polluted soil	Ghanem *et al*. (2010)
Stenotrophomonas maltophilia	Soil	Khan *et al*. (2010)
Serratia marcescens B4A	Shrimp culture ponds	Zarei *et al*. (2011)
Bacillus licheniformis TH-1	Shrimp waste	Akhir *et al*. (2009)
Massilia timonae U2	Soil sample	Faramarzi *et al*. (2009)
Paenibacillus sabina strain JD2	Sea dumps	Patel *et al*. (2007)
Streptomyces sp.	NM	Nawani and Kapadnis (2005)
Enterobacter sp. NRG4	NM	Dhar and Kaur (2009)
Pantoea dispersa	Chitinous waste disposal	Gohel *et al*. (2007)
Alcaligenes xylosoxidans	NM	Vaidya *et al*. (2003)

[a]NM, Not mentioned.

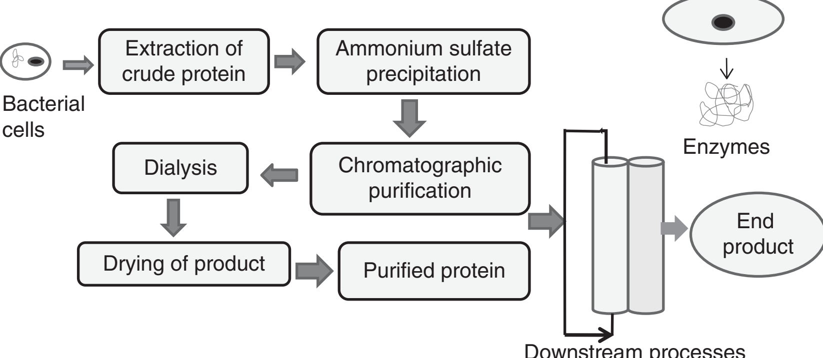

Fig. 21.5. Schematic diagram showing some of the processes involved in chitinase purification.

factors to be considered with regards to the introduction of chitinase into industrial processes and products. The characterization of these properties includes study of: (i) optimum pH and pH stability; (ii) optimum temperature and thermostability; (iii) the effect of metal ions, chelating agents, solvents and inhibitors; and (iv) the nature and concentration of the substrate.

pH

Each enzyme has an optimal pH range at which it is in its normal configuration. A change in pH can alter the ionization of the side chains and disrupt the tertiary structure and normal configuration, and sometimes it denatures the enzymes. Denatured enzymes cannot interact with a substrate. The optimum pH for chitinase produced by microorganisms mostly ranges from pH 7 to 10. However, some chitinases are optimally active outside this range, even at extreme alkalophilic pH (Loni and Bajekal, 2011) or at slightly acidic pH (Nawani *et al*., 2002). Many studies on chitinases from *Streptomyces* sp. M-20, *Paenibacillus* sp. D1, *Alcaligenes xylosoxidans* and *Aeromonas* sp. showed optimum activity in an acidic range.

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Temperature

The optimum temperature is the temperature at which an enzyme shows maximum activity and above that its catalytic activity decreases or is lost leading to its denaturation. Denaturation occurs due to damage in the three-dimensional structure of the enzyme (Harris and Angel, 1990). The temperature optima of chitinases may also be very variable, often depending on the source and origin of the microbial isolates. The optimum temperature of chitinase from *Microbispora* sp. was 60°C (Nawani *et al.*, 2002) and from *Aeromonas* sp., *Pseudomonas aeruginosa* K-187, *Cellulomonas flavigena* NTOU1 and *Bacillus* sp. BG-11 was 50°C (Chen *et al.*, 1997; Wang and Chang, 1997; Bhushan and Hoondal, 1998; Lien *et al.*, 2007). Thermophilic membrane-associated chitinase, Chi70, isolated from Archaea is optimally active at 70°C (Andronopoulou and Vorgias, 2003).

Metal ions and inhibitors

The effect of metal ions and inhibitors on chitinase activity has been investigated by various researchers. Some metal ions may protect the enzyme against thermal denaturation. Generally, the presence of divalent metal ions such as Ca^{2+}, Mg^{2+}, Zn^{2+} and Fe^{+2} often stimulate chitinase activity whereas transition and heavy metals such as Mn^{2+} (Wang *et al.*, 2010) and Hg^{2+} (Wang *et al.*, 2002; Kim *et al.*, 2003) generally caused inhibition of chitinolytic enzymes.

Molecular mass

Microbial chitinases vary widely in their size, ranging from as low as 20 kDa to about 120 kDa, with little consistency (Wang and Chang, 1997). Bacterial chitinases have a size range of 20–100 kDa, and 35–45 kDa have been reported for fungal chitinases (Ulhoa and Peberedy, 1991; Gunaratna and Balasubramanian, 1994; Sakurada *et al.*, 1996).

Microbial chitinases have an optimum activity at specific temperatures and pH, but they are stable over a wide range of temperatures and pH (Table 21.8). Due to these broad ranges of temperature, pH and substrate stability chitinases have great potential for biotechnological and industrial applications.

21.6 Applications of Chitinase

21.6.1 Antifungal properties of chitinase

The fungal phytopathogen poses severe problems on a global level in the cultivation of commercial plants, particularly in tropical and subtropical regions. Chitinolytic enzymes have the ablity to lyse the cell wall of various fungi. The microorganisms that produce these chitinolytic enzymes are capable of controlling fungal diseases that are a major problem for global agricultural production (Table 21.9).

Chemical fungicides are routinely used to reduce fungal pathogens. However, the excessive use of these compounds, which has increased almost threefold over the past 40 years, has led to problems related to contamination and degradation of the natural environment, along with induced pathogen resistance. These substances can be lethal to beneficial insects and microorganisms in the soil, and may also enter the food chain (Budi *et al.*, 2000). Biological control of plant pathogens by soil bacteria is a well-established phenomenon and production of chitinase by microbes may be an important factor in the suppression of various diseases (Hong and Hwang, 2006). Yan and co-workers (Yan *et al.*, 2008) reported direct evidence that some plants defend themselves against fungi by producing an enzyme called chitinase that potently inhibits fungal growth. Yan *et al.*, (2008) demonstrated that the antifungal activities of chitinases against four different plant pathogenic fungi were different, and it was directly correlated to the chitin content in the fungal cell wall. This study was useful as it provides information on the antifungal mechanism of the recombinant chitinase enzyme and its application for crop protection. Bolar *et al.*, (2001) studied the synergistic activity of endochitinase and exochitinase from *Trichoderma atroviride* (*T. harzianum*) against the pathogenic fungus *Venturia inaequalis* in transgenic apple plants. The protein, referred to as dolichin isolated from field beans (*Dolichos lablab*), exhibited antifungal activity against the fungi *Fusarium oxysporum*, *Rhizoctonia solani* and *Coprinus comatus* (Yea *et al.*, 2000). *Streptomyces coelicolor* A3 has 13 chitinase genes in which 11 encode family 18 chitinases and two encode family 19 chitinases but of these only Chi19F exhibited significant antifungal activity (Kawase *et al.*, 2006).

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Table 21.8. Characterization of purified chitinase (published from 2000 onwards).

Microorganism	Optimum temperature (°C)	Optimum pH	Inhibitors[a]	Molecular mass (kDa)	Reference
Streptomyces rimosus	40–45	7	Hg^{2+}, Pb^{2+}	63	Brzezinska *et al*. (2013)
Streptomyces hygroscopicus	30	6–7	NM	78, 76	Haggag and Abdallh (2012)
Streptomyces sp.	NM	NM	Hg^{2+}	25	Subramaniam *et al*. (2012)
Gliocladium catenulatum	60	6	Ag^{+}, Cu^{2+}, Fe^{2+}	51	Ma *et al*. (2012)
Streptomyces violaceusniger	50	5	Hg^{2+}, Ag^{+}	56.5	Nagpure and Gupta (2012)
Aspergillus niger	40	6–6.5	Hg^{2+}, Pb^{2+}	43	Brzezinska and Jankiewicz (2012)
Stenotrophomonas maltophilia MUJ	45	6.8	Hg^{2+}, Cu^{2+}	52	Jankiewicz *et al*. (2012)
Bacillus licheniformis	37	7	Mg^{2+}, PMSF	52	Zhang *et al*. (2011)
Bacillus firmus SBPL-05	37	10	NM	NM	Loni and Bajekal (2011)
Alcaligenes faecalis	37	8	NM	36	Annamalai *et al*. (2011)
Streptomyces sp.IK	37	6.7	NM	71	Margino *et al*. (2010)
Paenibacillus sp. D1	50	5	NM	56.5	Singh and Chhatpar (2011)
Bacillus sp.	60	7	Co^{2+}, Fe^{2+}, Zn^{2+}	79, 71, 48, 43	Natsir *et al*. (2010)
Micrococcus sp. AG84	45	8	EDTA	33	Annamalai *et al*. (2010)
Streptomyces sp. DA11	50	8	Fe^{2+}, Ba^{2+}	34	Han *et al*. (2009)
Bacillus licheniformis	70	5	Mg^{2+}, Co^{2+}	NM	Khiyami and Masmali (2008)
Aeromonas sp.	50	5	Ba^{2+}, Hg^{2+}, Mg^{2+}, Ag^{+2}	36	Lien *et al*. (2007)
Bacillus subtilis W-118	37	6	NM	20	Kim *et al*. (2003)
Enterobacter sp. NRG4	45	5.5	Cu^{2+}, Co^{2+}, Ag^{2+}, Hg^{2+}	60	Dahiya *et al*. (2005)
Streptomyces sp. M-20	30	5	Hg^{2+}, Hg^{+}	20	Kim *et al*. (2003)
Monascus purpureus	40	7	Hg^{2+}	81	Wang *et al*. (2002)
Streptomyces thermoviolaceus	60	4	NM	30	Tsujibo *et al*. (2000)
Vibrio sp.	45	6	Fe^{2+}, Cu^{2+}	98	Park *et al*. (2000)

[a]NM, Not mentioned; PMSF, phenylmethanesulfonylfluoride; EDTA, ethylenediaminetetraacetic acid.

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Table 21.9. Bacterial agents for biocontrol of fungal pathogens (published from 2000 onwards).

Microorganism	Pathogen	Reference
Penicillium ochrochloron	*Fusarium oxysporum*	Patil *et al*. (2013)
Streptomyces sp.	*Candida* sp.	Subramaniam *et al*. (2012)
Aspergillus niger LOCK 62	*Fusarium culmorum*, *Fusarium solani*, *Rhizoctonia solani*	Brzezinska and Jankiewicz (2012)
Streptomyces hygroscopicus	*R.solani*, *F. oxysporum*, *Alternaria alternata*, *Aspergillus niger*, *Aspergillus flavus*, *Sclerotinia scleotiorum*, *Phytophthora parasitica*, *Botrytis cinerea*	Haggag and Abdallh (2012)
Bacillus pumilus SG2	*Alternaria brassicicola*, *Fusarium graminearum*, *B. cinerea*	Dehestani *et al*. (2010)
Serratia marcescens B4A	*R. solani*, *Bipolaris* sp., *Alternaria raphani*, *Alternaria brassicicola*	Zarei *et al*. (2011)
Bacillus subtilis	*A. niger*, *A. flavus*, *Penicillium chrysogenum*	Karunya *et al*. (2011)
Enterobacter sp. KB3	*R. solani*	Velusamy and Kim (2011)
S. marcescens	*R. solani*	Jaganmohan *et al*. (2010)
S. marcescens	*F. solani*, *A. flavus*	Zeki and Muslim (2010)
Streptomyces griseus	*F. oxysporum*, *A. alternata*, *R. solani*, *F. solani*, *A. flavus*	Anitha and Rabeeth (2010)
Streptomyces sp. DA11	*A. niger*, *Candida albicans*	Han *et al*. (2009)
S. hygroscopicus	*Sclerotium rolfsii*, *Colletotrichum gloeosporioides*	Anitha and Rabeeth (2010)
Bacillus licheniformis	*R. solani*	Kamil *et al*. (2007)
Trichoderma harzianum	*R. solani*	Prabavathy *et al*. (2006)
Streptomyces venezuelae	*A. niger*, *A. alternata*, *Helminthosporium sativum*	Mukherjee and Sen (2006)
Enterobacter sp. NRG4	*Fusarium moniliforme*, *A. niger*, *Mucor rouxi*, *Rhizopus nigricans*	Dahiya *et al*. (2006)
Bacillus thuringiensis	*Aspergillus terreus*, *A. flavus*, *Nigrospora* sp., *Rhizopus* sp., *A. niger*, *Fusarium* sp., *Aspergillus candidus*, *Absidia* sp., *Helminthosporium* sp., *Aspegillus fumigatus*, *Curvularia* sp.	Reyes-Ramirez *et al*. (2004)
Pseudomonas fluorescens	*Pyricularia grisea*	Radjacommare *et al*. (2004)
T. harzianum	*S. rolfsii*	El-Katatny *et al*. (2000)

21.6.2 Chitinases and transgenic plants

A lot of literature is available on the molecular cloning of the chitinase gene in order to increase biocontrol efficiency. Some transgenic plants are produced that are resistant to pathogens due to overexpression of the introduced chitinase gene. For example the gene responsible for chitinase production *chiA* is produced by *S. marcescens* and this was introduced into endophytic *Pseudomonas fluorescens* in order to be used as a potential agent for the biocontrol of plant diseases caused by different phytopathogenic fungi (Downing and Thomson, 2000). Similarly a chitinase gene from *Enterobacter agglomerans* was introduced into *Escherichia coli* (Chernin *et al*., 1997). Some transgenic plants expressing the chitinase gene are shown in Table 21.10.

21.6.3 Chitinases as a biopesticide

Pesticides are applied in agricultural systems for protecting crops from damage caused by insects

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Table 21.10. Transgenic plants expressing the chitinase gene.

Plant	Gene/agent	Target pathogen/disease	Reference
Oil palm leaves (*Elaeis guineesis*)	*EgCHI1*, *EgCHI2*, *EgCHI3*	*Ganoderma boninense*, *Trichoderma harzianum*	Naher *et al*. (2012)
Chickpea (*Cicer arietinum*)	*RIP 30* and*Chi 26*	*Ascochyta rabiei*	Shahid *et al*. (2009)
Wheat	Chitinase gene	*Fusarium graminearum*	Shin *et al*. (2008)
Broccoli	Endochitinase	*Alternaria* sp.	Mora and Earle (2001)
Elite indica rice	PR-3 chitinase (RC_7)	*Rhizoctonia solani*	Datta *et al*. (2001)
Indica rice	Class-1 chitinase (Chi11)	*R. solani*	Datta *et al*. (2000)
Wheat	Chitinase (chi11)	*Fusarium graminearum*	Chen *et al*. (1999)
Potato	Endochitinase (chit42)	*Alternaria alternata*, *Botytis cinerea*, *R. solani*, *Sphaerotheca humuli*	Lorito (1998)
Cucumber	Chitinase (RCC_2)	*Botrytis cinerea*	Tabei *et al*. (1998)
Rose (*Rosa hybrida* L.)	Chitinase gene	Black spot disease (*Diplocarpon rosae*)	Marchant *et al*. (1998)
Tobacco	Bean chitinase promoter	*B. cinerea*, *R. solani*, *Sclerotium rolfsii*	Roby *et al*. (1990)

and disease. The application of chemical pesticides has also resulted in significant harmful effects to public health and the environment. Control of crop pests by the use of biological agents, such as chitinase from microorganisms, holds great potential as an alternative to the use of chemicals (Kramer *et al*., 1997).

In insects, chitin functions as a scaffold material, supporting the cuticles of the epidermis and trachea as well as the peritrophic matrices lining the gut epithelium. Insect growth and morphogenesis are strictly dependent on the capability to remodel chitin-containing structures. For this purpose, insects repeatedly produce chitin synthase and chitinolytic enzymes in different tissues. Coordination of chitin synthesis and its degradation requires strict control of the participating enzymes during development (Merzendorfer and Zimoch, 2003). However, as already mentioned, microbially produced chitinase can be used as a biopesticide in order to control insect pests. For example *Bacillus thuringiensis* base biopesticide supplemented with chitinase from *B. circulans* enhanced the effectiveness of *B. thuringiensis* subsp. *kurstaki* against diamondback moth larvae (Wiwat *et al*., 1999). Koga (2005) sprayed chitinase directly on to strawberry plants and observed that the plants were free from insects or pathogenic fungi. The chitinase genes expressed in baculovirus showed an increased killing rate of insect pathogens (Gopalakrishnan *et al*., 1995). Chitinase from other microbes, along with various applications, are summarized in some other reports in the literature (e.g. Gadelhak *et al*., 2005; Yanhua *et al*., 2007; Chandrasekaran *et al*., 2012).

Similarly, larval chitinases from tomato moth (*Lacanobia oleracea*) and tobacco hornworm (*Manduca sexta*) were shown to have insecticidal activity in transgenic plants (Wang *et al*., 2005). Kabir *et al.* (2006) isolated *Bombyx mori* chitinase (Bm-CHI), and found that it serves as a biocontrol agent against Japanese pine sawyer (*Monochamus alternates*). These findings open up the possibility of using insect chitinase as a biopesticidal agent with agronomic potential for insect control.

21.6.4 Isolation of protoplasts

Chitinases help in protoplast isolation because the enzyme accelerates the degradation of the cell wall which contains chitin. This property indirectly helps in strain improvement of various fungi produced by protoplast fusion and development of new economically viable strains which may be used in various biotechnological industries. The fungi *Rhizopus oligosporus*, *Penicillium* sp., *Aspergillus oryzae*, *Streptomyces phaerochromogenes*,

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Aspergillus niger, *Paecilomyces* sp. and *Trichoderma viride* have been used in the study of lysis of the cell wall for protoplast production by using chitinase. Waghmare *et al.* (2011) isolated protoplasts from *A. niger* using crude chitinase produced from *Oerskovia xanthineolytica* NCIM 2839 and results were confirmed by using scanning electron microscopy. A crude mycolase with a high chitinase activity was prepared from *Streptomyces olivaceoviridis* culture that was suitable for the formation of fungal protoplasts with good yield (Romaguera *et al.*, 1993). Bekker *et al.* (2009) isolated protoplasts from *A. niger* by using a cocktail consisting of lysing enzymes from *T. harzianum*, chitinase from *S. griseus* and β-glucuronidase from *Helix pomatia.* Rokem *et al.* (1986) isolated protoplasts from the mycelium of *A. niger* when it was treated with a mixture of chitinase and glucanase in the ratio of 1:1.4. Borba *et al.* (1994) generated protoplasts from yeast cells (*Paracoccidioides brasiliensis*) by using a three-enzyme system (novozym 234, chitinase and zymolyase 20T) and found that the osmotic stabilizer used was probably one of the factors responsible for the cytoplasmic changes observed by transmission electron microscopy in yeast phase cells and in their protoplasts. Two thermostable enzymes (chitinase and laminarinase) produced by the fungus *Paecilomyces varioti* were used to isolate protoplasts from the thermophilic fungus *Malbranchea sulfurea* and the frequency of regenerating protoplasts was considerably higher than that obtained using commercial lytic enzymes (Gautam *et al.*, 1996).

21.6.5 Medical applications of chitinase

In recent years, production of chito-oligomers from chitin by enzymatic hydrolysis is of great interest due to the broad medical applications of chito-oligomers such as their hypocholesterolaemic, antihypertensive activity, antitumour activity and immuno-enhancing effects (Liang *et al.*, 2007). Chitinase can also be used as: (i) potential additives in antifungal creams and lotions as well as a bone strengthener in osteoporosis (Ratanavaraporn *et al.*, 2009); (ii) a vector for gene delivery (Koping-Hoggard *et al.*, 2004); (iii) an antibacterial agent (Rhoades *et al.*, 2006); (iv) an antifungal agent (Oliveira *et al.*, 2008); (v) an anti-malaria agent or a haemostatic agent in wound dressings (Aam *et al.*, 2010); (vi) a food quality enhancer (Shahidi *et al.*, 1999); (vii) a food additive due to their sweet taste and stability (Deng *et al.*, 2005); and (viii) an agent for lowering serum glucose levels in diabetics (Lee *et al.*, 2003).

Enterotoxigenic *E. coli* K88 is a major cause of diarrhoea and death in neonatal and weaned pigs (Francis *et al.*, 1998). Liu and colleagues (2010) reported that dietary chito-oligosaccharide can replace antibiotics as a means of reducing infection-associated signs after *E. coli* K88 challenge in weaned pigs.

Chitinase can degrade chitin to generate chito-oligosaccharides such as chitohexaose and chitoheptaose, and both of these have been reported to have antitumour activity (Tokoro *et al.*, 1988; Patil *et al.*, 2000). Chitotetraose was found to have strong stimulating activity towards natural killer cells (Bezouska *et al.*, 1994) and more recently Park and Kim (2010) have indicated that chito-oliogosaccharides may reduce metastasis and tumour growth in cancer through immunomodulation (Fig. 21.6).

In addition, the GlcNAc itself (produced by the action of chitinase on chitin) is also an anti-inflammatory agent useful for the treatment of ulcerative colitis (Russell, 1999). Moreover, GlcNAc has been used as a nutritional substrate for paediatric chronic inflammatory bowel disease (Salvatore *et al.*, 2002).

21.6.6 Production of chito-oligosaccharides

Production of GlcNAc and *N*-acetyl chito-oligosaccharides from chitin is important for the food, agriculture, medicinal and biotechnology sectors.

GlcNAc and $(GlcNAc)_2$ can be produced by the acid hydrolysis of chitin, but this procedure is very costly and results in low yields and acidic waste (Inouye *et al.*, 1956). Recently, GlcNAc and $(GlcNAc)_2$ have been produced by enzymatic hydrolysis of chitin with good yields, low costs and also free from acidic waste (Kuk *et al.*, 2005). GlcNAc and *N*-acetyl chito-oligosaccharides were produced from colloidal chitin by application of crude enzyme obtained from *Paenibacillus illinoisensis* (Jung *et al.*, 2007).

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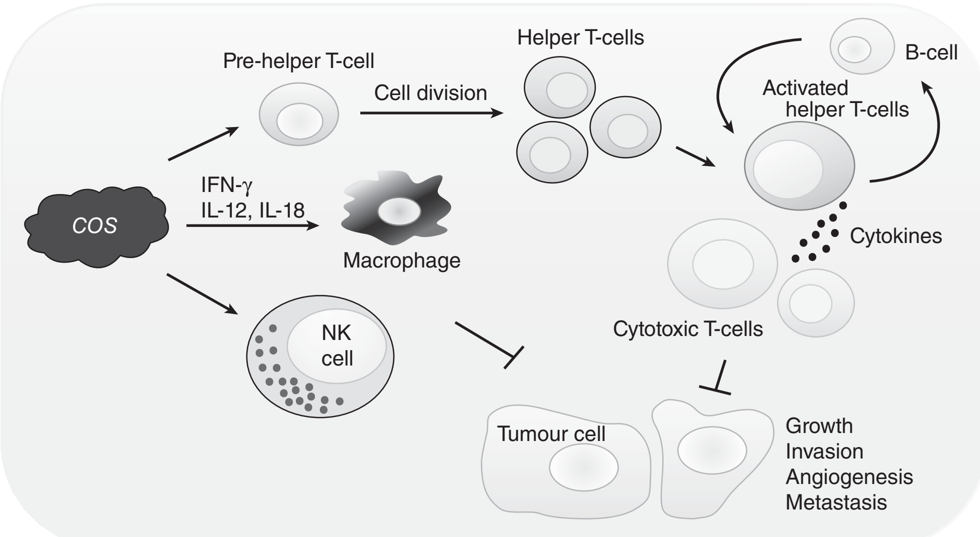

Fig. 21.6. Chito-oligosaccharides (COS) can display antitumorigenic activity through immunomodulation. IFN-γ, Interferon gamma; IL, interleukin; NK, natural killer cells. (From Park and Kim, 2010.)

The enzymatic production of chito-oligosaccharides with a high degree of polymerization was carried out using an ultrafiltration membrane reactor system by Jeon and Kim (2000). Several methods have been used to determine the content of chitin hydrolysed products, such as thin layer chromatography (Tanaka *et al*., 2003) and high-performance liquid chromatography (Sashiwa *et al*., 2003). Sashiwa *et al*. (2002) performed selective and efficient methods to produce GlcNAc using a mixed enzyme preparation with high GlcNAc-ase/endochitinase ratio. Shadia *et al.* (2012) reported production of GlcNAc by the application of chitinase of *Bacillus alvei* NRC-14.

21.6.7 Chitinase as a mosquitocidal agent

Chitinases can hydrolyse chitin in the peritrophic membrane of organisms and then cause pore formation, thus making insect larvae more susceptible to other toxins or infection. To investigate the potential value of chitinase in mosquito control, the activity of chitinase against mosquitoes and the synergistic activity of chitinase and crystal proteins to mosquitoes were studied. The crude chitinase (*Aeromonas hydrophila* SBK1) was tested for mosquitocidal agent against *Culex quinquefasciatus* larvae with LD_{50} (lethal dose, 50%) 0.60 IU/ml at 48 h (Halder *et al*., 2012).

21.6.8 Chitinase as a nematicidal agent

In many studies it has been found that chitinase enzymes show a nematicidal effect by acting on the eggshell of nematodes and preventing the egg from hatching. The nematicidal effect of chitin on plant-parasitic nematodes was first investigated by Mankau and Das (1974) who found that chitin amendments controlled the citrus nematode *Tylenchulus semipenetrans* and the root-knot nematode *Meloidogyne incognita.* Later, chitin amendments were used to control *Meloidogyne arenaria* (Mian *et al*., 1982), *Meloidogyne javanica* (Spiegel *et al*., 1987) and *Heterodera avenae* (Spiegel *et al*., 1989). Chitinase killed *Tylenchorhynchus dubius* by producing structural changes in the nematode cuticle (Miller and Sands, 1977). Purified chitinase inhibited egg hatch of *Globodera rostochiensis* by up to 70% *in vitro*, and the chitinase-producing bacteria *Stenotrophomonas maltophilia* and *Chromobacterium* sp. reduced egg hatch of nematodes both *in vitro* and

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in soil (Cronin *et al*., 1997). The chitinolytic fungus, *Paecilomyces lilacinus*, destroyed nematode eggs and efficiently controlled *M. incognita* (Morgan-Jones *et al*., 1984; Velusamy and Kim, 2011). *Pseudomonas chitinolytica*, with strong chitinolytic activity, reduced *M. javanica* infection and improved growth of tomato (*Lycopersicon esculentum*) (Spiegel *et al*., 1991).

21.6.9 Treatment of chitinous waste

Fish production is a significant contributor to the economy of our planet. According to the Food and Agriculture Organization of the United Nations (FAO), total world capture fisheries production in 2002 was 133 million tons (FAO, 2015). Almost 10% of the capture consists of species rich in chitinous material. India alone produces 60–80 thousand tons of chitinous waste annually that causes an environmental hazard and poses a serious problem (Dutta *et al*., 2004). Chitin can decompose by various methods such as conventional chemical and biochemical methods but these are harmful to the environment because gases are released into the environment that cause global warming. So, alternatively a biological method is used for degradation of chitinous waste by using chitinase-producing microbes. Biological methods are considered to be safe for the environment due to their extensive applications in biotechnology, medicine, agriculture and food industries (Arbia *et al*., 2013) (Table 21.11).

Nowadays chitin extracted from chitinous waste is used as a substrate and carbon source for chitinase production. Many reports are available in which microorganisms can degrade chitinous waste and produce chito-oligosaccharides, GlcNAc and single cell proteins. Mejia-Saules and co-workers (Mejia-Saules *et al*., 2006) used crude shrimp waste for chitinase production. The fermentation process has been studied for various crustacean shells including crab shells (Jung *et al*., 2007), shrimp waste (Xu *et al*., 2008) and prawn shells (Shirai *et al*., 1998).

21.7 Recent Patents and their Significance

In the area of chitinase and its applications several patents have recently been published (Table 21.12). For example in a recent patent (2013), a novel insecticidal chitinase protein was isolated from fern and used for insect control. The protein defined as the insecticidal protein is toxic to at least one of the following insects: (i) whitefly (*Bemisia tabaci*); (ii) cotton boll worm (*Helicoverpa armigera*); (iii) aphid (*Aphis gossypii*); and (iv) *Spodoptera litura*. The insecticidal activity includes a range of antagonistic effects such as mortality (death), growth reduction and deterring feeding (WO2013098858A2). Another patent, 'Biocontrol formulation containing *Streptomyces* sp. Method for preparing the relevant use' provided a biocontrol formulation containing a high concentration of *Streptomyces* spp. spores (US2007/0148755A1). Additionally, various patents on chitinase as an antifungal protein, insect repellent, biocide, antimicrobial and anti-nematode agent are available.

Table 21.11. Microbial degradation of chitinous waste.

Microbial	Substrate	End product[a]	Reference
Kurthia gibsonii MB126	Prawn shell powder	Chitinase	Paul *et al*. (2012)
Trichoderma harzianum	Chitinous waste	GlcNAc	Das *et al*. (2012)
Bacillus alvei NRC-14	Sugarcane molasses	GlcNAc	Shadia *et al*. (2012)
Bacillus licheniformis	Shrimp shell waste	Antifungal chitinase	Abirami *et al*. (2012)
Bacillus subtilis W-118	Shrimp powder	Chitinase	Wang *et al*. (2006)
Aspergillus sp. SI-13	Shrimp shellfish waste	Chitinase	Rattanakit *et al*. (2002)
Beauveria bassiana	Prawn waste	Chitinase	Suresh and Chandrasekaran (1999)

[a]GlcNAc, *N*-acetyl-D-glucosamine.

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Table 21.12. Some important patents on chitinase.

Year	Particulars	Patent number
2013	A novel insecticidal chitinase protein, its encoding nucleotide and application	WO2013098858A2
2013	A chitinase from *Brevibacillus laterosporus*, its production and use	WO2013050867A2
2013	Pest-resistant plants containing a combination of a spider toxin and a chitinase	US20130097731
2012	GH (Glycoside hydrolases) 18 family chitinase gene cloning method	CN102653768
2012	Chitinase gene and chitinase encoded by gene	WO/2012/111810
2012	Protein having chitinase activity and uses thereof	WO/2012/063338
2011	Pest-resistant plants containing a combination of a spider toxin and a chitinase	WO/2011/158242
2011	Recombinant biocatalyst which is prepared using chitinase gene	KR1020110117556
2011	Chitinase *chicd3*, encoding gene thereof and application thereof	CN102199583
2011	*Pseudomonas* sp. strain and method of producing chitinase	US20110207200
2011	Microorganism which expresses a recombinant chitinase gene for effective pest killing	KR101048626
2011	Chitin syntase, chitin deacetylase and chitinase gene sequence isolated from *Flammulina velutipes* KACC 42777	KR1020110052765
2011	Strain for producing chitinase and method for producing chitinase in high yield	CN101942401
2010	Use of nematode chitinase genes to control plant parasitic nematodes	US20100095404A1
2010	Method for improving plant disease resistance by using *Beauveria bassiana* chitinase gene	CN101812476
2010	Stable chitinase and preparation method thereof	CN101805729
2010	Chitinase genes of insects and application	CN101805746
2010	Method for preparing recombinant chitinase	CN101775405
2010	Method for manufacturing a *Poria cocos* extract using chitinase	KR1020100072900
2010	Method for detecting chitinase activity	JP2010130959
2010	*Trichoderma* for efficiently expressing chitinase coding gene and beta-1,3-glucanase coding gene as well as application thereof	CN101724573
2010	Cosmetic use of chitinase-type proteins	US20100056424
2010	New chitinase of bacterial origin with broad fungicide spectrum	WO/2010/023341
2009	Combined chitinase for deactivating *Varroa destructor* and application thereof	CN101613685
2009	Chitinase for deactivating *Varroa destructor*, *Serratia marcescens* and application thereof	CN101613684
2009	Chitinase Chib for deactivating *Varroa destructor* and application thereof	CN101613683
2009	Chitinase capable of degrading spore walls of Myxosporea and encoding genes thereof	CN101603039
2009	Cosmetic use of chitinase-type proteins	101600414
2009	Use of nematode chitinase genes to control plant parasitic nematodes	EN2126095
2009	Tick chitinase	US20090274715
2007	Biocontrol formulation containing *Streptomyces* sp. Method for preparing the relevant use	US2007/0148755A1
2006	Preparation of antibodies against the human chitinase-like protein GL008	EP1661915 A1
2006	Isolated nucleic acids encoding proteins with chitinase activity and uses	US7087810 B2
2004	*N*-acetyl-D-glucosamine (GlcNAc) and process for producing GlcNAc	US6693188
2004	Chitinase-encoding DNA molecules from cotton expressed preferentially in secondary wall cell during secondary wall deposition and a corresponding promoter	US2004/0049808A1
2002	Methods of using worm castings for insect repellence	US 6475503
2000	Diagnosis of fungal infections with a chitinase	US6093552
1999	Recombinant chitinase and use thereof as a biocide	US5866788
1997	Chitinase-producing plants	US5633450
1996	Diagnosis of fungal infections with a chitinase	US5587292
1992	Purified chitinases and use thereof	US5173419
1990	Novel chitinase-producing bacteria and plants	US4940840

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22 Characteristics of Microbial Inulinases: Physical and Chemical Bases of their Activity Regulation

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Abstract
This chapter is devoted to the analysis of the physical and chemical properties of inulinases from various microbial producers. Special attention is given to: (i) the description of functional features of these enzymes in the conditions of a various microenvirons; (ii) identification of optimum system parameters for their functioning; (iii) the characteristics of their stability; and (iv) resistance to temperature influences and extreme pH values. Perspectives of the development of biotechnological processes with the use of free and immobilized inulinases are discussed.

22.1 Introduction

Inulinases (inulases, 2,1-β-D-fructan-fructanhydrolases, EC 3.2.1.7) split inulin and fructooligosaccharides into fructose. Study of the physical and chemical properties of inulinases in conditions of various microenvirons has theoretical and applied significance. These enzymes participate in carbohydrate metabolism of plants and microorganisms, are present as some of the most important components of signal pathways of the communication system, and play one of the key roles in the controlling processes of cell differentiation, growth and development of the indicated organisms. Also they can be used in cycles of production of sugars with different degrees of polymerization, in particular fructose and inulooligosaccharides – inalienable components of functional nutrition, lowering the risk of the emergence of diabetes mellitus, caries and obesity. At the present time there are only a small number of review reports in the literature on inulases.

The catalytic activity of enzymes is determined by the degree of mobility of the protein molecule, and even small changes in the conformation of the polypeptide chain in enzymes can result in a major change in catalytic ability. For this reason the aim of our work was analysis of the physical and chemical properties of inulinases isolated from various microbial producers, description of the functional properties of these enzymes in conditions of various microenvirons, and discussion of the prospects of development of biotechnological processes with the use of inulinases in free and immobilized states.

22.2 Physical and Chemical Properties of Inulinases

It is well known that high temperatures and low pH values prevent risk of infection of fructose syrups with undesirable microflora, improve solubility of some substrates, including inulin, and can

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reduce the extent of colouring of products. At the same time temperature increase can lead to denaturizing of the enzyme and decrease in its catalytic function. Besides, taking into account that inulin possesses good solubility only at temperatures from 50°C and above, for industrial production of fructose from inulin-containing vegetable raw materials it is expedient to use thermotolerant microorganisms and their enzymes.

Research of the physical and chemical properties of inulinases from various microorganisms is necessary in order to expand the horizons of their effective practical application. The majority of microorganisms synthesize inulinase with a temperature optimum in the range of 45–55°C. Lower values are given also, for example: (i) 35°C for enzymes from *Bacillus polymyxa*; (ii) 37°C for those from *Bifidobacterium infantis*; and (iii) 40°C for those from *Rhizopus* species (Ohta *et al*., 2002; Warchol *et al*., 2002; Kwon *et al*., 2003).

Temperatures applied in production cycles do not always coincide with the maximum for the enzyme's activity. Often it is necessary to use lower values at which the enzyme is stable for a long time, therefore it is expedient to choose those inulinases which have an optimum of functioning that exceeds the estimated temperature in the bioreactor. The most useful species from an industrial application perspective are thermophilic microorganisms, such as *Kluyveromyces marxianus*, *Pichia guilliermondii*, *Aspergillus awamori*, *Aspergillus fumigatus*, *Aspergillus niger*, *Streptomyces* species, *Arthrobacter* species including *Arthrobacter ilicis* and *Cladosporium cladosporioides* which produce inulinase with a functioning optimum up to 60°C. Data on inulases with a higher temperature optimum are available, in particular for inulinase from some *Bacillus* species it is in area above 65°C, and for the enzymes from *Arthrobacter ureafaciens* and *Bacillus smithii* it is 70°C (Ferreira *et al*., 1991; Yokota *et al*., 1991; Tsujimoto *et al*., 2003; Gill *et al*., 2004; Cazetta *et al*., 2005, 2006a, b, c; Gao *et al*., 2007, 2009; Gong *et al*., 2008).

The temperature and pH optimum for inulinase from *K. marxianus* CBS 6556 differed when using sucrose (70°C and pH 3.5) and inulin (50°C and pH 4.5–5) as the substrate. This phenomenon was also characteristic for β-fructosidase from other strains of *Kluyveromyces* and other yeasts (Beluche *et al*., 1980; Workman and Day, 1983; Rouwenhorst *et al*., 1990).

Inulinases contain a large number of ionized groups therefore they can be in various ionic forms, however, their catalytic activity, as a rule, is shown in a narrow range of pH values (Kovaleva *et al*., 2008). Distribution of enzyme molecules between these ionic forms depends on constants of ionization (pK) of the separate groups and the value of the isoelectric point (pI) of the protein. The isoelectric points of the majority of inulinases are in the range pH 3.8–5.4. An exception is the enzyme from *A. fumigatus* (isoform I) which has a pI value of 8.8 (Xiao *et al*., 1989; Pessoa and Vitolo, 1997; De Roover *et al*., 1999a, b; Arand *et al*., 2002; Warchol *et al*., 2002; Moriyama *et al*., 2003; Van der Ende *et al*., 2003; Gill *et al*., 2004, 2006a, b, c).

Inulinases from various producers differ on a pH optimum. Fungi and yeast enzymes have the maximum activity in an acid environment (pH 4.0–5.5), while bacterial enzymes show maximum activity in more neutral environments (pH 5.5–7.0). Enzymes of bacteria are stable in the range pH 4.5–7.5, while enzymes of fungi are stable in the range pH 3.0–8.0, and those of yeast at pH 3.0–6.5. It is known that pH strongly influences the ratio of monosaccharides and oligosaccharides in solution: in an acid environment (pH 4.0) the content of fructose and inulobiose increases whereas at pH 6.0 the content decreases considerably. For industrial processes it is more expedient to choose those inulinases at which the optimum of functioning is in an interval pH 4.0–5.0, coinciding with a zone of the greatest stability of fructose.

22.3 Correlation Between Amino Acid Sequences of Inulinases and their Physical and Chemical Properties

Computer analysis of amino acid sequences of inulinases from various producers can create the premise to compose forecasts regarding the molecular peculiarities of the enzyme, the functional groups of its active centre, the mechanism of catalysis, and the physical and chemical properties. Detailed analysis of protein macromolecules on all levels of their organization in combination with classical approaches of biochemistry and biophysics reveal the structural

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and functional peculiarities and molecular mechanisms of inulinase action. At the present day the possibility to predict the properties of a protein from its amino acid sequence is one of the main problems of modern integrative biology.

Information about the primary structures of inulinases was obtained from the National Center for Biotechnology Information (NCBI, 2014). It has been established by us that the content of hydrophilic and especially hydrophobic residues is characteristically constant at 31.53–40.88% and 51.72–59.11%, respectively. The core of inulinases is formed by 21.79–36.97% of amino acids that are dependent on the producer, while the composition of ordered structures (α-helixes and β-sheets) varies from 61.25% (in the enzyme from *Penicillium* species) to 88.95% (in the enzyme from *A. niger*) of residues.

It is logical to suppose that an optimal value of temperature for the functioning of inulinases depends on the ratio of the number of amino acid residues disposed in ordered structures to those that form loops, however, such correlation was not disclosed by us (Artyukhov *et al*., 2013).

Remarkably, no direct connection was revealed between the quantity of amino acid residues forming separate α-helixes and β-sheets and the values of temperature and pH optima of inulinase. The number of residues in α-helixes correlates only with the species of producer, in particular in enzymes from *P. guilliermondii*, *Cichorium intybus* and *K. marxianus* it constitutes 36.4–41.2% of amino acid residues, in enzymes from *Arthrobacter* species 44.8–48.8%, for those from *Aspergillus* species it varies in the range 39.3–42.3%, while from *Bacillus* it is between 47% and 48%.

Practically in all inulinases the ratio of the number of amino acid residues forming β-sheets to the number of residues disposed in loops has a value close to 1. However, in all enzymes from *K. marxianus* it is somewhat lower than in enzymes from *Aspergillus*, which partially explains the higher temperature optimum of functioning for inulinases from fungi. Interestingly, a ratio greater than 1.1 is characteristic of proteins from *Pseudomonas mucidolens*, *P. guilliermondii* and *Geobacillus stearothermophilus*, for which the optimal conditions are pH 6.0 and temperatures of 55°C, 60°C and 60°C, respectively. The highest value (1.21) is observed for inulinases from *Bacillus polymyxa* and *Paenibacillus polymyxa*, which have a pH optimum of 7.0, sharply differing from other producers' enzymes, presenting a distinguishing peculiarity of enzymes from these species of bacteria (Artyukhov *et al*., 2013).

We also did not observe a direct correlation between temperature optima of inulinases and the ratio of content of hydrophobic and hydrophilic amino acid residues. The only tendency disclosed was that the given index is higher in inulinases of bacterial origin, somewhat lower in fungal enzymes, and still lower in yeast enzymes.

Thus, investigation of amino acid sequences of inulinases from various producers appears useful as a first step in studying the structural and functional properties of these enzymes, but it is insufficient for composing reliable prognoses regarding the optimal conditions of functioning, stability, and duration of storage for inulinases and the prospects of their application in industrial cycles. Consequently, this work must be supplemented and deepened by investigation of the biophysical and biochemical character of inulinases.

22.4 Immobilization – One of the Ways of Regulating Inulinase Activity

Investigations of structural and functional properties of inulinases in conditions of various microenvirons deserve special attention. Upon immobilization of an enzyme its microenvironment presents as a system of regulation, control and stabilization of enzymatic activity. In connection with this, immobilizing enzymes results in highly stable biocatalysts. Further, it is generally recognized that in industrial-scale catalytic processes heterogeneous technologies (i.e. food technologies) are economically more profitable than homogeneous technologies, because the entire industrial cycle is significantly simplified and so is less costly.

In the last 10 years definite results have been achieved in the field of immobilization of inulinases. Makino *et al*. (2005) managed adsorption of inulinase from *K. marxianus* on to anionite Streamline DEAE resin, while Kalil *et al*. (2005) achieved adsorption of its molecules on to cation exchange resin Streamline SP. Inulinase from *Arthrobacter* was successfully adsorbed on to anion exchange resins. An analogous operation was conducted for recombinant proteins from a

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genetically modified *Escherichia coli* strain (Letca *et al*., 2004) and there were attempts to include inulinase from *E. coli*/pMSiftOptRM and *A. ureafaciens* ATCC 21124 into an alginate hydrogel. Jahnz *et al*. (2003) investigated adsorption of inulinase (from the preparation Fructozyme) in an ion exchange column, with the use of two types of carrier – cationite and anionite. Immobilized on gelatin, inulinase from *K. marxianus* var. *bulgaricus* was used for hydrolysis of sucrose with the aim of obtaining fructose syrups (Silva and Santana, 2000; de Paula *et al*., 2008). A biosensor was created on the basis of fructose dehydrogenase and inulinase immobilized on gold nanoparticles of cysteamine for determination of the content of inulin in food. This sensor exhibited high selectivity to carbohydrates (Manso *et al*., 2008).

We investigated the quality of various carriers for immobilization of inulinase from *K. marxianus* and used the following as carriers: ion exchange resins AV-17-2P, KU-2, IMAC-HP, AV-16-GS, AM 21A and PUROLITE and ion exchange fibre VION KN-1 (Kovaleva *et al*., 2007, 2009a). It is known that the catalytic activity of an enzyme is determined by the degree of mobility of the protein molecule, therefore attachment of an enzyme to a carrier usually leads to weakening of the functional properties of the catalyst as compared with the native protein. Upon adsorption and immobilization of inulinase from *K. marxianus* on the synthetic ionites, a higher degree of preservation of specific enzymatic activity was observed in preparations with the carriers AV-17-2P and KU-2 (75.5% and 61.7%, respectively) than with the other ion exchange polymers which were less promising (Table 22.1).

Table 22.1. Preservation of enzymatic activity of inulinase from *Kluyveromyces marxianus* immobilized on various carriers.

Carrier	Activity (%)[a]
AV-17-2P	75.5
KU-2	61.7
VION KN-1	27.5
IMAC-HP	24.7
AV-16-GS	17.8
AM 21A	14.5
PUROLITE	9.9

[a]100% is the activity of the native enzyme (without immobilization).

We investigated the dependency of the catalytic activities of the free and immobilized inulinase on the hydrolysis temperature and pH. For the enzyme activities immobilized by adsorption on ion exchangers KU-2 and AV-17-2P, the optimal temperature increased; the greatest activities were observed at 70°C, which is 20°C higher than for the native enzyme. A similar increase in optimum temperature was observed during inulinase immobilization on other supports. According to our data, the pH optimum for both free and immobilized inulinase was 4.5–4.7. Retaining the pH optimum was observed in other yeast inulinase preparations as a result of immobilization on various synthetic ion exchangers and fibres (Kovaleva *et al*., 2007, 2009a). The pH optimum for inulinases of plant origin, however, expands.

Possibly, the increase in the optimum temperature for the reaction of inulin hydrolysis by the immobilized enzyme preparation is caused by absorption of the enzyme on to the carrier, which results in an increase in stability in relation to temperature of the protein (enzyme) molecule responsible for the catalytic transformation of the substrate.

On immobilization the enzyme molecule is surrounded by a microenvironment that differs from that of the water solution of the enzyme and this difference is caused by the existence of the functional groups of the carrier matrix. Expansion of a pH optimum for inulinases at immobilization can be explained by the distinction between the local pH values of the microenvirons of the active centre and the pH measured in the volume of solution as a whole.

The main statements of the heterogeneous catalysis theory can be effectively applied to immobilized enzymes. The immobilizaion causes, as a rule, changes in kinetic and thermodynamic parameters of enzymatic reactions, therefore V_{max} and K_m, two parameters which define the kinetic behaviour of an enzyme, can be established (and V_{max}', K_m' and so on). V_{max} is the rate of reaction at maximum velocity and K_m is the concentration of substrate which permits the enzyme to achieve half V_{max}; an enzyme with a high K_m has a low affinity for its substrate so needs a higher concentration of substrate to increase the rate of reaction. For the majority of enzymes, the value K_m' increases as a result of immobilization, whereas V_{max}' is reduced.

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Transformation of the curves of the V(S) function (V = velocity S = substrate concentration) in the Lyneweever–Birk coordinates permitted determination of K_m and V_{max} (and also K_m' and V_{max}') in the reaction of inulin hydrolysis by the investigated enzyme preparations (Table 22.2).

Stability of immobilized inulinase in reactors of periodic and continuous action was investigated. It was shown that despite repeated use of the immobilized preparation in both types of reactors the activity of inulinase practically did not change. Thus, an enzyme preparation of immobilized inulinase from *K. marxianus* with high activity has been obtained and the optimum conditions for inulin hydrolysis have been established.

22.5 Mechanisms of Interaction between Inulinase and the Matrices of Ion Exchange Materials

For investigation of structural changes in the inulinase molecule and study of the mechanism of enzyme adsorption on ion exchange resins and fibres of synthetic origin we used the method of infrared (IR) spectroscopy. Further, we investigated the degree of alteration of secondary structure for inulinase from *K. marxianus* upon its adsorption on synthetic ionites (Table 22.3).

Along with reduction of catalytic ability, upon immobilization of inulinase from *K. marxianus* on polymeric synthetic carriers AV-17-2P, KU-2 and VION KN-1 there is a decrease in the amount of unordered structures in the enzyme molecule, which points, probably, to compactization of the protein globule in the process of adsorption and emergence of steric hindrances for penetration of a molecule of inulin to the active centre of the enzyme.

Analysis of IR spectra of inulinase, carriers and heterogeneous enzyme preparations has shown that sorption of protein molecules on a matrix of ion exchange resins and fibre takes place in the main at the expense of electrostatic interactions, and such interactions are needed as a definite contribution in the formation of

Table 22.2. K_m and V_{max} values in the reaction of inulin hydrolysis by free and immobilized inulinase.[a]

Enzyme preparation	K_m (K_m') (mM)	V_{max} (V_{max}') (μM/(mg × min))
Free inulinase	0.22	102
Inulinase immobilized on AV-17-2P	0.32	54
Inulinase immobilized on VION KN-1	0.33	35
Inulinase immobilized on KU-2	0.36	34
Inulinase immobilized on AB-16-GS	0.54	19
Inulinase immobilized on AM-21-A	0.61	15

[a]V_{max}, The rate of reaction at maximum velocity; K_m, concentration of substrate which permits the enzyme to achieve half V_{max}.

Table 22.3. Content of secondary structure types in the molecule of inulinase from *Kluyveromyces marxianus* in free and immobilized states.

Conformation	Fraction of structure in native inulinase (%)	Fraction of structure in immobilized inulinase (%) on						
		AV-17-2P	KU-2	VION KN-1	IMAC-HP	AV-16-GS	AM 21A	PUROLITE
Ordered structures (α-helixes and β-sheets)	55	57	66	64	56	55	54	55
Unordered structures	45	43	34	36	44	45	46	45

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an enzyme–ionite complex made by hydrogen bonds. Upon immobilization of inulinase, it appears, there takes place compactization of the enzyme molecule, and also egress to the surface of an additional number of charged side amino acid residues. Formation of a more densely packed hydrophobic core in the immobilized protein as compared with its native form, probably presents the main cause of reduction of catalytic activity of heterogeneous preparations of inulinase (Holyavka *et al*., 2014).

From the experimental data obtained by us it follows that the mechanisms of interaction of inulinase from *K. marxianus* with a matrix of cation and anion exchange polymers differ from each other as a result of differences in the parts of the enzyme molecule being involved in the process of adsorption with different polymers, which stipulates the different conformational rearrangements in the protein molecule. In particular, the charged negatively functional groups of cationites (e.g. sulfogroups of KU-2 and carboxylic groups of VION KN-1) may interact with positively charged residues of lysine, arginine and histidine residing on the surface of the protein molecule, while the charged positively functional groups of anionites (secondary and tertiary aminogroups of AV-17-2P and AV-16-GS) probably bind with negatively charged residues of aspartic and glutamic amino acids.

22.6 Conclusion

Having considered a large number of works and based on results of our own investigations, we are convinced that immobilization is one of the ways of regulating inulinase activity, but it may lead to significant loss of activity of the enzyme preparation. However, by selecting a corresponding carrier and method of formation of connection between the interacting components, we can significantly decrease the unfavourable influence of the matrix on the structural and functional properties of the enzyme and thereby elevate the specific activity of the immobilized biocatalyst. In addition, immobilization allows the temperature optimum for functioning of inulinase to be increased, stabilizes the conformation of its molecule, and protects the spatial structure of the enzyme from the impact of denaturing agents (Kim *et al*., 1982; Bajpai and Margaritis, 1985; Wenling, 1999; Kovaleva *et al*., 2009b, 2012; Artyukhov *et al*., 2010).

Analysis of data from the literature allows us to suggest that mechanisms of stabilization of immobilized enzymes in conditions of extreme values of pH and temperature, and also with other denaturing agents, operate by changing the degree of mobility of protein tertiary structure responsible for formation of an enzyme–substrate complex, and consequently, also of the reaction products.

Immobilization is often not economical since the price of the carrier and for immobilization will not be compensated. Therefore work on selection and modification of carriers, and also development of methods for immobilization are of special importance.

A universal immobilization method does not exist. Each method has its advantages and disadvantages that have to be considered for every biocatalyst and enzymatic reaction. Comparative analysis of various methods of inulinase immobilization makes it possible to note that the most widespread way to immobilize inulinase is inclusion of the enzymes in hydrophobic gels, as this appears to work for the majority of enzymes. The most difficult and labour-consuming method is covalent binding, and the most simple and available method is adsorption. On the whole the choice of method used for immobilization of the biocatalyst depends on the direction of use for the enzyme preparation (i.e. the specifics of scientific research or type of industrial production).

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23 Microbial Resources for Biopolymer Production

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Abstract
Growing concern about the environment, rapid depletion of petroleum resources and new environmental regulations have prompted the search for new environmentally compatible materials obtained from natural resources, including biopolymers. Currently the research related to the biopolymers (e.g. cellulose, xanthan gum, polyhydroxyalkanoates, chitin and pullulan) obtained from microorganisms is rapidly expanding due to their biodegradability and abundant availability, and easy methods are being developed to grow and harvest them for use in numerous industrial and medical applications. Many microbial biopolymers have been described in the last few decades having promising functional properties potentially suitable for different applications. This chapter deals with the main aspects of the biotechnological production of microbial biopolymers, the conventional methods, biosynthesis, along with the tailor-made synthesis, applications and the future prospects of those biopolymers.

23.1 Introduction

Over the last 20 years, interest in the production of microbial biopolymers – polymeric substances synthesized by microorganisms – has expanded (Rehm, 2010). Depending on their composition and molecular weight, microbial polymers have applications that range from rheology modifiers of aqueous systems to bioplastics, which make them useful in many industrial sectors (e.g. agro-food, cosmetics, pharmaceuticals, textile, paper and oil recovery industries). The wide array of applications of biopolymers derives from their structural, conformational and functional diversity (Morris and Harding, 2009; Pereira *et al.*, 2009).

The extensive use of non-renewable petroleum-based polymers has negatively impacted fossil fuel reserves, which has contributed to global warming as well as other environmental and health problems. To overcome these issues and achieve sustainable world development, scientists and technologists have started to explore the use of renewable biopolymers in various areas. Biopolymers that originate from plants, animals and microbes, such as polysaccharides (cellulose and starch), protein, oil, chitin, DNA and RNA, are particularly interesting. So far, biopolymers of plant and animal origin have been investigated for numerous industrial and medical applications. As a result, several products have been developed and commercialized for use in food and non-food products in the areas of biodegradable plastics, pharmaceuticals, electronics, acoustic devices, biofuels, cosmetics, agriculture and biomedicine. The increasing demand for renewable polymers and the need to

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cope with the decline of plant and animal resources have led researchers to isolate various polymers from abundant bacterial, fungal and yeast sources (Rathna and Ghosh, 2011).

Microbial polymers offer a number of advantages. The fact that they originate from renewable resources under controlled environmental conditions ensures both the quantity and the quality of the final products; in addition, it is possible to tailor their properties for specific applications. However, several factors have limited their widespread use in food products, mainly: (i) production costs, which are higher as compared with the production costs involved in the synthesis of most of the traditional polymers; (ii) restrictions related to approval for their use in food products or processes; and (iii) difficulty in obtaining public acceptance. If the biopolymer is not 'generally recognized as a safe' (GRAS) status material, it is necessary that the certified authorities (e.g. US Food and Drug Administration (FDA) and the European Food Safety Authority (EFSA)) evaluate it and determine: (i) its safety under the conditions of its intended uses; (ii) its specifications, including purity and physical properties; and (iii) limitations in the conditions of the intended application (Freitas *et al.*, 2011).

This chapter outlines the main aspects of the biotechnological production of microbial biopolymers, focusing on conventional methods (extraction/isolation), biosynthesis and tailor-made synthesis. It also describes the main current applications of microbial biopolymers in products and processes. For the sake of readability, the content has been divided as follows: the most important biopolymers produced by bacteria and the most important biopolymers produced by fungi and yeasts.

23.2 Biopolymer Production by Bacteria: Biosynthesis and Applications

Many bacteria can produce polymers with potential application in the food, pharmaceutical and medical materials industry. To reduce the use of plant and animal resources and to meet the increasing demand for renewable polymers, researchers have turned to abundant bacterial resources in an attempt to isolate various polymers. The main biopolymers obtained from bacteria include alginate (*Pseudomonas* sp. and *Azotobacter vinelandii*), bacterial cellulose (BC) (*Aerobacter*, *Acetobacter*, *Achromobacter*, *Agrobacterium*, *Alcaligenes*, *Azotobacter*, *Pseudomonas*, *Rhizobium* and *Sarcina*), dextran (*Leuconostoc mesenteroides*, *Lactobacillus* sp. and *Streptococcus mutans*), gelan (*Pseudomonas elodea*, *Aureomonas elodea* and *Sphingomonas paucimobilis*), xanthan (*Xanthomonas campestris*), polyhydroxyalkanoates (PHAs) (*Alcaligenes* sp. and *Amphibacillus* sp.) and polyhydroxybutyrate (*Pseudomonas pseudomallei*) (Rathna and Ghosh, 2011).

The use of bacterial biopolymers is important because these polymers can decrease the world dependence on oil-based polymers. Although the latter polymers cost less, biopolymers present advantages such as biodegradability, better performance and defined structural variability. Furthermore, they originate from inexpensive substrates, consist of natural non-toxic compounds and are inherently biocompatible (Rehm, 2010).

Bacterial biopolymers have found application in countless industrial, biomedical and agricultural segments. In the biomedical field, some polysaccharides have been extensively used in controlled-release drug delivery systems by encapsulation. In the industry, polyesters can help to treat wastewater and serve as biodegradable packaging. Moreover, polysaccharides like cellulose, xanthan gum and dextran can be applied as healthy beverages, rheology modifiers and food additives. Cellulose, xanthan gum, both of which are exopolysaccharides, and PHAs constitute the most important bacterial polymers with valuable application as polymeric material for industrial, food and medical purposes (Rathna and Ghosh, 2011).

23.2.1 Bacterial cellulose (BC)

Cellulose is an abundant polymer in nature, and it is the main constituent of plant cell walls. It presents a complex structure that comprises hemicellulose, lignin and other impurities (Chen *et al.*, 2010). Various fields such as the medical, food, textile, paper and cosmetic industries employ pure cellulose (Jahan *et al.*, 2012; Gomes *et al.*, 2013). The traditional method used to remove hemicellulose and lignin and thus extract

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cellulose from natural fibres requires mechanical and/or chemical treatment (e.g. acid hydrolysis) (Hyun *et al.*, 2014).

A clean strategy to obtain chemically purer cellulose is to turn to microorganisms like bacteria, fungi and algae that can produce microbial cellulose. In this context, the main producers of cellulose are bacteria belonging to the genera *Acetobacter* (*Gluconacetobacter*), *Rhizobium*, *Agrobacterium*, *Aerobacter*, *Pseudomonas*, *Escherichia* and *Sarcina*, which generate the so-called BC (Cheng *et al.*, 2011a; Hyun *et al.*, 2014).

BC bears sets of parallel chains of β-1, 4-D-glucopyranose units interlinked by intermolecular hydrogen bonds. Because it is free of lignin, pectin and hemicellulose, BC displays many unique properties, namely higher purity, higher degree of polymerization and a larger crystallinity index, as compared with cellulose originating from the plant cell walls. Furthermore, BC has high water absorbing and holding capacity, large tensile strength and strong biological adaptability. In addition, the fibre dimensions are significantly lower for BC as compared with plant-derived cellulose (about 100 times thinner), which makes it a highly porous material (Hu and Catchmark, 2010; Ge *et al.*, 2011; Hong *et al.*, 2011; Gayathry and Gopalaswamy, 2014; Shi *et al.*, 2014).

Although various microorganisms can synthesize BC, bacteria of the genera *Gluconacetobacter* (formerly *Acetobacter*) stand out in this matter (Table 23.1). More recently, *Gluconacetobacter xylinum* has received special attention, because it is potentially applicable for industrial and commercial purposes (Karahan *et al.*, 2011; Guo *et al.*, 2013). *G. xylinum* is a Gram-negative, aerobic bacterium that produces cellulose in the form of interwoven extracellular ribbons as part of primary metabolism. This bacterium uses a wide variety of substrates to grow and to generate cellulose, and it is devoid of cellulase activity (Gayathry and Gopalaswamy, 2014). Typical carbon sources for the production of BC include glucose, fructose, sucrose and mannitol, among others (Castro *et al.*, 2012). To replace the carbon or nitrogen source, authors have employed Hestrin-Schramm (HS) medium (Hestrin and Schramm, 1954) both in its original and modified forms (Table 23.1).

However, BC production involves expensive culture media, and strains furnish relatively low BC yields. These drawbacks have prevented large-scale BC generation for industrial purposes and extended applications, since the fermentation medium can account for almost 30% of the total cost of microbial fermentations (Rivas *et al.*, 2004; Hong *et al.*, 2011). To reduce production expenses, it is possible to substitute some substrate components; for example, inexpensive non-conventional sources, such as cheap agricultural products or waste, can serve as carbon source. Several authors (Table 23.1) have investigated low-cost feedstock, including wheat straw (Hong *et al.*, 2011), dry olive mill residue (Gomes *et al.*, 2013), molasses, starch hydrolysate, sugarcane juice, coconut water, coconut milk, pineapple juice, orange juice, pomegranate juice (Hungund and Gupta, 2010), makgeolli sludge filtrate (Hyun *et al.*, 2014), corn steep liquor (Cheng *et al.*, 2011a; Lin *et al.*, 2014a), coffee cherry husk (Rani and Appaiah, 2013) and grape (Rani *et al.*, 2011) as substrates in the fermentation medium.

For fermentation purposes, first, it is essential to hydrolyse alternative substrates to simple sugar by pretreating them with acids or enzymes. Because enzymatic hydrolysis is very expensive, chemical hydrolysis is usually the treatment of choice. Unfortunately, efficient conversion of cellulose and hemicellulose to sugars releases a broad range of compounds that inhibit the fermenting microorganism, and BC production and yield heavily depend on the composition of the growth medium. Hence, some authors have attempted to obtain compounds free of toxins from lignocellulose via a process called detoxification. This process employs H_2SO_4, NaOH and $Ca(OH)_2$, among others (Hong *et al.*, 2011; Guo *et al.*, 2013).

It is possible to produce BC using either static or agitated cultivation. The production method influences the quality and quantity of produced cellulose (Chen *et al.*, 2010). The traditional process for BC generation is the static fermentation method, in which BC pellicles originate on the surface of the static culture. This method requires a large area and long culture time (Jung *et al.*, 2010). None the less, this scheme might be advantageous when pellicle-formed cellulose is the ultimate target or a relatively higher degree of crystallinity is preferable. In the agitated cultivation method, BC accumulates in a dispersed suspension containing irregular masses, such as granules,

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Table 23.1. Microorganism, substrate and fermentation conditions during bacterial cellulose (BC) production as reported in the literature.

Microorganism	Substrate	Fermentation conditions	Reference
Acetobacter xylinum JCM 9730	Dextrose and 1-methylcyclopropene	Shaken fermentation	Hu and Catchmark (2010)
Enterobacter amnigenus GH-1 isolated from rotten apple	Hestrin-Schramm (HS) medium with different carbon sources (glucose, molasses, starch hydrolysate, sugarcane juice, coconut water, coconut milk, pineapple juice, orange juice and pomegranate juice)	Static fermentation	Hungund and Gupta (2010)
Acetobacter sp. V6	Molasses and corn steep liquor (CSL)	Shaken fermentation	Jung *et al.* (2010)
Acetobacter xylinum (*Gluconacetobacter xylinum*)	Yeast extract, peptone and wheat straw hydrolysate as the sole carbon source	Shaken fermentation	Hong *et al.* (2011)
A. xylinum (*G. xylinum*)	CSL with fructose (CSL-Fru) medium and carboxymethylcellulose	Agitated fermentation	Cheng *et al.* (2011a)
Gluconacetobacter sp. A06O2 obtained from Turkish vinegar	HS medium	Static fermentation	Karahan *et al.* (2011)
Gluconacetobacter sacchari	HS medium and grape skins aqueous extract, cheese whey, crude glycerol and sulfite pulping liquor	Static fermentation	Carreira *et al.* (2011)
Gluconacetobacter swingsii	Sugarcane juice, pineapple peel juice and HS medium	Static fermentation	Castro *et al.* (2011)
Gluconacetobacter hansenii UAC09	HS medium	Static fermentation	Rani and Appaiah (2011)
Gluconacetobacter sp.	Grape medium	Static fermentation	Rani *et al.* (2011)
G. hansenii PJK (KCTC 10505BP) and *A. xylinum* (ATCC 23769)	Single sugar α-linked glucuronic acid-based oligosaccharide (SSGO)	Static and shaken fermentation	Ha and Park (2012)
Gluconacetobacter sp. F6 isolated from rotten fruit	HS medium with different carbon sources	Static and shaken fermentation	Jahan *et al.* (2012)
Gluconacetobacter medellensis	HS medium with different carbon sources	Static fermentation	Castro *et al.* (2012)
G. sacchari	Dry olive mill residue	Static fermentation	Gomes *et al.* (2013)
G. hansenii UAC09 (isolated from contaminated grape wine)	Coffee cherry husk medium	Shaken fermentation	Rani and Appaiah (2013)
A. xylinum (*G. xylinum*) (isolated of sugarcane juice)	HS medium	Static fermentation	Gayathry and Gopalaswamy (2014)
G. hansenii P2A (isolated of fruit)	HS medium	Static, shaken, and agitated fermentation	Aydin and Aksoy (2014)
A. xylinum (*G. xylinum*)	HS medium and makgeolli sludge filtrate (MSF)	Static fermentation	Hyun *et al.* (2014)
G. hansenii CGMCC 3917	Pretreated waste beer yeast	Static fermentation	Lin *et al.* (2014a)
G. xylinum (ATCC 700178) and *G. xylinum* (ATCC 23769)	CSL-Fru medium	Agitated fermentation	Lin *et al.* (2014b)

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stellates and fibrous strands. The agitated culture method is more suitable for the industrial production of BC and finds commercial applications in various fields. However, cellulose-deficient mutants sometimes appear in these agitated cultures and might interfere with BC fermentation (Chen *et al.*, 2010; Aydin and Aksoy, 2014).

Downstream processing involves separation of the biomass from the culture supernatant, usually by filtration, followed by a product-specific separation process (Rehm, 2010). In the case of BC, most of the downstream processes involve treatment with NaOH at low concentrations and high temperature, washing with water and centrifugation (Hungund and Gupta, 2010; Carreira *et al.*, 2011; Cheng *et al.*, 2011a; Karahan *et al.*, 2011; Ha and Park, 2012; Jahan *et al.*, 2012; Gomes *et al.*, 2013; Gayathry and Gopalaswamy, 2014; Hyun *et al.*, 2014).

23.2.2 Xanthan

Xanthan gum is a commercially important exopolysaccharide that various strains of pathogenic bacterium such as *Xanthomonas campestris* as well as some other *Xanthomonas* species produce as an extracellular slime (Table 23.2) (Sutherland, 2005). Xanthan gum presents a particularly complicated molecular structure – its main chain consists of glucose molecules connected by β-1,4 glycosidic links. This chain resembles the main chain of cellulose; however, every second glucose unit carries a side chain containing β-D-mannose, β-1,4-D-glucoronic acid and α-1,2-D-mannose together with a pyruvic acid unit (Ben Salah *et al.*, 2010; Schatschneider *et al.*, 2013).

The rheological properties of xanthan gum make it a useful suspension-thickening and stabilizing agent as well as a potent emulsifier in the food, cosmetics and pharmaceutical industries (Faria *et al.*, 2010). According to Palaniraj and Jayarman (2011), discovery of this important gum occurred in 1963 at the Northern Regional Research Center (now called The National Center for Agricultural Utilization Research) of the United States Department of Agriculture (USDA). Considerable commercial production began in early 1964.

Extensive research on the toxicological and safety properties of xanthan gum has been conducted with a view to food and pharmaceutical

Table 23.2. Microorganism, substrate and fermentation conditions during bacterial xanthan gum production as reported in the literature.

Microorganism	Substrate	Fermentation conditions	Reference
Xanthomonas campestris pv. *campestris* NRRL B-1459	Sugarcane broth	Agitated fermentation	Faria *et al*. (2011)
X. campestris NRRL B-1459	Sugarcane broth	Agitated fermentation	Faria *et al*. (2011)
X. campestris	Cheese whey	Agitated fermentation in continuous fermentation systems	Zabot *et al*. (2012)
Xanthomonas axonopodis pv. *citri* 306	Morpholinopropane sulfonate (MOPS) medium	–	Rojas *et al*. (2013)
X. campestris pv. *campestris* strain B100	Synthetic medium (glucose)	Agitated fermentation	Schatschneider *et al*. (2013)
Native bacteria no. 1866 of *X. campestris campestres*	Green coconut shell	Shaken fermentation	Rocha Nery *et al*. (2013)
X. campestris mangiferaeindicae 2103	Crude glycerin	Shaken fermentation	Brandao *et al*. (2013)
X. campestris (NCIM 2954)	Tapioca pulp pretreated with sulfuric acid	Shaken fermentation	Gunasekar *et al*. (2014)
X. campestris mangiferaeindicae 2103	Crude glycerin	Agitated fermentation	Assis *et al*. (2014a)
X. axonopodis pv. *manihotis*	Fermented agro-industrial waste	Shaken fermentation	Assis *et al*. (2014b)

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applications. Xanthan is non-toxic and does not inhibit growth. Therefore, in 1969, the FDA authorized the use of xanthan gum in food products without any specific quantity limitations, which marked the introduction of the first industrially produced biopolymer in the food industry. Since then, the demand for xanthan gum produced from *X. campestris* has increased progressively, at an annual rate of 5–10% (Ben Salah *et al.*, 2010; Faria *et al.*, 2011).

Factors such as pH, temperature, nitrogen source, mass transfer and carbon source affect the production and molecular structure of xanthan gum (Ben Salah *et al.*, 2010; Faria *et al.*, 2010). In this respect, several studies have focused on a variety of nutrients, particularly the nitrogen and carbon sources, and have frequently employed glucose and sucrose to promote microorganism growth. According to Zabot *et al.* (2012), factors such as the type of bioreactor and the mode of operation (batch or continuous) influence microorganism growth and xanthan production, too. Most of the recent research works have used batch fermentation instead of continuous fermentation.

The cost of the carbon source, which is often solely glucose or fructose, is one of the factors that determine production costs (Brandao *et al.*, 2013), and because of this research into xanthan production from inexpensive substrates is an important topic. Researchers have recently tested various agricultural wastes, which contain complex polysaccharides such as cellulose, hemicellulose, lignin and starch, as low-cost substrates, aiming to reduce the expenses related to raw material. Other less complex wastes have also been investigated (see Table 23.2).

On the other hand, Morris and Harding (2013) have stated that the raw material costs are typically small for a fermentation product, and that it is the recovery of the gum from the culture medium that accounts for the major expenses. Similarly, Palaniraj and Jayarman (2011) have argued that the very stringent FDA purity standards for foods make xanthan gum relatively expensive. In the case of food-grade xanthan gum, up to 50% of the production costs correspond to downstream purification steps, many of which would not be necessary for non-food applications.

Because the broth remaining at the end of the fermentation contains xanthan, bacterial cells and many other chemicals, some downstream purification steps are necessary. To recover xanthan from the fermentation medium, first it is necessary to remove cells, mainly by centrifugation or even filtration. Further purification may include precipitation in the presence of water-miscible solvents such as ethanol or acetone, addition of certain salts and pH adjustments. After precipitation, the product has to undergo mechanical dewatering and drying (Ben Salah *et al.*, 2010; Faria *et al.*, 2010, 2011; Brandao *et al.*, 2013; Rocha Nery *et al.*, 2013; Assis *et al.*, 2014a, b; Gunasekar *et al.*, 2014).

23.2.3 Polyhydroxyalkanoate (PHA)

PHAs constitute a group of biodegradable, bioabsorbable and biocompatible biopolyesters produced by microbes. PHAs have large market potential – their thermoplastic properties resemble the properties of a number of conventional polyolefins (e.g. polypropylene and polyethylene), which makes them very promising bulk materials to produce biodegradable plastics (Albuquerque *et al.*, 2010; Povolo *et al.*, 2010; Lan *et al.*, 2013; Fradinho *et al.*, 2014).

PHA synthases belong to different classes depending on their size, subunit composition and substrate specificity (Lan *et al.*, 2013). To date, the literature has reported on more than 150 types of biologically produced PHAs. Three major types of PHAs exist: (i) short-chain-length PHAs (SCL-PHAs), which consist of monomer units of C3–C5; (ii) medium-chain-length PHAs (MCL-PHAs), which bear monomer units of C6–C14 and are elastomeric, but not crystalline; and (iii) long-chain-length PHAs (LCL-PHAs), with $C \geq 15$, which are crystalline, but stiff and brittle (Höfer *et al.*, 2011; Li *et al.*, 2011). Researchers have devoted special attention to the production of PHAs bearing terminal double bonds in their side chains, because they can lead to high-yield production processes (Höfer *et al.*, 2011).

A wide variety of environmental bacteria can synthesize various types of PHAs under natural conditions. These PHAs serve as intracellular energy storage materials and carbon sources, and they can accumulate as granules (Hori *et al.*, 2011; Fradinho *et al.*, 2014). PHA production can reach amounts as high as 90% of the dry

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weight of the cell in microorganisms (Shahid *et al.*, 2013). The Gram-negative bacterium *Cupriavidus necator* produces approximately 85% of the dry weight of is cell as polymer in medium containing excess glucose (Passanha *et al.*, 2014). When nutrient supplies are imbalanced, many other genera of bacteria can synthesize PHAs to store carbon and energy, such as *Burkholderia* sp. and *Pseudomonas* sp. (Hori *et al.*, 2011; Pan *et al.*, 2012; Chen *et al.*, 2014; Obruca *et al.*, 2014) among others (Table 23.3).

Several factors affect PHA production, including: (i) the microorganism strain; (ii) the duration of fermentation; (iii) the growth rate; (iv) the nature and concentration of the carbon source; and (v) environmental stress such as nitrogen, phosphorus or oxygen limitations. PHAs participate in stress tolerance. They accumulate when the nitrogen, phosphorus or oxygen levels limit bacterial growth while excess carbon source still exists in the medium (Shahid *et al.*, 2013; Passanha *et al.*, 2014).

PHAs play an important role in the production of bioplastics because they exhibit thermoplastic properties. However, PHAs have not yet entered the bulk materials markets due to high production costs; indeed, current industrial PHA production relies on pure culture systems operated under aseptic conditions and supplied with chemically defined mediums. Therefore, the costs associated with these operational conditions increase PHA prices, which limits the application of PHA as a substitute for traditional plastics (Albuquerque *et al.*, 2010; Berezina, 2013), as already mentioned in the case of the synthesis of BC and xanthan gum.

Given that about 45% of the total costs involved in PHA production are ascribed to carbon sources, such as refined glucose or sucrose (Obruca *et al.*, 2014), to circumvent the economic issue of PHAs, researchers have focused on developing alternative cost-effective processes for PHA production, including the use of low-value substrates such as agricultural or industrial waste or surplus feedstocks (cheese whey, canola oil, spent coffee grounds oil and crude glycerin; Table 23.3).

Another strategy to reduce costs is to use open mixed microbial cultures (MMCs) for a more cost-effective and sustainable PHA production process. MMCs reduce the costs relative to equipment purchase, maintenance and control, sterilization and use of expensive refined substrates. In addition, MMCs can be operated in open systems in the presence of cheap industrial fermentable by-products (Fradinho *et al.*, 2013; Queirós *et al.*, 2014). The combined use of MMCs and substrates from renewable sources reduces operational costs, because the process employs a cheaper substrate and less energy.

The application of repeated cycles of feast and famine dismisses the operation of MMCs for PHA production. During periods of excess external carbon (feast), the bacteria store PHA as carbon and energy reserves; under carbon starvation (famine), the bacteria use the accumulated PHA to grow, a feature that determines the survival of PHA-accumulating bacteria as compared with non-PHA-accumulating bacteria (Carvalho *et al.*, 2014; Fradinho *et al.*, 2014).

23.2.4 Summary of bacterial polymers

The production of bacterial biopolymers such as cellulose, xanthan gum and PHA is very important for food, industrial, medical and agricultural applications. Nevertheless, production costs are still very high, and it is particularly difficult to achieve quantitative upgrade of the corresponding extraction and biotechnology for substantial biopolymer production. More research into techniques, microorganisms and substrates are necessary to promote large biopolymer generation at lower costs.

23.3 Biopolymer Production by Fungi and Yeasts: Biosynthesis and Applications

Microorganisms like fungi and yeasts have attracted researchers' attention because they can synthesize polymers with physicochemical and biodegradability properties that make them suitable substitutes for plant-derived polysaccharides. Although the main interest in these polymers lies on their use as an anti-infection component or for adhesion purposes, research works have focused on their applications in the food, agricultural, chemical, energy production, pharmaceutical and biomedical industries (Singh *et al.*, 2008; Cheng *et al.*, 2011b). A few fungal

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Table 23.3. Microorganism, substrate and fermentation conditions during bacterial polyhydroxyalkanoate (PHA) production as reported in the literature.

Microorganism	Substrate	Fermentation conditions	Reference
Cupriavidus necator and *Escherichia coli*	Minimal salts medium DSMZ81 containing lactose as the sole carbon source or a medium prepared from cheese whey permeate	Shaken fermentation	Povolo *et al*. (2010)
C. necator	Volatile fatty acids	Agitated fermentation	Wang *et al*. (2010)
Recombinant *E. coli*	Luria-Bertani (LB) medium	Static fermentation	Li *et al*. (2011)
Recombinant *Methylobacterium extorquens*	Methanol	Shaken fermentation	Höfer *et al*. (2011)
Pseudomonas aeruginosa IFO3924 and *Pseudomonas putida* IFO14164	Basal salt medium (BSM) supplemented with a fatty acid	Shaken fermentation	Hori *et al*. (2011)
Wautersia eutropha	Canola oil	Agitated fermentation	López-Cuellar *et al*. (2011)
Burkholderia cepacia ATCC 17759	Xylose	Shaken fermentation	Pan *et al*. (2012)
C. necator DSM 545	Mineral medium, Bonnarme's medium, Mandel's medium and LB medium	Shaken fermentation	Berezina (2013)
Bacillus megaterium DSM 509	Glucose, glycerol, lactose, citric, acetic, butyric, succinic, pentanoic or octanoic acids	Shaken fermentation	Shahid *et al*. (2013)
C. necator DSMZ 545	Acid mixture of pure acetic and butyric acids (99%)	Agitated fermentation	Passanha *et al*. (2013)
C. necator DSMZ 545	Culture medium, NaCl and some antifoam. High purity acetic acid (99%) was used as the carbon source	Agitated fermentation	Passanha *et al*. (2014)
B. cepacia CCM 2656 (ATCC 17759)	Spent coffee grounds hydrolysate (SCGH)	Shaken fermentation	Obruca *et al*. (2014)
C. necator DSM 428	Spent coffee grounds oil	Agitated fermentation	Cruz *et al*. (2014)
Pseudomonas mosselii TO7	Mineral salt (MS) medium containing gluconate, fructose, sucrose, hexanoic acid, octanoic acid, decanoate, dodecanoate, soybean oil or palm kernel oil	Shaken fermentation	Chen *et al*. (2014)
C. necator IPT 026	Crude glycerin	Shaken fermentation	Campos *et al*. (2014)

exopolysaccharides have gained industrial importance and become well known, including:

- pullulan (*Aureobasidium pullulans*) (Sugumaran *et al.*, 2014);
- chitin and chitosan (*Aspergillus* spp., *Trichoderma* spp., *Rhizopus* spp., *Absidia* spp., *Fusarium* spp., *Mucor* spp., *Gongronella butleri*, *Cryptococcus neoformans*, *Pencillium notatum*, *Saccharomyces cerevisiae*, *Schizosaccharomyces pombe*, *Zygosaccharomyces rouxii* and *Candida albicans*) (Lenardon *et al*., 2010; Ruiz-Herrera, 2012a; Kaur and Dhillon, 2014);
- glucan (*Aspergillus* spp., *Penicillum* spp., *S. cerevisiae*) (Ruiz-Herrera, 2012b);

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- the chitin–glucan complex (CGC) (*Aspergillus niger* and *Pichia pastoris*) (Roca *et al*., 2012);
- schizophyllan (*Schizophyllum commune*) (Zhang *et al*., 2013);
- glycans (*Morchella esculenta* and *Sarcodon aspratus*) (Costas and Ioannis, 2006); and
- PSK, a protein-bound polysaccharide (*Coriolus versicolor* and *Tricholoma lobayense*) (Oh *et al.*, 2007).

Researchers have extracted biopolymers from the cell walls, mycelia and spores of fungi and yeast. Through fermentation by culturing fungi, the microorganisms listed above can biosynthesize polymers from diverse renewable carbon sources, such as agricultural waste. Biowaste constitutes an infinite and economical source. Its use in bioprocesses aggregates value to agricultural residues and reduces environmental pollution. Compared with polymers obtained from plant sources, fermentation offers the advantages of a well-controlled production process. Indeed, it is possible to control and optimize parameters such as pH, temperature, nitrogen and carbon concentration, aeration and stirring (Costas and Ioannis, 2006; Ravella *et al.*, 2010).

In recent studies, chitin and chitosan, pullulan and CGC have become the most important functional polysaccharides obtained by the action of fungi and yeast. They constitute a particularly interesting group of bioactive compounds for the food and biomedical industry (Cheng *et al.*, 2011b; Freitas *et al.*, 2013; Sugumaran *et al.*, 2014).

23.3.1 Chitin and chitosan

Chitin (poly-β-(1,4)-*N*-acetyl-D-glucosamine) is a natural and biodegradable polymer. In terms of biomass, after cellulose, chitin is the most abundant organic compound in nature (Cohen, 2010). In its pure form, chitin is a linear homopolymer of *N*-acetyl-D-glucosamine linked by β-1,4 glycosidic bonds (β-1,4-*N*-acetyl-D-glucosamine). It is structurally similar to cellulose, mainly in its solubility and low chemical reactivity. In many fungi, chitin replaces cellulose as the structural polysaccharide stabilizing the cell wall (Merzendorfer, 2011; Andrade and Rojas, 2012). Chitosan (β-1, 4-D-glucosamine) is a homopolymer that originates from the deacetylation of chitin, which undergoes partial deacetylation in concentrated sodium hydroxide by means of a thermochemical process (Ravi Kumar, 2000; Logesh *et al.*, 2012). The different crystallinity between the structures of chitin and chitosan, as demonstrated by X-ray diffraction analysis, determines the solubility of these polysaccharides. Chitin is highly crystalline and extremely insoluble in water and most organic solvents. In contrast, chitosan is poorly crystalline and is soluble in acid aqueous medium; it is the polysaccharide of choice in several applications (Ravi Kumar, 2000; Ruiz-Herrera, 2012a; Kaur and Dhillon, 2014).

Chitin and chitosan are extracellular polysaccharides with important physiological functions in prokaryotes and eukaryotes. A broad variety of living organisms of different taxonomic groups can synthesize these molecules. In nature, these biopolymers primarily occur as essential structural components in the exoskeleton of arthropods or in the cell walls and septum structures; they also emerge during spore formation in yeasts and most filamentous fungi (Table 23.4) (Rinaudo, 2006; Cohen, 2010).

Chitin and chitosan have potentially useful properties like biodegradability, biocompatibility, non-toxicity and antibacterial, antifungal and

Table 23.4. List of microorganisms having chitin/chitosan in their cell walls.

Microorganism		References
Fungi	*Aspergillus* spp., *Trichoderma* spp., *Rhizopus* spp., *Absidia* spp., *Fusarium* spp., *Mucor* spp., *Gongronella butleri*, *Lentinus edodes*, *Phycomyces lakesleeanus*, *Pleurotus sajo-caju*, *Cryptococcus neoformans*, *Caprinus cinereus*, *Mortierella isabellina*, *Neurospora crassa*, *Ophiostoma ulmi*, *Pencillium notatum*, *Phycomyces blakesleeanus*	Lenardon *et al*. (2010), Ruiz-Herrera (2012a, b), Kaur and Dhillon (2014)
Yeast	*Saccharomyces cerevisiae*, *Schizosaccharomyces pombe*, *Zygosaccharomyces rouxii*, *Candida albicans*	

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antitumour activities. Their chemical modification products cover a large array of applications in the food, pharmaceutical, cosmetics, chemical and textile industries, as well as in food packaging, agriculture and water treatment, not to mention their health benefits (Teng *et al.*, 2001; Andrade and Rojas, 2012; Logesh *et al.*, 2012; Vendruscolo and Ninow, 2014).

Chitin and chitosan extraction from the exoskeleton of crustaceans affords these polysaccharides for commercial purposes. However, this process presents disadvantages, such as the use of concentrated alkaline or acid solutions at high temperatures and the seasonality of the raw material. Microorganisms that produce these polysaccharides in their cell walls, such as fungi, or in their ascospores, like yeasts, could become an environmentally friendly strategy to obtain chitin and chitosan (Vendruscolo and Ninow, 2014). Ascomycetes, zygomycetes and basidiomycetes are the main classes of microorganisms that bear chitin and chitosan in their cell wall and septa. In filamentous fungi, the proportion of chitin can be as great as 40–45% of the dry weight of the cell, whereas in yeast (ascomycetes), chitin accounts for only 1–2% of the cell wall constituents. Zygomycetes (e.g. *Absidia*, *Mucor*, *Rhizopus* and *Gongronella*) stand out as the class with the largest amount of these biopolymers in their structure (Suntornsuk *et al.*, 2002; Kaur and Dhillon, 2014).

Chitin deacetylation, to give chitosan, usually takes place via alkaline and acid treatments. Indeed, chitin fibrils are associated with minerals, proteins, lipids and pigments, so isolation of this polymer requires extraction of the latter compounds. An environmentally friendly approach would be to employ a proteolytic microorganism, especially fungi and yeast (e.g. *Candida albicans*, *Cryptococcus neofarmans*, *Wangiella dermatitidies*, *Aspergillus* sp. and *S. cerevisiae*) that secrete enzymes such as chitin deacetylases, to deacetylate chitin, dismissing the need for chemical reagents (Lenardon *et al.*, 2010; Kaur and Dhillon, 2014).

The production of fungal biomass is usually associated with fermentation processes, which can be solid state fermentation (SSF) and submerged fermentation (SmF). The quality and quantity of the chitosan produced during biotechnological processing of fungal cultures will depend on the fermentation conditions, such as microorganism strain, culture medium composition, pH, temperature, aeration, stirring and extraction time. Therefore, SmF could be advantageous over SSF, because it allows for easier control of the fermentation parameters (Ravi Kumar, 2000; Kaur and Dhillon, 2014; Vendruscolo and Ninow, 2014). According to some researchers, SmF furnishes larger amounts of biopolymers as compared with SSF. On the other hand, SSF generates biopolymers with lower molecular weight, which enhances solubility. The solubility of chitosan in aqueous solutions opens new opportunities for its application as a solution, gel, film and fibres (Nwe *et al.*, 2002; Rinaudo, 2006). The downstream process used to extract chitosan usually involves centrifugation, decantation or filtration, in separate, in sequence, or in combination (Freitas *et al.*, 2013). Table 23.5 summarizes some methods through which fungi and yeasts produce chitin and chitosan.

Another strategy to reduce the environmental impact and production costs of biopolymers is to search for unconventional substrates to obtain fungal biomass. Agro-industrial waste contains: (i) carbohydrates such as cellulose, hemicelluloses and lignin; (ii) reducing sugars (glucose, fructose and sucrose); (iii) protein; (iv) essential amino acids; (v) vitamins; and (vi) minerals. Due to its high sugar content, this waste can be a potential carbon source for microorganism growth, which could add value to this subproduct (Teng *et al.*, 2001; Merzendorfer, 2011; Vendruscolo and Ninow, 2014).

The production of chitin and chitosan from underutilized resources has attracted great attention, because these polymers constitute new potential functional materials with application in various fields. Countries such as India, Poland, Japan, the USA, Norway, Australia, Chile and Brazil already produce chitin and chitosan for the local market (Andrade and Rojas, 2012).

23.3.2 Pullulan

Microorganisms produce pullulan, an exohomopolysaccharide, from starch. Biodegradability is the characteristic that makes this material particularly attractive (Freitas *et al.*, 2013). Pullulan is a neutral, unbranched, linear glucan containing maltotriose units, also known as α-D-glucan. Three glucose units in maltotriose are connected via α-1,4 glycosidic bonds, whereas consecutive

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Table 23.5. Studies about the production of fungal biomass to obtain the biopolymers chitin and chitosan.

	Microorganism culture			
Microorganism	Substrate	Bioprocess	Downstream processes	Reference
Rocella montagnei	Potato dextrose broth (PDB)	Submerged fermentation (SmF)	Centrifugation	Logesh *et al*. (2012)
Aspergillus niger	Shrimp shell	Solid state fermentation (SSF)	Sedmentation and decantation	Teng *et al*. (2001)
Rhizopus oryzae, *Zygosaccharomyces rouxii* and *Candida albicans*	Soybean and mungbean	SSF	Centrifugation	Suntornsuk *et al*. (2002)
Gongronella bluteri	Apple pomace	SmF and airlift bioreactor	Filtration and centrifugation	Vendruscolo and Ninow (2014)
G. bluteri	Sweet potato pieces with mineral solution	SSF	Filtration and centrifugation	Nwe *et al*. (2002)
	Extract of sweet potato pieces with mineral solution	SmF		

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maltotriose units are linked by α-1-6 glycosidic linkages. It is a soluble biopolymer that is insoluble in some organic solvents (Kachhawa *et al.*, 2003; Sugumaran *et al.*, 2014).

At low concentration, pullulan affords a highly viscous solution that is also colourless, non-toxic, odourless, tasteless and non-hygroscopic. These features have raised interest in the use of this polymer in several commercial segments such as the agricultural, chemical and pharmaceutical industries. More recently, the European Union has approved pullulan as a food additive with recognized GRAS status. This polymer yields oil-resistant, transparent and oxygen-impermeable thin films with potential applications as a coating and packaging material or an adhesive, an encapsulating agent, a starch substitute in low-calorie food formulations and for cosmetic emulsions (Kachhawa *et al.*, 2003; Wu *et al.*, 2009a; Singh *et al.*, 2012; Sugumaran *et al.*, 2013a).

Fermentation by certain strains of polymorphic fungi such as *Cryphonectria parasitica* (Kang *et al.*, 2011), *Tremella mesentrica*, *Cytaria* spp., *Teloschistes flavicans* and *Rhodototula bacarum* (Singh *et al.*, 2008; Cheng *et al.*, 2011b) provides pullulan. Most studies on pullulan production have focused on the yeast-like fungus *Aureobasidium pullulans*, due to its high production yield, but not all strains are capable of producing them (Ravella *et al.*, 2010; Freitas *et al.*, 2013). *A. pullulans* has been isolated from different environmental sources. This microorganism is usually classified as non-pathogenic, even though some strains may harm human health and be pathogenic to plants (Cheng *et al.*, 2011b). Depending on the microorganism strain, age and culture-medium conditions, *A. pullulans* can exhibit five different polymorphic cells: (i) yeast-like cells; (ii) young blastospores; (iii) swollen blastospores; (iv) chlamydospores; and (v) mycelia. In the literature, this fungus is known as 'black yeast', because it generates a black melanin pigment, an undesirable characteristic feature of this fungus. Nevertheless, not all the *A. pullulans* strains produce this pigment, and today studies have concentrated on the isolation of strains that give high yield of polysaccharides while producing reduced amounts of melanin. Interestingly, these strains only occur in tropical and subtropical zones (Singh *et al.*, 2008, 2012; Ravella *et al.*, 2010; Sugumaran *et al.*, 2013b).

Pullulan is produced by SmF and/or SSF in the presence of numerous carbon sources, including starch, glucose, sucrose, mannose, galactose and fructose (Cheng *et al.*, 2011b). SSF offers advantages over SmF, such as less water and energy consumption and generation of high-quality biopolymers at a lower cost (Sugumaran *et al.*, 2013a). Interestingly, Cheng *et al.* (2011b) have reported successful implementation of SmF for commercial pullulan production.

The yield of pullulan produced by *A. pullulans* depends on several bioprocess parameters such as moisture content, pH (5.5–7.5), temperature (25–30°C), fermentation time, aeration (oxygen), stirring (rpm) and concentration of carbon, nitrogen and nutrient sources (starch hydrolysates, yeast extract, K_2HPO_4, NaCl, $MgSO_4$, $(NH_4)_2SO_4$ and $ZnSO_4$) as well as the bioreactor design (batch, fed-batch, immobilized and biofilm systems, as well as other designs). High viscosity of the fermentation broth and the melanin pigmentation (black colour) are the difficulties encountered during pullulan production (Kachhawa *et al.*, 2003; Wu *et al.*, 2009a; Ravella *et al.*, 2010; Cheng *et al.*, 2011b; Kang *et al.*, 2011; Singh *et al.*, 2012; Sugumaran *et al.*, 2013b).

Environmental concerns have prompted researchers to explore cheaper strategies such as pullulan production from agricultural waste and alternative carbon sources such as cassava bagasse (Sugumaran *et al.*, 2014), sweet potato (Wu *et al.*, 2009b), palm kernel, wheat bran, rice bran, coconut kernel (Sugumaran *et al.*, 2013b) and jack fruit seed (Sugumaran *et al.*, 2013a). Besides offering environmental benefits, these substrates are cost-effective – they constitute highly nutritional waste for microorganism growth and reduce fermentation costs (Wu *et al.*, 2009b; Sugumaran *et al.*, 2014).

As for the pullulan extraction process, it involves initial separation of the cells from the solid particles by cool centrifugation. The addition of cold ethanol elicits precipitation of the exopolysaccharide. After removal of residual ethanol, dialysis of the precipitate with neutral water (at about 80°C for 48 h) removes small molecules from the solution. Then, further addition of cold ethanol precipitates the material. Finally, exopolysaccharide drying at approximately 80°C yields pullulan (Kachhawa *et al.*, 2003; Wu *et al.*, 2009b; Singh *et al.*, 2012; Sugumaran *et al.*, 2014).

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Table 23.6 shows some investigations into the production of this exobiopolymer.

Pullulan has great economic importance and has found an increasing number of applications in several industrial segments. However, its large-scale production is still limited. Pullulan is comparatively more expensive than other polymers, such as xanthan and dextran; more specifically, it is three times more expensive. Improvements in the pullulan production process, including engineering innovations, improved strains that provide higher product yield, and identification of cheaper and more effective carbon and nitrogen sources could help to reduce costs (Kang *et al.*, 2011; Sugumaran *et al.*, 2013b).

23.3.3 Glucan and the chitin–glucan complex (CGC)

The classification of glucans relies on their structure (α-glucans and β-glucans). Some glucans have specific names, for example cellulose, glycogen and starch. Although these polysaccharides consist solely of glucose, their chemical and physical characteristics vary. They may exist as microfibrils or in the mucilaginous form. Some glucans are water soluble, whereas others are only soluble in alkali or are even completely insoluble. Their function and location also vary. All these features enable extraction of these biopolymers from the cell wall of fungi and yeasts by the cold alkali method (Ruiz-Herrera, 2012b). Both α- and β-glucans occur in a large number of fungi. β-Glucans are the most abundant polysaccharides in the fungal cell wall, mainly in basidiomycete species; α-glucans occur in species of ascomycote (Costas and Ioannis, 2006). Some glucans have already received specific names according to their structure and to the microorganism in which they exist (Table 23.7). Several kinds of glucans occur in nature; they display different physical properties and have important and varied functions in the cell wall of fungi and yeasts. Many authors have studied the physiological functions of these polymers. They usually have applications in the biomedical and pharmaceutical areas. Some exhibit antitumour, immunomodulatory or antiviral effects, whereas others have hypocholecterolaemic, hypolipidemic, hypoglycemic, antioxidant or anticoagulatory properties (Costas and Ioannis, 2006).

The CGC is another polysaccharide synthesized by fungi and yeast that has gained importance. The CGC is a natural and structured material composed of two types of biopolymers: chitin (*N*-acetylglucosamine units) and β-glucans (glucose units), which yields a water and alkaline insoluble complex. CGC is a valuable biopolymer for several applications. Recently, the CGC

Table 23.6. Studies of the optimal conditions for the production of the exopolymer pullulan.

Bioprocess		Optimal conditions			
Substrate	Process	Initial pH	Temperature	Fermentation time	Reference
Sweet potato	Submerged fermentation (SmF) with agitation	5.5	28°C	96 h	Wu *et al.* (2009b)
Palm kernel	Solid state fermentation (SSF)	6.5	–	168 h	Sugumaran *et al.* (2013b)
Cassava bagasse	SSF	5.5	Room temperature	96 h	Sugumaran *et al.* (2014)
Medium (g/l): sucrose, peptone, yeast extract, K_2HPO_4, $MgSO_4.7H_2O$, NaCl	SmF with agitation (200 rpm)	–	28°C	96 h	Ravella *et al.* (2010)

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Table 23.7. Some α-glucans and β-glucans and the main producing microorganisms.

Classification	Biopolymer name[a]	Source (fungi and yeast)	References
α-Glucan	Elsinan	*Elsinoe leucospila*	Costas and Ioannis (2006), Skorik *et al.* (2010), Roca *et al.* (2012), Ruiz-Herrera (2012b), Freitas *et al.* (2013)
	Nigerans	*Aspergillus* spp., *Penicillium* spp.	
	N/A	*Polyporus betulinus*	
	N/A	*Paracoccidioide brasiliensis*	
	N/A	*Saccharomyces cerevisiae*	
	N/A	*Schizosaccharomyces pombe*	
β-Glucan	Lentinan	*Lentinus edodes*	
	Curdlan	*Alcaligenes faecalis*	
	Zymosan	*S. cerevisiae*	
	Grifolan	*Grifola fondosa*	
	Gllomerellan	*Glomerella cingulata*	
	Glucan I	*Auricularia auricula-judae*	
	Pachyman	*Poria cocos*	
	Schizophyllan	*Schizophyllum commune*	
	Scleroglucan	*Sclerotium glucanicum*	
	N/A	*Coprinus lagopus*	
	N/A	*Monilinia fructigena*	
	N/A	*Schizosaccharomyces pombe*	
	N/A	*Candida albicans*	

[a]N/A, Not available.

isolated from the cell wall of the fungus *A. niger* and of the yeast *P. pastoris* have been granted GRAS status, and their use as functional fibre, a food supplement and an additive in food products and in processes such as wine clarification has been approved. The products derived from the fungus *A. niger* and from the yeast *P. pastoris* present similar physicochemical characteristics (Roca *et al.*, 2012; Freitas *et al.*, 2013). CGC production from *P. pastoris* constitutes an economically viable and sustainable production process – this microorganism can efficiently use a wide range of substrates, including inexpensive raw materials such as glycerol, glucose and methanol (Roca *et al.*, 2012).

23.3.4 Summary of fungal polymers

Biopolymers obtained from fungi and yeasts by the fermentation process have attracted much attention from both academia and industry. These materials constitute multipurpose compounds applicable in many fields, including the food and pharmacy industries, not to mention numerous possible agricultural and biomedical applications. To obtain their production in high yields, more studies about the conditions of the bioprocess and exploration of renewable sources are still necessary which will help to reduce production costs and will provide an environmentally friendly process.

23.4 Final Remarks and Future Prospects

Microorganisms are a versatile source of biopolymers – they can synthesize many different polymeric structures from a wide range of feedstocks, under controlled conditions, to ensure satisfactory quantity and quality of the final products. Owing to their new or improved properties, microbial biopolymers can replace plant, algal and animal products that currently dominate the market or even give rise to new applications. Moreover, manipulation of microbial processes can drive production towards specifically tailored polymer compositions that will fit the intended product applications.

Although many microbial biopolymers possess valuable properties that make them useful in products or processes, only a very limited number of these biopolymers have

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found widespread use. The main limitations are the high production costs, which can be reduced by optimization of the bioprocesses and/or use of low-cost feedstocks, and the difficulty in obtaining permission from the competent authorities for commercialization, as well as gaining public acceptance. While keeping their thoroughness, the evaluation process for new products should be more flexible and less time-consuming, to allow new polymers to reach the market and prove themselves as valuable materials for industrial, food and medical applications.

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24 Microbial Metabolites in the Cosmetics Industry

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Abstract

The cosmetics industry is one of the fastest growing sectors and is a multibillion dollar business. Over the last few decades, people have shown increased interest in using cosmetics of natural origin, replacing in part the extensively used chemicals in this industry. Natural cosmetics are usually considered as safer, more biocompatible and with fewer side effects. For a long time, natural cosmetics have used plant or animal extracts as the basal material. However, microorganisms such as bacterial cells are now considered as potential sustainable sources of functional ingredients or additives in order to improve the quality of such products. Microorganisms in the appropriate conditions produce a variety of low-molecular-weight chemical compounds, including several organic acids, alcohols, proteins and polysaccharides which can be used for various applications in the pharmaceutical, nutraceutical and cosmeceutical industries. Such compounds are extensively used in the cosmetic field either as the main functional active ingredients for protection against ultraviolet (UV) radiation, or as anti-ageing or antibacterial agents and immunomodulators, or as binding, thickening, colouring and stabilizing agents. This chapter reviews the main groups of microbial metabolites currently used in the cosmetics industry either as active ingredients or as additives.

24.1 Introduction

Microorganisms play a significant role in generating various metabolites with potential applications in industries such as agriculture, energy development, the chemical industry, feed and food manufacturing and the pharmaceutical industry. They are the main sources of various enzymes, organic acids, amino acids, antibiotics, vitamins, single cell protein and a variety of chemicals that could be commercialized as valuable products. Various metabolites produced by microbes are commonly used as ingredients in cosmeceuticals because they are natural, non-toxic, biocompatible and lack immunogenicity. Nowadays chemical ingredients play a major role in cosmetics. However, the awareness of consumers regarding the side effects of some of the chemicals which are banned or have restricted use in some cosmetic products have led to an increase in the demand and manufacture of products that use natural active ingredients with no harmful chemicals. These microbially produced metabolites may be used as the main active ingredient or as additional material that are used as filling materials, stabilizing agents, flavour and aroma enhancers or for their colouring properties (El-Enshasy and Sarmidi, 2012).

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Examples of such metabolites include chitosan, a linear polysaccharide produced by yeast cells, $C_{18}H_{34}O_4$ or octadecandionic acid, xanthan gum commonly used as an agent stabilizer or thickening agent, exopolysaccharides, exfoliation-promoting enzymes and natural pigments (El-Enshasy and Sarmidi, 2012). In addition, some compatible solutes or low-molecular-weight microbial metabolites such as ectoine are currently used as the active ingredient in lotions and creams. Other ingredients used in formulations are used on the basis of their activity in preventing or avoiding dryness of the skin, protecting the skin against the damage caused by ultraviolet (UV) rays from the sun (acting as a shield) and for their anti-ageing activities. Also mushrooms have great potential as a source of natural ingredients in cosmeceutical product formulation. Generally, mushrooms are rich in bioactive, low-molecular-weight compounds such as phenolics, terpenoids, organic acids and polysaccharides which may be used as immunomodulators (enhancing the immune system), antioxidants and anti-microbials active against several microorganisms and with anti-cancer activities.

24.2 Microbially Produced Compatible Solutes in Cosmetic Applications

The term compatible solutes refers to organic compounds that can be accumulated in cells at high concentrations and they are represented by several classes of organic compounds, including sugars, polyols, betaines, amino acids and ectoine and its derivatives (hydroxectoine) (Lentzen and Schwarz, 2006; Kurz, 2008; Pastor *et al.*, 2010; Shivanand and Mugeraya, 2011). They are low-molecular-weight compounds of high water solubility. These characteristics allow their accumulation in the cytoplasm in high concentrations and they have the ability to stabilize protein against various osmotic stress conditions. Ectoine and its hydroxyl derivatives are compatible solutes. According to their functionality as osmotic balancing agents, ectoines are used in the biotechnology area, especially in cosmetic applications as protecting agents for enzymes, DNA, membranes and cells against stress conditions. Based on the wide range of their applications in medicine and the cosmetics industry, ectoines are among the most industrially important chemicals. These compounds are produced on an industrial scale using different types of halophilic bacteria. According to Oren (2010) the process for ectoine and hydroxyectoine production on an industrial scale was developed for *Halomonas elongata* and *Marinococcus* M52, respectively, by using a bacterial milking strategy (Sauer and Galinski, 1998; Oren, 2010). The first success for industrial production of ectoine was achieved by the German company Bitop in cooperation with Merck by using *H. elongata* as the producer organism (Khmelenina *et al.*, 2010).

24.2.1 Ectoines

Ectoine (Chemical Abstracts Service number (CAS#) 96702-03-3) and hydroxyectoine (CAS# 165542-15-4) are 'compatible solutes' or osmo-protectant compounds produced by *H. elongata*. Their chemical structure was investigated and defined as 1,4,5,6-tetrahydro-2-methyl-5-hydroxy-4-pyrimidinecarboxylic acid (ectoine) and 4,5,6-tetrahydro-2-methyl-4-pyrimidinecarboxylic acid (hydroxyectoine). An ectoine compound was first discovered by a scientist and friend (Galinski *et al.*, 1985) produced by extreme halophilic bacteria, namely phototropic *Ectothiorhodospira halochloris*. Currently, ectoine is produced biologically by other halophilic bacteria, photosynthetic bacteria and chemoheterotrophs. Recently, Tanimura *et al.* (2013) successfully produced ectoine from a genetically engineered strain of *H. elongata* using lignocellulosic-biomass-derived sugars. Ectoine and hydroxyectoine were identified as zwitterionic low-molecular-weight compounds of high solubility in water with strong water-binding properties (Galinski *et al.*, 1985; Galinski, 1993; Graf *et al.*, 2008; Harishchandra *et al.*, 2010). The molecular weight of ectoine and hydroxyectoine was determined by nuclear magnetic resonance (NMR) and they have the chemical formula $C_6H_{10}N_2O_2$ and $C_6H_{10}N_2O_3$, respectively (Kanapathipillai *et al.*, 2005). Figure 24.1 shows the basic chemical structure of ectoine and hydroxyectoine and Table 24.1 summarizes their molecular characteristics.

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24.2.2 Ectoines in the cosmetics industry

Ectoine and hydroxyectoine have found interesting applications as ingredients in cosmetic preparations and formulations. Both are great microbial metabolites for the biotechnology industry, especially in the agricultural, biotechnological and pharmaceutical industries. For cosmetics, ectoine and hydroxyectoine are excellent as: (i) moisturizers and are better than glycerol (Graf *et al.*, 2008); (ii) acting as a chiral building block specifically for the care of aged, dry and irritated skin (Galinski, 1997; Zanjirband *et al.*, 2008; Shivanand and Mugeraya, 2011); and (iii) as protection for healthy cells during chemotherapy (Jebbar *et al.*, 1992; Buenger and Driller, 2004; Graf *et al.*, 2008; Khmelenina *et al.*, 2010; Oren, 2010). Also ectoine can be administrated orally (as a nutricosmetic).

Fig. 24.1. The chemical structure of (a) ectoine and its derivative (b) hydroxyectoine.

Table 24.1. Chemical and physical characteristics of ectoine and hydroxyectoine.

Characteristics	Ectoine	Hydroxyectoine
Molecular weight	142.2 g/mol	158.2 g/mol
Chemical formula	$C_6H_{10}N_2O_2$	$C_6H_{10}N_2O_3$
Microorganism	*Halomonas elongata*	*Marinococcus* M52
Storage temperature	20°C	20°C
Melting point	280°C	280°C
Solubility in water	4.0 mol/l	4.2 mol/l
Solubility in methanol	0.3 mol/l	0.05 mol/l
pH range	1–9	1–9

Ectoine (Ectoine™) is widely applied in the growing skin-care products area of extremolytes (Lentzen and Schwarz, 2006). Table 24.2 shows some of the applications of ectoine in the cosmetics industry.

24.3 Kojic Acid (CAS# 501-30-4)

Kojic acid, also known as 5-hydroxy-2-(hydroxymethyl)-4H-pyran-4-one (CAS# 501-30-4) is a low-molecular-weight organic acid, which is widely used in the cosmeceutical and nutraceutical industries. Kojic acid was produced biologically by several types of fungus by an aerobic process using different types of substrate (Wakisaka *et al.*, 1998; El-Aasar, 2006; Mohamad *et al.*, 2010). According to Yabuta (1924) the name kojic acid was derived and discovered in Japan by Saito in 1907, from the fungus *Aspergillus oryzae* grown on fermented rice, namely koji (a starter culture used in food fermentations for many centuries). This compound has tyrosinase inhibitor activity. Therefore, it inhibits melanin formation, and can also act as an antioxidant and metal-binding agent. At the early stage of discovery, kojic acid was applied as an antioxidant and used by the cosmetic industry as an alternative skin-whitening agent to hydroquinone. Kojic acid is also known for its antibacterial, antifungal and high antioxidant activities. Therefore, it is used in many commercialized cosmetics in the market in different concentrations from 0.1% to 4%. Besides its application in cosmeceutical industries, kojic acid is widely used in the Japanese diet to eliminate free radicals, to

Table 24.2. Applications of ectoine in the cosmetics industry.

Function	References
Ultraviolet (UV)A-skin protector	Buenger and Driller (2004), Oren (2010)
Treatment of the mucous membranes of the eye	Shivanand and Mugeraya (2011)
Anti-ageing	Graf *et al*. (2008)
Anti-wrinkling	Graf *et al*. (2008)
Natural cell protect (acts as potent moisturizer with long-term moisturizing efficacy)	Graf *et al*. (2008)

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strengthen cell activity and also act as a food preservative during fruit and vegetable storage.

24.3.1 Characterization, preparation and application of kojic acid

The appearance of kojic acid as a final product is one of pale yellow to almost white crystals. The chemical structure of kojic acid is depicted in Fig. 24.2. The hydroxyl groups mean that the compound acts as a weak acid and if different types of metals are added, such as sodium, zinc, copper or cadmium, salts are formed. It also contains a polyfunctional heterocyclic ring, which is the oxygen-containing skeleton, and this enables additional reactions such as oxidation and reduction, alkalytion and acylation, substitution and other related reactions (Brtko *et al*., 2004).

From physical observations, kojic acid is soluble in water and other solvents such as ethanol, acetone, ethyl acetate, chloroform and pyridine, but insoluble in benzene (Brtko *et al*., 2004). Kojic acid has the chemical formula $C_6H_6O_4$ with a molecular weight of 142.1 g/mol. The melting point of kojic acid lies between 151°C and 154°C. Kojic acid determination is usually carried out using UV absorption spectra between 280 nm and 284 nm (Ohyama and Mishima, 1990).

In the cosmetics industry, kojic acid is used as a basic material for the preparation and production of creams and specifically: (i) for skin whitening as it suppresses pigmentation that occurs in human skin through the inhibition of tyrosinase (Brtko *et al*., 2004); (ii) for skin-protective lotions to prevent the incidence of skin cancer and because it has antifungal and antibacterial properties; (iii) in whitening soaps; and (iv) as an ingredient for toothcare products. However, in addition to its importance in the cosmetics industry, kojic acid also has many potential pharmaceutical applications as it is antibacterial (Nohynek *et al*., 2004), antifungal (Balaz *et al*., 1993) and a painkiller (Beelik, 1956). In the food industry, kojic acid is used as a flavour enhancer (Burdock *et al*., 2001), an antioxidant and in the production of maltol and ethyl maltol (Chen *et al*., 1999). In the agricultural sector, it is also applied as an anti-melanosis compound (Chen *et al*., 1999) and it acts as an insecticide activator (Dowd, 1998). In addition, kojic acid is used in the chemical industry as an iron-chelating agent and for synthesis of 2-methyl-4-pyrone and chitosan conjugates (Mohamad *et al*., 2010).

O
OH
HO
O

Fig. 24.2. The chemical structure of kojic acid.

24.4 Botulinum Toxin (CAS# 93384-43-1)

Different types of living organism including microorganisms, plants and animals are able to produce a wide range of toxic compounds. Toxins were differentiated according to their source as mycotoxins, phytotoxins, zootoxins (e.g. snake venom) and bacterial toxins (Kant *et al*., 2009). Nowadays, bacterial toxins are used in the cosmetics industry for regenerative skin products (anti-ageing and anti-wrinkling), targeted mostly at women.

Clostridium botulinum is a bacterium that produces botulinum toxin which contains both protein and neurotoxin (Dressler *et al*., 2005). *C. botulinum* is a Gram-positive, rod-shaped and spore-forming bacterium which is widely distributed in soil and water (Dowell, 1984). According to Davis (1993) botulinum toxin is odourless and tasteless. Botulinum toxin was purified into a crystalline structure by Dr Edward and his researcher in the early 1950s. In 1953, a physiologist Dr Vernon Brooks successfully applied small amounts of this toxin to hyperactive muscle and blocked the muscle contraction and caused relaxation. Moreover, in 1960, Dr Alan B. Schott also applied type A toxin to an animal (monkey) for the treatment of strabismus. In 1978, Schott received approval from the US Food Drug Administration (FDA) for the application of this toxin to humans. Microbial botulinum neurotoxin can be used to treat a variety of diseases including movement disorders, spasticity, ophthalmic disorders, pain, pelvic floor muscles and gastrointestinal disorders as well as having cosmetic applications (Botox treatment of facial wrinkle lines).

The protein structure of this toxin was determined by NMR. Botulinum toxin (Botox)

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structure consists of a complex mixture of some proteins such as botulinum neurotoxin and others without toxicity (Sakaguchi *et al*., 1984). According to its structure, Botox consists of a heavy chain (binding chain) and a light chain (inhibitory chain) linked together by a single disulfide bond (Dressler *et al*., 2005). Botox or botulinum toxin is available in the form of seven different serotypes which are recognized as serotype A, serotype B, serotype C, serotype D, serotype E, serotype F and serotype G (Dressler *et al*., 2005). Among these seven serotype neurotoxins, toxins A and B are preferred for use because of their stability, action, ease of production and they have approval from the FDA for human use for many cosmetic applications. In addition, toxin A is used for therapeutic purposes because it rarely results in systemic side effects and there is less tissue destruction. With regards to the physical characteristics of toxin A, the length, molecular weight without surface proteins and molecular weight with surface proteins are 1295 amino acids, 149.3 kDa and 900 kDa, respectively (Bali and Thakur, 2005). For toxin B, the length is 1290 amino acids, and the molecular weight without surface proteins and the molecular weight with surface proteins are 150.6 kDa and 700 kDa, respectively (Bali and Thakur, 2005). The molecular formula and molecular weight of botulinum toxin are $C_{6760}H_{10447}N_{1743}O_{2010}S_{32}$ and 149,322 Da, respectively. This toxin is relatively heat labile and completely inactivated at 100°C (Bali and Thakur, 2005).

24.5 Microbial Polysaccharides

A wide range of polysaccharides of microbial origin have been used as the main ingredients of cosmetics. Microbial polysaccharides have great diversity in terms of structure and function and are regarded as excellent functional compounds as emulsifying, stabilizing and binding agents. They are produced by different types of microbes including bacteria, cyanobacteria, actinomycetes, lower fungi and higher fungi (mushrooms). These polysaccharides include hyaluronic acid (HA), alginate, kefiran, dextran, pullulan, xanthan and mushroom glucans/peptidoglucans among many others.

24.5.1 Hyaluronic acid (HA) (CAS# 9004-61-9)

HA is also known as hyaluronan or hyaluronate. HA was first isolated from ox vitreous humour by Karl Meyer and John Lamer. They found that this compound is a polymer of uronic acid and amino sugar. Therefore, they called the polysaccharide 'hyaluronic acid' which is derived from hyaloid (vitreous) and uronic acid. The structure of this biopolymer has recently been found to be made up of the linear dissaccharide repeats of D-glucuronic acid (GlcUA) and *N*-acetylglucosamine (GlcNAc) units joined alternately by β-1,3 and β-1,4-glycosidic bonds (Fig. 24.3). HA has a molecular formula $(C_{14}H_{21}NO_{11})n$ with a molecular weight that varies between 1×10^4 Da and 1×10^7 Da. Over the years, HA has been produced and extracted from animal tissue consisting of the vitreous body, umbilical cord or rooster combs. Nowadays, HA is produced mainly by microbial cells. Initially, the wild-type strain of *Streptococcus equi* subsp. *zooepidemicus* was used as a biofactory for the production of HA as it produces HA as an extracellular product in the fermentation broth during submerged fermentation. Based on the original strain with low fermentation and high market demand, HA is now produced on an industrial scale using recombinant strains of *Escherichia coli*, *Bacillus subtilis* and *Lactobacillus lactis* (Liu *et al*., 2011). The molecular

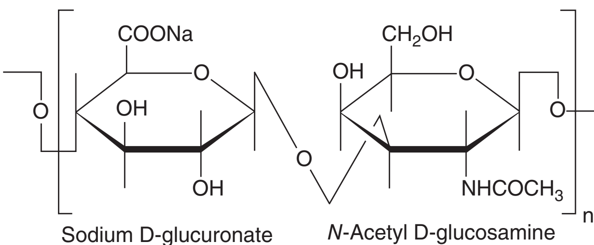

Fig. 24.3. Chemical structure of hyaluronic acid.

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weight of HA can be controlled in order to achieve the desired range by changing the parameters of the fermentation and extraction methods (Liu *et al.*, 2011; Manasa *et al.*, 2012). HA has an excellent water-holding property and it is very effective at retaining moisture in the eyes, joints and skin tissues.

24.5.2 Xanthan (CAS# 11138-66-2)

Xanthan or the commonly named xanthan gum is a high-molecular-weight polysaccharide secreted by the plant-pathogenic bacterium *Xanthomonas campestris.* Nowadays, this polysaccharide is widely used as a stabilizing, binding and skin-conditioning agent, as well as a food additive and rheology modifier in aqueous-based systems. Xanthan gum is known to be non-toxic and has been approved by the FDA. Therefore, it is used in cosmetics as a stabilizer to prevent phase separation during product formulation. The xantham gum broth is pseudoplastic, soluble in cold and hot water and shows a high degree of viscosity even in low concentrations. Xanthan gum has the molecular formula $C_{35}H_{49}O_{29}$ (monomer) and it is a heteropolysaccharide with the basic structural unit consisting of two glucose and mannose units and one glucoronic acid, in the molar ratio 2.8:2.0:2.0 (Fig. 24.4).

Commercially, xanthan gum is available as an odourless cream-coloured powder. It is characterized by high water solubility and high resistance to heat degradation. This biopolymer is also highly stabile across a wide acid and alkali range between pH 5.5 and 8.5 (Prajapati *et al.*, 2013). The molecular weight of xanthan gum varies within a very wide range, from 2×10^6 Da up to 20×10^6 Da. The biopolymer molecular weight could be controlled by adjusting the fermentation parameters during production in a submerged culture system.

24.5.3 Pullulan (CAS# 9057-02-7)

Pullulan is a non-ionic, water-soluble, white polymer. It is produced as an extracellular polysaccharide by the yeast strain *Aureobasidium pullulans*. This polysaccharide polymer is composed of 1-6 linked maltotriose residues connected by α-(1,4) glycosidic bonds (Fig. 24.5). This polysaccharide has a molecular weight which ranges between 100 kDa and 250 kDa. It is characterized by its high water solubility in solutions of pH 5–7. Pullulan produced by microbial fermentation is a non-toxic, safe compound with

Fig. 24.4. Chemical structure of xantham gum.

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Pullulanase

Pullulan
Linear polymer of α-(1-6)-linked maltotriose units

Fig. 24.5. Chemical structure of pullulan.

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non-immunogenic, non-mutagenic and non-carcinogenic properties. Therefore, in addition to its application in the food industry, it has many biomedical and cosmeceutical applications as an agent for gene delivery, targeted drug therapy, tissue engineering and grafts, wound healing and medical imaging (Prajapati *et al.*, 2013).

24.5.4 Kefiran (CAS# 86753-15-3)

Kefiran is a water-soluble biopolymer and is considered to be one of the most promising polysaccharides for many food, cosmetic and pharmaceutical applications. The polysaccharide comprises a hexasaccharide repeating unit of D-glucose and D-galactose (Fig. 24.6). This polysaccharide was first extracted from kefir grains and characterized as a metabolic product of the bacteria *Lactobacillus kefiranofaciens*. Kefiran generally was commercialized for food applications as a thickener, stabilizer, emulsifier, fat substitute and gelling agent. It has also been reported that kefiran confers beneficial health effects such as antitumour activity (Shiomi *et al.*, 1982). In addition, kefiran has the potential to enhance the production of interferon β from animal cells, which is suppressed by the stress hormones cortisol and norepinephrine (formerly known as noradrenaline) (Kabayama *et al.*, 1997). Therefore, kefiran has gained more interest recently and is currently being studied for mass production. Nowadays, kefiran is produced industrially as extracellular polysaccharides (EPSs) either from a pure culture of *L. kefiranofaciens* or from a mixed culture of *L. kefiranofaciens* and *L. lactis*. The polysaccharide could be either secreted into the medium, or produced as an amorphous layer of polysaccharides known as capsular polysaccharides (or CPSs) around the cells (Cheirsilp *et al.*, 2001).

24.5.5 Alginic acid (CAS# 9005-32-7)

Alginic acid, also called algin or alginate, is a linear polysaccharide with a molecular formula $(C_6H_8O_6)n$. Alginic acid has a molar mass between 10,000 Da and 600,000 Da. This polysaccharide is composed of linear chains of 1-4 linked co-polymers of β-D-mannuronic and its C-5 epimer, α-L-guluronic acid, in various proportions. Based on biocompatibility characteristics, biodegradability and safety, alginate is one of the most abundant polysaccharides used in the food, pharmaceutical and cosmetic industries as a thickening agent, stabilizer, preservative, gelling agent and emulsifier. Recent research has shown the ability of alginate to act as an anti-inflammatory and wound-healing agent. In addition, alginate is widely applied as an outstanding cell matrix for enzyme immobilization for use either in research or industry.

At first, alginate was isolated from various brown algae species such as *Macrocystis pyrifera*, *Laminaria hyperborea* and *Laminaria digitalis.* Besides the algae, different species of bacteria such as *Azotobacter* sp. and *Pseudomonas* sp. have been used for industrial production of alginate. These bacteria produce alginate as an extracellular product during the late exponential growth phase. At the present, *Azotobacter vinelandii* is one of the most popular strains used for the production of alginate and it is recognized as safety level 1 GRAS (generally recognized as safe) by the FDA (Then *et al.*, 2012).

24.6 Medicinal Compounds from Mushrooms Used for Cosmetic Applications

Recently, medicinal compounds isolated from fruiting bodies of various species of wild and greenhouse mushrooms have found many applications as remedies and in cosmetics. Among potential bioactive products from fungi or mushroom species, polysaccharides have been used in the pharmaceutical and cosmeceutical area especially as agents for topical application on the skin such as creams, lotions and massage oils. Hyde *et al.* (2010) showed the potential application of mushroom derivatives for production of various products such as anti-ageing, antioxidant, skin-revitalizing and/or -whitening agents and hair treatment applications. From a medicinal viewpoint, mushroom derivatives were able to treat various diseases such as cancer and diabetes. In addition, they were also effective against viral and bacterial infections, may act as antitumour agents and stimulate the immune system.

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Fig. 24.6. Chemical structure of kefiran.

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24.6.1 Mushroom cosmeceutical products

Grifola frondosa or maitake is a basidiomycete that has been used widely by traditional Chinese and Japanase herbal practitioners as a precious and good food as well as a medicinal food. The structure of of glucan of *G. frondosa* was reported to be a backbone of β-(1-6)-linked glucose residues with side chains of β-(1-3)-glucose residues and Minato *et al.* (2001) revealed that the glucan secreted from *G. frondosa* is a type of biological response modifier and possess an immunomodulating effect. In addition, the glucan was discovered to have the potential to accelerate the process of collagen biosynthesis and deliver photoprotection. For this reason polysaccharides derived from this mushroom contribute as functional materials in the development and production of valuable products in the cosmetic industry (Lee *et al.*, 2003; Bae *et al.*, 2005; Kim *et al.*, 2007a). According to a study by Kim *et al.* (2007b), *G. frondosa* polysaccharides have the potential to reduce matrix metalloproteinase (MMP) activity in the skin after solar exposure and stimulation and this is a good indicator of the potential of these polysaccharides extracted from mushrooms for new applications in cosmetic products. In addition, β-(1-3)-D-glucan, a polysaccharide extracted from *Pleurotus ostreatus*, has anti-cancer properties and its use in cosmetics is well known.

24.6.2 Mushroom tyrosinase inhibitors

Mushroom tyrosinase inhibitors are are group of enzyme inhibitors produced in nature by *Agaricus bisporus*. The enzyme inhibitor from mushroom is extensively used in cosmetic and pharmaceutical products due to the potential preventive effect on pigmentation disorders so it is used as a whitening agent for the skin, to lessen and avoid the development of pigment and for depigmentation of the skin after contact with UV rays (i.e. to treat sunburn).

24.6.3 Chitin–glucan

Chitin–glucan is a fibre from fungal cell walls that has several characteristics such as it is very easily incorporated into different formulations to cause thickening and when added to skin-care products it provides stability to the different components without the need for further additives or preservatives. Gautier *et al.* (2008) showed that formulation of novelty skin-care cosmetic creams with chitin–glucan had added value as it produced a very good moisturizer with specific use in anti-ageing products by increasing the firmness of superficial skin, enhancing water binding and improving skin barrier function as well.

24.7 Microbial Derivatives as Functional Cosmetics

24.7.1 Depigmenting material from microbial derivatives

Melanogenesis is the formation process of melanin, a group of dark macromolecular pigments. Melanin is formed through a combination of enzymatically catalysed and chemical reactions (Chang, 2005). Melanogenesis involves two steps in the skin pigmentation process (*de novo* synthesis) starting by production of melanin in melanocytes and transfer of melanosomes (melanin-packed vesicles) to neighbouring keratinocytes, which darken the skin tone (Seiberg *et al.*, 2000; Hearing, 2005). Melanosomes contain three types of enzymes: (i) tyrosinase; (ii) tyrosinase related protein 1 (TRP1); and (iii) dopachrome tautomerase (DCT)/tyrosinase related protein 2 (TRP2) (Kobayashi and Hearing, 2007). Tyrosinase is a rate-limiting enzyme involved in melanin synthesis that hydroxylates tyrosine, a kind of phenylalanine, to L-3, 4-dihydroxyphenylalanine (L-DOPA) and oxidizes L-DOPA to DOPA quinine (Lentzen and Schwarz, 2006). Hydroxylation and oxidation of tyrosine form DOPA quinone and excessive amounts of DOPA quinone result in DOPA chrome, which conditionally exhausts cysteine, and the consequence is the synthesis of the black and brownish pigment eumelanin. Pheomelanin, another type of melanin, is produced through formation of 3- or 5-cysteinyl DOPA in the presence of cysteine (Kim *et al.*, 2006; Miyamura *et al.*, 2007; Yamaguchi *et al.*, 2007). These three enzymes play an important role in the synthesis of melanin into eumelanin or pheomelanin. Accordingly, skin

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colour can be determined by the ratio between the two types of melanin, and the extent of transferring melanosomes to keratinocytes (Costin and Hearing, 2007). Melanin mainly plays a photoprotective role; however, the abnormal accumulation of melanin in different parts of the skin might become an aesthetic problem. Therefore, several studies have focused on the inhibition of tyrosinase activity and the prevention of abnormal pigmentation (Chang, 2005; Ding *et al*., 2011; Lin *et al*., 2011).

Imokawa (2004) reported that some cytokines and growth factors play important regulatory roles in melanogenesis. α-Melanocyte stimulating hormone (α-MSH) binds to its receptor, melanocortin-1 receptor (MC1R), on the membrane of melanocytes and stimulates melanogenesis via the GPCR-cAMP-MITF (G protein-coupled receptor-cyclic adenosine monophosphate-microphthalmia-associated transcription factor) pathway where the melanogenesis-related enzymes, including tyrosinase and TRP1 and TRP2 are upregulated (Hearing, 2005; Lin *et al*., 2011). Therefore, molecules blocking the signal pathway would exhibit depigmentation (Mun *et al*., 2004; Sato *et al*., 2008; Tai *et al*., 2009; Chang and Lin, 2010; Chang and Chen, 2011).

Active research to search for novel depigmenting agents has been made with little success. From an industrial aspect, cosmetics can lighten skin colour which is one of the important functions of cosmetics. Specifically, the development of novel depigmenting agents from microbial derivatives has become a new popular trend recently. These depigmenting materials as the active ingredient in cosmetic products are effective for treatment of melanogenic disorders and melasma (Surace *et al*., 2000; Tomita and Suzuki, 2004). The most popular skin-lightening agent used in many cosmetic formulations is kojic acid, chemically known as 5-hydroxy-2-(hydroxymethyl)-4-pyrone (Lim, 2009). It is a chelation agent produced by several species of fungi, especially *A. oryzae*, and is a by-product of malted rice fermentation used to manufacture sake (Yabuta, 1924). Kojic acid is a well-known skin-lightening ingredient that inhibits tyrosinase activity by binding to copper (Garcia and Fulton, 1996; Policarpio and Lui, 2009) and by so doing it inhibits melanogenesis. To enhance the skin-lightening effect of kojic acid, it may be used together with glycolic acid as a penetration enhancer.

Before the discovery of kojic acid, hydroquinone was the only ingredient used for skin whitening. Hydroquinone causes skin irritation and therefore dermatologists often recommend kojic acid as an alternative ingredient for treatment of skin discoloration. This is despite the fact that those with very sensitive skin may still develop symptoms such as redness or itching from the use of kojic acid, but overall this ingredient is better tolerated than hydroquinone. Kojic acid has been reported to have the same or just slightly less efficacy as hydroquinone. Besides having skin-lightening effects, kojic acid is also an antioxidant. This class of nutrients has the ability to counteract free radicals in the air, which have the potential to cause oxidative damage to skin cells. In a nutshell, kojic acid helps to prevent the signs of ageing that occur by the effects of free radical damage to the cells that produce the skin's vital structural proteins.

Kojic acid is also an antibacterial agent. Kojic acid interferes with the processes by which bacterial cells thrive and reproduce and hence causes the death of bacteria. The use of low concentrations of kojic acid has been recommended by some dermatologists to address acne blemishes caused by bacterial infections in the pores. Conflicting studies have been reported about the long-term safety of the ingredient since its discovery. Results in some clinical trials have established a link between the ingredient and some forms of cancer; on the other hand, others have found that kojic acid has no carcinogenic effects. Generally, experts do agree that only a high quantity of kojic acid exposure in the body will lead to cancer. The cancer-causing concentration limit of kojic acid greatly exceeds the amount of kojic acid that is found in skin-care products.

Other microbial derivatives that contribute to the whitening effect and inhibiting melanogenesis are polysaccharides such as kefiran. Another microbial derivative such as ectoine also helps in boosting the skin's built-in protection system and shields skin cells against environmental stress.

24.7.2 Functional cosmetics and photoageing caused by UVB irradiation

Skin changes are among the most visible signs of ageing. Evidence of increasing age includes

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wrinkles and sagging skin. Chronic exposure to UV irradiation is thought to be the major cause of skin damage that leads to premature ageing of the skin called photoageing (extrinsic ageing), characterized by coarse wrinkles, roughness, laxity and pigmentation (Weiss *et al*., 1988; Jenkins, 2002; Kang *et al*., 2003). Cell-surface growth factors and cytokine receptors at keratinocytes and fibroblasts are activated by UV irradiation and hence stimulate mitogen-activated protein kinase (MAPK) signal transduction pathways which involve ERK (extracellular signal-regulated kinase), JNK (c-JUN-NH(2)-terminal kinase) and p38 kinases (Rittie and Fisher, 2002; Xu and Fisher, 2005). Signal transduction through an MAPK pathway is dependent upon an active transcription factor AP-1 (activator protein 1), a heterodimeric protein composed of c-FOS and c-JUN that enhances the expression of the matrix metalloproteinase (MMP) genes and reduces collagen synthesis through collagenesis (Fisher and Voohees, 1998; Fisher *et al*., 2002). Increased expression of MMPs and decreased collagen synthesis are among the major causes contributing to wrinkle formation in the skin (Fisher *et al*., 1997). In addition, higher levels of reactive oxygen species (ROS) stimulated by UV irradiation seem to be involved in the activation of the MAPK signalling pathway (Shin *et al*., 2005). UV-induced ROS can lead to skin damage including skin cancer, autoimmune disease and inflammation while induced MMPs expression can cause collagen degradation (Camhi *et al*., 1995; Rittie and Fisher, 2002). The mechanism of photoageing is shown in Fig. 24.7.

MMPs are a large family of calcium-dependent zinc-containing endopeptidases, which are responsible for tissue remodelling and degradation of the basement membrane (BM) and extracellular matrix (ECM), including collagens, elastins, gelatin, matrix glycoproteins and proteoglycan. They are regulated by hormones, growth factors and cytokines, and are also involved in the expression of several inflammatory cytokines (McCawley and Matrisian, 2001; Lauer-Fields *et al*., 2002; Visse and Nagase, 2003). In particular, MMP-1 is the main reason for the degradation of dermal collagen during the ageing process of human skin (Brennan *et al*., 2003; Visse and Nagase, 2003). Type 1 collagen is the primary constituent of the ECM and reduction of type 1 pro-collagen is closely related to skin ageing (Fisher *et al*., 2000; Pinnell, 2003; Jung *et al*., 2007). Therefore analysis of MMP-1 expression levels along with collagen production has become a primary screening process for anti-ageing

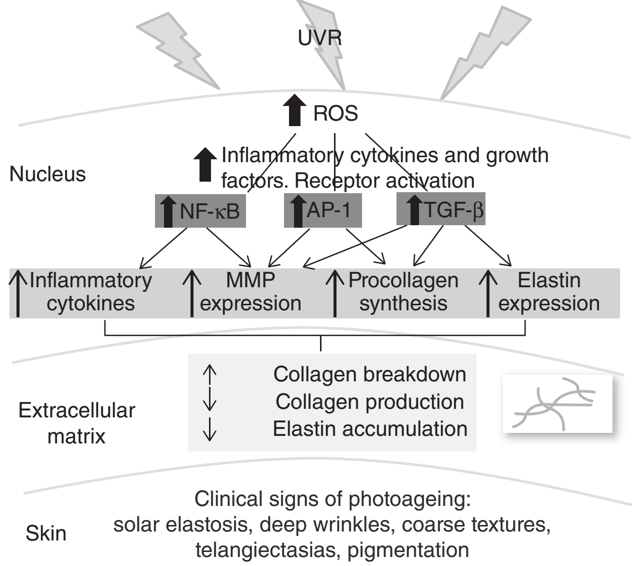

Fig. 24.7. Mechanism of photoageing. AP-1, Activator protein 1; MMP, matrix metalloproteinase; NF-κB, nuclear factor kappa B; ROS, reactive oxygen species; TGF-β, transforming growth factor beta; UVR, ultraviolet radiation. (Adapted from Hearing, 2005.)

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cosmetic ingredients. In UV-induced skin, MMP-9 is known to degrade collagen type IV, which is a vital component of the BM in skin's dermal–epidermal junction. Thus, regulation of collagen type IV plays a key role in the skin ageing process (Amano *et al*., 2005). In addition, MMP-9 is also implicated in wound healing, metastasis and angiogenesis. Expression of MMP-9 by keratinocytes after UV exposure has been determined by the expression level of several inflammatory cytokines such as tumour necrosis factor (TNF)-α and interleukin (IL)-1β (McCawley and Matrisian, 2001; Onoue *et al*., 2003; Kim *et al*., 2007b). Other workers have similarly found that UVB-irradiated keratinocytes stimulate the expression of numerous pro-inflammatory cytokines such as TNF-α, IL-1α, IL-1β, IL-6, IL-8, IL-10 and prostaglandins (PG) including prostaglandin E2 (PGE2) (Grone, 2002; Pupe *et al*., 2002; Pillai *et al*., 2005). These pro-inflammatory cytokines are associated with various inflammatory skin diseases as well as the progression of photodamage. UVB exposure prominently enhances the level of COX-2 (cyclooxygenase-2) expression in cultured keratinocytes, leading to the UVB-induced synthesis of the inflammatory mediator PGE2 and photocarcinogenesis (Ahn *et al*., 2002; Tsoyi *et al*., 2008).

Ectoine as a naturally compatible solute synthesized by halophilic bacteria and it is a neutral non-ionic, strong water-binding, organic molecule of low molecular weight (Galinski, 1993). The topical application of ectoine before sun exposure can protect the skin against intense sun irradiation, preventing the UV-induced effects described above (Pfluecker *et al*., 2005). Furthermore, ectoine can prevent oxidative damage in skin as it shows singlet oxygen quenching properties. It also has a preventive effect on UVA-induced large-scale mutations of the mitochondrial DNA in human dermal fibroblasts. It is strongly supposed that this ingredient exhibits an effect against skin wrinkle formation (Berneburg *et al*., 1997, 1999; Buenger and Driller, 2004).

Another microbial derivative used for anti-ageing is botulinum toxin (Botox). The American pharmaceutical company Allergan manufactured botulinum toxin A, a strain of botulinum neurotoxin that is produced by the organism *Clostridium botulinum* and introduced the trade name of Botox. Botulinum neurotoxin is able to cause paralysis of muscles by blocking the nerves and this lasts typically for 3–4 months. Subsequently, the nerve endings are then replaced and repaired (Lipham, 2004; Ting and Freiman, 2004). Hence, botulinum toxins A and B are used for a wide range of human health conditions, such as treatment of hyperactive muscles, involuntary muscle contractions and tremors and spasms in the face, trunk and limbs. Despite its fame for treating facial wrinkles, the clinical use of Botox originated with treating strabismus (crossed eyes) and blepharospasm (uncontrolled blinking) (Lipham, 2004; Mandeville and Rubin, 2004; Ting and Freiman, 2004). It was during such use that the Canadian ophthalmologist Jean Carruthers discovered a side effect of Botox which is it could reduce the appearance of frown lines (Benedetto, 2003; Mandeville and Rubin, 2004; Ting and Freiman, 2004). During the 1990s, this finding drove research into botulinum toxin A as to its effectiveness in treating various facial lines, especially those on the forehead and around the eyes (Lipham, 2004). According to the American Society for Aesthetic Plastic Surgery (2009), approximately 2.8 million procedures were performed in the USA in 2007. In the same year, Australians reportedly spent around AUS$300 million on non-surgical procedures including Botox (Cogdon, 2009).

The global market for dermal fillers is also booming, and people spent approximately US$759 million during 2009 (Liu *et al*., 2011). Nowadays, there are almost 100 different dermal fillers on the market and about half of them are based on HA (hyaluronic acid). The American Society for Aesthetic Plastic Surgery reports that about 23,000 dermatologists, plastic surgeons and cosmetic surgeons in the USA performed more than 11.8 million surgical and non-surgical cosmetic surgery procedures in 2004, generating US$12.5 billion in fees. In the USA, the dermal filler market is expanding at an annual rate of more than 25% through 2011 and 20% throughout the rest of the world, reaching US$1.5 billion in global sales (Liu *et al*., 2011).

24.8 Conclusion and Future Prospects

Microbial biofactories are a sustainable resource of metabolites which have highly diversified

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applications for the cosmeceutical industry. Bearing in mind that less than 5% of the world's microbes have been isolated and characterized, the rest of the members of microbial world are still to be discovered so it may be expected that many more microbial bioactive molecules may be isolated for cosmeceutical applications in the near future. Compared with plants, microbial cells can produce large quantities of the desired metabolite in higher concentrations, in a shorter time scale and under closed cultivation system independent of the effects of climate change. Therefore, microbes are considered as a huge resource for the production of safe, natural and functional cosmeceutically important molecules.

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25 Fungi of the Genus *Pleurotus*: Importance and Applications

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Abstract

The systematic classification of the genus *Pleurotus* has been much debated due to changes in the names of the species, however, biochemical, chemical and molecular tools support the classification by morphological characteristics. The main species are *Pleurotus ostreatus* (Jacq.:Fr) Kummer, *Pleurotus florida* Eger, *Pleurotus eryngii* (DC.:Fr) Quel, *Pleurotus sajor-caju* Fr.:Fr., *Pleurotus cystidiosus* O.K.Mill (*P. abalonus* Han, Chen & Cheng), *Pleurotus cornucopiae* (Paulet) Rolland, *Pleurotus djamor* (Rumph. ex Fr.) and *Pleurotus pulmonarius* (Fr.) Quél. In general, there is great interest in the study and production of these fungi since the nutritional value of their fruiting bodies (in particular for the protein content, including essential amino acids, fibre, vitamins and minerals) is widely documented. Furthermore, these fungi could be considered as a nutraceutical food since it has been reported that they contain biomolecules which help in certain diseases, including acquired immune deficiency syndrome (AIDS), autoimmune diseases (e.g. rheumatoid arthritis) and hypercholesterolemia, and which have anti-hypertension and antitumour activity. Molecules of major importance are statins, lectins, antioxidant compounds, glucans and enzymes. The latter are of industrial importance: (i) the hydrolases of *Pleurotus* can be used in several industries, including the food sector; and (ii) the phenoloxidases have been extensively studied as they could be used in the bioremediation processes to degrade xenobiotic compounds such as derivatives of paper processing, wastewater treatment and pesticides. The genus *Pleurotus* is of great importance as a food and as a source of many bioactive compounds.

25.1 Introduction

The genus *Pleurotus* is a group of basidiomycetes that have great economic importance and they are commonly cultivated around the world as they are able to grow in a wide temperature range using various substrates. As they have few nutritional requirements and adapt easily to different growing conditions, a food rich in protein is obtained from conversion of lignocellulosic residues (mainly from agro-industrial waste) which are generated on a large scale worldwide. Nutritionally they have a high protein content that is almost equal or greater than some vegetables and they also possess all the essential amino acids for human functions and high levels of vitamins and minerals. Likewise, consumer interest for purchasing products free of fertilizers, preservatives and other chemical compounds, as well as a food with high protein content and other nutrients, converts these fungi into a sought-after food with growing demand. On the other hand, the potential of these fungi as a medicinal source is also known across a number of cultures. Several

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medicinal and therapeutic properties have been reported such as anti-cancer/antitumour activity, hypocholesterolaemic, antiviral, antibacterial, immunomodulatory, antioxidant, antihypertensive, anti-inflammatory and anti-diabetic activity as well as protecting the liver.

25.2 Lignocellulosic Biomass

Through photosynthetic processes around 155 billion t of organic matter are produced on the surface of the planet per year (Rajarathnam and Bano, 1991). However, only a portion of this organic matter is directly edible by man and animals; most of it, taking various forms, is inedible and often becomes a major source of environmental pollution. Moreover, a huge amount of lignocellulosic wastes are generated through the practice of agriculture and forestry. The burning of lignocellulosic waste causes environmental pollution of the atmosphere and this problem increases year after year due to the steady growth of the human population and increase in land used for cultivation. It has been estimated that approximately 3 billion t of cereal straw or other vegetable fibre materials are produced worldwide each year (Mosier *et al.*, 2005). Approximately 1.5 t of straw are produced for every ton of grain harvested and as the world produces over 1 billion t of cereal a year this means that approximately 1.5 billion t of straw are available each year (Howard, 2003).

Wood is composed of cellulose, hemicellulose and lignin mainly, and due to the difficulty in the degradation of lignin and some of its subunits, the exact chemical composition and structure are still uncertain. Lignin generally contains three aromatic alcohols (coniferyl, sinapyl and ρ-coumaryl). Furthermore, dicots present large amounts of phenolic acids such as ferulic acid and ρ-coumaric acid, which are esterified with alcohol groups to each other and with other alcohols such as sinapyl and ρ-coumaryl. Lignin is further attached to the hemicellulose and cellulose in various amounts depending on the source, forming a physical seal around the last two components as an impenetrable barrier to solutions and enzymes (Howard *et al.*, 2003). Hemicellulose is a polymer of pentoses (xylose and arabinose), hexoses (mainly mannose) and sugar acids, whereas cellulose is a polymer of glucose. Biomass has been considered as the mass of organic material from any biological material and, by extension, any large body of biological material. There are a wide variety of biomass resources available on our planet for conversion to organic products. These can include the whole plant, plant parts (seeds, stems), plant components (starch, lipid, protein and fibre), the processing of by-products (distillers grains, soluble corn), animal by-products, and municipal and industrial wastes (Smith *et al.*, 1987). These resources can be used to create new biomaterials, however, a thorough knowledge of the composition of the raw material is required (i.e. whether it is the whole plant or components) so that the desired functional elements can be obtained to produce bioproducts.

Some of these wastes have been used for biotransformation into ethanol. However, this requires the appropriate infrastructure for carrying out the conversion. For example in the first stage of this process the waste needs a pretreatment which can be biological or chemical, and then, microorganisms with ligninolytic enzymes are used, which is a process with a low energy demand and low production of waste that may have few effects on the environment, contrary to chemical treatment. White-rot fungi have been utilized, such as fungi in the genus *Pleurotus*, due to their ligninolytic enzyme complex capable of degrading a variety of lignocellulosic wastes (Dashtban *et al.*, 2009).

25.2.1 *Pleurotus* production using lignocellulosic biomass as a substrate

Another important alternative use of lignocellulosic waste that has economic benefits has been obtaining fruiting bodies of the edible mushroom *Pleurotus*, which grows on straw-based substrates or hardwood and has been shown to improve the nutritional value of these residues for ruminants (Alemawor *et al.*, 2009). A product of high nutritional and medicinal value to humans is obtained, generating a more digestible and protein-rich residue for animal feed or as a bio-fertilizer in agriculture. The basic structure of the plant material consists of three polymers: cellulose, hemicellulose and lignin, and the relative proportion

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of these depends on the origin of the plants, so that the concentration ranges are 30–50% cellulose, 19–45% hemicellulose and 5–35% lignin (Fang *et al.*, 2010). These products have been used for feeding ruminants, however, their use is limited due to high levels of lignin and silica which makes them difficult to digest for cattle. Many of these wastes also contain anti-nutritional factors such as tannins and saponins, which interfere with normal digestion, absorption and metabolism of food (Wardlaw and Insel, 1995). Lignin is the main obstacle for use of agro-industrial wastes. During the hydrolysis of hemicellulose and cellulose, lignin generates compounds that inhibit the fermentation process (Wyman *et al.*, 2005). So fungi from the genus *Pleurotus* are used for their ability to preferentially remove lignin in the wood, leaving the hemicellulose and cellulose intact; the presence of the complex ligninolytic enzymatic system has made this type of fungi of particular interest in biotechnology, due to their possible use in delignification processes (Cardona and Sánchez, 2007), giving added value to agricultural residues.

Grape pomace (agro-industrial waste) can be used as animal feed especially in the dry season, however, its use is limited (about 30%) due to its low nutritional content and the content of phenolic compounds which inhibit symbiotic organisms in ruminants (Mole *et al.*, 1993). Sánchez *et al.* (2002) used the agro-industrial waste generated in winemaking as a substrate to grow *Pleurotus*, which produced fruiting bodies 40 days after inoculation. Furthermore, the winemaking waste fermented by the fungus has been used as food for ruminants, noting that the nutritional value of agro-industrial waste increases and some anti-nutritional factors such as phenolic compounds decreased by between approximately 21.3% and 57%. Bisaria *et al.* (1997) examined the bioconversion of straws of rice and wheat by *Pleurotus sajor-caju* to increase the nutritional value when used as ruminants' feed. After 20 days following inoculation, rice straw showed a 17.9–35.8% decrease in cellulose, a 9.5–17.2% decrease in lignin and an increase in *in vitro* digestibility of dry matter (IVDDM) of 19.7–29.8%. In wheat straw a similar trend was observed and IVDDM increased from 27.2% to 36.8%. By supplementing rice straw with a nitrogen source, the degradation of hemicellulose and cellulose was increased, but this decreased the degradation of lignin.

Coconut shell has a high lignin content (48%) and is a particularly hard material that is highly resistant to biological degradation. Shashirekha and Rajarathnam (2007) used coconut shell to grow *Pleurotus florida* and reported that lignin was actively decomposed (8.2% decrease) during mycelial growth and at 12 days the fungus started fruiting where the lignin content was 1.8%, and the fungus retained the organoleptic characteristics reported on other substrates.

Coffee is one of the most important beverages in the world with an annual production of approximately 1 million t in over 50 countries. The processing of the coffee cherry involves removal of the husk and mucilage, which cause serious problems because they contain toxic compounds such as caffeine, phenols and polyphenols (tannins). To prepare the drink 9.5% of the fresh fruit weight is used and 90.5% remains as residue. The content of caffeine and phenolic compounds in coffee pulp is 0.75% and 3.7% and in the husk is 1.2% and 9.3%, respectively (Brand *et al.*, 2000). Traditionally, coffee pulp has been limited to application as a fertilizer, a livestock feed or as a compost (Pandey *et al.*, 2000). Fan *et al.* (2003) used husk of coffee as a substrate for growing strains of *Pleurotus* and reported that the concentration of caffeine in the coffee shell was reduced by 60.6% after the fungi bore fruiting bodies. This was not degraded, rather the caffeine was accumulated in the fruiting bodies, however, phenolic compounds were reduced significantly (79.1%) and these were not found in the fruiting bodies of the fungus, meaning that *Pleurotus* is able to degrade these toxic compounds.

Rodríguez and Zuluaga (1994) grew *Pleurotus pulmonarius* on fermented coffee pulp, and obtained a biological efficiency of 54.4% with a yield of 3.6 kg of fresh fungus/m^2. Waste from the production of fungi was used for the cultivation of the earthworm *Eisenia foetida*. This study estimated that for every ton of fresh coffee pulp (on a wet basis) an average of 82.10 kg of mushrooms were produced, 9.76 kg of earthworms and 135.3 kg wet vermicompost, per culture cycle (about 3 months). Cocoa husk is more than 70% of the ripe fruit, and it has been used as conventional low-cost food for cattle, however, due to its composition (14% lignin, 11% hemicellulose, cellulose 35% and low protein) the use is limited. Alemawor *et al.* (2009) used this waste to grow *Pleurotus ostreatus*, and reported that it

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increased in protein and carbohydrate content by 36%, and decreased in crude fibre and lignin by 17% and in tannins by 88%, thus increasing its nutritional value for ruminants. Martínez-Carrera *et al.* (2000), Croan (2003) and Mandeel *et al.* (2005) reported that *P. pulmonarius* and *P. ostreatus* grew on coffee pulp, waste paper, cardboard and softwood scrap and obtained a biological efficiency of 100%. Philippoussis *et al.* (2001) reported biological efficiencies of 75% and 100% on cotton waste and wheat straw, respectively. Shah *et al.* (2004) obtained efficiencies of 50–75% when they grew *P. pulmonarius* on cottonseed hulls, corn cob and sugarcane bagasse. Das and Mukherjee (2007) reported low yields in the production of *P. ostreatus* using weed as a substrate, but yields were increased by mixing it with rice straw. Cultivation of *P. ostreatus* has been shown to be a highly efficient way to transform wastes considered as contaminants in food of high nutritional content.

World production of mushrooms (edible and medicinal) in 1994 was 4,909,300 t (Chang, 1999), including the genera *Agaricus*, *Lentinula*, *Pleurotus* and *Auricularia*. The high protein value and medicinal properties of cultivated genera have made sales of these fungi increase in the international market. The production of fungi, especially of *P. ostreatus* has become an important activity of various families in rural communities because their culture is simple. Mushroom cultivation represents an alternative to earn money, improving the economy of families, and it is sustainable economically enabling them to achieve better living conditions. *Pleurotus* spp. are popular as cultivated edible mushrooms and some of them are commercially produced; these fungi can grow in a variety of lignocellulosic substrates (Sánchez and Royse, 2001; Chang and Miles, 2004). Currently, this genus comprises one-third of cultivated mushrooms in the world, after *Agaricus* and *Lentinula* (Chang and Miles, 2004). *Pleurotus* production in Mexico during 2002 was estimated at 4380 t (Lahman and Rinker, 2004), with a gradual increase to over 5000 t for 2005 (Gaitán-Hernández *et al.*, 2007).

25.3 *Pleurotus* Species

Singer (1986) described 38 species in the genus *Pleurotus* and assigned some new species based on morphological characters. However, techniques based on molecular studies of PCR-restriction fragment length polymorphism (RFLP) from the IGS2 region in combination with the study of ribosomal DNA sequences of the small subunit (SSU) rDNA revealed the existence of 12 complexes of biological species of the genus *Pleurotus* (Urbanelli *et al.*, 2002). The genus *Pleurotus* has several species with high economic importance, including *P. pulmonarius* (Fr.) Quél. (= *P. florida* Eger s. auct.; = *P. ostreatus* var. *florida* Eger) and *Pleurotus florida* (Buchanan, 1993; Guzmán *et al.*, 1993) which are discussed in more detail below.

Wild mushrooms of the genus *Pleurotus* are distributed worldwide (Zervakis and Balis, 1996). Of the more than 20 species recognized and varieties cultivated around the world (Buchanan, 1993), *P. pulmonarius* is commonly cultivated in temperate and subtropical regions (Petersen and Hughes, 1993), growing in Europe, Asia and North America (Buchanan, 1993; Guzmán *et al.*, 1993). *Pleurotus cystidiosus* is distributed in tropical and subtropical regions, while *Pleurotus eryngii* is found in Europe and most of Asia (except Korea and Japan), where the fungus is grown commercially. *P. ostreatus* is distributed in temperate areas and the species is very adaptable to different climates and substrates.

25.3.1 *Pleurotus ostreatus* (Jacq.:Fr) Kummer

P. ostreatus is a white-rot fungus that is widespread in temperate zones and its fruiting bodies are developed at relatively cool temperatures compared with other species of *Pleurotus*. It has been reported that this species requires a low temperature treatment (cold shock) to begin the formation of primordia. Currently, commercial strains are developed primarily by crossing and selection, as well as protoplast fusion and mutagenesis (Curvetto *et al.*, 2005).

25.3.2 *Pleurotus florida* Eger

P. florida is widespread in temperate, subtropical and tropical areas. This fungus was considered a subspecies of *P. ostreatus* because it is similar in

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appearance. There are two groups in *P. florida* at the level of subspecies, one group is sexually compatible with *P. ostreatus* and the other group with *P. pulmonarius*. At low temperatures, the colour of the pileus (cap) is slightly brown, but becomes pale with increasing temperature. Cold shock for fruiting induction is not required (Curvetto *et al.*, 2005).

25.3.3 *Pleurotus eryngii* (DC.:Fr) Quel

Wild mushrooms of this species are normally collected in southern Europe, North Africa and Central Asia. It has many subspecies and similar taxa such as *Pleurotus fuscus* var. *ferulae* from China. *P. eryngii* is more prone to disease and more sensitive to the growing conditions, and grows more slowly than *P. ostreatus*. This fungus requires cold shock for primordia formation; fruiting bodies develop at 13–18°C (Curvetto *et al.*, 2005).

25.3.4 *Pleurotus sajor-caju* Fr.:Fr.

This species is compatible with *Pleurotus sapidus*, but is different in appearance. This fungus is cultivated in subtropical and tropical areas because its optimal temperature range for the development of fruiting bodies is relatively high.

25.3.5 *Pleurotus cystidiosus* O.K. Mill (*P. abalonus* Han, Chen & Cheng)

This species is extensively distributed in subtropical and tropical regions. The unique feature that distinguishes this fungus from other *Pleurotus* species is the presence of conidia in the mycelium. Conidia are asexual spores that appear on the mycelium under light (Curvetto *et al.*, 2005).

25.3.6 *Pleurotus cornucopiae* (Paulet) Rolland

This species is distributed in Asia and throughout Europe, appearing on the stumps of broadleaved trees from summer to autumn. The cap is beige and is 4–12 cm wide and the pedicel is white (Curvetto *et al.*, 2005).

25.3.7 *Pleurotus djamor* (Rumph. ex Fr.)

This species has been described several times and has many synonyms. It has great phenotypic plasticity, especially in pileus colour, ranging from dark pink to light pink and even white (Vilgalys and Sun, 1994) and the species is widely distributed throughout the world. Corner (1981) recognized six varieties based on differences in colour of the pileus and lamellae, the shape of stem and the substrate. However, in the literature the nomenclature of these varieties is very confusing.

25.3.8 *Pleurotus pulmonarius* (Fr.) Quél.

P. pulmonarius was separated from *P. ostreatus* by intercompatibility studies (Hilber, 1982; Petersen and Hughes, 1993). *P. pulmonarius* is characterized by a pileus colour ranging from light brown to yellowish brown or violet tones, becoming yellow at maturity or when dry (Hilber, 1982; Buchanan, 1993; Petersen and Hughes, 1993; Hrouda, 2001).

25.4 Improvements to *Pleurotus* Strains

The industrial use of fungi requires the initial selection of the strain, and subsequent evaluation for production of biomass and secondary metabolites. There have been a series of programmes to increase mushroom production for the farming industry developed by various research groups in which tests have been developed to identify and use improved strains of the genus *Pleurotus* (Verma *et al.*, 2000; Kothe, 2001). Emphasis is placed on identifying and using genetic material to obtain high performance in mushroom production as well as manipulating environmental parameters and crop management practices. Among the techniques used for *Pleurotus* species is hybridization, an efficient method to obtain a combination of desirable characteristics. This process has been widely used in many

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cultivated mushrooms (Kumar, 1997). Genetic recombination can result in strains with special characteristics that can be used in the edible mushroom industry (Chang and Miles, 2004).

The industrial production of edible basidiomycetes increases each year as a response to the growing public demand for them, due to their nutritional properties. However, production is threatened by biotic and abiotic factors that make it necessary to improve the fungal strains currently used in industry. Improving edible fungi has been mainly through classical genetics because genetic transforming tools have not been widely developed. The main factors that must be considered in developing improved genetic systems are: (i) the system of genetic control of reproduction of fungi; (ii) the genomic structure and organization of the fungi; and (iii) identification of the genes involved in the control of quantitative traits.

25.5 Nutritional Importance of the Genus *Pleurotus*

Human food is varied and, depending on the region being considered, is composed mainly of animal protein, carbohydrates obtained from tubers and cereals, lipids, vitamins and minerals. Fungi have been considered as an important alternative food because of the nutrient content for humans (fungi contain nine essential amino acids that are required). Edible and medicinal mushrooms of the genera *Pleurotus* and *Lentinula*, and some *Agaricus* species, have nutritional and nutraceutical properties and are distributed in supermarkets and other retail centres.

As already mentioned, fungi in the genus *Pleurotus*, from order Agaricales, are a group that are well distributed in nature, growing on live or dead plant parts and several species are of economic importance as they are cultivated for food. The cultivation of *Pleurotus* spp. began in the 20th century and has developed rapidly, growing on a wide variety of substrates, including a variety of agricultural wastes, and as such this fungus is considered to be of great ecological importance.

Fungal biomass is easily digestible and the chitin-containing cell wall provides an important source of dietary fibre and the fruiting body of *Pleurotus* is an excellent source of protein. The crude protein content is high, but is affected by factors such as the species and stage of development (Longvah and Deosthale, 1998). The amino acid content is about 34–47 g/100 g protein and *P. ostreatus* contains all the essential amino acids (alanine, glutamic acid and glutamine mainly). The percentage of protein as a proportion of the dry weight can vary between 10% and 30% but can be up to 40%. The level of free amino acids in edible fungi is low (7.14–12.3 mg/g dry weight) but this contributes flavour properties (Sugahara *et al.*, 1975; Maga, 1981).

The carbohydrate content of the fungi varies with species ranging from 35% to 70% (Mau *et al.*, 2001a). Fungi contain high levels of oligosaccharides and only a low level of total soluble sugars (Bano and Rajarathnam, 1988). Within the carbohydrates are pentoses, hexoses, sucrose, alcohol-sugars, sugar-acids, methyl-pentoses and amino-sugars such as chitin (Breene, 1990). The crude fibre content for the genus *Pleurotus* is reported to be between 14% and 57%, which is 47% of dietary fibre.

The level of fatty acids in fungi is generally low, at about 2–8%. The level of polyunsaturated fatty acids (constitutes over 75% of total fatty acids) is higher than the level of saturated fatty acids. Oleic and linoleic acids are the most representative polyunsaturated fatty acids (Ribeiroa *et al.*, 2009), while palmitic acid is the main saturated fatty acid. The lipid content in *Pleurotus* is about 3–5%, and because the fatty acids are mainly unsaturated and easily digestible, the fungus has been defined as a cholesterol-free food. Lipids are found mainly in the stem, and include monoglycerides, diglycerides and triglycerides, sterols, sterol-esters and phospholipids. In general, neutral lipids constitute 20–30% of the total, 10% glycolipids and 60–70% phospholipids. Linoleic acid is the most abundant (up to 80% of total fatty acids) and phosphatidylcholine and phosphatidylethanolamine are the main phospholipids (Breene, 1990). Rajarathnam and Bano (1991) reported that volatile compounds are derived by enzymatic action on oleic and linoleic acids and they assume that these are largely responsible for the flavour and aroma characteristics of the fungus.

The level of vitamin B2 present in mushrooms is generally higher than in plants, and in some varieties the level is equivalent to that

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found in cheese and eggs (Mattila *et al.*, 2001). Mushrooms contain riboflavin, niacin and high amounts of folate, at levels similar to those found in plants (300–1412 mg/100 g) (Clifford *et al.*, 1991). *Pleurotus* contains thiamine, riboflavin and niacin at levels of 4.8–7.8 mg/100 g, 4.7–4.9 mg/100 g and 55–109 mg/100 g, respectively. Cultivated mushrooms also contain small amounts of vitamins C, B1, B12 and traces of D2 (Mattila *et al.*, 2001). Ascorbic acid (vitamin C) is an important source of antioxidants and its content has been reported to be up to 36–58 mg/100 g. Fungi absorb all the minerals from the substrate where they are grown, and usually contain good amounts of phosphorus and potassium, and calcium in smaller quantities. Zinc, copper and magnesium and a small proportion of iron, manganese, aluminium and sodium are also found in *Pleurotus* spp. (Breene, 1990).

25.6 Medicinal and Therapeutic Properties of *Pleurotus* Species

The medicinal use of higher fungi has a long tradition in South-east Asia, while in the western hemisphere their pharmacological use has increased significantly over the last few decades. In the USA the commercial value of medicinal basidiomycetes and their derivatives was US$1.2 billion in 1991 and was estimated at US$6 billion by the year 1999 (Chang, 1996; Wasser *et al.*, 2000). Currently, between 80% and 85% of all medicinal fungal products are from fruiting bodies, which are commercially produced or collected in the wild (Lindequist *et al.*, 2005). Still, the number of species of basidiomycetes that have been investigated is relatively low, considering the knowledge of the great potential of fungi for the production of drugs, the experience of ethnomedicinal use of fungi and the ecological capacity of these organisms to produce bioactive secondary metabolites (Barros *et al.*, 2008). Many species of edible mushrooms are a source of a wide variety of bioactive compounds. The use of edible fungi with therapeutic properties has resulted in an increase in the overall interest shown by the scientific and clinical community, because edible fungi have been shown to be effective against many diseases and metabolic disorders such as cancer or degenerative diseases (Poucheret *et al.*, 2006). Among the metabolites produced by edible fungi are terpenoids, steroids, fatty acids, proteins, lectins, polysaccharides and proteoglycans. *Pleurotus* in particular produces a large number of biomolecules with important biological activities, including lectins, proteins, polysaccharides and glycoproteins that have antiproliferative and anti-cancer activity (Wang and Ng, 2000).

25.6.1 Antioxidants

Many studies have reported that edible mushrooms have antioxidant activity. Chang and Miles (2004) found antioxidant activity in fractions extracted by ethanol of 150 fungi. Methanol extracts had an inhibitory effect on lipid peroxidation and ferrous chelating capacity (Kasuga *et al.*, 1993). Similar studies in *Dictyophora indusiata*, *Grifola frondosa*, *Hericium erinaceus*, *Flammulina velutipes*, *Lentinula edodes*, *P. cystidiosus* and *P. ostreatus* showed that these fungi also possess antioxidant properties (Mau *et al.*, 2001b; Yang *et al.*, 2002). Antioxidant activity in methanolic and aqueous extracts from *Lentinula edodes* (hongo shiitake), *Pleurotus tuber-regium* and *Volvariella volvacea* was assessed by the method of α-carotene bleaching and haemolysis of erythrocytes (Lee *et al.*, 2007).

Dichloromethane and ethylacetate extracts from *Lentinula edodes*, *P. tuber-regium* and *V. volvacea* indicated that these fungi have strong antioxidant activity (Cheung, 2001). Jayakumar *et al.* (2009) reported that 10 mg/ml of the ethanol extract from *P. ostreatus* showed antioxidant activity when compared with a commercial antioxidant such as butyl hydroxyl toluene. Recent studies have reported that extracts of *Pleurotus* species showed antioxidant activity, which may be due to the high concentration of phenolic compounds. It has been observed that the polyphenolic compounds have inhibitory effects on carcinogenesis and mutagenesis in humans, when a diet rich in fruits and vegetables is ingested. A recent study reported that *Pleurotus* spp. extract shows the presence of saponins, alkaloids, flavonoids, steroids, anthraquinones and phlobatannins (Adebayo *et al.*, 2014). The amount of total phenols, flavonoids and ascorbic acid in the methanolic extract of *P. sajor-caju* was determined to establish the putative antioxidant components. Phenolics are important compounds

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with scavenging ability due to their hydroxyl groups and they participate directly in antioxidant activity. The total phenolic content of *P. sajor-caju* was higher (52.20 mg/g) than the reported values in other mushrooms such as *Ganoderma lucidum* (47.25 mg/g) and *Ganoderma tsugae* (51.28 mg/g) (Mau *et al*., 2001b). Consumption of oyster mushrooms (*P. ostreatus*) might be beneficial to protect the human body against oxidative damage due to the presence of radical scavenging activity (Wong *et al*., 2009).

25.6.2 Hypocholesterolaemic agents

Cardiovascular disease is associated with atherosclerosis, hypercholesterolaemia and oxidation of lipoprotein of low density, therefore the regulation of cholesterol is very important to prevent this disease. Statins are inhibitors of 3-hydroxy-3-methyl-glutaryl-CoA (HMG-CoA) reductase, in the cholesterol synthesis pathway (Shimada *et al*., 2003). Edible mushrooms have been used for the prevention of such disease because of their high fibre and low fat contents (Ishikawa *et al*., 1984). It has been reported that plovastina and mevinolin (lovastatin), both present in mushrooms, prevent cardiovascular disorders and hypercholesterolaemia (Gunde-Cimerman and Cimerman, 1995; Mizuno, 1999; Wasser and Weis, 1999). It has been reported that an aqueous extract from *P. pulmonarius* has hypoglycemic effect (Badole *et al*., 2006; Badole and Bodhankar, 2007), while *P. ostreatus* reduces the size of arteriosclerotic plaques in rabbits (Bobek *et al*., 1998). Wasser and Reshetnikov (2002) patented the methodology for the production of several molecules of *Pleurotus* sp. that reduce cholesterol. It was reported that the addition of 2% and 4% of the fruiting body of *P. ostreatus* to a hyperlipidemic diet can avoid the accumulation of cholesterol and triglycerides in the liver and serum of rats with hyperlipidaemia which was genetically induced (Bobek *et al*., 1991, 1993). A decrease in serum cholesterol was observed (up to 80%) when the rats were fed with the fruiting body of *P. ostreatus*, water and 30% ethanol extract of the mushroom. In another study, dietary fibre extracted from *P. cornucopiae* had an anti-atherosclerotic effect *in vitro* but also patients with coronary artery disease showed a decrease in atherogenic activity (20–40%) in their serum after consumption of this fungus, which confirms that cholesterol was reduced (Ryong *et al*., 1989). Lovastatin is produced by many species of the genus *Pleurotus*, mainly in the fruiting body (lamellae).

25.6.3 Antitumour agents

The main problem in the use of chemical agents in cancer treatment is the potential toxicity of these drugs to cells. One way to solve this problem is the use of inhibitors of tumours that are of natural origin. It has been reported that several natural extracts possess antitumour and anticancer activities. Natural extracts or purified compounds have been of interest in the study of some aspects related to cancer. Several recent studies have indicated that the polysaccharides from fruiting bodies and the mycelia of *P. ostreatus*, *P. sajor-caju*, *P. florida* and *Pleurotus citrinopileatus* can inhibit the growth of several types of cancer (Wang *et al*., 2005). In a recent study it was reported that aqueous extracts of two Brazilian fungi (*Lentinula edodes* and *P. sajor-caju*) exert inhibitory activity against the proliferation of the human tumour cell lines laryngeal carcinoma and cervical adenocarcinoma (Finimundy *et al*., 2013). Knowledge of the antitumour effect of plant lectins has increased interest in this field.

The *in vivo* contribution of lectins, as well as that of other proteins from fungi with antitumour effect, has not yet been completely elucidated. However, fruit bodies of *P. ostreatus* reduced the carcinogenic effects and decreased the toxicity of cyclophosphamide in rats, and *Ganoderma* showed antitumour activity (Wang *et al*., 2002; Wasser *et al*., 2002). In another study, it was found that *G. lucidum*, *Phellinus rimosus*, *P. florida* and *P. pulmonarius* showed antioxidant and antitumoural activities (Thekkuttuparambil and Kainoor, 2007). Jayakumar *et al*. (2006) used *P. ostreatus* extracts on CCl4-induced liver damage in male Wistar rats. They reported that when rats with CCl4-induced hepatotoxicity were treated with the extract of *P. ostreatus*, serum levels of glutamic oxaloacetic transaminase, glutamic pyruvate transaminase and alkaline phosphatase levels reverted to near normal. In another study, an extracellular polysaccharide composed of mannose:3-*O*-methyl-galactose:galactose:glucose

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(44.9:16.3:19.8:19) was obtained from *P. sajor-caju*. This polymer showed total antioxidant capacity, reducing power and ferric chelating, as well as superoxide radical scavenging, but it did not possess anticoagulant or antiproliferative activities (Telles *et al.*, 2011).

25.6.4 Importance of glucans

Fungal glucans are carbohydrates localized, together with chitin and proteins, in the cell wall. They are important due to their potent antitumour action; several linear glucans (1,3-β-glucan) and some branched glucans are widely known for their powerful inhibition of mutations, which allow their potential use in treatment of leukocyte diseases (Arango and Nieto, 2013). It has been reported that the β-glucans content in *P. ostreatus* and *Lentinula edodes* was 2.2–5.3 mg/g dry matter and 2 mg/g dry matter, respectively (Finimundy *et al.*, 2013). Manzi *et al.* (2001) reported on the β-glucan concentration in three different species of fungi: in *Agaricus bisporus* it was 1.2–1.7 mg/g dry matter, in *P. ostreatus* 139.2 mg/g dry matter and in *Boletus* 548.8 mg/g dry matter. The structural variability of these metabolites allows flexibility in the regulatory mechanisms of different cell–cell interactions in higher organisms (Daba and Ezeronye, 2003). Fucomannogalactans (Alquini *et al.*, 2004), xylomannans (Smiderle *et al.*, 2008) and mannogalactans (Rosado *et al.*, 2003) are some of the polysaccharides reported, however, the main ones are glucans, some having a main chain linked by β-(1–3) glycosidic bonds, β-(1–4) or mixed β-(1–3), β-(1–4) with β-(1–6) glycosidic bonds. The heteroglucans have glucuronic acid, galactose, mannose, arabinose or xylose bound to the main chain (Daba and Ezeronye, 2003).

There are several reports of fungal β-glucans as effective ingredients due to their beneficial influence on health, for example β-glucans appear to be immunomodulatory agents that have a positive effect on cancer and various infectious bacteria (Vetvicka and Yvin, 2004). Fungal β-glucans can directly activate leukocytes, stimulating their phagocytes and antimicrobial activity. Smiderle *et al.* (2008) reported that the β-glucans have anti-inflammatory and glucocorticoid activity similar to non-steroidal drugs. Therefore, it was suggested that the glucans downregulate pro-inflammatory cytokines such as interleukin-1β and tumour necrosis factor β. The mechanism of action includes induction of apoptosis, inhibition of proliferation, induction of cell cycle arrest, inhibition of invasive behaviour and removal of certain tumours including those of prostate cancer (Mahajna *et al.*, 2009).

Fungal polysaccharides exert their antitumour action mainly through the activation of the immune response of the host organism (immunoenhancement activity), therefore they do not directly kill tumour cells but instead help the host to adjust to different biological conditions and exert a non-specific action in the host, supporting some or all of the major systems. These polysaccharides cause no harm to the body and are considered as biological response modifiers (Zhang *et al.*, 2007). Gu and Sivam (2006) reported that a water-soluble extract of *P. ostreatus* showed antitumour activity against androgen-independent cancer (prostate cancer) PC-3 cells. Exposing the PC-3 cells to 150 μg/ml of the extract of *Pleurotus* rapidly induced apoptosis and reduced tumour colony formation to 4.5% (60 μg/ml) and 0.5% (120 μg/ml) compared with growth without the fungal extract (defined as 100%). Jedinak and Sliva (2008) reported that extracts of *P. ostreatus* significantly inhibited cell proliferation of the breast cancer cell line MCF-7 and colon cancer cells HT-29 by G0/G1 cell cycle arrest. Cell cycle arrest in MCF-7 cells was achieved by regulating the expression of the *p53* and *p21* genes, while cell cycle arrest in HT-29 cells was induced by the regulation of expression of the *p21* gene. This showed that the consumption of *P. ostreatus* inhibited growth of the cells of breast and colon cancer, without affecting normal cells.

Glucans are also considered as prebiotics because they are not digestible but have beneficial effects to the host by selectively stimulating the growth and activity of a limited number of bacteria in the colon (Aida *et al.*, 2009). Digestive enzymes secreted by the pancreas of mammals are unable to hydrolyse β-glycosidic bonds. This means that β-glucans remain indigestible by the human digestive enzymes (Van Loo, 2006). Synytsya *et al.* (2009) reported that extracts of *P. eryngii* and *P. ostreatus* stimulated the growth of probiotics, such as *Lactobacillus* spp. (four strains), *Bifidobacterium* spp. (three strains) and *Enterococcus faecium* (two strains). Pleuran is a β-glucan extracted from *Pleurotus* sp.; it is a polysaccharide with a backbone of β-D-glucopyranosyl molecules

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linked by glycosidic bonds β-(1-3) and every fourth residue is substituted with a single β-D-glucopyranosyl group at the carbon 6 by glycosidic bonds β-(1-6). The polysaccharide contains a small proportion (7%) of interior residues linked by β-(1–6) and β-(1–4). It has been demonstrated that pleuran is efficient in promoting the survival of susceptible mice to bacterial infections (Karácsonyi and Kuniak, 1994). Brizuela *et al.* (1998) reported that the antibacterial activity of fungi of the genus *Pleurotus* is due to acetylene-type compounds; while Iwalokun *et al.* (2007) reported that organic extracts of *P. ostreatus* had activity against Gram-positive and Gram-negative bacteria, indicating that terpenoids and phenolic compounds were responsible for the antibacterial effect. It has also been reported that a 3-*O*-mannangalactan from *P. pulmonarius* has an analgesic effect, and another β-glucan isolated from *P. pulmonarius* has inflammatory and analgesic properties (Smiderle *et al.*, 2008).

25.7 Production of Industrial Enzymes by *Pleurotus*

Enzymes have been used since the beginning of humanity in food manufacturing, for example enzymes from yeasts and bacteria in cheese making. Also enzymes isolated from microbes have many applications in the chemical industry, for example they are used in the production of pure amino acids, sugars (fructose) and penicillin derivatives. Large volumes of industrial enzymes generally are not purified, but are used as liquid concentrates or granular dry products. *Pleurotus* spp. grow on lignocellulosic materials, such as agricultural wastes (Stemets, 2000), and these organisms have an enzymatic complex including laccases, manganese peroxidise (MnP), versatile peroxidise (VP) and various hydrolases (Straatsma *et al.*, 2000).

25.7.1 Oxidase enzymes

Detoxification of organic compounds by various fungi is through oxidative coupling which is mediated by enzymes referred to as oxido-reductases (Gianfreda *et al.*, 1999). Microorganisms obtain energy by the biochemical reactions that these enzymes catalyse, by breaking chemical bonds and electron transfer from an organic substrate (proton donor) to another chemical (proton acceptor); with this reaction (oxidation–reduction) many organisms can degrade many pollutant compounds (phenolic compounds, dyes, chlorinated compounds, etc.) and convert them into less toxic or harmless compounds. Peroxidase enzymes catalyse the oxidation of phenolic compounds including lignin in the presence of hydrogen peroxide (H_2O_2) and a mediator. Such peroxidases may be haem and non-haem proteins. The main role of these enzymes in fungi appears to be degradation. They have been widely studied because of their wide potential to degrade toxic substances in nature.

Manganese peroxidase (MnP)

MnP is an extracellular haem peroxidase. MnP converts Mn^{2+} to Mn^{3+}, which is stabilized by an organic acid (i.e. chelators; malonate, oxalate, glyoxylate, etc.) which act as low molecular weight mediators. Mn^{2+} induces the production of MnP and acts as its substrate. The Mn^{3+}, produced by MnP, acts as a mediator for oxidation of various phenolic compounds. The release of Mn^{3+} from the active site of the enzyme is also facilitated by organic acids. Many groups of fungi are able to produce MnP in multiple forms; the most common genera are *Pleurotus* (Asada *et al.*, 1995), *Trametes* (Johansson and Nyman, 1993), *Phlebia* (Karhunen *et al.*, 1990) and *Bjerkandera* (de Jong *et al.*, 1992). The traditional cycle of MnP begins by oxidation of the enzyme in its native form by a molecule of H_2O_2, then the oxidized enzyme form (compound I) is generated with an oxidation state of two top levels more than the native form and a water molecule. Compound I is subsequently reduced by oxidation of Mn^{2+} generating Mn^{3+} and compound II, in a state of higher oxidation than the native enzyme. Finally a further reduction of the enzyme is produced to recover the ferric form. This new reduction involves the oxidation of another Mn^{2+} to Mn^{3+}. Compound II, in excess of H_2O_2, results in an inactive form (compound III). MnP is a manganese-dependent enzyme (Wariishi *et al.*, 1988; Kuan *et al.*, 1993). The source of H_2O_2 for initiation of the catalytic cycle of the enzyme can come from two sources: (i) the direct reduction of O_2 by other enzymes of the fungus itself, such as alcohol aryl oxidases for *Bjerkandera*

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adusta or *P. eryngii* (or glyoxal oxidase in the case of *Phanerochaete chrysosporium*) (Gómez-Toribio *et al.*, 2001); and (ii) through the generation of superoxide radicals (O_2^-) coming from the auto-oxidation reaction product (Guillén *et al.*, 1997).

Versatile peroxidase (VP)

VP is a peroxidase enzyme such as MnP, lignin peroxidase (LiP) and other peroxidases, having the ability to directly oxidize Mn^{2+}, methoxybenzenes and aromatic compounds. VP has a wide range of substrates and carries out the catalysis in the absence of manganese. It has been reported that the VP oxidized phenolic and non-phenolic compounds in the structure of lignin (Ruiz-Dueñas *et al.*, 2007). VP combines the catalytic properties of MnP and LiP, so MnP and VP can oxidize phenols. It is known that veratryl alcohol (VA) increases the ability of LiP to oxidize substrates with high redox potential and phenols (but its role as mediator or enzyme stabilizer is a controversial topic), whereas VP can oxidize compounds that LiP cannot oxidize in the absence of VA, such as various dyes (Heinfling *et al.*, 1998). VP has been reported in species of *Pleurotus* and *Bjerkandera*, so it is a very important enzyme for biotechnology applications.

Laccases

Laccases are major enzymes in fungi ligninolytic systems, involved in lignin degradation (Gianfreda *et al.*, 1999). Laccases belong to a group of enzymes called blue copper oxidases capable of oxidizing phenols and aromatic amines by reducing molecular oxygen to water (Solomon *et al.*, 1996). Fungal laccases are primarily extracellular and inducible and are present in multiple isoforms in almost all fungal species, including *P. ostreatus* (Giardina *et al.*, 2010). Laccases catalyse the removal of a hydrogen atom of the hydroxyl group in *ortho* and *para* positions, in monophenolics, polyphenols and aromatic amines, by removal of an electron to form free radicals capable of depolymerizing, re-polymerizing, methylating or synthesis of quinones (Abadulla *et al.*, 2000). The initial product is typically unstable and can undergo a second enzyme-catalysed oxidation (conversion of phenol into quinones) or a non-enzymatic reaction (such as hydration or deprotonation) and/or may participate in a polymerization reaction, resulting in an insoluble amorphous product such as melanin. Laccases have potential use in multiple biotechnological and industrial processes. These enzymes are used for dye degradation, pretreatment of lignocellulose in bioethanol production, xenobiotic transformation and detoxification and pulp bleaching (Kunamneni *et al.*, 2008).

25.7.2 Hydrolase enzymes

Hydrolytic enzymes break down chemical bonds in toxic molecules leading to decreased toxicity. This process is effective in the biodegradation of organophosphate insecticides and oil spills. Organochlorine insecticides such as DDT (dichlorodiphenyltrichloroethane) and heptachlor are stable in well-aerated soil, but are rapidly degraded in the anaerobic environment (Williams, 1977). The hydrolases also catalyse reactions such as condensations and alcoholysis. The main advantages of hydrolases are that: (i) they are readily available; (ii) they lack a stereoselectivity cofactor; (iii) they can tolerate the addition of water-immiscible solvents; and (iv) they can be classified according to the type of bond hydrolysed. Hydrolytic enzymes such as amylases, proteases, lipases and xylanases, among others, have potential applications in different areas such as the food industry, as feed additives, in the biomedical sciences and chemistry (Sánchez-Porro *et al.*, 2003). Hemicellulase, cellulase and glucosidase are very important due to their application in biomass degradation (Schmidt, 2006).

Cellulases

Cellulases are enzymes with great potential in the conversion of cellulose residues in food to meet the needs of a growing population and are the subject of intense research (Bennet *et al.*, 2002). Extracellular pectinases, hemicellulases and cellulases have been reported to be constitutive enzymes and are expressed at very low levels by some fungi and bacteria (Adriano- Anaya *et al.*, 2005). There are studies about the presence of isoenzymes of cellulases. At least, three major groups of cellulases are reported: (i) endoglucanase (endo-1,4-D glucanohydrolase) that strikes the regions of low crystallinity in the cellulose fibre;

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(ii) exoglucanase, also called cellobiohydrolase (1,4-β-D-glucan cellobiohydrolase), that degrades the cellulose molecule by removing cellobiose units in the chain free ends; and (iii) β-glucosidase which releases glucose units from cellobiose. Cellulases are capable of degrading crystalline cellulose to glucose. These enzymes have been used in detergents since the early 1990s. Cellulases are used to remove cellulose microfibrils formed during the washing and use of cotton. In the paper and pulp industry, cellulases have been used for removing the ink for paper recycling. Also cellulases are added during preparation to enhance delivery of juice from fruit pulp and for ethanol production from cellulosic wastes (Leisola *et al.*, 2006).

Xylanases

The enzymatic degradation of xylan is effected by a complex set of enzymes generically called xylanases. These enzymes are produced by bacteria and fungi. The distribution of the side chains in xylans and their physical shape is of great importance for the enzymes that degrade these substrates. Hydrolysis of the xylan structure is performed by the action of endo-β (1,4)-xylanase that releases xylo-oligosaccharides of different lengths and decreases the degree of polymerization of the substrate (Anthony *et al.*, 2003; Monti *et al.*, 2003).

Xylanases have a growing number of applications, individually or in combination with other enzymes. There are many investigations into endoxylanases for the production of xylans and their use in biotechnological processes. Application of xylanases in the paper industry for the bleaching of paper is presented as an alternative to reduce the use of chlorinated compounds (Anthony *et al.*, 2003). Along with other hemicellulase enzymes, xylanases enable xylo-oligosaccharides to be obtained from agricultural waste (saccharification of lignocellulosic biomass) for use as food additives and sweeteners. Also xylanases are used together with cellulases for modifying bakery products as they have been found to improve the bread volume, crumb structure and reduce stickiness and they are also used to improve the diets of birds and ruminants, since enzymatic hydrolysis of arabinoxylans (in forage and grain) increases their digestibility (Topakas *et al.*, 2003). Additionally, xylanases have been tested for their applications in: (i) extracting juices, flavours, essences and pigments; (ii) clarification of juices and wines; and (iii) improvement of textile fibres (Monti *et al.*, 2003).

Proteases

Proteases belong to the group of enzymes that hydrolyse peptide bonds in aqueous medium. Proteases have applications in the production of leather, food and detergents and in the pharmaceutical industry (Beena and Geevarghese, 2010). Proteases are classified as exopeptidases and endopeptidases. The exopeptidases act only near to carboxylic or amino terminals in the chain. Endopeptidases have been grouped according to the position of the active site, such as cysteine peptidase, serine endopeptidase, aspartate endopeptidases and metallopeptidases. The proteases which act on a free carboxyl group or terminal amino group are called as carboxypeptidase and aminopeptidase, respectively. The endopeptidase acts on the inner regions of the peptide chain.

Proteases have been used in the detergent industry and in the manufacture of cheese for many years. Alkaline proteases are used in the leather industry for removing hair and animal parts that are present in the skin. Proteases have been used to produce aspartame, which is a calorie-free artificial sweetener. In the pharmaceutical industry, proteases are used in the development of effective therapeutic agents, for example subtilisin, a non-specific protease, is used in conjunction with broad-spectrum antibiotics in the treatment of burns and wounds (Rao *et al.*, 1998).

25.8 Bioremediation of Contaminated Soils and Water Using *Pleurotus*

Toxic chemicals or their by-products that contaminate the environment are classified into organic and inorganic compounds. Organic contaminants include aliphatic, alicyclic, polycyclic aromatic hydrocarbons (PAH), including halogenated and non-halogenated compounds, explosives and pesticides. Inorganic contaminants can be metals, such as: Ag, Al, As, Be, Cd, Cr, Cu, Hg, Fe, Ni, Pb, Sb, Se, Zn, radioactive elements

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and their derivatives (Cluis, 2004). The main problem of pollutants is their toxicity and the risk to humans, so it is important to prevent these toxic compounds contaminating soils, surface water and groundwater. There are several processes, physical, chemical or combinations thereof, which are feasible for the treatment and management of wastes. However, some of these physico-chemical methods are very expensive and cause secondary pollution problems. So currently, bioremediation, involving the enhanced degradation of toxic compounds, transforming them into harmless substances, specifically CO_2 and water, is of great importance. It can be carried out *in situ* with autochthonous microorganisms or by introducing strains of fungi or bacteria, or by *ex situ* bioremediation where, for example, excavated contaminated soil is treated above ground to enhance degradation by the indigenous microbes in order to achieve complete detoxification of hazardous compounds.

Dyes are widely used in the food, pharmaceutical, cosmetics, textile and leather industries. During industrial processing, over 40% of the dye used is released into water (Vaidya and Datye, 1982). Dyes in the wastewater affect the aesthetics and transparency of the water, and the solubility of the gas in the water. This is important, particularly in the textile industry because as a result of the volume of the discharges and the composition of the effluent this industry can be considered one of the most polluting of all industries, thus requiring an appropriate treatment technology (O'Neill *et al*., 1999). Synthetic dyes and pesticides have been introduced into the environment through agriculture, as textile dyes, and in the sanitation, refinery and electrical industries; these contaminants are abundant and toxic, being carcinogenic and teratogenic to both animals and humans. So far white-rot fungi have been found to be the most efficient microorganisms in the degradation of synthetic dyes. White-rot fungi have enzymes, mainly peroxidase, giving them an advantage over bacteria which require pre-conditioning to grow on any recalcitrant medium (Brar *et al*., 2006). Faraco *et al*. (2009) reported that in a model wastewater, *P. ostreatus* resulted a 40% bleaching in 1 day of treatment in the absence of nutrients (malt extract) and more than 60% and 66% bleaching was achieved after 7 days and 14 days, respectively, in the presence of nutrients. Spectrum analysis showed that the fungus efficiently degraded acid-type dyes. Pesticides are highly recalcitrant, and have been reported to be transformed by *P. pulmonarius* (Beaudette *et al*., 1998).

PAH are formed by the incomplete combustion of fossil fuels and can enter the soil through atmospheric deposition. Local pollution with PAH is due to industrial activity such as from the gas-production industry or wood-preservation industry. PAH degradation is limited because of its low water solubility (Sack and Günther, 1993). Biotransformation of PAH can form compounds that are carcinogens. The main microorganisms involved in the oxidation and subsequent mineralization of these compounds are soil bacteria and white-rot fungi; bacteria degrade molecules of low molecular weight and fungi can oxidize PAH molecules with more than six aromatic rings and low water solubility and thus reduce the toxicity (Wolter *et al*., 1997). Bhattacharya *et al*. (2013) reported that *P. ostreatus* PO-3 is capable of degrading 71.2% of benzo[a]pyrene in the presence of copper ions; moderate degradation was observed in the presence of zinc and manganese. Byss *et al*. (2008) reported that *P. ostreatus* grew at 15°C for 120 days in creosote-contaminated (50–200 mg/kg PAH) soil originating from a wood-preserving plant. *P. ostreatus* degraded PAH (four to six aromatic rings) better than the endemic microbial flora. Wolter *et al*. (1997) showed the ability of *P. florida* to mineralize benzo-pyrene (39% of 5–125 mg/kg). Rodríguez *et al*. (2004) analysed the degradation of two pollutants by *P. ostreatus*, *P. eryngii*, *P. pulmonarius* and *P. sajor-caju*. The four strains degraded the two pollutants with high rates of transformation: 100 mM 2,4-dichlorophenol was easily transformed by the four strains, disappearing within 24 h after its addition to the liquid medium, but the 100 mM benzo-pyrene required 2 weeks for *P. eryngii* and *P. pulmonarius* to reduce it by 75%. Bioaugmentation studies with white-rot fungi frequently have reported low levels of transformation of organic pollutants compared with laboratory results. D'Annibale *et al*. (2005) reported that *P. ostreatus* and *Phanerochaete chrysosporium* degraded PAH compounds such as naphthalene, dichloroaniline isomers and diphenylether, by up to 100%. However, although *P. ostreatus* showed high colonization of the soil, this resulted in the inhibition of the

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native soil bacteria. This could involve several mechanisms including production of antibiotics, lysing the cell wall of prokaryotes or the activity of β-(1,3)-glucanase activity against other fungi, and it has been suggested that *Pleurotus* species have a higher apparent hydrophobicity. These results showed that the fungus was able to grow and detoxify the soil despite unfavourable conditions such as competition with the indigenous microflora, non-optimal pH and high acidity and heavy pollution by aromatic hydrocarbons associated with high concentrations of heavy metals. This ability has been associated with fungal enzymes such as laccases, MnP and aryl alcohol oxidases.

Another major problem is radioactive waste. Despite efforts to store radioactive waste, there is the possibility that caesium may be present in the environment. This pollutant is stable but it can have a great impact on the physiological processes of organisms as the toxicity of this compound is in micromolar concentrations, and in plants may cause competition between the Cs^+ and K^+ through the cell membrane (Hampton *et al.*, 2004). Bystrzejewska-Piotrowska and Bazała (2008) reported that the use of *P. eryngii* can perform caesium micro-extraction from an organic medium, achieving removal of approximately 60% of the initial caesium. Favero *et al.* (1991) reported that *P. ostreatus* accumulated 20 mg/g cadmium in dry weight from liquid medium containing 150 ppm, with at least 20% of cadmium deposited intracellularly.

25.9 Conclusion

Edible mushrooms of the genus *Pleurotus* have enormous potential in many areas such as food, pharmaceuticals and therapeutics, bioremediation and also in the production of many metabolites such as enzymes. Successful use lies mainly in finding the conditions under which the largest amount of metabolites are produced, either by manipulation of the conditions for fungal growth or by genetic manipulation.

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26 Useful Microorganisms for Environmental Sustainability: Application of Heavy Metal Tolerant Consortia for Surface Water Decontamination in Natural and Artificial Wetlands

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Abstract

Accelerated population growth and the concomitant industrial development increase the flow of pollutants to the environment and reduce the availability of natural resources. Recent research has focused on the implementation of systems that act as the natural counterparts, particularly the use of microorganisms as tools to enhance the recovery of degraded ecosystems. This approach is sustainable since it is a low-cost option and substitutes the use of chemical agents, electrical energy and other inputs associated with traditional clean-up strategies devised in most industrialized countries. This contribution is part of a larger project that aims to solve the problem of municipal wastewaters polluted with dissolved metals in toxic concentrations (an issue of concern in most countries with emerging economies) by applying microorganisms that are adapted to this type of environment. The microorganisms were isolated, characterized and identified prior to being inoculated into laboratory-scale systems simulating artificial or constructed wetlands. The metal-tolerant strains forming a consortium were able to remove, from synthetic wastewater with an initial concentration of organic matter equivalent to 500 mg COD (chemical oxygen demand)/l, three heavy metals, namely mercury, lead and chromium, in an overall removal efficiency of 50% from original concentrations of 0.106 mg/l Hg (II), 16.5 mg/l Cr (VI) and 26 mg/l Pb (II) using up to about 80% of the organic matter. These results indicate that it is possible to implement improved artificial wetland systems that in a very short time are able to diminish dissolved heavy metal concentrations from wastewater if inoculated with consortia of metal-tolerant strains. The strategy to be followed includes the isolation of promising microorganisms from natural sources, their characterization concerning the interaction between them and the pollutants of interest, the identification and selection of the best strains and the design of the consortia. Once these are ready, they can be inoculated into existing or new systems to bioaugment their efficiency to remove both the organic pollutants and the potentially toxic ones. A future challenge is to thoroughly study the cell mechanisms involved for microorganisms to be able to remove metals from the aquatic medium in order to make them more efficient.

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26.1 Introduction

The history of humankind since the industrial revolution has been characterized by population increase, thanks to rapid industrialization, with living patterns changing from rural to urban societies with a drastic negative effect on the environment. The implications are that by the year 2030 about 60% of the 9000 million human beings on the earth will be in urban areas with a net flux of pollutants from dwellings on both a local and a global scale (UN Population Division, 2008).

Among the effects of this industrialization is the presence and availability of heavy metals in the environment, from domestic and industrial wastes and from non-identified sources. Aquatic environments receive a net flux of them through wastewaters from residential areas, as well as from the manufacture of batteries and the paper, tanning and electroplating industries (Fu and Wang, 2011; Mansouri and Ebrahimpour, 2011). Some of them, for example cadmium (Cd), chromium (Cr), lead (Pb) and mercury (Hg), are crucial for the environment due to their effects on ecosystems (Brown *et al.*, 2000; Dickson, 2006). Mercury and lead may create autoimmunity problems in human beings resulting in damage to the circulatory and nervous systems, and even brain damage (Amuda *et al.*, 2007).

It is clear then that citizens must take the lead to start changing the present living conditions of the majority of the population (two-thirds of it), since typhoons, hurricanes and even floods, tornadoes and droughts occur as a result of climate changes, promoting more evaporation of ocean water as well as changes in temperature gradients in the atmosphere (Durán-de-Bazúa, 2013). Researchers have the ingenuity to apply scientific knowledge to look for sustainable solutions to the environmental problems that mankind is facing that can be directly applied by communities.

For many centuries natural systems have been put to work by human beings using microbes' ability to clean the environment. Microorganisms act in treatment systems, such as septic tanks and activated sludge, and have been used to clean up a variety of wastewaters. Taking these statements into consideration and recent approaches in relation to the sustainability of the environment, cleaner technologies have to be devised to maintain surface water that is safe to use (Akpor and Muchie, 2010).

Bioremediation is one of these strategies that use biological systems, microorganisms and other living organisms to remove pollutants from the environment (De Olivera Franco *et al.*, 2004; Okoh and Trejo-Hernandez, 2006; Labra-Cardón *et al.*, 2012). Phytoremediation and biosorption using plants and microorganism consortia that proliferate on their roots are an ingenious natural system that if put to work in artificial wetlands may become a sustainable technique to clean up surface water. According to the type of plants and microbes, microorganism consortia used for bioremediation may possess operational flexibility, become economically viable and be easy to operate and maintain in rural, suburban or even urban conditions, and are environmentally friendly.

Microbiological consortia possess a rich diversity both in physiological and in metabolic activities that allow them to assimilate, transform and immobilize pollutants and/or residues, even those considered to be toxic. There are several studies that present results on the ability of microbial consortia in the mitigation of hydrocarbon spills or pesticide degradation that in spite of its persistence have been used as substrates by these microorganisms (López *et al.*, 2005; Maila and Cloete, 2005; Salgado-Bernal *et al.*, 2012b) transforming its carbon compounds into CO_2 and water. If plants are present, they can take up CO_2 and convert it into glucose using solar energy (Bernal-González, 2013; Chapter 32, this volume). Others are related to the clean up of sites and water polluted with heavy metals and metalloids (Rathnayake *et al.*, 2010; Xie *et al.*, 2010); even those that in very small amounts or traces are necessary for vital functions of organisms, such as cobalt (Co^{2+}), copper (Cu^{2+}), iron (Fe^{2+}, Fe^{3+}), zinc (Zn^{2+}) and chromium (Cr^{6+}), in higher concentrations are deletereous or even lethal for organisms. Among those that seem to have no essential functions in living organisms are cadmium (Cd^{2+}), mercury (Hg^{2+}), lead (Pb^{2+}) and arsenic (As^{3+}) that are definitely toxic even at very low concentrations. Even in these cases, some microorganisms seem to contribute to their removal from aquatic and terrestrial ecosystems in spite of the fact that, contrary to what happens with organic compounds, these metals and metalloids are not biodegradable but accumulate in the microbial biomass.

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Bacteria and archaea have several mechanisms of interaction with metals and metalloids. Some even have certain tolerance to their presence and are able not only to survive and develop but can accumulate them through the induction of mechanisms codified in plasmids or chromosomes (Vullo *et al.*, 2005). Other mechanisms include the formation and sequestering of metals and metalloids in structural group complexes, the reduction of a metal to a less toxic species, and its flow outside the cell (Tietzel and Parsek, 2003), as well as bioadsorption and bioaccumulation. For bioadsorption immobilization takes place by concentrating the metals using cell-surface compounds such as structural and extracelullar polymers with a high content of polyanions such as carboxyls, amines, amides, hydroxyls, phosphates and sulfides, that constitute functional groups that interact with metals and metalloids trapping them within the cell structure (Kogej and Pavko, 2001). For bioaccumulation, an active process takes place where the metal/metalloid–microorganism interaction renders an enrichment of metal/metalloid ions within the cell (Anhalya *et al.*, 2004; Hussein *et al.*, 2004). These ions form high affinity ligands present in the cytosol, such as metallothioneins, as well as amino and organic acids (malate, glutamate, citrate, etc.) (Avilés *et al.*, 2005). Energy required for this mechanism is generated through the H^+-ATPase system, indicating the dependence of their capture on the cell physiological activity and the specific tolerance of the microorganism to the toxicity of these elements within the cell (Gadd, 2004).

Several studies such as the ones by Carballo *et al.* (2009), Orellana (2009), Rathnayake *et al.* (2010) and Xie *et al.* (2010) mention that bacteria can interact with heavy metals in ecosystems. These interactions may be due not only to the presence of a single organism but to the metabolic, genetic, physiological and structural capabilities of several of them acting in consortia.

These systems may be more competent to act on a variety of compounds and elements rendering selective advantages in a determined environment according to these capabilities. Presently, microbial monocultures are the most extensively studied biosorbents. Thus, future work should be focused on the isolation, characterization and identification of new microbial organisms with these capabilities, either working in a single manner or in consortia to evaluate potential usefulness to clean polluted sites, either terrestrial or aquatic, in order to obtain bioproducts that are safe, stable and efficient for bioremediation processes. Bioaugmentation or seeding of these single organisms or consortia in a particular contaminated site (Cerqueira *et al.*, 2012; Tyagi *et al.*, 2011) might become the ultimate target for this type of research, since not all of them possess a native population in large enough quantities to be able to clean up the surrounding area, either terrestrial or aquatic. The most common options are the addition of pure pre-adapted single organisms, the addition of pre-adapted consortia, the addition of genetically modified microorganisms, and the incorporation of groups of genes relevant to the contaminant of interest using a vector to be transferred to the indigenous organisms.

In this chapter, a joint project between researchers of a Cuban and a Mexican university is presented: the Universidad de La Habana and the Universidad Nacional Autónoma de México. The project focused on the isolation, characterization and identification of new microbial organisms with metal tolerance, working in a consortium, and on evaluating its potential usefulness to clean polluted wastewater containing heavy metals. The goal was to obtain consortia that are safe, stable and efficient for bioremediation processes.

26.2 Study Case

This study is focused on the removal of heavy metals in an artificial wetland model on a laboratory scale, using isolated strains of microorganisms pre-adapted to the presence of heavy metals and capable of removing them from the aqueous system. These artificial wetland systems include the presence of vegetation, a relatively inert support media for both plants and microorganisms (Cui *et al.*, 2010), and the metal-tolerant microorganisms. These systems have the advantage to be low-energy and low-maintenance installations, since they resemble natural wetlands. In these combined systems that have been extensively studied in the last few years, dissolved metals have been removed from the aqueous phase. In general, it has been assumed that most of the removal is carried out by

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the plants (Groudeva *et al.*, 2001; Munteanu and Munteanu, 2005; Higueras *et al.*, 2007; Kumar *et al.*, 2008; Sas-Nowosielska *et al.*, 2008; Wenzel, 2009; Romero *et al.*, 2010; Ruiz-López *et al.*, 2010; Panizza-de-León *et al.*, 2011; Willis *et al.*, 2011; Anjum *et al.*, 2012), and thus, one of the purposes of this study is to assess the capability of the metal-tolerant bacteria to remove them too (De Souza *et al.*, 1999; Cañizares-Villanueva, 2000; Cervantes *et al.*, 2006; Ma *et al.*, 2009a; Ma *et al.*, 2009b).

26.3 Methodology

26.3.1 Toxicity test

The concentrations of metals added to the systems were previously assessed using toxicological tests, both with germination of lettuce seeds (*Lactuca sativa*) and with the metal-tolerant bacterial consortia previously isolated. Table 26.1 presents the initial metal concentrations that were based on those reported for industrial aqueous effluents coming from installations that use and process these metals (Loredo *et al.*, 2003). Methodology is presented elsewhere (Amábilis-Sosa *et al.*, 2013; Amábilis-Sosa, 2015).

26.3.2 Reactors

Six reactors of 34 cm height, 20 cm in diameter and a height:diameter ratio of 1.7:1, with a total volume of 10.68 l were set up (the effective volume was 4 l). These physical proportions are recommended in the literature (Wood, 1995; Lüderitz, 2001; Winter and Goetz, 2003; Puigagut *et al.*, 2006; Yalcuk and Ugurlu, 2009; Zhao *et al.*, 2009). Four of the reactors simulated artificial wetlands, and two were blanks without hydrophytes. The plant species used was *Phragmites australis*, an indigenous aquatic species of the former Mexico City lakes area. Its rhizosphere was sterilized following the technique proposed by De Souza *et al.* (1999). Once the reactors were built (Fig. 26.1), three of them operated as controls without any addition of bacteria, and the other three were inoculated with metal-tolerant bacteria isolates described by Salgado-Bernal *et al.* (2012a), tolerant to mercury, lead and chromium as shown in Table 26.2.

Table 26.1. Concentrations of heavy metals in mixtures applied for the toxicity test.

	Combination of concentration of heavy metals		
Concentration level	Chromium (mg/l)	Lead (mg/l)	Mercury (mg/l)
0	0	0	0
1	0.525	0.825	0.003
2	3.5	5.5	0.02
3	10.5	16.5	0.06
4	21	33	0.12
5	42	66	0.24
6	70	110	0.4

Reactors were operated in a batch or discontinuous mode maintaining a hydraulic residence time of 4 days. Volcanic scoria, known as *tezontle*[1] in Mexico, was used as inert packing material support for the vegetation and for the establishment of the active microbial biofilm. It had an average diameter of 3.8–4.5 mm, passing through No. 5 ASTM mesh.

Its porosity was 38% (EPA, 2000), that has been demonstrated as suitable for the operation of laboratory- and pilot-scale artificial wetlands that avoids becoming compacted. Since the effect of the inoculation with specific organisms is one of the objectives, *tezontle* was washed and sterilized before filling the reactors with it (Amábilis-Sosa *et al.*, 2013; Amábilis-Sosa, 2015).

Once the highest possible metal concentration was assessed, metals were added to tap water to form the synthetic wastewater as follows: carbon (500 mg/l), nitrogen (34 mg/l), phosphorus (5 mg/l) and potassium (30 mg/l), in the form of sucrose ($C_{12}H_{22}O_{11}$), ammonium sulfate $(NH_4)_2SO_4$, sodium phosphate (Na_3PO_4) and potassium phosphate (KNO_3) (Orduña-Bustamante *et al.*, 2011). These compounds were all dissolved in the tap water (which included the trace metals normally contained in it).

To this solution the calculated amounts of heavy metal salts were added as follows: potassium dichromate ($K_2Cr_2O_7$) for chromium, lead nitrate ($Pb(NO_3)_2$) for lead, and mercuric chloride ($HgCl_2$) for mercury. After the toxicological experiments described above, the amounts were as follows: 0.11 mg Hg/l, 26 mg Pb/l and

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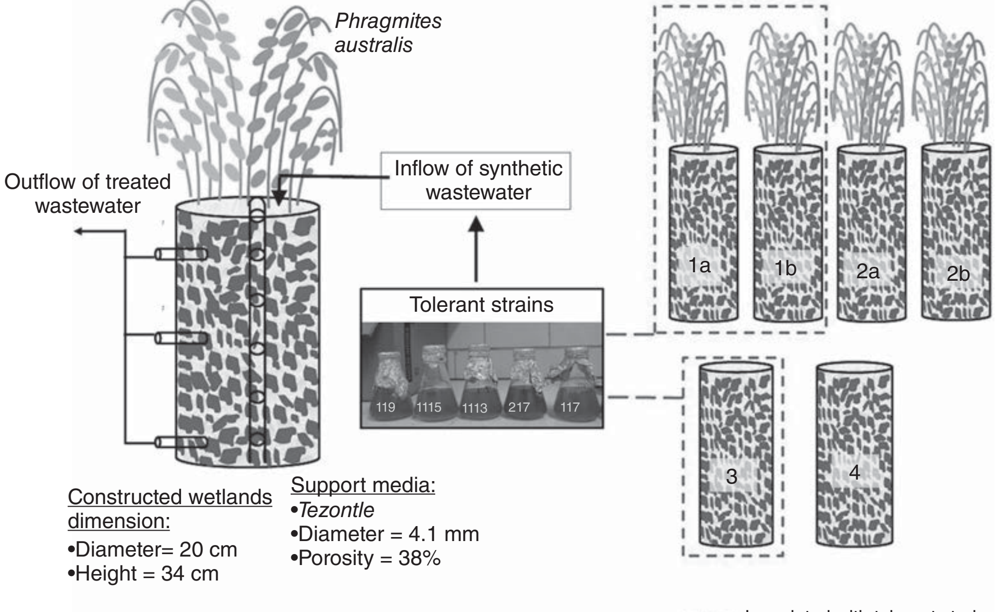

Fig. 26.1. Complete scheme of laboratory-scale artificial or constructed wetlands implemented for the study. Three reactors were inoculated with metal-tolerant strains of bacteria and three reactors were controls without bacteria (from the two groups of reactors there was one blank with no hydrophyte). Numbers in the Erlenmeyer flasks indicate the isolated strains that were mixed in to form the inocula using exactly the same amount of each one.

Table 26.2. Treatment using six reactors (1a, 1b, 2a, 2b, 3 and 4).

	With inoculum[a]	Without inoculum (controls)
With hydrophyte	Reactor 1 + replicate (i.e. 1a, 1b)	Reactor 2 + replicate (i.e. 2a, 2b)
Without hydrophyte	Reactor 3	Reactor 4

[a]Inoculated with metal-tolerant bacteria (tolerant to mercury, lead and chromium).

Table 26.3. Characteristics of the synthetic wastewater influent of the laboratory-scale constructed wetlands.

Compound	Concentration (mg/l)
Nitrogen	34
Carbon (expressed as chemical oxygen demand, COD)	500
Phosphorus	5
Potassium	30
Mercury	0.11
Lead	26
Chromium	16.5

16.5 mg Cr/l (Table 26.3) (Amábilis-Sosa *et al*., 2013; Amábilis-Sosa, 2015).

Samples of influent and effluent from each reactor were microfiltrated in order to measure the heavy metals content in the liquid. The filters with the trapped solids or biomass of the effluents were also digested in acid conditions to extract the metals present in them (EPA, 1996; Amábilis-Sosa *et al*., 2013; Amábilis-Sosa, 2015).

On the other side, the assimilation of organic matter by microorganisms was determined using Method 410.4 suggested by the Environmental Protection Agency (EPA) (1993) for chemical oxygen demand (COD). The rest of the parameters followed during the experiments are shown in Table 26.4, including references for the methodology.

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Table 26.4. Analytical methodologies used during this investigation.

Determination	Method
Values of pH, temperature and ORP (oxidation–reduction potential)	NMX-AA-008-2000 (DOF, 2000)
Carbon (expressed as chemical oxygen demand, COD)	Method 410.4 (EPA, 1993)
Mercury, chromium and lead	Method 3005A (EPA, 1996) atomic adsorption spectroscopy
Extraction of heavy metals soluble in *aqua regia*	Method 11466 (ISO, 1995)

26.3.3 Inoculation of reactors 1 and 3

Once the metal-tolerant strains were selected according to their ability to remove the studied metals, the volume of each of the selected microbial strains in its own culture medium was divided equally between the three reactors – so that each reactor had the same microbial consortium. Thus, the total effective volume of each reactor (4 l) was made up by the addition of the strains' volume, each with its own culture medium, and completed with synthetic wastewater if needed.

26.3.4 Statistical analysis

Covariance analysis was used to improve the precision of the experiments (Montgomery, 2012), considering removal of heavy metals. The statistical package Minitab 15 (Minitab Inc., 2007) was applied to analyse the results and this included the general linear model.

26.4 Results and Discussion

26.4.1 Toxicity test

None of the concentrations tested were lethal, neither for the metal-tolerant biomass consortia nor for the *L. sativa* seeds. However, some inhibitory effects were detected in both the lettuce plants and the metal-tolerant consortia: there was a reduction in the length of roots and hypocotyls for *L. sativa* (Fig. 26.2) and reduction in the density of the bacterial biomass consortia as the concentration of heavy metals increased (Fig. 26.3a). The concentration of heavy metals that reduced the length of roots and hypocotyls and the biomass density by 50% were the following: 0.106 mg/l Hg (II), 16.5 mg/l Cr (VI) and 26 mg/l Pb (II). These concentrations were added to the synthetic wastewater influents of the reactors.

26.4.2 Reactor experiment

Once the reactors started to operate, colonization reached about 37×10^6 c.f.u./ml and 80% COD removal was observed after 17 days. Figure 26.3(b) presents data on removal of organic matter measured as the COD for both types of reactors (reactors with heavy-metal tolerant strains and control reactors without tolerant strains) considering the initial concentration of 500 mg COD/l. Artificial wetlands as wastewater treatment systems are characterized considering their ability to remove pollutants from the aqueous media.

The purpose of this system inoculated with metal-tolerant consortia was to corroborate if the consortia besides withstanding the metals, could remove them from the aqueous media using the organic matter as a source of energy. This might be the case where municipal sewage receives wastewater from an industrial origin that is to be treated in an artificial wetland system. After 80 days of operation, it was observed that the reactor and its replicate with the inoculum of metal-tolerant consortia both reached stability and were able to maintain it (with variations of ± 5%) (Fig. 26.3b).

On the other side, the control without inoculum varied by ± 12% and no more than 60% of the organic matter was removed as measured by the COD and it seemed that the non-adapted consortia growing in reactor 2 and its replicate were not able to withstand the metal presence as time proceeded.

26.4.3 Heavy metals removal

Mercury removal

Figure 26.4(a) shows the data obtained for the removal efficiency of mercury in the effluent

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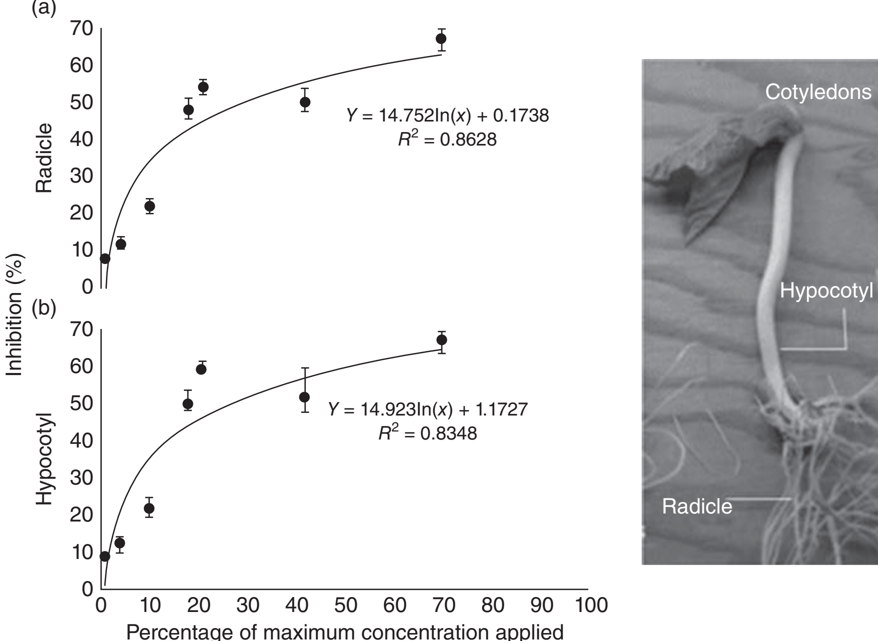

Fig. 26.2. Dose–response curves for phytotoxicity test using *Lactuca sativa* seedlings treated with a toxic heavy metal mixture: (a) radicle length inhibition; and (b) hypocotyl length inhibition.

of the reactors studied. Mercury seemed to be removed by the consortia at the beginning, but then showed a pattern of diminishing removal followed by a smaller rise in the percentage of mercury removed; further research is needed to explain this. Nevertheless, after around 80 days (day 74) the two systems showed significant differences ($P < 0.05$), with the non-adapted consortia reducing their removal efficiency. In the last 30 days of operation, the metal-tolerant consortia maintained their removal efficiency, whereas the non-adapted ones continued decreasing it. Xie *et al*. (2010) mentioned that there is the possibility for microorganisms to develop the ability to tolerate and even proliferate through generational adaptation and this is perhaps what happened with the non-adapted consortia up to day 90.

Figure 26.5(a) presents the data for mercury leaving the reactors together with the bacterial biomass and solids. During the stable operation days, the suspended matter in reactor 1 and its replicate was about 8% whereas in the non-inoculated reactor (reactor 2) and its replicate it was 2%.

Lead removal

Figure 26.4(b) presents the data from days 15–150, where removal efficiency of lead for the metal-tolerant consortia was about 20% higher than for the conventional microbial community. Once this period elapsed, the behaviour of the system was quite stable until the end of the operation, and variations were less than 5%. On the other side, the systems with conventional microbial communities presented an opposite tendency with respect to the ones with metal-tolerant consortia, there being a gradual decline in the percentage of lead being removed, which occurred from day 70 onwards. After this gradual decline, the percentage of lead removed became relatively stable particularly in the last 20 days.

The percentage of lead removed was about half of that obtained by metal-tolerant consortia. These results show the ability of the metal-tolerant consortia to adapt to lead-contaminated media and to continue interacting with the hydrophytes promoting lead removal from the wastewater. This behaviour has been also reported by Vacca *et al.* (2005) and Rathnayake *et al.* (2010).

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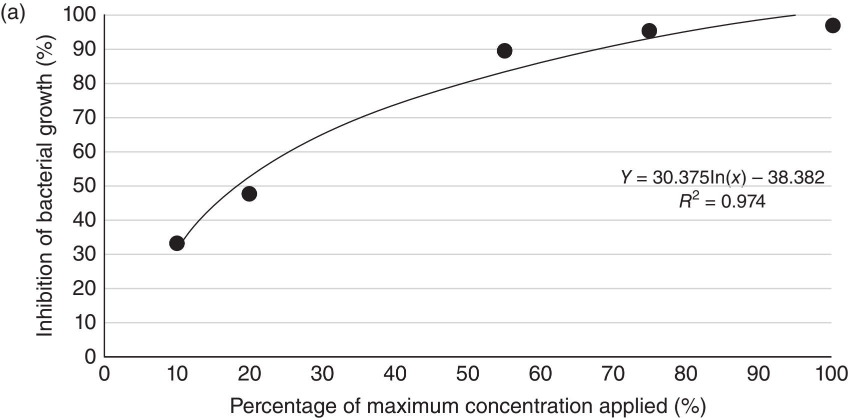

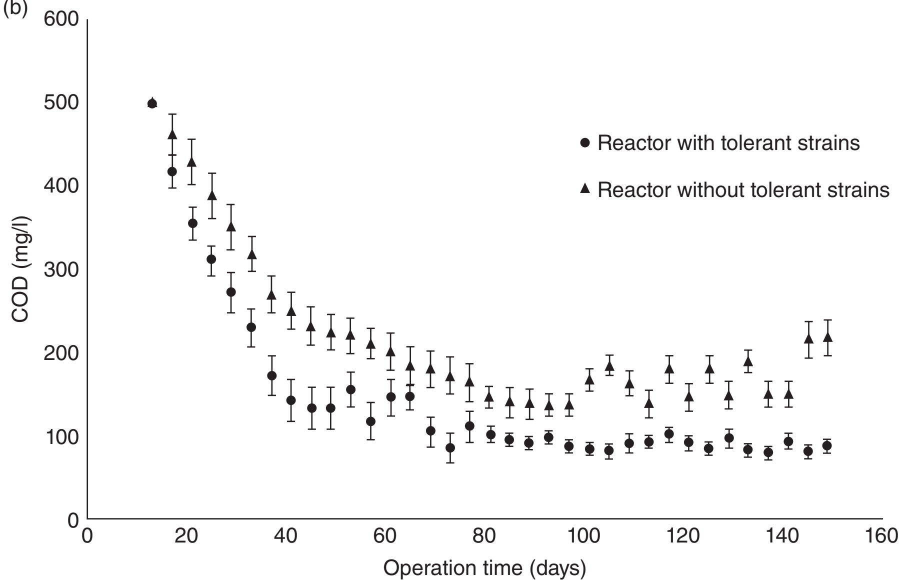

Fig. 26.3. (a) Dose–response relationship between heavy metals mixture and bacterial growth. (b) Removal of the chemical oxygen demand (COD) in the two types of artificial or constructed laboratory-scale simulated wetlands comparing their ability to remove heavy metals, Hg, Pb and Cr, from synthetic wastewater.

Figure 26.5(b) shows the lead contents in the biomass and solids leaving with the effluent. It was observed that after day 60, the amount of lead contained in the microorganisms and solids suspended in the effluent increased by up to 25% of the total removed, a difference with the non-inoculated systems that represented 5% of the total lead removed. Just as for mercury, the difference suggests that metal-tolerant microorganisms possess the mechanism to remove the metal from the aqueous media absorbing it and keeping it in their biomass.

Chromium removal

Finally, the tendency for the removal of chromium was the one that presented a more defined behaviour for both types of systems. In Fig. 26.4(c) it can be clearly seen that the reactors inoculated with metal-tolerant strains started the removal with about 60%, decreasing

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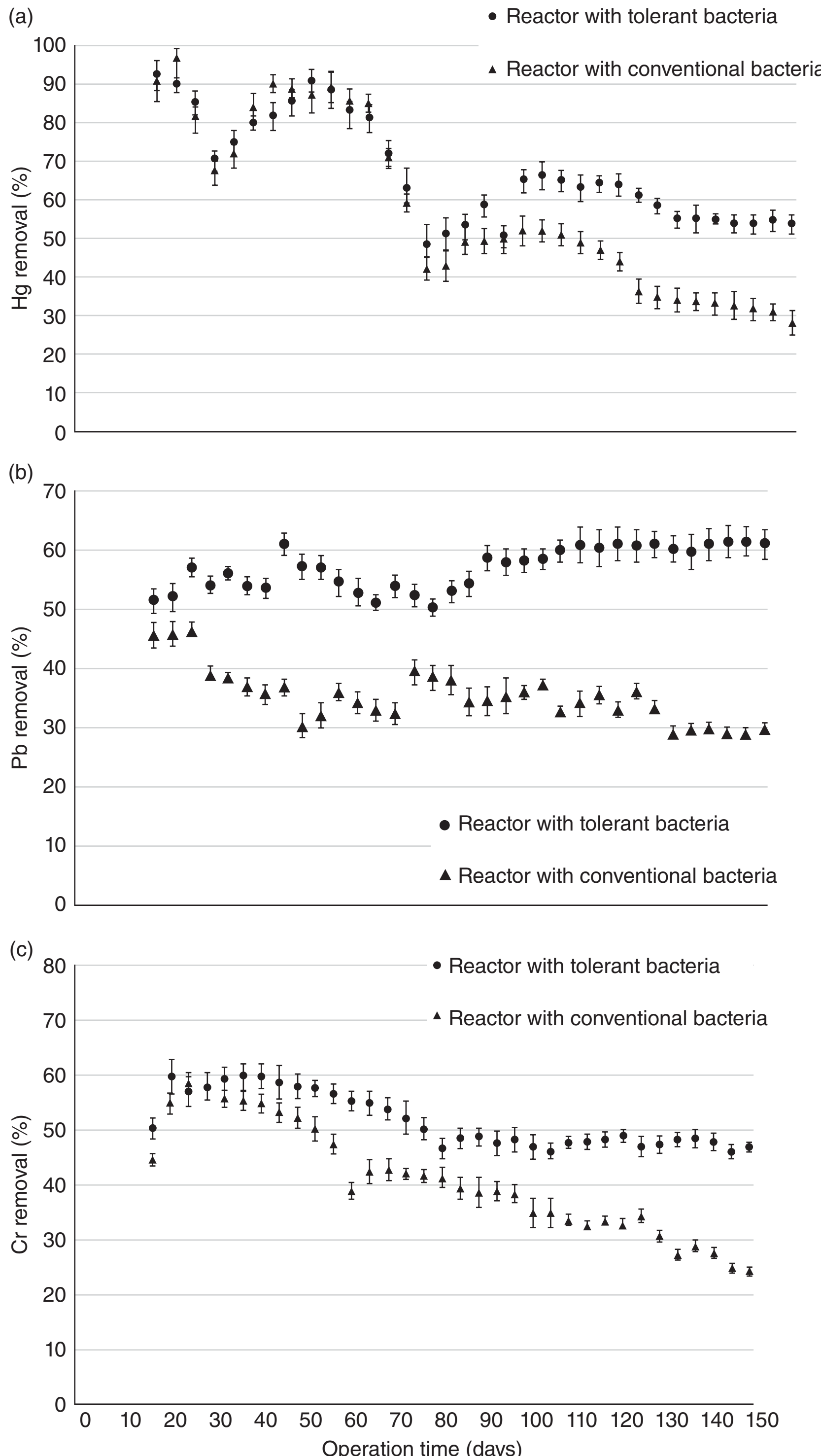

Fig. 26.4. Trend of heavy metals removal from synthetic wastewater in the two types of artificial or constructed wetlands. (a) Mercury removal, (b) lead removal and (c) chromium removal.

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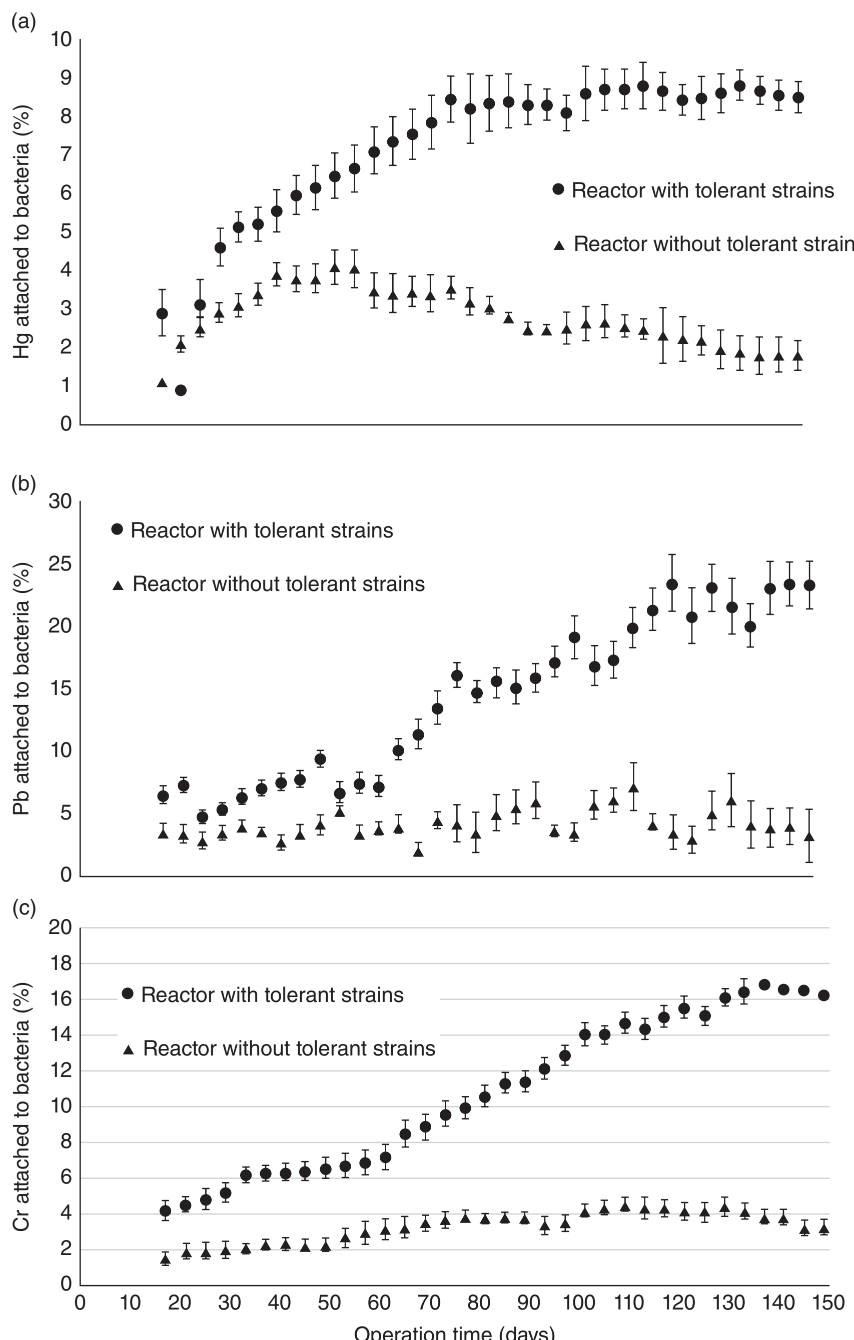

Fig. 26.5. Trend of heavy metals attached to the bacterial biomass suspended in the effluent of the two types of artificial or constructed wetlands evaluated. (a) Mercury, (b) lead, and (c) chromium attached to bacterial biomass.

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down to 47% on day 80. After this point removal remained stable with variations of ± 10%. This behaviour was not followed by the non-adapted consortia that steadily decreased its removal efficiency for chromium as the time elapsed. Results clearly indicate there were differences between the two types of systems ($P < 0.05$) for chromium removal after day 40 of operation.

With respect to the removal of chromium in the suspended matter coming out with the effluent of the reactors, Fig. 26.5(c) shows that for the metal-tolerant consortia, the chromium content removed tended to increase as the operation days elapsed, reaching approximately 16% removal after day 125.

Concerning the non-inoculated reactors, values reached on average 3% removal of chromium, although in the last 30 days the amount of chromium in the suspended matter tended to decrease.

26.5 Conclusion

Artificial or constructed wetlands simulated through laboratory-size reactors inoculated with metal-tolerant strains forming a consortium were able to remove three heavy metals, namely mercury, lead and chromium, from synthetic wastewater with an initial concentration of organic matter equivalent to 500 mg COD/l. There was an overall removal efficiency of 50% from original concentrations of 0.106 mg/l Hg (II), 16.5 mg/l Cr (VI) and 26 mg/l Pb (II) using up about 80% of the organic matter, and these values were within the range of those found in Europe (Kolb and Wilderer, 1997).

These results indicate that it is possible to implement an improved artificial wetlands system that in a very short time is able to start removing dissolved heavy metals if inoculated with consortia of metal-tolerant strains.

The strategy to be followed includes the isolation of promising microorganisms from natural sources, their characterization concerning the interaction between them and the pollutants of interest, the selection of the best strains and their identification, and the design of the consortia. Once these are ready, they can be inoculated into existing or new systems to bioaugment their efficiency to remove both the organic pollutants and the potentially toxic ones.

26.6 Final Remarks

The use of microorganisms to remediate wastewater polluted with heavy metals seems feasible, as indicated by how the reactors performed in this experiment.

This contribution is part of a larger project directing efforts to solve the problem of municipal wastewaters polluted with dissolved metals that may represent a toxicological problem (an issue ever present in most countries with emerging economies) through the use of microorganisms that are adapted to this type of environment. The knowledge gained from the strategy outlined in this chapter, in which consortia of the best strains of microorganisms to degrade the pollutants are tested for their usefulness in laboratory-scale systems of artificial or constructed wetlands, will allow the design of economically friendly, environmentally friendly and stable bioproducts based on microbial biodiversity that might be used to bioaugment remediation systems. This research will contribute to close the technological and social gaps between the *in vitro* results and the potential applications to solve real problems. The ability to bioaugment remediation systems may be easily applied in countries with emerging economies, allowing the local population to start solving their water pollution problems, an urgent issue just recently considered by the United Nations in 2013. As mentioned in the Introduction (section 26.1), this approach is sustainable since it does not require huge investment and does substitute the use of chemical agents, electrical energy and other inputs associated with traditional clean-up systems used in most industrialized countries.

A future challenge is to thoroughly study the cell mechanisms involved that enable microorganisms to remove metals from the aquatic medium, in order to make them more efficient.

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Note

[1] *Tezontle* is a Nahuatl or Aztec word that means stone light as hair (*tetl* = stone and *tzontli* = hair). It is a porous, igneous rock with a reddish colour that is used extensively in construction in Mexico.

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27 Exopolysaccharide (EPS)-producing Bacteria: an Ideal Source of Biopolymers

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Abstract
Polysaccharides are the most abundant carbon sources in the biosphere, thus it is no surprise that their uses and applications are wide ranging. Extended use of polysaccharides has initiated a need to focus research on the isolation of polysaccharides from natural resources. Among the natural resources used for polysaccharide isolation interest has focused on bacterial extracellular polysaccharides as they are candidates for many commercial applications in different industrial sectors such as food, petroleum, cosmetics and pharmaceuticals. These polysaccharides have enabled the industry to replace the traditionally used gums from plants and algae due to the possibility of easy and quick mass production. Bacterial polysaccharides are eco-friendly in nature and are susceptible to natural biodegradation causing little damage to the environment and thus diminishing pollution. These polysaccharides are also not vulnerable to changes in climate and geographical barriers. Thus this chapter encompasses the various sources for isolating exopolysaccharides (EPS) producers, screening methods used for their isolation, polysaccharide recovery, quantification and purification methods.

27.1 Introduction

Biopolymers are natural biodegradable polymers produced by living organisms that form a protective layer around the cells and protect them from the harsh environmental conditions such as osmotic stress, temperature stress and radiation stress and as these biopolymers store carbon-based macromolecules, cells can also get energy by their reduction. The biopolymers can be classified on the basis of their monomer composition as polysaccharides, polyamides, nucleic acids, polyesters, polyphosphates, etc. Since polymers of biological origin are superior to synthetic polymers, as they are eco-friendly and exhibit both biocompatible and biodegradable properties, they are gaining commercial importance all over the world. Bio-based polymers, especially polysaccharides, can provide answers to the challenges faced by the world in terms of global sustainability. Increase in research on such polysaccharides will lead to development of applications that may open new fields and markets (Persin *et al*., 2011). The next decade of research will probably be focused on identifying novel bio-polysaccharides.

Most of the polysaccharides that are being used are mainly obtained from natural sources such as plants, seaweeds and animals. The most widely used plant polysaccharides include starch and cellulose while hyaluronic acid and chitin are the commonly used polysaccharides of animal origin. A large number of plant- and seaweed-based gums (Margaritis and Pace, 1985; Lewis

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et al., 1988) such as gum arabic, ghatti, karaya and tragacanth (McChesney *et al.*, 2007; Lam, 2007) have been in use for many years. The disadvantage of plant- and seaweed-based polysaccharides is that these gums suffer from lack of reproducibility in their properties, purity, supply, cost and quality. Supplies of such gums are also affected by environmental factors such as seasonal variation and eutrophication (Lawson and Sutherland, 1978). Continuous market demand and extended use of polysaccharides has therefore initiated a need to focus research on the isolation of polysaccharides from other easily available and cheap natural resources.

The advancement of technology has made it possible to produce biopolymers from renewable and easily available resources like microbes. Microorganisms are ubiquitously distributed in nature and thus can be explored for polysaccharide production at a commercial level. Microbial sources are known for numerous classes of polysaccharides (Crescenzi, 1995) which are comparable to the plant and animal products such as cellulose, algin, chitin and hyaluronic acid. Microbes produce two types of polysaccharides, namely capsular polysaccharides and extracellular polysaccharides (Crescenzi, 1995). The cell surface of pathogenic microorganisms contain capsular polysaccharides which protect these microorganisms from immune system defences by preventing them from bacteriophage infection while extracellular polysaccharides or exopolysaccharides (EPS) form a slime layer loosely attached to the cell surface or secreted into the environment (Madigan *et al.*, 1997). Capsular polysaccharides are difficult to separate from cell biomass and require various costly purification procedures. On the other hand, extracellular polysaccharides constantly diffuse into the culture medium making it slimy and viscous, hence these are easy to isolate from the culture medium and are free from protein and cell debris.

The usefulness of bacterial extracellular polysaccharides was recognized with the discovery of dextran and xanthan (Sutherland, 1998). Dextran was the first-produced commercial polysaccharide (Kang and Pettit, 1993). It plays an important role in the confectionary industry as it inhibits sugar crystallization as well as improves viscosity and moisture retention. In ice creams and pudding mixes it acts as a crystallization inhibitor and improves mouthfeel. In the medical field it is commonly used in blood transfusions as a blood plasma expander. Dextrans like Sephadex are used for making chromatography matrices in size-exclusion chromatography (Patel and Patel, 2011). Apart from dextran the most extensively used polysaccharide in today's global market is xanthan isolated from *Xanthomonas campestris* which acts as a good suspending and stabilizing agent for oil/water emulsion, removing the rock particles released on drilling, oil recovery and in salad dressings (Sutherland, 2001).

Microbial biopolymers possess good rheological properties (Kawai *et al.*, 1992) hence EPS-producing bacteria are therefore emerging as a new and industrially important source of polymeric materials. A number of bacterial strains such as species of *Xanthomonas*, *Pseudomonas*, *Rhizobium*, *Erwinia* and *Vibrio* (Simonnet *et al.*, 2000; Ding *et al.*, 2003; Goksungur *et al.*, 2004; Brahmachari and Dubey, 2006) as well as number of lactic acid bacteria (LAB) like *Leuconostoc mesenteriodes*, *Lactobacillus plantarum* (Sanni *et al.*, 2002), *Lactobacillus sake* (Berg *et al.*, 1995), *Lactobacillus debrueckii* and *Lactobacillus casei* (Bukola *et al.*, 2008) have been shown to produce EPS with various compositions and functionalities (Dogsa *et al.*, 2005). It is the diversity of microbial sources and their types that appeals to the industrial users of such gums and offers hope to develop new polysaccharides with properties superior to those of the existing polymers.

The novel functionality, different chemical and physical properties, stable supply and cost are regarded as some of the advantages of microbial extracellular polysaccharides over plants or marine macroalgal polysaccharides (MacCormick *et al.*, 1996). The rheological and physiological properties of microbial EPS are quite different from those of natural gums or synthetic polymers (Ahn *et al.*, 1998) and the physicochemical characteristics vary depending on the microbial source and culture conditions. Additionally, microbial extracellular polysaccharides are non-toxic, biodegradable and environmentally friendly (Shoda and Sugano, 2005). Since, these polysaccharides are also not vulnerable to changes in climate and geographical barriers (Roseiro *et al.*, 1992), they can be used as active ingredients in pharmaceutical products, cosmetics or as raw materials for industrial synthesis

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of aromas (Crescenzi, 1995), as thickeners, stabilizers, emulsifiers, gelling agents and water-binding agents in the food, cosmetics, bioplastics, pharmaceuticals and oil industries (Sutherland, 2002). There are reports of the presence of unique physiological activities in some polysaccharides such as antitumour, antiviral and anti-inflammatory activity as well as acting as an inducer for interferon, platelet aggregation inhibition and colony stimulating factor synthesis (Wiley, 2003). The various industrial applications of microbial EPS are discussed below.

27.1.1 Gelling agent

Pseudomonas species were reported to produce a new gelling polysaccharide called gelrite which possesses good thermal stability and clarity. This polysaccharide was reported to be superior to agar (Lin and Casida, 1984) as it forms a brittle, firm and optically clear gel upon deacetylation using mild alkali (Kang *et al.*, 1982). *Sphingomonas paucimobilis* was reported to produce an anionic extracellular polysaccharide called gellan (Kang *et al.*, 1982). This polysaccharide can withstand three to four autoclaving cycles and hence can be used as a substitute to agar. It is also resistant to a wide range of pH and could also tolerate high salt concentrations. These properties render the use of these polysaccharides as thickening agents, substances that accelerate gelling in foods (used in icing and glazes) and as an agar substitute (Omoto *et al.*, 1999). It also finds its application as a low-cost substitute for guar in soil erosion control products (Kimberlin, 2004).

27.1.2 Medical applications

Okutani (1992) reported the antitumour, antiviral and immunostimulant activities of polysaccharides produced by marine *Vibrio* and *Pseudomonas* species. *Alteromonas infernus* isolated from deep-sea hydrothermal vents produced a low-molecular-weight heparin-like EPS exhibiting anticoagulant properties (Colliec *et al.*, 2001). An L-fucose-containing polysaccharide, clavan, possesses the ability to prevent tumour cell colonization in the lung. It was also seen that this polysaccharide aids in the synthesis of antigens for antibody production thereby controlling the formation of white blood cells. Clavan is also reported to be used in the treatment of rheumatoid arthritis and also finds application in cosmeceuticals as a skin-moisturizing agent (Vanhooren and Vandamme, 2000). An extracellular polysaccharide secreted by *Vibrio diabolicus* when evaluated in an experimental model found application as a strong bone-healing material (Zanchetta *et al.*, 2003).

27.1.3 Source of monosaccharides

The bacterial extracellular polysaccharides (homo- and hetero-polysaccharides) beyond their own properties have increased market value because they are capable of producing the rare sugars which are difficult to obtain by chemical synthesis or extraction from plant or animal tissues. Exopolymers are regarded as a valuable source of monosaccharides such as L-fucose, L-rhamnose, L-altrose and D-mannose, which are otherwise difficult to obtain because it is laborious and expensive and they are often in scant supply. The extracellular polysaccharide from *Enterobacter cloaceae* has been reported to be rich in fucose (Iyer *et al.*, 2005b), *Clavibacter* species produce clavan, which is rich in D-fucose (Vanhooren and Vandamme, 2000) and *Klebsiella pneumoniae* produces a polysaccharide which on hydrolysis releases a monosaccharide rich in L-fucose (Vanhooren and Vandamme, 1998).

27.1.4 Emulsifiers

Increasing attention has been paid to microbial surfactants and emulsifiers because of their biodegradability and possible production from renewable resources. *Acinetobacter calcoaceticus* produces an EPS called emulsan (Rosenberg *et al.*, 1979) which was reported to form stable emulsions with a number of hydrocarbons by forming a strong film on the interface. The stability of the emulsion layer proves that the EPS can be used more efficiently than the commercially available emulsifiers (Iyer *et al.*, 2006). EPS isolated from *Sphingomonas paucimobilis*, *Pseudomonas* and *Bacillus* species were found to stabilize emulsions

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more effectively than commercial gums such as arabic, tragacanth, karaya and xanthan (Ashtapure and Shah, 1995).

27.1.5 Heavy metal removal

Heavy metal contamination in the environment is a major concern due to the health risks posed to humanity and animals. Cell-bound polysaccharides produced by the marine bacterium *Zooglea* sp. have been reported to adsorb metal ions like chromium, lead and iron in solution (Kong *et al.*, 1998). Iyer *et al.* (2004, 2005a) reported the biosorption of heavy metals by *E. cloaceae*.

27.1.6 Enhanced oil recovery

The advantage of microbes in oil recovery is a well-known phenomenon. Microbes can enhance oil recovery in different ways. They produce gases like carbon dioxide, organic solvents and some surface active agents (biosurfactants). All these can help oil mobilization in reservoirs. Wells (1977) reported the *in situ* production of a microbial xanthan-like polysaccharide in oil-bearing strata which is used as a means of aiding tertiary oil recovery. *Volcaniella eurihalina* F2-7 is known to synthesize an EPS which is stable to pH and inorganic salts, making it a suitable candidate for enhanced oil recovery (Calvo *et al.*, 1995). *Alcaligenes* has been reported to produce a high viscosity polysaccharide with excellent suspending and heat stability properties which is useful in oilfield drilling and enhanced oil recovery fluids (Baird *et al.*, 1983).

27.2 Sources of EPS-producing Isolates

Microorganisms producing EPS are found in various ecological niches. The development of a microbial population does not require any sophisticated arrangements and their metabolites can be produced at high rates using controlled production parameters. Bacterial strains are generally more used as they offer higher activities and tend to have alkaline or neutral pH and are thermostable. Environments with a high carbon:nitrogen ratio are known to contain polysaccharide-producing bacteria (Morin, 1998). Over the years, extensive studies on EPS production have focused on bacteria isolated from marine habitats, fermented food products, soil and wastewater sources.

Marine bacteria have been largely explored in the search for polysaccharides as the marine environment offers novel microbial biodiversity that produces varied and promising EPS. Extreme habitats such as deep-sea hydrothermal vents, polar regions, hypersaline ponds, cold seeps and coastal hot springs represent a large source of unknown and uncultivated bacteria that need to be explored. The extreme nature of hypersaline environments are expected to harbour unusual microorganisms of biotechnological interest, thus, for the last few years there has been a wide research programme examining microorganisms from such habitats to find new EPS with different characteristics (Martinez-Canovas *et al.*, 2004; Quesada *et al.*, 2004; Saravanan and Jayachandran, 2007; Vu *et al.*, 2009; Poli *et al.*, 2010). It still remains possible that new polysaccharide-producing bacteria could be found in these habitats.

The bacteria in extreme habitats adopt special metabolic pathways to survive in extreme conditions and thus have the ability to produce bioactive compounds. A Gram-negative bacterium *Vibrio diabolicus*, isolated from a deep-sea hydrothermal vent polychaete annelid *Alvinella pompejana*, produces EPS which contains uronic acids and hexosamines (Raguenes *et al.*, 1997).

A new *Alteromonas* species, *A. infernus*, isolated from a hydrothermal vent in the Guaymas region produced extracellular polysaccharide which was modified chemically by sulfation to obtain a new heparin-like compound which possessed the same anticoagulant properties as heparin (Colliec *et al.*, 2001).

LAB play an important role in food fermentation as the products obtained with their aid are characterized as 'generally recognized as safe' (GRAS). *Lactobacillus* EPS have attracted increasing attention because of their importance in food product development. EPS produced by LAB can not only be applied as natural additives but can also be produced *in situ*. Extracellular polysaccharide-producing LAB are isolated from dairy products, fermented meat and fermented foods (Cerning *et al.*, 1986; Doco *et al.*, 1990;

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Makela, 1992). LAB producing significant EPS belong to the genera *Streptococcus*, *Lactobacillus*, *Lactococcus*, *Leuconostoc* and *Pediococcus*. Apart from these some strains of the genus *Bifidobacterium* are also reported positive for biopolymer production (Abbad-Andaloussi *et al*., 1995; Roberts *et al*., 1995; Hosono *et al*., 1997). A review of the literature suggested that EPS produced by LAB contribute to human health as prebiotics, with antitumour, anti-ulcer, immunomodulating or cholesterol-lowering activities (De Vuyst *et al*., 2001; Ruas-Madiedo *et al*., 2002).

EPS from LAB confer beneficial rheological and functional properties to foods (e.g. yoghurts) as natural thickening agents, giving the product a suitable viscosity and a better mouthfeel and longer retention time in the mouth. The LAB produce both neutral and charged EPS which have distinct functional properties. The screening, identification and optimization of fermentation media for EPS production from LAB isolated from fermented foods, milk and milk products is gaining importance among researchers (Smitinont *et al*., 1999; Desai *et al*., 2006; Bauer *et al*., 2009; Xu *et al*., 2010). Total yield of EPS produced by LAB can be influenced by the composition of the medium and growth conditions (Degeest *et al*., 2001b).

Scientific developments in recent years have opened new frontiers and enabled a better understanding of the polysaccharides secreted by various microbes. Read and Costerton (1987) purified and characterized adhesive EPS from *Pseudomonas putida* and *Pseudomonas fluorescens*, both isolated from freshwater epilithic communities, with respect to their chemical composition, biosynthesis and their role in adhesion. Domenico *et al*. (1989) reported on the quantitative extraction and purification of EPS from *Klebsiella pneumonia*.

Over the last 20 years, extensive studies on EPS production have been focused mainly on *X. campestris*, *Rhizobium* spp., *Klebsiella* spp., *Pseudomonas* spp., *Acetobacter* spp. and *Escherichia coli* (Sutherland, 1990). Matsuyama *et al*. (1999) isolated a new polysaccharide-producing bacterium *Microbacterium kitamiense* from the wastewater of a sugarbeet factory in Kitami, Hokkaido, Japan. Matsuyama *et al*. (2003) isolated novel EPS producer *Novosphingobium rosa* from wastewater of a winery in Ikeda town, Hokkaido and reported the conditions of EPS production. Tallgren *et al*. (1999) reported EPS-producing bacteria from sugarbeets collected from different parts of Finland. Guezennec (2002) isolated bacterial EPS of biotechnological interest from the deep-sea hydrothermal vents. Fusconil *et al*. (2006) isolated an EPS-producing strain of *Gordonia polyisoprenivorans* from groundwater contaminated with landfill leachate in a subtropical area in Brazil. Bueno and Cruz (2006) has also reported optimization of polysaccharides production by bacteria isolated from soil. Asker *et al*. (2007) have reported the isolation, purification, fractionation and structure features of the acidic polysaccharide produced by *Bacillus polymyxa*.

The screening of various sources for isolating EPS producers is thus promising because of the enormous range of microorganisms that have yet to be adequately explored. Therefore, it still remains possible that new EPS-producing bacteria could be isolated from various other habitats.

27.3 Isolation of EPS-producing Bacteria

The culture conditions and the composition of the culture media influence the EPS yield and the molecular characteristics of the biopolymers. There is no specific media for the production of extracellular polysaccharides as various organisms differ in their carbon and nitrogen source utilization, mineral requirements, temperature and pH optima, which are the critical factors for maximum EPS production (Sutherland, 1972; Williams and Wimpenny, 1977). The yield and quality of microbial EPS are affected by the nutritional and environmental conditions and an increase in polymer production is possible by manipulating the culture conditions. Therefore, the choice of an adequate EPS production medium is of great importance in polysaccharide production.

EPS-producing organisms can be isolated using complex media containing nutrients like yeast extract, peptone and salts such as magnesium sulfate, ammonium nitrate and potassium dihydrgen phosphate (Tallgren *et al*., 1999; Matsuyama *et al*., 2003; Asker *et al*., 2007). Nutrient broth containing different carbon sources like sucrose, dextrose, maltose or mannitol were also used for isolating polysaccharide producers

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(Patil *et al*., 2009). *Lactobacillus delbrueckii* spp. *bulgaricus* RR when cultured on de Man, Rogosa and Sharpe (MRS) broth supplemented with yeast extract, beef extract and proteose peptone accounted for 94% of the total background EPS (Kimmel and Roberts, 1998) compared to normal MRS media.

Lee *et al*. (2001) used a medium containing sea water, peptone, yeast extract and iron phosphate to isolate EPS-producing marine bacteria *Hahella chejuensis* from Marado, Cheju Island, Republic of Korea. Quesada and Calvo (1993) used maltose yeast (MY) medium supplemented with 7.5% w/v marine salts to isolate an EPS-producing halophilic bacterium *Halomonas maura*. *Bacillus megaterium* isolated from fresh water hydrodynamic sediment was found to produce EPS when grown in organic nitrogen-free glucose mineral salt medium (Chowdhury *et al*., 2011).

The polysaccharide producers are identified on the basis of colony morphology as mucoid and ropy on the culture media. Ropiness of the culture can be defined as its resistance to flow through graduated pipettes (Vedamuthu and Neville, 1986). The ropy character is genetically unstable and non-ropy variants can be detected after several passages in culture. Morin (1998) stated that no direct correlation exists between morphological characteristics of colonies on solid medium and the ability of a culture to produce polysaccharides in liquid medium.

Some polysaccharides form stable complexes with water-soluble dyes such as aniline blue and ruthenium red, hence these dyes can be incorporated into the media which can be used as a screening tool. Stingele *et al*. (1996) used a ruthenium red-milk agar plate method to check the stability of ropy phenotypes. Ruthenium red stains the bacterial cell wall, producing pink colonies from non-ropy strains. Jung *et al*. (2007) used a media containing aniline blue to isolate glucan-producing *Paenibacillus polymyxa* from soil. Aniline blue staining is highly specific for β-1, 3-glucans (Nakanishi *et al*., 1976).

27.4 EPS Recovery

The most important aspects in polysaccharide production are the recovery of polysaccharides from the culture broth. The recovery steps should be carried out in such a manner that the functional properties of the EPS are not altered or affected. Extracellular polysaccharides from the culture broth can be recovered by centrifugation or filtration of the broth for removal of cells followed by biopolymer precipitation from the supernatant using a precipitating agent consisting of a water-miscible solvent in which the polymer is insoluble (e.g. methanol, ethanol, isopropanol, acetone, etc.). The precipitated polymers are dried by freeze drying in the laboratory or if it is on an industrial scale by drum drying (Rosalam and England, 2006; Bajaj, 2007; Pena, 2008; Freitas *et al*., 2011).

The culture medium used for EPS production influences the purification steps necessary to remove the protein content as a contaminant from the isolated EPS and other components in the final EPS preparation. Microorganisms from the culture broth can also be separated by heat treatment when the EPS is thermally stable. Heat treatment partially kills the bacterial cells and their enzymes secreted into the broth thereby lowering the viscosity of the media. In the case of xanthan, it was observed that heat treatment enhances the viscosity even though this effect is pH dependent (Sutherland, 1990). Apart from heat treatment the commonly used method for separation of EPSs from culture broth is centrifugation. The nature and viscosity of the polysaccharide decides the speed and duration of centrifugation. The cells and their debris can be removed by ultracentrifugation on the laboratory scale (Morin, 1998). The EPS isolated from a complex medium usually contains protein as a contaminant that can be removed by trichloroacetic acid precipitation followed by centrifugation (van Calsteren *et al*., 2002; Harding *et al*., 2003). Milk proteins like casein present in the culture media of dairy starters must be removed prior to EPS isolation. In such cases, the culture broth is digested with proteolytic enzymes (e.g. Pronase E, protease type XIV) in the presence of 0.1% merthiolate to prevent further microbial growth.

EPS can be recovered from the cell-free supernatant by solvent precipitation of the broth. Organic solvents that are miscible with water are used for recovery of EPS from the cell-free supernatant as these solvents favour EPS separation by lowering their solubility. During this separation, proteins and salts of the medium may also precipitate along with EPS which can be separated by dialysis. The proportion of solvent

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used is variable, which can be one, two or three volumes of the culture broth, although two volumes are most often used. They may also serve to remove colour and to extract low-molecular-mass fermentation products and medium components (Kumar *et al.*, 2007). Often three to five volumes of ethanol (Kaplan and Rosenberg, 1982; Otsuji *et al.*, 1994), isopropanol (Edward *et al.*, 2011) or acetone (Williams and Wimpenny, 1977) are used to precipitate out the EPS from the culture supernatant. Azeredo and Oliveira (1996) reported water-miscible organic solvents like cetyltrimethylammonium bromide (CTAB) and 3,5,6-triphenyl-2,3,5,6-tetraaza bicyclo-1-hexene (commercially known as nitron) for recovery of polysaccharides. Recovery of about 99% has been reported using only 0.1 volume of a 10% solution of nitron in 3% acetic acid as the precipitating agent of neutral (e.g. hydroxyl propyl starch) and acidic (e.g. sodium alginate) polysaccharides.

The solvent-precipitated EPSs are usually harvested by centrifugation and then dissolved in a minimal amount of distilled water followed by dialysis against distilled water for 24 h at 4°C. The polysaccharide solutions are dialysed extensively to ensure the removal of small-molecular-weight salts or enzymatic degradation products, residual sugars or other medium components and then freeze-dried or dried with inert gas and stored at 4°C (Cerning *et al.*, 1988; Morin, 1998). The final EPS products after freeze drying are off-white to white, depending on their purity. Ultrafiltration and reverse osmosis are also reported to be used for reducing the water content of EPS preparations (Sutherland, 1990).

27.5 Quantification of EPS

The extraction methods described above usually result in solutions containing a mixture of components (i.e. other polysaccharides as well as non-carbohydrate material). Thus the recovered EPS needs to be analysed for the amount of carbohydrates present in it. The lyophilized powder obtained is weighed, which is the simplest indication of the EPS yield in terms of dry weight (De Vuyst *et al.*, 1998; Van Geel *et al.*, 1999; Degeest *et al.*, 2001a).

Colorimetric procedures have been employed for the determination of sugars and their related compounds. The most commonly used and reliable method for EPS quantification is the phenol-sulfuric method described by Dubois *et al.* (1956). This method has been applied widely to quantify EPS recovered by various isolation processes (Cerning *et al.*, 1986; Abbad-Andaloussi *et al.*, 1995; Mozzi *et al.*, 1995, 1996; Roberts *et al.*, 1995; Stingele *et al.*, 1996; Macedo *et al.*, 2002). Carbohydrates on dehydration with concentrated sulfuric acid and phenol produce furfural derivatives which give an orange-yellow colour. This method thus quantifies the total carbohydrates including low-molecular-weight carbohydrates. Subtracting the total sugar fraction measured by the phenol-sulfuric acid method from the reducing sugar measured by the dinitro salicylic acid method has also been reported for quantification of polysaccharide (Ruijssenaars *et al.*, 2000). Anthrone reagent was also used by several workers for quantification of sugars (Morris, 1948; Berg *et al.*, 1995; Rimada and Abraham, 2001).

27.6 Purification of EPS

Several types of chromatographic techniques can be used for separating polysaccharides from each other and from non-carbohydrate contaminants. Ion exchange chromatography on DEAE-Cellulose, DEAE-Sepharose, etc. was reported for separating acidic and neutral polysaccharides (Asker *et al.*, 2007). The former pass through the column without binding, whereas the latter, because of their negative charge, are retained on the column and can be eventually eluted with buffers of increasing ionic strength or pH. Acidic polysaccharides were eluted using a continuous gradient of various molarities of sodium chloride (Iyer *et al.*, 2005b; Asker *et al.*, 2007; Chowdhury *et al.*, 2011; Yang *et al.*, 2012).

Gel filtration chromatography has been reported suitable for purification of both neutral and anionic polysaccharides. It separates these biopolymers based on their molecular weight and hydrodynamic volume. For example, arabinoxylans and arabinogalactans can be effectively separated by gel filtration chromatography using Sepharose columns (Fincher *et al.*, 1974). Asker *et al.* (2007) used a Sephadex G-150 column for molecular weight determination of acidic polysaccharide produced by *Bacillus polymyxa.*

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The crude EPS isolated from deep-sea psychrotolerant bacterium *Pseudoalteromonas* sp. was purified through gel-filtration chromatography, through a column of Sephadex G-100 (Qin *et al.*, 2007).

Affinity chromatography, based on specific non-covalent interaction between the binding ligand attached to the column and polysaccharides, is one of the most powerful techniques for purification of certain carbohydrate polymers. Lectins are natural plant proteins known for their carbohydrate-binding activity and, therefore, are used as ligands in affinity chromatography columns. Although many lectins recognize and bind to simple sugars, such as glucose, mannose, galactose, *N*-acetylgalactosamine, *N*-acetyglucosamine or fucose, they have higher affinity for oligosaccharides. Concanavalin A (Con A), a lectin obtained from *Canavalla ensiformis*, exhibits specific affinity towards D-mannopyrans.

The most extensively used technology for the analysis of the monomer composition of EPS is gas chromatography/mass spectrometry. The EPS molecules are hydrolysed generally with trifluoroacetic acid or sulfuric acid. In the presence of a strong acid and heat, the glycosidic bond between monosaccharide residues in a polysaccharide is cleaved. However, not all glycosidic linkages are cleaved at the same rate and the hydrolysis time must be sufficient to hydrolyse all linkages in the sample. These two needs must be balanced; the need for hydrolysis of sufficient strength and length to permit complete hydrolysis, but not so long so as to lead to sample degradation. Sulfuric acid is difficult to remove after post-hydrolysis and its presence can interfere with some analyses. Triflouro acetic acid is volatile and can be easily removed prior to an HPLC (high performance liquid chromatography) analysis (Brummer and Cui, 2005). The monosaccharides released by acid hydrolysis are derivatized to alditol acetates (Blakeney *et al.*, 1983) or trimethyl silylated glycosides (Gerwig *et al.*, 1978, 1979), which are then separated by gas chromatography using different gas carriers (He, N_2, H_2), columns and temperature programmes. The partially methylated deuterated alditol acetates obtained can be identified by GCMS (gas chromatography mass spectrometry) (Faber *et al.*, 1998, 2001a, b; van Casteren *et al.*, 1998, 2000; Harding *et al.*, 2003).

27.7 Examples of Some Commercially Used Bacterial EPS

27.7.1 Dextran

Dextran is a complex branched homopolysaccharide made of many glucose molecules with α-1,6 glycosidic linkages between glucose molecules. It was the first industrial polysaccharide to be commercialized and approved for food use. LAB has been considered as the most beneficial probiotic organisms because of their GRAS status. LAB like *Leuconostoc* species produced dextran when sucrose was used as the carbon source (Hucker and Pederson, 1930). Various species of bacteria are reported to produce dextran but two strains of *Leuconostoc*, namely *Leuconostoc mesentecosides* and *Leuconostoc dextranicum*, are commonly used for commercial dextran production.

Dextran solutions provide colloidal stability and visco-elasticity which ensures its various industrial applications. It acts as a gelling agent in confectionary and crystallization inhibitor in ice creams. Its biocompatibility, easy chemical modification, low cost, low toxicity and slow degradation by human enzymes allow it to be used in biomedical applications. It has been in use in blood transfusions as a plasma volume extender and antithrombolytic agent. It has also found application in nanoscience technology. The trade name of a cross-linked dextran gel manufactured in bead form is Sephadex used in size exclusion chromatography matrices (Qader *et al.*, 2005).

27.7.2 Xanthan gum

Xanthan gum was the first industrially produced biopolymer widely studied and accepted for commercial purposes (Rottava *et al.*, 2009). In the mid-1950s The Northern Utilization Research and Development Division of the US Department of Agriculture discovered an EPS produced by the bacterium *Xanthomonas campestris*. Xanthan gum can be produced commercially by submerged aerobic batch fermentation in a media containing glucose, sucrose and starch. It is a heteropolysaccharide with a primary structure consisting of repeated pentasaccharide units formed by two glucose

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units, two mannose units and one glucuronic acid unit. The main chain of xanthan consists of β-D glucose units at the 1 and 4 position which resembles that of cellulose. Trisaccharide side chains contain a D-glucuronic acid unit between two D-mannose units linked at the O-3 position of every other glucose residue in the main chain.

The extraordinary physical properties of xanthan gum such as high viscosity at very low concentrations and stability at both acidic and alkaline pH and across variations in temperature have made it one of the top food and industrial biopolymers. It is used in salad dressings and dips to stabilize emulsions, and in the petroleum industry to drill fluids and enhance the oil recovery process. The initial viscosity of xanthan solutions is rebuilt even after high shear rates making it pseudoplastic in nature which enhances its sensory qualities such as mouthfeel, flavour, etc. It also finds application in pharmaceutical combinations and textile and agricultural products.

27.7.3 Curdlan

Curdlan is the third microbial EPS to be approved for food use in the USA. The polysaccharide produced by *Alcaligenes faecalis* var. *myxogenes* was discovered by Harada *et al*. in 1966. Curdlan production was also detected in a few *Rhizobium* strains (Ghai *et al*., 1981) and in species of the Gram-positive *Cellulomonas*, including *Cellulomonas flavigena* (Kenyon and Buller, 2002). It is a neutral gel-forming linear homopolymer of D-glucose with β-1,3 glucosidic linkage. Curdlan polysaccharide consists of as many as 12,000 glucose units and is insoluble in water, alcohols and most organic solvents, but dissolves in dilute bases (0.25 M NaOH), dimethylsulfoxide (DMSO) and formic acid (Yotsuzuka, 2001). Curdlan forms a weak gel on heating above 55°C followed by cooling. Further heating to 80–100°C increases the gel strength and produces a firm, resilient gel, while autoclaving at 120°C converts the molecular structure to a triple helix. Thus curdlan has the capacity to form thermoreversible gels when heated. When combined with other polysaccharides like xanthan, guar gum or locust bean gum it forms tasteless, colourless, odourless hydrogels. It is also stable over a pH range of 2–12. Curdlan also finds it applications in the pharmaceutical and food industries and in the field of construction. Due to its rheological property and non-toxicity it is used in the food industry as a bio-thickner. Curdlan and its derivatives possess antiviral, antimalarial and antitumorogenic activities. Curdlan hydrogels were also used as a drug-delivery vehicle for bioactive macromolecules.

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28 Microbial Process Development for Fermentation-based Biosurfactant Production

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Abstract
The development of fermentation processes of microbial bioproducts has come a long way, with scientists modifying existing technologies and inventing new and more efficient methods. Surfactants, especially those produced by biological systems, are highly useful and unique molecules that have a variety of applications in various industries and aspects of human life. Biosurfactants are produced mainly by microorganisms of which *Pseudomonas* species were one of the first that were discovered to produce such molecules as secondary metabolites. Biosurfactants are generally classified based on the charge they carry on their surfaces as anionic, cationic, amphoteric or non-ionic surfactants. They can also be classified based on their chemical structures as glycolipids, lipopeptides, oils or polymers. Production of biosurfactants can be achieved by batch, fed-batch or continuous fermentation processes but are, however, quite expensive. Optimization of the production parameters, such as the nutrient content, can lead to more efficient production and reduced cost. Varying the carbon to nitrogen content and altering some parameters, like the need for oxygen or the pH, can greatly affect the production of biosurfactants and lead to a better yield.

28.1 Introduction

Surfactants are surface-active molecules which have the ability to lower the surface tension of liquids due to the molecules' unique structure which contains both hydrophilic and hydrophobic regions (Gutnick and Bach, 2011). This structure enables surfactants to display a variety of surface activities that allow the solubilization of hydrophobic compounds (Satpute *et al.*, 2010) due to the reduction in surface and interfacial tension. As a result of this unique property, surfactants have been widely used as detergents, emulsifiers, de-emulsifiers, dispersants, wetting agents and foam retardants, but these chemically produced surfactants are derived from waste products of the petrochemical industry and are toxic and not easily degraded in the environment.

28.2 Biosurfactants

Biosurfactants are receiving increasing interest and attention in the last decade in an attempt to compete with chemically derived surfactants (Satpute *et al.*, 2010; Khopade *et al.*, 2012a, b; Luna *et al.*, 2013). These are a structurally diverse group of surfactants synthesized by microorganisms such as bacteria and yeast, and they are surface-active biomolecules known as amphiphilic compounds. They are great detergents

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with foaming and emulsification properties. Their properties in general are: (i) reduction of surface tension between different phases; (ii) their ability to reduce critical micelle concentration (CMC); and (iii) their ability to reduce interfacial tension between aqueous and hydrocarbon mixtures. Biosurfactants can be classified based on the charge they carry as either negatively charged molecules known as anionic biosurfactants or positively charged molecules known as amphoteric biosurfactants. In between these two groups are the non-ionic and cationic biosurfactants that are either a polymerization product or possess a positively charged quaternary ammonium group, respectively. Biosurfactants can also be classified based on their molecular weight (Rahman and Gakpe, 2008). These biologically produced surfactants have the same properties as chemical surfactants but they have shown several advantages over the latter such as lower toxicity and biodegradability. In addition to these desirable properties they have also shown high activity and stability at extreme temperatures, pH and salinity and have shown better environmental acceptability (Luna *et al.*, 2013; Jain *et al.*, 2013). These traits have all contributed to the increased interest in biosurfactant production as many industries are looking for greener alternatives to many of their products and processes.

Classification based on their chemical structure is another method that is finding ground. Table 28.1 shows this classification mode. Biosurfactants gained their popularity and

Table 28.1. Examples of biosurfactants based on their chemical structure classification. (From Müller *et al.*, 2012.)

Structural classification	Examples
Glycolipids	Rhamnolipids (RL), sophorolipids (SL), mannosylerythritol lipids (MELs), trehalose lipids (TL)
Lipopeptides/ lipoamino acids	Surfactin, ornithine lipids, lysin lipids
Polymers	Polysaccharides, lipopolysaccharides, proteins, lipoproteins
Oil/membranes	Fatty acids, glycerolipids and phospholipids

importance in the area of oil recovery and environmental bioremediation. They have also been used extensively in the food processing industry and as pharmaceuticals. They are produced by bacteria, yeasts and fungi by fermentation using renewable carbon sources. The increase in interest in the application of biosurfactants is due mainly to their highly desirable characteristics, which include higher biodegradability compared with chemical surfactants. This can be said to be the singular most important reason as to why the microbial surfactants have gained their popularity. Another property that is unique to biosurfactants compared with their chemical counterparts is that they have lower toxicity when used, especially in the environment. Microbial surfactants are also a better choice as they are effective at extremes of pH, temperature and salinity (Mukherjee *et al.*, 2009).

In addition to the environmental benefits of biosurfactants, they also have other benefits over chemical surfactants such as their stability in extreme conditions which would make them suitable replacements for conventional chemical surfactants. Microorganisms produce biosurfactants in order to assist in solubilization of hydrophobic compounds in the environment to facilitate their use as substrates by the microorganism. A few examples of biosurfactants and their structures can be seen in Fig. 28.1; this figure includes monorhamnolipid, di-rhamnolipid, lipopeptide and sophorolipid. In order for biosurfactants to become a commercially viable product, they need to compete with currently utilized chemical surfactants in terms of cost, function and production capacity (Rocha e Silva *et al.*, 2014). Therefore, to successfully meet these demands and successfully compete with chemical surfactants, microorganisms with suitable metabolic pathways that are easily culturable and are capable of producing a high yield of effective biosurfactant need to be identified through bioprospecting. Bioprospecting for a suitable microorganism to meet the current industrial demand may be a lengthy and costly process but a lot of research is currently being conducted into this (Najafi *et al.*, 2010; Satpute *et al.*, 2010; Khopade *et al.*, 2012a, b).

The marine environment presents an enormous diverse environment and it is estimated that less than 0.1% of the marine microbial

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Fig. 28.1. Different biosurfactant molecules. (From Gutnick and Bach, 2011.)

world has currently been explored (Satpute *et al.*, 2010). This gives great potential for important microorganisms which may contain unique metabolic pathways to be discovered, which could not only influence the production of biosurfactants but could potentially have a huge impact on all areas of bioprocessing. Due to the complexity of the marine environment and the extreme conditions that some marine microorganisms exist under, not all microbes currently collected are able to be cultured in a laboratory environment for analysis and have only been identified using molecular methods. Due to the necessity during oil recovery and remediation of oil spills of a bacteria and its biosurfactant to be halotolerant, it is mandatory to screen and develop potential biosurfactant producers from marine environments (Khopade *et al.*, 2012b). Different screening methods are currently utilized in order to identify biosurfactant-producing microorganisms but as each biosurfactant has different functional and chemical properties it is difficult to obtain biosurfactant-producing microorganisms using only one screening method (Satpute *et al.*, 2010). Several marine microbial identification strategies and their effectiveness as marine biosurfactant producers have been reviewed. Khopade *et al.* (2012a) isolated marine *Streptomyces* species B3 and characterized its biosurfactant after optimization of the culture medium and fermentation process. Khopade *et al.* (2012b) isolated marine *Nocardiopsis* species B4 and characterized its biosurfactant after optimization of the culture medium and fermentation process. Identification of the strains studied in Khopade *et al.* (2012a) and Khopade *et al.* (2012b) was achieved using 16S rDNA technology, scanning electron microscopy, biochemical and cultural characterization.

28.3 Characteristics of Biosurfactants

Microbial surfactants act by reducing interfacial and surface tensions in much the same way as chemical surfactants. The ability to reduce the

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CMC is another characteristic of biosurfactants as stated previously. These molecules are usually produced by microorganisms where they either remain adherent to the cell surface of microbial cells or are secreted into the culture broth. Microbial surfactants are diverse and can occur as a variety of chemical structures like glycolipids or lipopeptides, which are the two most commonly occurring molecules. They can also occur as fatty acids, phospholipids or particulate structures (Müller *et al.*, 2012).

The advantages of using microbial biosurfactants far outweighs using chemical surfactants as the latter are produced from petroleum feedstock which has detrimental effects on the environment and, moreover, the source is deemed expendable. On the other hand, microbial surfactants are from sustainable sources and the technology to produce them in large quantities is readily available.

Another important advantage of microbial surfactants over chemical surfactants is that not only are the microbial agents renewable but also they utilize low-cost feedstock.

28.4 Fermentation Requirements

There are three different types of fermenter operation processes which are frequently used for culturing bacteria. These are batch, fed-batch and continuous fermentation processes.

Batch fermentation is the process of culturing when all of the ingredients (e.g. nutrients) required for fermentation are added to the fermenter at the start of the fermentation process, before inoculation with the seed culture, and the process is run until all of the nutrients are exhausted and the broth is then harvested. Batch fermentation has the advantage of being simple and having low risk of external contamination as no further additions are required except for pH stabilizers. The process is best for fermentations of cultures with high yield and for substances that can tolerate high initial nutrient conditions.

Fed-batch fermentation is similar to batch fermentation but only starts with some of the required nutrients at the inoculation stage, in order to prevent inhibition of product production at high concentrations of substrate. Further nutrients are added as the fermentation progresses in order to maintain the substrate concentration for the production of the desired product. The advantages of fed-batch fermentation include reduction of substrate and product inhibition and this can decrease the overall fermentation time allowing a higher concentration of product without being inhibited by high levels of nutrients in the broth (Anderson, 2009; Chang *et al.*, 2012). Fed-batch fermentation, however, carries the risk of potential contamination due to the addition of nutrients through a sterilizer, and the increased costs for specialized sterilization equipment. Batch and fed-batch fermentations can be repeated using the same fermenter system after harvesting the culture by leaving a small amount of the previous batch in the fermenter as inoculums. This adds the risk of contamination, and degradation of the culture limits the number of repeat batches to about two or three before the fermenter must be cleaned and sterilized.

Continuous fermentation processes start with the medium and inoculum in the fermenter, and after the culture has grown, the broth is withdrawn at the same rate as the fermenter is fed nutrients in order to maintain a constant volume of broth in the fermenter. Under ideal conditions the dilution rate will be the same as the culture growth rate, and when this balance is maintained for long enough there are no changes in the conditions within the reactor; this is called steady state operation (Brethauer and Wyman, 2010). Compared with batch fermentation processes, continuous fermentation reduces the time needed for cleaning and sterilization between batches. Although continuous fermentation cannot be run indefinitely, fermentations of several hundred hours can be completed under aseptic conditions. Continuous fermentation has better control at steady state operation which in turn reduces costs (Brethauer and Wyman, 2010), but contamination from adapted cultures is difficult to avoid as they can grow back through the continuous harvest line (Anderson, 2009). Khopade *et al.* (2012b) completed their investigations using shake flasks in batch fermentation.

When designing and optimizing a fermentation process, the optimum growth conditions of the isolated microorganism need to be identified. This is most effectively achieved at small

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scale using shake flasks by measuring the optical density of the culture medium throughout the culture time to produce a growth curve for each of the variables such as temperature, salinity and medium composition. Parameters such as pH, O_2 content and O_2 uptake and other environmental factors cannot be as easily monitored and controlled on a small scale (Smith, 2009). Following this, optimum conditions can be established for culture of the microorganism. It should be noted that optimum conditions for growth of the microorganism may not be the optimum conditions for the production of the desired product. Following the optimization in shake flasks, the process can be scaled up to larger volumes for further optimization and development for potential use at an industrial scale.

The development of a suitable growth medium depends on the nutritional requirements of the microorganism to be cultured. In order to ensure that the production of biosurfactants is economical, low-cost substrates with sufficient nutritional value need to be used as this can account for 10–30% of the overall costs (Silva *et al.*, 2010). Khopade *et al.* (2012b) chose to optimize the carbon and nitrogen source available for utilization in order to obtain higher productivity of the biosurfactant. This was done using several carbon sources while keeping the nitrogen source constant, then using the optimum carbon source, varying nitrogen sources were compared and the optimums were chosen. The optimal growth conditions required for high cell density is not the same as the optimum conditions for biosurfactant production, as previously noted, and in the case of *Pseudomonas aeruginosa*, when producing rhamnolipid, fed-batch fermentation with the carbon source in the feed produces a very low dry cell weight concentration (g/l) whereas the rhamnolipid concentration is at its highest, producing over 3.5g/l (Ghomi Avili *et al.*, 2012).

28.5 Production of Biosurfactants

Biosurfactants, in general, are diverse in nature with about 60 different congeners and homologues and are produced at different concentrations by various species of bacteria, yeast and fungi especially those with filaments. The major group of microorganisms that produce biosurfactants are the *Pseudomonas* species that were the first to be discovered to produce such secondary metabolites. Other microorganisms that produce biosurfactants are *Bacillus subtilis*, *Candida bombicola*, *Acinetobacter calcoaceticus* and *Arthrobacter protophormiae*.

Pseudomonas species are well known as potential marine and terrestrial bacteria that produce a variety of bioactive metabolites. A report by Bhatnagar and Kim (2012) showed that these novel bacteria produce about 800 bioactive molecules ranging mainly from antibiotic agents to others with diverse properties. The main biosurfactant produced as exoproducts by *Pseudomonas* spp. are the glycolipid-type surfactants known as rhamnolipids (Bhatnagar and Kim, 2012).

Biosurfactants can be produced on a large scale using bioreactors and cheap substrates as sources of nutrient. A lot of studies have shown that biosurfactants can be produced by growing bacteria like *Pseudomonas* known for their production of rhamnolipids as shown by rhamnolipids experts like Rahman and Gakpe (2008) (Table 28.2).

28.6 Monitoring of Biosurfactant Production

Optimization of the production of biosurfactant can be achieved by testing the biosurfactant production throughout the fermentation process while changing the variables accordingly. Using a tensiometer in order to monitor any changes in surface tension is a good indicator of biosurfactant production. Foaming in shake flasks during culturing is also a good indicator of the presence of biosurfactants in the media and this can be analysed further by testing the emulsification index of the biosurfactant produced as described in Shavandi *et al.* (2011). It can be noted that fed-batch fermentation is more effective than batch fermentation processes in order to produce higher concentrations of rhamnolipid by *P. aeruginosa*, when the carbon source is limited by the feed process (Shavandi *et al.*, 2011; Ghomi Avili *et al.*, 2012). This higher concentration does not mean that the biosurfactant produced cannot be isolated and studied at lower concentrations when using the batch fermentation process, however,

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Table 28.2. Different biosurfactants and their microbial sources. (Adapted from Rahman and Gakpe, 2008.)

Type of biosurfactant	Source microorganism(s)
Alasan	*Acinetobacter radioresistens*
Arthrofactin	*Arthrobacter* sp.
Biosur PM	*Pseudomonas maltophila*
Cellobose lipids	*Ustilago maydis*
Diglycosyl diglycerides	*Lactobacillus fermentii*
Fatty acids (corynomycolic acid, spiculisporic acid)	*Penicillium spiculisporum*, *Corynebacterium lepus*, *Arthrobacter paraffineus*, *Talaramyces trachyspermus*, *Norcadia erythropolis*
Glycolipids	*Alcanivorax borkumensis*, *Arthrobacter* sp., *Serratia marcescens*, *Tsukamurella* sp.
Lichenysin A, lychenysin B	*Bacillus licheniformis*
Lipopolysaccharides	*Acinetocbacter calcoaceticus* (RAG1), *Pseudomonas* sp., *Candida lipolytica*
Ornithine, lysine peptids	*Thiobacillus thiooxidans*, *Streptomyces sioyaensis*
Particulate surfactant(PM)	*Pseudomonas marginalis*
Phospholipids	*Acinetocbacter* sp.
Polyol lipids	*Rhodotorula glutinous*, *Rhodotorula graminus*
Rhamnolipids	*Pseudomona aeruginosa*, *Pseudomonas* sp., *Serratia rubidea*
Sophorolipids	*Candida apicola*, *Candida bombicola*, *C. lipolytica*, *Candida bogoriensis*
Streptofactin	*Streptomyces tendae*
Sulforyl lipids	*T. thiooxidans*, *Corynebacterium alkanolyticum*, *Capnocytophaga* sp.
Surfactin	*Bacillus subtilis*, *Bacillus pumilus*
Trehalose lipids	*Arthrobacter paraffineus*, *Corynebacterium* sp., *Mycobaceterium* sp., *Rhodococcus erythropolis*, *Norcardia* sp.
Viscosin	*Pseudomonas fluorescens*

which may be more cost-effective during initial screening for suitable biosurfactants.

28.7 Downstream Processing of Biosurfactants

In order to successfully characterize the biosurfactant produced by the fermentation process, it must be separated from the cells and the broth and then purified. Separation from the cells can be achieved by centrifugation at 5000–10,000 ***g*** for 10–20 min (Nayak *et al.*, 2009; Shavandi *et al.*, 2011; Luna *et al.*, 2013; Rocha e Silva *et al.*, 2014), the cell pellet can then be removed and dried in order to measure the dry cell weight for the culture and the supernatant can be further purified in order to characterize the biosurfactant more accurately. Purification of the biosurfactant from the supernatant can be achieved by acidifying the supernatant with hydrochloric acid to pH 2.0 and then precipitating the biosurfactant with methanol. The precipitate can then be centrifuged before being separated and the precipitate is then washed with methanol and dried at 37°C. This method is described in Luna *et al.* (2013) and yields pure biosurfactant for further study. Another separation method described recently by Ismail *et al.* (2013) used solid phase extraction (SPE) by centrifuging the broth followed by filtration of the supernatant to remove excess biomass, then the leftover solution was loaded onto SPE cartridges and the crude biosurfactant was eluted from the cartridge using methanol. Both of these extraction methods are able to produce crude biosurfactant for further analysis and have both proved to be effective in estimating yield by weighing the residual biosurfactant (Ismail *et al.*, 2013; Luna *et al.*, 2013).

Characterizing the purified biosurfactants can be done in a number of ways in order to identify structure, functional groups and properties. When using a tensiometer with a Du-Nouy ring, the CMC can be calculated (as seen in Khopade *et al.*, 2012b) and the surface tension and interfacial tension (mN/m) are determined by the maximum force exerted by the solution and at the CMC a sudden change in surface tension can be observed (Shavandi

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et al., 2011). This value can be determined by plotting a graph showing the surface tension observed for different concentrations of biosurfactant (log of mg/l or g/l). Fourier transform infrared spectroscopy (FTIR) analysis can be completed to characterize the structure of the biosurfactant produced. In this the sample is freeze dried and then analysed using an infrared spectrophotometer, and the resulting spectrum can then be analysed. The bands and peaks on this spectrum can be used to indicate the functional groups and chemical bonds in the molecular structure of the biosurfactant (Aparna *et al.*, 2012; Khopade *et al.*, 2012b; Jain *et al.*, 2013).

Biosurfactants such as those shown in Table 28.3 have the potential to be utilized in a number of different processes including land and water remediation, oil extraction, medical processes and a number of other industrial applications. In order for them to be successfully integrated into current industrial processes they must be produced at a reasonable price and have a yield high, enough to compete with currently utilized chemical surfactants. Even though biosurfactants are a greener alternative which is an attractive attribute, they need to be more cost-effective in order to secure their place in the industrial marketplace. Some scientists are now trying to overcome the cost and capital issues by not only focusing on biosurfactants being more attractive as a greener alternative, but by showing their higher productivity and ability to outperform current chemical surfactants even in extreme environments such as high salinity, variable pH and extremes in temperature (Aparna *et al.*, 2012; Khopade *et al.*, 2012a, b; Marti *et al.*, 2014).

28.8 General Applications of Biosurfactants

Biosurfactants, as mentioned earlier, are produced in different quantities by a variety of microorganisms. These molecules are produced by microorganisms to fulfil a number of functions like self-defence or the ability to feed. The same principles are used in developing them for various applications. As stated above, the diversity in their chemical structures gives rise to a variety of functions which include the reduction of surface tension and thereby the reduction of interfacial tension. They also increase surface areas which have been proved useful in water insoluble-hydrophobic compounds. Their ability to disperse or dissolve hydrophobic compounds has made them useful in a variety of industries such as the pharmaceutical, food and energy industries.

They are also capable of quorum sensing with the ability to initiate cell–cell signalling. These novel substances have the ability to bind heavy metals. They can be pathogenic to bacteria and also have the ability to form biofilms. The sectors where they are used are presented below with examples of their applications.

Table 28.3. Examples of biosurfactant-producing microorganisms, culture process in batch condition and biosurfactant properties.

Microorganism	Culture time (h)	Fermenter type[a]	Biosurfactant[b]	Surface tension (mN/m)	Yield (g/l)[b]	Reference
Pseudomonas cepacia	144	SF	NR	27.5	5.2	Rocha e Silva *et al.* (2014)
Nocardiopsis B4	96	SF	Rhamnolipid	30	NR	Khopade *et al.* (2012a)
Pseudomonas sp. 2B	168	SF	Rhamnolipid	29.7	4.97	Aparna *et al.* (2012)
Streptomyces sp. B3	216	SF	Glycolipid	29	NR	Khopade *et al.* (2012b)
Bacillus subtilis	72	SF	Surfactin	27.4	6.2	Marti *et al.* (2014)
B. subtilis	33	BR	Surfactin	27.4	2.5	Marti *et al.* (2014)
Bacillus licheniformis	10–72	BR	NR	28	NR	Joshi *et al.* (2013)
Pseudoxanthomonas sp. PNK-04	Up to 120	SF	Rhamnolipid	29	2.8	Nayak *et al.* (2009)

[a]SF, Shake flask; BR, bioreactor.
[b]NR, Not reported.

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28.8.1 Environmental applications

Since their discovery and isolation in 1965 up until the current time, biosurfactants have been employed in a variety of applications chief among which is as bioremediation agents in the environmental clean-up of oil-contaminated areas. This technique is known as microbial enhanced oil recovery (MEOR) as they are surface-active agents with low toxicity and excellent emulsifying capabilities which are stable even under extreme conditions, as shown by research. In a study by Xia *et al.* (2011) biosurfactants from three bacteria were compared for their bioremediation activity and they all showed good promise in oil recovery even when used at extreme levels of pH, temperature, metal ions and salinity. The three bacteria used were *P. aeruginosa*, *Bacillus subtilis* and *Rhodococcus erythropolis* and *P. aeruginosa* showed the highest emulsification index of 80%. These highly efficient bioremediation agents are being used to clean up land contaminated by hydrocarbons and heavy metals as it has been found that only about 30% of oil contaminants can be removed by conventional primary and secondary techniques.

28.8.2 Agricultural applications

Biosurfactants have been used in agriculture as a measure against pests. This use has been made possible due to their antimicrobial effects, especially against plant pathogens. An added advantage of using biosurfactants in agriculture is their safety margin compared with synthetic surfactants that leave residues on agricultural produce. This use has been demonstrated by the application of rhamnolipids extracted from *Pseudomonas* sp. EP-3 as a pesticide against the green peach aphid (Kim *et al.*, 2011). Another study on the use of biosurfactants in agriculture showed that rhamnolipids are recognized as conferring immunity to plants by triggering signalling pathways known as microbe-associated molecular patterns (MAMPs). This activation of MAMPs confers immunity to the plants in a way similar to how the pathway works in mammals (Vatsa *et al.*, 2010).

28.8.3 Food industry applications

Another area where biosurfactants are being used is the food industry where they are used as antimicrobial agents to prevent food-borne pathogenic infections. A recent study published in 2013 showed that rhamnolipids in particular are active against a wide range of Gram-negative and Gram-positive bacteria which include *Salmonella typhimurium*, *Escherichia coli*, *Enterobacter aerogenes*, *Staphylococcus aureus*, *Clostridium perfringens* and *Bacillus suptilis*. Rhamnolipids also showed fungicidal activity against *Phytophthora infestans*, *Phytophthora capsici*, *Mucor* spp., *Botrytis cinerea* and *Fusarium graminearum*. The research showed the remarkable effect of rhamnolipids against *Listeria monocytogenes*, a Gram-positive bacterium that is one of the most virulent food-borne pathogens responsible for an unprecedented number of deaths related to food contaminated by this organism. The study, however, showed that although the rhamnolipids effect was bacteriostatic, the addition of nisin remarkably increased the effectiveness of the biosurfactant (Magalhães and Nitschke, 2013).

28.8.4 Cosmetic industry applications

Biosurfactants have been used in the cosmetic industry for quite some time and they have been found to be safer alternatives compared with some chemical surfactants. These novel molecules are used in the cosmetics industry due to their excellent wetting, emulsifying, dispersing, solubilizing, foaming and most especially detergent effects. A number of cosmetic products containing rhamnolipids have been patented for use as anti-ageing products and anti-acne products. Their use has been successful as they are compatible with the skin and cause very low or no irritation when used as personal care products (Lourith and Kanlayavattanakul, 2009).

28.8.5 Application as antimicrobials

Biosurfactants have been found to be excellent algaecides as shown by a research on their algicidal activity. The harmful algal bloom (HAB)

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species of algae, *Heterosigma akashiwo*, *Prorocentrum dentatum* and *Gymnodinium* sp., are known to be harmful to human health and research using rhamnolipids from *P. aeruginosa* showed that it was able to inhibit their growth at a low concentration (0.4–3.0 mg/l) causing cell lysis. The study showed that rhamnolipids were able to inhibit these harmful organisms by breaking down their plasma membranes thereby irreversibly damaging their inner structures leading to loss of function (Wang *et al.*, 2005).

28.9 Conclusion

Biosurfactants are unique biomolecules with a variety of functions that are fast becoming a more efficient and greener alternative to their predecessor, chemical surfactants. Biosurfactants occur in various forms and they can be characterized based on either the charge they carry on their surfaces, their chemical structure or even their molecular weight. They occur as rhamnolipids, glycolipids, sophorolipids, surfactin and viscosin. They can also occur as lipopolysaccharides and fatty acids. They are surface-active biomolecules that act by reducing surface tension between different layers of liquid surfaces. They are great detergents and have excellent foaming properties and as such they are used in a number of industries that include the food, agricultural and pharmaceutical industries. The environment and the oil industry are the two areas where the impact of biosurfactants have had the most effect as they have been used and studied extensively in bioremediation and environment clean-up of oil spillages. They are also used in the cosmetic industry due to their history of safety and low toxicity.

Biosurfactant production can be a costly process which can be made less so by varying production modalities and parameters. Production can be either by batch, fed-batch or continues fermentation methods when down streaming and growth-controlling factors, especially the nutrient, can be altered to optimize production. Biosurfactants can therefore be produced in adequate quantities using bioreactors and cheap feedstock as nutrient sources. Knowledge of biosurfactants, their characteristics and uses is expanding and more invaluable research is being conducted into optimizing productivity and reducing costs. The benefits of biologically produced surfactants cannot be denied and they surpass conventional chemical surfactants in many ways but there are major limitations still facing their industrial application. Their low yield and high cost when compared with chemical surfactants has started to receive more biotechnological research in order to successfully overcome these limitations. If momentum is maintained, we will start to see commercially available biosurfactant products being utilized by industries such as oil recovery, fuel extraction and medicine within the next decade.

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29 Recent Developments on Algal Biofuel Technology

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Abstract
Microalgae provide great promise for the production of biofuels as they have the highest growth rates of all photosynthetic organisms and have demonstrated high levels of desirable products required for biofuel production without competing for land or resources from the agriculture industry. It has been established that the type of algae chosen to cultivate is very important. This can affect which techniques may be best to use for harvesting and converting algae to biofuels. It is important to consider the lipid content of the algae and its growth rate as a high yield shall be needed from the process, but it must also be of high quality. The research that has been conducted so far has proven very useful, however, there is still much to learn when it comes to the best techniques to use for the production of biofuels. Any process utilizing microalgae needs to be cost-effective to be commercially viable and be able to fit into the current fuel demand without altering distribution or storage processes. In order to achieve a positive energy balance which will be important when considering cost-effective processes, a highly optimized production system is required. In recent scientific discussions, many of the environmental impacts and cost-performance points raised produce both challenges and opportunities that would require further research and the potential integration of other processes. Once a gold standard has been established for the cultivation of algal biomass and lipid extraction methods, commercialization of algal biofuel production will soon follow.

29.1 Introduction

There have been many new developments in the field of biotechnology over the past 5 years, and marine biotechnology has been one of the most researched disciplines since it was recognized in the 1980s (Burgess, 2012). Marine biotechnology is a discipline where resources come from ocean habitats rather than land habitats. The ocean has a high biological and chemical diversity; this is due to the ocean having so many unique habitats and these have existed for so many more years than terrestrial habitats. Recent advances such as whole genome sequencing and metagenomics are revealing greater biodiversity than previously thought in the marine environment, diversity that remains largely untapped (Ritchie *et al*., 2013). Extreme environmental conditions such as temperature, light and the change in oxygen (O_2) concentration have resulted in adaptation of many organisms with unique features where new bioactive compounds have been created.

Biofuel is a renewable energy source produced from biomass which can be used as a substitute for petroleum fuels (Demirbas, 2010). The ever-growing requirement for biofuels is somewhat demanding, relying on the ability for

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them to be able to fit into the existing infrastructure for storage and distribution on a huge scale without competing with food crops for land or the food industry for biomass (Leite, *et al.*, 2013; Li *et al.*, 2013). Due to the growing concern associated with the depletion of non-renewable fuel sources, many sources of biomass have been proposed as biofuel feedstock, these include vegetable oil, jatropha and microalgae (Perez-Garcia *et al.*, 2011; Suali and Sarbatly, 2012). This depletion has increased in recent years due to rapid development growth in newly developing countries and also higher consumption rates of currently utilized fuels as a result of an increase in industrial growth (Adenle *et al.*, 2013). This suggests that we need to find a way of creating these fuel types manually, but also create them in a way that is environmentally friendly and reproducible on a commercial scale.

Microalgae are aquatic photosynthetic organisms with the simple composition $CH_{1.7}O_{0.4}N_{0.15}P_{0.0094}$, and they are able to convert carbon dioxide (CO_2) and water (H_2O) into biomass using sunlight (Demirbas, 2010; Zhu *et al.*, 2014). Algae have shown the ability to synthesize and accumulate a variety of high energy molecules including fatty acids and triacylglycerols. This accumulation has shown a lipid content of up to 73.4% which mostly consists of these high energy molecules which can be converted through the process of transesterification into biodiesel (Suali and Sarbatly, 2012; Leite *et al.*, 2013). It has also been found that some microalgal species can double their biomass in as short a time as 3.5 h. Microalgae have a higher growth rate than terrestrial plants and require less water in order to grow. In addition to this, microalgae can potentially be produced in an economical and sustainable manner (Demirbas, 2010; Lam and Lee, 2012; Suali and Sarbatly, 2012).

There are several main hurdles facing the economical and sustainable production of algal biofuel including cultivation, lipid yield, extraction, purification and cost. Lam and Lee (2012) critically discussed the concerns and limitations of processing microalgae for the production of biofuels and the potential way forward. The discussion focused around two alternatives for the cultivation of photoautotrophic algae: (i) open pond systems; and (ii) photobioreactors (PBRs). Environmental concerns that they highlighted were also described as having positive potential if the processes are integrated with other industrial processes such as water remediation, heat and CO_2 from power generation, toxicological, chemical and biomedical research. One concern that was raised is that leakage and transfer of genetically modified algae or natural algae into the environment is unavoidable (Lam and Lee, 2012). Fuels have already been developed from the fermentation and esterification of some crops in the USA and some European countries (Adenle *et al.*, 2013). These types of fuel are known as first-generation biofuels; however, such first-generation biofuels are not entirely supported as cultivating biofuel crops competes with food production for land and resources. This makes algae the perfect option as they can produce high lipid yield so can potentially create a large amount of biofuel without depleting or competing with any food crops. Crops could continue to be used as a fuel source, but in order to do so vastly increases in the amount of crops grown would need to be seen in order to cover the consumption of these crops in both the food and the fuel industry. This would be incredibly expensive due to the amount of manpower and land that would be needed. An additional problem with crops being used as a fuel source is that the competition for materials between the two markets would force prices to rise for consumers.

Microalgae have been promoted as one of the most promising third-generation biofuels for their ability to create such a large amount of lipid, to grow in low quality water and divide at a rapid pace (Rogers *et al.*, 2014). They have been found to be more efficient in converting sunlight to biochemical energy than terrestrial plants and green microalgae are known to have the same photosynthetic machinery as C3 plants; they can convert 4.6% of solar energy that they receive into biomass energy and have been known to reach up to 8.3% conversion (Chisti, 2013).

29.2 Algal Growth

There are several different methods of converting microalgae lipid into biofuel, but the first thing to consider is the growth of the microalgae and their lipid yield. There are two ways in which microalgae grow: (i) phototrophic growth; and (ii) heterotrophic growth. In phototrophic growth the algae uses light and CO_2 and releases O_2

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through the process of photosynthesis. For this process the algae need a light environment with plenty of CO_2, however, the growth of the algae is slow and their lipid content tends to be low. In heterotrophic growth the microalgae use sugars and O_2 and release CO_2. The algae need a dark environment. This type of growth tends to produce high lipid content in the algae and a fast growth rate (Fig. 29.1).

29.2.1 Open ponds

There are several culturing methods to consider for the growth of algae depending on the type of growth that is decided upon. Raceway ponds and circular ponds are the two main types of open pond currently used (Leite *et al.*, 2013). They are a simple and cheap way of culturing algae. Open pond cultivation has been in use since the 1950s (Christenson and Sims, 2011) and is the oldest and simplest method of mass cultivation of microalgae (Demirbas, 2010), however, only a select few species can be cultured in conventional open systems as the control of physico-chemical conditions is limited (Silva Benevides *et al.*, 2013). Open pond cultivation is carried out in shallow basins in order to prevent self-shadowing. The ponds are also open to the environment and this can present potential problems due to environmental contamination, contamination with unwanted algal species and inefficient use of CO_2 due to evaporation (Wen *et al.*, 2011). Open ponds are equipped with a device which creates hydrodynamic conditions to mix algal cells along a light path (Hadiyanto *et al.*, 2013). Sunlight is used as the main energy source for photosynthesis while CO_2 is injected into the pond system.

Raceways are one open pond method of algae cultivation. These ponds are closed-loop systems which are typically quite shallow to ensure that light penetrates all levels of the pond. In these ponds paddle wheels are used to mix the algae constantly; one paddle wheel is enough to mix algae in raceways covering very large areas and this maintains aeration and nutrient dispersion with low cost and low energy consumption while preventing sedimentation (Chisti, 2007; Jorquera *et al.*, 2010; Leite *et al.*, 2013). As the cultivation of algae depends heavily on large volumes of water, a large amount of land and water are needed. Lack of water due to evaporation can cause major sustainability issues if the process was to be scaled up (Rogers *et al.*, 2014), although evaporation of the water is essential in cooling to keep the appropriate pond temperature. Here is where covered raceways can be used, as any evaporated water could be collected and then redeposited into the raceways. Covered raceways have been found to support 4.2 times the production capacity compared with uncovered raceways. Most algal raceways use channels which have a constant depth and width, which end in hairpin bends with one or more paddle wheels to circulate the water (Liffman *et al.*, 2013).

Circular open ponds work in very much the same way as raceway ponds but the pond is

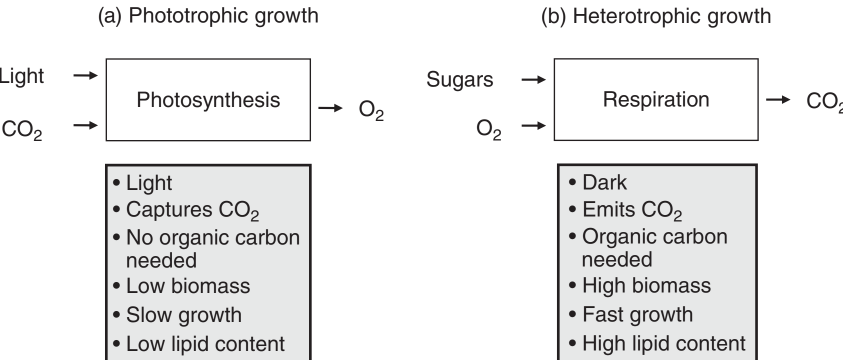

Fig. 29.1. Comparison of (a) phototrophic and (b) heterotrophic algal cultivation. (From Tang and Lewis, 2012.)

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entirely circular and mixing is maintained by a paddle which is fixed at the centre of the pond to the perimeter edge and rotates slowly. In comparison to raceway ponds, circular open ponds have poor mixing and light distribution properties (Suali and Sarbatly, 2012).

29.2.2 Photobioreactors (PBRs)

PBRs tend to be a mass of connected suspended compartments which contain algae and water that are stirred by mechanical stirring and/or bubble sparging. This method of cultivation heightens the absorbance as sunlight is reflected off the clear surfaces and back onto the algae (Lee *et al.*, 2014). PBRs, unlike open ponds, permit the culture of a single species of microalga and prevent environmental contamination of unwanted algal species and potential contamination of the environment with genetically modified microalgae (Leite *et al.*, 2013). In addition to this they also have high productivity per unit area and enable the control of pH, temperature, light intensity and nutrient availability (Chisti, 2007; Leite *et al.*, 2013). The most widely used PBR design is tubular which has a large surface area to volume ratio to maximize exposure to sunlight and sunlight penetration (Wen *et al.*, 2011). In closed systems like PBRs, the O_2 produced during photosynthesis will build up until it inhibits growth and potentially poisons the algae (Wen *et al.*, 2011; Suali and Sarbatly, 2012). This build-up of O_2 can be alleviated using a degassing zone where air is bubbled through the broth to remove excess O_2 (Chisti, 2007; Wen *et al.*, 2011).

These bioreactors can also be kept in the dark for heterotrophic growth (Fig. 29.2). Varied lipid contents were noted using heterotrophic culture methods by Suali and Sarbatly (2012) ranging from 44.8% when using glucose as the carbon source, to 73.4% using different carbon sources such as crude glycerol or sweet sorghum in the culture medium. Despite this high lipid content, heterotrophic microalgae cultivation would not be as cost-effective as phototrophic culture when the carbon source has to be purchased. Research has found that *Chlorella* spp. can be manipulated to utilize CO_2 (Suali and Sarbatly, 2012). This is seen as a promising source of organic carbon, when CO_2 produced by power plants burning fossil fuels is often available at little or no cost (Chisti, 2007). This could provide a suitable alternative as a carbon source using a monoculture under heterotrophic conditions. In addition to this *Chlorella* spp., especially *Chlorella pyrenoidosa*, *Chlorella ellipsoidea* and *Chlorella vulgaris* have been found to be suitable for further research for microalgae cultivation for biofuel production. This is due to their successful cultivation both in heterotrophic and in photoautotrophic conditions while still producing a high lipid content (Han *et al.*, 2012).

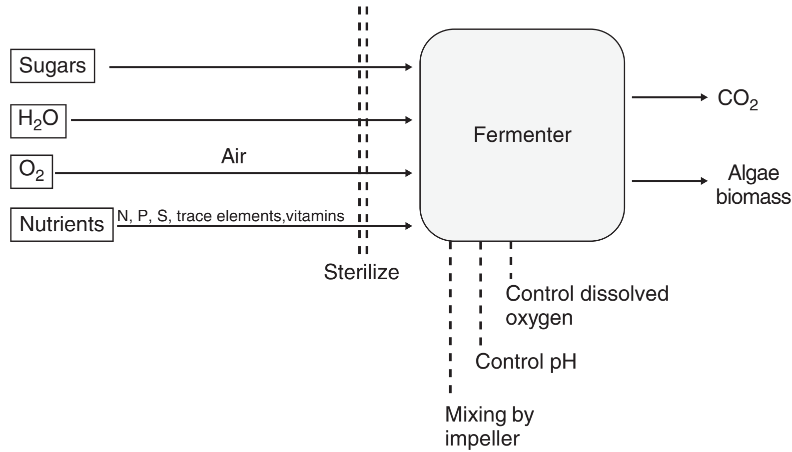

Fig. 29.2. Algal cultivation in heterotrophic (dark) system. (From Tang and Lewis, 2012.)

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29.3 Separation Techniques

There are several harvesting methods used for the separation of the microalgae from the solution but there is currently no gold standard or universal algae harvesting method widely available (Mata *et al.*, 2010). Harvesting the algae from the cultivation stage can be very costly, costing between 20% and 30% of the total cost of production, as cell concentration is very dilute and therefore a large volume of medium needs to be treated in order to harvest suitable quantities of biomass for lipid extraction (Wen *et al.*, 2011). Harvesting methods can be chemically, mechanically or to a lesser extent electrically based. Examples of these include flocculation, filtration, centrifugation, sedimentation, flotation or ultrasound techniques (Suali and Sarbatly, 2012). Variations or sequences of these methods are also commonly used. These methods all discriminate on a size and density basis (Mata *et al.*, 2010) which can hinder specific techniques when using different algae species due to the natural variation in size. Flocculation is not a critical step in separating algal cells from the medium as processes have been described just involving sedimentation, filtration or centrifugation alone (Wen *et al.*, 2011) but the aggregated cells become easier to harvest as flocculating agents lead to the coagulation of the algae (Suali and Sarbatly, 2012). Biological methods are also being researched as a more eco-friendly and cost-effective way of harvesting algae.

29.3.1 Flocculation

Chemical flocculation is mostly used as a pretreatment for small algal cells to increase the particle size before using another harvesting method such as flotation or filtration (Christenson and Sims, 2011). The use or potential use of flocculation as part of a biomass harvesting technique has been described in recent research (Christenson and Sims, 2011; Wen *et al.*, 2011; Lam and Lee, 2012; Suali and Sarbatly, 2012; Leite *et al.*, 2013). Ideal flocculants would have to be low cost, non-toxic and effective at low concentrations in order to be commercially viable due to their high cost (Wen *et al.*, 2011). Flocculation can speed up or facilitate other separation techniques although many flocculating agents are described as being toxic and so prevent the use of the biomass after lipid extraction as animal feed or for other processes (Leite *et al.*, 2013). The use of metal salts for coagulation and flocculation is cautioned when considering a downstream process. Methanogenic activity of acetogenic and methanogenic bacteria fed wastewater sludge has been found to be inhibited by aluminium and sulfate. Application of sludge treated by aluminium has been seen to increase heavy metal uptake and cause phosphorus deficiencies in plants, however natural polymers may also be used as flocculants but this had not been as extensively researched (Christenson and Sims, 2011). The efficiency of flocculation depends highly upon the type of algal cells used. The amount of cells flocculated tends to depend on the concentration of the algal cells. Cationic starches are an ecologically friendly and effective alternative for the processing of algae to biofuel (Gerde *et al.*, 2014). More recently, bioflocculants have been described in Kim *et al.* (2013) as being environmentally friendly and demonstrating successful harvesting of 95% of biomass. This could potentially overcome the inherent problem of toxic contamination when using traditional chemical flocculation and is certainly something that requires further investigation.

29.3.2 Sedimentation

The use of sedimentation as a harvesting method is very low cost and can typically give concentrations of 1.5% solids (Christenson and Sims, 2011), but reliability is low due to the fluctuation in algal cell sizes. It is also a slow process as the algal cells are left to drift to the bottom of the culturing media naturally, and this could affect the quality of the algae if it is left for a period of time. Sedimentation is achieved by some algae strains that have the property of natural sedimentation in the absence of mixing. The capital and operational costs of this harvesting method are low but the process can be time-consuming (Wen, *et al.*, 2011; Leite *et al.*, 2013). The sedimentation process can be speeded up and increased in algae with low sedimentation properties by the addition of a flocculation agent (Wen *et al.*, 2011).

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29.3.3 Centrifugation

The most reliable and quick method of harvesting algae is possibly the use of a centrifuge, which separates the suspended algae due to the density of the mixture. Centrifuges with nozzle-type disks are easily cleaned and sterilized, making them ideal for any type of algal separation. The samples are kept pure and uncontaminated ensuring the end product will remain of good quality. However, this form of harvesting is very expensive, making it an unrealistic permanent harvesting method. The process also involves exposing cells to high gravitational forces which can damage the cell structure (Knuckey *et al.*, 2006). Centrifugation provides an effective separation of biomass from the medium within a short time but incurs a high cost due to the initial start-up costs and power consumption (Kim *et al.*, 2013). This harvesting technique is currently used for the recovery of high-value products to avoid bacterial contamination or fouling of the raw product but it is claimed that it would not be cost-effective for the recovery of biomass for biofuels due to their lower value (Mata *et al.*, 2010; Kim *et al.*, 2013). It has, however, been discussed that if the volumetric throughput was increased there is potential for it to become more cost-effective but with lower efficiency (Leite *et al.*, 2013).

29.3.4 Filtration

In past studies low-cost filtration has been used to harvest filamentous algae strains at a high rate from algal ponds (Christenson and Sims, 2011). Microscreening is used to retain larger cells and wash out smaller cells of algae that are non-filamentous. However, the use of filamentous algae has been found to be unrealistic as they have a low lipid content. For algae of a smaller size the method that is considered most reliable is that of tangential filtration. Filtration is currently widely used in biotechnology for extraction of products from a liquid medium because there is no requirement for chemicals, it is a continuous operation and it has high separation efficiency. It also enables the recycling of the culture medium which may contain valuable nutrients for reuse (Kim *et al.*, 2013). Most microalgae are too small for filtration (Leite *et al.*, 2013) and those that are small cause plugging of the filter which is a major limitation for filtration (Wen *et al.*, 2011). Plugging can be overcome by backwashing to maintain the efficiency of the membrane but this is time-consuming (Suali and Sarbatly, 2012). A lot of filtration processes have been increasing in cost due to the need for pumping, backwashing, additional steps and possible replacement of membranes on a commercial scale (Mata *et al.*, 2010).

29.3.5 Ultrasound

Ultrasound presents the possibility of both algal harvest and lipid extraction (Kim *et al.*, 2013) although further research needs to be completed in order to upscale this process for industrial use. In open ponds ultrasound doesn't just have the ability to cause aggregation of the microalgae but it can also affect other sediments such as mercury (Suali and Sarbatly, 2012). At particular wavelengths ultrasound can enable the microalgae to aggregate with no shear stress at low frequency but high amplitude ultrasound has the potential to rupture the cells, releasing their contents (Kim *et al.*, 2013).

29.3.6 Biologically based harvesting methods

Research into biologically based harvesting methods has shown that algae can flocculate spontaneously without the use of chemicals. Research has shown that increasing the natural nutrients plants use to flocculate can increase the flocculation rate by up to 100% when the nutrients in the culture medium were doubled (Silva and Silva, 2007). It would be incredibly beneficial to control this natural development as it would be much more eco-friendly and much less costly, however, further research needs to be conducted to explore all novel biological harvesting options.

29.4 Drying Methods

Once the algae have been harvested they need to be dried before the oil can be extracted. Various

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drying methods such as sun drying, freeze drying, spray drying, hot air oven and drum drying are used to remove excess water from the biomass, as solvent-based lipid extraction is difficult when wet biomass is used (Wen *et al.*, 2011). Oil yield is generally much lower with wet biomass compared with lipid extraction after a dewatering stage (Suali and Sarbatly, 2012). One drying method used is solar drying which was originally designed for the drying of seaweed for the possible use as biofuel. Solar power has become a very popular source of energy over the past few years when it comes to housing and industrial use. Most marine products require a drying stage in the process to produce a quality product (Fudholi *et al.*, 2014). Extraction of lipids from the harvested algal biomass is receiving a great deal of research, as lipid extraction methods that do not require a very low water content (and hence a drying stage) would make the harvesting process much simpler and cheaper (Leite *et al.*, 2013). The biomass must be processed rapidly in order to avoid the biomass from spoiling due to the enzymes within the algae hydrolysing the cellular lipids into free fatty acids (Wen *et al.*, 2011). It is important that during the oil extraction methods the lipids do not get damaged, however, the method still needs to be quick, effective and easily scalable (Pragya *et al.*, 2013).

29.5 Lipid Extraction

For oil to be extracted from the algal cells, the cells need to be lysed and the cell membranes broken. Current commercial oil extraction methods from crops such as palm oil use heating under high pressure before mechanical pressing. This technique requires no chemical solvents so preserves the quality of the extracted oil, but it does have limitations when producing biofuels from algae due to the small size of microalgal cells (Suali and Sarbatly, 2012).

29.5.1 Supercritical CO_2 extraction

Supercritical CO_2 is one method of effective oil extraction that is non-toxic, has a low critical temperature, easy separation after extraction and a high diffusivity and low surface tension (Lam and Lee, 2012). Despite these advantages of supercritical CO_2 extraction, it has high costs and safety issues which have made CO_2 extraction of microalgae oils expensive and unsuitable until recently (Wen *et al.*, 2011; Lam and Lee, 2012; Suali and Sarbatly, 2012).

29.5.2 Electrical extraction

Some extraction methods use cell disruption by ultrasound or osmotic shock but each of these methods normally uses further chemical extraction using solvents such as hexane or a hexane-ethanol mixture which contaminates the remaining biomass (Mata *et al.*, 2010; Suali and Sarbatly, 2012). One such solvent extraction method is Soxhelt extraction, which uses hexane to extract lipids from algal cells. This technique can be used on its own or alongside another technique such as the oil press/expeller pressing method. After the lipids have been initially extracted by the expeller the remaining lipids can be extracted using cyclohexane which dissolves oil into itself so that the pulp can be removed (Pragya *et al.*, 2013).

29.5.3 Solvents

Extraction of lipids from the algal cells can be done through the use of solvents. The idea with using a solvent is that non-polar organic solvents disrupt hydrophobic interactions between non-polar/neutral lipids (Pragya *et al.*, 2013) and through shifting the pH of the mixture to be more alkaline it can disrupt the hydrogen bonds between polar lipids. The type of solvent used depends on the algae that are being used, though it should be kept in mind that using a solvent that is expensive would not be viable for large-scale production. The solvent would also need to be volatile, non-toxic, non-polar and a poor extractor of other non-lipid parts of the algal cells. When Ramluckan *et al.* (2014) came across this issue they used an ultraviolet light to determine the amount of chlorophyll in the extracted oil. They also used a chromatographic method to identify the quantity of useable lipids. Although these additional methods solve the problems with the unwanted extracted lipids it would also make the process much more expensive and time-consuming.

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A solvent which has both polar and non-polar properties has recently been described in the literature (Suali and Sarbatly, 2012; Leite *et al.*, 2013). This extraction method uses switchable solvents which can be changed from polar to non-polar or hydrophilic to hydrophobic when bubbled with CO_2 (Suali and Sarbatly, 2012; Leite *et al.*, 2013). This enables the solvent to enter the algal cell when miscible with water and then when switched they extract the lipid out of the cells and out of the liquid. Following extraction the solvent can be easily separated from the biomass and can consequently be reused, reducing production costs. This form of extraction may be suitable to use on undried biomass but future research is required to investigate this.

29.6 Lipid Conversion

From the extracted lipids biodiesel is produced by transesterification of the triglycerides into esters, which is the final biodiesel product, and glycerol, which is a by-product. The glycerol by-product can be used as a carbon source for heterotrophic algal growth so this process produces no waste (Leite *et al.*, 2013; Zhu *et al.*, 2014). Biodiesel is already produced using oilseeds and the reagents used for transesterification in these processes can easily be transferred and applied to microalgae biodiesel production (Leite *et al.*, 2013). Transesterification is thought of as the most useful method of converting oil to biodiesel, as the process of converting the fatty acids to biofuel have the closest characteristics to those of diesel fuel (Pragya *et al.*, 2013). Through the process of transesterification, raw lipids extracted from algae are converted to fatty acid alkyl esters which have a lower molecular weight. A major drawback to this method, however, is the use of methanol to drive the reaction as this affects the cost and sustainability of the conversion method (Daroch *et al.*, 2013).

Lipids from algae may also be converted to biodiesel through a process called pyrolysis, which is a thermochemical technique which uses heat and a lack of oxygen to break down the lipids. The product of this process is a liquid that has similar chemical components to that of petroleum diesel (Pragya *et al.*, 2013). The known problems with this technique are temperature, particle size, sweep gas flow rate and reactor types (Hu *et al.*, 2013).

29.7 Alternative Products

As well as the production of biodiesel there are other possible fuels that could be created through the use of different processes. Wen *et al.* (2011) also looked into biofuel from microalgae. One such fuel that they researched was biogas, such as methane produced from anaerobic digestion, which can be used as a heat source or to supply electricity (Fig. 29.3). They mentioned bio-oils and syngas as products from algae through the process of thermochemical conversion, which can also be categorized as gasification, pyrolysis or thermochemical liquefaction. The end products of these processes can vary between solid, liquid and gas forms. From this it appears that biodiesel is not the only useful fuel that can be created from algae.

Other potential products from microalgae include biogas, fertilizer, cosmeceuticals, nutraceuticals and pigments (Suali and Sarbatly, 2012) all of which have a much higher value when sold on so the production of these in a closed-cycle biorefinery concept would increase the potential of industrial-scale production of biodiesel and biofuels. Bioethanol can be obtained by the fermentation of sugars which are produced by hydrolysing the carbohydrates in microalgal cells, and the production of bioethanol can be performed simultaneously with production of biodiesel but bioethanol production is still in the preliminary research phase (Lam and Lee, 2012; Zhu *et al.*, 2014).

29.8 Concerns, Limitations and Further Thoughts

Wen *et al.* (2011) discovered that it would still be too expensive to use algae as a biofuel due to the cost of oil extraction and biodiesel production, which would make it too much to commercialize. It is also highlighted that biogas production would be much more beneficial as it is much cheaper than the production of other biofuels, and it could largely contribute to a widely used

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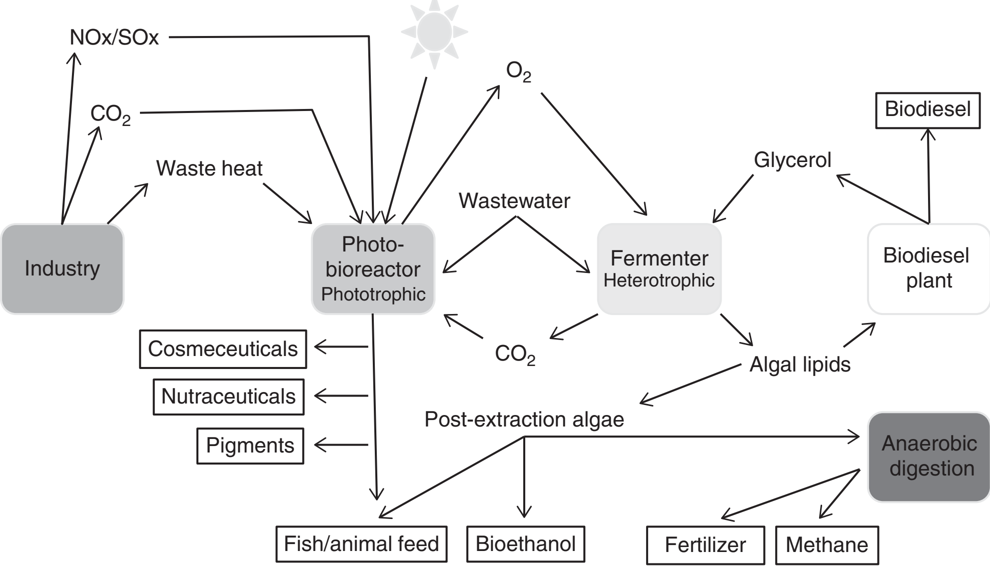

Fig. 29.3. Biorefining and closed-cycle concept. (From Tang and Lewis, 2012.)

fuel source such as electricity. However, biodiesel is a very important fuel source as it could be used to power transport vehicles, which would cause much less destruction to the environment than normal diesel/petrol used now. This puts across the point that new technologies need to be developed that can extract the oil from algae but also turn it into biodiesel more cheaply. Waste products from this process could be sold as animal feed, as it is full of proteins and carbohydrates. On the other hand these authors also had the thought that if the raw biomass was used for methane production instead of biofuel production, this would massively reduce the costs as there would be no oil extraction or production of biodiesel.

Suali and Sarbatly (2012) concluded that microalgae are feasible as a biofuel feedstock, and the production could be made cheaper by using a solvent that can be reused for the extraction of lipids from the microalgae. It would also be cheaper than other crops grown for biofuel production as less water is needed for the process, algae can reproduce more rapidly than normal plants and they have a high lipid content. Along with microalgae being used to create an alternative fuel source, they can also be beneficial in wastewater treatment and the production of pharmaceuticals. Nutrients are an essential element of algal growth, the sustainability and costs of which depend on the source of the nutrients and the recyclability (Rogers *et al*., 2014). CO_2, nitrogen and phosphorus will need to be supplemented as biofuel technology develops, possibly through locating algal farms close to power plants and wastewater treatment plants in order to utilize their waste products.

Zheng *et al.* (2012) looked into the use of both phototrophic and heterotrophic growth being used together. They established that using open ponds and runways to culture algae was difficult as the algae take so long to multiply. Using other culturing methods such as PBRs is extremely costly, however, creating a problem when it comes to high yield production. Their main idea was to use a two-stage process involving phototrophic and heterotrophic growth, which would take the advantage of low cost from phototrophic growth and high efficiency from heterotrophic growth. They determined that heterotrophic culturing can be used as a better process to produce seed cells from the algae in large open systems, as its productivity was much higher but had the same standard of performance. Organic waste can also be used as a feedstock for heterotrophic growth making it good for the environment. Zhu *et al*. (2014) also discussed the potential of a recycling system in a

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microalgae biorefinery chain but did not consider other potential products such as cosmetics, pharmaceuticals or nutritional supplements. Suali and Sarbatly (2012) briefly discussed the potential for pharmaceutical products that can be obtained from microalgae and also the possibility of combining wastewater treatment plants with microalgae culture methods in order to remove and utilize waste products to the benefit of the culture system.

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30 Microbial Lipases: Emerging Biocatalysts

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Abstract

Microbial lipases (EC 3.1.1.3) are an important group of biotechnologically valuable enzymes. They are enantioselective, chemoselective and stereoselective in nature, which makes them an important tool in various medical and industrial fields. Lipases also have unique characteristics as they act at the oil/water interface. Microbial lipases can be distinguished from each other in structure, substrate specificity and the biochemical environment in which they are secreted, and therefore can be classified under different categories. Due to their commercial importance and ecological as well as economical significance, the market value of these enzymes is continuously growing and new or improved enzymes will be a key element in the emerging realm of biotechnology. The current need is isolation of novel microbial lipases from various sources and their characterization for development of safer and cheaper formulations for industrial and medical prospects. Maximum utilization of such lipases will be possible only by detailed study of their isolation, production, molecular structure and kinetics. In addition to this, physical and chemical factors and sources that affect lipase production and activity also have to be considered. This chapter presents an overview of the microbial lipases with emphasis on their classification, sources, structure, mechanism of lipolysis and the various applications of these biocatalysts.

30.1 Introduction

The history of modern enzymes can be assumed to have started in the late 19th century, when the Danish chemist Christian Hansen produced rennet by extracting it from calf stomach with saline solution. Since then researchers all over the world have been working on discovery, isolation, characterization and application of enzymes in all walks of life, but the industrial biotechnology sector experienced a major breakthrough when it was understood that enzymes could be exploited commercially. Nowadays a significant number of enzymes are being produced and sold for various purposes and the upcoming industrial enzyme market is one of the biggest revenue generators in the life-sciences-based industry sector. According to a report given in 2012 by global strategic business, the market for major product segments like carbohydrases, proteases and lipases all over the world is supposed to exceed by US$29 billion (ReportLinker, 2012).

Of the various enzymes exploited commercially, lipase is one of the most important enzymes. Lipases responsible for lipolytic activity of microorganisms (EC 3.1.1.3) are reported to be found in bacteria, fungi and archea (Saxena *et al*., 1999, 2003; Gupta *et al*., 2004; Verma *et al*., 2012).

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The International Union of Biochemistry and Molecular Biology (Tipton, 1994) classifies lipolytic enzymes under the Enzyme Classification (EC) number EC 3.1.1. (carboxylic ester hydrolases) according to which lipase typically acts at a water/lipid interface, on the carboxylic ester bonds in substrates such as mono, di and triglycerides, phospholipids, thioesters, cholesterol esters, wax esters, cutin, suberin, synthetic esters or other lipids mentioned in the context of EC 3.1.1.

Various food samples have been screened for the presence of lipolytic bacteria. Presence of lipolytic bacteria has also been reported in dairy products (Ruban *et al.*, 1978; Griffiths *et al.*, 1981; Cempirkova and Mikulova, 2009; Samaržija *et al.*, 2012). In addition to these, existence of lipolytic bacteria has also been described in spoiled frozen marine fish (Joseph *et al.*, 2007). Santos *et al.* (1996) described the presence of lipolytic bacteria from Villalón cheese (a fresh variety of Spanish sheep's milk cheese).

The psychrotrophs found in refrigerated bulk milk are mainly Gram-negative rod-shaped bacteria of the genera *Pseudomonas*, *Achromobacter*, *Alcaligenes* and *Enterobacter* (Luck, 1972; Ray *et al.*, 2013), but Gram-positive psychrotrophs of the genus *Bacillus* have also been isolated (Thomas, 1974; Hantsis-Zacharov and Halpern, 2007). Most of the psychrotrophic bacteria (excluding *Bacillus* spp.) are killed by pasteurization (Witter, 1961), but these bacteria secrete extracellular enzymes which are extremely thermostable (Cocas, 1977).

The presence of lipases in bacteria had been observed as early as 1901 for *Bacillus prodigiosus*, *Bacillus pyocyneus* and *Bacillus fluorescens* (Jaeger *et al.*, 1994) which represent some of today's best studied lipase producers, and are now named, *Serratia marcescens*, *Pseudomonas aeruginosa* and *Pseudomonas fluorescens*, respectively. Lipolytic bacteria have already been purified from soil, or spoiled food material that is enriched with vegetable oils. Different types of lipase have been purified from taxonomically close strains. There are many microorganisms known to produce different lipases (Iizumi *et al.*, 1990). Highly alkaliphilic or thermophilic microbial lipases have been reported from *Alcaligenes* (Kakusho *et al.*, 1982). *Pseudomonas fragi* (Iizumi *et al.*, 1990) *Pseudomonas nitroreducens* (Watanabe *et al.*, 1977) and other *Pseudomonas* strains that produce lipase having different characteristics were isolated from soil. Choo *et al.* (1998) isolated a cold-adapted lipase-secreting psychrotrophic *Pseudomonas* strain from Alaskan soil. *Aeromonas hydrophila* secretes a lipolytic enzyme that has several properties in common with the mammalian enzyme lecithin-cholesterol acyltransferase (Brumlik and Buckley, 1996).

Commercialization of many microbial lipases has been done by industrial giants like Novozyme (Denmark), Amano Enzyme Inc (Japan), Biocatalysts (UK), Unilever (The Netherlands) and Genencor (USA). A few examples are: (i) lipases produced from the genera *Burkholderia* and *Pseudomonas*; (ii) Lipase PS and Lipase AK produced by *Burkholderia cepacia* and *P. fluorescens*, respectively, are sold by Amano; and (iii) Lipase SL and Lipase TL isolated from *B. cepacia* and *Pseudomonas stutzeri* have been commercialized by the Japanese company Meito Sangyo (Sangeetha *et al.*, 2011).

30.2 Three-dimensional Structure of Bacterial Lipase Enzyme

It is very important to understand the mechanisms underlying gene expression, folding and secretion for high-level production of these biocatalysts. Transcription of lipase genes may be regulated by quorum sensing and two-component systems: lipases can be secreted either via the Sec-dependent general secretory pathway or via the ATP-binding cassette (ABC) transporters. In addition, to achieve a secretion-competent conformation, some lipases need folding catalysts such as the lipase-specific foldases and disulfide-bond-forming proteins. Resolving the three-dimensional structure of bacterial lipases is helpful in order to understand the catalytic mechanism of lipase reactions. Structural characteristics involve an α/β hydrolase fold, a catalytic triad containing a nucleophilic serine located in a highly conserved Gly-X-Ser-X-glypentapeptide, and an aspartate or glutamate residue that is hydrogen bonded to a histidine. Four substrate-binding pockets were also identified for triglycerides: an oxyanion hole and three pockets accommodating the fatty acids bound at positions *sn*-1, *sn*-2 and *sn*-3. The enantio preference of a lipase depends on the differences in size and the hydrophilicity/hydrophobicity of these pockets.

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30.3 Mechanism of Lipolysis

Lipases have the ability to catalyse reactions such as hydrolysis, esterification, transesterification and lactonization (intra-molecular esterification) (Jaeger and Eggert, 2002; Joseph *et al.*, 2008). Since, the hydrolysis of fats and oils is a reversible reaction, modification of reaction conditions can direct the reaction towards synthesis. The water content of the reaction mixture controls the equilibrium between forward and reverse reactions. In non-aqueous environments, lipases catalyse the ester synthesis reaction. The ester synthesis reaction can be classified into simple esterification, transesterification and interesterification, depending on the nature of the reaction. The esterification reaction involves synthesis of glyceryl esters from glycerol and fatty acid. In transesterification, in place of fatty acid the acyl donar is an ester. Transesterification can be further divided into glycerolysis and alcoholysis, involving transfer of the acyl group from the triglyceride to either glycerol or an alcohol. In interesterification, the acyl group is exchanged between a glyceride and either a fatty acid (acidolysis) or a fatty acid ester. Interesterification requires a small amount of water, in addition to the amount needed for the enzyme to maintain an active, hydrated state. This flexibility in various reactions, associated with the possibility of different substrate specificity among the different lipases, gives these enzymes an enormous potential for applications (Gandhi *et al.*, 2000). Interfacial activation, a characteristic property of lipase, is a phenomenon in which the enzymatic activity of lipase is greatly increased when the substrate is presented at an oil/water interface. This phenomenon is a result of the unique structural characteristics of this class of enzymes (Jaeger *et al.*, 1999; Fabricio *et al.*, 2003). The three-dimensional configuration of lipase determines the enzyme stability. Structurally lipases possess a hydrophobic surface, often containing a helical 'lid' region associated with the active site. The main function of this helical 'lid' is to protect the active site (Aloulou *et al.*, 2006; Adlercreutz, 2013), and it is predicted that this surface associates with the hydrophobic phase at the interface between hydrophilic and hydrophobic fluids. The interfacial activation of lipase occurs primarily from conformation changes of the 'lid' surrounding the active site in response to the solvent conditions. This conformation change leads to exposure of the active site and provides a hydrophobic surface for interaction with the lipid substrate; therefore the substrate can access the active site more freely (Jaeger *et al.*, 1999).

Lipases have a characteristic catalytic property and particularly act on water-insoluble fatty substrates. Emulsion is the most popular lipid system used for estimation of lipase activity in laboratories. A 'core' or a bulk lipid phase surrounded by a surface monolayer of amphipathic molecules, is a characteristic property of emulsions. In the absence of other system components, the dispersed lipid droplets tend to coalesce in order to minimize the apolar surface exposed to water. In a typical biological system, the surface components of oil emulsions can be lipids, denatured proteins or other types of amphipathic compounds. Lipolysis by lipases occurs entirely at the lipid/water interface in an emulsion, implying that the concentration of substrate molecules at this interface (expressed in mol/m^3) directly determines the rate of lipolysis.

Lipases generally act on tri-acylglycerols, cholesterol esters and wax esters, which are insoluble in water. Tri-acylglycerols are the main substrates of lipases. They are uncharged lipids. The tri-acylglycerols with long-chain fatty acids esterified with glycerol are insoluble in water, although those with short-chain fatty acids are sparingly soluble in water. The maximum amount of solute that can be dissolved in solvent is called its saturation value. Formation of an emulsion from tri-acylglycerol occurs at concentrations greater than their saturation value. Phospholipids are natural substrates for phospholipases. Phospholipids are also insoluble in water, but they form micelles when exceeding the maximum concentration of dissolved monomer at a point called the critical micelle concentration.

The kinetics of lipase cannot be described with the Michaelis-Menten model because this model is valid only in the case of a single homogenous phase of soluble enzyme and substrate. A new model was projected by Verger *et al.* (1980) to explain catalysis by lipolytic enzymes. The model involved two successive equilibria. According to this, the water-soluble enzyme first penetrates into an interface. This is followed by a second equilibrium, in which one molecule of penetrated enzyme gets bound to one substrate

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molecule, forming the complex. This is comparable in two dimensions to the classical Michaelis-Menten equilibrium. Once the complex is formed, the catalytic reaction takes place, regenerating the enzyme along with the liberation of the products.

30.4 Lipase Production

Mostly bacterial lipase formation is induced by some form of oil, fatty acid alcohol or fatty acid ester and fatty acids (Borgstrom and Brockman, 1984; Shah and Bhatt, 2011). However, there are a few reports of constitutive lipase production by bacteria (Elwan *et al.*, 1983; Gao *et al.*, 2000). Lipases are usually secreted out into the culture medium although there are a few reports of the presence of intracellular lipases (Lee and Lee, 1989; Dulermo *et al.*, 2013) as well as cell-bound lipases (Large *et al.*, 1999). However, the onset of lipase production is organism specific, but, mostly it is released during late logarithmic or stationary phase (Makhzoum *et al.*, 1995; Mishra *et al.*, 2011). Normally, a cultivation period of 5–168 h is needed for lipase production but fast-growing organisms were found to secrete the lipase within 12–24 h (Steur *et al.*, 1986; Lee and Lee, 1989; Chartrain *et al.*, 1993; Lee and Rhee, 1993; Lin *et al.*, 1996; Imamura and Kitaura, 2000). Patents have been found on production of lipases from bacteria, however, published work is rather scanty (Gawel and Chen, 1977; Nakanishi and Ikeda, 1986; Inoue *et al.*, 1987; Holmes, 1990; Ishida *et al.*, 1995; Ishikawa *et al.*, 1995; Lawler and Smith, 2000). Research has been done on systematic medium optimization and fermentation studies for lipase production. A complex medium containing a carbon source (usually oil), a nitrogen source (organic/inorganic), a phosphorus source (sodium or potassium phosphate) and mineral salts, supplemented with micronutrients ($MgSO_4$ or $CaCl_2$) is used to grow organisms. The pH of the medium is generally maintained around 7.0. A pH range between 8.0 and 10.0 has been used for lipase production by alkaliphilic bacteria. Lipase production has been done using a variety of non-conventional carbon sources such as beef tallow, wool-scour effluent, whey, n-hexadecane and n-paraffins and Tweens (Gilbert *et al.*, 1991; Kosugi and Suzuki, 1998; Fonchy *et al.*, 1999; Papanikolaou and Aggelis, 2010).

30.5 Secretion of Extracellular Lipase

Several Gram-negative bacteria are known to secrete extracellular lipases, especially *Pseudomonas* and *Burkholderia* species. Four main secretion pathways have been identified in *P. aeruginosa* among which extracellular lipases use the type II pathway. Once they are secreted through the inner membrane via the Sec machinery, they reach the periplasm where they fold into an enzymatically active conformation. This action requires periplasmic folding catalysts comprised of specific intermolecular chaperones called Lif proteins (lipase-specific foldases). The chaperones ensure correct folding and proper secretion of lipases. Recently, a lipase variant from *Pseudomonas* species KFCC 10818 carrying just the single amino acid exchange Pro112Gln folded into its active conformation displayed 63% of the wild-type enzymatic activity, even in the absence of its cognate Lif protein. This finding may have important consequences for development of novel high-yielding strains.

In Gram-positive bacteria only a single cytoplasmic membrane has to be crossed by the secreted enzymes. Usually, these proteins contain a signal sequence, which directs their translocation via the Sec machinery. A second translocation pathway has also been described to operate in both Gram-negative and Gram-positive bacteria, named the Tat pathway since proteins using this pathway contain a unique *T*win *a*rginine *t*ranslocation motif in their signal sequence. In the *Bacillus subtilis* genome, 188 proteins have been identified as being potentially secreted. These include two lipases, of which LipA contains a Tat signal sequence, whereas the highly homologous enzyme LipB contains a Sec signal sequence.

30.6 Applications of Bacterial Lipase

Lipase activity usually depends on the available surface area. Lipases are the most adaptable biocatalysts and they bring about a range of bioconversion reactions such as hydrolysis,

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interesterification, esterification, alcoholysis, acidolysis and aminolysis. Table 30.1 enumerates a few of the most significant industrial applications of microbial lipases. Medical applications are another promising area where microbial lipase can be used as digestive aids. To date, only pancreatic enzyme (lipase) therapy is used to treat fat malabsorption in cystic fibrosis and pancreatitis patients. However, pancreatic lipase is susceptible to low pH (acidic gastric environment) and protease which can render the lipase inactive. Therefore, it is proposed that an alternative bacterial lipase might have the ability to retain its activity under acidic conditions and also be protease tolerant. In addition, these probiotic lipolytic lactic acid bacteria could also be very helpful to reduce the serum triglyceride level and may thus be beneficial for people suffering from higher cholesterol levels.

30.6.1 Hydrolysis of oils and fats

Several patents pertaining to the use of lipases as catalysts for the hydrolysis of oils and fats to produce fatty acids and glycerol have been granted. Examples include US patent numbers 4,629,742 and 4,678,580. Enzymatic hydrolysis of triglycerides can be used as a low-energy-requiring substitute to the present steam (Colgate-Emery) process for hydrolysing fats and oils. This may also result in the formation of products with better odour and colour. Use of immobilized enzyme may further reduce the cost of production. For the process to be successful, ideally the enzyme should have an operating temperature range which is above the melting point of the feedstock, such as BFT (bleachable fancy tallow). Unfortunately, the non-specific lipase from *Candida* sp. used in the above-mentioned patents has an optimal temperature of less than 42°C. Hence, there is scope for isolating new enzymes from more varied sources.

30.6.2 Interesterification of oils and fats

The composition and physical properties of triglyceride mixtures used in the oils and fat industries are modified by the interesterification process. For example products similar to cocoa butter are produced by 1,3-specific lipase-catalysed

Table 30.1. Applications of bacterial lipases.

Source	Application
Acinetobacter sp., *Acinetobacter calcoaceticus*	Waste management – heating oil/furnace oil, removal of fats, oils and greases (Mrin *et al*., 1995; Wakelin and Forster, 1997)
Arthrobacter sp.	Pesticide pyrethroids (Danda *et al*., 1991) and insecticide synthesis (Mitsuda *et al*., 1988)
Bacillus subtilis	Biomedical applications – cephalosporin derivative (Usher *et al*., 1995)
Chromobacterium viscosum	Biomedical applications – precursors of vitamin D (Fernandez *et al*., 1995), Verlukast – synthesis of a leukotriene D4 (LTD4) antagonist (Hughes *et al*., 1993)
Pseudomonas sp.	Biomedical applications – e.g. synthesis of (-)-indolmycin (Akita *et al*., 1997), pesticide synthesis – triazole/morpholine (Akita *et al*., 1995), lipase as detergent additive (Yokoe and Mase, 1988; Mukoyama and Umehara, 1989)
Pseudomonas cepacia	Biomedical applications – e.g. syntheisis of rapamycin-42 (Adamezyk *et al*., 1994), pesticide synthesis – nikkomycin-B (Akita *et al*., 1995), pyrenophorin (Sugai *et al*., 1995), fenpropimorph (Avdagic *et al*., 1994), racemic morpholine (Bianchi *et al*., 1992a, b), cyanohydrin acetate (Inagaki *et al*., 1992), pyrethroids (Inagaki *et al*., 1992)
Pseudomonas fluorescens	Biomedical applications – hydantoins (Yokomatsu *et al*., 1995), lamivudine (3TC) (Milton *et al*., 1995), racemic 2-tetradecyloxirane-carboxylate (Jimenez *et al*., 1997), pesticide synthesis – tetraconazole (Bianchi *et al*., 1990)
Staphylococcus warneri, *Staphylococcus xylosus*	Food industry – production of flavour esters (Talon *et al*., 1996)
Streptomyces sp.	Pancreatic lipase inhibitor – panclicins production (Yoshinari *et al*., 1994)

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interesterification of 1,3-dipalmitoyl-2-oleoyl glycerol (POP), which is a major triglyceride of palm oil, with either stearic acid or tristearin.

30.6.3 Esterification of fatty acids

Lipases are a category of enzymes that also have the ability to catalyse reversible reactions. This property of lipases makes it possible to use them for catalysis of the formation of esters from alcohol and fatty acids. One of the methods of production of glycerides is to incubate microbial lipases with a mixture of fatty acids and glycerol water.

30.6.4 Flavour development in dairy products

Most dairy products develop their specific flavour due to the degrading action of lipolytic enzymes on milk fat. Due to this property, lipolytic activity has guided enhancement of flavour in cheeses and butter and is very commonly used. In addition, cheese flavour preparations from butter oil and whole milk powder are also prepared in this manner. Recently, US patent number 4,726,954 has been granted for some fungal lipases that can be substituted for animal pre-gastric-esterases as a flavouring agent to enhance the flavour in cheese manufacturing.

30.6.5 Applications in the detergent industry

Detergents, especially those which boast of biological action for their activity, usually contain lipases along with proteases and celluloses. However, other enzymes such as amylases, peroxidases and oxidases are also reported to be added in detergent preparations (Kottwitz *et al.*, 1994). Removal of oil or fatty deposits by lipase is attractive owing to its suitability under milder washing conditions. To be a suitable additive in detergents, lipases should be both thermostable as well as alkaliphilic and capable of functioning in combination with the other components of washing powder formulations (Jaeger *et al.*, 1994). Lipase preparations from *Pseudomonas medocina* and *Pseudomonas alcaligenes* have been used to formulate the washing powders Lumafast and Lipomax, respectively. Lipases have been manufactured by Genencor International USA as a detergent additive (Jaeger *et al.*, 1994; Reetz and Jaeger, 1998).

Recently the fungal lipase gene from mesophilic *Humicola lanuginosa* has been introduced into *Aspergillus* spp. (Boel *et al.*, 1999). The enzyme thus produced removes fatty stains at temperatures from 15°C to 60°C and has a pH optimum of 9 or higher. This enzyme is primarily used in detergent formulations and is commercially available in Japan. Microbial lipases have also found application in the removal of fatty deposits in dishwashing products, leather manufacture and sewage treatment.

30.6.6 Lipases in the food industry

The modern food industry usually incorporates lipases in various processes (Momsia and Momsia, 2013). A large number of processes which use enzymes to improve the traditional chemical processes of food manufacture have been developed in the past few years. Yoneda *et al.* (1996) have been granted a patent on the use of *Pseudomonas* lipase in food processing and oil manufacture. Alcoholysis of cod liver oil for the production of omega-3 polyunsaturated fatty acids was investigated by using *Pseudomonas* lipase (Zuyi and Ward, 1993). Chang (2001) described the synthesis of fatty acid esters by a recombinant *Staphylococcus epidermidis* lipase. Lipases from *Chromobacterium viscosum* were found to have good potential for the quick generation of aroma and flavour compounds and could be stored for at least 1 month. In this case, the lipase activity was immediately regenerated on dehydration (Carlile *et al.*, 1996).

30.6.7 Lipases in biomedical applications

Lipases possess an extraordinary ability for specific region-selective reactions in a variety of organic solvents with broad substrate recognition.

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Due to this property they have emerged as an important biocatalyst in biomedical applications. Akita *et al*. (1997) have used immobilized *Pseudomonas* sp. lipase for enzymatic hydrolysis in organic solvents for the kinetic resolution of water-insoluble acetyloxy esters to produce chiral intermediates for the synthesis of the antibiotic (-)-indolmycin. A method was developed by Jimenez *et al*. (1997) to synthesize methyl (R)- and (S)-2-tetradecyloxiranecarboxylate through sequential kinetic resolution catalysed by *Pseudomonas* sp. lipase. Both the enantiomers are potent antidiabetic and antioxidant agents.

30.6.8 Lipases in the synthesis of pesticides

A variety of pesticides (insecticides, herbicides, fungicides or their precursors), made with the applications of lipases, are presently in use (Pandey *et al*., 1999). The most important application of lipases is the organic synthesis of pesticides for the production of an optically active compound (Reddy, 1992). Generally, the resolution of racemic mixtures of alcohol or carboxylic esters lead to the production of these compounds; stereospecific synthesis reactions have also been employed. Mitsuda *et al*. (1990) have given the use of *Achromobacter* lipase for enantioselective hydrolysis of the acetic acid ester of racemic α-cyano-3-phenoxybenzyl alcohol (CPBA) for the production of (S)-CPBA, an active insecticidal stereoisomer.

30.6.9 Lipases in the leather industry

Removal of subcutaneous fat (which may be done with the use of lipases), de-hairing and stuffing are the main processes in the production of leather. Tanning processes are usually done in an alkaline environment, so it would be worth investigating the possible use of alkaliphilic microbes that produce lipase for use in these processes. Many *Bacillus* sp. strains, which flourish successfully under highly alkaline conditions, were found to be useful in leather processing (Haalck *et al*., 1992).

30.6.10 Lipases in environmental management

Lipases have also found application in the degradation of wastewater contaminants such as olive oil from oil mills (Vitolo *et al*., 1998). For this purpose, lipase-producing microbial strains are cultivated in the effluents. Wakelin and Forster (1997) investigated the microbial treatment of waste from fast-food restaurants for the removal of fats, oils and greases. They cultured pure and mixed microbial flora known to produce lipases and other enzymes. *Acinetobacter* sp. was the most effective of the pure cultures, typically degrading 60–65% of the fatty material.

30.6.11 Lipases in the cosmetics and perfume industry

Monoacylglycerols and diacylglycerols, prepared by the lipase-catalysed esterification of glycerol, are useful as surfactants in the cosmetics industry (Pandey *et al*., 1999). Monoacylglycerol synthesis has been reported using *Pseudomonas* sp. LP7315 monoacylglycerol lipase.

30.7 Conclusion

Lipases are the most versatile biocatalysts and bring about a range of bioconversion reactions using a variety of substrates, ranging from lipids to a wide array of esters. In addition, lipases have the unique property of working at the lipid/water interface. The most important property of lipases is their enantioselective nature. Owing to the wide substrate specificity of lipases, a number of assay protocols are employed. Determination of lipase activity at the lipid/water interface is indicative of a true lipase. However, structural studies on several lipases point towards the fact that a number of these differ with respect to their properties. Various microbal lipases have been commercialized but there is still a need to search for novel microbial lipases that have multidimensional properties. The development of enzymes with novel properties by searching the wide diversity of known microorganisms or by creating newer enzyme variants, hold a pivotal position in future enzyme technology.

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31 Bioremediation of Gaseous and Liquid Hydrogen Sulfide Pollutants by Microbial Oxidation

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Abstract

Sulfide emissions into the environment, both in gas and in liquid phase, have increased due to the rapid increase in industrial operations. Although physico-chemical treatment methods have been developed and applied successfully at an industrial level of operation their ability to remove sulfide compounds from the environment is questionable. Biological sulfide oxidation processes are promising methods as the microorganism used in these processes uses sulfide as part of the metabolic mechanism and thus eliminates the compounds from the sulfur cycle changing them into non-toxic end products. This chapter discusses a few developments in the area of biological sulfide oxidation and in particular their application in reactor systems.

31.1 Introduction

Hydrogen sulfide, either in gaseous or liquid form, is toxic to both living and non-living things upon exposure from low to high concentrations. In the biotechnological process, dissolved sulfide (HS^-) is converted to elemental sulfur by the aerobic metabolism of sulfur-oxidizing bacteria (SOB) for example by *Thiobacillus* spp. under microaerophilic conditions (Buisman *et al.*, 1990a, 1991; Janssen *et al.*, 1995). The metabolic products of SOB, insoluble sulfur or biologically produced sulfur, can easily be removed from the process as the biologically produced sulfur settles at the bottom in reaction vessels or bioreactors. Conversion of sulfide to elemental sulfur is a sensitive step and is dependent on the dissolved oxygen concentration alongside other critical parameters of importance such as temperature, sulfide loading rate and pH in the aerobic sulfide oxidation process. Figure 31.1 shows the possible sulfur cycle by various metabolic pathways and their end products (Frigaard and Dahl, 2009; Gregersen *et al.*, 2011).

31.2 Biological Conversion of Sulfide into Elemental Sulfur

Sulfide-containing waste streams are generated by a number of industries such as petrochemical plants, tanneries, viscose rayon, coal-based power plants and wastewater treatment by sulfate-reducing bacteria or anaerobic bacteria (Rinzema and Lettinga, 1988; Brimblecombe and Lein, 1989; Kuenen and Robertson, 1992). Treatment

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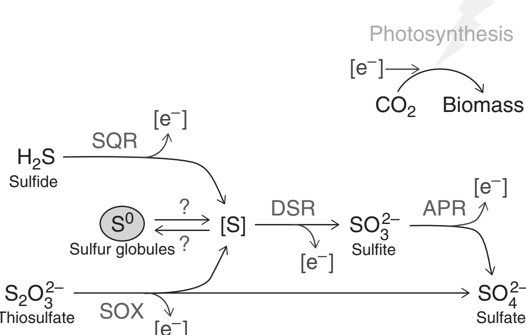

Fig. 31.1. Pathways in the sulfur cycle. APR, Adenosine phosphosulfate reductase; DSR, dissimilatory sulfite reductase; SOX, sulfide oxidation; SQR, sulfide:quinone oxidoreductase. (Diagram courtesy of Dr Niels-Ulrik Frigaard, University of Copenhagen.)

of sulfide-containing waste streams is essential for several reasons including: (i) its inhibitory effect on methane-producing bacteria; (ii) the corrosive effect on materials; (iii) the unpleasant odour; (iv) the contribution to the chemical oxygen demand (COD); and (v) the sludge bulking problem in post-aerobic treatment. Biological sulfide oxidation offers many advantages over physico-chemical processes such as low energy requirement, low chemical and residual disposal costs, and selective conversion of sulfide to elemental sulfur with less sulfate and thiosulfate formation. SOB produce elemental sulfur as an intermediate in the oxidation of H_2S to sulfate. Sulfur globules, located both inside and outside the cell membrane, store the sulfur formed by the metabolism of these microorganisms. Sulfur globules have colloidal characteristics and also globules produced by various organisms have varied surface properties.

Phototropically produced sulfur consists of long sulfur chains terminated with organic groups whereas sulfur produced by chemotrophic bacteria will give sulfur rings. Elemental sulfur (S^0) formation is preferred over the formation of sulfate (SO_4^{2-}) because there are several advantages. One major advantage is the non-toxic and non-corrosive nature of elemental sulfur as it contains more sulfur per unit mass than any other form. Elemental sulfur can be used as feedstock in different industries including fertilizer and chemical industries. Elemental sulfur is a desired end product of sulfide oxidation because of its ability to settle out at the bottom of reaction vessels, lower oxygen requirement and the possibility of its reclamation and reuse as a valuable by-product in industrial applications and metal bioleaching processes, even though oxidation of sulfide to sulfate yields more energy for the bacteria than the formation of sulfur.

Biological treatment of sulfidic-spent caustic produced in refineries could be an inexpensive alternative for the physico-chemical process. In the biotechnological process, dissolved sulfide (HS^-) is converted to elemental sulfur. The insoluble sulfur can easily be removed from the water stream and can be reused as a soil fertilizer or fungicide. Biological conversion of sulfide into elemental sulfur by using SOB is shown schematically in Fig. 31.2.

As an alternative to the Claus and/or Stretford processes for gas desulfurization, different work done by Cork and team (Cork, 1982; Cork and Garunas, 1982; Cork and Ma, 1982; Cork *et al*., 1983) has proposed a microbial process for the removal of H_2S from a gas stream using a photosynthetic bacterium *Chlorobium thiosulfatophilum.*

In nature sulfide can be oxidized biologically in three different ways (Kuenen, 1975): (i) anaerobic oxidation by photosynthetic bacteria; (ii) oxidation by denitrifying organisms; and (iii) oxidation with oxygen by the colourless sulfur bacteria.

Suspended reactors with photoautotrophic bacteria has resulted in the removal of sulfide at sulfide loading rates up to 100 mg/h/l whereas higher removal rates were observed with chemoautotrophs at higher loading rates. Gangagni Rao *et al.* (2003) developed a stripper which was capable of removing sulfide (60–70%) from anaerobically treated wastewater, before aerobic

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Fig. 31.2. Biological conversion of sulfide into elemental sulfur by sulfur-oxidizing bacteria (SOB). (Diagram courtesy of Dr Niels-Ulrik Frigaard, University of Copenhagen.)

treatment, without altering the chemical characteristics of the wastewater. It was a physical system in which air and wastewater were passed as counter currents. H_2S could be efficiently removed by coupling this type of stripper to existing anaerobic systems. The treated wastewater from the stripper, which contains fewer sulfides, may be post-treated in the aerobic system before final discharge. Ravichandra *et al.* (2006) studied a fluidized bed bioreactor to test the biological conversion of sulfides using immobilized cells of *Thiobacillus* sp. that were isolated from aerobic sludge of a distillery and dairy effluent treatment plant using standard methods. Experiments conducted on the batch fluidized bed bioreactor using calcium-alginate-immobilized cells of isolated *Thiobacillus* sp. revealed that 100% sulfide oxidation was achieved with an initial sulfide concentration of 150 mg/l by the continuous supply of sterile air as oxidant. The photosynthetic bioreactor operated in continuous mode resulted in complete removal of S^{2-}, with more than 90% being converted into elemental sulfur (Henshaw *et al.*, 1998). Whereas in chemotropic reactors (Buisman *et al.*, 1990a), 100% of the S^{2-} eliminated from the effluent was converted into S^0. Kobayashi *et al.* (1983) proposed photosynthetic bacterium, *C. thiosulfatophilum*, for the sulfide removal, with sulfur as the final product. However, the requirement of radiant energy for photosynthentic bacteria is economically a disadvantage. Sublette and Sylvester (1987a, b) investigated the use of denitrifying bacteria for sulfide oxidation and sulfur as the end product. This system is not widely applicable because nitrate is additionally needed as an oxidizing agent.

The most important bioconversion in an aerobic sulfide-oxidizing bioreactor is (Kuenen, 1975):

$$2HS^- + O_2 \rightarrow 2S^0 + 2OH^- \qquad (31.1)$$

Studies conducted on biological sulfide oxidation using suspended and submerged attached growth systems have shown that biological sulfide oxidation is a complex phenomenon. In the suspended growth system, sulfide oxidation was limited due to the difficulty of maintaining an adequate biomass level, whereas in the attached growth system an uneven oxygen profile in the bioreactor resulted in the formation of more sulfate and thiosulfate. Moreover, in the submerged attached growth system, anaerobic conditions often prevail within the biofilm core part that leads to bioconversion of sulfur to sulfide again by heterotrophic sulfur-reducing bacteria. Both biomass-limiting conditions and uneven oxygen distribution could be eliminated by employing a biotrickling filter. Various researchers have studied the biological oxidation of H_2S, primarily using different organic carbons as substrates and under heterotrophic conditions (Yang and Allen, 1994a, b; Chung *et al.*, 1996, 1998; Chitwood *et al.*, 1999; Koe and Yang, 2000; Chitwood and Devinny, 2001; Cox and Deshusses, 2002; Gabriel and Deshusses, 2003; Soreanu *et al.*, 2008, 2009; Potumarthi *et al.*, 2009). Gabriel and Deshusses (2003) achieved a high removal efficiency of 98% for H_2S with inlet concentrations of 30 ppmv (parts per million volume) in a biotrickling filter using *Thiobacillus* sp. The removal efficiencies for methyl mercaptan, carbon disulfide and carbonyl sulfide were 67, 35 and 44 ppbv (parts per billion volume) with inlet concentrations of 67, 70 and 193 ppbv, respectively.

Sercu *et al.* (2005) studied the aerobic removal of hydrogen sulfide using a biotrickling filter packed with 1 l polyethylene rings (73% volume free) inoculated with *Acidithiobacillus thiooxidans* ATCC-19377. The performance of the system was not affected by the influent H_2S concentration and the airflow rate (400–2000 ppm and 0.03–0.12 m^3/h, respectively) and 100% removal efficiency was obtained. Soreanu *et al.* (2005) developed a laboratory-scale biotrickling system using anaerobic sludge, packed with polypropylene balls (90% volume) for the removal of H_2S generating from digester biogas using anaerobic conditions. The removal efficiency of the system was observed to be greater than 85% for an H_2S inlet concentration of 500 ppm and a gas flow rate of 0.05 m^3/h. Sodium sulfite was added to the nutrient solution as an oxygen-scavenging agent.

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Biogas having more than 2000 ppm of H_2S which was generated during anaerobic treatment of wastewater was treated in a bioscrubber followed by an aeration tank with 99% H_2S removal efficiency (Nishimura and Yoda, 1997) using SOB. This system could prevent the air from mixing in the biogas. Pagella and De Faveri (2000) introduced a two-stage process for the treatment of H_2S from gas streams, using the bacterium *Thiobacillus ferrooxidans*. H_2S was absorbed in a ferric solution in the first stage (chemical step). Ferrous ion in the solution was oxidized by the biological action of *T. ferrooxidans* in the second stage (biological step). An integrated bioscrubber system developed by Mesa *et al*. (2002) aimed to treat H_2S present in the biogas using a combination of chemical and biological processes. Ferric sulfate solution was used to absorb H_2S producing ferrous sulfate and sulfur. Further ferric sulfate was regenerated by the metabolic activity of *Acidithiobacillus ferrooxidans*.

Koe and Yang (2000) reported that a fixed-film bioscrubber could remove H_2S and odour with efficiencies greater than 99% using bacteria from the genus *Thiobacillus* with an H_2S loading rate below 90 g $H_2S/m^3/h$. A continuous flow fixed-film bioreactor using green photosynthetic sulfur bacterium *C. thiosulfatophilum* was used by Henshaw and Zhu (2001) to remove H_2S from synthetic industrial wastewater. The sulfide removal rates and elemental sulfur recovery rates were found to be 82–100% and 75–95%, respectively, and the maximum sustainable sulfide loading rate was found to be 286 mg/h/l which is a much higher value (nearly 2.5 times) than previous reports.

Oxygen concentration and sulfide loading rate along with flow velocity strongly influence the sulfide oxidation (Annachhatre and Suktrakoolvait, 2001). Also the system efficiency is dependent on anaerobic conversion of the sulfur particles because it leads to: (i) electron donor consumption increases; and (ii) accumulation of sulfide levels in the bioreactor which causes the inhibition of biomass activity (Koster *et al*., 1986).

31.3 Sulfur-oxidizing Bacteria (SOB)

The desirable bacterium for the bioprocess should readily convert H_2S to elemental sulfur and require a minimum of nutrient input and produce S^0 that is easily separable from the biomass.

Most of the SOB are spherical, ovoid or rod-shaped organisms. The SOB are often embedded in a gelatinous mass and range from less than 1 μm to several tens or hundreds of micrometres in length. All contain a type of bacteriochlorophyll, a pigment related to the chlorophyll found in plants, algae and cyanobacteria. Cell masses may be yellow-green-brown, purple-violet-red or green in colour. SOB live in special tissues in invertebrates such as *Riftia pachyptila* (vestimentiferan tube worms) and *Calyptogena magnifica* ('giant' white clams) that live around deep-sea hydrothermal vents. SOBs provide energy to invertebrates by oxidizing reduced sulfur compounds. They also reduce the organic matter resulting into carbon dioxide (CO_2) and other organic compounds. Metabolism for sulfur bacteria is shown in Fig. 31.3.

Recognized groups of SOB are mainly phototrophic bacteria and chemotrophic bacteria. Phototrophic bacteria (green or purple in colour) use light as the energy source to reduce CO_2 to carbohydrates under anaerobic conditions by using reduced sulfur compounds as an electron donor. Phototrophic *Chlorobium limicola* has the ability to grow ideally under anaerobic conditions using a light source and an inorganic substrate to carry out the H_2S removal process and to generate elemental sulfur extracellularly. The chemotrophic sulfur bacteria (colourless bacteria) obtain energy from the chemical oxidation of reduced sulfur compounds under aerobic conditions (i.e. spontaneous reaction of H_2S or S^0 with dissolved oxygen at the water surface). SOB can also be classified into lithotrophic bacteria, which use inorganic substances as the source for hydrogen and organotrophic bacteria, which use organic substances as the source for hydrogen. The process for sulfide removal is based on aerobic oxidation by the group of colourless sulfur bacteria (Kuenen and Beudeker, 1982) with widely different types of physiology and morphology. Genera belonging to the group of colourless sulfur bacteria are: *Thiobacillus*, *Thiomicrospira*, *Thermothrix*, *Thiothrix*, *Thiospira*, *Pseudomonas*, *Thioovulum*, *Sulfolobus*, *Beggiatoa* and *Thioploca*. *Thiobacillus ferrooxidans*, *Thiobacillus thiooxidans*, *Thiobacillus novellus*, *Thiobacillus thioparus* and *Thiobacillus denitrificans* are examples of major sulfide-reducing bacteria.

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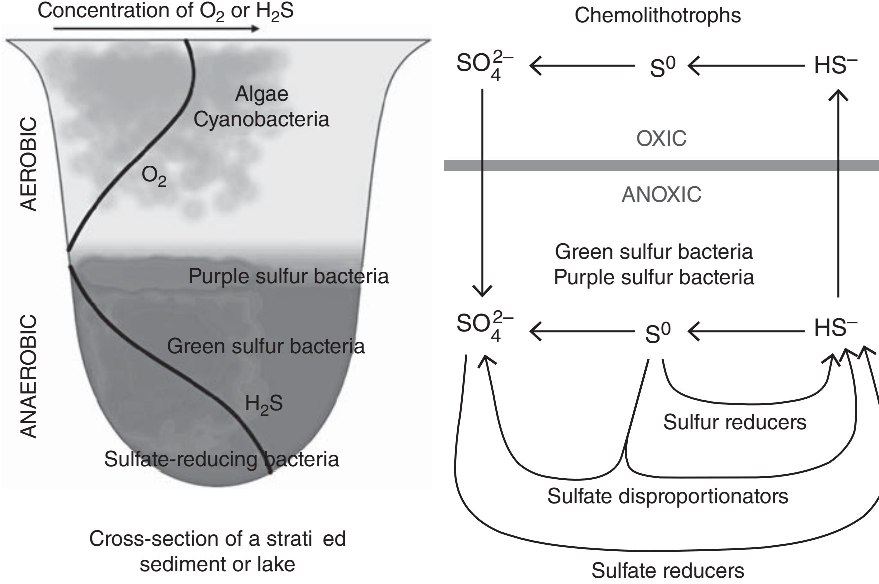

Fig. 31.3. Metabolism for sulfur bacteria. (Diagram courtesy of Dr Niels-Ulrik Frigaard, University of Copenhagen.)

Visser *et al*. (1997a) isolated the dominant autotrophic sulfide-oxidizing strain present in S^0-producing bioreactors, which is found to be a new *Thiobacillus* species, designated *Thiobacillus* sp. W5. This organism has been used as a model organism for studying sulfur production by thiobacilli in wastewater treatment reactors (Visser *et al*., 1997b). Interestingly, the end product of sulfide oxidation at a given sulfide loading rate by *Thiobacillus* sp. W5 is almost exclusively S^0, whereas a very closely related bacterium, *Thiobacillus neapolitanus*, converted only 50% of the sulfide to S^0 while the other 50% is completely oxidized to sulfate. Comparison of the metabolic properties of *Thiobacillus* sp. W5 with those of *T. neapolitanus* revealed that *Thiobacillus* sp. W5 has a competitive advantage over *T. neapolitanus* in bioreactor environments because its sulfide-oxidizing capacity is double that of *T. neapolitanus*. Interestingly, their maximum specific oxygen uptake rates are very similar and no other significant biochemical differences were observed between the two organisms. This means that the limited sulfide oxidation rate of *T. neapolitanus* gives this species a competitive disadvantage, as it can oxidize only 50% of the incoming sulfide to S^0. Thus, these bioreactor environments select species such as *Thiobacillus* sp. W5. Table 31.1 shows the characteristics of some microorganisms used in the removal of H_2S or other sulfur compounds.

31.4 Factors Affecting Sulfide Oxidation

There are several factors that affect sulfide oxidation including: (i) oxygen rate; (ii) pH; and (iii) temperature.

31.4.1 Effect of oxygen rate

The sulfide is biologically oxidized to elemental sulfur under oxygen-limiting conditions (Buisman and Prins, 1994). Kuenen (1975) has postulated that the existence of the sulfur produced enhances its chance of further oxidation to sulfate or the formation of a linear polymer, polysulfide, respectively. Therefore the sulfur produced should be simultaneously removed from the reactor.

$$2HS^- + 4O_2 \rightarrow 2SO_4^{2-} + 2H^+$$

$$n/8\,S_8 + HS^- \rightarrow S_{n+1}^{2-} + H^+$$

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Table 31.1. Characteristics of some microorganisms implicated in degradation of H_2S or other sulfur compounds. (Adapted from Syed *et al.*, 2006 with permission.)

	Microorganisms								
Conditions	*Thiobacillus ferrooxidans*	*Thiobacillus thiooxidans*	*Thiobacillus novellus*	*Thiobacillus thioparus*	*Thiobacillus denitrificans*	*Thermothrix azorensis*	*Thiothrix nivea*	*Thioalkalispira microaerophila*	*Thiomicrospra frisia*
pH growth range	–	0.5–0.6	5.7–9.0	5–9	–	6.0–8.5	6–8.5	8–10.4	4.2–8.5
Optimum pH	1.3–4.5	2.0–3.5	7.0	7.5	6.8–7.4	7.0–7.5	–	10	6.5
Temperature growth range (°C)	10–37	10–37	10–37	–	–	63–86 Thermophile	–	–	3.5–39
Optimum temperature (°C)	30–35	28–30	30	28	28–32	76–78	15–30	–	32–35
G+C content of DNA molecule (%)[a]	56–59	–	67.2	62	63	39.7	44–55	58.9	39.6
Cell type	Gram-negative	Gram-negative	Gram-negative	Gram-negative	–	Gram-negative	Gram-negative or Gram-variable	Gram-negative	Gram-negative
Group	–	–	α-2 Proteobacteria	β-Proteobacteria	β-Proteobacteria	β-Proteobacteria	γ-Proteobacteria	γ-Proteobacteria	γ-Proteobacteria
Spore formation	None	None	None	–	–	None	–	–	–
Motility	May have none(0) or up to several polar flagella; or peritrichous flagella	–	Non-motile	Motile	Motile by means of a polar flagellum	Motile	No flagella	Motile by means of a single polar flagellum	Motile
Shape and size	Rod, 0.5–1.0 μm	Rod, 0.5 × 1.1–2.0 μm	Rod, 0.4–0.8 × 0.8–2.0 μm	Rod, 0.9–1.8 μm	Rod, 0.5 × 1.0–3.0 μm	Rod, 0.3–0.8 × 2–5 μm	Rod, 0.7–2.6 × 0.7–5.0 μm	Spirillum, 0.3–0.45 × 1–4 μm	Bent-rod, 0.3–0.5 × 1.0–2.7 μm
Trophy	Obligate chemoautotroph	Obligate chemoautotroph	Mixotroph (facultative Chemoautotroph)	Obligate chemoautotroph	Obligate chemoautotroph	Obligate chemoautotroph	Mixotroph (facultative chemoautotroph)	Obligate chemoautotroph	Obligate chemoautotroph

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Examples of energy sources	Ferrous ion and reduced sulfur compounds	Hydrogen sulfide, polithionates, elemental sulfur	Hydrogen sulfide, methyl mercaptan, dimethyl sulfide, dimethyl disulfide	Thiosulfate, sulfide	Thiosulfate, tetrathionate, thiocyanate, sulfide, elemental sulfur	Thiosulfate, tetrathionate, hydrogen sulfide, elemental sulfur	Inorganic sulfur compounds, simple organic compounds, sugars	Sulfide, polysulfide, elemental sulfur, thiosulfate	Thiosulfate, tetrathionate, sulfur, sulfide
Oxygen requirement	Facultative anaerobe[b]	Strict aerobe	Strict aerobe	Strict aerobe	Facultative anaerobe[c]	Strict aerobe	Strict aerobe and microaerophile	Strict aerobe and microaerophile	Strict aerobe
Sulfur deposit	–	–	–	Extracellular	–	Intracellular	Intracellular	Intracellular	Extracellular
Reference	Colorado School of Mines (2006)	Takano *et al*. (1997)	Cha *et al*. (1999); Kelly *et al*. (2000)	Vlasceanu *et al*. (1997)	Kelly and Wood (2000)	Odintsova *et al*. (1996)	Howarth *et al*. (1999); Prescott *et al*. (2003)	Sorokin *et al*. (2002)	Brinkhoff *et al*. (1999)

[a]G, Guanine; C, cytosine.
[b]Under anaerobic conditions, *T. ferrooxidans* can grow on elemental sulfur using ferric iron as an electron acceptor.
[c]Grows as an anaerobic chemoautotroph by using nitrate, nitrite or nitrous oxide.

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Previous studies by Janssen *et al*. (1995) have demonstrated that the molar sulfide to oxygen ratio for sulfur production would be around 0.6–1.0. However, in practical situations such as a real wastewater treatment plant, it is difficult to maintain a narrow sulfide to oxygen ratio. On the other hand, maintaining the optimum redox potential could control sulfide oxidation more precisely (Janssen *et al*., 1998; Khanal and Huang, 2003). During sulfide oxidation, when the dissolved oxygen (DO) concentration in the reactor is higher than 0.1 mg/l, the main product is sulfate, whereas sulfur is the major end product when the DO is less than 0.1 mg/l.

31.4.2 Effect of pH

Due to sulfate formation in gas phase bioreactors when sulfur compounds are biologically degraded, there is a substantial drop in pH resulting in decreased biological activity. This significantly affects the H_2S removal efficiency of the system. Various researchers working on biological oxidation systems have reported a maximum sulfur recovery in a suspended growth system with pH between 7 and 8 (Sublette, 1987; Buisman *et al*., 1989, 1990b, 1991; Lee and Sublette, 1993; Hasan *et al*., 1994; Janssen *et al*., 1995, 1997; Ravichandra *et al*., 2007a, b, 2009). Yang and Allen (1994a, b) observed removal efficiencies exceeding 99% under a wide range of heterotrophic conditions in a compost biofilter but sulfide removal efficiencies were affected due to a drastic decrease in pH. This is in contrast to the studies of Cox and Deshusses (2002), who observed that operating a bioreactor at either pH 4.5 or 7.0 did not affect the biotrickling filter's performance significantly, when feeding H_2S and toluene simultaneously. However, they observed that at pH 4.5, the start-up phase for toluene degradation was relatively long and that a sudden pH drop may cause temporary poor removal of H_2S and toluene. The H_2S removal efficiency increased as the pH of the nutrient feed increased in the pH range of 2.0–6.0. However, an opposite trend was observed for the pH between 6.0 and 7.0. The optimum pH for the autotrophic population was near 6.0.

In a reverse fluidized loop reactor (RFLR) studied by Krishnakumar *et al*. (2005), up to 19 kg sulfide/m^3/day loading, the sulfide removal is nearly 100%, in which sulfur recovery is around 80% and sulfate is around 2–3% of total sulfide-S when the pH is controlled at 8. However, the optimum pH is 7–8 for chemotrophic SOB such as the *Thiobacillus denitrificans* (Sublette, 1987; Sublette and Sylvester, 1987a; Buisman *et al*., 1989; Janssen *et al*., 1995; Krishnakumar *et al*., 2005). But the biomass experienced inhibition and toxicity at a pH of 9–9.5 at the same sulfide loading compared with that of pH 8. The presence of higher residual sulfide and transitional products such as $S_2O_3^{2-}$ and polysulfide are a clear indication of decline in SOB activity in the RFLR (Krishnakumar *et al*., 2005) under alkaline pH. Use of alkaliphilic SOB, as reported recently (Banciu *et al*., 2004; Sorokin *et al*., 2004), in future bioreactor studies may help to remove sulfide under more extreme conditions.

31.4.3 Effect of temperature

SOB exist in environments with temperatures up to 100°C (Tributsch, 2003). It was observed that there was no big difference in sulfide oxidation efficiency at either ambient room temperature or 35°C. An increase in the temperature from 35°C to 50°C increased sulfide oxidation from around 38–48% whereas at 60°C, sulfide oxidation is highest at around 51% (Brierley, 2003).

31.5 Conclusion

In the biotechnological process, dissolved sulfide (HS^-) is converted to elemental sulfur by the aerobic metabolism of SOB. The insoluble sulfur can easily be removed from the water stream. Conversion of sulfide to elemental sulfur is a sensitive step and is dependent on dissolved oxygen concentration. Temperature, sulfide loading rate and pH are the other critical parameters in aerobic sulfide oxidation process. Table 31.2 shows the performance of different biological sulfide oxidizing systems including both phototrophic and chemotrophic systems.

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Table 31.2. Performance of different biological sulfide oxidizing systems.

System	Scale	Sulfide in wastewater/ gas/others	Type of biomass	Sulfide concentration (mg/l)	Sulfide loading rate (kg/m³/day)	Sulfide removal (%)	Sulfur recovery (%)	Reference
Phototrophic	Lab	Wastewater	Suspended	20–30	4.4	100	100	Henshaw *et al*. (1998)
	Lab	Gas	Immobilized	Nm[a]	1.77	100	Nm	Kim *et al*. (1992)
	Lab	Gas	Suspended	Nm	2.5–3.3	100	99	Basu *et al*. (1996)
	Lab	Wastewater	Suspended	2.5	0.43	Nm	70	Khanna *et al*. (1996)
	Lab	Wastewater	Attached	1.0	0.87–1.1	95	Nm	Kobayashi *et al*. (1983)
Chemotrophic	Lab	Gas	Flocculated	< 1	25 mM/g biomass/h	100	Nil	Sublette (1987)
	Lab	Wastewater	Flocculated	Nm	0.8–3.4	–	Nm	Lee and Sublette (1993)
	Lab	Synthetic wastewater	Suspended	240	6	99	Nm	Buisman *et al*. (1990b)
	Lab	Anaerobic effluent	Sulfur sludge	240	14	90	80	Janssen *et al* (1997)
	Pilot	Caustic liquid	Attached	Nm	6	99	100	Buisman *et al*. (1993a)
	Pilot	Papermill effluent	Attached	60–240	Nm	70	60–90	Buisman *et al*. (1993b)
	Lab	Synthetic wastewater	Fluidized bed	240	7.5–30	100–90	90–65	Krishnakumar *et al*. (2005)
	Lab	Synthetic wastewater	Fluidized bed and immobilized	150	0.868–1.736	90–95	85–95	Potumarthi *et al*. (2008)

[a]Nm, Not measured.

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32 Archaea, a Useful Group for Unconventional Energy Production: Methane Production From Sugarcane Secondary Distillation Effluents Using Thermotolerant Strains

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Abstract

Some kinetic data indicate that thermophilic archaea might be of use in anaerobic reactors where microbial biota can metabolize contaminants in secondary effluents of industrial processes and transform them into methane-rich biogas, producing natural gas as a cleaner energy source and bioremediating the effluents. This chapter discusses research into the employment of thermotolerant archaea to convert the organic matter dissolved in vinasses (a by-product of the sugar industry) transforming it into methane and identifying the methanogenic organisms involved in the process. Upflow anaerobic sludge blanket reactors were designed and inoculated with biomass adapted to the vinasses substrate. The reactors operated under thermophilic conditions. The strict anaerobic archaebacteria present in the reactors were identified using culture-dependent and culture-independent methodologies, highlighting denaturing gradient gel electrophoresis (DGGE). Methanogenic archaea of the genus *Methanobacterium* and sulfate-reducing bacteria (*Desulfotomaculum*) were isolated and identified. This suggests that these microorganisms can grow in culture media in the presence of vinasses and under thermophilic conditions. Methanogenic archaea identified by total DNA extraction and DGGE included members of the genus *Methanoculleus* and others related to unclassified methanomicrobia, which belong to the order *Methanomicrobiales*. Also sequences related to *Methanobacterium* and *Methanothermobacter*, belonging to the order *Methanobacteriales*, were recovered. The analysed sequences corresponded to hydrogenotrophic methanogenic archaea, proposing their ability to reduce CO_2 to CH_4 using H_2 as the electron donor.

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32.1 Introduction

One of the main issues relating to human development is the negative effect on the environment. To date the human population stands at over 7000 million (PRB, 2015) and associated environmental problems have become evident including climate change and emergent diseases. Mankind has known the usefulness of microbes to deal with environmental problems for a long time, for example civil and sanitary engineers have used septic tanks since the 19th century and activated sludge using consortia of biota have been used in enormous wastewater treatment plants in the 20th century.

Presently, the use of bioremediation technologies includes systems designed to perform the treatment of wastewaters while rendering useful by-products for other human activities without affecting the environment. One example is the use of anaerobic reactors where the microbial biota is able to use the contaminants present in secondary effluents of industrial processes and transform them into methane (CH_4)-rich biogas that can substitute natural gas as a cleaner energy source. When the original source is a terrestrial plant, for example sugarcane (*Saccharum officinarum*), photosynthesis drives the transformation of inorganic carbon (carbon dioxide, CO_2) to produce biomass with a very efficient yield (80–150 t/ha). Humans consume the energy-rich metabolites as sucrose or sugar, an edible carbohydrate that is converted into available energy (ATP), and by using microbial organisms such as yeasts (e.g. *Saccharomyces cerevisiae*) its by-products can be used to produce another energy-rich substance, ethyl alcohol for internal combustion engines, which makes this a highly efficient green-engineering solution (Fig. 32.1) (Bazúa and Wilke, 1977).

A limitation to the use of sugarcane secondary distillation has been the formation of the distillation by-product, vinasses. Thus, the objectives of this research were to use thermotolerant archaea to convert the organic matter dissolved in vinasses, transforming it into an energy-rich substance, CH_4, for electricity and other useful types of energy for human beings and returning CO_2 to the atmosphere for sugarcane to reuse it for its biomass, and to

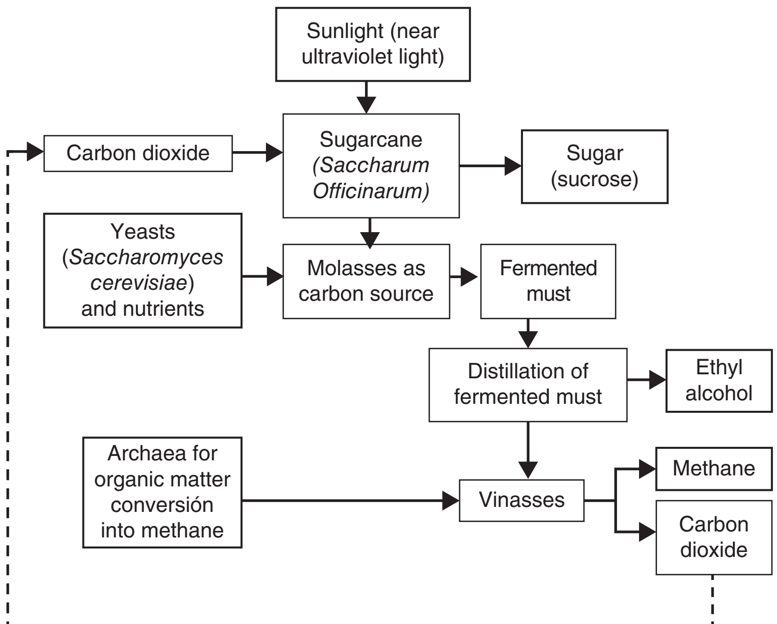

Fig. 32.1. Simplified diagram of sustainable conversion of solar energy into sugar (energy for human beings), ethyl alcohol (energy for motors) and methane (energy for industry and electricity production) using living organisms. (Modified from Bazúa and Wilke, 1977.)

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identify the methanogenic organisms involved in the process.

32.2 Theoretical Considerations

32.2.1 Classification of living organisms

Eubacteria and archaebacteria have intense enzymatic activity, and for this reason they are amply used in the bioreactions[1] industry. Some are located in the stomachs of mammals to help them in the digestion of their foodstuffs (Aron and Grasse, 1969), and to date, it is understood that they represent the most diverse and metabolically rich component of the biosphere (Brock and Madigan, 1993).

For classification, several features are considered besides morphology, including the capacity to sporulate, cell wall structure (identified using a specific stain as Gram-positive or Gram-negative), metabolism (aerobic, anaerobic, anoxic or facultative), and the reactions carried out to obtain energy (e.g. sulfobacteria oxidating hydrosulfide ions, ferrobacteria oxidating carbonates and iron sulfates, and sulfate-reducing organisms (SRO) that will be considered in this research). Archaea were named and classified by Woese *et al*. (1978). Studies of archaeal ribosomal RNA sequences indicate similarities in size to Eubacteria; however, the majority of the proteins in Archaea have the characteristics of the Eukarya domain, indicating a close relationship during evolution. It is suggested that Archaea and Eukarya had a common ancestor rooting back to the proposed last common ancestor to all three domains of life (Woese and Fox, 1977; Woese *et al*., 1990). Among the main characteristics for Archaea that make them distinct from Bacteria, are the following (Whitman *et al*., 2006):

- Methane synthesis is the major source of energy for growth of methanogenic archaea.
- They have the capacity to live in extreme environments, including thermophily.
- Their antibiotic sensitivity is different from that of bacteria.
- The cell wall is made of protein, glycoprotein or pseudomurein.
- Lipidic stereochemistry is 2,3-sn glycerol.
- Their lipid compounds are glycerol ethers, isoprenoids and tetraethers.

A distinctive feature is that they preserve the capacity to live in reducing conditions such as those prevailing on the primitive earth: a carbon dioxide-rich atmosphere, with little hydrogen and no free oxygen. Consequently, they can proliferate in extreme conditions (Woese, 1987; García, 1990).

The Archaea domain is formed by a heterogeneous group of microorganisms. The distinctive general characteristics were described by Woese *et al*. (1990). From those characteristics, two are important for this research:

1. Archaebacteria that have pseudomurein in their cell walls are Gram-positive.
2. The main physiologic groups are: (i) halophilic archaea; (ii) thermophilic archaea; and (iii) methanogenic archaea.

32.2.2 Methanogenic archaea

One of the most common habitats of methanogens is anoxic sediments from swamps, lakes or saturated soils with high moisture contents. These organisms are also found in the digestive tracts of animals such as cows, sheep, camels, deer and even cellulolytic insects (Madigan *et al*., 2003). They are also present in wetlands, anaerobic bioreactors and hydrothermal submarine currents, but this last place has been less studied (Whitman *et al*., 2006). Methanogens belong to Archaea or archaebacteria, but even in 1972 a report on six methanogenic organisms (*Methanococcus vanniellii*, *Methanobacterium ruminantium*, *Methanobacterium mobilis*, *Methanobacterium formicicum*, *Methanosarcina barkeri* and *Methanobacterium thermoautotrophicus*) referred to them as bacteria, even though they presented unconventional characteristics not at all related to bacteria.

Methanogens are presently divided into five orders: *Methanobacteriales*, *Methanococcales*, *Methanomicrobiales*, *Methanosarcinales* and *Methanopyrales* (Brochier-Armanet *et al*., 2008). Table 32.1 shows the class, order, families and genera of the methanogenic archaea, and Table 32.2 mentions some of their morphological characteristics.

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Table 32.1. Classification of methanogenic archaea. (From Boone *et al.*, 2001.)

Class	Order	Family	Genus
Methanobacteria	*Methanobacteriales*	*Methanobacteriaceae*	*Methanobacterium*, *Methanosphaera* *Methanobrevibacter*, *Methanothermobacter*
		Methanothermaceae	*Methanothermus*
Methanococci	*Methanococcales*	*Methanococcaceae*	*Methanococcus*, *Methanothermococcus*
		Methanocaldococcaceae	*Methanocaldococcus*, *Methanotorris*
	Methanomicrobiales	*Methanomicrobiaceae*	*Methanomicrobium*, *Methanoculleus* *Methanogenium*, *Methanolacinia* *Methanofollis*, *Methanoplanus*
		Methanocorpusculaceae	*Methanocorpusculum*
		Methanospirillaceae	*Methanospirillum* *Genera incertae sedis*, *Methanocalculus*
	Methanosarcinales	*Methanosarcinaceae*	*Methanosarcina*, *Methanococcoides* *Methanolobus*, *Methanohalophilus* *Methanohalobium*, *Methanosalsum*, *Methanomicrococcus*
		Methanosaetaceae	*Methanosaeta*
Methanopyri	*Methanopyrales*	*Methanopyraceae*	*Methanopyrus*

Table 32.2. Characteristics of methanogenic archaea. (From Whitman *et al.*, 2006.)

Family	Characteristics
Methanobacteriaceae	Long and short bacilli. Methanogenesis substrates: $H_2 + CO_2$, formate or alcohols. Gram-positive, contain pseudomurein
Methanothermaceae	Bacilli. Methanogenesis substrates: $H_2 + CO_2$. Gram-positive, contain pseudomurein. Extremely thermophilic
Methanococcaceae	Irregular cocci. Methanogenesis substrates: $H_2 + CO_2$ and formate
Methanomicrobiaceae	Irregular cocci, bacilli. Methanogenesis substrates: $H_2 + CO_2$ and in some cases formate and alcohols. Gram-negative
Methanocorpusculaceae	Small and irregular cocci. Methanogenesis substrates: $H_2 + CO_2$ and in some cases formate and alcohols
Methanosarcinaceae	Irregular cocci, short bacilli. Methanogenesis substrates: $H_2 + CO_2$, acetate, methylated compounds. Gram-positive and Gram-negative

32.2.3 Main substrates used by methanogenic archaea

According to Whitman *et al.* (2006), methanogenic archaea obtain their energy using different substrates. The main electron donors are H_2, formate or some alcohols, and the electron acceptor is CO_2, which is reduced to methane; although most of the methanogens use H_2 as the electron donor (Whitman *et al.*, 2006).

Other energy substrates are the methyl-containing C-1 compounds. The electron acceptors

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are the remaining methyl groups that are also reduced to methane. Less frequently, trimethylamine oxide forms trimethylamine, while methionine and dimethyl-sulfoniopropionate produces dimethylsufide (Whitman *et al*., 2006). Acetate is the third major source of methane particularly for *Methanosarcina* and *Methanosaeta* (*Methanotrix*). As acetate is present in many environments, methane synthesis occurs by an acetoclastic reaction, with methyl carbon reduced to methane and carboxyl carbon oxidized to CO_2. Table 32.3 describes the reactions implicated in methanogenesis (Whitman *et al*., 2006).

32.2.4 Molecular tools and denaturing gradient gel electrophoresis (DGGE)

By 1976, scientists were far from establishing a reliable classification of living organisms. The main reason for this was that prokaryotes and eukaryotes seemed not to share comparable traits, a fundamental issue necessary for trustable phylogenetic relationships (Sneath and Sokal, 1973).

However, previous studies in proteins by Zuckerkandl and Pauling (1965) suggested that molecules could be used to establish these connections. Thus, with the development of molecular biology tools and the study of DNA/RNA molecules, Woese and Fox (1977) proposed a phylogenetic structure for the prokaryotic domain and its relationship with Eukarya. This approach was based on ribosomal RNA sequences and now a reliable classification system exists in addition to several culture-independent methodologies to better study the now established domains of Archaea and Bacteria.

Culture-independent methodologies to profile mixed and natural bacterial populations became available by the mid-1980s. At the beginning, 5S rRNA sequences were employed as molecular markers (Stahl *et al*., 1984, 1985) and eventually they were replaced by a more informative molecule, the 16S rRNA (Giovannoni *et al*., 1990; Ward *et al*., 1990). Since then, 16S rRNA gene surveys have become widely used to explore the diversity and taxonomic affiliation of bacterial and archaeal communities in several habitats, and this has resulted in the discovery of several previously unreported organisms (Head *et al*., 1998). Other molecular markers have also been employed to study particular functional communities by targeting function-specific sequences: for example, aerobic ammonia-oxidizing communities can be detected by targeting the *amo*A gene, a gene encoding for the alpha subunit of the ammonia monooxygenase, a key enzyme in the process (Sinigalliano *et al*., 1995; Rotthauwe *et al*., 1997). In a similar way, methanogenic archaea can be studied by targeting *mcr*A, a gene encoding the alpha subunit of the methyl coenzyme M reductase, the enzyme that converts the coenzyme M binding methyl group to methane in the last step carried out by all methanogenic archaea (Hales *et al*., 1996).

Several strategies have been developed to study complex microbial populations from a culture-independent approach, mainly by employing molecular cloning and electrophoresis techniques. Denaturing gradient gel electrophoresis (DGGE) separates DNA fragments based on

Table 32.3. Main substrates for methanogenic archaea. (From Whitman *et al*., 2006; ATCC, 2011.)

Substrate	Reaction	ΔG°' (kJ/mole methane)
$H_2 + CO_2$	$4\ H_2 + CO_2 \rightarrow CH_4 + 2H_2O$	–135.6
Formate	4 Formate $\rightarrow CH_4 + 3CO_2 + 2H_2O$	–130.1
Propanol	4 2-Propanol + $CO_2 \rightarrow CH_4$ + 4 Acetone + $2H_2O$	–36.5
Ethanol	2 Ethanol + $CO_2 \rightarrow CH_4$ + 2 Acetate	–116.3
Methanol	Methanol + $H_2 \rightarrow CH_4 + H_2O$	–112.5
Methanol	4 Methanol $\rightarrow 3CH_4 + CO_2 + 2H_2O$	–104.9
Methylamine	4 Methylamine + $2H_2O \rightarrow 3CH_4 + CO_2 + 4NH_4^+$	–75.0
Dimethylamine	2 Dimethylamine + $2H_2O \rightarrow 3CH_4 + CO_2 + 2NH_4^+$	–73.2
Trimethylamine	4 Trimethylamine + $6H_2O \rightarrow 9CH_4 + 3CO_2 + 4NH_4^+$	–74.3
Dimethylsulfide	2 Dimethylsulfide + $2H_2O \rightarrow 3CH_4 + CO_2 + H_2S$	–73.8
Acetate	Acetate $\rightarrow CH_4 + CO_2$	–31.0

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their composition, thus band patterns are generated by the different mobility of partially melted DNA chains in a chemical denaturing gradient of urea/formamide in polyacrylamide gels (Muyzer *et al*., 1993; Muyzer and Smalla, 1998). This technique offers advantages such as studying community complexity and changes under different environmental conditions, monitoring the enrichment of certain organisms, sequencing the separated DNA bands, and when the experimental conditions are established, the community dynamic can be observed in a reasonable amount of time, less than is needed for other techniques such as molecular cloning (Muyzer and Smalla, 1998). The disadvantages include those related to molecular methods including: (i) biases by DNA extraction protocols and polymerase chain reactions (PCR); (ii) difficulties separating fragments > 500 bp (Myers *et al*., 1985); and (iii) misrepresentation of low-abundance species (Muyzer *et al*., 2004).

32.3 Methodology

32.3.1 Assessment of vinasses composition

The composition of vinasses leaving distillation towers in which yeast-biodegraded molasses from sugar production is distilled into ethyl alcohol was assessed with regards to the following parameters: (i) pH; (ii) temperature; and (iii) salts composition. This was done in order to establish an understanding of the environmental conditions in which methanogenic microorganisms have to exist. The vinasses containers were transported to the laboratories and stored in a cool room at 7 ± 1°C.

32.3.2 Upflow anaerobic sludge blanket (UASB) reactors

Three UASB reactors made of glass with a working volume of 2.4 l were the systems used to convert dissolved organics into methane-rich biogas (Fig. 32.2). As distillation by-products came to boiling temperature, thermophilic conditions were studied in each reactor at 45°C, 55°C and 65°C.

The heating was provided by glass helicoidal tubing with hot water circulating through it to maintain the temperature at 45°C, 55°C and 65°C for each reactor from automatic Grant baths with temperature controlling systems (0–150 ± 0.004°C) using integrated pumps to recycle the water. Due to the low pH value of vinasses (pH 4–5), a buffering formula composed of 600 mg NaOH/l and 1200 mg $NaHCO_3$/l was added to the vinasses before feeding them into the reactors. The three reactors were each inoculated with: (i) 50% of their volume with biomass (800 ml) adapted to the vinasses substrate (Castro-González *et al*., 2004); (ii) 400 ml of a special culture medium proposed by Bryant *et al*. (1971) that was adapted to thermophilic conditions; and (iii) 400 ml of vinasses. The three reactors were closed and left at constant temperature (45°C, 55°C and 65°C, respectively) without further feeding for 14 days. Three peristaltic type bombs (Masterflex) with a line L/S™ 13 were

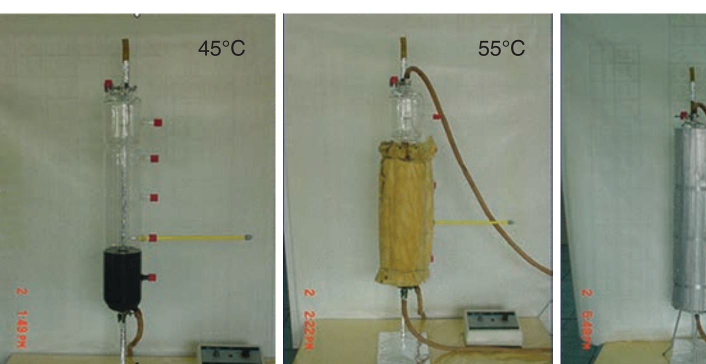

Fig. 32.2. Laboratory-scale upflow anaerobic sludge blanket (UASB) reactors, operating at 45°C, 55°C and 65°C. (From Rincón-Acelas, 2008.)

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coupled to a motor (Cole-Palmer) with a capacity of 1–100 rpm to feed vinasses as an influent stream into the reactors. The upper part of each UASB had a gas-liquid separator built with a funnel and a plastic baffle. The funnel was situated at an angle of 55° with respect to the horizontal where the liquid effluent is separated from the biogas so that any biomass lifted up by the pressure of the bubbles of biogas was retained (reducing elutriation).

Biogas was collected and measured in a Wet Test Meter (Precision Petroleum Instruments) for high wet gas tests (with a precision of up to 680 l/h). Methane, CO_2, H_2S and N_2 were measured in the gaseous effluent using a Perkin Elmer Autosystem gas chromatograph. The chromatographic conditions to measure N_2, CH_4, CO_2 and H_2S were: carrier gas, helium at 85 kPa (*c*.12.5 psig); oven temperature of 30°C for 4 min, 45°C for 1 min, 100°C for 2 min; injection of samples of 25 μl to an injector at 150°C; thermal conductivity detector to 150°C. The column used was a GS-GasPro (30m × 0.32 mm ionization detector). To corroborate the methane measurement a single measurement was devised, as follows: carrier nitrogen at 70 kPa (*c*.10 psig); oven temperature of 70°C for 3 min; injector with split 1:5.2, injection of samples of 10 μl to an injector at 150°C; flame ionization detector temperature at 150°C. A more detailed description is presented elsewhere (Castro-González and Durán-de-Bazúa, 2002; Castro-González, 2004). Vinasses were provided by a cooperating sugarcane processing factory located in the Mexican state of Veracruz. The highest organic load of vinasses was 120,000 mg total chemical oxygen demand (CODt)/l and the lowest was 80,000 mg CODt/l. The sulfates concentration in vinasses varied from 10,000 mg sulfate/l to 20,000 mg sulfate/l (CODt:sulfates ratios of 12:4).

32.3.3 Isolating, identifying and establishing the growth kinetics of methanogenic organisms

Isolation of these strict anaerobic organisms is complicated and should be performed very carefully and in an anaerobic environment. For this reason when a sample of sludge was obtained from a UASB reactor every effort was made to avoid contact of the sample with ambient air. To isolate the organisms enrichment media are recommended as a starting step, using several carbon sources or preferably substrates that are unique for methanogenic archaea. In this work general culture media suggested by Bryant *et al*. (1971) (Table 32.4A) were prepared. Then for the genera *Methanobacterium* and *Desulfotomaculum* specific culture media (Table 32.4B) were used (Balch *et al*., 1979; Soto *et al*., 1993; Flaherty *et al*., 1998). Finally, selective culture media were used for each species as follows: (i) the one proposed by Balch *et al.* (1979) for *Methanobacterium formicicum*; (ii) the one suggested by Belyaev *et al*. (1983) for *Methanobacterium ivanov*; (iii) the culture medium cited by Winter *et al*. (1985) for *Methanobacterium wolfeii*; and (iv) the medium employed by Klemps *et al*. (1985) for *Desulfotomaculum nigrificans* (Table 32.5).

Selection of the optimum working dilution

To establish the optimum working dilution, 9 ml general culture media were placed in sterile tubes in anaerobic conditions, and inoculated with 1 ml of UASB reactor bottom sludge from the three temperatures (45°C, 55°C, 65°C). Serial dilutions from 10^{-1} to 10^{-8} were taken from them and incubated for 5 days at the corresponding temperatures.

The dilution tubes were removed from the incubators after 5 days. A sample of the liquid media from each dilution tube was streaked onto a glass slide and fixed in a Bunsen flame. A Gram tinction was made for each fixed sample, adding a drop of crystal violet (dissolving 0.5 g crystal violet or gentian violet in 100 ml deionized water, forming a homogeneous solution). After 1–2 min excess dye was washed out with deionized sterile water. A drop of Lugol's iodine (dissolving 0.3 g iodine and 0.6 g KI in 100 ml deionized sterile water) was added, and after 1 min was washed away. Ethanol at 95% was added, left for 20 s before washing, before adding safranin (0.25 g safranin in 100 ml deionized sterile water). After 1 min, the slides were dried in the open air in a clean environment. To select the optimum dilution, the nine samples corresponding to each dilution and temperature were observed under an optical microscope (Olympus BH-2; clear field, objective 100×).

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Table 32.4. Culture media. (From Bernal-González *et al*., 2012.)

A. General culture media, incubation time (T_i) = 5 days (Bryant *et al*., 1971)			B. Specific culture media for methanogenic (M) activity and sulfate-reducing organisms (SRO) (T_i = 30 days) (Balch *et al*., 1979; Soto *et al*., 1993; Flaherty *et al*., 1998)			
Solutions to be added to 1 l of final volume		Contents (g/l) added to distilled sterile water	Specific culture media for M		Specific culture media for SRO	
Mineral solution 1	50 ml	6 g K_2HPO_4	Mineral solution 1	10 ml	Mineral solution 1	50 ml
Mineral solution 2	50 ml	6 g KH_2PO_4 6 g $(NH_4)_2SO_4$ 12 g NaCl 2.6 g $MgSO_4.7H_2O$ 0.16 g $CaCl_2.2H_2O$	Mineral solution 2	50 ml	Mineral solution 2	50 ml
Mineral solution 3	500 ml	0.67 g KCl 5.5 g $MgCl_2.2H_2O$ 6.9 g $MgSO_4.7H_2O$ 0.5 g NH_4Cl 0.28 g $CaCl_2.2H_2O$ 0.28 g K_2HPO_4	Oligo-elements solution	10 ml	Resarzurine (0.1%)	1 ml
NaCl	18 g		Vitamins solution	10 ml		
NH_4Cl	1.25 g					
Mineral traces	10 ml	1.5 g nitrilotriacetic acid 3 g $MgSO_4.7H_2O$ 0.5g $MnSO_4.2H_2O$ 0.1 g NaCl 0.1 g $FeSO_4.7H_2O$ 0.1 g $CoSO_4$ or $CoCl_2$ 0.1 g $CaCl_2.2H_2O$ 0.1 g $ZnSO_4$ 0.001 g $CuSO_4.5H_2O$ 0.01 g $AlK(SO_4)_2$ 0.01 g H_3BO_3 0.01 g $Na_2MoO_4.2H_2O$	$FeSO_4$ $7H_2O$ (2% solution) $NiCl_2$ solution (5 mg/100 ml) Resarzurine (0.1%)	1 ml 50 ml 1 ml	$FeSO_4.7H_2O$ (2% solution)	0.5 ml/l
Oligo-elements solution	10 ml	0.002 g Biotin 0.002 g Folic acid 0.010 g Pyridoxine 0.005 g Thiamine 0.005 g Riboflavin 0.005 g Nicotinic acid 0.005 g Calcium pantotenate 0.00001 g Vitamin B12 0.005 g Para-aminobenzoic acid 0.005 g Lipoic acid	Yeast extract	1 g/l	Cysteine	0.5 g/l
$FeSO_4.7H_2O$	2 mg		Sodium bicarbonate	3 g/l	Bromo-ethanol sulfuric acid	75 mM
$Fe(NH_4)_2(SO_4)_2.7H_2O$	0.02 g					
$NaHCO_3$	7.5 g					
Sodium acetate	2.5 g		Caseine-peptone	1 g/l	Neutralized lactic acid solution	8.5 ml/l
Sodium formate	2.5 g					
Yeast extract	2.0 g					
Tripticase	2.0 g					
Cysteine	0.6 g					
$Na_2S.9H_2O$	0.6 g					

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Table 32.5. Selective media (T_i = 15 days) used to culture *Methanobacterium formicicum*, *Methanobacterium ivanov*, *Methanobacterium wolfeii* and *Desulfotomaculum nigrificans*. (From Balch *et al*., 1979; Widdel, 1998.)

Component	*M. formicicum*	*M. ivanov*	*M. wolfeii*	*D. nigrificans*
Mineral solution 1 (ml)	50		50	
Mineral solution 2 (ml)	50	25		
Mineral solution 3 (ml)				50
NH_4Cl (g)		1.25	1.00	
Mineral traces 1 (ml)	10	10	10	10
Mineral traces 2 (ml)	10	10	5	10
$FeSO_4.7H_2O$ (g)	0.002		0.0006	
$Fe(NH_4)_2(SO_4)_2.7H_2O$ (g)		0.02		
$NaHCO_3$ (g)	5.0	7.5		2.4
Sodium acetate (g)	2.5			
Sodium formate (g)	2.5			
Yeast extract (g)	2.0			
Tripticase (g)	2.0			
Cysteine (g)	0.5	0.6	0.125	
$Na_2S.9H_2O$ (g)	0.5	0.6		0.7
$Na_2HPO_4.7H_2O$			2.1	
$MgCl_2$ $6H_2O$			0.2	
Resarzurine (0.2%)	0.25	0.25	0.25	0.25
Penicillin G (g) (6%)			0.1	

General culture media incubation

From the selected dilutions for each temperature, inocula were taken from the three reactors and added to 200 ml of general culture media, incubated for 5 days at temperatures of the corresponding UASB reactors.

Specific culture media incubation

To select for isolates from the genera *Methanobacterium* and *Desulfotomaculum*, 60 culture tubes were divided into two groups of 30: (i) one group filled with 9 ml of specific culture media for the methanogenic archaea; and (ii) the other group with 9 ml of specific culture media for SRO (see Table 32.4B). The tubes were inoculated with 1 ml of the pre-seeded inoculum in general culture media for the three studied temperatures, sealing the tubes hermetically. The procedure was performed in an anaerobic chamber.

The inoculated tubes were left to incubate for 30 days at the corresponding temperatures. Daily, a tube of each temperature and specific culture media was removed, a sample taken, fixed on a glass slide and a Gram tinction applied to observe under the microscope (Olympus BH-2 objective 100×) to check for growth.

Selective culture media incubation

A volume of 0.1 ml of the last culture tube using specific culture media for methanogenic bacteria and SRO at each temperature (45°C, 55°C, 65°C) was taken and restreaked in triplicate in Petri dishes containing the selective culture media for each species (Table 32.5). These were then incubated for 15 days. Daily, a Petri dish for each temperature and medium was removed to observe the development of the colonies directly using the stereoscope BH2-RFC, quantifying them during the experiment. At the end of the experiment (day 15), two colonies from each Petri dish per species and temperature were selected. A colony was isolated and fixed using a 3% glutaraldehyde solution, to observe its morphology using a scanning electronic microscope (SEM) (JEOL Mod. JSM-5800 Microanalysis System Oxford Mod. ISIS 486el; SEM procedure). The other colony was isolated in a phosphate buffered saline solution to verify if the microorganisms had external structures (cilia and/or flagella) by examination using an Olympus FluoView ver. 17c viewer.

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Quantification using the most probable number (MPN) technique

Methanogenic archaea and SRO were quantified using the MPN technique described by Girard and Rougieux (1965). Specific culture media for methanogenic archaea and SRO were prepared as indicated in Table 32.4B. One millilitre of the general culture media seeding was inoculated in the specific culture media for each temperature studied (45°C, 55°C and 65°C) and reseeded in the specific culture media, with 16 dilutions (10^{-1}, 10^{-2}, 10^{-3}, 10^{-4}, 10^{-5}, 10^{-6}, 10^{-7}, 10^{-8}, 10^{-9}, 10^{-10}, 10^{-11}, 10^{-12}, 10^{-13}, 10^{-14}, 10^{-15},10^{-16}) by triplicate. The whole procedure was carried out in an anaerobic chamber. A Durham tube was placed over each tube in order to detect any gas produced by the microorganisms. The tubes were hermetically sealed and incubated for 30 days at the three temperatures. After day 30, the positive tubes were counted for each dilution and temperature obtaining an MPN index that was multiplied by the sample volume and divided by the corresponding dilution of the first positive tube, obtaining the cell number per millilitre of sample.

Direct plate counting method

To establish the quantities of the species *Methanobacterium formicicum*, *Methanobacterium wolfeii*, *Methanobacterium ivanov* and *Desulfotomaculum nigrificans* the direct plate counting method described by Arias *et al.* (2005) was used. Selective culture media were prepared and mixed using general agar BD BIOXON in proportions 50:50. The mixture was set in sterile Petri dishes (60 mm × 15 mm). One millilitre of sludge from each reactor was inoculated in the specific culture media to obtain an optimum dilution (previously selected) and 100 µl of this dilution were reseeded in triplicate in the Petri dishes with the selected culture media, and incubated for 15 days at the working temperatures. Daily, a Petri dish for each temperature and medium was removed and observed in the stereoscope BH2-RFC, to count the number of colonies that developed in each dish.

Growth kinetics for each selective culture medium

An equation for the growth kinetics was obtained from the number of colonies established by the daily direct plate counting method. These data were used to obtain the number of organisms with respect to time (Brock and Madigan, 1993; Tortora *et al.*, 1993). To calculate the generation time of a species (*G*) first the number of bacteria per millilitre was established using the following equation:

$$\text{Number of bacteria (bacteria/ml)} = \text{number of colonies} \times \text{specific environmental concentration} \quad (32.1)$$

The time taken for the population to double was calculated using equation 32.2:

$$y = 2nx \quad (32.2)$$

where
x = the number of bacteria at time 0
y = the number of bacteria at time t
t = time of growth (‘growth’) exponential
At time 0, $y = x$
Solving equation 32.2 to n (the number of generations), results in equation 32.3:

$$\log y = \log x + n \log 2 \quad (32.3)$$

Substituting equation 32.3 gives the number of generations:

$$n = \log y - \log x - \log y/\log 2 \quad (32.4)$$

In equation 32.4 the value of log 2 is substituted by 0.3010, resulting in 1/0.3010 = 3.3. Using this, the number of generations can be calculated, where the initial population x is known, after time t:

$$\text{n} = 3.3 \log y/x \quad (32.5)$$

The generation time at G equals (time to reach the exponential phase of x and y) divided by the number of generations n:

$$\text{G} = t/n \quad (32.6)$$

Confocal microscopy and scanning electron microscopy

Some cells have natural fluorescent substances that help in studying these cells (Doddema and Vogels, 1978). Methanogenic archaea have autofluorescence, and thus, confocal microscopy is a very useful technique to detect their presence in a pure culture and/or in an environmental sample with archaea without any previous isolation. This property of autofluorescence in methanogenic archaea is due to the abundance

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of coenzyme F_{420}, implicated in its metabolism (methanogenesis). Coenzyme F_{420} is derived from flavin that participates in the process of electron transport in the cytoplasm of archaea. It was isolated in 1972 from methanogenic archaea and its chemical structure was established 6 years later. This coenzyme receives such a name because of the absorption of ultraviolet light at a wavelength of 420 nm. For this reason, to have a successful fluorescence for methanogenic archaea, excitation should be adjusted to a wavelength between 350 nm and 420 nm (green or blue colour) (Madigan *et al*., 2003).

In confocal microscopy, fluorescein staining allows the observation of outstanding cellular structures as cilia, flagella, pili, fimbriae, etc. The procedure followed for this staining was to collect a small sample from each Petri dish colony on selective culture medium after 15 days of incubation, using an Eppendorf tube, performing the process for each species and temperature. Phosphate buffer solution was then added, as well as 1 ml of fluorescein solution, shaking vigorously and the tube was incubated at the working temperature for 1 h, centrifuged and the supernatant was removed. The precipitate was placed on a slide, previously cleaned with 70% ethanol, and it was spread across the surface in a thin layer. Then, it was allowed to dry. The prepared slide was wrapped in foil for protection and kept refrigerated until analysis of cellular structures using a confocal microscope (Olympus FluoView & trade1000).

For scanning electron microscopy a colony was selected from the selective culture medium in the Petri dish and placed in an Eppendorf tube by adding 1 ml of phosphate buffer. The sample was homogenized, left to stand for 10 min and centrifuged. Then, the supernatant was removed and the precipitate left with the microorganisms. The pellet was resuspended in 3% glutaraldehyde and 0.1 ml of 1 N solution osmium tetroxide, stirred vigorously and left to stand for 2 h. Carefully, the precipitate was fixed to a steel disc (10 mm in diameter) and placed inside the critical point dryer (CPD 7501, SPI Supplies) to dry to the 'critical' point for 30 min. Finally, the steel disc with the sample was mounted on the object holder and covered with a layer (20 nm) of gold layer. The method of coating the slide with the sample was by evaporation using a Balzers metallizer (SCD 004). Morphological observations were done by scanning electron microscopy (JEOL, mod. JSM-5800, Jeol, Japan) with a microanalysis system OXFORD (mod. ISIS 486).

32.3.4 Molecular analysis of the methanogenic community composition

To study the methanogenic community, DNA was extracted from sludge samples taken from the three reactors used in the biomethanation process with sugarcane secondary distillation effluents.

Three sludge subsamples (*c*.5 g) were taken from each UASB reactor, pooled in a sterile Falcon tube and kept at -20°C until DNA extraction. DNA was extracted from 750 μg biomass using the Power Soil® DNA Isolation Kit (MoBio Laboratories) according to the manufacturer's instructions, with slight modifications. DNA was washed twice with 500 μl ethanol-based wash solution (Solution C5) to remove PCR inhibitors. After a centrifugation step to remove residual ethanol (10,000 ***g***, 1 min), DNA was then eluted with 40 μl molecular grade water (Sigma Aldrich). Samples were kept at -20°C until PCR amplification.

PCR amplification

DNA amplification was carried out using archaeal 16S rDNA and *mcr*A specific primers, following nested amplification protocols for both genes. The first round of amplification was used to increase the number of gene copies in the sample with the primers described below. Meanwhile, the second round of PCR amplification was used to generate amplicons containing a CG-clamp to increase the melting point of PCR products for DGGE purposes; thus reverse primers –1492R and ME2– contained a 5′ attached GC-clamp (5′-CGC CCG GGG CGC GCC CCG GGC GGG GCG GGG GCA CGG GGG-3′) and were used exclusively in the second-round PCR mixtures. For 16S rDNA amplification, the first PCR round was done with primer 25F (5′-CYG GTT GAT CCT GCC RG-3′) and 1492R (5′-GGT TAC CTT GTT ACG ACT T-3′) (Dojka *et al*., 1998). Meanwhile, the second PCR round was carried out using the reverse primer 1492R and a modified version of the primer A1098F (Reysenbach and Pace, 1995), A1098Fa (5′-GGR AAC GAG CGA

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GAC CY-3′). The *mcr*A gene was amplified with ME1 (5′-GCM ATG CAR ATH GGW ATG TC-3′) and ME2 (5′-TCA TKG CRT AGT TDG GRT AGT-3′) (Hales *et al*., 1996). For *mcr*A fragments, the same primers were used for both amplification rounds, but a 5′ GC-clamp was attached to the reverse primer ME2. Reaction mixtures (25 μl) contained: (i) 10–100 ng of metagenomic DNA; (ii) 1 × ViBuffer S (Vivantis); (iii) the appropriate primers at 0.5 μM each; (iv) dATP, dCTP, dGTP and dTTP, each at 200 μM; and (v) 0.5 μg/μl bovine serum albumin (Biolabs) and 1 U of Taq DNA polymerase (Vivantis). Additional dimethyl sulfoxide (5%) was used in the 16S rRNA amplification mixture. The PCR protocol to amplify 16S rRNA partial sections included an initial denaturation cycle at 94°C (2 min); followed by 30 amplification cycles at 94°C (30 s), 55°C (30 s) and 72°C (90 s); and a final extension step at 72°C (5 min). For ME1/ME2, the amplification protocol was the same as mentioned before but primers were annealed at 50°C during the amplification process. Triplicate PCR reactions were done and the results were pooled to avoid PCR bias.

DGGE

Resultant amplicons were separated with a DGGE system (DGGE-2001, CBS Scientific Company). A GM-40 gravity flow gradient maker (CBS Scientific Company) was used to pour the 1 mm thick, 15 cm × 20 cm polyacrylamide gels (7.0% acrylamide-bisacrylamide (37.5:1)). The denaturing gradient for 16S rRNA amplicons was 40–60% and 40–70% for *mcr*A (100% denaturant mixture consisted of 7 M urea and 40% formamide). DGGE gels were run in 0.5 × TAE (1×, 40 mM Tris-acetate, 1 mM EDTA, pH 8) at 60 V for 20 h at 60°C, stained with 1:10,000 (v/v) SYBR Green 1 Nucleic Acid Gel stain (Sigma-Aldrich), and visualized and digitized with the MiniBIS Pro gel documentation system (DNR Bio-Imaging Systems).

Reamplification, sequencing and phylogenetic analysis

To obtain the separated DNA sequences, DGGE bands were excised and eluted in 40 μl molecular-grade water (Sigma-Aldrich). Reamplifications were done using as template 4 μl of each eluted DGGE band, following the same amplification conditions mentioned above with the appropriate primer pair. DNA sequences were obtained with the ABI 3730xl DNA analyser (Applied Biosystems). Sequences were compared to those reported in databases, reference and obtained sequences were then aligned and edited with SeaView and Clustral W (Galtier *et al*., 1996; Chenna *et al*., 2003), and neighbour-joining trees constructed with SeaView using the K-2 parameter model (1000 bootstraps).

32.4 Results and Discussion

32.4.1 Vinasses composition

Results from the average vinasses composition during the experiments are presented in Tables 32.6 and 32.7 (Bernal-González *et al*., 2012).

It can be seen that salts contents, pH and temperature after leaving the distillation towers where ethyl alcohol is concentrated up to 96% in volume are definitively high. Thus, the reactors receiving this liquid influent can be considered an extreme environment.

32.4.2 Identification and growth kinetics of methanogenic organisms

Archaea – traditional identification

Application of the methodology proposed to identify archaea during the anaerobic treatment of waste from sugarcane molasses fermented and distilled to obtain methane as a non-conventional source of energy gave the following results. The general culture media proposed by Bryant (1978) was adequate to grow methanogenic archaea and SRO, at the three selected temperatures, 45°C, 55°C and 65°C, for 5 days. On the other side, the optimum dilution selected was 10^{-2}, permitting a clear appreciation of the well-defined morphological structures, and the existence of an adequate development of the microorganisms, with separated, isolated colonies without any agglomeration.

The Balch *et al.* (1979) general culture media favoured the development and proliferation of methanogenic archaea, according to the authors' description of characteristics, such as typical morphology of elongated bacilli, Gram-positive

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Table 32.6. Average composition of vinasses.

Parameter[a]	January–May	May–December	Parameter[a]	January–May	May–December
pH	4.34	4.28	DVS (mg/l)	70,429	78,463
BOD (mg/l)	66,825	98,250	FDS (mg/l)	6,376	5,220
CODt (mg/l)	99,879	116,250	Sulfates (mg/l)	7,057	8,150
BOD:COD ratio	0.67	0.85	Sulfides (mg/l)	24	69
TS (mg/l)	85,340	91,227	Chlorides (mg/l)	4,700	5,230
VTS (mg/l)	63,745	63,792	Nitrogen (%)	5.35	6.1
FTS (mg/l)	21,595	27,435	Carbon (%)	4.55	4.47
TSS (mg/l)	8,834	7,591	Hydrogen (%)	5.64	6.77
VSS (mg/l)	6,625	5,884	Sulfur (%)	$7.28*10^{-3}$	$9.85*10^{-3}$
FSS (mg/l)	2,208	1,706	Electric conductivity (μS/cm)	21,867	26,500
DTS (mg/l)	76,806	83,683			

[a]BOD, Biochemical oxygen demand; CODt, total chemical oxygen demand; TS, total solids; VTS, volatile total solids; FTS, fixed total solids; TSS, total suspended solids; VSS, volatile suspended solids; FSS, fixed suspended solids; DTS, dissolved total solids; DVS, dissolved volatile solids; FDS, fixed dissolved solids.

Table 32.7. Elements and metals present in vinasses.

Element/metal	January–May	May–December
Cu (mg/l)	9.5	1.1
Ni (mg/l)	0.8	1.1
Co (mg/l)	0.7	1.0
Mn (mg/l)	15.2	20.4
Fe (mg/l)	30.6	38.2
Zn (mg/l)	6.7	9.0
Ca (mg/l)	2008.3	2083.3
Na (mg/l)	2153.0	711.0
K (mg/l)	8354.5	9940.4
Mg (mg/l)	1163.9	1935.1
As (mg/l)	61.9	109.4
Pb (mg/l)	0.7	4.5
Al (mg/l)	0.7	6.1

and rod bacilli, whereas the temperature does have an influence in the population reflecting more development at 45°C of Gram-positive cocci.

At 55°C and 65°C, the population was scarce. On the other side, the specific culture media for SRO presented bacilli that had their internal structure dyed as Gram-positive, whereas the reddish cocci were present in higher quantities as Gram-negative. The morphology of the SRO followed that reported by Flaherty *et al.* (1998). This indicates that the specific culture media used was adequate for the development and proliferation of SRO at the three studied temperatures.

According to Bryant *et al*. (1971), Bryant and Wolin (1975), Belyaev *et al*. (1983) and Boone *et al*. (1986, 1989, 2001), the optimum temperature for proliferation of *Methanobacterium formicicum* is found between 35°C and 45°C. The morphology they present is of long rod bacilli that are 0.4–0.8 μm wide and 2–15 μm long. They do not possess external structures, indicating that no movement is performed. The observations found in this study indicated that at 45°C and 55°C there were rod bacilli that did not aggregate, and that were dyed as Gram-positive. This means that their cell wall possessed a thick layer of peptidoglucan defining the species. Thus, only the first dye can cause a tinction, and the layer is not decolorized by the other solutions utilized in this procedure. A fluorescein dye and the use of a SEM allowed the observation of the micromorphological characteristics (i.e. shape, size, grouping, presence or absence of surface structures) but not the internal structure, corroborating that no motility exists. The species at 45°C had an approximate size of 3 μm long and 0.3 μm wide. Due to the morphological characteristics obtained in this study, and compared with the cited authors, it was corroborated that the species in the study was *M. formicicum*. It was also corroborated that these archaea tolerated temperatures up to 55°C, with more growth than at 45°C. These results do not agree with those reported by Bryant *et al*. (1971), Bryant and Wolin (1975), Belyaev *et al.* (1983) and Boone *et al.* (1986, 1989, 2001), who indicate that methanogenic archaea (*M. formicicum*) do not proliferate at 55°C. It is an interesting finding and will be studied further.

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The species *Methanobacterium ivanov* is described in the literature in a very detailed manner by authors König (1984), Kneifel *et al.* (1986) and Jain *et al.* (1987). They mention that its optimum temperature is 45°C and the morphology is short rod bacilli, Gram-positive, measuring approximately 0.2–0.5 μm wide and 1–15 μm long, without motility. For this research the organisms were also rod bacilli, Gram-positive, without external structures (meaning no motility) and measuring 4 μm long and 0.2 μm wide (Table 32.8). They were only present at 45°C, whereas at 55°C and 65°C no presence was detected. Thus, this organism is present in the UASB reactors at a temperature of 45°C.

Species *Methanobacterium wolfeii* is thoroughly described by Winter *et al.* (1985), Winter and Zellner (1987) and Zellner *et al.* (1989): long rod bacilli, Gram-positive, with a length of 2.4–2.7 μm and a width of 0.4–1 μm, without external structures, and thus, no motility. Its optimum growth temperatures are 55°C and 65°C. In this study, besides these temperatures it was also found at 45°C, with morphological characteristics of short and long rod bacilli, some with curving or rounded ends, dyed as Gram-positive, with no external structures, and measuring approximately 0.9 μm long and 0.3 μm wide. At 55°C, the species had better growth, and its morphology was of long rod bacilli, 1.5 μm long and 0.5 μm wide. For 65°C, the species formed straight short bacilli, 7 μm long and 0.3 μm wide. Table 32.9 shows its detailed morphology. It was the only species proliferating at the three studied temperatures. According to its morphology it can be confirmed that it is *M. wolfeii*.

The sulfate-reducing species *Desulfotomaculum nigrificans* is characterized by the morphology described by Flaherty *et al.* (1998), as Gram-negative irregular cocci, with motility, and dimensions of *c.*0.3–0.5 μm wide and 3–6 μm long. The optimum growth temperature is 65°C. These characteristics can be compared with the ones found in this study, in which some organisms showed, at 55°C, Gram-negative cocci, with a thin layer of peptidoglycan (murein) that was easily dyed and easy to decolorize, that took the safranin final colour, confirming the organism to be Gram-negative. The size, 1 μm long and 0.4 μm wide, was slightly smaller than the reported characteristics, with motility due to peritrichous flagella, although such structures were observed on a limited number of occasions, since they were not adequately dyed. Table 32.10 shows identification of this organism at 55°C and 65°C. At a temperature of 65°C the same characteristics as for

Table 32.8. Methanogenic archaea species (*Methanobacterium ivanov*) at 45°C. (Modified from Bernal-González *et al.*, 2012.)

Microscopic observations	Characteristics
Optical microscopy with clear field (Gram dye, 100×)	Short rod-type bacilli, Gram-positive, grouped in great quantities
Confocal microscope (fluorescein dye, 600×)	Short rod-type bacilli, no internal structure
Scanning electron microscope (SEM) (15,000×)	Short rod-type bacilli, curved, with rounded edges, grouped clustered themselves, size 4 μm long, 0.2 μm wide

Table 32.9. *Methanobacterium wolfeii* at 45°C, 55°C and 65°C. (Modified from Bernal-González *et al.*, 2012.)

Microscopic observations	*Methanobacterium wolfeii*		
	45°C	55°C	65°C
Optical microscopy with clear field (Gram dye, 100×)	√	√	√
Confocal microscope (fluorescein dye, 600×)	√	√	√
SEM (15,000×)	√	√	√

Table 32.10. *Desulfotomaculum nigrificans* at 55°C and 65°C. (Modified from Bernal-González *et al.*, 2012.)

Microscopic observations	*Desulfotomaculum nigrificans*	
	55°C	65°C
Optical microscopy with clear field (Gram dye, 100×)	√	√
Confocal microscope (fluorescein dye, 600×)	√	√
SEM (15,000×)	√	√

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55°C were found, but the organisms are slightly bigger, 3 µm long and 0.4 µm wide. It may be said that these organisms grow better at temperatures higher than 55°C (i.e. at 65°C) as reported by Flaherty *et al.* (1998).

Table 32.11 presents the characteristics for *M. formicicum* (Bryant *et al.*, 1971; Bryant, 1978; Belyaev *et al.*, 1983; Boone *et al.*, 1986); *M. ivanov* (König, 1984; Kneifel *et al.*, 1986; Worakit *et al.*, 1986; Jain *et al.*, 1987; Patel *et al.*, 1990); *M. wolfeii* (Winter and Zellner; 1987; Zellner *et al.*, 1989) and *D. nigrificans* (Campos, 2001). It can be said that these species share many phenotypic features.

Methanogenic archaea and SRO quantification using the MPN technique: growth kinetics

As mentioned before, quantification of methanogenic archaea and SRO was done using the Girard and Rougieux (1965) MPN technique with specific culture media at the three temperatures studied, during 30 days. Data found for methanogenic archaea were 150,000 MPN/ml at 45°C, whereas SRO, were only 200 MPN/ml. At 55°C, methanogenic archaea were 15,000,000 MPN/ml, compared with 12,000 SRO MPN/ml. At 65°C, 4600 methanogenic archaea MPN/ml and 11,000 SRO MPN/ml were found. These results indicate that, at 65°C, methanogenic archaea decreased considerably whereas SRO maintain their growth as at 55°C. These findings are interesting from the biotechnological point of view, since vinasses come out of the distillation towers at temperatures higher than 65°C, but if diluted, its temperature may be reduced down to 55°C so that methanogenic archaea are the preferred organisms growing in order to produce methane and to inhibit the production of H_2S, corroborating the results previously obtained (Castro-González, 2004).

Results obtained for growth kinetics of each of the species studied are presented in Fig. 32.3. Comparing these results it can be seen that *M. formicicum* maximum value was 21,100 organisms/ml at 55°C (Fig. 32.3a), whereas *M. ivanov* only appears at 45°C with 37,800 organisms/ml (Fig. 32.3b), and *M. wolfeii* can grow at the three temperatures presenting the highest growth at 55°C with 20,800 organisms/ml (Fig. 32.3c). The sulfate-reducing species *D. nigrificans* proliferates at 55°C with 11,300 organisms/ml, and a higher abundance, 15,900 organisms/ml, at 65°C (Fig. 32.3d). Definitely, with these results, to promote methane production from vinasses, the maximum operating temperature in the UASB reactors should be 55°C.

Comparison of data obtained in this study with previous data

Table 32.12 presents the overall data obtained by isolation from the inocula obtained from the

Table 32.11. Methanogenic archaea and sulfate-reducing organisms (SRO) characteristics. (From García *et al.*, 1995; Campos, 2001.)

Property	*Methanobacterium formicicum*	*Methanobacterium ivanov*	*Methanobacterium wolfeii*	*Desulfotomaculum nigrificans*
Morphology	Bacillus (rod)	Bacillus (rod)	Bacillus (rod)	Cocci and irregular bacilli
Cell width (µm)	0.4–0.8	0.5–0.8	0.4	0.3–0.5
Cell length (µm)	2–15	1.2	2.4–2.7	3–6
Filaments[a]	+	+ (size 2–15 µm)	+	–
Gram stain	+	+	+	–
Optimum pH	6.6–7.8	7.0–7.4	7.0–7.5	7.3–7.6
Optimum temperature (°C)	35–45	45	55–65	55–80
Substratum	H_2	H_2	H_2	Low molecular mass
Autotrophic	+	+	+	+
Type of strain	DSM 1535	DMS 2611	DSM 2970	ATCC 7946

[a]+, Filaments present; –, filaments not observed.

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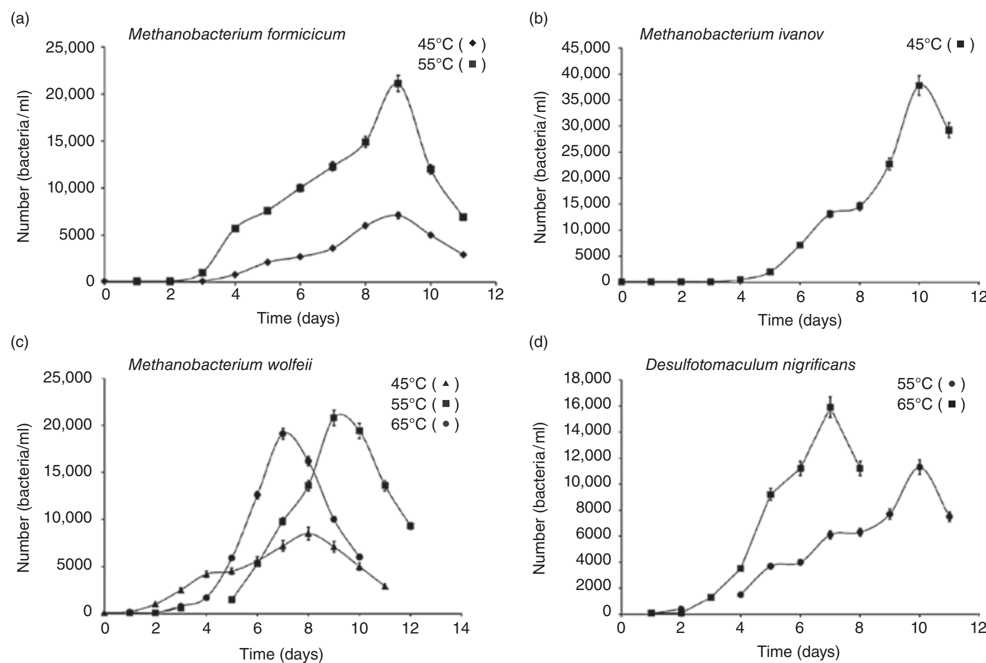

Fig. 32.3. Growth kinetics of (a) *Methanobacterium formicicum*, (▲) 45°C, (■) 55°C; (b) *Methanobacterium ivanov*, (■) 45°C; (c) *Methanobacterium wolfeii*, (▲) 45°C, (■) 55°C, (●) 65°C; and (d) *Desulfotomaculum nigrificans*, (●) 55°C, (■) 65°C. (Modified from Bernal-González *et al*., 2012.)

Table 32.12. Comparison of the data obtained in this study* with methane production reported in the literature**. (From Castro-González, 2004; Bernal-González *et al*., 2012.)

	Inoculum from UASB reactor at		
Parameter[a]	45°C	55°C	65°C
Methanobacterium formicicum (MPN/ml)	7,100*	21,100*	–[b]
Methanobacterium ivanov (MPN/ml)	37,800*	–	–
Methanobacterium wolfeii (MPN/ml)	8,500*	20,800*	19,100*
Desulfotomaculum nigrificans (MPN/ml)	–	11,300*	15,900*
MPN for MA and SRO (MPN/ml)	150,000 MA, 200 SRO	15,000,000 MA, 12,000 SRO	4,600 MA, 11,000 SRO
*Methane production (mMol/day)	45	50	46
**Methane production (mMol/day)	120	165	Not done
**H_2S production (mMol/day)	10	10.7	Not done

[a]MPN, Most probable number; MA, methanogenic archaea; SRO, sulfate-reducing organisms.
[b]–, Organism not found.

three UASB reactors operating at 45°C, 55°C and 65°C in the present study. According to this data, and the comparison with the information previously found in these reactors operating at 45°C and 55°C, ethanol producers may use the carbonaceous compounds found in vinasses as substrate for archaea, enhancing the methane-producing organisms that grow at 55°C.

32.4.3 Molecular analysis of the methanogenic community composition

The amplification of methanogenic molecular markers from metagenomic DNA was achieved for all samples (Fig. 32.4). The primer 25F-1492R selectively amplified *c*.1500 bp for the region 16S rDNA of the archaea found in these

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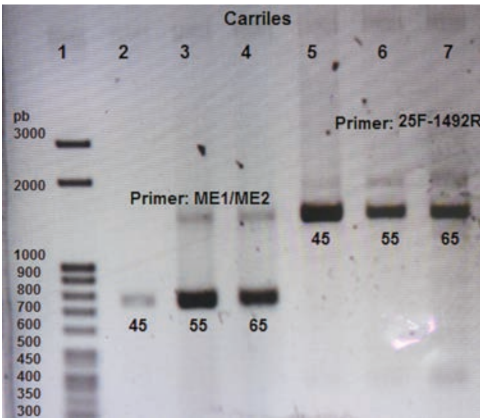

Fig. 32.4. Agarose gel electrophoresis showing *mcr*A and 16S rDNA amplicons. PCR products were obtained using metagenomic DNA extracted from three UASB reactors operating at 45°C, 55°C and 65°C. Lane 1 presents the molecular marker (in bp). Lanes 2, 3 and 4 correspond to *mcr*A amplicons and lanes 5, 6 and 7 to 16S rDNA sequences. Numbers below DNA bands represent the temperature (in °C) of the UASB reactor from which amplicons were amplified.

extreme environments of pH, temperature and salts concentrations represented by the three reactors studied. The amplification of 750 bp from the *mcr*A gene implies that the last step of methanogenesis from methylated compounds was also present.

The DGGE electrophoresis analysis was carried out using the *mcr*A and the 16S rDNA fragments amplified as mentioned above (Fig. 32.5). The community fingerprint showed several phylotypes (bands) in the reactor sludge, suggesting the presence of at least two main methanogens in each reactor.

Phylogenetic reconstructions for methanogenic archaea in the UASB reactors

All methanogenic archaea sequenced included members of *Methanoculleus*, *Methanothermobacter* and others related to unclassified Methanomicrobia (Fig. 32.6). Predominating archaea are related to the genus *Methanoculleus* (45_1, 55_2 and C4). Sequences C1, C2 and C3 are closer to archaeons belonging to the order *Methanomicrobiales* found in sediments and/or biodigestors. Analysed sequences correspond to hydrogenotrophic methanogens (which reduce CO_2 to CH_4 utilizing H_2 as the electron donor). This metabolic route starts with the formation of N-carboximethanofuran from CO_2 and methanofuran, that is immediately reduced to formyl-methanofuran, thanks to cofactor F_{420} (8-hydroxi-5-deazaflavin) and the hydrogenases enzymes (Deppenmeier *et al*., 1996).

According to Kudo *et al*. (1997), the most common habitats for *Methanomicrobiales* are the anaerobic reactors and the activated sludge systems, particularly for *Methanoculleus*, *Methanofollis*, *Methanocorpusculum*, *Methanospirillum* and *Methanomicrobium*. Liu (2010) confirms that several species belonging to these orders have been isolated from these habitats. Results analysed in Fig. 32.6 demonstrate that the use of 16S rRNA as a phylogenetic molecular marker is adequate to study the methanogenic community, confirming the results presented by Nettmann *et al*. (2008). Table 32.13 shows some physiological characteristics of the strains referenced in Fig. 32.6.

For *mcr*A, more information from the phylogenetic relationships of the methanogenic community was obtained (Fig. 32.7). In this topology it can be observed that D1, D2, E1, E2 and F2 were sequences related to species of *Methanobacterium* and *Methanothermobacter*, belonging to the order *Methanobacteriales*. According to the literature, these species have a hydrogenotrophic metabolism, as also seen in the species of the order *Methanomicrobiales* order (sequence F1). Their optimal temperature for growth ranges from 40°C to 65°C (Smith *et al*., 1997). Most isolated strains from these genera have come from anaerobic bioreactors, oil fields, marine sediments and intestinal tracts of ruminants.

A relevant result is that the methanogens in these UASB-type reactors is not highly diverse, as seen in other bioreactor systems, where conditions are managed to select for specific strains. Microbial composition in natural and man-made communities depends on environmental characteristics that include pH, substrate availability and temperature, among others (Nettmann *et al*., 2008; Chenguang *et al*., 2011).

Although it was not possible to recover acetoclastic methanogenic archaea sequences, it must be considered that DGGE gives a general overview of community composition, but more recent techniques, including next generation

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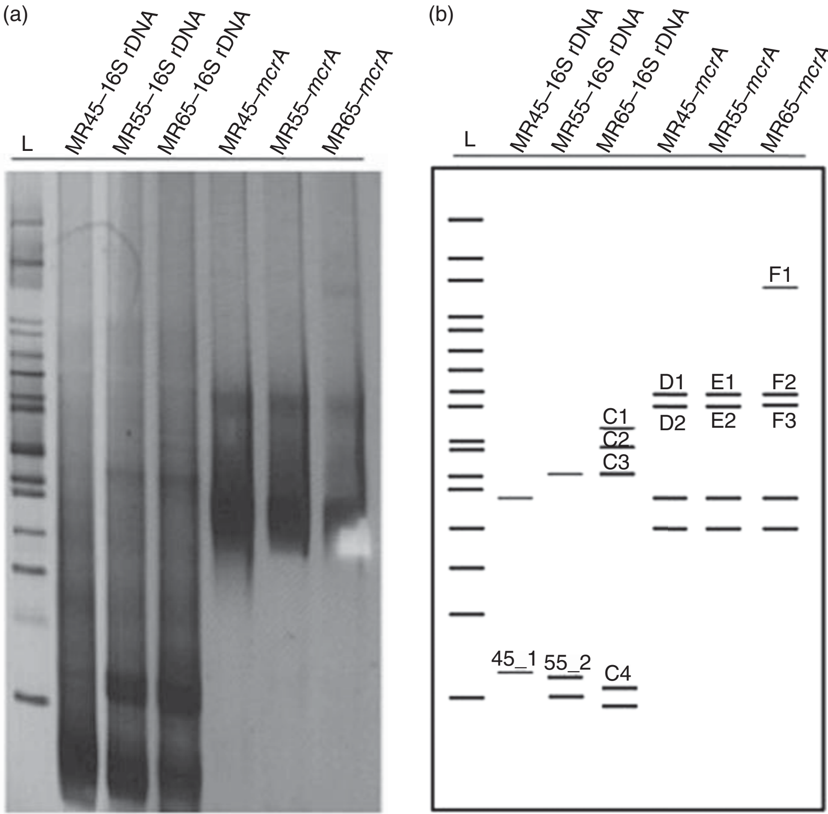

Fig. 32.5. Methanogenic community fingerprint from three UASB reactors. (a) DGGE analysis of the methanogenic community in a UASB reactor at 45°C (MR45), 55°C (MR55) and 65°C (MR65); using 16S rDNA (-16S rDNA) and *mcr*A (-*mcr*A) as molecular markers. A reference ladder was also included in the analysis (L). (b) Schematic representation of the DGGE analysis, in letters, showing the excised and sequenced bands.

sequencing, are a more powerful tool to estimate microbial diversity (Mardis, 2008).

32.5 Concluding Remarks

The use of culture media (Bryant, 1978) is optimum to obtain good growth of methanogenic archaea (*Methanobacterium*) and SRO (*Desulfotomaculum*), using as inoculum 1 ml of sludge (diluted to 50% with tap water) from each UASB reactor, operating at 45°C, 55°C and 65°C, fed with vinasses from an ethanol-producing plant that uses sugarcane molasses as yeast substrate. The best dilution to observe the morphology of the isolated colonies was 10^{-2}.

The media proposed by Balch *et al.* (1979) and Flaherty *et al.* (1998) for methanogenic archaea and SRO, respectively, were adequate, particularly using incubation periods of 30 days.

The isolation of the species that were identified as *Methanobacterium formicicum*, *Methanobacterium ivanov*, *Methanobacterium wolfeii* and *Desulfotomaculum nigrificans* was possible thanks to the selective culture media employed.

Methanogenic archaea identified with total DNA extraction and DGGE included members of the genus *Methanoculleus* and others related to unclassified Methanomicrobia, which belong to the order *Methanomicrobiales*. The predominating archaea are related to genus *Methanoculleus.* The analysed sequences correspond to hydrogenotrophic methanogenic archaea suggesting their ability to reduce CO_2 to CH_4 utilizing H_2 as the electron donor.

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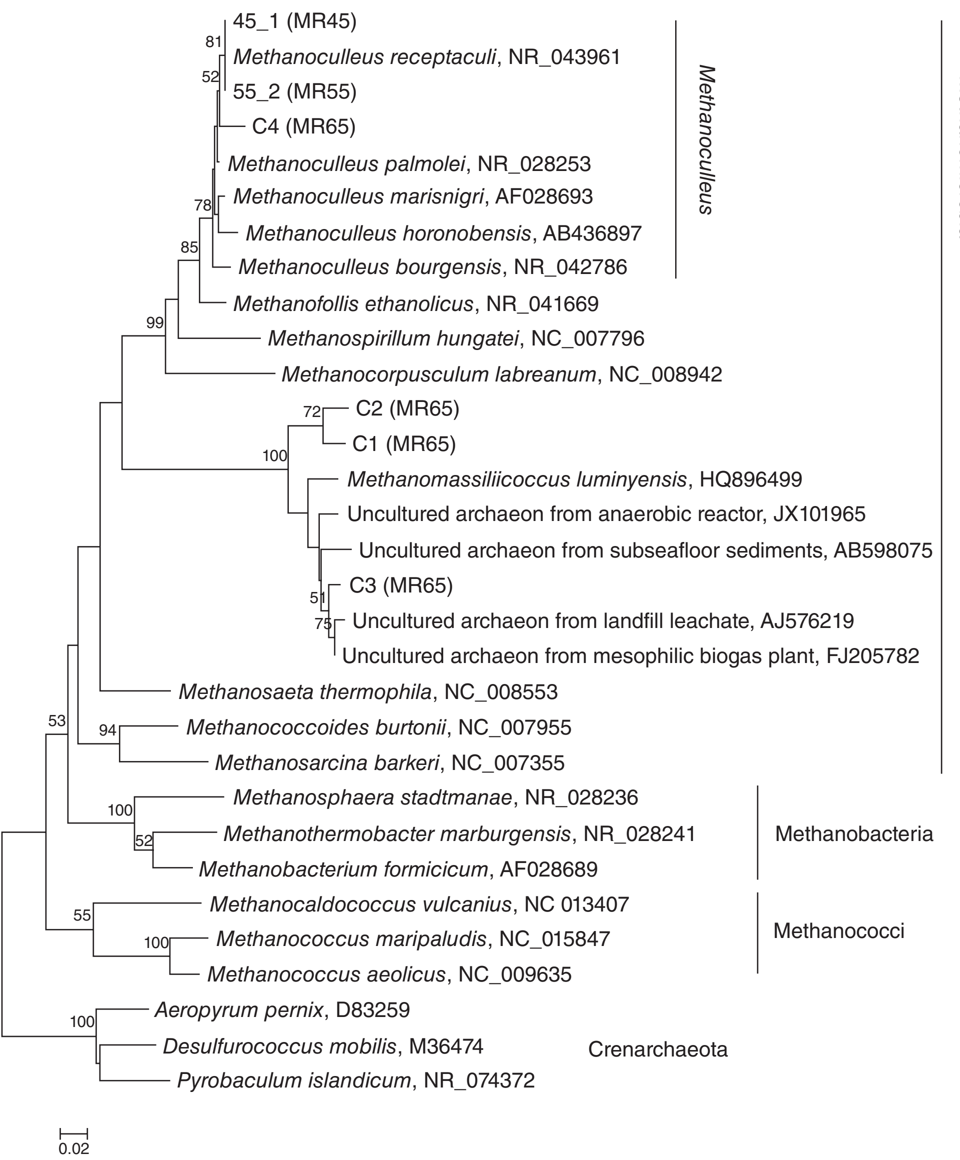

Fig. 32.6. Phylogenetic relationships of 16S rRNA sequences from methanogenic phylotypes found in the studied upflow anaerobic sludge blanket (UASB) reactors. As an external group, three members of the phylum Crenarchaeota were used. Bootstrap values > 50% are mentioned (1000 replicates) and the scale bar below represents 2% divergence. The GenBank accession number is given after the species name.

Sequences related to *Methanobacterium* and *Methanothermobacter*, belonging to the order *Methanobacteriales*, were also recovered. The above shows the relevance of including molecular tools in the characterization of microbial consortia.

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Table 32.13. Characteristics of some species found in the phylogeny with 16S rDNA and *mcr*A.

Methanogenic archaea (GenBank accession number)	Dimensions (μm)[a]	Isolation source[a]	Substrates for methanogenesis	Growth temperature (optimum) (°C)	pH (optimum)	Reference
Methanoculleus receptaculi (NR_043961)	0.8–1.7	Oil field	$H_2 + CO_2$, isobutanol, butanol	(50–55)	7.5–7.8	Cheng *et al*. (2008)
Methanoculleus bourgensis (NR_042786)	1–2	Biodigester (anaerobic reactor)	$H_2 + CO_2$, formate	37–45 (35–40)	5.5–8.0 (6.7)	Liu (2010)
Methanoculleus marisnigri (AF028253)	1.3	Marine sediment	$H_2 + CO_2$, formate, 2-propanol, 2-butanol	10–45 (20–25)	5.8–7.6 (6.2–6.6)	Liu (2010)
Methanoculleus chikugoensis (BAF46709)	1–2	Rice cultivation soil	$H_2 + CO_2$, formate, 2-propanol, 2-butanol, cyclopentanol	15–40 (25–30)	6.7–8.0 (6.7–7.2)	Liu (2010)
Methanoculleus thermophilus (BAF56662)	0.6–1.8	Nuclear plant sediment	$H_2 + CO_2$, formate	37–65 (55–60)	6.2–7.8 (6.5–7.2)	Liu (2010)
Methanoculleus palmolei (NR_028253)	1.25–2	Biodigester (anaerobic reactor)	$H_2 + CO_2$, formate, 2-propanol, 2-butanol, cyclopentanol	22–50 (40)	6.5–8.9 (6.9–7.5)	Liu (2010)
Methanobacterium thermoautotrophicum (1HBM)	1–2	ND	$H_2 + CO_2$	>65	(6.5–7.5)	Pihl *et al*. (1994)
Methanobacterium alcaliphilum (BAI67095)	2	Lake sediment	$H_2 + CO_2$	35–45 (37)	7.8–9.2 (8.4)	Worakit *et al*., (1986)
Methanobacterium sp.(ADM52196)	0.3–0.5 × 2–5	Lake sediment	$H_2 + CO_2$, formate	35–45 (37)	6.9–7.5	Zhu *et al*. (2011)
Methanothermobacter thermautotrophicus (NP_276292)	1–2	ND	$H_2 + CO_2$	>65	(6.5–7.5)	Pihl *et al*. (1994)
Methanothermobacter wolfeii (BAL72749)	1–2	River sediment	$H_2 + CO_2$	55–65	6.5–7.9 (6.9–7.5)	Winter *et al*. (1985)
Methanospirillum hungatei (AAK16835)	0.4–0.5	Activated sludge	$H_2 + CO_2$, formate	(30–37)	(6.6–7.4)	Liu (2010)
Methanofollis ethanolicus (NR_041669)	2–3	Lotus field	$H_2 + CO_2$, formate, 1-propanol, 1-butanol	15–40 (37)	6.5–7.5 (7.0)	Imachi *et al*. (2009)

Continued

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Table 32.13. Continued.

Methanogenic archaea (GenBank accession number)	Dimensions (μm)[a]	Isolation source[a]	Substrates for methanogenesis	Growth temperature (optimum) (°C)	pH (optimum)	Reference
Methanocorpusculum labreanum (NC_008942)	0.4–2.0	Lake sediment	$H_2 + CO_2$, formate	<45 (37)	6.5–7.5 (7.0)	Liu (2010)
Methanomassiliicoccus luminyensis (HQ896499)	ND	Human faeces	$H_2 + CO_2$, methanol	(37)	(7.6)	Dridi *et al*. (2012)
Non-cultivable archaeon (JX101965)	ND	Biodigester (anaerobic reactor)	$H_2 + CO_2$	(35)	(7.0)	Cardinali-Rezende *et al*. (2012)
Non-cultivable archaeon (AB598075)	ND	Marine sediment	$H_2 + CO_2$	(10)	(7.0)	Imachi *et al*. (2011)
Non-cultivable archaeon (AJ576219)	ND	Leachates (garbage deposition)	$H_2 + CO_2$	ND	8.0–8.4 (8.2)	Huang *et al*. (2003)
Non-cultivable archaeon (FJ205782)	ND	Biogas plant	$H_2 + CO_2$, alcohols unidentified and acetate	(40)	ND	Kröber *et al*. (2009)
Non-cultivable archaeon (ADC29459)	ND	Oil field	$H_2 + CO_2$, alcohols unidentified	(55)	ND	Gieg *et al*. (2010)
Non-cultivable archaeon (BAD21104)	ND	Biodigester (anaerobic reactor)	Acetate	(37)	(7.4)	Shigematsu *et al.* (2004)

[a]ND, Not determined.

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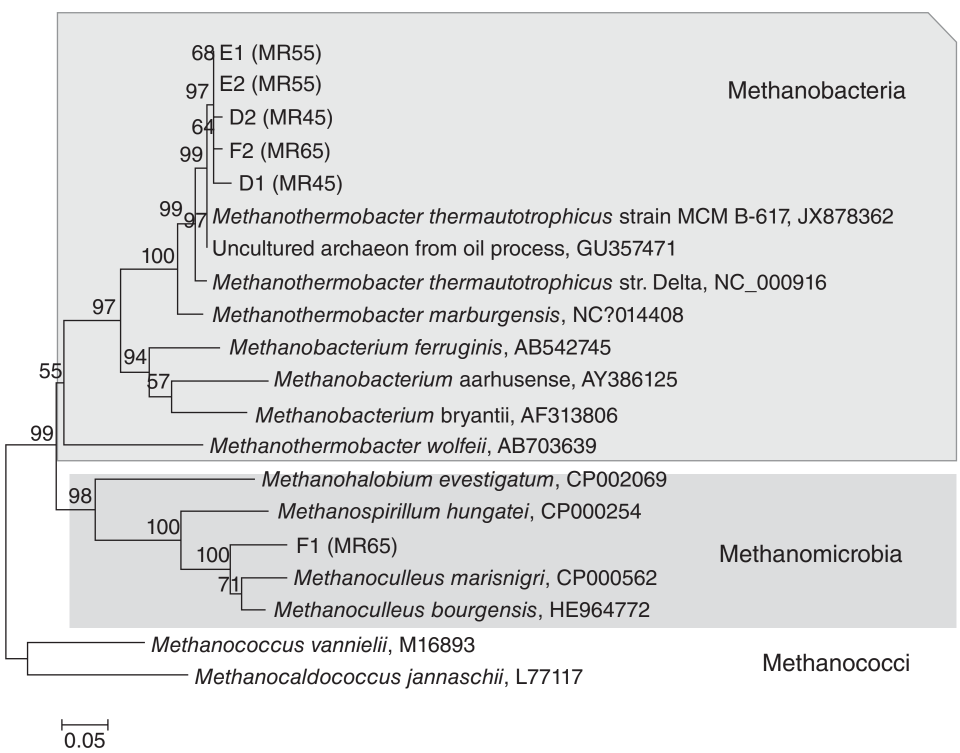

Fig. 32.7. Phylogenetic relationships of *mcr*A sequences from methanogenic phylotypes found in the studied upflow anaerobic sludge blanket (UASB) reactors. Bootstrap values > 50% are mentioned (1000 replicates) and the scale bar below represents 5% divergence.

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Note

[1] Some authors use the word fermentation instead of bioreaction. Louis Pasteur gave the name of fermentation to the bioreaction of yeast *Saccharomyces cerevisiae* with glucose in anaerobic conditions to produce ethyl alcohol and carbon dioxide (Fig. 32.1). However, all bioreactions, not just this one, should be called bioreactions not fermentations.

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33 Industrial Additives Obtained Through Microbial Biotechnology: Biosurfactants and Prebiotic Carbohydrates

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Abstract

Industrial biotechnology has expanded considerably in recent years bringing new possibilities for the production of industrial additives. Among the most studied compounds are the the non-digestible oligosaccharides (NDOs) that are related to the bioavailability of minerals, modulation of the immune system, prevention of gastrointestinal infections, regulation of metabolism disorders related to obesity and type 2 diabetes, and many other important health allegations. There is an increasing market for health-promoting NDO food ingredients whose industrial applications are focused on dairy products, beverages, milk products and many others. Additionally, another class of additives that are very important for industry, and that have been extensively studied by means of biotechnological processes, is biosurfactants synthesized by fungi, yeasts and bacteria, which exhibit high surface activity and emulsifying activity. Considering their characteristics and physico-chemical properties, the biosurfactants present interesting applications, such as for bioremediation, enhancement of oil recovery, industrial applications in food additives, cosmetics and others. In this perspective, this chapter is focused on the biotechnological production of surfactants and prebiotic carbohydrates, and aims to show their potential to industry, especially when obtained by means of biotechnological processes. Additionally, this chapter includes an extensive and recent review of the literature, highlighting key advances in the production of additives through microbial biotechnology, the potential and challenges of this area and the future prospects.

33.1 Biotechnology: Useful Products for the Future of Industry

The basis for much of the growth of modern science surrounds developments in biotechnology, considering that over the last few years it is possible to observe an incredible expansion in the biological sciences including the ability to use genetic information to produce new pharmaceutical products, and genetically modified microorganisms for industrial and environmental developments (Colwell, 2002).

Biotechnology is versatile and has been assessed as a key technology for a sustainable chemical industry (Lievonen, 1999), since it operates at lower temperatures, produces less toxic

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waste and there are fewer emissions and by-products compared with conventional chemical processes. In this sense, environmental concerns help drive the use of biotechnology in industry, not only to remove pollutants from the environment but also to prevent pollution (Gavrilescu and Chisti, 2005).

Modern biotechnology can be divided into four major market segments: (i) biomedical; (ii) agricultural; (iii) environmental; and (iv) industrial (Colwell, 2002). The last of these is the focus of this chapter, an area that is evolving and searching for new products and processes to meet the demands of a broad market.

Industrial fermentation is a multi-step processes where cultivation is increased in scale in a staged approach. Today most industrial fermentation products are produced at the largest scale via fed-batch strategies. Limiting a carbon source in this way by feeding is used to slow down the fermentation in the later stages, with the advantage that oxygen supply and heat removal can be managed. Nevertheless, for aerobic fermentations the size of an individual unit is limited to around 200 m^3, making it hard to gain the benefits of economies of scale seen in conventional petrochemical processes. For anaerobic fermentation the yield of product is often, although not always, lower (since more of the carbon goes into the cells and other products). Nevertheless, the final scale can be as high as 500–1000 m^3 (Woodley, 2013). Several chemical products are produced by fermentation today including organic acids and alcohols (Miller and Nagarajan, 2000; Whited *et al.*, 2010; Weusthuis *et al.*, 2011).

In fact, the chemical industry has used traditional biotechnological processes (e.g. microbial production of enzymes, antibiotics, amino acids, ethanol, vitamins; and enzyme catalysis) for many years (Chisti, 1999; Demain, 2000; Schmid, 2003). In addition, traditional biotechnology is widely used in producing fermented foods and treating waste (Jördening and Winter, 2004).

Industrial segments has been largely favoured by developments achieved in genetic engineering and molecular biology techniques, that have been used to obtain many modified enzymes with enhanced properties compared with their natural counterparts (Demain, 2000). Metabolic engineering, or molecular level manipulation of metabolic pathways in whole or part, is providing microorganisms and transgenic crops and animals with new and enhanced capabilities for producing chemicals. Nowadays, the development of this area shows that most fine chemicals being manufactured in multi-tonnage quantities using biotechnology, such as bioethanol, organic acids, antibiotics, enzymes, vitamins and others are now made using engineered biocatalysts (Gavrilescu and Chisti, 2005).

Additionally, the development of recombinant DNA (rDNA) technology enables several possibilities for the real exploitation of the biocatalysts used in these processes. First, it has provided a cheap way to produce a given biocatalyst. The desired enzyme (or enzymes) can now be overproduced meaning that it represents a much bigger fraction of the available protein in the cell. This not only reduces the required scale of the fermentation (and consequently the feedstock and energy required, as well as the waste produced) but for isolated enzyme applications also reduces the downstream burden prior to catalysis. Secondly, a given gene may be expressed *in vivo* only in a poor host for production (e.g. the host may be pathogenic or grows only under conditions far from those used for application) (Woodley, 2013). In the near future, industrial biotechnology will expand as an important area, bringing new possibilities for the production of industrial additives.

With this perspective, this chapter is divided into two parts and focuses on a comprehensive review of the biotechnological production of additives with industrial and commercial interests. The first part concentrates on the biotechnological production of prebiotic carbohydrates, while the second part reviews the bioproduction of surfactants. These additives were targeted in this study for their future potential to the industry, especially when obtained by means of biotechnological processes. Therefore, this chapter includes an extensive and recent review highlighting key advances in the production of additives through microbial biotechnology, the potential and challenges of this area and the future prospects.

33.2 Prebiotic Carbohydrates

Oligosaccharides are carbohydrates characterized by small chains with between two and

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ten monosaccharide residues, joined through glycosidic linkage (Anadón *et al*., 2010; Wolf *et al*., 2012). They occur free or in bound form and can be obtained from natural sources, or produced by physical, chemical or enzymatic processes (Anadón *et al*., 2010; Wolf *et al*., 2012; Moreno and Sanz, 2014). Some oligosaccharides exhibit not only nutritional or sweetener functions, but also physiological activity being considered as functional food ingredients (Macfarlane *et al*., 2008; Hernandez and Pandinella, 2013; Díez-Municio *et al*., 2014). Generally these compounds are classified as digestible or non-digestible oligosaccharides (NDOs) depending on how they are metabolized in the gastrointestinal tract (GIT), and this information can help to understand their physiological functions (Hernandez and Pandinella, 2013).

The NDOs increase the growth or activity of beneficial bacteria in the colon, mainly bifid bacteria and lactobacilli, and thus can be recognized as prebiotics (Mussatto *et al*., 2007; Rastall, 2010; Slavin, 2013). These compounds play an important role in maintaining homeostasis in the body and their physiological properties are associated with the fermentation and production of short-chain fatty acids (SCFAs) such as acetate, propionate and butyrate (Macfarlane and Macfarlane, 2011; Nicholson *et al*., 2012; Al-Sheraji *et al*., 2013; Di Bartolomeu *et al*., 2013). These molecules provide energy and nutrients for the growth of these beneficial bacteria besides stimulating blood flow and fluid and electrolyte uptake (Roberfroid *et al*., 2010; Di Bartolomeu *et al*., 2013; Rosenberg and Zilber-Rosenberg, 2013). Basically, they have a positive influence on the cell cycle of the intestinal epithelium as well as other metabolic effects (Al-Sheraji *et al*., 2013; Di Bartolomeu *et al*., 2013). The effects of prebiotics on the GIT are shown in Fig. 33.1.

In addition, these compounds are related to the bioavailability of minerals, modulation of the immune system, prevention of the incidence or improvement to the severity and duration of gastrointestinal infections, modification of inflammatory conditions, regulation of metabolism disorders related to obesity and type 2 diabetes; neutralization of toxic products and decreased frequency of colon cancer (Sousa *et al*., 2011; Brownawell *et al*., 2012; Charalampopoulos and Rastall, 2012).

33.2.1 Chemical structure and types of non-digestible oligosaccharides (NDOs)

By definition, oligosaccharides are polymers of monosaccharides with a low degree of polymerization linked by *O*-glycosidic bonds such as (1→2)-, (1→3)-, (1→4)- and (1→6)- (Witczak, 2008; Martínez-Villaluenga and Frías, 2014; Panesar *et al*., 2014). NDOs have the anomeric carbon atoms (C1 or C2) of the monosaccharide units (glucose, galactose, fructose and xylose) in two possible configurations (α- or β-) that make their osidic bonds non-susceptible to the hydrolytic enzymes in GIT (Ferreira *et al*., 2011; Preedy, 2012; Benekeblia, 2014; Martínez-Villaluenga and Frías, 2014).

The market of functional oligosaccharides is growing through the continuous development of technologies (Noomhorm *et al*., 2014). They are categorized according to common criteria as established prebiotics, emerging prebiotics and those with huge applications (Huckle and Zhang, 2011; Patel and Goyal, 2011; Lam and Cheung, 2013).

Established prebiotics include inulin-based fructose oligomers, galactooligosaccharides (GOS) and lactulose (Sabater-Molina *et al*., 2009; Gänzle, 2012). Compounds such as soybean oligosaccharides, xylooligosaccharides (XOS), genitiooligosaccharides, raftiloses, raftiline, isomaltooligosaccharides and mannanoligosaccharides are categorized as emerging prebiotics (Crittenden, 2006; Aachary and Prapulla, 2011; Lam and Cheung, 2013). Other NDOs and sugar alcohols such as mannitol, maltodextrin, raffinose, sorbitol, arabinose and arabinoxylan-oligosaccharides (AXOS) have huge applications as prebiotics (Sanchez *et al*., 2009; Siok-Koon and Liong, 2010; Lamsal, 2012). A brief summary of physico-chemical properties and methods of production of the main NDOs are provided in Table 33.1.

33.2.2 Technologies for the production of NDOs

There are essentially three processes to obtain NDOs: (i) direct extraction of natural oligosaccharides from plants; (ii) controlled hydrolysis of natural polysaccharides; and (iii) enzymatic synthesis using hydrolases and/or glycosyl transferases from plants or of microbial origin (Lee and

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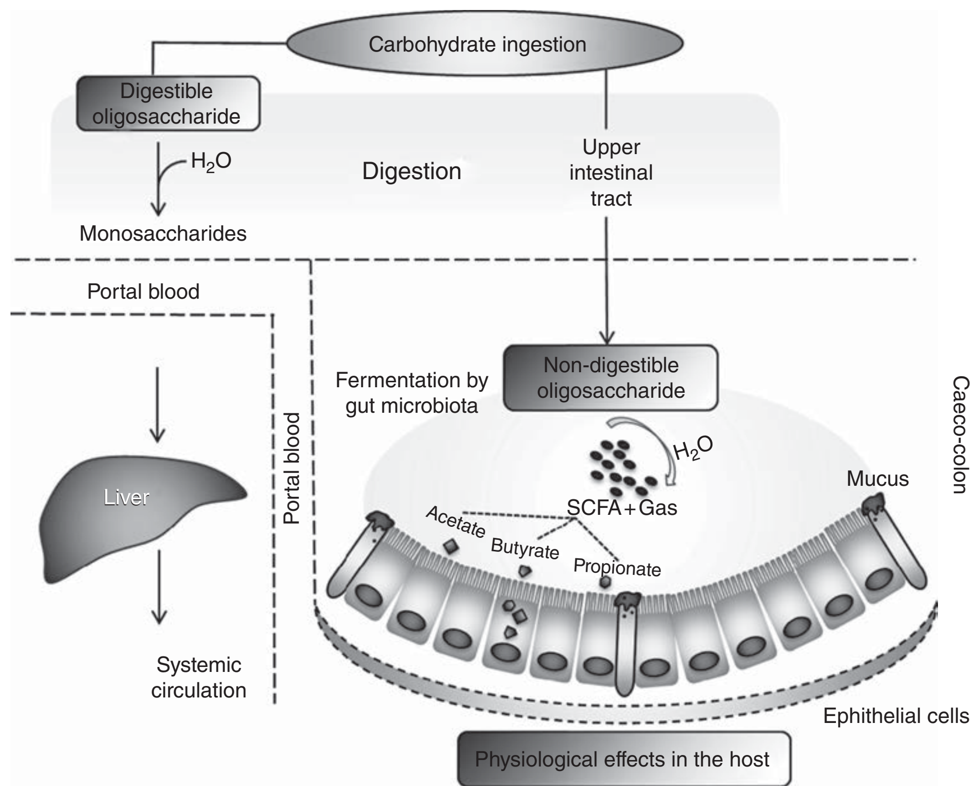

Fig. 33.1. Schematic representation of the physiological properties of non-digestible oligosaccharides (NDOs). Most oligosaccharides are broken by hydrolysis in the upper part of the gastrointestinal tract **(**GIT) and the resulting monosaccharides are transported via the portal blood to the liver and, subsequently, to the systemic circulation. However, NDOs reach the caeco-colon because their chemical structure does not allow them to be absorbed in the upper part of the GIT or hydrolysed by human digestive enzymes. In the caeco-colon NDOs are hydrolysed to small oligomers and monomers, which are further metabolized by bifidobacteria and lactobacilli and their fermentation results in a decrease in pH, the production of gases (H_2, CO_2, CH_4) and short-chain fatty acid (SCFA) production (acetate, propionate, butyrate) which serve as fuels in different tissues and may play a role in the regulation of cellular processes. (Adapted from Mussatto and Mancilha, 2007.)

Salminen, 2009; Bicas *et al.*, 2010; Cho and Finocchiaro, 2010; Boler and Fahey, 2012). However, the main industrial processes involving carbohydrates include biotechnological transformations due to the requirements of specificity and stereo-selectivity (Buchholz and Seibel, 2008). They can also be synthesized from monosaccharides and disaccharides by chemical or enzymatic process and from polysaccharides by enzymatic hydrolysis (Mudgil and Barak, 2013; Tymczyszyn *et al.*, 2014). This section will focus on the most recent advances in the biotechnological production of fructooligosaccharides (FOS), GOS and XOS.

Fructooligosaccharides (FOS)

FOS are oligosaccharides of fructose containing a single glucose moiety and are mainly composed of 1-kestose (GF_2), nystose (GF_3) and 1-β-fructofuranosyl nystose (GF_4), in which fructosyl units (F) are bound at the β(2→1) position of the sucrose molecule (GF), as may be seen in Fig. 33.2 (Sabater-Molina *et al.*, 2009; Sanchez *et al.*, 2009). They are industrially produced from sucrose by inulin or the action of enzymes with transfructosylation activity (i.e. fructosyltransferase (EC 2.4.1.9) and/or fructofuranosidase (EC 3.2.1.26) derived from plants and

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Table 33.1. Physico-chemical properties and methods of production of the main non-disgestible oligosaccharides (NDOs). (From Playne and Crittenden, 2009; Patel and Goyal, 2011; Fu and Wang, 2013.)

NDO[a]	Natural source	Chemical structure	Production method	Applications	Manfactor/trade name/product description
FOS Fructose	Fruits and vegetables (chicory, onions, banana, garlic, etc.)	β(2→1) linked fructans	Transfructosylation from sucrose or hydrolysis of chicory inulin	Improves gut absorption of Ca and Mg, prevents urogenital infections, sweetener in beverages, has an effect on lipid metabolism, reduces risk of colon cancer	• GTC Nutrition, Westchester, USA/ **NutraFlora**®**P**-95 – a powder containing 95% short-chain fructooligosaccharides consisting of GF, GF23 and GF4 molecules on a dry basis • Mitushi Pharma, Gujarat, India**/FOS-G** – a syrup containing 55% of fructooligosaccharides
GOS Galactose	Human milk	β(1→4) and β(1→6) linked galactose	Enzymatic lactose transgalactosylation	Promotes beneficial cutaneous flora, used in dermocosmetic industry	• Yakult, Acton, London, UK/**Oligomate**® – a syrup containing 75% (w/v) solids. Oligosaccharides make up 55% of solids • Nissin Sugar Mfg. Co. Ltd, Tokyo, Japan/**Cup-Oligo H-70** – a syrup containing 75% (w/v) solids • Clasado Inc., Milton Keynes, UK/ **Bimuno**® – a powder
XOS Xylose	Bamboo shoots, fruits, vegetables, maize (corn) cobs, milk and honey	β(1→4) linked xylose	Enzymatic hydrolysis of xylan. Enzyme treatments of native lignocellulosic materials. Hydrolytic degradation of xylan by steam, water or dilute solutions of minerals acids	Used in cosmetics, as a plant growth regulator, antioxidant, gelling agent and for treatment of diabetes, atherosclerosis and colon cancer	• Suntory, Japan/**Xylo-oligo 95** – a powder containing 95% of oligosaccharides • Shandong Fegyuan Zhongke Ecology Technology Co. Ltd, Shandong, China/**XOS-70S** – a syrup containing 70% of xylobiose-xyloheptaose

[a]FOS, Fructooligosaccharides; GOS, galactooligosaccharides; XOS, xylooligosaccharides.

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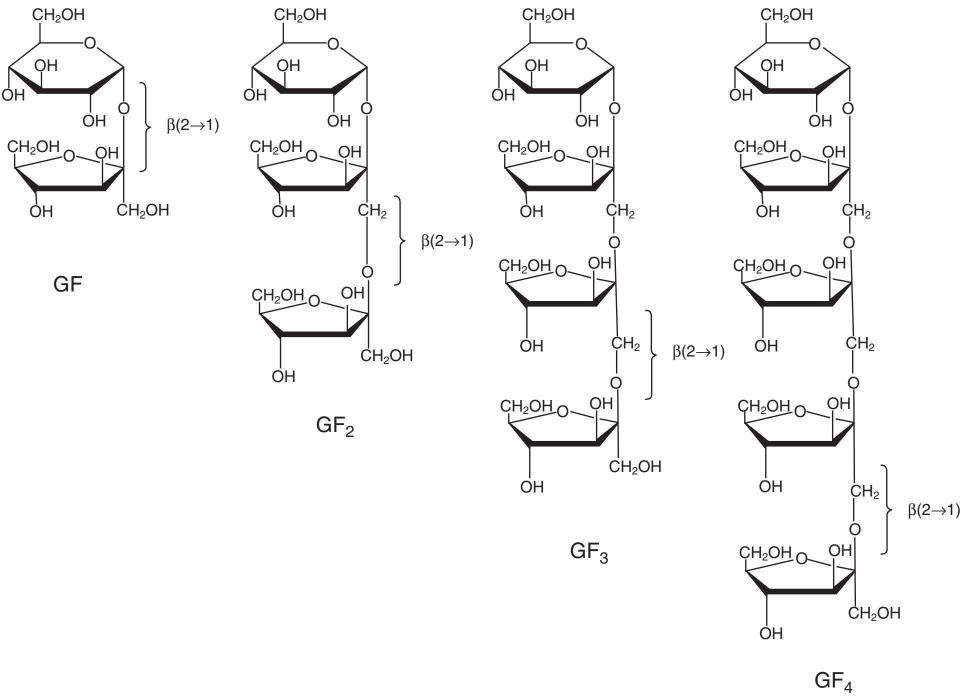

Fig. 33.2. Molecular structure of fructooligosaccharides **(**FOS). FOS are defined as a mixture of GF_2, GF_3 and GF_4 and these oligosaccharides consist of short chains of fructose units linked by β(2→1) glycosidic bonds and the degree of polymerization of oligofructose ranges from two to four. GF, Sucrose; GF_2, 1-kestose; GF_3, nystose; GF_4, 1-β-fructofuronosyl nystose.

microorganisms) (Mussatto and Teixeira, 2010; Dominguez *et al.*, 2012; Mussatto *et al.*, 2012). However, most of these enzymes that are used are from members of the fungi kingdom, especially from the genera *Aureobasidium*, *Aspergillus*, *Penicillium* and *Fusarium*, and they provide a cost-effective and convenient alternative to chemical synthesis (Mussatto and Teixeira, 2010).

Conventionally, FOS are industrially produced by a two stage process: (i) microbial production by fermentation; and (ii) enzymatic reaction with sucrose as the substrate, using immobilized enzyme or whole cells (Monsan and Ouarné, 2009; Mussatto *et al.*, 2009; Ning *et al.*, 2010). The FOS yield depends on the relative rate of synthesis, that in the case of transfructosylation reactions ranges from 55% to 86% under optimal conditions (Mussatto *et al.*, 2009; Mussatto and Teixeira, 2010; Singh and Singh 2010). Figure 33.3 illustrates the industrial process for the production of FOS using microorganisms as biocatalysts.

Although this exists as a well-established method of producing FOS, many studies are focusing on biotechnological processes to improve enzymatic reactions and yields on the industrial scale using immobilized-cell systems (Casci and Rastall, 2006; Mussatto *et al.*, 2009).

Galactooligosaccharides (GOS)

GOS naturally occur in human milk and their structure is composed of galactose units linked mostly by β(1→4) and β(1→6) bonds and a terminal glucose residue; also dimers are considered as GOS (Gänzle, 2012; Lamsal, 2012; Vera *et al.*, 2012). They are usually produced from concentrated lactose syrup using the transgalactosylase activity of the enzyme β-galactosidase (EC 3.2.1.23), that can involve crude enzymes, purified enzymes, recombinant enzymes, whole cells, toluene-treated cells, immobilized enzymes and immobilized cells (Casci and Rastall, 2006; Park and Oh, 2010; Boler and Fahey Jr., 2012;

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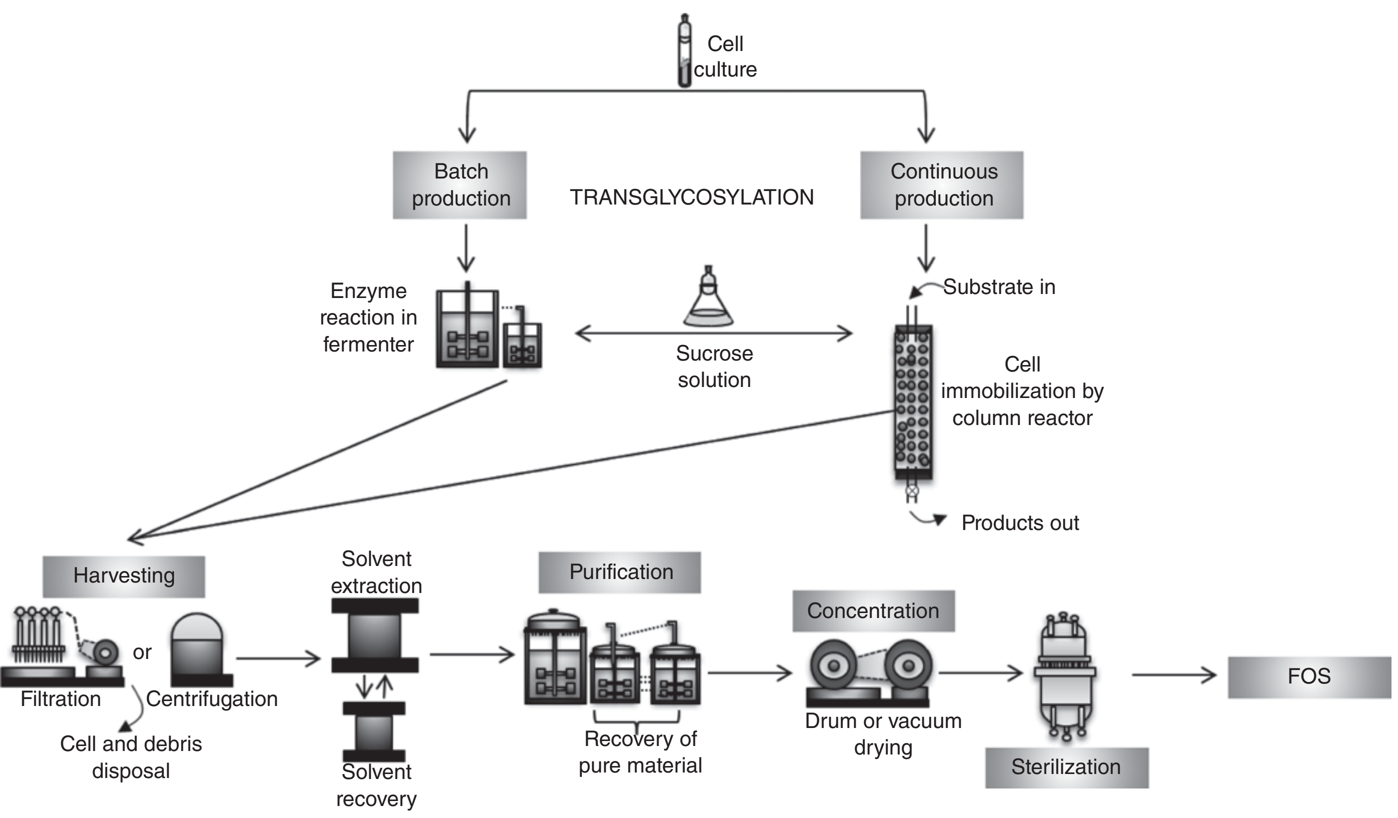

Fig. 33.3. Ilustration of industrial process for the production of fructooligosaccharides (FOS) by microorganisms.

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Manera *et al*., 2012). Figure 33.4 illustrates the industrial process for the production of GOS by microbial means.

β-Galactosidase can be isolated from many sources (plants, animal tissue, yeast, bacteria or fungi), and among them yeasts such as *Kluyveromyces lactis* represent a promising source of this enzyme in food applications (Casci and Rastall, 2006; Manera *et al*., 2010; Vera *et al*., 2012). Additionally, *Aspergillus oryzae* and *Bacillus circulans* could also be suitable for GOS biosynthesis (Huerta *et al*., 2011; Rodriguez-Colinas *et al*., 2012; Vera *et al*., 2012).

Basically, the reaction mechanism includes hydrolysis and a transglycosylation reaction (Manera *et al*., 2010, 2012; Palai *et al*., 2012). In other words, there are a complex of processes in which lactose or other sugar components present in the mixture act as galactosyl acceptors, followed by several degrees of polymerization of glucose and galactose joined by glycosidic bonds resulting in GOS (Otieno, 2010; Sangwan *et al*., 2011; Rodriguez-Colinas *et al*., 2012).

The main drawback of oligosaccharide synthesis by these enzymes is that the reaction equilibrium is shifted to favour hydrolysis over synthesis in aqueous systems, which leads to a low yield in GOS production (Otieno, 2010). Generally, GOS yield improves with increasing lactose concentration that under optimal conditions (enzyme source/activity, glucose and galactose concentration, temperature and the length of reaction) can present yields ranging from 30% to 40% (Otieno, 2010; Sangwan *et al*., 2011; Rodriguez-Colinas *et al*., 2012).

Many approaches to increase production of GOS have been attempted such as ultrafiltration techniques, temperature change, microwave irradiation, protein engineering technology and changes in specific amino acids in the active site of the enzyme or the enzyme anchored on the cell surfaces of engineered microorganisms (Otieno, 2010; Sangwan *et al*., 2011).

Xylooligosaccharides (XOS)

XOS are sugar oligomers made up of xylose units, and mainly consist of xylobiose, xylotriose and xylotetraose, which occur naturally in honey, bamboo shoots, fruits, vegetables and milk (Casci

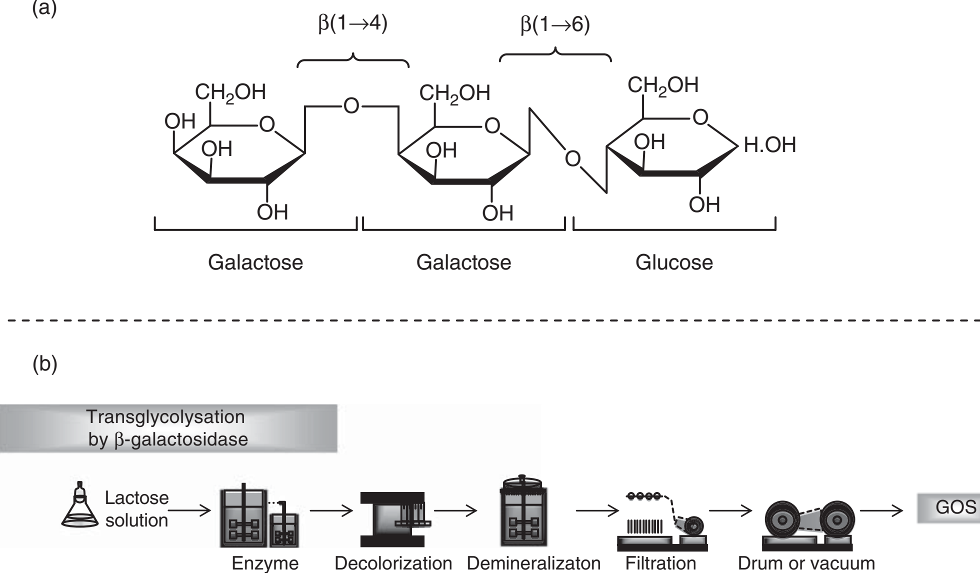

Fig. 33.4. (a) Molecular structure of galactooligosaccharides (GOS). GOS are defined as oligosaccharides linked by β(1→4) or β(1→6) glycosidic bonds. (b) Ilustration of industrial process for the production of GOS by microorganisms, produced from lactose syrup using the transgalactosylase activity of the enzyme β-galactosidase.

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and Rastall, 2006; Boler and Fahey Jr, 2012). They can be produced on an industrial scale through using a variety of lignocellulosic substrates such as maize (corn) cobs, almond shells, olive stones, rice husks, wheat straw and barley straw agroresidues, whose hemicellulosic fraction is mainly made up of xylan, a polymer made up of a β(1→4)-D-xylopyranose backbone (Nabarlatz *et al.*, 2007).

Generally, XOS are obtained by either chemical treatments or, more commonly, enzymatic hydrolysis of xylan-rich lignocellulosic materials (Gilad *et al.*, 2010; Chapla *et al.*, 2012; Qing *et al.*, 2013). But it is also possible to use other combined approaches such as chemical-enzymatic autohydrolysis or autohydrolysis-enzymatic methods (Sabiha-Hanim *et al.*, 2011). Figure 33.5 illustrates the industrial process for the production of XOS.

The autohydrolysis process, for example, involves the deacetylation of xylans to produce acetic acid which hydrolyses the hemicelluloses and subsequent purification using high temperature and pressures (Akipinar *et al.*, 2009). The products obtained by this method contain a variety of undesirable components such as lignin, monosaccharides, furfural and others, thereby requiring further purification (Carvalho *et al.*, 2013).

Alternatively, XOS can be produced by acid hydrolysis of xylan where the fragmentation of xylan can be easily accomplished by partial acid hydrolysis using dilute sulfuric acid (0.1–0.5 M) (Akpinar *et al.*, 2009; Qing *et al.*, 2013). The degree of polymerization of the XOS depends on the acid concentration, the temperature and the reaction time (Qing *et al.*, 2013). Like autohydrolysis, this method produces a large amount of monosaccharides and their dehydration products (Akpinar *et al.*, 2009; Qing *et al.*, 2013). One disadvantage of acid hydrolysis is low yields of oligomers compared with monomers as well as production of furfural and other degradation products. In contrast, the major advantage is the rapid kinetics observed (Qing *et al.*, 2013).

Another approach is the enzymatic hydrolysis that has been used to degrade xylan into

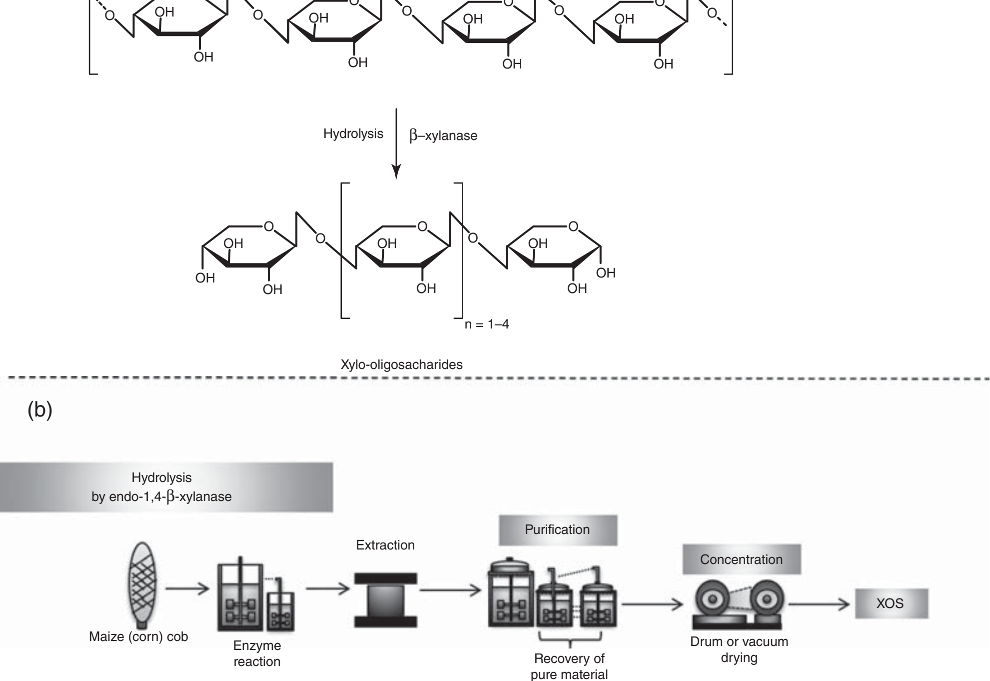

Fig. 33.5. (a) Molecular structure of xylooligosaccharides (XOS). XOS are defined as a mixture of oligosaccharides formed by xylose residues linked by β(1→6) glycosidic bonds. (b) Illustration of industrial process for the production of XOS, produced by enzymatic hydrolysis.

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monosaccharide through the synergistic action of enzymes such as: (i) endo β-1-4-xylanase (β-1-4-D-xylan xylanohydrolase; EC 3.2.1.8) that cleaves glycosidic bonds to produce XOS; (ii) β-xylosidase (1,4-xylan xylohydrolase; EC 3.2.1.37) responsible for converting small xylo-oligosaccharides into monomeric xylose; and (iii) debranching enzymes (esterases) required to breakdown side groups (Maalej-Achouri *et al*., 2009; Manisseri and Gudipati, 2010; Chapla *et al*., 2012; Bajpai, 2014).

The recovery yields and the degree of purification are dependent on the type of solvent employed for extraction as well as the lignocellulosic raw material, which control the XOS substitution pattern and the possible presence of stabilizing non-saccharide components, especially comparatively high proportions of uronic groups and/or compounds in autohydrolysis liquors that are influenced by the XOS substitution pattern (Qing *et al*., 2013).

33.2.3 Main applications: research and industry

There is an increasing market for health-promoting NDO food ingredients that can be formulated either as a powder or as a syrup and the industrial applications are focused on dairy products, beverages, milk products, infant formula and weaning foods, bakery products, confectionary, dietary supplement foods, animal feeds and others (Mussatto and Mancilha, 2007; Charalampopoulos and Rastall, 2012; Patel and Goyal, 2012; Fu and Wang, 2013).

There exists an array of prebiotics that differ in source or chemical properties. In food formulations, for example, they exhibit considerably lower sweetness than sucrose, are more stable and are practically imperceptible in the food product enriching the organoleptic and physico-chemical characteristics (Wang, 2009; Boler and Fahey Jr, 2012; Charalampopoulos and Rastall, 2012; Patel and Goyal, 2012). In addition, some non-food applications have also been proposed for oligosaccharides including use in the pharmaceutical, cosmetics and agrochemistry industries (Mussatto and Mancilha, 2007; Patel and Goyal, 2011).

Several NDO such as inulin, FOS and GOS have 'generally recognized as safe' (GRAS) status, as their history of safe use is sufficient to guarantee safety to public authorities, users and consumers (Malcata *et al*., 2014). However, many countries have no requirement for pre-market approval of prebiotics, because there is no established or implemented system for health claims, although scientific substantiation should be available on request by authorities (Brownawell *et al*., 2012).

33.3 Biosurfactants

Natural surfactants, including biosurfactants, are important biological active agents produced by plants, microorganisms and animals, including humans, and are able to reduce the surface and interfacial tension at the interfaces between liquids, solids and gases (Li *et al*., 2002; Aparna *et al*., 2011; Makkar *et al*., 2011; She *et al*., 2011; Al-Wahaibi *et al*., 2014).

Biosurfactants, in particular, are considered compounds synthesized by a wide variety of microorganisms such as fungi, yeasts and bacteria which exhibit high surface activity and emulsifying activity (Mulligan, 2005; Rodrigues *et al*., 2006a; Cameotra *et al*., 2010; Marchant and Banat, 2012). These compounds comprise molecules with different chemical structures of low and high molecular weight, and are classified as glycolipids, lipopeptides and lipoproteins, phospholipids and fatty acids, neutral lipids, polymeric surfactants and particulate surfactants (Biermann *et al*., 1987; Desai and Banat, 1997; Mukherjee *et al*., 2006). They affect the survival of microorganisms since they are associated with nutrient acquisition, mobility, differentiation and cell signalling, biofilm formation and other physiological phenomena (Van Hamme *et al*., 2006).

The low-molecular-weight biosurfactants (LMB), such as rhamnolipids from *Pseudomonas aeruginosa*, viscosin from *Pseudomonas fluorescens* and surfactin from *Bacillus subtilis*, are associated with the reduction of surface and interfacial tension between air–water or oil–water (Kim *et al*., 2010; Nitschke *et al*., 2011; Kumar *et al*., 2012; Alsohim *et al*., 2014). An approximate 50% reduction in the surface tension can be observed at the air–water interface, decreasing from 70 mN/m to below 30 mN/m (Gutnick and Bach, 2011). The high-molecular-weight biosurfactants (HMB), like emulsan from *Acinetobacter* sp. and liposan from *Candida lipolytica*, are associated with emulsifying properties (Nitschke and Costa, 2007; Su *et al*., 2009; Gutnick and Bach, 2011; Rufino *et al*., 2014; Santos *et al*., 2014).

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Structurally, biosurfactants are amphiphilic compounds and have hydrophobic and hydrophilic domains. The hydrophilic domain can have amino acids, peptides or glycosylated residues and the hydrophobic domain has unsaturated or saturated fatty acids which together are capable of lowering the surface and interfacial tension (Mehta *et al*., 2010; Aparna *et al*., 2011).

Currently the use of biosurfactants as an alternative to the use of chemical surfactants is due to the fact that they have several advantages, especially regarding: (i) lower toxicity; (ii) biodegradability; (iii) compatibility with the environment; (iv) activity even in extreme temperature, pH and salinity conditions; and (v) synthesis from renewable raw materials such as agro-industrial residues (Souza-Sobrinho *et al*., 2008; Banat *et al*., 2010).

Considering these characteristics and their physico-chemical properties, biosurfactants present interesting applications, for example bioremediation, enhancement of oil recovery, desorption of contaminants from soil, as a biocontrol agent, for wastewater treatment, industrial applications as food additives and in cosmetic and detergent formulations (Singh *et al*., 2007; Cerqueira *et al*., 2011; Makkar *et al*., 2011; Damasceno *et al*., 2012; Zou *et al*., 2014). Besides that, some biosurfactants show biological activities and can be applied in the medical field (Rodrigues *et al*., 2006a).

The rhamnolipids produced by *P. aeruginosa* are glycolipids composed of one or two rhamnose molecules linked to one or two fatty acid alkyl chains and they have low toxicity and surface active properties that can be useful for the food processing industry (Magalhães and Nitschke, 2013) (Fig. 33.6). Furthermore, in recent studies this biosurfactant has demonstrated antimicrobial activity against fungal and bacteria strains and anti-proliferative activity (Vatsa *et al*., 2010; Sha *et al*., 2011; Zhao *et al*., 2013).

Among all biosurfactants, surfactin, a cyclic lipopeptide produced by *B. subtilis*, is particularly interesting because of its high surface activity, antimicrobial, antitumour and anti-inflammatory activities (Fig. 33.6). Therefore, many studies have been conducted in order to assess other biological activities of this compound (Kim *et al*., 2007; Park *et al*., 2010; Son *et al*., 2011; Park *et al*., 2013b).

Park *et al.* (2013a) showed that surfactin inhibits the amyloid β-induced inflammatory response of microglial cells and confers protection against indirect neurotoxicity to hippocampal cells, indicating that the biosurfactant may have therapeutic potential for Alzheimer's disease as well as other neurodegenerative disorders which involve neuroinflammation. In another study, also performed by Park *et al.* (2013b), it was proved that surfactin significantly inhibited excessive production of the pro-inflammatory mediators tumour necrosis factor (TNF)-α, interleukin (IL)-1β, IL-6, monocyte chemoattractant protein-1 (MCP-1), prostaglandin E2 (PGE2), nitric oxide (NO) and reactive oxygen species (ROS), and suppressed the expression of matrix metalloproteinase-9 (MMP-9), inducible NO synthase (iNOS) and cyclooxygenase-2 (COX-2) (Park *et al*., 2013a, b).

Therefore, biosurfactants are valuable economically and have attracted research investigations with the purpose of increasing their yields in large-scale production, which is, nowadays, the main challenge to inserting these compounds in the market.

33.3.1 Production of biosurfactants from residues and application of statistical methods for process optimization

The demand for biologically produced compounds, such as biosurfactants, in industry has grown significantly considering economic and environmental concerns. However, the production and use of these compounds is limited due to the application of expensive substrates, associated with low yields and low product concentrations, formation of product mixtures and the several complex extraction and purification steps required in the final process (Syldatk and Hausmann, 2010; Makkar *et al*., 2011; Al-Bahry *et al*., 2013).

On the other hand, new strategies like the use of genetic engineering tools and alternative substrates are promising approaches to enhance the viability of biosurfactant production (Van Bogaert *et al*., 2007; Saerens *et al*. 2011; Gutnick and Bach, 2011; Marchant and Banat, 2012). In addition, the industrial-scale production through cheaper processes depends on several strategies, including the application of statistical methods to optimize the media components and process conditions, and the use of cheaper raw

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Rhamnolipids

Surfactin

Fig. 33.6. Chemical structures of some biosurfactants with biological activities.

materials such as agro-industrial by-products to reduce the cost of production and to enhance the yield of biosurfactants (Nitschke *et al.*, 2004; Joshi *et al.*, 2008; Liu *et* al., 2010; Pereira *et al.*, 2013; Santos *et al.*, 2014).

Agro-industrial residues and by-products with high content of carbohydrates, proteins or lipids are suitable as the raw material for biosurfactant production (Cameotra and Makkar, 2004). Some examples include the use of cassava wastewater, sludge palm oil, cashew apple juice, olive oil mill effluent, plant oil extracts and waste, distillery and whey wastes, potato process effluent, restaurant frying oil and lignocellulosic wastes (Makkar *et al.*, 2011). Other residues have also been used as a carbon source for the production of biosurfactants by several bacteria strains, fungi and yeasts, such as molasses, maize (corn) powder, animal fat, corn steep liquor, vegetable fat waste, and others (Sammai *et al.*, 2011; Gusmão *et al.*, 2010; Santos *et al.*, 2013; Jain *et al.*, 2013). In this context, many studies have focused on evaluating the potential of various low-cost substrates for the production of surfactants, and in some cases satisfactory yields have been obtained,

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demonstrating that these processes have a great development potential.

Biosurfactant production using maize (corn) powder, potato peel powder, *Madhuca indica* and sugarcane bagasse as substrate has also been reported. Among the carbon sources tested, the production of biosurfactant was found to be the highest with maize (corn) powder (15.40 ± 0.21 g/l) (Jain *et al*., 2013).

The use of mixed substrates obtained from vegetable processing showed higher yields compared with the use of a single substrate. Daverey and Pakshirajan (2009) achieved production of approximately 24g/l of sophorolipids from the yeast *Candida bombicola* (Daverey and Pakshirajan, 2009). Recently, Chooklin *et al.* (2014) described high production of lipopeptide biosurfactant (5.30 g/l) by *Halobacteriaceae archaeon* AS65 when the cells were grown on a minimal salt medium containing 35% (w/v) banana peel and 1 g/l commercial monosodium glutamate after 54 h of cultivation.

The most influential variables relating to biotechnological processes, and in particular for the control and optimization of biosurfactant production, are temperature, medium pH, medium composition (including residues) and salinity. It is known that these conditions need to be maintained within a certain range, so maximum production of biosurfactant can be achieved (Najafi *et al*., 2011). Thus, the application of statistical methods to optimize the process has shown interesting results in biosurfactant production. Santos *et al*. (2014) used a 2^3 full factorial design to determine the maximum production of biosurfactants by *Candida lipolytica* UCP0988. This yeast was cultivated in a low-cost fermentative medium based on 5% animal fat and 2.5% corn steep liquor. They concluded that optimal conditions to reduce the surface tension and biomass were 200 rpm of agitation speed, allowing 144 h for the process and 0 vvm of aeration (Santos *et al*., 2014).

Najafi *et al*. (2011) reported the combination of a central composite rotatable design (CCRD) and response surface methodology (RSM) to optimize biosurfactant production and four parameters (pH, temperature, glucose and salinity concentrations) were selected for optimization of growth conditions for the bacterial strain *Paenibacillus alvei* ARN63 isolated from an Iranian oil well. The results showed that maximum reduction in surface tension was obtained under the optimal conditions of 13.03 g/l glucose concentration, 34.76°C, 51.39 g/l total salt concentration and the medium being held at pH 6.89 (Najafi *et al*., 2011).

33.3.2 Promising environmental applications of biosurfactants

The use of biosurfactants has also been proposed for various industrial applications and recent studies have mostly focused on environmental and bioremediation aspects. In this context, the biological methods for remediation of polluted areas have been recognized as a cost-effective and promising strategy (Szulc *et al*., 2014).

Bioremediation can be considered as the degradation of toxic pollutants through microbial assimilation or enzymatic transformation to less toxic compounds, and is considered an alternative to problems of environmental pollution by petroleum and petrochemical products (Singh *et al*., 2008; Cameotra and Makkar, 2010). Therefore, many studies have focused on the application of biosurfactants in the bioremediation of polluted environments and in the petroleum industry, showing that microbially enhanced oil recovery (MEOR) can be advantageous (Marchant and Banat, 2012; Nikolopoulou *et al*., 2013).

Cappello *et al*. (2011, 2012) showed that the biosurfactant exopolysaccharide EPS2003 has significant potential to be applied as an oil-spill remediation agent in marine environments. Petrikov *et al*. (2013) described the production of glycolipids with high surface activity by an oil-degrading bacteria used in bioremediation preparations with surface tension ranging from of 31 mN/m to 34 mN/m, two times lower than the surface tension of the reference solution that was 77 mN/m. In this work, it was observed that rhamnolipid B and its homologues were produced by *Pseudomonas* sp. and trehalose tetraesters by *Rhodococcus* sp. (Petrikov *et al*., 2013).

Recently, Noparat *et al*. (2014) investigated the application of biosurfactant from *Sphingobacterium spiritivorum* AS43 using molasses as a substrate and fertilizer to enhance the biodegradation of used lubricating oil (ULO). They found that the maximum degradation of ULO (62%) was observed when 1.5% (w/v) of biosurfactant or fertilizer was added (Noparat *et al*., 2014).

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The surface activity of the lipopeptide biosurfactant produced by *Acinetobacter baylyi* ZJ2, isolated from a crude-oil-contaminated soil sample in China, was evaluated in MEOR by Zou *et al*. (2014). In this study, the biosurfactant was able to decrease the water surface tension from 65 mN/m to 35 mN/m, and interfacial tension against oil from 45 mN/m to 15 mN/m, characterizing a powerful surface-active agent (Zou *et al*., 2014).

In this context, recent studies have been directed to the study of how to increase the scale of production of these compounds, bringing a challenge to researchers and requiring larger investments to achieve the scale required for industrial production.

33.4 Concluding Remarks

This chapter has discussed future advances in modern biotechnology that have directed efforts for the industrial production of prebiotic carbohydrates and surfactants.

Knowledge about the health benefits of NDOs associated with biotechnological processes, as well as microbiology and enzymatic technologies, have improved the design of new sugars and resulted in their large-scale employment in several industry sectors, especially the food ingredients segment. These insights aim to optimize the conditions of production for maximum yield while keeping costs low during manufacture. However, the yields achieved must be improved through chemical or biological approaches to increase the portfolio of products available in the market. NDOs can be incorporated into many food products but there still remain questions about efficiency and safety that need to be resolved.

Several NDOs such as inulin, FOS and GOS have been allocated GRAS status, as their history of safe use is sufficient to guarantee safety to public authorities, users and consumers. However, many countries have no requirement for pre-market approval of prebiotics, because there is no established or implemented system for health claims, although scientific substantiation should be available on request by authorities. Thus, these oligosaccharides present a good opportunity to explore effects on the human nutrition profile and physiopathology. Their future prospects highlight the use of alternative sources of substrates for production, such as agro-industrial residues as substrates for new sugar production.

The physico-chemical properties of biosurfactants present interesting applications such as for bioremediation and enhancement of oil recovery and applications in the food and cosmetics industries. Studies devoted to increase yields in large-scale production of biosurfactants by microorganisms and the utilization of low-cost substrates for biosurfactant production were discussed. These approaches, along with the promising environmental applications and potential use in the petroleum industry, are certainly the most promising ways to establish these valuable compounds in the market. However, more research is still necessary in order to gain better control of the production technologies and improvement of the overall process.

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34 Industrial Additives Obtained Through Microbial Biotechnology: Bioflavours and Biocolourants

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Abstract

Biotechnology offers unique opportunities to produce natural food ingredients, and this area has expanded considerably in recent years bringing new possibilities for the production of industrial additives. Aroma compounds are remarkable for their volatility and chemical diversity, such as lactones, hydrocarbons, alcohols, ketones, aldehydes, acids or esters, with an important application and market. The biotechnological production of these compounds has shown several advantages compared with chemical production, such as the elevated velocity for reactions, multi-step synthesis and high stereospecificity. In the same approach, recent data makes clear the advantages of using microorganisms to produce biocolourants. These compounds have been added to food, drinks, clothes, paints, cosmetics and pharmaceuticals, for example, enhancing their appeal and acceptability by consumers. In this perspective, this chapter is focused on the biotechnological production of bioflavours and biocolourants, and aims to show their potential to industry, especially when obtained by means of biotechnological processes. Additionally, this chapter aims to cover an extensive and recent review of the literature, highlighting key advances in the production of additives through microbial biotechnology, the potential and challenges of this area and the future prospects.

34.1 Biotechnology: Useful Products for the Future of Industry

Food biotechnology has been developed empirically since ancient history, when humanity began to dominate the techniques for manufacturing fermented products, for example beer, wine, cheese and bread. Ever since, fermentation technology has evolved as one of the main tools for the food industry, which is applied by aiming at the preservation of food products or the modification of their sensory attributes (aroma, flavour, texture, etc.). Food science and technology have also begun to understand the phenomenology behind these processes and, today, the use of microbial and enzymatic processes for the production of food ingredients is increasingly being developed.

With the advent of biotechnological processes, a new division was proposed to cover compounds obtained on an industrial scale, defined ‘white biotechnology’, and considered as a modern version of biotechnology ‘based on the

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use of renewable resources and clean production and less pollution and less energy intensive processes in biological systems, such as whole cells or enzymes, used as reagents or catalysts' (Berger, 2008). Although many additives are already industrially produced following this division of modern biotechnology, many investments and efforts should also be devoted to research other important additives which are yet to reach the scale of industrial production, such as the case of bioflavours and biocolourants.

Flavours and fragrances have a wide application in the food, feed, cosmetic, chemical and pharmaceutical sector (Vandamme, 2003). Many flavour compounds on the market are still produced via chemical synthesis or via extraction from plants. However, the growing market for flavoured and fragranced products requires novel strategies for aroma chemical production (Krings and Berger, 1998), and a rapid switch towards the bioproduction and use of flavour compounds using biotechnological origin has been observed (bioflavours).

Additionally, another rapidly expanding area aims to produce natural colourants by microbial fermentations. Colourants have been added to food, drinks, clothes, paints, cosmetics, pharmaceuticals and plastics for a long time (Malik *et al.*, 2012; Boer, 2014). Besides that, colour is added to replace colour in cases where it is lost during processing, to enhance colour already present, to minimize variation between batches and to supplement food with nutrient, making the product more attractive for the consumer (Mortensen, 2006; Chattopadhyay *et al.*, 2008; Rymbai *et al.*, 2011).

Therefore, biotechnology offers unique opportunities to produce natural food ingredients. However, although it is apparently an exhausted subject due to its long history, the biotechnological production of food and food additives remains an active field of research and there are many promising investigations that are still to be developed in this area.

With this perspective, this chapter is focused on the biotechnological production of bioflavours and biocolourants, and aims to show their potential to industry, especially when obtained by means of biotechnological processes. Additionally, this chapter aims to cover an extensive and recent review of the literature, highlighting key advances in the production of additives through microbial biotechnology, the potential and challenges of this area and the future prospects.

34.2 Bioflavours and Aroma Compounds Obtained by Microbial Biotechnology

The concept of aroma and flavour compounds involves organic substances with distinctive attributes related to odour and taste (Labuda, 2009). Aroma compounds are remarkable for their volatility and chemical diversity, such as lactones, hydrocarbons, alcohols, ketones, aldehydes, acids or esters, for instance (Bicas *et al.*, 2010b). The proportion of volatile molecules in a product directly influences the acceptance of consumers (Bicas *et al.*, 2011a).

The market profile concerning the flavour and fragrance industries has presented great potential prospects through a continuous increase over the last 3 years. In 2013, the forecast for sales for the ten most successful industries in this field was US$24 billion, higher than any of the previous 4 years (Leffingwell & Associates, 2014).

Extraction from natural sources is not regarded as an appropriate technique as key aroma compounds are often present only in trace amounts in nature (Serra, 2011). Moreover, this method is often restricted to geographical availability and seasonality.

Obtaining flavouring substances by physical, enzymatic or microbial processes from vegetable, animal or microbiological origin enables their classification as natural according to European Community regulation (Pöttering and Le Maire, 2008). Furthermore, there is a remarkable preference of consumers for ingredients labelled as natural, instead of those chemically synthesized, resulting in attractive marketing for the attribute 'natural'. These have been encouraging facts for the production of flavour and aroma compounds through biotechnology processes (Berger *et al.*, 2010).

Even though the costs regarding the use of biocatalysts along the processes could be higher compared with chemical processes, some relevant benefits associated should be cited including: (i) the utilization of renewable starting materials; and (ii) the decrease of hazardous

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substances involved. Furthermore, efficiency concerning biocatalysis has improved as a consequence of the development of genetic engineering techniques (Serra, 2011).

Biocatalysts have been considered valuable tools for industries willing to encourage sustainable production of valuable chemicals such as flavour and fragrance compounds. Beyond environmental compatibility, further advantages can be described compared with chemical synthesis such as: (i) elevated velocity for reactions; (ii) multi-step synthesis (making it possible, for example, to use different enzymes for each step in a sequence of reactions from designated starting compound to desired product); and (iii) high stereospecificity. The last can be considered an important feature regarding aroma compounds production, once chiral configuration and the presence of interfering compounds exert influence over sensory properties (Berger *et al.*, 2010).

34.2.1 Bioflavours: microbial production and potential

This section focuses on recent developments in the production of these compounds, covering only the last few years of progress. For further information on the subject, the recent review by Bicas *et al.* (2009) is widely recommended, which is focused in the industrial perspective of bioflavour production.

Noted for its sweet, lilac-like and floral notes (Labuda, 2009), α-terpineol is regarded as an important monoterpene alcohol and can be obtained by biotransformation of limonene (Chizzola, 2013). A promising anaerobic bioprocess resulting in nearly 130 g/l of α-terpineol, including a biphasic medium was reported: *Sphingobium* sp. cells were provided in aqueous phase, while sunflower oil constituted the organic phase. The hydratase enzyme presented biotransformation capability for both R-(+) and S-(-) substrate enantiomers (Bicas *et al.*, 2010a).

Remarkable for rose-like notes, and used in the beverages industry, two monoterpenoids can be obtained by reduction of citral by yeasts: nerol and geraniol. For instance, *Saccharomyces cerevisiae* has been used as the biocatalyst to produce nerol (Labuda, 2009).

In order to improve the profile of volatile compounds in Muscat wine, three immobilized glycosidases were obtained from a commercial preparation of *Aspergillus niger*. Some monoterpenoids usually bound to sugar units were released in consequence of these biocatalytic activities. Thus, some pleasant fruity and floral notes were better detected from the wine. The previously cited compound, geraniol, was significantly increased from 98 ± 11 μg/l to 438 ± 26 μg/l by using the described enzymes (González-Pombo *et al.*, 2014).

Recognized as a sesquiterpenoid with noteworthy odour characteristics, (+)-nootkatone has been valuable for both food and cosmetics industries due to a pleasant grapefruit-like note (Fraatz *et al.*, 2009). Biotechnological production of nootkatone has been stimulated because extraction from vegetal sources results in low yields and the alternative, chemical synthesis, applies environmentally noxious methods (Wriessnegger *et al.*, 2014).

The metabolically engineered yeast *Pichia pastoris* reached 208 mg/l of (+)-nootkatone by biotransformation of the sesquiterpene (+)- valencene, using bioreactor systems (Wriessnegger *et al.*, 2014). This substrate is available in the essence oil of Valencia orange (*Citrus sinensis*) (Fraatz *et al.*, 2009).

Biocatalysis of (+)-valencene by the basidiomycete *Pleurotus sapidus* dioxygenase also enabled production of approximately 280 mg/l (+)-nootkatone, and the authors revealed a lipoxygenase-type enzyme able to receive a terpene hydrocarbon as substrate in place of fatty acid (Krügener *et al.*, 2010). This valencene dioxygenase has been already expressed in *Escherichia coli*, and also presented catalytic activity, and the presence of a surfactant was considered advantageous. The authors also reported the alcohols α- and β-nootkatols as products of biotransformation as well as (+)-nootkatone (Zelena *et al.*, 2012).

Corynespora cassiicola DSM 62475 performed the biotransformation of linalool into linalool oxides, remarkable for their lavender notes. This microbial transformation bioprocess resulted in an overall yield of 4600 mg/l. The system was composed of hydrophobic absorbers in order to reduce to substrate toxicity and simultaneously keep the necessary amount of substrate available for biocatalysis (Bormann *et al.*, 2012).

Widely used for industries and recommended as an additive by the US Food and Drug Administration, γ-decalactone presents a pleasant peachy

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aroma (Moradi *et al*., 2013). *Yarrowia lipolytica*, remarkable for metabolic pathways adapted to hydrophobic substrates, has presented potential for γ-decalactone production. *Y. lipolytica* W29 yielded 5.4 ± 0.5 g/l of γ-decalactone from ricinoleic acid originating from castor oil triglycerides. This result was possible after 25 h of experiment, using 60 g/l of cells and substrate concentration (Braga and Belo, 2014). Some sulfur volatile substances relevant in cheeses produced by *Y. lipolytica* have also been reported (Zinjarde, 2014).

Benzaldehyde has been described for its cherry and almond notes (Labuda, 2009). This compound has been regarded as the second most-used compound in the flavour industries, after vanillin. The utilization of polymers as a sequestering phase in two-phase partitioning bioreactors presented potential advantages concerning biotechnological production of benzaldehyde. As the performance of the biocatalyst *P. pastoris* ATCC 28485 was influenced by the effects of product inhibition, further experiments involving partitioning concepts and sequestering polymers were performed. The use of the polymer Kraton D1102K to decrease toxicity along the bioprocess allowed superior results, such as the total obtainment of product (14.4 g) and promising volumetric productivity (97 mg/l/h), compared with the single phase experiment (Craig and Daugulis, 2013). The chemical structures of some bioflavours that are remarkable for their pleasant notes are shown in Fig. 34.1.

β-Ionone, an aroma compound characterized for floral, fruity and sweet notes, has been obtained by a yeast (34.0 mg/l) and a bacterium (42.4 mg/l) after 72 h and 24 h, respectively (Uenojo and Pastore, 2010).

As an alternative process to achieve natural β-ionone, the enzymatic oxidative cleavage of β-carotene was achieved with 60% of the substrate being converted. *In situ* separation of the product using organophilic pervaporation was performed, once the activity of *Arabidopsis thaliana* carotenoid cleavage dioxygenase 1 (AtCCD1) could be inhibited (Nacke *et al*., 2012). Recently, an innovative method to achieve β-ionone production by yeast has been presented: the utilization of polycistronic expression in *S. cerevisiae* would enable the use of glucose as substrate. The engineered strain included not only three genes related to β-carotene biosynthesis, originating from *Xanthophyllomyces dendrorhous*,

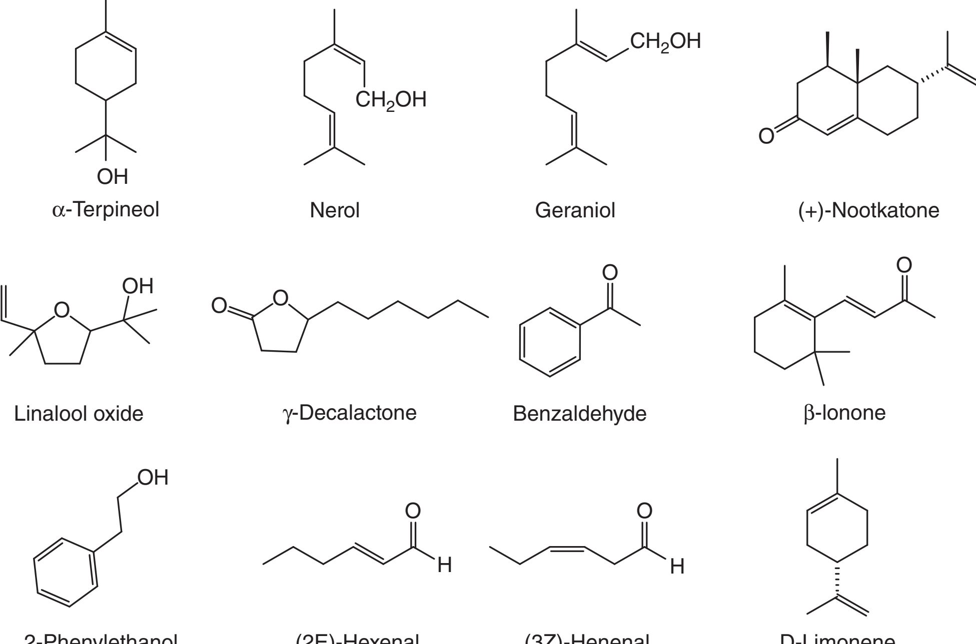

Fig. 34.1. Aroma or flavour compounds and their respective chemical structures.

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but also a dioxygenase from raspberry (*Rubus ideaus*), in order to provide β-carotene cleavage (Beekwilder *et al.*, 2014).

Microbial obtainment of phenylethanol (PEA), a compound with a remarkable rose-like aroma that is present in several essential oils, is possible from phenylalanine biocatalysis by yeasts according to the Ehrlich pathway (Serra, 2011; Wang *et al.*, 2011). This alcohol has been widely used in the perfume and cosmetic industries (Wang *et al.*, 2011).

Some articles have described product inhibition as one of the most challenging barriers to achieve high efficiency during the biotechnological obtainment of PEA. Thus, separation processes were recently tested to integrate the production of this compound. For instance, *S. cerevisiae* strain R-UV3, achieved 0.90 g/l/h of 2-phenylethanol, reported as the highest space–time yield obtained for biotechnological production of this compound at the time when the paper was published. A high concentration of yeast cells was maintained continuously during this experiment, as was *in situ* product removal technology (ISPR) using macroporous resin columns as adsorbents, achieving good results (Wang *et al.*, 2011).

An improvement of 2-phenylethanol production from L-phenylalanine using *S. cerevisiae* was described during some experiments using continuous removal of the product using a hybrid system and membrane separation techniques. The previous output of 4 g/l without product removal was overcome and higher yields of 7.1 g/l and 7.5 g/l were obtained for the hybrid system and membrane separation techniques, respectively. Ethanol presence was relevant as a side product in this experiment and caused yeast inhibition and limited the performance of the biotechnological process (Mihal *et al.*, 2012).

Metabolic engineering has also presented advances in relation to 2-phenylethanol. Recently, recombinant *E. coli* yielded 285 mg/l of PEA from glucose. Genes from *S. cerevisiae* S288c (*adh1*) and *P. pastoris* GS115 (*kdc*) were tested, but the best result was achieved only after the coordinated expression of these and another two genes (*aroF* and *pheA*) was optimized (Kang *et al.*, 2014). Recently, approximately 335 mg/l of 2-phenylethanol has been reported for another engineered strain, *Enterobacter* sp. CGMCC 5087 after a 12 h experiment, using the same monosaccharide previously cited as the carbon source (Zhang *et al.*, 2014).

The recombinant M15 *E. coli* expressing hydroperoxide lyase from the *BvHPL* gene was able to produce C6-volatile aldehydes (approximately 6.5 mM) such as (2E)-hexenal and (3Z)-hexenal, which are characterized by their green leaf notes. This yield was only possible by performing modulated substrate (13-HPOT) addition (Gigot *et al.*, 2012).

In order to develop environmentally friendly methods for biotechnological processes, several by-products have been tested as substrates. Basidiomycetes were challenged to transform apple pomace, broken waffle and broken cake (by-products from the food industry), cocoa shells, cocoa powder, coffee grounds and wine pomace. *Tyromyces chioneus* metabolized apple pomace resulting in 3-phenyl-propanal, 3-phenyl-1-propanol, benzyl alcohol and benzaldehyde after 4 days, reaching yields of 290 μg/l, 270 μg/l, 100 μg/l and 160 μg/l, respectively. (E)-cinnamic acid was suggested as a precursor of the first two compounds. Sweet and plum-purée sensory notes were reported by the authors (Bosse *et al.*, 2013).

In another approach, immobilized cells of *S. cerevisiae* performed *de novo* synthesis of some volatile esters remembered for their fruity notes, from orange peel hydrolysate. Immobilization technology was used as a strategy to reduce the toxic effects of some compounds such as D-limonene present in the substrate (Lalou *et al.*, 2013).

In a study to establish better conditions to obtain phenethyl esters, as an alternative to obtain rose-like compounds, 80% enzymatic esterification of butter oil and 2-phenethyl alcohol was achieved (Li *et al.*, 2014).

Further functionalities concerning bioflavours have been studied recently, elucidating promising features about α-terpineol. This aroma compound available in wine has presented chemopreventive potential against genotoxic damage caused by contaminants available in the environment. Moreover, indirect antioxidant activity was suggested for its strong ferrous ion chelating capability (Di Sotto *et al.*, 2013). A cytostatic effect against some cancerous cell lines has been reported, even though the authors declare that *in vivo* experiments are still required to verify

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the antitumour potential for this monoterpenoid. Limonene, the biotransformation precursor of α-terpineol, has been already recognized for its preventive activity *in vivo* over cancer (Bicas *et al.*, 2011b).

Considering the promising economic market for aroma and flavour industries, as well as the evident preference of consumers for natural ingredients, relevant efforts have been presented in the scientific field in order to overcome the challenges related to biotechnology. Thus, biotechnological advances for production of aroma compounds have been reported, some of them through engineered biocatalysts, others testing by-products as substrates. The yields of some compounds such as α-terpineol, linalool oxide, γ-decalactone and 2-phenylethanol have exceeded 1 g/l during biotechnological production. Achievements in some articles were associated with the utilization of technologies related to product recovery, as excess of several industrially important aroma compounds exert inhibition effects over biocatalysts productivity. Moreover, reports of further properties of bioflavours suggest in some cases potential functions beyond sensory improvements, which largely increases the interest in their production and application.

34.3 Biocolourants

Colourants have been added to food, drinks, clothes, paints, cosmetics, pharmaceuticals and plastics for a long time (Malik *et al.*, 2012; Boer, 2014). In food, they are considered the main feature, since colour enhances the appeal and acceptability by the consumers (Rymbai *et al.*, 2011), and an important function of colour is the identification and recognition of spoilage (Boer, 2014). Besides that, colour is added to replace colour in cases where it is lost during the processing, to enhance colour already present, to minimize variation between batches and to supplement food with nutrient, making the product more attractive for the consumer (Mortensen, 2006; Chattopadhyay *et al.*, 2008; Rymbai *et al.*, 2011).

Nowadays, most of the colourants used are synthetic or made from non-renewable sources, such as fossil oil. The production of synthetic colourants is advantageous because it is economically efficient and technically advanced, with a whole colour spectrum, but there are some challenges for the production of synthetic colourants and also consumers increasingly prefer natural additives (Soetaert and Vandamme, 2006; Boer, 2014). The production of synthetic dyes depends on non-renewable resources, can be toxic for the environment and this kind of product has been associated with allergenic reactions, behavioural problems in children, cancer and even bad taste. In this context, there is worldwide interest in the food, cosmetics and pharmaceutical industries for the development of a process by which safe pigments can be obtained and from natural sources (Gupta *et al.*, 2011; Chaudhari, 2013; Shahid *et al.*, 2013).

Natural colourants are mostly obtained from plants, animals, insect tissues and microorganisms (Chaudhari, 2013; Santos-Ebinuma *et al.*, 2013). The term biocolourants refers to products derived from biological sources and they can be classified as natural or nature identical, the latter being obtained through biotechnological processes (Chattopadhyay *et al.*, 2008). Natural colourants can be more expensive to produce, less stable to heat and light and restricted in colour range, but they have been gaining the preference of the consumers (Boer, 2014). The best-known biocolourants are carotenoids (yellow-red), flavonoids and anthocyanins (blue-red), chlorophyll (green) and tannins (brown-red) (Ahmad *et al.*, 2012). These compounds can also show anticancer, antimicrobial and antioxidant activities and prevent some human diseases, and due to these properties they are preferred as food additives (Aberoumand, 2011; Gharibzahedi *et al.*, 2013). Figure 34.2 presents the chemical structure of some important biocolourants.

The extraction of natural colourants from plants is a well-known process, but the yields are very low and it has the disadvantage of seasonality and variability between and within species (Gupta *et al.*, 2011; Lu *et al.*, 2013). Although several pigments can be obtained using agro-industrial wastes, such as residues of processing of tomato (lycopene), grape (anthocyanins) and palm (carotenoids), the production of these compounds using microorganisms is still the most advantageous method of production (De Carvalho *et al.*, 2014).

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Fig. 34.2. Chemical structures of some of the most used biocolourants.

34.3.1 Biocolourants: microbial production and potential

Microorganisms are able to grow fast, can produce a large variety of dyes, do not depend on weather conditions and the fermentation has high yields and also microbial colourants are more stable and soluble than plant-derived ones. The production of colourants by microorganisms can occur on a large scale and use cheaper material as substrate, decreasing production costs (Chaudhari, 2013; Lu *et al.*, 2013; Santos-Ebinuma *et al.*, 2013).

Productivity data makes clear the advantages of using microorganisms to produce biocolourants. One example is the production of β-carotene in carrots. The vegetative cycle of carrots is around 100 days and leads to 70 mg of β-carotene/kg of biomass. For microorganisms, a slow-growing cycle is about 5 days and the final concentration of carotenoid reaches around 250 mg of β-carotene/kg of biomass (De Carvalho *et al.*, 2014). Most research studies nowadays have focused on the production of yellow and red pigments, although red, yellow and blue pigments can be produced by microorganisms (Gupta *et al.*, 2011).

Various species of bacteria, fungi, yeasts and algae are coloured and these natural colours can be extracted from the microorganisms using simple and effective protocols. Therefore, there are many efforts to isolate new microorganisms as

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a source of biocolourants and ways to optimize the extraction of the colour (Gupta *et al.*, 2011; Ahmad *et al.*, 2012).

Rymbai *et al.* (2011) showed in their review some isolated microorganism sources of biocolourants, such as: (i) bacteria from the genera *Bradyrhizobium* and *Halobacterium* capable of producing canthaxanthin (Lorquin *et al.*, 1997; Asker and Ohta, 1999); (ii) the fungi *Blakeslea trispora* and *Phycomyces blakesleeanus* able to produce β-carotene; and (iii) fungi from the genus *Monascus* that are already used for commercial production of red and yellow pigments (Fabre *et al.*, 1993; Ootaki *et al.*, 1996; Kim *et al.*, 1997; Rymbai *et al.*, 2011).

The yellow pigment riboflavin can be produced through biotechnological processes using fungi (*Ashbya gossypii* and *Candida famata*) and bacteria (*Bacillus subtilis*) and is already used in various kinds of foods. Bacteria such as *Serratia marcescens*, *Vibrio psychoerythrus* and *Rugamonas rubra* are known for producing pigment. They are able to produce prodigiosin, a red pigment with antibacterial, antimalarial and antibiotic activity (Malik *et al.*, 2012).

Monascus purpureus has been widely used in Asia as a food colourant (Lin *et al.*, 2008; Shahid *et al.*, 2013). This fungus is able to produce six azaphilone pigments: ankaflavin and monascin (yellow), monascorubrin and rubropunctatin (orange), and monascorubramine and rubropunctamine (purple–red). However, this species co-produces a mycotoxin called citrinin that has hepato-nephrotoxic effects in humans (Pisareva *et al.*, 2005). In this context, many research studies have focused on alternatives to minimize the production of citrinin or to develop strains that are incapable of co-producing this compound. Fungal strains of *Penicillium* and *Epicoccum* spp. have been selected in order to produce the polyketide azaphilone pigment, since they are non-toxigenic fungi and some strains can secrete extracellular pigments into the liquid media (Mapari *et al.*, 2009). Besides these advances, there is a need to understand the mechanisms involved in the colourant production and the products must be tested for toxicity before approval is granted as a food additive (Mapari *et al.*, 2010).

Yeasts and bacteria are also known for producing carotenoids. Yeast species from the genera *Rhodotorula*, *Rhodosporidium*, *Sporobolomyces* and *Phaffia* are able to produce carotenoids naturally and the products are very important commercially. Among these species, the ones from genera *Rhodotorula* and *Phaffia* are considered potential pigment sources due their ability to produce β-carotene, torulene, torularhodin and astaxanthin (Frengova and Beshkova, 2009). Some authors describe the production of carotenoids by *Rhodotorula* sp. in a co-cultivation method, using milk whey as substrate. In this method, lactose-negative yeasts (*Rhodotorula glutinis* and *Rhodotorula rubra* strains) and lactose-positive yeasts (*Kluyveromyces lactis*) are used, allowing production using lactose as the carbon source and reaching carotenoid yields of nearly 10 mg/ml (Frengova *et al.*, 2004).

Dharmaraj *et al.* (2009) isolated a *Streptomyces* (AQBWWS1) strain from the marine sponge *Callyspongia diffusa* and observed the ability of this strain to produce carotenoids on fermentation under fluorescent white light. The compounds obtained were analysed through thin layer chromatography and high performance liquid chromatography and were found to be lycopene and an unidentified pigment (Dharmaraj *et al.*, 2009). Bose *et al.* (2013) also used a *Streptomyces* strain for the production of β-carotene under light induction, reaching yields of 23–30 μg/g using about 50% of the total carbon source added, when the strain *Streptomyces* sp. T1027 was grown under optimized conditions.

In another study, a psychrotrophic XT1 strain was isolated by Lu *et al.* (2009) from a glacier in China's Xinjiang province. This strain was able to produce violet pigments and was identified as *Janthinobacterium lividum*. The main pigment identified was violacein which exhibits cytotoxic activity in human colon cancer cells, antileishmanial, antiviral, antibiotic and antitumour activities. The authors also determined that a temperature lower than 20°C is required for cell growth, sucrose was the best carbon source and casein the best nitrogen source, reaching the maximum pigment productivity of 0.8 g of pigment per dry cell weight (Lu *et al.*, 2009).

For large-scale production the production of carotenoids by yeasts or bacteria is convenient because of their unicellular nature and high growth rate (Frengova and Beshkova, 2009). Table 34.1 summarizes the microorganisms and the pigments they produce that are described in this chapter.

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Table 34.1. Some pigment-producing microorganisms exhibiting future potential.

Microorganism	Pigment	Colour	Reference
Bradyrhizobium sp.	Canthaxanthin	Orange	Rymbai *et al*. (2011)
Halobacterium sp.	Canthaxanthin	Orange	Rymbai *et al*. (2011)
Blakeslea trispora	β-Carotene	Orange	Rymbai *et al*. (2011)
Phycomyces blakesleeanus	β-Carotene	Orange	Rymbai *et al*. (2011)
Monascus spp.	Ankaflavin and monascin	Yellow	Lin *et al*. (2008), Gupta *et al*. (2011)
	Monascorubrin and rubropunctatin	Orange	
	Monascorubramine and rubropunctamine	Purple-red	
Candida famata	Riboflavin	Yellow	Malik *et al*. (2012)
Bacillus subtilis	Riboflavin	Yellow	Malik *et al*. (2012)
Serratia marcescens	Prodigiosin	Red	Ahmad *et al*. (2012), Malik *et al*. (2012)
Vibrio psychoerythrus	Prodigiosin	Red	Malik *et al*. (2012)
Rugamonas rubra	Prodigiosin	Red	Malik *et al*. (2012)
Rhodotorula sp.	β-Carotene, torulene, torularhodin and astaxanthin	Yellow, orange	Frengova and Beshkova (2009), Ahmad *et al*. (2012)
Phaffia sp.	β-Carotene, torulene, torularhodin and astaxanthin	Yellow, orange	Frengova and Beshkova (2009)
Streptomyces sp.	Lycopene, β-carotene	Red, orange	Dharmaraj *et al*. (2009), Bose *et al*. (2013)
Janthinobacterium lividum	Violacein	Purple	Lu *et al*. (2009)
Chromobacterium violaceum	Violacein	Purple	Ahmad *et al*. (2012)
Streptomyces coelicolor	Prodigiosin	Red	Ahmad *et al*. (2012)

Besides the efforts directed to identify new pigment-producing strains, there is an interest in finding new kinds of compounds that can be used as colourants. Lu *et al.* (2013) isolated and identified a novel yellow pigment, called cordycepoid A, from the entomogenous fungus *Cordyceps bifusispora*. The pigment cordycepoid A was found to be water soluble, with low toxicity and high stability, being a potential safe, edible pigment (Lu *et al.*, 2013).

One interesting way to improve biocolourant production is to optimize the culture conditions. There are a lot of factors that affect their production, such as light, temperature, aeration and carbon source. Shatila *et al*. (2013) isolated a strain of *Exiguobacterium aurantiacum* FH from air able to produce carotenes and xanthophylls. The products were considered stable and the conditions that lead to maximum pigment production were evaluated. The maximum pigment production achieved was 534.51 μg pigment/g dry weight when *E. aurantiacum* FH was cultivated at 30°C, 150 rpm in 40 ml of LB (Luria Bertani broth) medium, at pH 7, for 3 days in light (Shatila *et al.*, 2013).

In order to identify the effects of nine nutrient parameters that influence the production of red pigments by *M. purpureus* MTCC 369, Ahmad and Panda (2011) used a Plackett-Bruman experimental design. It was found that dextrose, peptone, NH_4Cl, $MnSO_4.H_2O$ and malt extract had a significant effect on red pigment production (Ahmad and Panda, 2011).

Santos-Ebinuma *et al.* (2013) achieved a 78% increase in the production of a red colourant using the fungus *Penicillium purpurogenum* DPUA 1275, by optimizing the concentration of sucrose and yeast extract using a 2^2 central composite design. Other variables such as pH, temperature, orbital stirring speed and incubation

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time have already been optimized by the same group and further investigation is necessary in order to characterize the red colourant produced (Santos-Ebinuma *et al.*, 2013).

One important variable related to biocolourant production is the presence or absence of light. As shown before, *S. marcescens* is able to produce prodigiosin, a red pigment. When the culture is kept in the dark by wrapping the flask with aluminium foil, a more intense red colouration is observed compared with cultures grown in the presence of light (Ahmad *et al.*, 2012).

Velmurugan *et al.* (2010) showed the effect of darkness and different coloured light (blue, green, yellow, red and white) on extracellular and intracellular pigment production of five fungi: *M. purpureus*, *Isaria farinosa*, *Emericella nidulans*, *Fusarium verticillioides* and *P. purpurogenum*. They found out that incubation in darkness leads to an increase in pigment production, being the most effective, followed by red, blue and white light. The maximum yield of colourant (expressed as optical density (OD) per gram of dry substrate) obtained in darkness by the fungi were 36.75 OD/g with *M. purpureus*, 29.70 OD/g with *I. farinosa*, 32.20 OD/g with *E. nidulans*, 30.80 OD/g with *F. verticillioides* and 32.90 OD/g with *P. purpurogenum*. On the other hand, yellow and green light decreased the production of pigment by the fungi (Velmurugan *et al.*, 2010).

With recent advances in molecular biology and genetic engineering, some studies focused on creating new strains able to produce pigments by heterologous expression of the biosynthetic pathways and on trying to make modifications in an already-known pigment-producing strain in order to improve the yields (Dawson, 2009; Shahid *et al.*, 2013). Due to its medical benefits, the improvement of prodigiosin production was the objective of some studies. El-Bialy and El-Nour (2014) used ultraviolet (UV) radiation and ethyl methanesulfonate (EMS) in order to create new variants of *S. marcescens* able to produce large amounts of prodigiosin. The strains obtained through UV mutagenesis S2, S3 and S4 showed the highest pigment productivity (45.93 mg/g, 44.8 mg/g and 43.74 mg/g, respectively). When EMS mutagenesis was used, the best productivity was found to be 137.08 mg/g and 50.77 mg/g, for the strains S26 and S30. The variant S26 was used in further studies to characterize the strain and the pigment produced (El-Bialy and El-Nour, 2014). A UV mutant of *Rhodotorula gracilis* obtained by Vijayalakshmi *et al.* (2001) showed 1.8 times higher carotenoid synthesizing activity compared with the wild-type strain. Another mutant strain of *Rhodotorula* was able to produce 2.9 mg of carotenoids/g dry cell, which is 24-fold higher than the wild-type strain of *Rhodotorula glutinis*, and in a shorter time of culture (Bhosale and Gadre, 2001; Frengova and Beshkova, 2009).

Das *et al.* (2007) showed how progress in metabolic engineering helps the development of new carotenoid-producing microorganisms from non-carotenogenic ones. The main host used is *E. coli* because it has a well-known fermentation process and it can be easily modified genetically. These new strains are usually obtained by cloning and expressing genes from the carotenoid biosynthetic pathway present in bacteria, fungi and higher plants that naturally produce pigment, and in some cases the yields reached with these strains are higher than the ones obtained with native carotenogenic microorganisms (Das *et al.*, 2007). Besides the advantages of using genetically modified microorganisms, the consumer acceptance for products obtained by these methods is still low (Boer, 2014).

The production of biocolourants through biotechnological processes and using microorganisms is very interesting and has been actively investigated. The reasons to produce natural biocolourants include: (i) the fact they are preferable to consumer; (ii) they do not show undesirable side effects; (iii) they can have antioxidant, antitumour and antimicrobial activity; (iv) production does not depend on seasonal and political factors; and (v) production can be cheaper than synthetic production and allows the production of biocolourants on a large scale (Ahmad *et al.*, 2012; Boer, 2014). Therefore, many efforts from academics and from industries have been made in order to improve yields and varieties of colour produced, using microorganisms as biocolourant sources.

34.4 Trends and Prospects for New Industrial Additives

Additives represent a significant area in which innovations reflect opportunities for the development of several products. Industrial additives are used with the main objective of optimizing

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organoleptic properties, making products in the cosmetics, pharmaceutical and food industries more interesting from a sensory standpoint. Although the goals are challenging, the efforts to reach them brings continuous results in the development of unprecedented technologies (Vialta, 2010).

Intense competition in the world market has led to the food industry expanding the products they offer and modifying them in order to appeal to consumers, and consumers in turn have become are more exigent with regard to the quality of food products and the nature of food additives. According to an estimate of the Food Additives and Ingredients Association (FAIA), the world market has been increasing over the past few years, exceeding £17 billion in 2013 (FAIA, 2014).

Within recent developments of industrial additives investigated in this chapter, we will focus on describing the trends and prospects for new organoleptic additives.

34.4.1 Additives fitting the sensory trend

This trend reveals that the market tries to preserve the maximum organoleptic properties of products, and in particular this applies to products in the food industry but it also applies to products in the cosmetic industry too. Because of this principle, the challenge to industry is to avoid changing the sensory properties of the products they produce, as well as restoring them when these properties are lost during processing.

The industry uses intensifier compounds to recover organoleptic features lost during processing, or to improve the natural qualities of the products. Many of the products introduced by the food and drink industry contain reduced levels of their original constituents, most commonly sugar, fat and salt or sodium. Usually, the fact that these substances are reduced directly affects the sensory properties of the products, reducing their attractiveness and also affecting the demand for these products (Vialta, 2010).

The restriction of fat has an adverse impact on the consistency and aroma perception of drinks and food. Another aspect is that the introduction of some functional ingredients can also alter the organoleptic profile of the product. To supplement this loss, different types of additives may be required, such as aromas, flavouring and colouring compounds (Pastore *et al.*, 2013).

The aroma or flavour is one of the main components that influence the preference of a product, considering that an important part of the taste is determined by the flavour component, as previously discussed. The large variety of options of aromas available in the industry makes products attractive and enables their acceptance by the market. Bioaromas or natural aromas are currently preferred by the consumer because they are considered to be natural in comparison to synthetic compounds which bring aspects of artificiality (Molina *et al.*, 2013).

The demand for natural aromas should continue to grow in the next few years and will probably be influenced by bioflavours, which are produced by enzymes or fermentation, using several strains of microorganisms. Although there are some difficulties involved in the process of obtaining these products, bioaromas represent important advancements in this field. For the cost of production of a bioflavour by microorganisms to be feasible, the market value should be above US$200/kg (Vialta, 2010).

Focusing on functional, diet and light products (e.g. low-fat, fat-free, low-sugar products), taste modulators are used that optimize the salty taste, overlaying the undesirable off-flavours, thus retrieving the Japanese concept of taste umami (Molina *et al.*, 2013). Thus, the taste modulators provide the product with features that the market does not want to give up.

Emulsifiers or surfactants promote a uniform mixture of two or more unmixable substances, and for this reason these compounds are widely used throughout the cosmetic and food industries and there has been an increased demand for modified starches over the past decade (Vialta, 2010; Pastore *et al.*, 2013).

There is increasing interest in biosurfactants that has become greater year after year. Everything indicates that biosurfactants produced by microorganisms will have a promising future because they are non-toxic and biodegradable (Vasel, 2009). Nowadays, the most important limiting factor is the high costs of their production. However, with the development of new processes and the genetic improvement of promising microorganisms, along with

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the use of agro-industry residues for the reduction of costs in fermentation processes, this problem will tend to be solved in the near future and the use of biosurfactants is expected to be common practice in the cosmetic and food industries (Nitschke and Pastore, 2002).

34.4.2 Prospects for additives for the healthiness and wellness trend

There is increasing concern from consumers about their health and well-being and this has led to increasing demand for products in which sugar, salts, saturated and trans-fats have been reduced or eliminated. There is also demand for products that contain functional ingredients that, in addition to being nourishing, are beneficial to health.

There are a great number of product lines that have been developed with functional additives, besides there are several polyfunctional additives that are usually employed. With the development of biotechnology, there has been a change in food consumption patterns with consumers increasingly wanting foods in their diet that incorporate probiotics, prebiotics and symbiotic organisms that modulate the gut microbiota promoting beneficial effects to the consumer (Maróstica Jr *et al.*, 2013).

Probiotics are food additives that contain microorganisms which are available to the body by consuming fermented foods. The probiotics exert a beneficial effect on the health and well-being of the host by optimizing the viability and properties of the microbiotic cells. They regulate gut activity by controlling and stabilizing the gut microbiota, promoting digestion, increasing absorption of minerals and the production of vitamins and have the property of stimulating the immune system (Maróstica Jr *et al.*, 2013). Due to the introduction onto the world market of more probiotic products with specific beneficial effects, the consumption of these products is expected to increase over the next few years, mainly because of the benefits introduced for health and wellness (Vialta, 2010).

Prebiotics are compounds that are non-digestible in the gut but they stimulate the selective growth of desirable bacteria, changing the gut microbiota and establishing a healthier gut microbial balance. In addition, they inhibit the proliferation of pathogens and promote the stimulation of the immune system. Due to its low price, inulin is today the most extensively marketed prebiotic (Maróstica Jr *et al.*, 2013). Other prebiotics available on the market are oligosaccharides, especially fructooligosaccharides (FOS) derived from fruits and vegetables, as well as the galactooligosaccharide (GOS) from lactose (Fai *et al.*, 2014).

The human immune system reacts to infection through oxidative reactions related to different processes, such as stimulation of the white blood cells that produce enzymes linked to the presence of reactive oxygen species. These processes lead to oxidative stress, which may cause problems in humans (Vialta, 2010). Thus, it is wise to make use of food and drinks that contain antioxidants which are recently being produced by biotransformation processes using microorganisms (Vialta, 2010; Bicas *et al.*, 2011c).

34.5 Advances in Biotechnology for New Industrial Additives

Biological additives will transform the pharmaceutical, cosmetic and food industries, carrying significant technological changes in products and introducing more benefits related to this area. The biotechnology industry provides the basis for research and development for several sectors of the economy, including the pharmaceutical, cosmetic and food industries. The biotechnology world market has grown by more than 10% per year in the last decade and there has also been an increase in the number of products available in the USA and European Union (Vialta, 2010).

Modern biotechnology uses tools such as genetic engineering in order to obtain genetically modified organisms (GMOs), which may be mutants and/or transgenic organisms if they have a gene in their DNA from another species (Malavazi and da Cunha, 2013).

Several works have described closer ties between GMOs and economic, environmental sustainability and social aspects. After many years of harvests of, for example, genetically modified soybeans and maize, several studies have shown the security of the use of GMOs and thus demystified the fear of the unknown that surrounded

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these products. A large number of enzymes, amino acids, aromas, vitamins and organic acids are produced either directly by GMOs or from derivatives of genetically modified microorganisms and have wide acceptance in the world market (Malavazi and da Cunha, 2013; Molina *et al.*, 2013).

Another promising field of biotechnology is bioremediation and biofortification. The development of these products has a positive impact on the additive industry, and there is a strong trend to increase the active ingredients and nutrient levels of products derived from animals and plants on special diets (Bhamra, 2014).

34.6 Use of 'Omics' Tools in Searching for New Bioproducts

Genome mapping has allowed for the systems level analysis of cells, and the information extracted from genomics analyses now serves to predict new engineering targets for the design of fermentation strategies. For example, transcriptome data that reveal gene expression levels on a global scale using DNA microarrays helped identify a global regulator and exporter as the amplification targets in an L-valine-producing *E. coli* strain (Park *et al.*, 2007; Bution *et al.*, 2014). Transcriptome data have been useful in analysing the cell and re-designing experiments for drug production.

In another case, transcriptome data and metabolite profiling were combined to identify the cytotoxic causes of accumulated 3-hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA) in engineered *E. coli* that heterologous expresses the mevalonate pathway and overproduces isoprenoids, precursors of the antimalarial drug artemisinin (Kim *et al.*, 2007). This analysis revealed that HMG-CoA inhibits the fatty acid metabolism of the host and suggested a new fermentation strategy for providing palmitic and oleic acids in the medium.

Proteomics and metabolomics data can be used in a similar manner to identify new engineering targets (Kizer *et al.*, 2008). As these high-throughput genomics analyses contribute to the systematic analysis of the cell, they are expected to be more frequently employed for drug production to identify and surmount the limitations of our knowledge and understanding of biology (Kizer *et al.*, 2008; Bution *et al.*, 2014).

Although not often currently practised, computational modelling and simulation are important tools for the metabolic engineering of microorganisms for drug production as they help characterize and engineer the cell at the systems level (Kim *et al.*, 2007; Park *et al.*, 2007; Bution *et al.*, 2014).

The computational analysis of metabolic characteristics on the genome scale allows for the prediction of appropriate gene targets to be engineered by considering many different components constituting a biological system and their interactions often provides more results (Alper *et al.*, 2005; Lee *et al.*, 2009).

One of the most popular computational methods for predicting gene targets to be engineered is constraints-based flux analysis, which begins with a genome-scale stoichiometric metabolic model that represents the mass balance information of metabolites in all cellular reactions (Kim *et al.*, 2007). Based on the assumption of a pseudo-steady state, this system can be simulated by optimization techniques, typically linear programming, with various objectives such as to maximize cell growth and product formation and to minimize by-product production. This would allow for estimation of the metabolic flux distribution in the whole cell (Pharkya *et al.*, 2004; Bution *et al.*, 2014).

This simulation method has proved to be a powerful tool for predicting metabolic engineering targets (Lee *et al.*, 2005). For instance, *in silico* knockout simulation using this method predicted three gene targets to be knocked out in an engineered L-valine-producing *E. coli*. By knocking out these three genes, the L-valine production yield could be increased to 148.7% (Park *et al.*, 2007); these knockout targets are non-intuitive ones, thus suggesting the usefulness of genome-scale modelling and simulation in metabolic engineering.

In addition, computational frameworks based on constraints-based flux analysis that guide the addition of heterologous reactions (Pharkya *et al.*, 2004) or upregulation and downregulation of homologous reactions have also been developed (Pharkya and Maranas, 2006; Bution *et al.*, 2014). These examples suggest that genome-scale computational analysis can be

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applied in the early discovery phase of fragrance and flavour development, including predicting drug, fragrance and food compound targets, as well as gene and protein targets.

34.7 Concluding Remarks

Additives represent a dynamic area in which innovations are introduced at an accelerated pace and often create opportunities for the development and launching of new products. Industry uses a vast array of industrial additives with the main objective of extending the shelf life of products and making them healthier and more attractive from a sensory standpoint mainly in the cosmetics, pharmaceutical and food industries.

The advantage of biotechnologically based products, as presented here, is that they can be labelled as 'natural' and in some cases may also present desirable functional properties. However, developments in bioprocess engineering and microbial genetic improvements should be considered to overcome low yields and high manufacturing costs that may hamper the commercial adoption of some of these processes.

In this perspective, recent developments in microbial biotechnology devoted to the production of important industrial additives will certainly guide the future of the industry, offering the most promising economic and environmental advantages and fitting the sensory, healthiness and wellness trends.

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35 Actinomycetes in Biodiscovery: Genomic Advances and New Horizons

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Abstract

Insights gained through genome sequencing revolutionized the approaches to biodiscovery and subsequent use of microbially derived products in biotechnology. Actinomycetes since the 1940s have provided biologically active compounds including most of the industrial-scale antibiotics, antitumour agents and immunosuppressors. Genome sequencing investigations on this group of bacteria have recently indicated possession of larger genomes, however, only a fraction of their coding capacity is utilized for secondary metabolite synthesis and the rest remain cryptic. Strategies thus targeting the trigger of full coding capacity will lead to the detection of new therapeutic agents that might become scaffolds of future potent drugs. Genomics has also brought together novel advances in the field of biodiscovery to increase hit rates and generate in-depth knowledge on the compound–pathogen interactions to render antibacterial compounds more effective therapeutic agents. Metagenomical approaches also generate new insights ranging from revealing true functional roles and chemo-diversity of the secondary metabolism of microflora to bacterial niche adaptation and antibiotic resistance. By combining genomic information with the systematic exploration of defined ecological niches using highly selective techniques of isolation and specifically composed media, a collection of representative strains from different parts of microbial ecosystems can be recovered. These approaches will thus ensure that a wide and diverse range of microorganisms are examined by facilitating improved understanding of associated chemo-diversity for targeted niches. This chapter provides an overview of the contributions of actinomycetes to the bio-industry and evaluates their future potential as producers of novel bioactive compounds in relation to current molecular advances.

35.1 Introduction

The onset of the genomics era, when rapid sequencing of genes and genomes became routine, increased in-depth understanding related to the metabolic and chemical diversity of microorganisms (Winter *et al*., 2011; Zotchev *et al*., 2012; Scheffler *et al*., 2013). In particular, the analysis of sequenced genomes from numerous *Streptomyces* species, the most bioactive group of bacteria, indicated that even a single species in this genus can carry more than 30 secondary metabolite gene clusters, reinforcing the superior biosynthetic potential of this genus waiting for further explorations (Aigle *et al*., 2014). These discoveries thus paved the way for design of genome-guided approaches for discovery of novel compounds deriving from genomic information on the actinomycete antibiotic biosynthesis genes (Zotchev *et al*., 2012). Moreover, genomics advances combined with powerful bioinformatics tools revealed previously untapped

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opportunities for drug discovery (Siezen and Khayatt, 2008; Boddy, 2014). As stated by Bansal (2005) examples include:

> target directed approaches in drug discovery and infection control (e.g. development of rational drugs and antimicrobial agents, development of better and easy ways to administer vaccines, development of protein biomarkers for various bacterial diseases, and better understanding of host–bacteria interaction to prevent bacterial infections) in the medical area, as well as applications in biotechnology (e.g. development of new enhanced bacterial strains for bioremediation and pollution control).

This chapter provides an overview of the contributions of actinomycetes to the bio-industry and evaluates their future potential as producers of novel bioactive compounds in relation to current molecular advances.

35.2 Contributions of Actinomycetes to Biodiscovery

Antibiotic discovery and commercial production was one of the most important advances in medicine in the 20th century and its golden era lasted from the 1940s to the late 1960s. Antibiotic research thus subsequently provided a wide range of chemically diverse and effective therapeutic agents for the treatment of a wide range of microbial infections (McDevitt and Rosenberg, 2001; Hopwood, 2007; Demain and Sanchez, 2009). Since the discovery of the anti-mycobactericidal antibiotic streptomycin in 1943 (Schatz *et al.*, 1944), actinomycetes have been a major source of important bioactive compounds (Table 35.1), finding uses in broad industrial applications such as the use of pharmaceutical and agrobiological compounds (Bérdy, 2005, 2012; Doroghazi and Metcalf, 2013).

35.2.1 Antibiotics: early years (1940–1974) to mid-era (1975–2000)

In the early years of antibiotic discovery, actinomycetes were an unexhausted source of antibiotics, with broad-target-directed therapeutic applications (Bérdy, 2005, 2012). In particular, members of the most talented family *Streptomycetaceae*, including ones from the genera *Streptomyces* and *Streptoverticillium*, were specifically targeted for isolation (Küster and Williams, 1964), characterization (Shirling and Gottlieb, 1966) and subsequent antibiotic discovery (Cassinelli *et al.*, 1967). Moreover, following the establishment of the International *Streptomyces* Project (ISP), with collaboration of over 40 laboratories from 18 different countries, over 400 bioactive streptomycete species were described (Nonomura, 1974; Szabó *et al.*, 1975; Szabó and Marton, 1976). Most of these identified species were producers of the key antibiotics of the golden era and the early years of biodiscovery (Table 35.2). These efforts were further complemented with the full numerical classification of *Streptomyces* and *Streptoverticillium* species (Locci *et al.*, 1981; Williams *et al.*, 1983a, b, 1985).

However, high rates of rediscovery of known compounds became a major hurdle by 1961 (Lancini, 2006) which constitutes a major obstacle even today in the biodiscovery programmes (Baltz, 2005b; Bérdy, 2005, 2012). Thus, new strategies targeting the isolation of bioactive actinomycetes other than streptomycetes proved fruitful. Examples include successful detection of new antibiotics (e.g. gentamicin from *Micromonospora* species at Schering in the USA) (Weinstein *et al.*, 1963). Discovery of many other antibiotics such as everninomicin, sisomicin, rosaramicin, verdamicin, megalomicin, microcin and micromonosporin subsequently

Table 35.1. Number of bioactive actinomycete metabolites from 1940 to 2010. (Adapted from Bérdy, 2012.)

	Early years (1940–1974)	%[a]	Mid-era (1975–2000)	%[a]	New age (2001–2010)	%[a]	Total
Actinobacteria	3,400	62	7,200	42	3,100	28.5	13,700
Streptomyces species	2,900		5,100		2,400		10,400
Other actinobacteria	500		2,100		700		3,300

[a]Percentage of the total number of bioactive molecules from actinomycetes.

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Table 35.2. Examples of actinomycete antibiotics (discovered from 1940 to 1980). (Compiled from Kurtböke, 2012c.)

Species	Product	Target use	Source
Streptomyces antibioticus	Actinomycin (Merck product Cosmogen®)	Wilm's disease of children	Waksman and Woodruff (1940), Strohl *et al*. (2001)
Streptomyces griseus	Streptomycin	Antimycobacterial	Schatz *et al*. (1944), Strohl *et al*. (2001)
Streptomyces aureofaciens	Chlortetracycline	Typhoid fever, typhus, invasive infections by *Streptococcus* pneumonia and beta-haemolytic streptococci	Projan *et al*. (2006)
Streptomyces garyphalus	Oxamycin	A competitive antagonist of the incorporation of D-alanine into a uridine nucleotide in *Staphylococcus aureus*	Keuhl *et al*. (1955), Strohl *et al*. (2001)
Streptomyces sphaeroides	Novobiocin (cathomycin)	A narrow-spectrum antibiotic, with activity against penicillin-resistant microorganisms	Kaczka *et al*. (1955), Strohl *et al*. (2001)
Streptomyces rimosus	Oxytetracycline	To treat acute bacterial infections (such as chest infections, urine infections, skin infections and mouth infections) and longer-term skin conditions (such as acne)	Projan *et al*. (2006)
Streptomyces fradiae	Fosfomycin	Broad-spectrum antibactericidal activity	Hendlin *et al*. (1969), Strohl *et al*. (2001)
Streptomyces erythreus	Erythromycin	Similar activity to penicillin and prescribed for those allergic to penicillin	Baltz (2005a)
Streptomyces avermitilis	Avermectin	Antiparasitic agent	Campbell *et al*. (1983), Strohl *et al*. (2001)
	Derivative	Ivomec® (round worm elimination in farm animals)	
	Derivative	Mectizan® (river blindness treatment)	

followed from this genus (Wagman and Weinstein, 1980; Begg and Barclay, 1995).

Search for new antibiotics increasingly targeted rare and previously undetected actinomycetes in the mid-era (1975–2000) (Table 35.3). The Central Institute of Microbiology and Experimental Therapy (ZIMET) and its successor the Hans Knöll Institute for Natural Products Research (HKI) (in former East Germany, DDR) contributed significantly towards description of bioactive and rare actinomycetes (e.g. *Actinomadura*, *Amycolatopsis* and *Nocardiopsis*; Gumpert, 2005).

However, effectivity of the then current screening methods failed and rediscovery of known compounds became even more frequent by the 1990s (Bérdy, 2012) and industry faced subsequently increased research costs and discovery of less promising leads (Bérdy, 2005, 2012; Baltz, 2006). A sharp decrease in the marketable new compounds was observed (e.g. from 20–30 new drugs per decade to three to four). Some relative success was obtained

Table 35.3. Bioactive compounds from actinomycetes by 2005. (Adapted from Bérdy, 2005.)

Actinomycete family	Number of bioactive compounds
Streptomycetaceae	8,345
Micromonosporaceae	1,061
Pseudonocardiaceae	327
Streptosporangiaceae	154
Thermomonosporaceae	657
Mycobacteriaceae	126
Other actinomycete taxa	81
Total	10,751

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through the use of third-generation semisynthetic agents or with the wide use of a few old and new compounds, such as daptomycin, but without any major breakthroughs (Bérdy, 2005, 2012; Baltz, 2006). From 1980 to 2003 pharmaceutical industrial profits decreased due to increasing research costs. The small number of new leads and regulatory obstacles also hampered the true breakthroughs (Bérdy, 2012).

As summarized by Baltz (2006), the lack of promising leads for that era was due to:

> (i) lack of the quality and quantity of compounds to be tested and/or screening methodologies at the front end of the pipeline, (ii) difficulties in getting bio-active compounds isolated and characterized, further evaluated and confirmed in a timely manner, and (iii) the lack of inspiration in discovery strategy or tactics.

However, new avenues have appeared with the advances in genomics and microbial genome mining for accelerated natural products discovery has been recognized as a renaissance in the making (Bachmann *et al.*, 2014). Genomics has also delivered well-validated, novel, target-based screening technologies to support the antibacterial discovery process (Mills, 2006; Pucci, 2006). Use of bacterial genomic data together with innovative natural products approaches can thus create a second 'golden age' of antibacterial agents including overcoming the current bacterial resistance obstacles (Singh and Barrett, 2006; Peláez, 2006; Demain, 2014).

35.2.2 New age (2001 onwards): genomics-inspired discovery of bioactive compounds

Genome sequencing exposed the unknown bioactive potential of many streptomycete species. The first breakthrough came after the full genome analysis of *Streptomyces coelicolor* A3(2) (Bentley *et al.*, 2002) revealing the enormous biosynthetic capabilities of this species, including those undetected by current fermentation and bioactivity testing methods (Zotchev *et al.*, 2012; Scheffler *et al.*, 2013). Reconstruction of its metabolic network at the genome level was also shown to be useful to fill gaps in genome annotation (Borodina *et al.*, 2005). Since then functional genomics combined with chemical biology has identified new targets of bioactive compounds (Ho *et al.*, 2011). With the advances in the field of natural product chemistry now large chemically diverse libraries of bioactive compounds can be generated and be used for therapeutic applications (Davies, 2011; Gomez-Escribano and Bibb, 2011). Moreover, the integration of chemical ecology and bacterial genome mining has also enhanced the detection of chemically diverse natural products in functional contexts (Vizcaino *et al.*, 2014).

Genomics combined with bioinformatics is now providing an even more powerful platform for biodiscovery and revealing the presence of novel bioactive metabolites. Previously undetected compounds under standard fermentation conditions are now being detected as well as quicker optimization for metabolite production is now possible (Winter *et al.*, 2011). The availability of genome sequences of clinically important antibiotic producers are also bringing improved insight into their biology and facilitate rational development of strains to generate high-titre production of antibiotics (e.g. erythromycin A producer strain *Saccharopolyspora erythraea* (NRRL23338)) (Oliynyk *et al.*, 2007).

The genome sequencing of *Streptomyces* species, most notably *S. coelicolor* and *Streptomyces avermitilis* revealed remarkably that each strain is genetically capable of the production of a large number (e.g. 23 and 32, respectively) of secondary metabolites (Bentley *et al.*, 2002; Ikeda *et al.*, 2003). These findings indicated that a large percentage (90%) of the biosynthetic potential of these organisms could not be detected by the conventional screening programmes (Alduina and Gallo, 2012).

The complete genome sequence of *Streptomyces griseus* (IFO 13350), producer of streptomycin, was also completed by Ohnishi *et al.* (2008). Their subsequent comparisons with the genomes of two related species, *S. coelicolor* A3(2) and *S. avermitilis*, revealed the full characteristics of the *S. griseus* genome but also provided information on the existence of 24 *Streptomyces*-specific proteins. The *S. griseus* genome-sequencing studies also indicated presence of 34 gene clusters or genes for the biosynthesis of known or unknown secondary metabolites.

Preliminary analyses of the *Streptomyces bingchenggensis* genome by Wang *et al.* (2010) also exposed the presence of at least 23 gene

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clusters for polyketide, non-ribosomal peptide or terpene biosynthesis. The other 20 gene clusters detected by them were suggested to dictate the biosynthesis of siderophore, geosmin, or absolutely unknown compounds waiting to be exploited by further endeavours.

35.3 Genome Mining

Examples of the genome sequences listed above are now providing valuable insight into silent genes or clusters and also into the ones that are poorly expressed when a specific trigger is absent (e.g. lack of appropriate chemical or physical signals to induce expression) (Zazopoulos *et al.*, 2003; Winter *et al.*, 2011). Transformation from a ‘phenotype-driven’ field to a ‘genotype-driven’ one in natural product discovery is currently taking place (Deane and Mitchell, 2014). These new approaches correlating biosynthetic gene clusters with the compounds they produce, currently facilitates the production and isolation of a rapidly growing collection of ‘reverse-discovered’ natural products (Deane and Mitchell, 2014). Examples include coelichelin, which had its structure predicted several years prior to its isolation from *S. coelicolor* (Challis and Ravel, 2000).

Current genome-mining campaigns for synthetic biology programmes are organized in two major categories: (i) eliciting expression in the encoding producing organism (homologous expression); and (ii) endeavouring to recapitulate pathways in non-producing hosts (heterologous expression). In the case of homologous expression, the power of secondary metabolic prescience combined with the foreknowledge of metabolic potential has unlocked a significant fraction of unknown metabolites (e.g. discovery of farnesylated benzodiazepinone EC O4601) (Bachmann *et al.*, 2014).

35.3.1 Cryptic secondary metabolite pathways

Following the genomics revolution, strategies for rapid strain improvement shifted from improved expression of well-known, highly productive, secondary metabolite fermentations to the expression of novel and often cryptic secondary metabolite pathways (Baltz, 2011; Gomez-Escribano and Bibb, 2014). As stated by Gomez-Escribano and Bibb (2014) for the full expression of cryptic secondary metabolite pathways the two main approaches used the following strategies:

> (1) activation of the expression of a transcriptionally-silent cryptic gene cluster by genetic manipulation of the producing organism (e.g. by deleting or over-expressing putative negative or positive transcriptional regulators, respectively) and (2) cloning a fragment of the genome of the producing organism containing the gene cluster and expressing it in a suitable heterologous host.

Examples for silent gene detection and activation include definition of 38 secondary metabolic gene clusters and 46 insertion sequences (IS)-like sequences on the genome of *Streptomyces avermitilis* by Ikeda *et al.* (2014), which have not been detected from this organism previously. Heterologous expression of more than 20 exogenous biosynthetic gene clusters from this organism was made possible by Ikeda *et al.* (2014). Similarly, recent genome mining of *Streptomyces ambofaciens* (ATCC 23877), producer of spiramycin and congocidine, resulted in the identification of 23 clusters potentially involved in the production of other secondary metabolites. Further research on these clusters led to the characterization of new bioactive compounds and of previously known compounds but never characterized in this *Streptomyces* species to date (Aigle *et al.*, 2014). Another recent approach included the utilization of rare earth elements as a novel method to activate these silent genes (Tanaka *et al.*, 2010; Ochi *et al.*, 2014).

Cloning is also expected to facilitate unconventional approaches to obtain metabolic product of a cryptic gene cluster from an organism that is difficult to culture or that is not genetically amenable. With this approach unnatural metabolites can be obtained by combining genes from different biosynthetic pathways or by expressing mutated gene clusters (Gomez-Escribano and Bibb, 2014). Examples for the heterologous expression include construction of the derivatives of *S. coelicolor* (M145) as hosts for the heterologous expression of secondary metabolite gene clusters by Gomez-Escribano and Bibb (2011). When the native actinorhodin gene cluster and of gene clusters for the heterologous

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production of chloramphenicol and congocidine were constructed by Gomez-Escribano and Bibb (2011), dramatic increases in antibiotic production compared with the parental strain were observed. Similarly, the biosynthetic pathway for rebeccamycin was dissected and reconstituted in *Streptomyces albus*, which provided an environment capable of supplying precursors without the need for further genetic manipulations (Van Lanen and Shen, 2006).

In another approach, Alduina and Gallo (2012) indicated that stable integration of artificial chromosomes into the *Streptomyces* genome can be feasible and be used as effective vectors to transfer entire biosynthetic gene clusters from intractable actinomycetes into more suitable hosts. Such an approach may thus create the possibility towards the access into the gene clusters from uncultured bacteria present in natural environments without the need to actually isolate them for the subsequent production of bioactive metabolites (Alduina and Gallo, 2012). In addition, orphan biosynthetic gene clusters for which the coded metabolite is still undiscovered, currently indicate enormous potential for novel bioactive compound discovery (Chiang *et al.*, 2011).

35.3.2 Diverse enzymology and biosynthetic pathways

Even the most extensively studied *S. coelicolor* following its complete genome sequence investigations revealed the possession of previously unknown specialized metabolite biosynthesis, including 17 chemically distinct classes of specialized metabolites (Challis, 2014). Many novel enzymes have been discovered in the course of elucidating the biosynthetic pathways for such metabolites, revealing remarkable diverse enzymology involved in bioactive natural product biosynthesis (Van Lanen and Shen, 2006; Challis, 2014). Currently, the metabolic products of several putative specialized metabolic pathways still remain unidentified in *S. coelicolor*, including unsolved mechanisms for specialized metabolite assembly and the biological functions of the majority of specialized metabolites (Challis, 2014). Their definition can provide novel 'parts' for synthetic biology approaches to the production of fine, commodity and specialty chemicals, as well as novel biocatalysts with potential applications in industrial biotechnology (Challis, 2014).

Ju *et al.* (2014) have indicated that studies on the phosphonate natural products, a rich source of useful pharmaceutical, agricultural and biotechnology products, can reveal numerous diverse enzymes that catalyse unprecedented biochemical pathways. Similarly, correlation of secondary metabolism and genome data is expected to bring new insights into terpene cyclases and bacterial terpene biosynthesis (Citron *et al.*, 2012), including detection of three new enzymes (E)-b-caryophyllene synthase, selina-3,7(11)-diene synthase and aristolochene synthase by Rabe *et al.* (2013), increasing the in-depth understanding of biosynthetic pathways involved in the known terpenes of actinomycetes. Hornung *et al.* (2007) studied halogenases, which code for the synthesis of halometabolites. They screened 550 randomly selected actinomycete strains using PCR and identified 103 novel putative genes coding for halogenases. Their subsequent phylogenetic analysis of the corresponding putative halogenases, followed by determination of their sequential context with mass spectrometric analysis of cultures filtrates, revealed a distinct correlation between the sequence and secondary metabolite class of the halometabolite. Their screening strategy facilitated rapid access to novel natural products with predetermined structural properties.

Synthetic biology approaches via the engineered supply of precursors are also resulting in the generation of novel antibiotics and the optimization of their yield (Davies, 2011; Wohlleben *et al.*, 2012).

35.3.3 Polyketides and non-ribosomal peptides

Many natural products with clinical utility are synthesized by polyketide synthases (PKSs) or non-ribosomal peptide synthetases (NRPSs) (Walsh, 2004). Both of these enzyme families create structurally and functionally diverse compound scaffolds. NRPSs and PKSs both contain numerous domains and synthesize natural products via multi-modular assembly lines (Walsh, 2004) and they have been an important

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source of drugs, toxins and virulence factors (Meier and Burkart, 2011). Over the last two decades substantial efforts have been made towards genetic characterization of the modular synthases (NRPS) that produce the abovementioned compounds (Meier and Burkart, 2011). With use of new PCR primers targeted to specifically amplify NRPS and PKS-I gene sequences from actinomycetes, Ayuso-Sacido and Genilloud (2004) observed that the wide distribution of such genes is a trait of *Streptomyces* species even extending into other minor lineages (e.g. *Saccharomonospora*). Interestingly, they noted that some members of the *Streptosporangiaceae*, *Thermomonosporaceae* and *Nocardiaceae* revealed high incidences of NRPS and PKS-I sequences despite the lack of NRPS and polyketide compound production in these analysed strains.

Advances in the proteomics field are also changing the scene, facilitating the identification and quantification of these enzymes via the implementation of new proteomic platforms. These platforms are called: '(i) orthogonal active site identification system (OASIS) and (ii) proteomic interrogation of secondary metabolism (PrISM)' (Meier and Burkart, 2011). With the complementary advanced mass spectroscopic data collection and analysis tools these technological improvements will thus enable and accelerate new discoveries in the natural products arena, ranging from drug discovery to pathway engineering (Siezen and Khayatt, 2008; Meier and Burkart, 2011; Poust *et al.*, 2014).

Strain-specific proteogenomics is also accelerating the discovery of natural products by aiding the development of full information into biosynthetic pathways (Albright *et al.*, 2014). A novel actinomycete strain de-replication approach based on the diversity of PKS and NRPS biosynthetic pathways has also been suggested (Ayuso *et al.*, 2005). Moreover, Bumfus *et al.* (2009) using mass spectrometry-based proteomics selectively detected NRPS and PKS gene clusters in microbial proteomes without requiring genome sequence information.

Baltz (2014) has also recently suggested that small chaperone-like proteins encoded by *Mycobacterium tuberculosis* might be utilized as unique probes or beacons to identify bioactive microorganisms encoding large numbers of diverse NRPS pathways, due to their unique function(s) and small size.

35.3.4 Ribosomal natural products

Velásquez and van der Donk (2011) have recently indicated that with the advantage of sequence homology studies among genes encoding precursor peptides or biosynthetic proteins, the discovery rate of diverse peptide natural products that are synthesized via the ribosomal pathway has also increased. Examples include recent lantipeptides, cyanobactins, linear thiazole/oxazole-containing peptides, microviridins, lasso peptides, amatoxins, cyclotides and conopeptide discoveries (Velásquez and van der Donk, 2011). Moreover, the combination of molecular biology advances and *in silico* mining has resulted in an increase in the discovery of new ribosomal natural products. The remarkable structural and functional diversity of these products leading to novel pharmaceutical applications have also been noted by Velásquez and van der Donk (2011). For these ribosomally synthesized peptides (e.g. lantibiotics and thiopeptides), new representatives have also been generated by site-directed mutagenesis of the corresponding structural genes, many of which retain antibiotic activities comparable with those of the parent molecule (e.g. actagardin produced by *Actinoplanes liguriae* ATCC 31048) (Donadio *et al.*, 2010).

35.3.5 Evolutionary systems biology

Generation of knowledge into the design principles and evolutionary mechanisms of biodiversity has been made possible via the 'evolutionary systems biology' approach following the information generated from genome sequences of evolutionarily related species/strains (Zhou *et al.*, 2012). Investigations into the patterns and functional roles of lineage-specific expansions (LSEs) gene families can also provide further valuable clues on the existence of strain-specific adaptive phenotypes. Such eco-functional activities can range from 'diverse secondary metabolism, to sophisticated morphological differentiation, pathogenesis, and effective responses to endogenous and exogenous signals' (Zhou *et al.*, 2012). Moreover, some of the crucial roles of natural products in the biology of certain genera (e.g. *Mycobacterium*, *Streptomyces* and *Frankia*)

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can be revealed through investigations into the conservation of natural product gene clusters (Doroghazi and Metcalf, 2013).

Zazopoulos *et al.* (2003) examined the biosynthetic pathways encoding enediyne antitumour antibiotics in different actinomycete taxa. Their comparative analysis of five biosynthetic loci as representatives of major structural classes of enediynes indicated the presence of a conserved cassette of five genes, including a novel family of PKS. Subsequent genome scanning analysis indicated the existence of a widely dispersed enediyne warhead cassette among actinomycetes, which under selective growth conditions might be induced to express. All this information suggested that the range of enediyne natural products may be much greater than previously thought (Zazopoulos *et al.*, 2003).

Evolution of new biosynthetic pathways yielding complex bioactive natural products while maintaining evolutionarily successful ones dating back millions of years continues in environmental microorganisms (Wright, 2007; Diminic *et al.*, 2014). From an evolutionary point of view, the sequencing of multiple actinomycete genomes will provide multiple copies of known pathways, thus allowing phylogenic calculations to determine the age of antibiotic biosynthetic and resistance genes (Baltz, 2010). Baltz (2005) estimated that the biosynthetic pathways for erythromycin, streptomycin and vancomycin emerged over 880, 610 and 240 million years ago, respectively. Even the relatively new antibiotic daptomycin was predicted to be at least 30 million years old (Baltz, 2005; Wright, 2007). In addition, a cluster of secondary metabolite biosynthetic genes highly related to those of daptomycin were located in *Saccharomonospora viridis*. Calculations in relation to the last common ancestor of the lipopeptide pathway in *S. viridis* and the original producer organism *Streptomyces roseosporus*, indicates that the daptomycin-like pathway may have evolved over a billion years ago (Baltz, 2010).

35.3.6 Bioinformatics

Biological data analysis and storage capabilities have significantly improved following the advances in computation speed and memory storage (Bansal, 2005; Pucci, 2006). For the development of rational drugs and antimicrobial agents target-directed approaches have been adopted. This approach resulted in the creation of enhanced bacterial strains for different industrial uses such as: (i) bioremediation and pollution control; (ii) development of better and easy-to-administer vaccines; (iii) the development of protein biomarkers for various bacterial diseases; and (iv) better understanding of host–bacterial interactions to prevent bacterial infections (Bansal, 2005; Pucci, 2006).

As noted by Bansal (2005) and Jensen *et al.* (2014) current research in bioinformatics is now providing support into:

> (i) *genomics*: sequencing and comparative study of genomes to identify gene and genome functionality, (ii) *proteomics*: identification and characterization of protein related properties and reconstruction of metabolic and regulatory pathways, (iii) cell visualization and simulation to study and model cell behaviour, and (iv) application to the development of drugs and anti-microbial agent.

35.3.7 Extracellular microbial environment and metabolomics

In a natural environment nutrients are consumed by microorganisms for the formation of many extracellular metabolites as by-products from the activity of microbial metabolism. As a result, changes in the metabolic activities of the microorganisms are directly reflected in the modification of the environment (Mapelli *et al.*, 2008).

The actual status of the environment will be defined by microbial metabolic status and, subsequently, by the metabolites that are released into the environment by the existing microbial community (Mapelli *et al.*, 2008). Co-inhabitants of the same environments trigger the production and secretion of competitor compounds (e.g. toxins and antibiotics) shaping the environment further (Mapelli *et al.*, 2008). Natural products are involved in metabolic exchange processes including cell-to-cell communication, growth promoters, nutrient scavengers, protection or induction of oxidative damage and cell differentiation (Dorrestein and Carroll, 2011).

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Comprehensive metabolomic studies are now being used to detect such microbial responses which may not be identifiable at the genomic level. It also aids towards increased understanding on phenotypes shaped by metabolite levels and interactions (Mapelli *et al.*, 2008). Advances like the metabolic foot-printing thus now represent a niche within metabolomics via the analysis of extracellular metabolites. Although metabolic foot-printing represents only a fraction of the entire metabolome, it provides important information for functional genomics and strain characterization, and it can also provide scientists with a key understanding of cell communication mechanisms, metabolic engineering and industrial biotechnological processes (Mapelli *et al.*, 2008; Olano *et al.*, 2008).

Under certain conditions secondary metabolites may provide selective advantages to the producing strains (Challis and Hopwood, 2003). Accordingly, production of these secondary metabolites occurs only under certain environmental conditions. Strategic exploration of this fact using the 'one strain/many compounds' (OSMAC) can lead to identification of new compounds from well-studied producers or multiple compounds from the same strain (Bode *et al.*, 2002; Ochi *et al.*, 2013; Rebets *et al.*, 2014).

35.3.8 Microbiomics

The host microbiome is directly affected by the abundance of small molecule and metabolite producing microorganisms within a host which in turn dictate the physiology of a host (Yang *et al.*, 2011). Knowledge on the function of these molecules, which are often produced for communication or defence by these microorganisms, will improve understanding of their beneficial and detrimental effects on the host. Moreover, utilization of multiple 'omics' strategies will generate further understanding in the diversity of microbiome-associated small molecules (both in diversity and function) (Yang *et al.*, 2011). Biochemical and genetic approaches have also been fundamental for determination of the modes of actions of these small molecules (Roemer *et al.*, 2012).

Recently, genomic technologies utilizing chemical-genetic strategies for in-depth analysis of compound–target relationships in the context of a living cell were reported by Roemer *et al.* (2012) to provide a systems biology view of both the cellular targets and the interdependent networks that respond to chemical stress. These studies highlighted the limitations of *in vitro* studies when information is subsequently used for generation of a complete understanding of drug behaviour in the cell (Roemer *et al.*, 2012). Microbiomics combined with knowledge of drug behaviour in a cell will undoubtedly lead to the development of effective treatment strategies.

35.3.9 Target-directed screening for antibiotic activity

Current genome transcription analyses is now generating in-depth understanding of the multiple effects that antibiotics exhibit at low concentrations on bacterial cells, including exhibition of the phenomenon of hormesis and provoking considerable transcription activation (Davies *et al.*, 2006). These analyses thus can be of value in providing information on antibiotic side effects, in bioactive natural product discovery and antibiotic mode-of-action studies. Therefore, detection of transcription modulation induced by low-molecular-weight organic molecules can now provide a unique approach to the screening of large numbers of natural products synthesized by microorganisms which would be missed in conventional drug-screening processes because of their low concentrations (Davies *et al.*, 2006).

In another approach, currently known antibiotics might be revived for use in different targets. Examples include the mannopeptimycins that were originally isolated in the late 1950s from *Streptomyces hygroscopicus* and have been recently revived because of their promising activity against clinically important Gram-positive pathogens, including *Streptococcus pneumoniae*, methicillin-resistant *Staphylococcus aureus* (MRSA) and vancomycin-resistant *Enterococci*. For the optimization of the mannopeptimycin activity profile multiple approaches were used. These approaches included the use of selective chemical derivatization, precursor-directed biosynthesis and pathway engineering (He, 2005; Koehn, 2008; Donadio *et al.*, 2010). Similarly, chemical modifications of second-generation vancomycin analogues and characterizations have generated

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insights into mechanisms of novel antibacterial action and pharmacokinetics for the drug candidates. Pace and Yang (2006) has stated that such candidates might carry unique beneficial properties for replacement drugs (e.g. near-term vancomycin replacements) or for the semisynthesis of analogues of importance. Accordingly, such developments in metabolic engineering can generate new chemical scaffolds for future glycopeptide antibiotics (Pace and Yang, 2006).

Incorporation of in-depth understanding of microbial physiology into experimental design and measurement of secondary metabolism will inevitably yield diverse metabolites (Knight *et al.*, 2003). Co-cultures and improvements in the mixed-culture fermentations currently provide novel insights into secondary metabolism (Knight *et al.*, 2003). Pettit (2009) has reported that another successful approach to more fully access the metabolic potential of cultivatable microbes is mixed fermentation, where the presence of neighbouring microbes may induce secondary metabolite synthesis. Mixed fermentation can result in: (i) increased antibiotic activity in crude extracts; (ii) increased yields of previously described metabolites; (iii) increased yields of previously undetected metabolites, analogues of known metabolites resulting from combined pathways; and (iv) induction of previously unexpressed pathways for bioactive constituents (Pettit, 2009).

35.4 Actinomycetes in Nature

Actinomycetes are widely distributed in both terrestrial and aquatic ecosystems. They constitute a significant component of the microbial population in most soils and counts of over 1 million/g are commonly obtained using conventional enumeration techniques (Goodfellow and Williams, 1983). The soil is also the most prolific source of bioactive isolates, streptomycetes being ubiquitous and most numerous and the producers of antibiotics and other useful metabolites. Actinomycetes, usually streptomycetes, are also capable of degrading many other polymers occurring in soil and litter, including hemicelluloses, pectin, keratin and chitin. Nocardiae have been implicated in the decomposition of humic materials (Goodfellow and Williams, 1983).

Conventional approaches in defining the many activities of actinomycetes documented that they were able to: (i) degrade plant, animal and microbial polymers in soil and litter; (ii) produce tastes and odour in natural portable waters; and (iii) fix nitrogen in a variety of non-leguminous plants (Goodfellow and Williams, 1983). Currently, with the utilization of novel molecular tools (e.g. metagenomics) it is now becoming possible to answer key ecological questions, including the functional roles of actinomycetes in nature. Information will enable scientists to relate specific functions to specific microorganisms within multispecies soil communities (Cowan *et al.*, 2005).

Zhou *et al.* (2012) assessed the mechanisms underlying genome plasticity and systems adaptation in closely related *Streptomyces* strains using the sets of available genome information. When they comprehensively analysed the core genomes of five *Streptomyces* species with distinct phenotypes, they were found to contain important genes for *Streptomyces* biology. These genes were important for gene regulation, secretion, secondary metabolism and morphological differentiation. Moreover, the core genome components were essential for a sustained successful life cycle of these organisms in the soil environment. Such observations revealed the true effects of both genome evolution and environmental stress on expressed phenotypes.

Cultivation-independent molecular studies including proteogenomics bring new insights into ecological understanding. An example is the most likely acquisition of proteasome genes by Gram-negative bacteria from actinobacteria, the only eubacteria previously known to contain proteasomes (De Mot, 2007).

35.4.1 Natural roles of antibiotics

Microbial metabolites exhibit a wide range of biological activities including defence, cell regulation and communication. In addition, chemical and physiological functions of these metabolites might also be diverse. These metabolites might act as information storage devices, hormones, pheromones and neurotransmitters (Bérdy, 2012). They also have diverse functions at the biological-cellular level including modulation of cellular transcription, phage induction,

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and inter- and intracellular signalling. Moreover, endless possibilities can exist for the exploitation of the diverse possible effects and activities of these compounds in industry (Davies, 2006; Bérdy, 2012).

Information on the true nature of natural interactions thus aids in development of further understanding and identifying the triggers that activate the production of antibiotics in nature (Zhu *et al.*, 2014). In nature, many antibiotics will only be produced after receipt of specific signals, such as from the environment (stress) or from surrounding microbes (symbionts or competitors) (Zhu *et al.*, 2014). Natural products also define cellular functions of living organisms and improved strategies are required to efficiently identify their functions (Dorrestein and Carroll, 2011).

Moreover, information on the sub-inhibitory concentrations of antibiotics inducing novel phenotypic and genetic responses in exposed organisms, including increased biofilm formation and expression of virulence genes indicates novel roles for antibiotics. One of those roles might be that antibiotics are 'collective regulators of the homeostasis of microbial communities', acting as signals or cues rather than weapons. Antibiotics acting as signals enable symbiotic relationships between different organisms resulting in each benefiting from either nutrition or protection (Zhu *et al.*, 2014).

35.4.2 Metagenomics

The vast amount of information held within the genomes of uncultured microorganisms can now be exposed using metagenomics via extraction, cloning and analysis of the entire genetic complement of a habitat (Handelsman *et al.*, 1998; Daniel, 2004; Schmeisser *et al.*, 2007). Metagenomical approaches currently allow investigation of a wide diversity of individual genes and their products as well as analysis of entire operons encoding biosynthetic or degradative pathways (Steele *et al.*, 2008; Wilson and Piel, 2013). In the light of these advances ecosystem diversification can easily be identified and talented microorganisms could be attempted to be selectively recovered from different natural environments such as the marine-associated ones (Doney *et al.*, 2004). Proteomics of strains within a community can also complement cultivation-independent genomic (metagenomic) analysis of microorganisms in the natural environment (Lo *et al.*, 2007).

The field of metagenomic gene discovery offers enormous scope and potential for both fundamental microbiology and biotechnological development (Cowan *et al.*, 2005).

35.4.3 Resistome concept in relation to antibiotic discovery

Studies of antibiotic resistance in soil were reported to indicate that environmental bacteria harbour antibiotic resistance genes independently of abiotic influences (Allen *et al.*, 2009). Currently, two different techniques are used to investigate antibiotic resistance in the environment:

> (i) functional metagenomics: which serves to find new antibiotics or new antibiotic resistance genes, and (ii) descriptive metagenomics, which serves to analyse changes in the composition of the microbiota and to track the presence and abundance of already known antibiotic resistance genes in different ecosystems.
> (Garmendia *et al.*, 2012)

Culturable bacteria in soil were found to harbour genes encoding enzymes that degrade or otherwise inactivate antibiotics, and/or use them as the sole carbon and nitrogen sources, such as *Streptomyces venezuelae* grown on chloramphenicol (Allen *et al.*, 2009). In a comprehensive study conducted by D'Costa *et al.* (2006) on antibiotic resistance, over 400 actinomycetes cultured from forest, agricultural and urban soils were found to have highly varied resistance profiles. Moreover, some exhibited resistotypes that had not been seen before, such as inactivation of telithromycin by a novel structural modification (Allen *et al.*, 2009). On the other hand, despite decades of clinical use, resistance to the β-lactam antibiotics has not yet emerged in group A streptococci (Wright, 2007). Increased understanding of the resistome combined with a better understanding of the resistance mechanisms related to redundancy will change the scene for drug discovery (Wright, 2007). The currently observed density of resistance mechanisms in the soil, including the data produced by recent metagenomic studies, suggests that

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many of these natural products function as antibiotics, or at least that they trigger a molecular response by bacteria that is equivalent to resistance. Therefore, organisms that have evolved under a constant barrage of increasingly complex chemical diversity might be the source of many antibiotic resistance genes, and comprise the bulk of the resistome (Wright, 2007). Accordingly, targeting antibiotic resistance might be an alternative to antibiotic discovery including the activation of natural products involved in counteracting antibiotic resistance mechanisms (Tomasz, 2006; O'Connell *et al.*, 2013; Zhou *et al.*, 2014). Moreover, the emergence of multidrug resistance among the latest generation of pathogens suggests that the discovery of new scaffolds should be a priority (Fischbach and Walsh, 2009).

One approach might be development of in-depth understanding on quorum sensing (QS) or cell-to-cell communication in bacteria. QS is achieved through the production and subsequent response to the accumulation of extracellular signal molecules called auto-inductors and the main role of QS is regulation of virulence factors in bacteria. Bacterial pathogenicity is often manifested by the expression of various cell-associated and secreted virulence factors, such as exoenzymes, toxin and biofilms (Gospodarek *et al.*, 2009). Accordingly, QS may be one physiological process that offers an interesting array of novel targets to explore as antibacterial agents (O'Connell *et al.*, 2013).

35.4.4 Target-directed search and recovery of bioactive microorganisms

Increased data on the genome sequences are resulting in the emergence of previously underexplored or neglected organisms as alternative resources for new drugs (Winter *et al.*, 2011). Metabolically specialized groups of microorganisms will grow and thrive in almost all ecosystems, including extreme and harsh ones. These unique ecosystems will thus contain microorganisms with unique metabolic pathways that have evolved to allow the microorganisms to adapt and survive in these harsh environments (Zhang, 2005; Banik and Brady, 2010). As a result, selective recovery of these microorganisms from these diversified ecosystems can provide rich resources of bioactive material for drug discovery (Zhang, 2005; Banik and Brady, 2010). Examples include: (i) metagenomic approaches to natural products from free-living and symbiotic organisms (Brady *et al.*, 2009); and (ii) isolation of novel biocatalysts and bioactive molecules (Daniel, 2004). The field of metagenomic gene discovery offers enormous scope and potential for both fundamental microbiology and biotechnological development (Cowan *et al.*, 2005).

Lack of systematic investigations into ecosystems for the discovery of unknown bioactive compounds led to random sampling and could not reveal the true bioactive potential of microbial niches in many regions of the world (Czárán *et al.*, 2002; Knight *et al.*, 2003). It also failed to provide comprehensive eco-taxonomical information. An untapped source of novel strains in all ecosystems is currently present in different environments as indicated by molecular studies which clearly reveal the discrepancy between the number of microorganisms detected by molecular methods and the number of their representatives in culture (Knight *et al.*, 2003; Harvey, 2008). However, still little effort has focused on the isolation and cultivation of difficult-to-culture microorganisms. Failure of culturing representatives of many rare taxa negatively impacts the generation of information on microbial physiology and *in vitro* growth requirements.

Information on chemical diversity can be used to detect its ecological basis, such as the production of cyclosporins, rapamycin, penicillin and rifamycin by microorganisms that originate from temperate ecosystems (Knight *et al.*, 2003). However, information on chemical diversity and genotypes are also still limited (Firn and Jones, 2000; Knight *et al.*, 2003). Recent studies on the most bioactive marine genus *Salinospora* also showed a clear correlation between species and classes of compounds produced, indicating a large degree of chemo-consistency exists within these types of organisms (Jensen *et al.*, 2005; Larsen *et al.*, 2005). Information generated in such studies will aid to improve target-directed search and recovery strategies for bioactive microorganisms.

In vitro culture conditions mimicking environmental conditions will also gain importance, including development of different methods ranging from strain-specific to globally applicable. So far, in routine screening methods the natural

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competitors and symbionts that are found in the natural habitat have been lacking (Zhu *et al.*, 2014). Co-cultivation methods or chemical mimicking of inter-species communication will thus be promising new approaches in the search for novel antibiotics (Lewis *et al.*, 2010; Zhu *et al.*, 2014).

Significant research has been directed towards the selective isolation of bioactive actinomycetes and this has been documented in different reviews (Goodfellow, 2010; Goodfellow and Fiedler, 2010; Kurtböke, 2010, 2011, 2012a, b; Tiwari and Gupta, 2012, 2013, 2014; Kurtböke *et al.*, 2014, 2015). Data from genome sequences indicate that previously untargeted organisms are realistic resources for new therapeutic agents (Winter *et al.*, 2011). Target-specific isolation techniques are also being implemented, examples include the use of diffusion chambers resulting in the isolation of previously undetected bacteria including rare and unusual species from the genera *Dactylosporangium*, *Catellatospora*, *Catenulispora*, *Lentzea* and *Streptacidiphilus* (Kaeberlein *et al.*, 2002; Gavrish *et al.*, 2008; Lewis *et al.*, 2010).

Post-isolation treatments of the isolates such as domestication of wild strains of rare or unusual bacteria after capturing them created variants that become capable of growth on standard media (Lewis *et al.*, 2010). With such strategies Lewis *et al.* (2010) were able to recover a rich diversity of bacterial species from the lifeless sands of Dry Valley in Antarctica. Their observations indicated that colony-forming species in the diffusion chamber could only grow on a Petri dish, if other species from the same environment were present. They concluded that uncultured bacteria only commit to division in a familiar environment, upon recognition of the presence of growth factors released by their neighbours (Nichols *et al.*, 2008; Lewis *et al.*, 2010).

Members of the *Nocardiaceae* and *Pseudonocardiceae* of the order *Actinomycetales* have been a source of key secondary metabolites since the early years of biodiscovery. Examples include: (i) rifamycins produced by *Amycolatopsis rifamycina* (Sensi *et al.*, 1959; Bala *et al.*, 2004) effective against mycobacteria and subsequently used to treat tuberculosis, leprosy and infections caused by the *Mycobacterium avium* complex; (ii) cephamycin from *Nocardia lactamdurans* (Miller *et al.*, 1972); and (iii) vancomycin (Brigham and Pittenger, 1956) produced by *Amycolatopsis orientalis* (Lechevalier *et al.*, 1986). This group of actinomycetes has also been implicated in the degradation of hydrocarbons (Atlas, 1981; Chaillan *et al.*, 2004; Quatrini *et al.*, 2008), as well as consuming the lignin component of the plant biomass (Davis *et al.*, 2012). They have extensively been implicated in the formation of stable foams in activated sludge plants (Goodfellow *et al.*, 1998; Pagilla *et al.*, 2002). Occurrence of these organisms in the Australian wastewater plants have also been frequently reported (Soddell and Seviour, 1990; Seviour *et al.*, 1990, 1994; Blackall *et al.*, 1991), including opportunistic pathogenic ones, for example *Nocardia farcinica* (Stratton *et al.*, 1996).

Quality and pollution control of surface waters is now a significant issue for public, scientists, politicians and policy makers (Schilling and Zessner, 2011). The quality of surface water, seepage water and groundwater should not be impacted by the pollutants by urban stormwater runoff (Göbel *et al.*, 2007). Over the last few years following heavy rains and storms, foaming events have been encountered along the shores of the Sunshine Coast Region of Australia, which might be due to floating pollutants in the environment (Kurtböke, 2008). Foaming can also happen due to other natural sources such as humic waters from rainforests (Schilling and Zessner, 2011).

The occurrence of *Nocardiaceae* and *Pseudonocardiceae* in the foaming shallow coastal waters has been investigated during the local foaming events of the Sunshine Coast Region of Australia. Use of selective isolation techniques directed to these taxa (Williams and Wellington, 1982) revealed the presence of a diverse number of species within these two families (Figs 35.1 and 35.2). Subsequent analysis of some of these species showed they possessed *alkB* gene and were capable of degrading alkanes. Isolates were also capable of *in vitro* formation of foam in the presence of a wide range of oils (e.g. motor, avocado, groundnut, tea-tree, vegetable oils) (K. Aitken, D. Matuccio, D. Powell, P. Brooks and D.I. Kurtböke, 2012, unpublished data).

Bacteriophages specific to the members of *Nocardiaceae* and *Pseudonocardiceae* have been reported (Thomas *et al.*, 2002). Such specificity can be a powerful tool during a targeted search for rare or previously uncultured actinomycetes by indicating the presence of the host in selected

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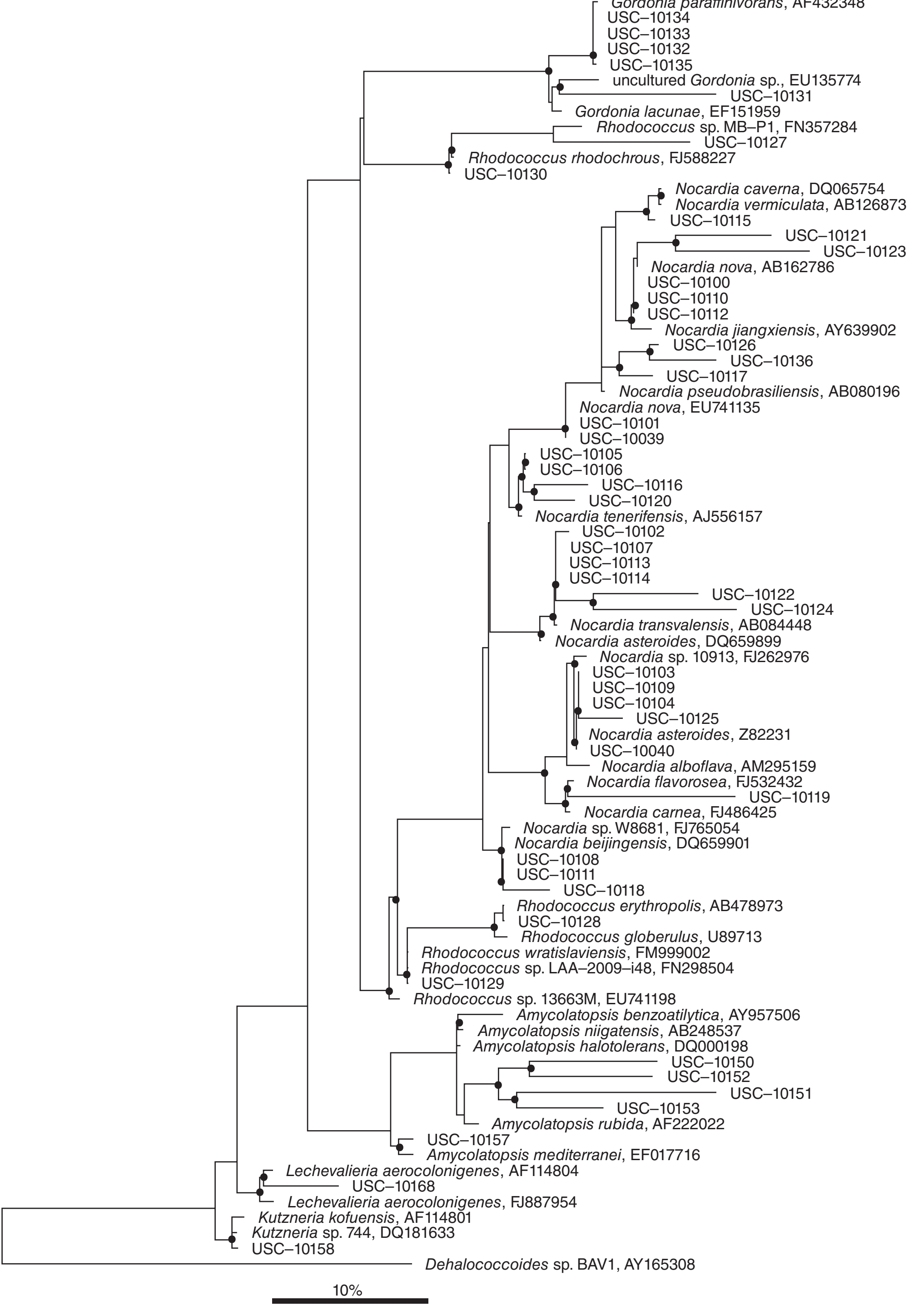

Fig. 35.1. Phylogenetic tree of the 16S rRNA gene sequences obtained from local isolates belonging to the families *Nocardiaceae* and *Pseudonocardiceae* in relation to their closest relatives. Bootstrap values > 50% are indicated at nodes as filled circles. The scale bar represents 10% sequence divergence. GenBank accession numbers of reference sequences are included at the end of each reference sequence description. Black bars and associated genus names are indicated at the left side of the tree when taxonomy for each sequence was consistent with tree-based phylogeny and taxonomy assignments generated using the Greengenes classifier. *Dehalococcoides* sp. strain BAV1 was used as an outgroup sequence to root the tree.

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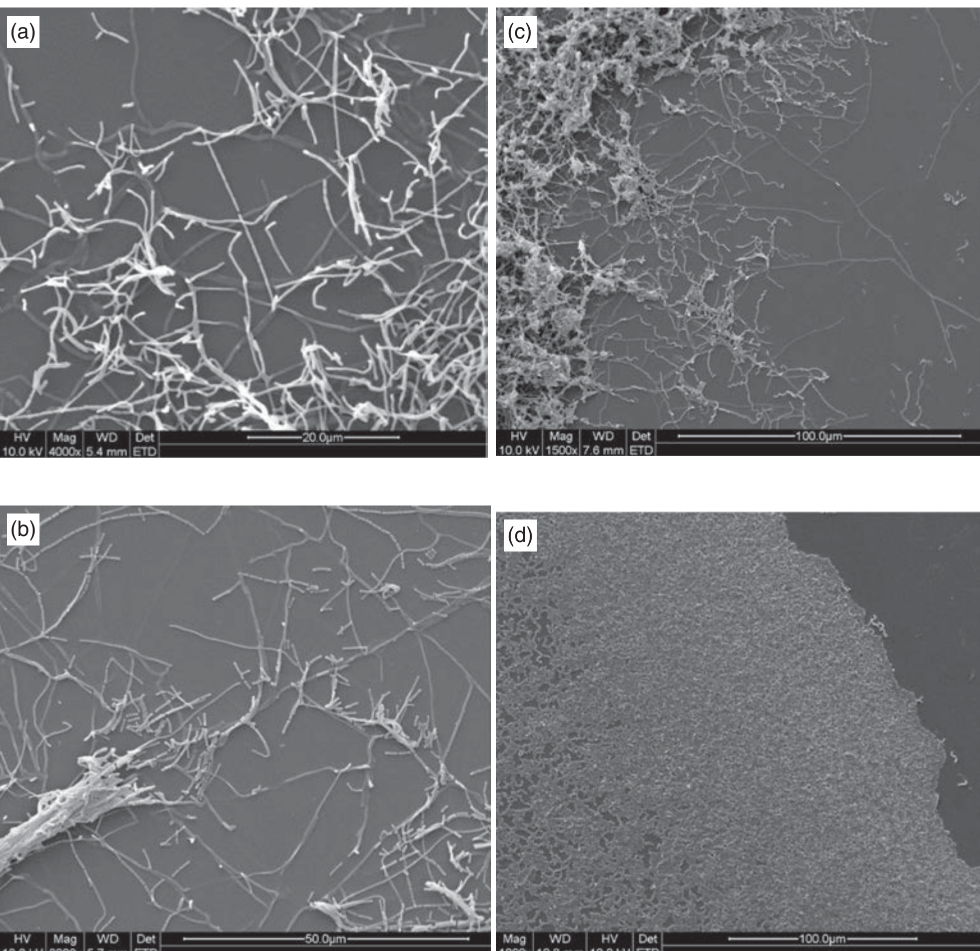

Fig. 35.2. Electron micrographs of the isolates from foaming coastal waters in the Sunshine Coast Region of Australia. (a) Isolate (USC-10157) related to the members of the genus *Amycolatopsis*. (b) Isolate (USC-10158) related to the members of the genus *Kutzneria*. (c) Isolate (USC-10102) related to the members of the genus *Nocardia*. (d) Isolate (USC-10128) related to the members of the genus *Rhodococcus*.

environmental samples (Kurtböke, 2003, 2012b). Near-shore beach environments including sand dunes were screened for the presence of the species belonging to the *Nocardiaceae* and *Pseudonocardiceae* previously detected in the foaming coastal waters in the same area using bacteriophages as indicators. Significant phage activity was found to be present in the screened area against the isolates and type strains indicating the presence of active hosts (Table 35.4 and Box 35.1). Species of *Nocardiaceae* and *Pseudonocardiceae* isolated from the nearby foaming shallow coastal marine waters might therefore have originated from the surrounding environments and been carried to the surface waters by wind and/or animal or human activities. Long-term monitoring and selective culturing not only provided information on the occurrence of the species of *Nocardiaceae* and *Pseudonocardiceae* in our region but also indicated the presence of different types of species with diverse metabolic activities such as pollutant degradation.

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Table 35.4. Examples of detected taxon-specific bacteriophage activity against the local isolates of the genera belonging to the families of *Nocardiaceae* and *Pseudonocardiceae*.

Genus[a]	Isolate code	Bacteriophage presence against local isolates in the surrounding natural environment (p.f.u./ml)
Nocardia	USC-10039	$\times 10^3$
	USC-10100	$\times 10^3$
	USC-10115	$\times 10^3$
	USC-10102	$\times 10^3$
	USC-10040	$\times 10^3$
Rhodococcus	USC-10127	$\times 10^2$
	USC-10128	$\times 10^3$
	USC-10129	$\times 10^2$
Amycolatopsis	USC-10150	$\times 10^2$
	USC-10157	$\times 10^2$
Gordonia	USC-10135	$\times 10^2$
	USC-10131	$\times 10^2$
Kutzneria	USC-10158	$\times 10^2$
Lechevalieria	USC-10168	$\times 10^2$

[a]Genus allocation of the local isolates was done according to their 16s rRNA sequences (see Fig. 35.1).

Box 35.1. Examples of cross infectivity of locally isolated phages on the type species of the genera belonging to the families of *Nocardiaceae* and *Pseudonocardiceae*.

Type strains of the *Nocardiaceae* and *Pseudonocardiceae* (and their codes) used to detect phage activity spectra

Nocardia asteroides (ACM 2963)
Nocardia brasiliensis (ACM 2964)
Nocardia cummidelens (DSMZ 44490)
Nocardia salmonicida (40472)
Nocardia takedensis (DSMZ 44802)
Nocardia pinensis (ACM 3335)
Amycolatopsis lurida (DSMZ 43134)
Amycolatopsis orientalis subsp. *orientalis* (DSMZ 40040)
Amycolatopsis mediterranei (DSMZ 43304)
Rhodococcus zopfii (DSMZ 44108)
Rhodococcus pyridinivorans (DSMZ 44555)
Rhodococcus ruber (DSMZ 44319)
Lechevalieria aerocoloniges (DSMZ 44217)
Gordonia rhizosphera (DSMZ 44383)

Target-directed screening and selective culturing strategies combined with information generated through metagenomics and functional diversity surveys will provide information on the true occurrence and diversity of the targeted taxa in natural environments.

35.5 Future Prospects

Microbial natural products will remain the most promising source of novel antibiotics, in particular the ones from actinomycetes (Watve *et al.*, 2001) and molecular advances will further improve the efficiency of the discovery process (Monaghan and Barrett, 2006; Peláez, 2006). An holistic approach will still be the most effective approach contributing to success in discovering antibiotics from microbial natural products, while taking account of the following: (i) metagenomic analysis of microbial bio- and functional diversity; (ii) genomic definition of biosynthetic genes; (iii) the influence of growth conditions on the production of secondary metabolites; (iv) objective

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and target-directed choice of screening steps; and (v) rapid isolation and identification of the active compounds (Peláez, 2006; Demain, 2014).

Genomic advances will redefine the regulatory stage of antibiotic production; however, detailed experimentation via 'good old-fashioned biochemistry, genetics and microbial physiology' will still facilitate generation of a complete understanding of the regulation of antibiotic production by actinomycetes (Cundliffe, 2006; Kurtböke *et al.*, 2015). Such in-depth understanding will then lead to development of effective drug design and development strategies.

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36 Molecular Strategies for the Study of the Expression of Gene Variation by Real-time PCR

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Abstract

All living organisms regulate their activities through the activation or repression of expression of their genes. The genetic expression of a target gene is generally proportional to the number of copies of messenger RNA (mRNA). Therefore, for detecting specific cell products it is crucial to identify the number of copies of mRNA when it is translated to form proteins. This measure allows obtaining data on the production of biological elements and the varying levels of expression of its genes in the cell in response to exposure to various effects. The identification of specific sequences (DNA or RNA) and detection of the level of genetic expression represents important data in several areas such as medicine, biotechnology and the food industry. A number of diverse molecular biology techniques are capable of detecting genetic expression, for example northern blotting, microarrays, capillary electrophoresis and real-time PCR. Real-time PCR has become one of the most widely employed methods for gene quantification. This technique provides a high data output with a high level of sensitivity that is able to detect specific sequences; it does not require post-amplification manipulation and a large number of samples can be processed using modern equipment that avoids laboratory contamination. Nevertheless, several considerations must be taken into account when working with real-time PCR, such as the choice of the strategy of quantification, the choice of fluorescent markers and how the data is to be interpreted. This chapter covers the basic concepts of the real-time PCR technique and handling the data obtained, including types of absolute and relative quantification, mathematical models available for relative quantification and amplification efficiency calculations, types of normalization, chemicals used and applications in different fields of research. In addition, various steps for starting real-time PCR in order to determine gene expression levels of 16SrDNA, *gyrB*, *bgl-A*, *bgl* and *CspA* from *Shewanella* sp. G5 cultures are evaluated and optimized.

36.1 Introduction

In all living organisms (prokaryotes and eukaryotes) the genetic code is universal. It contains all the information required for synthesis of all necessary proteins for physiology, pathophysiology, development and function. But not all genes are expressed at the same time in one cell or all of them. Gene regulation in the cell comprises different processes (chromatin condensation, methylation, DNA-associated proteins, etc.) that affect its metabolic activity by activation or repression of one or more genes, thereby regulating the functional products. This mechanism of action is based on control of access with RNA polymerase to nucleic acids (DNA). This type of control of gene expression is also known as epigenetic control (Berger *et al*., 2009). The processes

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consist of transforming the information encoded in the DNA into protein (amino acid sequences). Production of messenger RNA (mRNA) copied from the nucleotide sequence of the gene (DNA) is called transcription, and the mRNA transcript after this is transformed into protein. The variations in gene expression levels are generally proportional to the number of copies of mRNA of a given gene. This fact is crucial when identifying the presence of specific cell products as the mRNA is translated by ribosomes into proteins. Therefore, it is possible to obtain data relating to the production of biological elements in the gene expression of a cell under consideration. Genetic expression studies are uncovering broad patterns of genetic activity, providing new understanding of gene functions, and generating unexpected insight into transcriptional processes and biological mechanisms (Berg *et al.*, 2006).

The identification of specific sequences (DNA or RNA) and detection of the level of genetic expression represents important data in several areas such as medicine, biotechnology and the food industry. In order to study gene expression, a wide variety of molecular biology techniques have been developed, such as northern blotting, microarray and real-time PCR (Draghici *et al.*, 2006; Bustin *et al.*, 2009). Northern blotting is a method based on separation of RNA purified in an agarose gel by electrophoresis and then identifying the gene of interest with specific DNA probes (Freeman *et al.*, 1999). While this technique is still used to investigate cell gene expression, its main disadvantage is the need for high levels of mRNA, preventing its use when the amounts of mRNA are very low (Berger *et al.*, 2009).

DNA microarray technology can simultaneously measure the genetic expression level of thousands of genes within a particular mRNA sample (Schena, 2000). These assays consist of a highly ordered matrix of different DNA sequences that can be used to measure the variation in DNA and RNA levels in applications that include gene expression profiling, comparative genomics and genotyping (Harrington *et al.*, 2000). Such expression profiling can be used to compare the level of gene transcription in all research areas. It is very important to note the advances in human medicine which allow detecting changes in the genomes. Results obtained in the variation in the genetic expression level allow the classification of diseases, identification of diagnostic biomarkers, measurement of the response to therapy drugs and an understanding of the mechanisms involved in the genesis of disease processes. Therefore, DNA microarray techniques are considered important tools for discoveries in medicine. The microarray assay types can be usually classified according to the length of the probes, the manufacturing method, and the number of samples that can be simultaneously outlined on one array. When microarray experiments are conducted within their optimal dynamic range, measurements reflect the magnitude and direction of expression changes of about 70–90% of the gene (Harrington *et al.*, 2000). There is a sensitivity (detection limit) range between one and ten copies of mRNA per cell, depending on the specific technology employed or cell type (Draghici *et al.*, 2006). The magnitude of expression changes observed is often different from those measured with other technologies, such as real-time quantitative reverse transcriptome-polymerase chain reaction (RT-qPCR) (Freeman *et al.*, 1999; Ellefsen *et al.*, 2008; Bustin *et al.*, 2009). The sensitivity may be insufficient to detect biologically important changes for genes with low levels of expression, such as transcription factors (Holland, 2002; Strube *et al.*, 2008). To overcome this limitation, the technique of real-time PCR has been developed, which is also called real-time quantitative PCR (qPCR). To obtain this information, qPCR has been accepted as one of the most powerful and sensitive techniques to analyse gene expression since it allows accurate quantitative detection of mRNA levels (Valasek and Repa, 2005; Sellars *et al.*, 2007; Ellefsen *et al.*, 2008; Strube *et al.*, 2008). The qPCR technique provides fast and easy results, a high data output with a high level of sensitivity that is able to detect specific sequences; it does not require post-amplification manipulation and can process a large number of samples using modern equipment that avoids laboratory contamination that could be caused by nucleic acids (Wong and Medrano, 2005). The technique based on qPCR amplifies and simultaneously quantifies specific DNA molecules giving access to reliable and accurate data on gene expression in the cells under study (Valasek and Repa, 2005). One advantage of qPCR is that it can generate small amplicons (from 60 bp to 250 bp) making it ideal for the detection of

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quantitative changes in gene expression during the course of various cellular processes, such as alterations in pathological tissue samples, experimental crop damage or changes in a cell's DNA (Strube *et al*., 2008). Another important feature of qPCR is its wide dynamic range, which can analyse a large number of target genes and normalized genes with the same sensitivity and specificity (Sellars *et al*., 2007). In this test, the qPCR product is measured at the end of each cycle. Data can be analysed using computer software and the number of copies of mRNA, or relative gene expression between multiple samples, can be calculated (Valasek and Repa, 2005).

Wong and Medrano (2005) described in their review the following data on qPCR assays. The qPCR technique can be produce quantitative data with an accurate dynamic range of 7–8 log orders of magnitude, and qPCR is 10,000–100,000-fold more sensitive than RNase protection assays and 1000-fold more sensitive than dot blot hybridization. In addition, qPCR can even detect a single copy of a specific transcript and can reliably detect gene expression variations as small as 23% between samples and have lower coefficients of variation (SYBR Green at 14.2%; TaqMan at 24%) than end-point assays such as band densitometry (44.9%) and probe hybridization (45.1%). Another great advantage of qPCR is that it can discriminate between mRNA with identical sequences, and requires less RNA template compared with other methods of gene expression analysis. The major disadvantage about qPCR is that it requires expensive equipment and reagents.

36.2 Theory of Real-time PCR

qPCR refers to the process where the products of amplification are directly monitored during each amplification cycle. The qPCR data collection is achieved using fluorescent chemistries that provide a strong correlation between fluorescence intensity and PCR product abundance. Relative fluorescence intensities can be plotted against PCR cycle number providing a graphic representation of the PCR process (Wang *et al*., 2006). The qPCR process can be broken into four major basic phases: (i) the linear ground (also known as baseline) phase; (ii) the early exponential phase; (iii) the log-linear (also known as exponential) phase; and (iv) the plateau phase. During the initial baseline phase, fluorescence emission from PCR product accumulation is below background detection. These fluorescence readings provide baselines for each individual sample. At the early exponential phase the amount of fluorescence has reached a threshold where it is significantly higher. At the beginning of the third amplification phase, a threshold fluorescence intensity level can be identified as significantly higher than the baseline level and where PCR reactions for all samples are undergoing logarithmic amplification. Once qPCR product accumulation reaches a critical threshold, fluorescence emission rises above background and further increases linearly over the next several PCR cycles. The PCR cycle at which this occurs is generally known as the cycle threshold (C_t) or crossing point (CP). This value is representative of the starting copy number in the original template and represents the log-linear phase from which the C_t data are collected to calculate the final experimental results (Wong and Medrano, 2005). In an optimal PCR assay, this value is inversely related to the amount of starting target DNA material. During the final plateau phase, fluorescence intensity slowly reaches the upper limit and is no longer useful for data calculation because PCR reaction components become increasingly limited and the product amplification rate is no longer proportional to starting concentrations. It is noteworthy that it is this final phase, non-linear data are measured in traditional endpoint PCR (Valasek and Repa, 2005).

36.2.1 Chemistry: types of fluorophores

There are various fluorescence-based chemicals for qPCR detection, which can be classified into four categories: (i) hydrolysis probes such as TaqMan chemicals; (ii) hairpin probes such as molecular beacons; (iii) fluorescent labelled hybridization probes; and (iv) DNA intercalating dyes (Wang *et al*., 2006; Bustin *et al*., 2009). A widely used method due to its lower cost is the use of fluorophores that bind to double-stranded DNA (dsDNA), such as SYBR Green. SYBR Green binds non-specifically to dsDNA and fluoresces. These fluorescent dyes are not specific as they bind to each molecule of dsDNA, including primer dimers or non-specific amplification products.

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The use of SYBR Green involves a very careful design of the primers in order to prevent primer dimer formation and avoid amplification of contaminating genomic DNA in the cDNA sample, so it should be designed so that the primers contain amplicon sequences of a different region of DNA (Valasek and Repa, 2005; Bustin *et al.*, 2009). Another alternative, that is more expensive but recommended when there are specificity problems, is the use of specific fluorescent probes such as different types of TaqMan probe. These chemicals allow the quantification of the specific cDNA of interest even in the presence of non-specific amplification (e.g. first dimers, non-target DNA sequences).

36.2.2 Strategies for RNA quantification by real-time PCR

It has been observed that the relative quantification of mRNA has some limitations. First, a significant statistical bias can be introduced when there are large differences in the levels of expression of the gene under study and the normalizing gene which may lead to the wrong biological interpretation. Secondly, suitable reference genes must be selected for normalization studies (Vandesompele *et al.*, 2002). For these reasons it is recommended that multiple reference genes are used in order to have reliable research data (Theis *et al.*, 2007). One of two standard procedures is commonly used to quantify gene expression using real-time PCR: (i) absolute quantification; or (ii) relative quantification (Bustin, 2000; Livak and Schmittgen, 2001; Liu *et al.*, 2009).

Absolute quantification

Absolute quantification by qPCR technique is based on a standard calibration curve, which is prepared from samples of known template concentration, such as plasmid with insert target. The concentration of any unknown sample can be determined by relating the signal obtained from the sample to this standard curve. Calibration curves are highly reproducible and allow the generation of specific, sensitive data (Bustin, 2000). However, the model of external calibration curves must be carefully validated with absolute accuracy for the quantification of gene expression in the qPCR and depends solely on the accuracy of the standards used (Bustin *et al.*, 2009). Furthermore, the design of standard sequences, their production and accurate determination of their concentration and their long-term stability and storage factors are complex and may present some problems (Pfaffl *et al.*, 2004). These considerations make absolute quantification a laborious and expensive procedure that cannot always be carried out in all laboratories.

Relative quantification

Relative quantification is a method used to measure changes in genetic expression level and it is based on the comparison between a treated sample with another control sample (e.g. pesticide-treated samples to an untreated sample). For this method, normalization is requested, which is to use a housekeeping gene known as calibrator of the target gene (Livak and Schmittgen, 2001). When using a reference gene, the method allows the comparison of mRNA transcription levels in a test sample with the equivalent in a calibrator sample (Nolan *et al.*, 2006; Strube *et al.*, 2008); and the results are expressed as a target/reference ratio. According to Wong and Medrano (2005), there are numerous mathematical models available to calculate the normalized gene expression from relative quantification assays. These include the following methods of analysis: (i) amplification efficiency (E); (ii) standard curve; (iii) comparative C_t method (C_t $2^{-\Delta\Delta C_t}$; Livak and Schmittgen, 2001); (iv) Pfaffl model (rest software; Pfaffl *et al.*, 2002); (v) Q-Gene; (vi) Liu and Saint; and (vii) the amplification plot method. Commonly, when relative quantification analysis is performed standard concentrations are not required.

AMPLIFICATION EFFICIENCY. It is important to assume that different qPCR assays do not necessarily have ideal amplification efficiencies; the calculation without an appropriate correction factor may overestimate the starting concentration. Current mathematical models make assumptions of reaction kinetics and usually require its accurate measurement. The amplification efficiency (E) of a reaction is calculated employing C_t dilution data from a standard curve with the following formula:

$$E = [10^{-(1/\text{slope})}] - 1 \qquad (36.1)$$

It is noteworthy that the E of the qPCR is the ability of the reaction to double the number of

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copies of DNA strands, or cDNA in each cycle (Bustin, 2000; Botteldoorn *et al.*, 2006). A linear standard curve implies an E that is constant at different cDNA concentrations. The E value of the standard curve should be between 90% and 110% (corresponding to slopes of –3.6 and –3.1, respectively) and 100% of E indicates a perfect replication of the amplicon in each cycle (Botteldoorn *et al.*, 2006; Theis *et al.*, 2007). If the value of E is significantly less than 100% this implies a slow reaction at some stage, which may be the result of the presence of inhibitors in the reaction mixture, or an unsuitable set of primers or reaction conditions. On the other hand, an E value significantly higher than 100% indicates typical experimental errors, for example: (i) wrongly calibrated pipettes; (ii) degradation of the probe; or (iii) formation of non-specific products or primer dimers. Deviation in E may also be due to poor preparation of serial dilutions, concentrations that can inhibit PCR (high annealing concentrations), or exceeding the particular assay sensitivity (low amounts of DNA). It is important for this type of quantification to run the standard curve on the same plate as the unknown sample. Repetitions of the genetic expression tests may lead to variations (between 5% and 10%) in C_t value, when the assay conditions are done at different times; consequently, they would not be directly comparable with other qPCR tests (Ali-Benali *et al.*, 2005; Botteldoorn *et al.*, 2006). If the standard curve was generated from a sample of known abundance, the starting quantity of unknown samples can be inferred from its C_t relative to the standard curve. Even when the standard curve was generated from an abundant but unknown absolute quantity, as is often the case in practice, the relative abundance of different samples can be directly compared by calculating the position of their C_t values along the same standard curve.

STANDARD CURVE. Consequently, a standard curve can be generated by plotting C_t values against the log of the starting quantity over a dilution series. The standard curve must be calculated in the assay for each gene in which two important parameters are involved: r^2 and the slope. The r^2 is the Pearson correlation coefficient of C_t values and it is proportional to the linearity of the curve, and the square of r^2 should go to 1 (Botteldoorn *et al.*, 2006). The slope reflects the E of each qPCR assay (Ellefsen *et al.*, 2008), and can be calculated from the slope of the standard curve obtained after performing serial dilutions with the qPCR reactions (Pfaffl *et al.*, 2004; Bustin *et al.*, 2009) according to the formula (Eqn 36.1). When the qPCR assay is performed, a standard curve for each gene must be created to assess the E value of the qPCR reaction. Therefore, the quantity of each sample is determined using this standard curve and then expressed relative to a calibrator. This method is applied when the E of the target and reference gene are unequal, and because it requires no preparation of exogenous standards, no quantification calibrator sample and is not based on mathematics models (Wong and Medrano, 2005).

$2^{-\Delta\Delta C_T}$ METHOD. Calculations on relative quantification of gene expression were based on comparison of the C_t values using the E of the qPCR reaction as a correction factor. However, there is a model that does not require the reaction E to access a correction factor. This model assumes an optimal and identical E (corresponding to 100%) in the reaction in both the qPCR of the gene under study and the reference gene (Livak and Schmittgen, 2001, 2008). This $2^{-\Delta\Delta C_t}$ method is applicable only for a quick estimate of the relative proportion of gene expression of the gene under study (Pfaffl *et al.*, 2002). The $2^{-\Delta\Delta C_t}$ method expresses the ratio obtained from the relationship between the sample C_t values and the control C_t values, as shown in the following equation:

$$\text{ratio} = 2^{-[\Delta C_t\,\text{Sample} - \Delta\, C_t\,\text{Control}]} \tag{36.2}$$

$$\text{ratio} = 2^{-[\Delta\Delta\, C_t]} \tag{36.3}$$

PFAFFL MODEL. Another model for relative quantification analysis has been described by Pfaffl *et al.* (2002). In this model the different E of the qPCR gene for both the gene under study (target) and the reference gene are taken into account as shown in the following equation:

$$\text{Ratio} = \frac{(E_{target})^{\Delta C_t\text{target (Control–Sample)}}}{(E_{reference})^{\Delta C_t\text{reference (Control–Sample)}}} \tag{36.4}$$

This equation analyses the ratio of the expressed gene (or genes) under study from a sample versus a control in comparison to a reference gene.

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The E_{target} represents qPCR amplicon efficiency of the gene under study. The $E_{reference}$ represents the qPCR efficiency of the reference gene; ΔCt_{target} is the deviation in the control C_t value minus the sample gene of interest, and $\Delta Ct_{reference}$ is the deviation in the control C_t value minus the sample reference gene (Wong and Medrano, 2005).

36.2.3 Normalization

As previously mentioned, qPCR quantification studies need normalization analysis, which is important because it eliminates the variations and performs correction of the expression of target genes by the constitutive expression of the reference genes (Vandesompele *et al.*, 2002). However, depending on cell type or research experiments, variation in gene expression was observed in some of these genes. Furthermore, it has been shown that the use of a single gene as a reference gene is susceptible to errors in interpreting the results of qPCR (Huggett *et al.*, 2005). Accordingly, to normalize gene expression when working with qPCR analysis, it is important to perform normalization, in which the expression of two to six housekeeping genes should be evaluated and those that exhibit optimal characteristics for relative quantification can be selected (Huggett *et al.*, 2005). The correct choice of housekeeping genes for qPCR normalization is essential to reflect reliable data on the biological processes under study (Dheda *et al.*, 2005). For normalization studies in prokaryotes, Table 36.1 shows a variety of reference genes; by contrast in most eukaryotic organisms the housekeeping genes used include β-actin, glyceraldehyde 3-phosphate dehydrogenase, hypoxanthine guanine phosphoribosyl-transferase and 18S rRNA (Theis *et al.*, 2007). The total expression ratio of the genes of interest can be tested for significance by a randomization test implemented in the relative expression software tool rest (website: http://rest.gene-quantification.info/). Currently the right software for choosing housekeeping genes for each experiment is completed and can be found freely on the Internet. The relative expression data by RT-qPCR can be analysed by an Excel application for group comparison and statistical analysis (Pfaffl *et al.*, 2002).

The qPCR technique is widely used to obtain the magnitude of physiological changes in gene expression levels of a gene under study compared with one or more reference gene (Pfaffl *et al.*, 2004). It must be taken into account that the expression of reference genes should be constant in the studied cells. For this reason, the reference genes used in the relative quantification method are generally called 'housekeeping genes' (Table 36.1; Nielsen and Boye, 2005; Theis *et al.*, 2007). These are those genes whose expression is constitutive in the cell (i.e. which are essential for the operation

Table 36.1. Housekeeping genes for prokaryotes. (Modified from Nielsen and Boye, 2005; Theis *et al.*, 2007.)

Gene	Product	Pathway
adK	Adenylate kinase	
ccpA	Catabolite control protein A	
fabD	Malonyl CoA-acyl protein transacylase	Fatty acid biosynthesis
ftsA	Cell division protein	Cell division
glpT	Glycerol-3-phosphate permease	
glyA	Serine/glycine hydroxymethyl transferase A	Amino acid catabolism
gmk	Guanylate kinase	Nucleotide metabolism
gyrA	DNA gyrase subunit A	Replication
gyrB	DNA gyrase subunit B	Replication
proC	Pyrroline-5-carboxylate reductase	Amino acid biosynthesis
pyk	Pyruvate kinase	Glycolysis
pyrE	Orotate phosphoribosyltranferase	
recA	DNA replication and repair protein	Replication and repair
rho	Transcription termination factor Rho	Transcription
rpoD	RNA polymerase sigma factor RpoD	Transcription
rrsC	16S ribosomal rRNA subunit	Translation
sucC	Succinyl coenzyme A synthetase	
tpiA	Triosephosphate isomerase A	Gluconeogenesis

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and development of organisms) (Ellefsen *et al*., 2008). For accurate gene quantification analysis, normalization of qPCR data is essential to eliminate template variations due to differences between samples in the initial sample amount (Nolan *et al*., 2006). To date, normalization is most frequently achieved by the use of internal controls, often referred to as housekeeping genes (Theis *et al*., 2007; Vandesompele *et al*., 2002).

36.2.4 Optimization of qPCR

When qPCR experiments are performed a number of parameters must be considered so the results are reliable. Initially, if the case study that is being worked on is designed to measure the variation in the expression of target genes, it is necessary to conduct an analysis of the main factors and then optimize the reaction in the laboratory. To take into account the main factors, the following parameters are defined:

- the kind of quantification analysis (absolute or relative – the type of experiment to be performed is considered, taking into account the optimal characteristics of each analysis);
- what genes to assess (the primer design is essential in this type of study);
- the conditions to be assessed and standardization of the study (the test conditions and whether they will have a high or low impact for a cell will guide the selection of the appropriate reference gene);
- the design primers for the target and reference genes (all genes should be evaluated on a single platform, therefore the primers must meet rigorous requirements such as having a melting temperature (Tm) that will prevent the formation of primer dimers, etc.);
- selection of the chemical probe (SYBR Green or TaqMan probes – the cost of the specific reactions in the qPCR should be determined); and
- the laboratory experiment (e.g. the test conditions, obtaining mRNA, cDNA, primer testing through conventional PCR and qPCR optimizations).

Depending on the type of chemical being worked with, it is important to assess the primers by conventional PCR and by agarose gel electrophoresis. The results of these analyses provide data to correlate with the length of the product analysis, the peak melting curve and the possible presence of non-specific products as first dimers. Furthermore, to reduce the risk of contamination, a number of precautions should be taken that include: (i) distributing different parts of the work in different areas of the laboratory; (ii) adopting correct work habits that minimize risks; and (iii) using special equipment or anti-pollution systems to irradiate the laboratory equipment and surfaces such as ultraviolet light or chemical systems such as isoprenaline-2 or uracil-N-glycosylase (Rys and Persing, 1993).

It is important to remember that among the most important factors to optimize the qPCR are specificity, sensitivity, efficiency and reproducibility (Bustin *et al*., 2009). Optimization of qPCR makes different variations of the reactions that do not cause significant effects on the C_t values of the target genes and reference genes. The factors that must be optimized are reagent mixtures, concentrations of primers, concentration of fluorescent labels, cDNA concentration, as well as evaluating changes in the cycling conditions. Using the optimum concentrations of reagents, associated with the optimum qPCR cycling, produces optimal results that are observed in terms of the melting temperature curve and the amplification efficiency. It is important to mention that these tests should be performed with positive controls and calibrators.

36.2.5 Applications

qPCR has a wide range of applications, as it is a test of great sensitivity and specificity that can be carried out in less time and at lower cost than other forms of analyses. The applications include scientific research (basic and applied), use as a molecular diagnostic tool, use in molecular epidemiology and in forensic science, and for genotyping, food security, biotechnology and identification of mutations determining drug resistance or virulence, among many others.

Diarrhoea is a disease that can be caused by a wide variety of organisms, including bacteria (*Escherichia coli*, *Shigella* and *Salmonella*), mycobacteria (*Mycobacterium tuberculosis*), viruses (rotavirus, adenovirus, enterovirus, pestivirus

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and astrovirus), parasites (*Entamoeba histolytica*) and fungi. Some of the bacterial pathogens observed in communities around the world are *Aeromonas*, *Campylobacter*, diarrhoeagenic strains of *E. coli*, *Salmonella*, *Shigella*, *Vibrio* and *Yersinia* (Liu *et al.*, 2011a; de Boer *et al.*, 2012; Wang *et al.*, 2012). The microbiologic etiology of diarrhoea is usually not clinically evident and therefore laboratory diagnosis is important since many of the bacteria, such as *Shigella*, *Vibrio* and *Yersinia*, usually require specific antibiotic therapy (Guerrant *et al.*, 2001). Actually, the conventional culture methods are still routine techniques for the detection and identification of bacterial enteric pathogens in clinical laboratories, although studies have repeatedly shown poor performance and high cost (Guerrant *et al.*, 2001; Liu *et al.*, 2011b). Moreover, these methods of detection and identification of cultures require great ability, labour and time, late epidemiological research or treatment. Another difficulty with the conventional techniques is the viability of these bacteria meaning that many cannot be cultured (Alam *et al.*, 2010; de Boer *et al.*, 2010) and are greatly affected by antibiotics, a phenomenon that has been described with *Vibrio cholerae*, *Vibrio vulnificus*, *Salmonella enterica* serovar Enteritidis, *Shigella* and *Campylobacter jejuni*. Therefore, the detection of enteroinvasive bacteria cultures has been compared with insensitive immunosorbent ELISA assay methods and those based on qPCR (Cheyne *et al.*, 2010; Bessede *et al.*, 2011). The qPCR provides high sensitivity, specificity and speed in obtaining results. In particular, the implementation of PCR provides quantitative results and allows the detection, localization and classification of different types of pathogenic microorganism from a large number of samples, such as clinical samples (blood, faeces), environmental samples (water- and soil-contaminated) and food samples (Le Cann *et al.*, 2004; Monpoeho *et al.*, 2004; Pourcher *et al.*, 2005; Poma and Rajal, 2013). The detection and identification of multiple pathogens can be accomplished by qPCR using genetic markers, such as the multiple virulence and marker genes (VMGs) or toxin genes (Miller *et al.*, 2008; Cheyne *et al.*, 2010; de Boer *et al.*, 2012). The use of multiple markers, VMGs, for the same pathogen improves the specificity and reliability of the assay to determine the presence of the pathogen in a sample. Currently a large number of systems that amplify VMGs have been described and use of these genes can discriminate between bacterial pathogens, including *E. coli*, *Staphylococcus aureus*, *Aeromonas* spp., *Salmonella* spp., *C. jejuni*, *Pseudomonas aeruginosa*, *Vibrio* spp., *Shigella flexneri*, *Yersinia*, etc. The heredity of blocks of genes, called pathogenicity islands, is often the key to the expression of virulence factors in bacteria (Stedtfeld *et al.*, 2008; Chase and Harwood, 2011; Wang *et al.*, 2012). Among these genes present in the chromosome of the bacteria or plasmids are genes that encode for toxins and adhesins. Horizontal transfer of genes through these mobile genetic elements is an important force in evolution, indicating the strength and expertise of these pathogenic bacteria. Environmental factors such as temperature, pH, availability of iron, osmolarity and nutrients regulate the expression of virulence factors. The sensitivity and specificity of qPCR and the ability to obtain high yields mean that this method of analysis is necessary to detect such pathogenic virulence genes.

In the field of clinical microbiology, it is important to highlight the research advances in virology, bacteriology and pathogenic fungi that affect humans and animals. Infectious agents with great economic interest to several drugs companies for microbiological diagnosis include the human immunodeficiency virus (HIV), hepatitis C virus and hepatitis B virus. In this field of application qPCR is used in the identification of easily cultivable and uncultivable organisms whose rapid detection allows obtaining some benefit, such as clinical prevention. The qPCR is useful if a rapid and early diagnosis of the causative etiologic agent in the patient is required to initiate specific antibiotic treatment as soon as possible (Espy *et al.*, 2006). Reducing the time of diagnosis may improve the prevention of various infections and improve the efficiency and speed of treatment of children and adults. qPCR provides a quantitative method that is used both for detection of infected patients who should be treated, and for checking on the drug's effectiveness once treatment is started (Bustin *et al.*, 2009).

In the environmental field, faecal pollution represents a dangerous risk to human health (Costafreda *et al.*, 2006) and this is one of the major concerns in relation to water bodies used for drinking water supply, recreational activities and harvesting seafood as such water bodies are

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known to contain a wide range of pathogens, including viruses, bacteria and protozoa (Ahmed *et al.*, 2009; Harwood *et al.*, 2009; Balleste *et al.*, 2010). These pathogens come from infected animals or humans and can be introduced into the source waters through faeces and cause public health risks, environmental degradation and economic losses. A wide range of pathogenic microbes are uncultivated (i.e. not capable of being grown on culture media) and persist in the environment. Microbial source tracking (MST) is a rapid method to discriminate between human and non-human faecal contamination in water, based on the detection of 16S rDNA in *Bacteroides*. Therefore, several authors have developed qPCR-based assays to amplify the host-specific *Bacteroides* markers for humans and animals to monitor human faecal pollution in water (Kildare *et al.*, 2007; Balleste *et al.*, 2010; EPA, 2011).

An additional advantage of this qPCR technique is only a small amount of sample is needed. This makes it ideal when used in conjunction with laser microsurgery to biopsy tissue or specific cells in order to study them, for example in monitoring the behaviour of cancer cells (Edwards *et al.*, 2004). The qPCR is utilized to identify genetic mutations or polymorphisms using systems of specific probes and results in the analysis of changes in the melting curve. The qPCR has also been used to access relevant biodynamics of the disease organism's data. Another important application of this technique is the research of changes in genetic expression of cells through quantification of the cell's mRNA, allowing association of these changes in the cell with changes in physiological conditions, the presence and effects of several drugs, the presence of different infectious agents, and in biotechnological processes for the enhancements of biocatalysts (Espy *et al.*, 2006; Bustin *et al.*, 2009).

The qPCR is also being used in other fields such as forensic science and biosecurity. In these cases, the investigations are based on the identification of pathogens or saprophytic organisms disseminated in the environment. The characterization of small amounts of genetic material from such organisms can make it possible to identify the origin and dynamics. Another area in which the qPCR is used is food security. Here, the identification of genetically modified organisms in food or food additives is a need that can be met using this technology. The revised principles above can also access the quantification of these organisms, using reference genes to normalize the amount of cDNA admitted in the assays.

36.3 Conditions for Quantification of Gene Expression of *bgl-A, bgl* and *CspA* from *Shewanella* sp. G5 Cultures

Winemaking is a biotechnological process in which the use of exogenous enzyme preparations isolated from yeasts or bacteria help to overcome the problem of insufficient endogenous activity in the grapes (Palmeri and Spagna, 2007). Consequently, commercial preparations of glycosidase from *Aspergillus niger* are used to increase wine aroma (Spagna, 2000) and the techniques that allow these enzymes to be identified have been of fundamental importance in oenology for use in enzymatic treatments in the commercial preparation of wine (Barbagallo *et al.*, 2004). The enzymatic hydrolysis of glycosides occurs sequentially in two stages. In the first one the intersugar bond is cleaved by rhamnosidase (Rha, EC 3.2.1.40) and arabinosidase (Ara, EC 3.2.1.55). In the second, the β-glucosidase (βG, EC 3.2.1.21) releases aglycone, which is responsible for the increase in wine aroma (Cristóbal *et al.*, 2013). The Ara and βG combination can also be utilized in citrus-juice technology. In particular, in the debittering by naringin hydrolysis (Spagna, 2000), and in food processing for aromatization of fruit juices from passion fruit, apple, apricot, peach, tomato, pineapple, cherry, pear, papaya and banana (Spagna *et al.*, 1998; Iwashita *et al.*, 1999). The βGs have received a great deal of attention due to their extensive potential applications in different industrial fields such as food, feed, textile, detergents, pharmaceutical and bioethanol conversion industries (Yang *et al.*, 2013). As a major group among glycoside hydrolase enzymes, βGs have been mainly classified in the GH family 1 (GH1) and GH family 3 (GH3) based on their amino acid sequence identities (Henrissat *et al.*, 1996). *Shewanella* sp. G5, a psychrotolerant and Gram-negative bacterium, was used in the current study. It was isolated from the intestines of *Munida subrrugosa* and two cold-active βGs producers (EF141823 and DQ136044) were previously

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characterized (Cristóbal *et al*., 2008, 2009). To examine optimal conditions for use in biotechnological processes from *Shewanella* sp. G5, the highest level of gene expression of *bgl-A*, *bgl* and *CspA* genes was detected in response to different culture conditions using the RT-qPCR method. In the current work, various steps in the qPCR method for determination of genetic expression levels starting from cells of *Shewanella* sp. G5 were evaluated and optimized. The aim of this study was to detect the highest level of gene expression of *bgl-A*, *bgl* and *CspA* genes in response to different culture conditions using the RT-qPCR method. This information is of importance at the time for optimizing enzymatic production and reaction at an industrial scale. The study described a comparison method to establish the relative quantification of the gene tested (Cristóbal *et al*., 2015).

36.3.1 Methods

Culture conditions

In order to evaluate growth conditions in which glucosidases genes have high genetic expression, *Shewanella* sp. G5 was cultured in liquid Luria-Bertani medium (LB), supplemented with 20 g/l sodium chloride and 10 g/l of cellobiose (LBC) or glucose (LBG) as the carbon source, and Mineral Medium Brunner (MMB; DSMZ, 2012), modified by the addition of 20 g/l of sodium chloride and 10 g/l of cellobiose (MMBC) or glucose (MMBG). Flasks (1 l) containing 300 ml of the aforementioned media were inoculated with 100 µl of over-night cultures obtained in both media and incubated on an orbital shaker (150 rpm) at 10°C and 30°C, respectively. Growth was monitored with a Beckman (DU®640) spectrophotometer until an absorbance of about 0.8 was obtained.

RNA extraction and cDNA synthesis

RNA was extracted from 2 ml *Shewanella* sp. G5 liquid cultures (absorbance approximately 0.8). The biomass of each jar was recovered and used in the RNA Isolation System Kit (Promega) according to the manufacturer's protocol. A total of 50 µl RNA was obtained per sample, which was treated with DNAse provided by the manufacturer. Reverse-transcription (RT) was performed in 25 µl reaction volume using 10 µl RNA and 2 µl of random primers incubating at 70°C for 5 min and cooling down rapidly to 4°C; the following were then added: 5 µl 5X buffer (Promega), 4 µl dNTP (10 µM) and 3 µl of enzyme (AMV 10 U, Promega). This was homogenized and incubated at 37°C for 1 h. This last step allowed the primers to anneal, therefore the prolonged incubation was critical for successful cDNA synthesis from *Shewanella* sp. G5 cultures. To minimize potential effects of differential synthesis efficiency during the RT reaction, three separate RT assays were carried out and pooled for each RNA preparation analysed. All cDNA solutions samples were stored at –20°C until use for qPCR amplification.

Design of oligonucleotide primers to detect β-glucosidase genes by conventional PCR

Primers are essential to ensure that only a single specific PCR product is amplified. To detect target genes from *Shewanella* sp. G5, primers were designed for qPCR based on the sequences of the *bgl-A* (GenBank accession number DQ136044), *bgl* (EF141823) and *gyrB* (DQ268831) genes, that encode β-glucosidase A, β-glucosidase and gyrase subunit B, respectively. For *CspA*, primer sequences were designed on *Shewanella baltica* OS155 (CP001252). Universal primers for 16S rDNA (Muyzer *et al*., 1993) were also used. Oligonucleotide primers were designed based on bacteria nucleotide and amino acid sequences retrieved from the National Center for Biotechnology Information (NCBI). Sequences were analysed using the blast program and the GenBank databases (http://www.ncbi.nlm.nih.gov/). Multiple sequence alignments were conducted with ClustalW algorithm. Sequences with high similarity and low *E* values were selected for a final multiple sequence alignment. All primers, listed in Table 36.2, were designed using DNAMAN software version 4.03 (Lynnon BioSoft) and evaluated online with the *In silico* simulation of the molecular biology experiments program (http://insilico.ehu.es/). The predicted primer melting temperatures (Tm) were calculated between approximately 55°C and 60°C. The ideal amplicon sizes were about 186 bp, with an upper limit of 242 bp.

Extraction of genomic DNA (gDNA) from *Shewanella* sp. G5 was carried out according to a

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Table 36.2. Specific oligonucleotide primers used in qPCR to amplify *blg-A*, *bgl*, *CspA*, *gyrB* and 16S rDNA genes from *Shewanella* sp. G5.

Gene	Locus access[a]	Primer name	5′ to 3′ sequence	Tm (°C)[b]	Amplicon size (bp)[c]	Reference
bgl-A	DQ136044	F-gh1	GCATTAGCGCCAGAAGACAGA	64	218	Cristóbal
		R-gh1	ATAGGTTTGATTTAAAGAAAC			*et al*. (2015)
bgl	EF141823	F-gh3	ATCACGGTAATCCTTATTTATT	62	204	
		R-gh3	CTTGCGCGATAGTGTTTTCATA			
CspA	CP000961	F-cspA	TTACTGGTGTTGTTAAGTGGTTCA	64	186	
		R-cspA	TTACGTTTTCAGCTTGTGGACC			
gyrB	DQ268831	F-gyrB	TTTCCGTAGTGCGTTGACACGT	66	242	
		R-gyrB	GGTTTTCCAGCAGATAATCGTTC			
16S rDNA	AY398666	F357	ACTCCTACGGAGGGCAGCAG	57	200	Muyzer
		R518	ACGTATTACCGCGGCTGCTGG			*et al*. (1993)

[a]National Center for Biotechnology Information (NCBI) database GenBank accession number.
[b]Tm, Melting temperature of the primers according to DNAMAN software.
[c]Size of PCR product tested online with the *In silico* PCR simulation program from *Shewanella baltica* OS155 (CP001252).

protocol described previously (Cristóbal *et al.*, 2008). The *bgl-A*, *bgl*, *CspA*, *gyrB* and 16S rDNA genes were amplified by conventional PCR using specific primers designed for this study (Table 36.2). Amplification of each gene was performed in 25 μl reaction mixture containing 1 μl gDNA, 5 μl GoTaq 5X buffer, 0.5 μl of each primer (33 μM), 0.4 μl bovine serum albumin (10 mg/ml), 0.2 μl GoTaq (Promega) and water to complete the volume. PCR conditions for all genes consisted of an initial denaturalization step of 4 min at 94°C followed by 30 amplification cycles, comprising a denaturalization step of 1 min at 94°C, annealing at 56°C for 1 min and extension at 72°C for 30 s. Reactions were completed with 5 min at 72°C followed by cooling down to 4°C. Amplification reactions were carried out in an automated thermal cycler (Perkin-Elmer, model 9700). PCR products were separated by 1.0% (w/v) agarose gel electrophoresis, stained with ethidium bromide and then visualized using an Image Analyzer Gel Doc BIO RAD.

Real-time PCR assay

Real-time PCR was performed on a thermal cycler (GeneAmp® 5700 Sequence Detection System Applied Biosystems) using SYBR Green stain that binds dsDNA. PCR reactions were carried out in a total volume of 25 μl that contained 5 μl cDNA, 1 μl of forward and reverse primers (33 μM), 12.5 μl SYBR Green ER™ master mix (Invitrogen), and water to complete the volume. PCR amplifications were performed under the following conditions: incubation at 50°C for 2 min (uracil-N-glycosylase activation), at 95°C for 10 min (polymerase activation), and 40 cycles of 95°C for 15 s, 56°C for 30 s and 72°C for 1 min. After the amplification, melting curves were performed from 60°C to 95°C (0.3°C/s) generated with GeneAmp® 5700 SDS software (Applied Biosystems). Each sample was analysed in triplicate; negative controls with water instead of cDNA templates were included in all real-time plates. The C_t value, which is the number of cycles required to reach the threshold fluorescence value, was employed to calculate the expression level of each gene under study. The data expressed as C_t were imported into a Microsoft Excel data sheet for subsequent analysis.

Standard curves and normalization

From a series of dilutions from the cDNA, pooled standard curves for each amplification system were generated in order to evaluate the efficiency of amplification and the dynamic range and sensitivity of the system. The amplification efficiency (E, expressed as a percentage) was calculated according to Equation 36.1 (Theis *et al.*, 2007). The standard curves were used to quantify the analysed genes in all the samples from the growth of *Shewanella* under different culture conditions. The 16S rDNA and *gyrB* genes were used as constitutive expression of internal controls. Both genes were amplified and quantified

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simultaneously with the target genes, in order to normalize gene expression.

Relative quantification and data analysis

The 16S rDNA and *gyrB* genes were used as the endogenous reference and simultaneously amplified with the target genes. Target concentrations were adjusted and normalized by dividing the average concentration of the target gene by the average concentration of the reference DNA. The average C_t values were used to calculate the relative transcript quantification using the comparative C_t method ($2^{-\Delta\Delta C_t}$, according to Equation 36.3) described by Livak and Schmittgen (2001); each target was normalized against the *gyrB* gene. The *E* of reference and target genes must almost be equal, calculated from Equation 36.1. The relative quantification is expressed as a $2^{-\Delta\Delta C_t}$ value (Nielsen and Boye, 2005; Schmittgen and Livak, 2008).

36.3.2 Results and discussion

Analysis of designing primers for genes of interest

From the sequences of studied genes of *Shewanella* sp. G5, and according to the multiple alignments performed by the DNAMAN program, all primers were obtained for qPCR. Figure 36.1 shows two examples of the multiple alignments of the *gyrB* (Fig. 36.1a, b) and *bgl* (Fig. 36.1c, d) genes.

Then, the primers were analysed using the online program *In-silico* PCR that allows a virtual PCR to be carried out. First the genus and species of bacteria of interest are selected and then the designed primers are evaluated (Fig. 36.2). For this analysis, positive results were obtained from *S. baltica* OS155 utilized as a model; where the sizes and specific amplifications were detected. The analysis provided an estimated length of the theoretical amplification products of 214 bp, 204 bp, 242 bp and 186 bp for *bgl-A*-GHF1, *bgl*-GHF3, *gyrB* and *CspA*, respectively (Fig. 36.2).

Also, the analyses provides amplification specificity through a link (number), which directs to the position of the amplification product (gene) in the genome of *S. baltica* OS155 for all genes evaluated; therefore, these data provide precise information for the designed primers. On the other hand, the DNAMAN program (version 4.03) was employed to analyse the complementarity between the primers (dimer formation) and the melting temperature (Tm = GC + AT) with an average of 64°C for all genes.

Primer evaluation by conventional PCR

For all genes, positive amplifications were obtained by conventional PCR (Perkin Elmer) using primers especially designed for this study (Fig. 36.3). Agarose gel electrophoresis showed specific amplifications of all PCR products corresponding to the genes analysed, exhibiting fragments of the expected size (Table 36.2). Universal primers were used to amplify 160 bp of the internal region of 16S rDNA, which are shown in Table 36.2.

Optimization of real-time PCR

Three amplification protocols (A, B, C) were used for the qPCR optimization; they differed in the annealing temperature (52°C, 56°C and 60°C) and extension steps (Table 36.3). Positive C_t values were obtained for all the genes under all the studied conditions, with the exception of *CspA* gene that was not amplified under condition B. The expression of the 16S rDNA gene was remarkably higher than for the other genes in all cases. Instead, *gyrB* gene was expressed at the same level as the other genes, and as such this was selected as the reference housekeeping gene and condition C was found to be the optimum. Showing a C_t value close to the target genes, justifies the choice of the *gyrB* gene as the normalizer in qPCR reactions.

Condition C showed the average C_t values for all genes (being 13 and 24 for the 16S rDNA and *gyrB* genes, respectively) and consequently this protocol was selected for qPCR. The LBC10 condition was chosen to monitor expression of the *bgl-A*, *bgl* and *CspA* genes and two-step qRT-PCR reactions were carried out with SYBR Green I detection, as these steps avoid the formation of dimers (Wang *et al.*, 2006; Sellars *et al.*, 2007). In this work, absence of non-specific PCR products and primer dimer artifacts in the qPCR was established with melting curves. In all cases the curves showed a single peak for each gene indicative of the specific amplification (Fig. 36.4).

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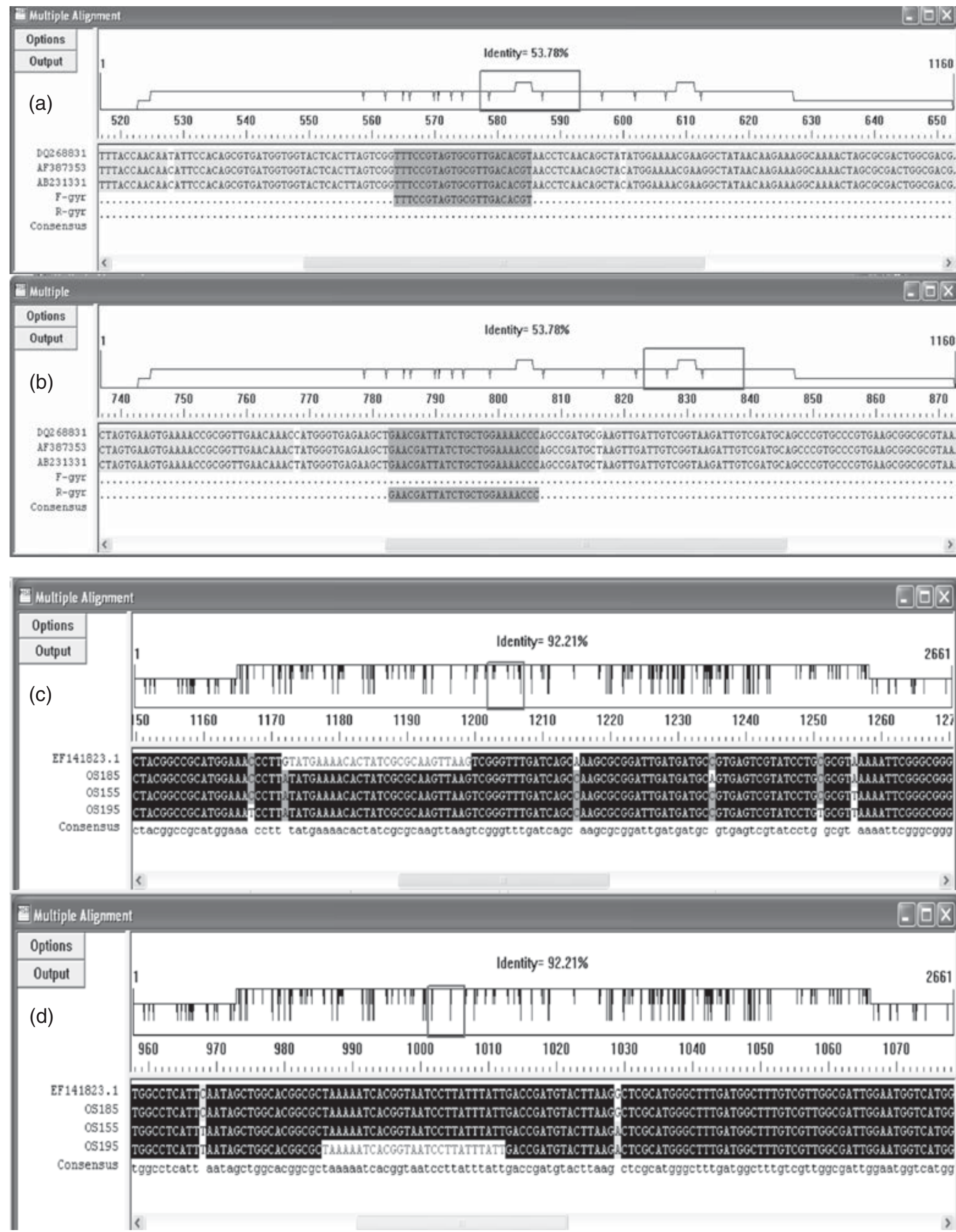

Fig. 36.1. Multiple alignment analyses for sequences of genes *gyrB* and *bgl* obtained by oligonucleotide consensus. For the *gyrB* gene (a) forward and (b) reverse; for *bgl* gene (c) forward and (d) reverse.

It is noteworthy, that a first qPCR analysis was evaluated with primers previously designed (data not shown) for inverted PCR assay in order to achieve complete gene amplification. Therefore, for genes *bgl-A* and *bgl*, two pairs of primers (forward and reverse) were used to evaluate the qPCR accuracy of amplification. Figure 36.5(a, b) shows the positive results obtained in the

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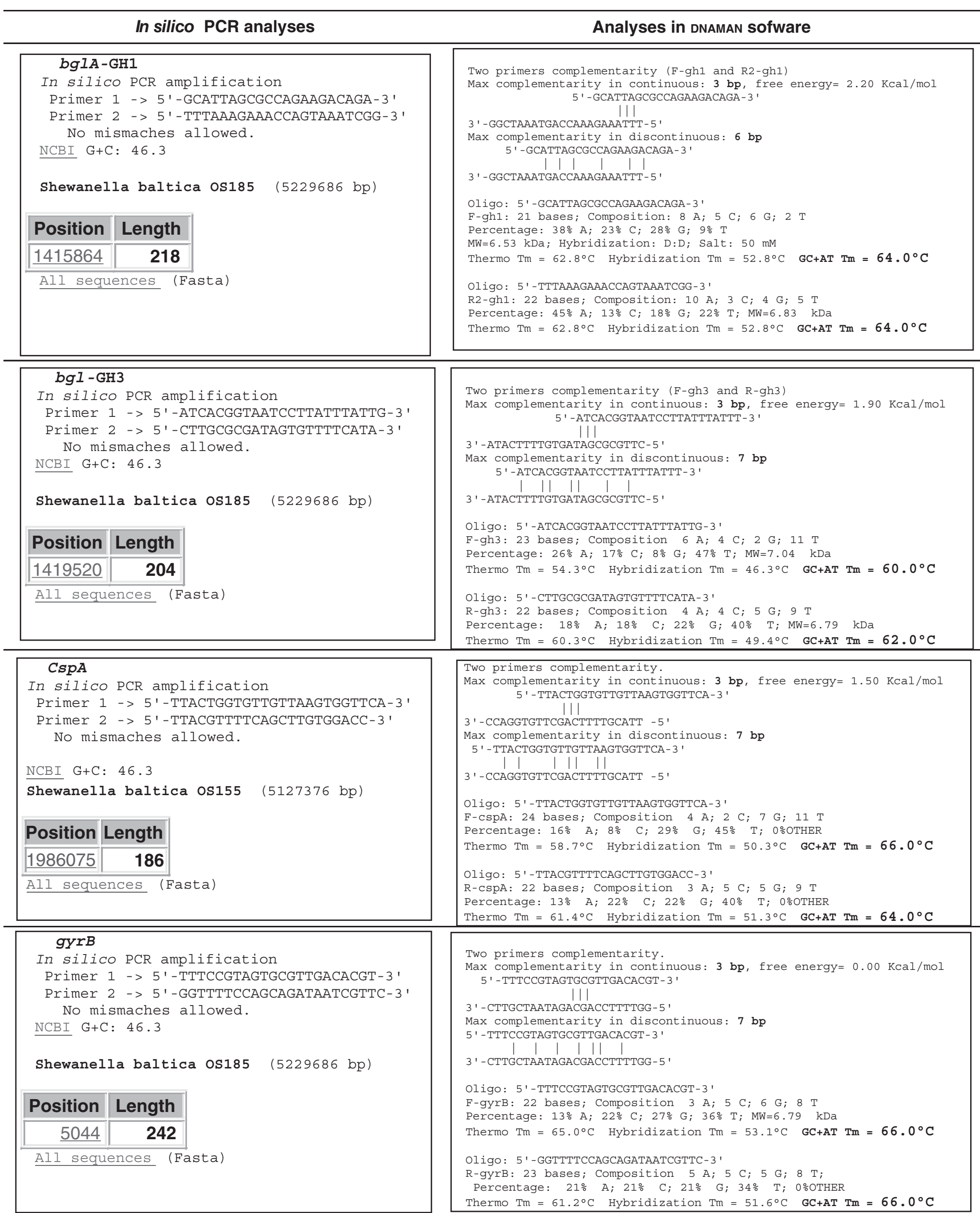

Fig. 36.2. Oligonucleotide primer design analysis for the genes under study in PCR real-time.

amplification curves for *bgl-A* and *bgl* genes, respectively. However, the melting curves for these two genes showed that these amplification data were inaccurate, because non-specific amplification or primer dimers were observed for both genes (Fig. 36.5c, d).

Normalization with the reference gene and generation of standard curves

Relative quantification is the most commonly used method, whereby the level of gene expression is normalized to that of an internal reference gene. In the current assay, differences in

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amplification were observed for the 16S rDNA and *gyrB* genes, resulting in the C_t value for the *gyrB* gene being similar to that of the target genes. An explanation could be that there are usually a greater number of 16S rDNA copies than the *gyrB* gene in the *Shewanella* sp. G5 genome. This justifies the choice of *gyrB* as the normalizer in the reactions by qPCR. The C_t values for *gyrB* and the genes assayed showed that their genetic expression did not exhibit any changes during the conditions examined.

The qPCR condition C was used to perform standard curves for each gene under study (Table 36.3). Standard curves were calculated in the present study for all systems, using the C_t value of the qPCR of each gene designed as standard points with assigned concentrations. An arbitrary threshold level was set and C_t values determined (Cristóbal *et al.*, 2015).

For this study, the standard curves allowed verification that the amplification efficiency (*E*; calculated from Equation 36.1), slope and r^2 were calculated for each system studied (Table 36.4), determining an optimal *E* for *gyrB*, *bgl-A*, *bgl* and *CspA* genes. However, the *E* assay for 16S rDNA was at 116%, reinforcing the selection of *gyrB* as the normalizing gene; and the r^2 value was used to assess linearity for which r^2 should be very close to 1 (≥ 0.995), as described Botteldoorn *et al.* (2006) and Ellefsen *et al.* (2008).

Before using the comparative $2^{-\Delta\Delta C_t}$ method (calculated from Equation 36.3) described by Livak and Schmittgen (2001), we verified that the *E* of the reference (*gyrB*, for this case) and target genes were almost equal. Using data from samples designed as standard points with assigned concentration numbers, an arbitrary threshold was set and C_t values for all qPCR samples were calculated, allowing the generation of standard curves. This type of standardization study was carried out to conduct quantification analysis of the genes of interest. Theis *et al.* (2007) and

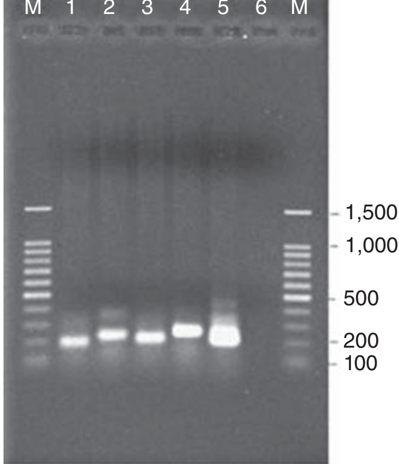

Fig. 36.3. Primer analysis by conventional PCR. Agarose gel electrophoresis of the PCR products from the genes studied with specially designed primers. Lanes: M, 100 bp DNA ladder (Promega); 1, *CspA*; 2, *bgl-A*; 3, *bgl*; 4, *gyrB*; 5, 16S rDNA; and 6, negative control.

Table 36.3. qPCR optimization. Average of the cycle threshold (C_t) values and standard deviation (± SD) obtained for LBC10 (Luria-Bertani medium + cellobiose incubated at 10°C) under different annealing temperatures and extension conditions. The level of expression is expressed in terms of the C_t value, which is the number of cycles required to reach a certain fluorescence value.

	qPCR conditions tested					
	A		B		C	
	95°C, 15 s; 60°C, 60 s		95°C, 15 s; 52°C, 30 s; 72°C, 1 min		95°C, 15 s; 56°C, 30 s; 72°C, 1 min	
Genes evaluated	C_t	± SD	C_t	± SD	C_t	± SD
16S rDNA	12.52	± 0.07	13.14	± 0.07	12.47	± 0.07
gyrB	24.61	± 0.17	25.68	± 0.07	24.53	± 0.17
bgl-A	29.02	± 0.59	26.44	± 0.22	23.97	± 0.31
bgl	29.31	± 0.44	26.31	± 0.05	24.13	± 0.16
CspA	24.19	± 0.16	Negative	--	25.32	± 0.07

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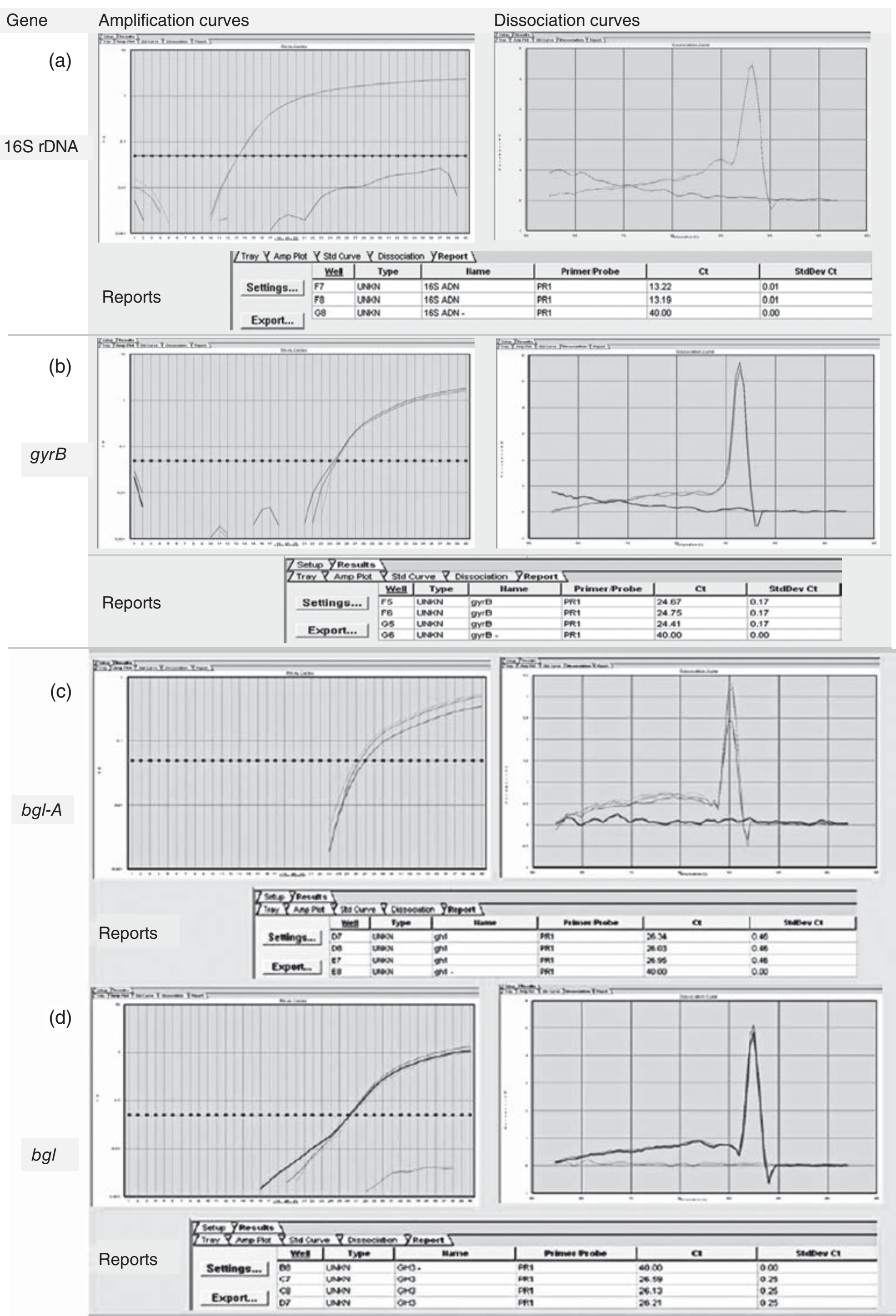

Fig. 36.4. The qPCR results: reports, amplification and dissociation curves generated with GeneAmp® 5700 SDS software (Applied Biosystems): (a) 16S rDNA, (b) *gyrB*, (c) *bgl-A*, (d) *bgl* and (e) *CspA*.

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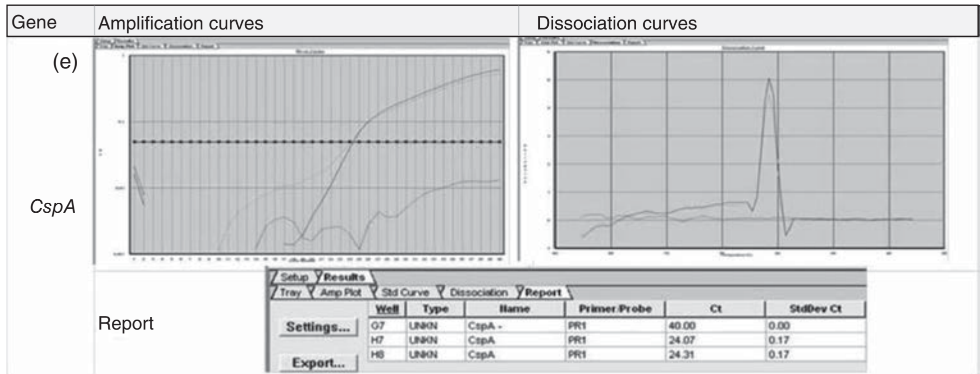

Fig. 36.4e. Continued.

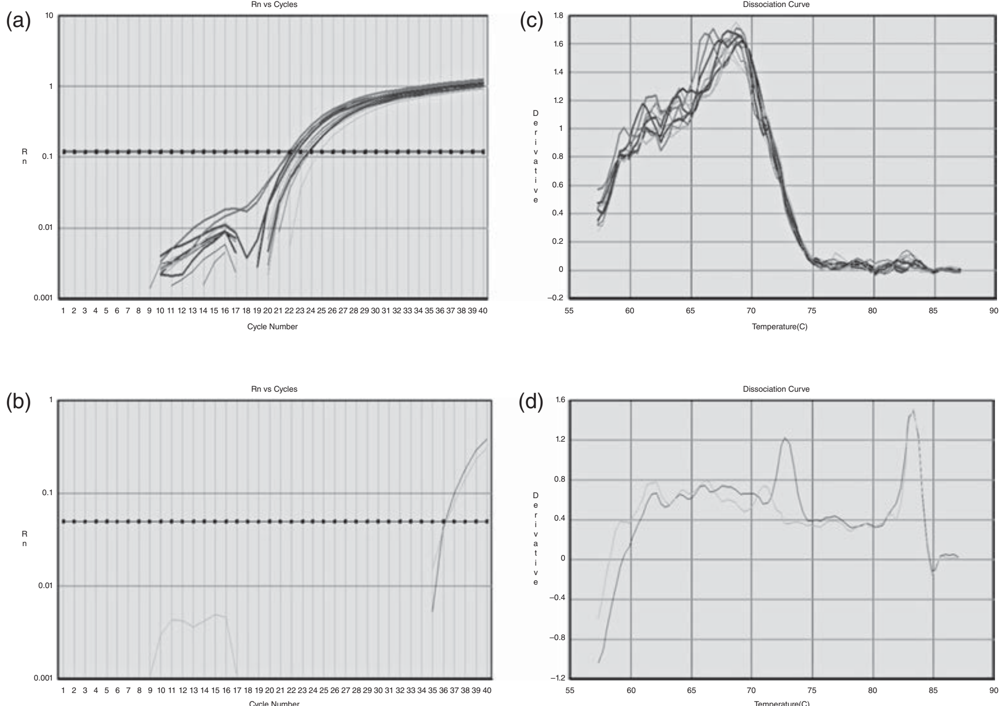

Fig. 36.5. First analysis of primers by qPCR generated with GeneAmp® 5700 SDS software (Applied Biosystems): amplification curves (a) *bgl-A*, (b) *bgl*; and dissociation curves (c) *bgl-A*, (d) *bgl*.

Ellefsen *et al.* (2008) have explained that normalization is an essential process to compensate for the error of the experimental procedure in qPCR. They studied several housekeeping genes (e.g. 16S rDNA, *gyrB*, *recA* and *rho*: Table 36.1), for which they determined the variation in the expression using the comparative C_t method, and they selected genes with similar C_t. In the current study, two housekeeping genes (*gyrB* and 16S rDNA) were studied, but *gyrB* showed a better performance regarding the level of genetic expression.

As indicated the presence of different β-glucosidase isoenzymes, induced by cellobiose in *Shewanella* sp. G5 and encoded by two genes, *bgl-A* and *bgl*, was determined in previous studies

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Table 36.4. Determination of the r^2 and E of all genes assayed.

Genes	Standard curves		
	r^{2} [a]	Slope	E (%) [b]
16S rDNA	0.999	–2,99	116.00
gyrB	0.997	–3,33	99.66
bgl-A	0.997	–3,29	99.66
bgl	0.996	–3,31	100.51
CspA	0.993	–3,34	99.25

[a]Correlation coefficient.
[b]Amplification efficiency.

(Cristóbal *et al*., 2008). Proteome re-arrangement can be expected after growth on different carbon sources (Cristóbal *et al*., 2009). Therefore, *Shewanella* sp. G5 provides an optimal system for studies of genetic variation in *bgl* genes that encode for different β-glucosidase isoenzymes. These studies show optimal conditions for enzyme production in *Shewanella* sp. G5, which will lead to further studies for large-scale production.

Tasara and Stephan (2006) reported the qRT-PCR studies for gene expression changes for 16 *Listeria monocytogenes* strains underlying stress adaptation. Validation of five housekeeping genes were studied as potential reference genes required for normalization. In this case, the 16S rRNA gene was consistently the most stably expressed in the different *L. monocytogenes* strains under all the experimental conditions. While the expressions of β-glucosidase (*bgl-A*), glyceraldehyde-3P-dehydrogenase (*gap*), RNA polymerase beta subunit (*rpoB*) and ribosomal protein L4 (*rplD*) were stable among the different *L. monocytogenes* strains, they were prone to significant variations under the different stress adaptation models.

Relative quantification analyses applying the $2^{-\Delta\Delta C_t}$ method

Genes are considered to be up- or downregulated if their levels of relative expression are at least twofold higher or lower, respectively, compared with the control sample or the calibrator. In the current work, variations in the levels of genetic expression of the genes assayed were observed, using the Excel program to load C_t data, the results were expressed as relative to genetic expression indices corresponding to the studied genes (Table 36.5). Therefore, standard analyses of the genes under study were performed with the constitutive *gyrB* gene as calibrator. In the analyses carried out, a gauge was provided that allows complete standardization of the genes under study.

This is an important condition to establish the relationship of genetic expression relative to the calibrator. For each condition, C_t values were obtained by qPCR and used for comparison of gene expression through the $2^{-\Delta\Delta C_t}$ method, allowing relative quantification. We observed that the culture conditions had significant effects on the expression of the genes under study.

The highest $2^{-\Delta\Delta C_t}$ values of relative quantification of *bgl-A* and *bgl* were 14, 15, 28 and 10, 12, 30, respectively, obtained under MMBC30 conditions (Fig. 36.6). Relative quantification is expressed as $2^{-\Delta\Delta C_t}$ and shown as a summary of the most salient results of the genetic expression in Fig. 36.6(a), which should be interpreted as follows: it can be observed that under MMBC30 conditions, expression of the *bgl-A* gene in $2^{-\Delta\Delta C_t}$ values were 6, 15, 6, 14 and 28 times higher than under LBC10, LBG10, MMBC10, LBC30 and LBG30 conditions, respectively. On the other hand, expression of *bgl* in $2^{-\Delta\Delta C_t}$ values were 4, 12, 10 and 30 times higher than under LBC10, LBG10, LBC30 and LBG30 conditions, respectively. These results suggest high genetic expression for *bgl-A* and *bgl* genes when *Shewanella* sp. G5 is grown under MMBC30 conditions. These were found to be the optimum conditions for expression of both genes compared with the remaining assay conditions, and highest relative expression was determined. The expression of individual genes assayed under each condition was calculated according to the comparative quantification method which provides a measure of reference (Cristóbal *et al*., 2015).

It is remarkable that under other conditions the *bgl-A* and *bgl* genes also showed a high level of genetic expression. Highest $2^{-\Delta\Delta C_t}$ values for *bgl-A* and *bgl* were obtained under MMBG30 conditions, which were 11 and 19 times more than under LBG30. Regarding MMBG10, $2^{-\Delta\Delta C_t}$ values were 9 and 19 times higher for *bgl-A* and *bgl*, respectively, than under LBG30, and for MMBC10 this was 5 and 16 times more than under LBG30 conditions (Fig. 36.6). For each of the genes *bgl-A*, *bgl* and *CspA* analysed variations in the levels of genetic expression are shown in full in Fig. 36.6(b), (c) and (d), respectively.

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Table 36.5. Matrix of gene expression related indices.

		Normalization study genes with housekeeping *gyrB* gene							
Samples[a]	Genes	LBC10	LBG10	MMBC10	MMBG10	LBC30	LBG30	MMBC30	MMBG30
LBC10	*bgl-A*	1	2.56	1.05	0.54	2.37	4.77	0.17	0.45
	bgl	1	2.95	0.48	0.38	2.49	7.49	0.25	0.39
	CspA	1	0.48	1.16	1.21	0.5	1	0.96	0.7
LBG10	*bgl-A*	0.39	1	0.41	0.21	0.93	1.87	0.07	0.18
	bgl	0.34	1	0.16	0.13	0.84	2.54	0.08	0.13
	CspA	2.08	1	2.42	2.53	1.04	2.08	2.01	1.46
MMBC10	*bgl-A*	0.95	2.44	1	0.51	2.26	4.55	0.16	0.43
	bgl	2.1	6.19	1	0.8	5.22	15.73	0.52	0.82
	CspA	0.86	0.41	1	1.05	0.43	0.86	0.83	0.6
MMBG10	*bgl-A*	1.87	4.77	1.96	1	4.42	8.91	0.32	0.84
	bgl	2.61	7.7	1.24	1	6.5	19.53	0.65	1.02
	CspA	0.82	0.4	0.96	1	0.41	0.13	0.79	0.58
LBC30	*bgl-A*	0.42	1.08	0.44	0.23	1	2.01	0.07	0.19
	bgl	0.4	1.19	0.19	0.15	1	3.01	0.1	0.16
	CspA	2.01	0.96	2.33	2.44	1	0.31	1.93	1.41
LBG30	*bgl-A*	0.21	0.54	0.22	0.11	0.5	1	0.04	0.09
	bgl	0.13	0.39	0.06	0.05	0.33	1	0.03	0.05
	CspA	1	0.48	1.16	1.22	0.5	1	0.97	0.7
MMBC30	*bgl-A*	5.8	14.83	6.08	3.11	13.74	27.67	1	2.62
	bgl	4.03	11.88	1.92	1.54	10.02	30.17	1	1.57
	CspA	1.04	0.5	1.21	1.26	0.52	1.04	1	0.73
MMBG30	*bgl-A*	2.21	5.66	2.32	1.19	5.24	10.56	0.38	1
	bgl	2.56	7.54	1.22	0.98	6.36	19.16	0.64	1
	CspA	1.42	0.68	1.65	1.73	0.71	1.42	1.37	1

[a]LBC10, Luria-Bertani (LB) medium + cellobiose incubated at 10°C; LBG10, LB medium + glucose incubated at 10°C; MMBC10, Mineral Medium Brunner (MMB) medium + cellobiose incubated at 10°C; MMBG10, MMB medium + glucose incubated at 10°C; LBC30, LB medium + cellobiose incubated at 30°C; LBG30, LB medium + glucose incubated at 30°C; MMBC30, MMB medium + cellobiose incubated at 30°C; MMBG30, MMB medium + glucose incubate at 30°C.

Relative quantification tests showed that the factors used under the different growth conditions had significant effect on the variation in expression of both genes. The best conditions were found to be for MMBC30, when the levels of genetic expression were highest for *bgl-A* and *bgl* genes compared with the other culture conditions assayed. These results show that relative quantification of genes using qPCR allows establishing optimal growth conditions. In the case of *Shewanella* sp. G5, the incubation temperature and carbon source affected the expression of *bgl* genes that encode β-glucosidases. The qPCR is an easy and fast technique to assay specific activity; β-glucosidase activity showed an increase when *Shewanella* sp. G5 was grown in mineral medium with cellobiose at 30°C.

Therefore, MMBC30 showed optimal growth conditions with excellent possibilities for enzyme production in bioreactors in future experiments. It is also remarkable that under MMBC10 conditions, *bgl-A* and *bgl* showed variations in the level of genetic expression with $2^{-\Delta\Delta C_t}$ values that were two, two and four (*bgl-A*) and six, five and 15 times (*bgl*) more compared with LBG10, LBC30, LBG30 conditions. It is noteworthy that under MMBG10 conditions the *bgl-A* and *bgl* genes showed several variations in the level of gene expression; the best $2^{-\Delta\Delta C_t}$ values observed were of nine, five and four (*bgl-A*) and 19, eight and six times (*bgl*) higher than LBG30, LBG10, LBC30 conditions, respectively (Fig. 36.6).

Under MMBG30 conditions, the *bgl-A* and *bgl* genes showed variations in the level of genetic expression with $2^{-\Delta\Delta C_t}$ values of six, five and 11 (*bgl-A*) and seven, six and 19 times (*bgl*) more than LBG10, LBG30, LBC30 conditions, respectively.

It is important to mention that under MMBG10 and MMBG30 conditions, the level of

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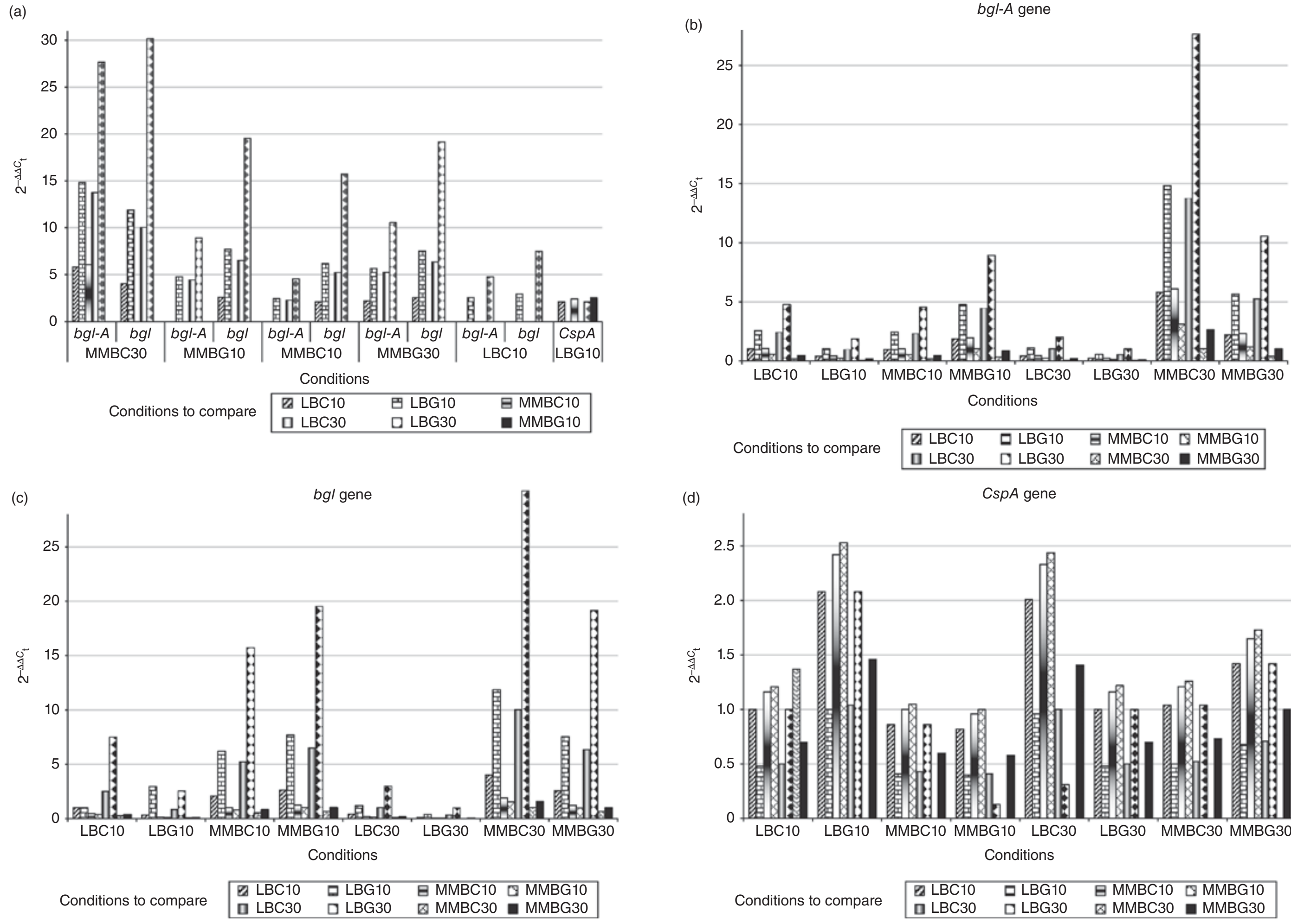

Fig. 36.6. Relative quantification of the level of genetic expression of the *bgl-A*, *bgl* and *CspA* genes obtained with the comparative $2^{-\Delta\Delta C_t}$ method. (a) Summary of the most salient results of the genetic expression. Full analysis of levels of genetic expression for (b) *bgl-A* gene, (c) *bgl* gene and (d) *CspA* gene.

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genetic expression of both genes was relatively high. Normally, cellobiose is the β-glucosidase enzyme inducer, but although glucose was used as the carbon source in the medium assayed, β-glucosidase was expressed. In previous studies with cellular fractions of *Shewanella* sp. G5, enzyme activity was quantified from intracellular protein obtained with cellobiose or glucose as the carbon source (Cristóbal *et al*., 2009).

Constitutive activity by the cell was observed in the presence of glucose, since β-glucosidase acts on two glucose units during hydrolysis of cellobiose. The sequential induction of isoenzymes has been associated with the presence of distinct metabolites. An accepted model, the induction of the cellulases is mediated either by low-molecular-weight soluble oligosaccharides that are released from complex substrates as a result of hydrolysis by constitutive enzymes (beta-glucosidase, xylanases, etc.). These metabolites enter the cell and signal the presence of extracellular substrates and provide the stimulus for the accelerated synthesis of constituent enzymes of the cellulase complex. This process is complex in view of the fact that many fungi and bacteria are known to functionally express multiple cellulases or hemicellulases in the presence of different carbon sources and conditions. However, the regulation of expression of these multiple isoenzymes is still not clear which necessitates further research regarding the sequential and differential expression of the enzymes (Singhania *et al*., 2013).

Similar results have been found by Tsukada *et al*. (2006), who reported on the expression of two genes, *bgl1A* and *bgl1B*, which encode for two family 1 β-glucosidases. Both enzymes were produced by *Phanerochaete chrysosporium* and their expression was monitored by RT-PCR with cellobiose and glucose in the culture medium; *bgl1A* was expressed constitutively in both media, whereas *bgl1B* was only expressed in cellobiose medium but it was repressed in the presence of glucose. Differential expression of various enzymes, such as endoglucanase and β-glucosidase, has been reported in response to the carbon sources supplied in the medium or the conditions of culture in *Aspergillus terreus* (Nazir *et al*., 2010). Karnchanatat *et al*. (2007) reported that β-glucosidase produced by *Daldinia eschscholzii* belongs to family 3 of glucosyl hydrolase, and glucose competitively inhibited this enzyme. Yoshida *et al*. (2004) have reported similar effects in relation to cellobiose metabolism in the basidiomycete *P. chrysosporium*, using transcriptional analysis of the β-glucosidase (*bgl*) and cellobiose dehydrogenase (*cdh*) genes performed by qPCR. The results showed that addition of glucose to the cellulose-degrading culture significantly decreased the amount of both transcripts. In contrast, addition of cellobiose only repressed transcription of the *bgl* gene but did not affect that of the *cdh* gene. Induction assays of the two genes showed that in cellobiose medium the level of *bgl* transcripts was considerably lower than in glucose medium, whereas that of *cdh* transcripts was 2.3-fold higher than that in glucose medium.

Other effects in the variation of the level of gene expression due to the carbon source were observed by Ohnishi *et al*. (2007) These authors found that in *Polyporus arcularius*, the *cel1* and *cel2* genes, encoding cellobiohydrolase, were induced by microcrystalline cellulose and cellopentaose but repressed by glucose, cellobiose, cellotriose and cellotetraose. These results suggest that *P. arcularius* cells constitutively express a very low level of cellulase that is able to degrade insoluble crystalline cellulose and that transcription of *cel1* and *cel2* in the cells is induced by products produced by cellooligosaccharides.

On the other hand, gene expression in the function of growth was performed by analysis of the expression every 30 min, starting at a cell density of $\pm 7\times10^6$ c.f.u./ml, using the standard qRT-PCR procedure previously described by Werbrouck *et al*. (2006). The cold shock protein (CspA) is a protein of approximately 70 kDa, which is expressed in *E. coli* when the temperature drops from 37°C to 15°C, and it has the hypothetical role of RNA chaperone (Jones *et al*., 1996). Our results show that the LBG10 conditions had a better effect on *CspA* gene expression and that the other conditions assayed did not significantly affect gene expression (Fig. 36.6d).

36.4 Conclusion

At present, the molecular biology-based research leads us in a wide range of possibilities for a better understanding of biological phenomena around us. The numerous applications of this new

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technology directs us to such diverse fields as medicine and its impact on human health, the environment and all efforts for its conservation and sustainability, finding biotechnological improvements, among other important applications. In this context the qPCR experiments contributes to the development of large areas of basic and applied research. The results are very promising because they provide reliable and accurate data in a short space of time on a wide range of organisms studied in which decisions can be made for improvements, such as health and industry. However, it requires a greater commitment from the public and private sector, in which the research and development of this technology is accessible and applied to studies of global concern such as water pollution, food and the search for tumour markers, and human pathogens (viruses, bacteria and parasites). Therefore, assays using qPCR address a wide range of molecular diagnostic tests, which makes this type of technology a first rate practical and preventive measure, impacting sectors such as health, social, economic, etc. of any country in the world. Knowledge of all the results should be employed to discover the quality of human welfare worldwide.

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37 Whole Genome Sequence Typing Strategies for Enterohaemorrhagic *Escherichia coli* of the O157:H7 Serotype

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Abstract

Escherichia coli of the O157:H7 serotype is the dominant Shiga-toxin-producing enterohaemorrhagic *E. coli* in North America that causes widespread and potentially lethal outbreaks of food-borne disease. Unlike other *E. coli*, the O157:H7 lineage features a genetically homogenous population structure, which hinders phylogenetic marker development. Historically classified using selective media, O157:H7 isolates were further differentiated using molecular and phenotypic typing strategies, such as pulsed-field gel electrophoresis or metabolic profiling. The introduction of cost-efficient and rapid next generation sequencing technologies allowed the field to transition from assessing the plasticity in only few selected loci to whole genome sequence typing approaches. The resulting enriched polymorphic marker base already provided increased phylogenetic accuracy and resolution. In this chapter, the whole array of established and latest typing assays that have been specifically developed for this lineage are discussed. The synergistic use of a wealth of sequence information combined with epidemiological and phenotypic metadata will open the avenue for genome-wide association studies that will not only allow to link bacterial genotype to disease severity, but is also crucial for biosurveillance, risk assessment and informed countermeasures in the event of an outbreak.

37.1 Introduction

In the USA each year food-borne diseases cause approximately 76 million illnesses, 325,000 hospitalizations and 5200 deaths with a total cost of US$10–83 billion/year (Bavaro, 2012). The O157:H7 serotype is the most common Shiga-toxin-producing *Escherichia coli* (STEC) serotype in North America, and has caused numerous food-poisoning outbreaks worldwide (Kaper *et al.*, 2004; Croxen *et al.*, 2013). It is estimated that in the USA there are an average of 17 outbreaks and 75,000 cases associated with STEC O157:H7 each year (Bavaro, 2012). In the developed world cattle are considered as a main reservoir of STEC O157:H7 (Chase-Topping *et al.*, 2008). Interestingly, *E. coli* O55:H7, the ancestor of STEC O157, or O157:H(-), the first strain to diverge within the STEC O157, are not found in cattle. This raises the question how cattle may have initially acquired STEC O157 serotypes (Bono *et al.*, 2012).

The pathogenicity of *E. coli* O157:H7 was initially recognized in 1982 when it was isolated in 47 patients in two US states, who developed bloody diarrhoea after consuming contaminated hamburgers (Riley *et al.*, 1983). Since then, *E. coli* O157:H7 has emerged as a major enteric

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pathogen, capable of causing localized as well as large outbreaks of gastrointestinal disease (Mead and Griffin, 1998). Data accumulated from 1982 to 2006 in the UK, Canada, the USA, Japan, Ireland and Scandinavia showed that 54% of the cases of *E. coli* infections from 90 recognized *E. coli* O157 outbreaks were associated with the ingestion of contaminated food products, whereas 20% of the reported cases were from secondary spread (person-to-person transmission) and 6% were from drinking water (Snedeker *et al*., 2009). A retrospective study of *E. coli* O157:H7 among cattle showed a wide range in prevalence with a general trend of higher occurrence in more recent studies, probably due to more sensitive detection methodologies (Hussein and Bollinger, 2005). Despite the occasional high prevalence only 6% of the human infections were caused by direct animal contact (Hale *et al*., 2012). This supports the potential hypothesis that a subset of *E. coli* O157:H7, found mainly in two lineages harboured by cattle, may be the source for the majority of human infection (Clawson *et al*., 2009; Bono *et al*., 2012). However, two other major pathogenic *E. coli* serotypes, O55:H7 and STEC O157:H(-), are not found in cattle (Bono *et al*., 2012).

The *E. coli* O157:H7 lineage has been the focus of numerous forensic, epidemiologic, genomic, microbiologic and diagnostic studies to reduce the global health impact of this major pathogen. According to the Centers for Disease Control, from 2000 to 2008 about 63,000 cases of STEC O157 were recorded in the USA each year with a mean of 2100 hospitalizations and 20 deaths (Scallan *et al*., 2011). The interval between exposure to contaminated produce and the start of diarrhoeal symptoms ranges between 2 days and 12 days (Tarr *et al*., 2005). The infection usually starts with 1–3 days of watery diarrhoea progressing into bloody diarrhoea (Griffin *et al*., 1988). Although bloody diarrhoea occurs in 90% of cases, some patients can progress to haemolytic uraemic syndrome (HUS) without prior symptoms of diarrhoea (Brandt *et al*., 1994; Miceli *et al*., 1999). Compared with other bacterial infections the patients are mainly afebrile (Wong *et al*., 2000), but with increased abdominal pain (Tarr *et al*., 2005). HUS patients display renal dysfunction known as haemorrhagic colitis (HC) and can have complications, such as cardiac dysfunction, anaemia or central nervous system failure with potentially lethal outcomes (Cimolai and Trombley, 1992; Brandt *et al*., 1994; Besser *et al*., 1999; Riley *et al*., 2003).

The majority of virulence factors of *E. coli* O157:H7 are present on mobile genetic elements, such as prophages and pathogenicity islands. One of the most important virulence markers are Shiga-toxin-producing prophages (*stx* phages), which have been linked to disease severity (Nishikawa *et al*., 2000; Tarr *et al*., 2005). They belong to the lambdoid phages and are mainly found in two forms, *stx1* and *stx2.* The organization of the *stx* prophages is quite similar, with the *stx* genes downstream of the anti-terminator Q that controls late gene expression. There is a considerable mosaicism between lambdoid phages due to the presence of a Red recombinase that contributes to their ecological fitness (Allison, 2007). The two Shiga toxins have only 60% identity and among *stx2* 13 variants have been described so far (Bertin *et al*., 2001; Leung *et al*., 2003). A new nomenclature for *stx2* has been proposed to avoid confusion with *stx2c* and *stx2d*: the prototypical EDL933 *stx2* is now called *stx2a* (Fuller *et al*., 2011). Fuller and colleagues have observed a striking difference in *in vitro* and *in vivo* potency of the different subtypes (Fuller *et al*., 2011). The Shiga toxin subunit B binds to the globotriaosyl ceramide receptor (Gb3) in the human gastrointestinal tract and subunit A enters the cell and prevents peptide elongation after cleavage. Another important virulence factor is the locus of enterocyte effacement (LEE) that mediates attaching-and-effacing (A/E) lesions by destruction of microvilli of epithelial cells through the intimate adherence of the bacteria to the host cells (Donnenberg *et al*., 1997). This locus is found not only in several *E. coli* pathovars but also in pathogenic *Enterobacteriaceae* (Muller *et al*., 2009; Schmidt, 2010).

This review will highlight the strategies in the pathogenome characterization of the *E. coli* O157:H7 lineage describing both historic and modern approaches for molecular typing. Additional genome sequencing of *E. coli* O157:H7 that have been become available with the introduction of next generation sequencing (NGS) technologies provided detailed insights into the evolution of this important pathogen. Comparative analysis of strains has increased enormously with the sequencing of bacterial genomes and allows for identifying regions within the genome

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that may be associated with differences in virulence as described above. In 1997 a derivative of *E. coli* K12 (MG1655) was sequenced (Blattner *et al.*, 1997) and a few years later concomitantly two O157:H7 isolates, EDL933 (Perna *et al.*, 2001) and Sakai (Hayashi *et al.*, 2001) were published. These genomes provided the first comparisons of pathogenic versus avirulent genomes of *E. coli*. Additional genome sequencing of *E. coli* O157:H7 rendered significant insight into the evolution of these important pathogens. Nowadays, with the advent of NGS technologies there has been a move from sequencing 'prototype' strains to sequencing 'collections' or 'outbreaks' to determine the molecular markers of these pathogens. This chapter will highlight the changes in molecular characterization of *E. coli* and O157:H7 serotype.

37.2 Typing Methodologies and Resolution Power

Initially, typing was provided by phenotypic traits, such as response to given antibodies (serology) (Kaufmann, 1943). Other phenotypic characteristics that have been used for classification are sorbitol fermentation (March and Ratnam, 1986), latex agglutination technique for a more detailed serotyping (March and Ratnam, 1989), and more recently broad-range carbon and nitrogen source panels (Bochner *et al.*, 2001). Genetic heterogeneity among STEC O157:H7 strains has been established using a broad panel of targeted-typing technologies, such as:

- multi-locus enzyme electrophoresis (MLEE) (Selander and Levin, 1980; Feng *et al.*, 1998);
- phage typing (Ahmed *et al.*, 1987; Ratnam *et al.*, 1988);
- pulsed-field gel electrophoresis (PFGE) (Bohm and Karch, 1992; Krause *et al.*, 1996; Gerner-Smidt *et al.*, 2005);
- octamer and PCR-based genome scanning (Kim *et al.*, 1999; Ohnishi and Hayashi, 2002);
- multi-locus sequence typing (MLST) (Reid *et al.*, 2000; Wirth *et al.*, 2006);
- multiple-locus variable-number tandem repeat analysis (MLVA) (Noller *et al.*, 2003a; Keys *et al.*, 2005);
- Shiga-toxin-producing bacteriophage insertion site typing (SBI) (Shaikh and Tarr, 2003; Bono *et al.*, 2012);
- microarrays (Jackson *et al.*, 2007);
- microarray-based comparative genome hybridization (CGH) (Zhang *et al.*, 2007);
- subtractive hybridization (Steele *et al.*, 2007, 2009); and
- optical mapping (Kotewicz *et al.*, 2007, 2008; Eppinger *et al.*, 2011b).

The role of these methodologies in determining the evolution of *E. coli* O157:H7 and discrimination between clonal subpopulations will be briefly described, emphasizing their power and limitations. Many of these techniques were developed simultaneously and often in combination, rather than one single technique being used to discriminate *E. coli* O157:H7 isolates from each other. An overview on the timeline detailing typing techniques can be found in Fig. 37.1.

37.2.1 Multi-locus enzyme electrophoresis (MLEE) and sequencing typing

Bacterial population genetic techniques were used for the first time to study *E. coli* population dynamics. MLEE revealed that given allele combinations occurred multiple times, suggesting a clonal population with little recombination (Selander and Levin, 1980). A more detailed analysis of strains with similar MLEE profiles by PCR of four 1 kb loci has proven that recombination is more frequent in *E. coli* than previously thought (Guttman and Dykhuizen, 1994). Thanks to MLEE, enterohaemorrhagic *E. coli* (EHEC) isolates could be separated into multiple clades despite being highly clonal, suggesting that there are multiple evolutionary paths to generate a fully virulent EHEC isolate (Whittam *et al.*, 1988). A first evolutionary model was proposed suggesting a stepwise evolution of *E. coli* O55:H7 to O157:H7. Allelic profiles of 20 enzymatic loci resulted in 15 electrophoretic profiles that were further used to establish relatedness between *E. coli* O157 and O55 serotype (Feng *et al.*, 1998).

One of the most powerful subtyping tools in molecular epidemiology is MLST. Together with MLEE it can be easily standardized and automated.

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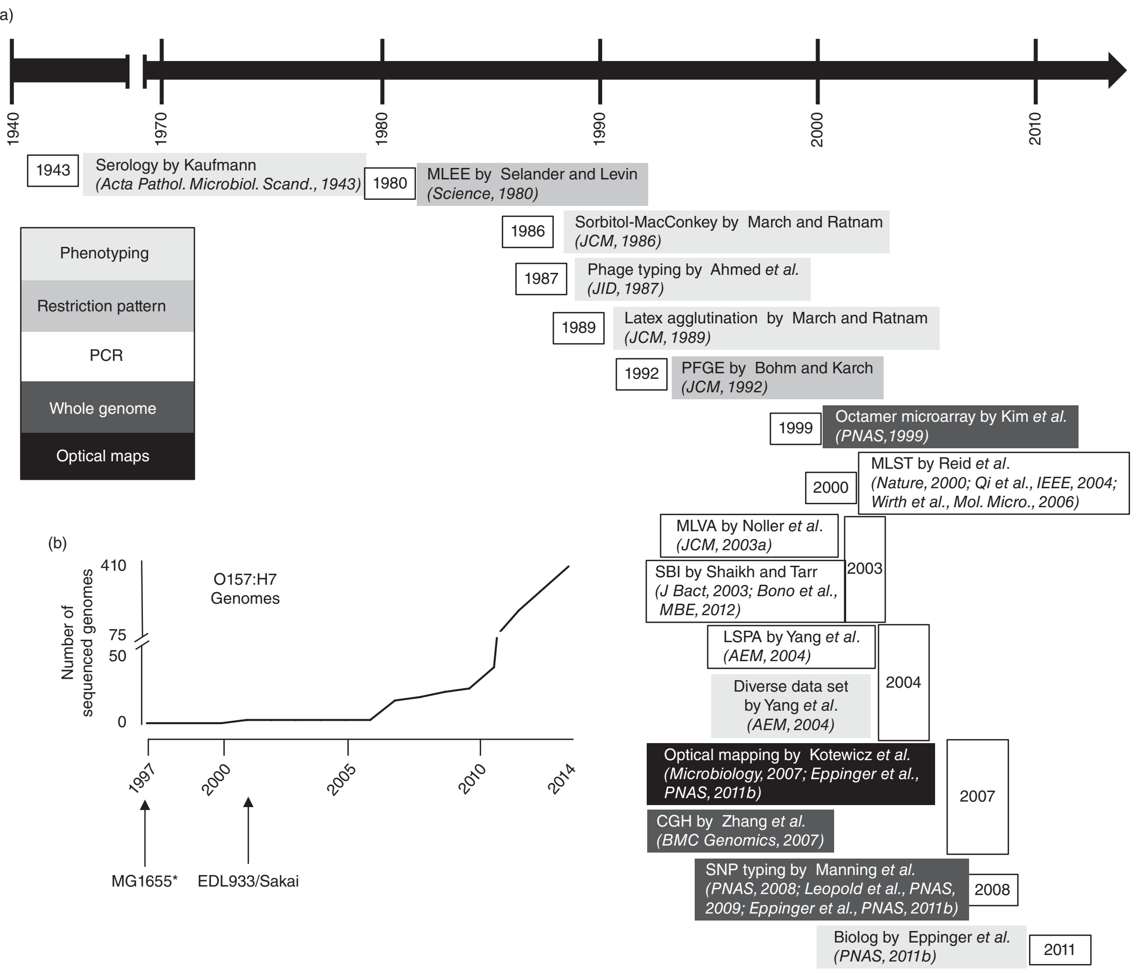

Fig. 37.1. Historical overview of typing methods for *Escherichia coli* O157:H7. (a) Typing methods are classified according to the main components of each method. Optical mapping can be considered as an optimization of restriction pattern analysis on the whole genome scale. MLEE, Multi-locus enzyme electrophoresis; PFGE, pulsed-field gel electrophoresis; MLST, multi-locus sequence typing; MLVA, multiple-locus variable-number tandem repeat analysis; SBI, Shiga-toxin-producing bacteriophage insertion site typing; LSPA, lineage-specific polymorphism analysis; CGH, comparative genome hybridization; SNP, single nucleotide polymorphism; PCR, polymerase chain reaction. (b) An exponential growth observed in publicly available *E. coli* O157:H7 genomes is driven by the introduction of next generation sequencing (NGS) technologies. *The K12 derivative MG1655 was the first completed *E. coli* genome.

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MLST was first developed for *Neisseria meningitidis* in 1998, as older molecular typing schemes had poor reproducibility (Maiden, 1998). The principle behind the MLST scheme is based on the sequencing of 400–500 bp stretches in multiple housekeeping genes (HKG). Each unique sequence (allele) is assigned a random integer number. The combination of alleles at each locus, an 'allelic profile,' defines the sequence type (ST). MLST was used to describe the population structure of *E. coli* O157 (Reid *et al.*, 2000). Seven genes were selected to determine the phylogenetic relationship. The MLST has been further expanded to include 15 genes and the MLST profiles can be found in *Ec*MLST an online database of MLST profiles (Qi *et al.*, 2004). Today three MLST schemes are available in publically curated databases: (i) the *Ec*MLST (Manning *et al.*, 2006: http://www.shigatox.net); (ii) the Pasteur (Brisse *et al.*, 2011: http://www.pasteur.fr/mlst); and (iii) the Achtman scheme (Wirth *et al.*, 2006). The Pasteur and Achtman are both available on PubMLST (Jolley, 2004: http://pubmlst.org). Recently, a public server that uses whole-genome sequencing to identify sequence types was set up (Larsen *et al.*, 2012: http://cge.cbs.dtu.dk/services/MLST). While these MLST methods are generally congruent, there can be different results observed depending on the gene set analysed. However, both MLEE and MLST cover only a fraction of the entire genome (few genes) and therefore do not have the resolution power to differentiate between closely related isolates (Noller *et al.*, 2003b).

37.2.2 Phage susceptibility assay

Two phage-based assays have been developed to distinguish *E. coli* O157:H7 strains, as assessed through susceptibility to phage lysis and alternative chromosomal phage insertion loci (Ahmed *et al.*, 1987; Shaikh and Tarr, 2003). The strains are grouped according to their phage susceptibility totalling 66 types (Smith and Scotland, 1993), however, screens including more strains showed a limited resolution, with the majority of human isolates falling into the same typing category, despite bearing different PFGE profiles (Krause *et al.*, 1996).

37.2.3 Pulsed-field gel electrophoresis (PFGE)

Among the molecular typing methods for epidemiological investigations of STEC O157:H7, PFGE has been considered as the 'gold standard' for the past 25 years. Changes in the *Xba*I restriction patterns of O157:H7 can be traced back to spontaneous genomic rearrangements or recombination of mobile elements providing information about the genomic backbone (Akiba *et al.*, 1999; Murase *et al.*, 1999). Different types (types I–V and ND) of O157:H7 isolates can be observed according to differences in the *Xba*I PFGE patterns (Izumiya *et al.*, 1997). For short-term epidemiological studies, PFGE patterns can be useful as an inclusion/exclusion criterion when examining *E. coli* O157:H7 isolates over a defined time window. However, PFGE technology does not allow us to decipher the underlying genetic composition of isolates (Frank *et al.*, 2011; Mellmann *et al.*, 2011; Rasko *et al.*, 2011; Rohde *et al.*, 2011; Brzuszkiewicz *et al.*, 2011; Grad *et al.*, 2012).

37.2.4 Metabolic typing

Metabolic typing is an important complement to genome-based typing. It used to be a cumbersome and expensive technique, where often only one characteristic (e.g. sugar metabolism) was tested. With so-called 'phenotypic microarrays' (PM) over 400 assays for N-source metabolism, 200 assays for C-source metabolism, 100 for P- and S-source metabolism can be tested at once (Bochner, 2009). This panel assays over 1200 metabolic and chemical sensitivity phenotypes (Omnilog) in less than 48 h (Bochner *et al.*, 2001; Bochner, 2003). The 96-well plates contain medium with a reducible tetrazolium dye and different C-, N-, S-, P-sources, and enables changes in ionic, osmotic or pH environment. The antibiotics and chemical inhibitors are tested at four different concentrations. Upon cell growth or respiration the tetrazolium dye will be reduced to a blue compound and the intensity is quantified (Bochner *et al.*, 2001; Bochner, 2003). The Omnilog microarray system was used to test a large collection of O157:H7 strains from food-borne outbreaks, finding a sucrose-positive, D-serine-negative phenotype common to most O157 strains

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(Mukherjee *et al*., 2006). In O157, a sucrose operon had inserted into the D-serine operon and the genome region proved to be a hot spot for genetic mosaicism (Jahreis *et al*., 2002). The PM analysis can also be used to highlight outbreak-specific phenotypic changes, such as the O157 isolates from a spinach outbreak in 2006 in the USA, which display a rare *N*-acetyl-D-galactosamine-negative (Aga) phenotype (Mukherjee *et al*., 2008). All other O157 strains were able to use Aga and the difference was traced back to a single nucleotide change in the *agaF* gene of the outbreak strains. Phenotypic analyses were used to determine factors relevant for adaptation to the bovine environment. Differences in C-source utilization between commensal and pathogenic O157:H7 isolates were observed, but could not be linked to specific traits that would offer advantages in the bovine environment (Durso *et al*., 2004). Finally, phenotypic analyses in combination with genotypic approaches can reveal and validate metabolic phenotypes (Eppinger *et al*., 2011b).

37.2.5 Octamer-based genome scanning (OBGS) typing assay

The fast accumulation of whole genome sequence data for O157:H7 has allowed for detection of differences among strains that were previously thought to be clonal. High-resolution subtyping methods that enable inter- and intra-species bacterial genome comparisons were derived from sequencing data. High-density octamer-based genome scanning (OBGS) analysis is based on PCR amplification of genomic segments that lie between over-represented, strand-biased octamers in the genome. Octamers are biased towards the leading strand and are fluorescently labelled and combined with unlabelled octamers that are biased towards the lagging strand. Fragment analysis allows for detecting size distribution down to the nucleotide level. OBGS first demonstrated that the *E. coli* O157:H7 clonal complex has diverged into two highly related lineages (lineages I and II) that were mainly found among human and bovine isolates, respectively (Kim *et al*., 1999). Restriction fragment length polymorphism (RFLP) of prophages detected five subtypes that are distributed accordingly to the lineages, suggesting that phage-mediated recombination events were the cause or consequence of the divergence of the two lineages (Kim *et al*., 1999).

37.2.6 Multiple-locus variable-number tandem repeat analysis (MLVA)

The poor discriminatory ability of MLST for *E. coli* O157:H7 prompted the development of an assay based on target short tandem repeats (TRs), which evolve rapidly within the genome. This technique was initially used to differentiate between eubacterial species and strains (Versalovic *et al*., 1991). Versalovic *et al*. (1991) demonstrated that PCR targeting the repeat regions could be used to discriminate between different Gram-negative enterobacteria and even among strains of *E. coli*. MLVA combines the analysis of the number of repeats at multiple loci, and was initially developed to distinguish the highly genetically homogeneous *Bacillus anthracis* (Keim *et al*., 2000). Noller *et al.* developed an MLVA for *E. coli* with seven loci and as seen for *B. anthracis*, observed a higher resolution than PFGE (Noller *et al*., 2003a).

37.2.7 Shiga-toxin-producing bacteriophage insertion site typing (SBI)

STEC O157:H7 strains harbour Shiga-toxin-encoding bacteriophages (*stx*) that are found integrated in the genome at different loci. Shaikh and Tarr found three different combinations of *stx* phages in O157:H7 isolated from patients. The *stx1* phage was always found inserted in the *yehV* gene, but in the majority of the strains it was truncated in the central region containing the *stx1A* and *stx1B* genes (Shaikh and Tarr, 2003). For the *stx2* bacteriophage the previously known *wrbA* insertion site was intact in the majority of the investigated strains. This led to three combinations: with a truncated stx_1 either with or without *stx2* in *wrbA* or a full-length *stx1* with *stx2* integrated in *wrbA* (Shaikh and Tarr, 2003). These initial investigations demonstrate the variability in *stx* prevalence and integration site. The location and composition of *stx* prophages appear to be linked to disease severity. However,

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more refined analyses with larger numbers of samples, analysing differences within *stx* prophages are still needed to elucidate the role of insertion sequence (IS) elements and rearrangements within the phage sequence on Shiga toxin expression and production.

In a CGH study by Wick *et al.* more than 85% of variability between O157:H7 was due to prophage or prophage-related elements (Wick *et al.*, 2005). Using the insertion sites pattern of *stx* prophages, a PCR was designed by Besser *et al.* for typing purposes. The *stx* bacteriophage insertion (SBI) typing expanded the insertion site patterns from the original three to 16, with some patterns specific to isolates from cattle or human (Besser *et al.*, 2007). This study further confirmed that given bovine genotypes are underrepresented in clinical isolates, suggesting differences in transmissibility and virulence (Besser *et al.*, 2007). SBI was used for samples from supershedders and no specific genotype could be correlated to this phenotype. Only half of the supershedder samples belonged to cluster 1 and 3, which are mainly found in clinical isolates (Arthur *et al.*, 2013). The classification scheme of SBI not only depends on the insertion sites, but also on the *stx* prophages present. The supershedder strains carried either *stx2a* or *stx2c* and never all three *stx* prophages. The distribution of the *stx* prophages correlated with the lineages obtained by lineage-specific polymorphism assay (LSPA) (Whitworth *et al.*, 2010; Arthur *et al.*, 2013). By combining SBI with LSPA and other virulence markers a Markov chain Monte Carlo (MCMC)-based model clustering divided clinical and bovine O157:H7 into five clusters. The distribution of the clusters was different depending on the country of origin, although the amount of samples tested for other countries besides the USA was limited (Whitworth *et al.*, 2010). Additional studies are required to investigate possible links between geographical cluster distribution and differences in occurrence of HUS between countries.

37.2.8 Lineage-specific polymorphism assay (LSPA)

The LSPA allowed a more refined distinction between isolates that ultimately partitioned *E. coli* O157:H7 strains into three lineages (I, I/II and II). LSPA is a PCR-based polyacrylamide gel separation assay using the difference in length at six genic and intergenic chromosomal loci (Yang *et al.*, 2004). The selection of the loci was based on previously identified polymorphic OBGS products and consisted of short insertions or deletions of 1–100 nucleotides located on the genome backbone (no prophage or plasmid region included). In O157:H7 EDL933 the markers are a nine-base insertion in two genes *Z5935* and *rtcB*, a nine-base insertion in an intergenic region spanning the *folD-sfmA* genes, a nine-base deletion in the *rbsB* gene, a 78-base insertion in the *yhcG* gene, as well as an 18-base insertion in the intergenic region spanning the *arp-iclR* genes. The insertion in the intergenic region of *folD-sfmA* is found in all lineage II strains and is a main lineage determinant. Lineage I is most common in both humans and bovine samples, but statistically more frequent in humans, while lineage II is more frequent in bovine compared with human samples (Yang *et al.*, 2004; Ziebell *et al.*, 2008; Sharma *et al.*, 2009). Distribution of Shiga toxin subtypes varies between these lineages, as well as polymorphisms in virulence factors (antiterminator Q). Lineage II carries *stx2c* and *stx1* prophages and no *stx2* and the insertion sites *argW* and *wrbA* of *stx2* are intact (Eppinger *et al.*, 2011a). Numerous novel lineage II-specific genome signatures, some of which could be intimately associated with the altered pathogenic potential and adaptation to the bovine intestinal tract have been catalogued (Clawson *et al.*, 2009; Eppinger *et al.*, 2011a, b; Bono *et al.*, 2012).

37.2.9 Whole genome mapping (WGM)

WGM was initially developed to create physical maps of *Saccharomyces cerevisiae* chromosomes (Schwartz *et al.*, 1993). The first optical map of *E. coli* O157:H7 was generated by Lim *et al.*, which provided a valuable scaffold for assembly of newly sequenced genomes (Lim *et al.*, 2001). The whole genome is positioned on an optical mapping surface, digested with restriction enzymes and fluorescently labelled. WGM can provide helpful information for contig ordering and gap closure by comparing *in silico* and *in vitro* maps (Latreille *et al.*, 2007). Detailed genome comparisons with WGM highlighted insertions,

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deletions and rearrangements between closely related *E. coli* O157:H7 strains (Kotewicz *et al.*, 2007). Prophages were commonly found next to inverted chromosomal regions, underlining the role of prophages in *E. coli* recombination and diversity. WGM was successfully used to determine *stx* allele prevalence and respective chromosomal insertions sites of *stx* prophages (Kotewicz *et al.*, 2007, 2008; Eppinger *et al.*, 2011b). WGM can assist in genome assembly for molecular epidemiology outbreak investigations (Eppinger *et al.*, 2011b; Miller, 2013).

37.2.10 Comparative genome hybridization

With the genome sequences of *E. coli* O157:H7 and non-pathogenic K12 available, oligomer microarrays comparative genome hybridization (CGH) based on the open reading frames (ORFs) were developed to define the evolutionary relationship of *E. coli* (Wick *et al.*, 2005). The phylogeny derived from CGH data confirmed the stepwise evolutionary history of *E. coli* O157:H7. It also revealed single nucleotide polymorphisms (SNPs) in the core genes that allowed distinguishing of more closely related strains (Wick *et al.*, 2005). Furthermore, it was used to define core genes shared by bacteria and the specific set of genes unique to each strain, as illustrated by the comparison of *E. coli* K12 and *Shigella* genus strains (Fukiya *et al.*, 2004). By classifying the ORFs according to functional classes, it was revealed that even within basic metabolic function, such as energy and central intermediary metabolism, the pathogenic strains differ greatly from *E. coli* K12 (Fukiya *et al.*, 2004). This technology provides a large amount of information about presence and absence of genes. However, it requires prior knowledge of genes present to develop the probes and cannot detect new genes that are specific to the analysed strains. In addition to absence/presence, CGH allows to determine the location of the genes on chromosomal segments, which gives insight into structural rearrangements and insertion/deletion events (Willenbrock *et al.*, 2006). CGH was used mainly because whole genome sequencing was too costly and time consuming and is now obsolete in the field of microbiology, but is still widely used for diagnostics of genetic disorders, cancer and prenatal diagnosis (Riegel, 2014).

37.2.11 Genome-wide association studies (GWAS)

GWAS have been widely used to reveal the underlying genetics of human disease. As more genotypic data is gathered for microbial species the correlation to phenotypic traits, such as differences in pathogenic potential, can be established and GWAS provide an optimal tool for it (Dutilh *et al.*, 2013). To obtain statistically significant data, Dutilh *et al.* recommend using at least 30 genomes and re-annotating genomes to better define orthologous groups. Orthologous genes are differentiated because of a speciation event and often conserve their function (Woods *et al.*, 2013). Once the genotypes and phenotypes are obtained, three approaches are used to determine GWAS: (i) statistical analysis for association; (ii) correlation tests; and (iii) machine learning with training data sets (e.g. random forest algorithm). Bayjanov and colleagues developed a web tool (Phenolink) based on the random forest algorithm to link phenotype to ~omics data (Bayjanov *et al.*, 2012). The advantage of using classification algorithms over correlation studies is that they can highlight features that are only relevant for a subset of the samples for a given phenotype. Forty-two *Lactobacillus plantarum* isolates were used to validate Phenolink with CGH data, sugar utilization and nitrogen-dioxide production phenotypes. Previously known genes as well as new candidates were found by this tool (Bayjanov *et al.*, 2012). GWAS was recently used to detect differences in antibiotic resistance in *Staphylococcus aureus* using a large SNP panel of over 50,000 SNPs (Alam *et al.*, 2014). Only one SNP in *rpoB* was found to be associated with increased vancomycin tolerance. The potential of GWAS is promising, but will require efforts for creating standardized collections of complete and accurate phenotypic data.

37.3 Historical View of *E. coli* O157:H7 Genomics

Among pathogenic *E. coli*, the O157:H7 was the first serotype where genomes of multiple isolates

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were available for genome-wide comprehensive analysis (Hayashi *et al*., 2001; Perna *et al*., 2001). The scope of microbial comparative genomics has rapidly evolved from sequencing microbial prototype isolates to a large number of divergent isolates within a group of pathogens. The avirulent model strain MG1655 was the first non-pathogenic bacterium with a completed genome sequence (Blattner *et al*., 1997). A considerable effort was made to provide a full map of the genome architecture with only minor gaps (Hayashi *et al*., 2001; Perna *et al*., 2001). Only few studies have provided closed O157:H7 genomes (Kulasekara *et al*., 2009; Eppinger *et al*., 2011b; Xiong *et al*., 2012). These studies enabled the definition of structural modifications between different strains. The following section briefly highlights the major findings that were obtained by the inter- and intra-genome comparison of closed O157:H7 genomes discerning the core genome from mobile genetic elements, which helped to define the genomic make up of EHEC genomes. Comparative analysis of strains allowed regions within the genome to be identified that may be associated with differences in virulence. The first *E. coli* to be sequenced was the MG1655 isolate (Blattner *et al*., 1997) followed by two O157:H7 isolates, EDL933 (Perna *et al*., 2001) and Sakai (Hayashi *et al*., 2001). Upon sequencing these three isolates two key aspects in *E. coli* evolution were addressed by the associated comparative analysis: (i) O157:H7 isolates were very similar to each other, but still had unique features; (ii) > 1 Mb of DNA was unique to the O157:H7 isolates compared with the laboratory strain K12 and consisted mainly of prophage and prophage-like elements. Twenty percent of the ORFs were unique to O157:H7 providing a large pool of candidate virulence genes. Perna *et al.* (2001) classified all except the *stx2* prophage as cryptic prophages because the lytic cycle could not be induced for those prophages. In Sakai the majority of the phages are integrated in tRNA genes, showing a clear bias in integration sites (Hayashi *et al*., 2001). Even though the lambdoid prophages pool is shared, their locations and composition varied substantially among the individual isolates (Eppinger *et al*., 2011b; Xiong *et al*., 2012), which has led to the development of the SBI typing assay (Bono *et al*., 2012).

These seminal genome studies were crucial to functionally characterize virulence genes in model systems. Additional studies with other members of the *E. coli* species allowed defining the core set common to all strains (Rasko *et al*., 2008; Touchon *et al*., 2009). The study by Rasko *et al.* (2008) provided the first sequenced genomes of enterotoxigenic *E. coli* (ETEC), enteroaggregative *E. coli* (EAEC), enteropathogenic *E. coli* (EPEC) and the first commensal isolate. The different pathovars and commensal bacteria share a common set of ~2200 genes in a pool of 13,000 genes. Touchon and colleagues confirmed the size of the core set and the additional sequencing expanded the gene pool to ~18,000 genes (Touchon *et al*., 2009), indicative of an open pangenome. Rather than finding shared virulence gene sets in the different pathovars that were absent in commensal strains, each pathovar featured specific gene sets, suggesting separate events of virulence acquisition for niche adaptation (Rasko *et al*., 2008; Touchon *et al*., 2009).

37.4 Whole Genome Sequence Typing

The number of available O157:H7 genomes is constantly increasing (Eppinger *et al*., 2011a, b), but a lack of markers for accurate typing and genotype–phenotype association studies prevents biological insight into epidemiology and disease mechanisms. With high-resolution phylogenomic approaches, the dynamics of pathogenome evolution can be followed with a high level of phylogenetic accuracy and resolution. The current molecular markers and discussed typing assays used by public health microbiology laboratories are adequate for routine surveillance and identification of *E. coli* O157:H7, but reach their limits with respect to clonal complexes that only have a few genetic polymorphisms. Especially SNPs have gained popularity since their discovery as they provide stable genetic markers to study evolution, but also offer improved phylogenetic resolution. *In silico* scoring of SNPs was successfully applied by our group to investigate the genetic heterogeneity in strains derived from a single outbreak of human disease (Eppinger *et al*., 2011b) and to define biologically relevant markers between strains from clinical and animal source (Eppinger *et al*., 2011a). High resolution typing of *E. coli* O157:H7 and

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ancestor O55:H7 helped to define trends and the dynamics of pathogenome evolution of EHEC with a high level of phylogenetic accuracy and resolution (Whittam *et al*., 1988; Manning *et al*., 2008; Leopold *et al*., 2011; Eppinger *et al*., 2011b; Bono *et al*., 2012; Jung *et al*., 2013). Routine surveillance and identification of *E. coli* O157:H7 can be achieved with the current molecular markers and typing assays used by public health microbiology laboratories, but these approaches often lack the discriminatory power. The rapidity and cost-efficiency of NGS technologies has allowed whole genome sequence typing approaches to be deployed. In particular PacBio sequencing (English *et al*., 2012) allows the sequencing of long fragments surpassing the Sanger technology and this was instrumental in investigation of the German O104:H4 outbreak (Rasko *et al*., 2011). This platform improves assembly of *E. coli* O157:H7 that is notoriously difficult due to the multiple highly homogenous lambdoid prophages. A refined phylogenomic framework for *E. coli* O157:H7 was obtained by several groups utilizing SNP discovery (Leopold *et al*., 2009; Eppinger *et al*., 2011b; Norman *et al*., 2012) that surpasses classical technologies in terms of phylogenetic accuracy and resolution. Ninety-six SNP loci were selected for a typing assay by Manning and colleagues and applied to > 500 clinical *E. coli* O157 isolates that further divided the LSPA-6 lineage classification into nine distinct clades, which exhibited *stx*-converting prophages frequency and distribution that correlated with varying types of clinical sequelae (Manning *et al*., 2008; Abu-Ali *et al*., 2010a, b). Among these clades, clade 8 appeared to comprise a high number of hypervirulent *E. coli* O157:H7 strains (Kulasekara *et al*., 2009; Abu-Ali *et al*., 2010a, b; Eppinger *et al*., 2011b). The power of SNP typing for evolutionary analysis can be observed in the tracing of the evolutionary origin and emergence of pathogenic O157:H7 lineages from ancestral EPEC O55:H7 serotype with the intermediate non-motile O157:H(-) (Leopold *et al*., 2009). These enriched mutational database resources obtained from SNP discovery will provide crucial information to better associate genotypic group profiles and virulence phenotypes within *E. coli* O157:H7 (Eppinger *et al*., 2011b). While SNP are robust phylogenetic markers, a physiological role has been established for only a few (Eppinger *et al*., 2011b). For example, the type three secretion system (T3SS) effector translocated intimin receptor (*tir*) has a T allele 255 T>A that is found in all lineage I and I/II strains, while the A allele is mainly found in lineage II of bovine origin (Bono *et al*., 2007). The *tir* protein is important for adherence of the bacteria to the epithelial cells as it integrates into the cytoplasmic membrane and allows close interaction with the intimin (*eae*) present on the bacterial surface. Despite the mutation, no differences in bovine intestinal colonization could be observed when position 255 in *tir* was changed to the clinical T allele (Bono *et al*., 2007; Arthur *et al*., 2013).

37.5 Impact of NGS Technologies

With sequencing costs plummeting, larger numbers of isolates can be sequenced in parallel, allowing for large-scale and whole-genome-based comprehensive analyses. This has provided a vast amount of high quality genomic data to address outbreak-specific genomic traits and detection of variants with higher pathogenic potential. Multiple isolates from the 'Spinach', 'Taco Bell' and 'Taco John' outbreaks of 2006 were sequenced to determine structural and mobilome changes that could be used for outbreak distinction (Eppinger *et al*., 2011b). Sequencing was complemented with optical maps to highlight structural changes and facilitate positioning of the highly homogenous lambdoid prophage pool.

A recent outbreak that proved the importance of genomic sequencing for epidemiological understanding and the impact of novel combinations of virulence factors was the 2011 *E. coli* O104:H4 outbreak in Germany with over 830 cases and 46 deaths (Brzuszkiewicz *et al*., 2011; Mellmann *et al*., 2011; Rohde *et al*., 2011). Although the isolates from the German outbreak carried the *stx* prophage, they lacked several typical EHEC traits, such as LEE and intimin and were more closely related to EAEC (Brzuszkiewicz *et al*., 2011; Mellmann *et al*., 2011). Initially identified as an 'entero-aggregative-haemorrhagic *Escherichia coli* (EAHEC)' (Brzuszkiewicz *et al*., 2011), this strain due to its chimerical features should be more appropriately referred to as an enteroaggregative, STEC O104:H4. This case highlights the issues with the current nomenclature of pathogenic *E. coli* that is mainly based

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on the identification of virulence-associated genes that are linked to floating mobile elements (Rasko *et al.*, 2011).

37.6 Typing Isolates Based on the Virulence Complement

Studies on pathogenesis often focus only on traditional and established virulence factors, such as LEE, Shiga toxin, adhesins and plasmid-encoded genes. The majority of these virulence traits are found on prophages or pathogenicity associated island (PAI) acquired through horizontal gene transfer (Ogura *et al.*, 2009). These transfers are crucial evolutionary events that transformed the commensal *E. coli* into the pathogenic STEC. The LEE PAI is a mosaic structure, which is fused to SpLE3 in non-O157 EHEC strains (Morabito *et al.*, 2003). As the SpLE3 contains a duplication of 2 kb at the 3β end of LEE, the separation observed in O157 is probably due to rearrangement within the previously intact LEE-SpLE3 locus. Three tRNA gene integration sites are known for LEE. The initial hypothesis favoured multiple separate acquisitions of LEE for different EHEC strains (Wieler *et al.*, 1997), but a more detailed analysis of the LEE locus points more towards an internal transfer among serotypes (Muller *et al.*, 2009). Likewise for LEE, the evolutionary history of the haemolysin-encoding plasmid is complex (Boerlin *et al.*, 1998). When comparing STEC, the haemolysin gene *ehxA* phylogeny showed two clusters and was linked to the presence of LEE that might simply mirror the two plasmid lineages found in EHEC (Boerlin *et al.*, 1998). Though to date no systematic analysis of other virulence factors found on the O157 genome, such as fimbrial and non-fimbrial adhesins, non-LEE-encoded T3SS effectors and iron uptake systems has been undertaken. For O157:H7, a specific pattern of T3SS effectors was found in O157:H7 that was not shared with other STEC or EPEC strains. While these provide STEC-specific typing markers their physiological role remains largely unknown. In each EHEC branch, isolates carrying *stx* were present concomitantly with strains lacking the prophages. This demonstrates once again that whole genomic comparison is a more accurate tool to establish phylogenetic relatedness than typing based on presence of virulence factors that are mainly found on mobile genetic elements. The non-uniform prevalence of *stx* is indicative of independent acquisition events resulting in STEC groups with different evolutionary backgrounds as previously suggested (Reid *et al.*, 2000; Hazen *et al.*, 2013).

37.7 Conclusions

As outlined, typing strategies have substantially evolved from testing a few biochemical or genetic loci to large-scale whole genome typing approaches that offer, in combination with more traditional methodologies, a superior phylogenetic resolution. The development of cost-effective NGS technology that also required the development of analysis pipelines was instrumental (Bertelli and Greub, 2013; Hayden, 2014). These enriched mutational database resources obtained from whole genome sequencing and typing will provide crucial information to promote GWAS studies to better associate epidemiological and virulence metadata collections with genotypic group profiles within *E. coli* O157:H7. To gather insight into the genetically distinct STEC genotype, association studies in broader panels of isolates from cattle and other animal host species and environmental isolates are necessary to enrich the mutational database in this homogenous lineage and as a prerequisite to determine differences in disease manifestation and ecological niche. Polymorphic markers are essential in the development of a robust typing system critical for diagnostic, forensic, epidemiological and evolutionary studies of *E. coli* O157:H7. The public health community and livestock industry are interested in determining the mechanisms that underlie differences in transmissibility, host prevalence and virulence phenotypes among strains from bovine and human sources (Ferens and Hovde, 2011; Arthur *et al.*, 2013). Polymorphic loci anchored both in the chromosomal backbone and in the mobilome provide many interesting candidate genes that might be involved with altered virulence phenotypes and host prevalence for bovine genotypes (Eppinger *et al.*, 2011a). The described whole genome typing principles are readily translatable and have been deployed to other emerging infectious *E. coli* pathotypes (Rasko *et al.*, 2011; Reeves *et al.*, 2011). Finally,

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the German O104:H4 outbreak demonstrated the importance of collaboratively sharing and rapidly releasing DNA sequence data that allow analyses of a bacterial outbreak by the research community (Rohde *et al*., 2011), which proved to be critical in limiting the spread and transmission of infection and in enacting effective risk management strategies.

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38 Microbial Keratinases: Characteristics, Biotechnological Applications and Potential

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Abstract
Keratinases are a group of proteolytic enzymes that can catalyse the cleavage and hydrolysis of the highly stable and fibrous proteins: keratins. A diverse range of microorganisms, including fungi, actinomycetes and bacteria, have been reported to produce keratinases that have biotechnological applications and potential. These keratinases have been usefully applied in agricultural, pharmaceutical, leather and textile processes as well as within environmentally friendly waste management solutions. Potential uses of keratinases include the fields of biomedicine, cosmetics, biological control and the generation of green energy. This chapter aims to provide an overview of the properties of this group of versatile enzymes, including the mechanisms of keratin degradation. The diversity of microbial sources of keratinases is discussed and the optimization of keratinase production examined. The chapter concludes with an assessment of the established biotechnological applications of keratinases in different industries and current research that highlights other promising potential uses.

38.1 Introduction

Keratinases are key proteolytic enzymes produced by dermatophytes; they hydrolyse both 'soft' keratins (cytoskeletal materials in epithelial tissues, containing up to 1% sulfur) and 'hard' keratins (protective tissues in hairs and nails, containing up to 5% sulfur) (Karthikeyan *et al.*, 2007). Hence, in the past few decades, a number of research projects have focused on the activities of keratinases and their role in the virulence of dermatophytes such as *Trichopyton* and *Microsporum* (Siesenop and Bohm, 1995; Monod, 2008). The potential of keratinases in the biotechnological context has gained substantial and significant recognition since the beginning of the 21st century: their substrate specificity and ability to attack highly cross-linked and recalcitrant structural proteins that resist common known proteolytic enzymes, such as trypsin and pepsin, make them valuable biocatalysts in industries that deal with keratinous materials. Novel applications of keratinases are continuously being discovered (see section 38.6 'Potential Applications of Keratinases'). A number of excellent reviews have been published charting the progress of our understanding of keratinases and their microbial sources, providing excellent overviews on the ecology, physiology and mechanisms of keratinolytic microorganisms (Korniłłowicz-Kowalska and Bohacz, 2011) and the applications of keratinases (Onifade *et al.*, 1998; Gupta and Ramnani, 2006; Karthikeyan *et al.*, 2007; Brandelli *et al.*, 2010; Gupta *et al.*, 2013a, b).

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This chapter aims to consolidate and update the information to provide a comprehensive review of this remarkable biocatalyst.

38.2 Characteristics and Properties of Keratinases

Keratinases are proteolytic enzymes that can hydrolyse keratins. Keratins belong to a super family of intermediate filaments. They are stable, insoluble and fibrous structural proteins that are found in epithelial tissues (soft epithelial keratins) and protective tissues such as hair, nails and horns (hard trichocytic keratins). Coulombe and Omary (2002) have developed a set of principles for defining the structures, functions and regulations of keratin. The primary function of keratins is to protect cells from mechanical and non-mechanical stress; they also have other roles such as cell signalling, regulating the availability of other abundant cellular proteins and as a stress protein.

In general, keratins can be classified as Type I (acidic keratins) or Type II (basic keratins). The strength and robustness of keratin is derived from the highly stable, tightly packed α-helix (in α-keratins such as hair) and/or β-sheet (in β-keratins such as horn and hooves) configurations. The keratin micro- and macro-filaments in these pleated sheets are supercoiled to form a highly stable left-handed superhelical motif (Voet and Voet, 1995) sustained by strong inter- and intramolecular hydrogen bonds and hydrophobic reaction of the polypeptides (Bradbury, 1973). In addition, all keratins contain a high degree of cysteine which confers rigidity and chemical resistance via the cross-linking of thermally stable disulfide bonds.The amount of cysteine plays a significant role in determining the nature of the keratin; in soft keratin, the amount of cysteine present (up to 2%) was much lower than the hard keratin (~22%; Korniłłowicz-Kowalska and Bohacz, 2011). Table 38.1 lists the cysteine content in different types of keratins.

Keratinases are predominantly secreted extracellularly into the growth medium containing keratin (Monod *et al*., 2002; Gupta and Ramnani, 2006; Brandelli *et al*., 2010). However, Wawrzkiewicz *et al*. (1987) noted that *Trichophyton gallinae* only produced intracellular keratinase, while Korniłłowicz-Kowalska (1999) and Al-Musallam *et al*. (2013) observed the production of both extracellular and intracellular keratinase in geophilic microscopic fungi (namely *Arthroderma quadrifidum*, *Arthroderma curreyi* and *Chrysosporium pruinosum*) and macroscopic fungi (*Coprinopsis* sp.), respectively. Gessesse *et al*. (2003) and Manczinger *et al*. (2003) reported the production of constitutive keratinase by *Nesterenkonia* sp. AL20 and *Bacillus licheniformis*, respectively, whereas Apodaca and McKerrow (1989) discovered that keratinase

Table 38.1. Cysteine content of keratin.

Types of keratin	Cysteine content	Example	Reference
Soft keratin	Up to 2%	Epithelial cells – low chemical resistance and mechanical strength	Korniłłowicz-Kowalska and Bohacz (2011)
Hard keratin			
α (40–68 kDa)	10–17%	Wool and hair	Filipello Marchisio (2000), Robbins (2012)
β (10–22 kDa)	5–10%	Scales and claws	Dalla Valle *et al*. (2010)
γ (Amporphic keratin)	~22%	Outer layer of hair cuticle; globular, about 15 kDa, high in sulfur content and acts as disulfide cross-linkers	Hill *et al*. (2010), Robbins (2012)
Feather (contains both α-helix and β-sheet[a]	8%	Feather	Akhatar and Edwards (1997)

[a]Feather mainly consists of feather-specific β-keratins; cellular and biochemical studies have shown that α-keratin plays an important role in the early formation of rachides, barbs and barbules (Alibardi and Toni, 2008).

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may be constitutively produced in *Trichophyton rubrum*in the absence of keratin.

The classification and nomenclature of all proteolytic enzymes are available in the MEROPS database (http://merops.sanger.ac.uk/cgi-bin/family_index?type=P#S). These proteases are grouped into: aspartic, cysteine, glutamic, asperagine, metallo, mixed, serine, threonine peptidases and those that are of unknown catalytic mechanisms. Microbial keratinases are predominantly of the metallo, serine or serine-metallo type (Brandelli, 2008) with the exception of keratinase from yeast which belongs to aspartic protease (Negi *et al.*, 1984; Lin *et al.*, 1993; Koelsch *et al.*, 2000). Both metallo and serine peptidases are endoproteases that cleave peptide bonds internally within a polypeptide.

Metalloproteases are highly diverse, having more than 90 families. A common feature of this type of enzyme is the involvement of a divalent ion (such as Zn^{2+}) for their catalytic activities which are inhibited by metal chelating agents, transition or heavy metals (Nam *et al.*, 2002; Riffel *et al.*, 2003; Thys *et al.*, 2004; Gupta and Ramnani, 2006). Serine proteases fall into two broad categories based on their structure: (i) chymotrypsin-like (trypsin-like); or (ii) subtilisin-like. The subtilisin subfamily are completely inhibited by phenylmethanesulfonyl fluoride (PMSF), antipain and chymostatin (Tyndall *et al.*, 2005).

38.2.1 Optimal pH and temperature

Keratinases belonging to the metallopeptidase group work best in neutral to mildly alkaline conditions (pH 7–8.5; Lee *et al.*, 2002; Sousa *et al.*, 2007; Riffel *et al.*, 2007; Bach *et al.*, 2011; Han *et al.*, 2012; Tork *et al.*, 2013) with the exception of the keratinase produced by *Bacillus thuringiensis* TS2 (Sivakumar *et al.*, 2013) where the optimal pH was 10 and one of the two metalloproteases isolated from an endophytic and keratinolytic *Penicillium* spp. Morsy1 which also has an optimum working pH range of 10–11 (El-Gendy, 2010).

Keratinases belonging to the serine peptidase group are mainly alkaline proteases that have pH optima in the alkaline range (pH 8–11; Yoshioka *et al.*, 2007; Cao *et al.*, 2009; Fakhfakh *et al.*, 2009; Jeong *et al.*, 2010; Lv *et al.*, 2010; Habbeche *et al.*, 2014). Some alkalophilic actinomycetes such as *Nocardiopsis* sp. strain TOA-1 (Mitsuiki *et al.*, 2004) and *Streptomycces* AB1 (Jaouadi *et al.*, 2010); and alkalophilic bacteria *Bacillus circulans* (Benkiar *et al.*, 2013) and *Bacillus halodurans* AH-101 (Takami *et al.*, 1999) have been found to produce keratinases that perform best in a highly alkaline environment (pH >11.5). Atypically, the serine keratinases produced by two fungal strains have an acidic optimal pH range: *Trichophyton mentagrophytes* at pH 4.5 (Tsuboi *et al.*, 1989) and *Purpureocillium lilacinum* at pH 6 (Cavello *et al.*, 2013).

Only a few keratinases belonging to the group serine-metalloprotease have been isolated: *Bacillus* sp. 50-3 (Zhang *et al.*, 2009), *Stenotrophomonas maltophilia* BBE11-1 (Fang *et al.*, 2013), *Streptomyces gulbargensis* (Syed *et al.*, 2009), *Streptomyces* SK1-02 (Letourneau *et al.*, 1998) and *Streptomyces* sp. 7 (Tatineni *et al.*, 2008). This group of keratinases also have an alkaline optimum pH range (9–11).

In general, microbial keratinases have a broad, thermally stable range where they can function and the optimal temperature is along the thermophilic range of 45–60°C (Bernal *et al.*, 2006a; Kojima *et al.*, 2006; Chao *et al.*, 2007; Kim, 2007; Riffel *et al.*, 2007; Cao *et al.*, 2008; Rai *et al.*, 2009; Sye *et al.*, 2009; Xu *et al.*, 2009; Lateef *et al.*, 2010; Cavello *et al.*, 2012; Jaouadi *et al.*, 2013; Sivakumar *et al.*, 2013; Tork *et al.*, 2013). A number of organisms such as *Actinomadura keratinilytica* Cpt29 (Habbeche *et al.*, 2014), *B. circulans* (Benkiar *et al.*, 2013), *Thermoactinymces candidus* (Ignatova *et al.*, 1999), *Thermoanaerobacter keratinophilus* (Riessen and Antranikian, 2001), *Fervidobacterium pennavorans* (Friedrich and Antranikian, 1996; Kluskens *et al.*, 2002) and *Fervidobacterium islandicum* (Gödde *et al.*, 2005) produce keratinases that work best at temperatures at or above 70°C. The highest optimal temperature (100°C) was recorded by Nam *et al.* (2002) from a serine keratinase produced by *F. islandicum* AW1, isolated from a geothermal hot spring. Mesophilic keratinses with a lower optimal temperature range (20–45°C) are predominately produced by pathogenic organisms, including *Kocuria rosea* (Bernal *et al.*, 2006a), *Myrothecium verrucaria* (Moreira-Gasparin *et al.*, 2009), *Scopulariopsis brevicaulis* (Malviya *et al.*, 1992), *Serratia marcescens* P3 (Bach *et al.*, 2012), *S. maltophilia*

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(Yamamura *et al.*, 2002; Cao *et al.*, 2009; Jeong *et al.*, 2010; Fang *et al.*, 2013) and *Trichophyton* sp. (Anbu *et al.*, 2008; Ismail *et al.*, 2012), and is probably indicative of the ecological niches they occupy.

38.2.2 Biochemical properties of keratinases

The majority of keratinases reported are monomeric enzymes with a diverse range of molecular weights (14–240 kDa; see section 38.3 'Sources of Microbial Keratinases'). The keratinase produced by *Bacillus pumilus* A1 has the lowest molecular weight (Fakhfakh *et al.*, 2013), whereas *K. rosea* produced keratinase of the highest molecular weight (Bernal *et al.*, 2006a). Although less common, multimeric keratinases have also been isolated in a number of microorganisms. Keratinase from fungal isolates of *Coccidioides immitis* produced seven distinct polypeptides ranging from 15 kDa to 65 kDa (Lopes *et al.*, 2008), *S. brevicaulis* and *Penicillium* spp. Morsy1 both produced two fractions when purified by SDS-PAGE (sodium dodecyl sulfate polyaccylamide gel electrophoresis) that were 24–45 kDa and 19–40 kDa, respectively (Malviya *et al.*, 1992; El-Gendy, 2010). Actinomycetous isolates of *Steptomyces* sp. strain 16 produced a keratinase that was comprised of four active polypeptides varying from 19 kDa to 50 kDa (Xie *et al.*, 2010). Multimeric keratinases were also detected in bacterial strains such as *Bacillus* sp. MTS (three fractions ranging from 16 kDa to 50 kDa; Rahayu *et al.*, 2012); *Chryseobacterium* sp. kr6 (three active fractions at 20–64 kDa; Riffel *et al.*, 2007; Silveira *et al.*, 2010); and *Chryseobacterium indologenes* TKU014 (three active fractions, 40–56 kDa; Wang *et al.*, 2008); *Kytococcus sedentarius* M17C (two fractions at 30 kDa and 50 kDa; Longshaw *et al.*, 2002) and *B. licheniformis* ER-15 (28 kDa and 30 kDa; Tiwary and Gupta, 2010).

Keratinase produced by *B. licheniformis* PWD-1 is the best studied and the entire nucleotide sequence of the coding and flanking regions of the keratinase structure gene, *kerA*, was determined (Lin *et al.*, 1997). Although many microorganisms are able to produce keratinase (see section 38.3 'Sources of Microbial Keratinases') and many have been sequenced (Gupta *et al.*, 2013b), few keratinase encoding genes have been cloned and expressed in heterologous systems (Porres *et al.*, 2002; Radha and Gunasekaran, 2007) except for *Bacillus megaterium*, which is a stable host to clone and express keratinase genes from heterologous origin (Radha and Gunasekaran, 2007). In contrast, in a comparative study using *Escherichia coli*, *B. subtilis* and *Pichia pastoris* as cloning hosts to express the keratinase gene from *B. licheniformis* BBE11-1, *B. subtilis* appeared to be the ideal host for keratinase production (Liu *et al.*, 2014).

38.2.3 Chemical properties of keratinases

The N-terminal sequences of a number of keratinases have been comprehensively analysed and reviewed by Gupta and Ramnani (2006) and Brandelli *et al.* (2010). Depending on the microbial source, keratinases produced by each class and group share a high degree of similarity in their N-terminal sequences (Table 38.2). Most keratinases isolated from *Bacillus* sp. belong to the subtilisin group and have very high (> 90%) N-terminal sequence homology with the subtilisin Carlsberg produced by *B. licheniformis*. Keratinases A (kerA) and RP (kerRP) from *B. licheniformis* PWD-1 and RPk, respectively, are almost identical to subtilisin Carlsberg (Jacobs *et al.*, 1985; Lin *et al.*, 1995; Fakhfakh *et al.*, 2009). The deduced amino acid sequence revealed that keratinase kerRP differs from kerA, subtilisin Carlsberg and a keratinase of *B. licheniformis* by two, four and 62 amino acids, respectively, but conserving the active site residues D32, H63 and S220 (Fakhfakh *et al.*, 2009). Keratinases from *B. licheniformis* MKU3 and MSK103 have over 99% and 87% similarity with kerA, respectively (Radha and Gunasekaran, 2007; Yoshioka *et al.*, 2007), and the keratinase from *B. circulans* showed more than 80% homology with *B. pumilus* K12 and *B. pumilus* CBS (Benkiar *et al.*, 2013). The N-terminal amino acid of keratinase KERUS of *Brevibacillus brevis* US575 differs from *B. pumilus* A1, *B. pumilus* CBS and subtilisin Carlsberg by only one amino acid – the Gln13 residue in KERUS was an Ala13 in the other enzymes. Similarly, keratinase isolated from *Streptomyces griseus*, *Streptomyces albidolavus* K1-02 and *Streptomyces fradiae* share comparable N-terminal sequences,

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Table 38.2. N-terminal amino acid sequences of a number of keratinases and their microbial sources.

Microorganism	Keratinase	N-terminal sequence	Reference
Bacteria			
Bacillus circulans DZ100	Keratinase SAPDZ	AQTVPYGMAQIKDPAVHGQGYKGAN	Benkiar *et al*. (2013)
Bacillus licheniformis	Subtilisin Carlsberg	AQTVPYGIPLIKADK	Jacobs *et al*. (1985)
B. licheniformis PWD-1	Keratinase A	AQTVPYGIPLIKADK	Lin *et al*. (1995)
B. licheniformis RPk	Keratinase RP	AQTVPYGIPLIKADK	Fakhfakh *et al*. (2009)
B. licheniformis MP1	Alkaline protease	AQTVPYGIPLIKAD	Jellouli *et al*. (2011)
Bacillus mojavensis A21	Serine proteases BM1	AQSVPYGISQIKA	Haddar *et al*. (2009)
	Serine proteases BM2	AIPDQAATTLL	
Bacillus pumilus	Keratinase A1	AQTVPYGIPQI	Fakhfakh-Zouari *et al*. (2010a, b)
B. pumilus	Keratinase CBS	AQTVPYGIPQIKAPAVHAQGY	Jaouadi *et al*. (2008)
Bacillus subtilis	Keratinase S14	AQSVPYGISQIKAPA	Macedo *et al*. (2005)
B. subtilis	Subtilisin E	AQSVPYGISQIKAPA	Stahl and Ferrari (1984)
B. subtilus	Keratinase KS-1	AZPVEWGISZ	Suh and Lee (2001)
Bacillus halodurans	Keratinase AH-101	SQTVPWGISFISTQQ	Takami *et al*. (1999)
Bacillus pseudofirmus	Keratinase FA30-01	XQTVPXGIPYIYSDD	Kojima *et al*. (2006)
Brevibacillus brevis US575	Keratinase KERUS	AQTVPYGIPQIKEPAVHAQGYK-GANVK	Jaouadi *et al*. (2013)
Pseudomonas aeruginosa	Keratinase Pa	AEAGGPGG	Lin *et al*. (2009)
Fervidobacterium pennivorans	Fervidolysin	STARDYGEELSN	Kluskens *et al*. (2002)
Vibrio metschnikovii J1	Serine protease	AQQTPYGIRMVQADQLSDVY	Jellouli *et al*. (2009)
Actinomycetes			
Streptomyces griseus	Protease B (SGPB)	ISGGDAIYSSTGRCS	Jurasek *et al*. (1974)
Streptomyces fradiae	Keratinase Sfase-2	IAGGEAIYAAGGGRC	Kitadokoro *et al*. (1994)
Streptomyces albidoflavus	Serine protease SAKase	XXGGDAIYSSXXRXS	Bressollier *et al*. (1999)
Norcardiopsis TOA-1	NAPase	ADIIGGLAXYTMGGX	Mitsuiki *et al*. (2004)
Fungi			
Paecilomyces marquandii	Keratinase Pm	ALTQQPGAPWGLG	Gradišar *et al*. (2005)
Doratomyces microsporus	Keratinase Dm	ATVTQNNAPWGLG	Gradišar *et al*. (2005)
Aspergillus fumigatus	Keratinase Af	ALTTQKGAPWGLGSI	Noronha *et al*. (2002)

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but are distinct from keratinases produced by other bacterial and fungal strains (Table 38.2).

A number of chemicals have been shown to inhibit keratinases (Table 38.3). Keratinases belonging to the metalloproteases group are inhibited by metal chelating agents (e.g. ethylenediaminetetraacetic acid, EDTA), organic ligands (e.g. 1,10-phenanthroline) and a number of heavy metals including Cu^{2+}, Hg^{2+}, Pb^{2+}, Ni^{2+}, Zn^{2+}, Co^{2+} and Mn^{2+} (Farag and Hassan, 2004; Thys *et al.*, 2006; Riffel *et al.*, 2007; Daroit *et al.*, 2011; Sivakumar *et al.*, 2013). Serine proteases are generally inhibited by PMSF (Xie *et al.*, 2010; Shrinivas *et al.*, 2012; Benkiar *et al.*, 2013; Jaouadi *et al.*, 2013) and some are also susceptible to Cd^{2+} and Hg^{2+} inhibition (Li *et al.*, 2007; Anitha and Palanivelu, 2013; Benkiar *et al.*, 2013; Chaudhari *et al.*, 2013). Keratinases that are serine-metalloproteases are sensitive to both chelating agents and PMSF (Tatineni *et al.*, 2008; Fang *et al.*, 2013; Tork *et al.*, 2013). The presence of Ca^{2+} and Mg^{2+} appeared to enhance keratinase activities in all protease groups (Farag and Hassan, 2004; Riffel *et al.*, 2007; Benkiar *et al.*, 2013; Jaouadi *et al.*, 2013; Sivakumar *et al.*, 2013). Interestingly, while Co^{2+} and Cu^{2+} are inhibitory to the metalloproteases produced by *Bacillus* sp. P45 (Dozie *et al.*, 1994), *Bacillus subtilis* NRC 3 (Tork *et al.*, 2013), *B. thuringiensis* (Sivakumar *et al.*, 2013) and some *Chryseobacterium* sp. (Riffel *et al.*, 2007; Chaudhari *et al.*, 2013), they improved the serine protease activities in *B. circulans* DZ100 (Benkiar *et al.*, 2013), *B. brevis* US575 (Jaouadi *et al.*, 2013), *B. licheniformis* BBE11-1 (Liu *et al.*, 2013) and *S. fradiae* var k11 (Li *et al.*, 2007). A small number of keratinases are stimulated by the presence of surfactants and detergents; metalloproteases of *Chryseobacterium gleum* (Chaudhari *et al.*, 2013), serine proteases of *Aspergillus parasiticus* (Anitha and Palanivelu, 2013), *Brevibacillus* sp. AS-S10-II (Mukherjee *et al.*, 2011) and *S. maltophilia* BBE11-1 (Fang *et al.*, 2013) are augmented by Triton X-100, Tween 20, Tween 80 and non-ionic surfactants.

Keratinases that are either active or stable in the presence of organic solvents, surfactants and bleaching agents have potential industrial applications. A keratinolytic serine protease secreted by *P. lilacinum* is found to demonstrate stable keratinolytic activities with dimethyl sulfoxide (DMSO), methanol and isopropanol; Triton X-100, SDS, Tween 85 or hydrogen peroxide (Cavello *et al.*, 2012). The keratinase produced by *B. pumilus* KS12 was found to exhibit both high detergent compatibility and oxidation stability with an eight- and fivefold enhancement of enzymatic activities in the presence of Triton X-100 and saponin, respectively (Rajput *et al.*, 2010). The keratinolytic proteases of *Meiothermus ruber* H328 was able to tolerate SDS at 30% (w/v) and organic solvents (methanol, ethanol, acetonitrile, acetone and chloroform) at 40% (v/v) at 60°C (Kataoka *et al.*, 2014). Similarly, the thermally stable keratinase isolated from *Meiothermus* sp. I40 also exhibited good stability in the presence of DMSO, ethanol, isopropanol and acetonitrile (Kuo *et al.*, 2012) and the keratinase produced by *B. halodurans* PPKS-2 was not inhibited by SDS, EDTA, H_2O_2 (15%) or other commercial detergents (Prakash *et al.*, 2010a).

It has been reported that reducing agents such as dithiothreitol (DTT), β-mercaptoethanol, cysteine and sodium sulfite stimulated keratinase activity as the thiol groups activate the keratinolytic enzymes (see section 38.2.5 ‘Mechanism of keratinolysis’; Gupta and Ramnani, 2006; Tatineni *et al.*, 2008; Xie *et al.*, 2010; Fang *et al.*, 2013). However, this phenomenon was not universal and did not apply to the keratinases isolated from *Brevibacillus* sp. AS-S10-II (Mukherjee *et al.*, 2011) and *Chryseobacterium* sp. kr6 (Riffel *et al.*, 2007); probably resulting from the chelation of essential ions that are necessary to maintain the structure and activity of the keratinase by DTT (Riffel *et al.*, 2007).

38.2.4 Keratinous substrates and their specificities

Microbial keratinases can be isolated from a number of sources and have diverse properties depending on the producer organisms (Brandelli *et al.*, 2010; see section 38.3 ‘Sources of Microbial Keratinases’ for details). For example, keratinases from fungi, actinomycetes and bacteria have a wide range of substrates: from soft keratin such as stratum corneum (Błyskal, 2009) to hard keratin such as feather keratin (Friedrich and Antranikian, 1996; Ichida *et al.*, 2001; Gousterova *et al.*, 2005; Mazotto *et al.*, 2013), sheep’s wool (Riessen and Antranikian, 2001;

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Table 38.3. Some chemical compounds that attenuate or stimulate keratinase activities.

Microbial source	Protease type	Inhibitors[a]	Stimulators[a]	References
Actinomadura keratinilytica Cpt29	Serine	PMSF, DFP, Ni^{2+}, Cd^{2+}, Hg^{2+}, Ba^{2+}, Fe^{2+}	H_2O_2, Tween 20, Tween 80, Triton X-100, Ca^{2+}, Mn^{2+}	Habbeche *et al*. (2014)
Aspergillus parasiticus	Serine	PMSF, Cd^{2+}, $Cu^{2+}$and Zn^{2+}	Ca^{2+}, Mg^{2+} and Mn^{2+}, non-ionic detergents and urea	Anitha and Palanivelu (2013)
Aspergillus oryzae	Metallo	EDTA, Pb^{2+}, Cd^{2+} and Hg^{2+}	Ca^{2+}, Ba^{2+}, Cu^{2+}, Na^{+}, K^{+}, Mg^{2+}	Farag and Hassan (2004)
Bacillus sp. P45	Metallo	EDTA, SDS, Zn^{2+}, Cu^{2+}, Co^{2+}	Ca^{2+}, Mg^{2+}	Daroit *et al*. (2011)
Bacillus circulans DZ100	Serine	PMSF, DFP, Ni^{2+}, Cd^{2+}, Hg^{2+}	Ca^{2+}, Mg^{2+}, Mn^{2+}, Zn^{2+}, Co^{2+}, Cu^{2+}	Benkiar *et al*. (2013)
Bacillus halodurans JB 99	Serine	PMSF		Shrinivas *et al*. (2012)
Bacillus licheniformis BBE11-1	Serine	PMSF	Mg^{2+}, Co^{2+}	Liu *et al*. (2013)
Bacillus pumilus	Serine	PMSF		Kumar *et al*. (2008)
Bacillus subtilis NRC 3	Serine-metallo	PMSF, EDTA, citric acid, 1-10-PA, Zn^{2+}, Cu^{2+}, Co^{2+}, Mn^{2+}	Na^{+}, K^{+}, Mg^{2+}	Tork *et al*. (2013)
Bacillus thuringiensis	Metallo	EDTA, Cu^{2+}, Zn^{2+}, Co^{2+}, Mn^{2+}, Ni^{2+}	Ca^{2+}, Mg^{2+}	Sivakumar *et al*. (2013)
Brevibacillus brevis US575	Serine	PMSF, DFP, Cd^{2+}, Hg^{2+}, Ni^{2+}	Ca^{2+}, Mg^{2+}, Mn^{2+}, Zn^{2+}, Co^{2+}, Cu^{2+}	Jaouadi *et al*. (2013)
Brevibacillus sp. AS-S10-II	Serine	PMSF, IAA, DTT	SDS, Triton X-100, Tween-20, H_2O_2	Mukherjee *et al*. (2011)
Chryseobacterium gleum	Metallo	EDTA, Cu^{2+}, Hg^{2+}	Triton X-100, Tween 80, MCE, Fe^{2+}, Fe^{3+}	Chaudhari *et al*. (2013)
Chryseobacterium sp. kr6	Metallo	EDTA, EGTA, PHEN, MCE, DTT, SDS, Cu^{2+}, Zn^{2+}	Ca^{2+}, Mg^{2+}, Cd^{2+}	Riffel *et al*. (2007)
Lysobacter NCIMB 9497	Metallo	EDTA		Allpress *et al*. (2002)
Microbacterium sp. strain kr10	Metallo	EDTA, PHEN, CMB, Cu^{2+}, Hg^{2+}, Zn^{2+}, Mn^{2+}		Thys *et al*. (2006)
Streptomyces fradiae var k11	Serine	PMSF, Co^{2+} and Cr^{3+}	Ni^{2+} and Cu^{2+}	Li *et al*. (2007)
Streptomyces sp. 16	Four different serine proteases	PMSF	EDAC, DTT, Na^{2+}	Xie *et al*. (2010)
Streptomyces sp. S7	Serine-metallo	PMSF, EDTA, SDS	DTT	Tatineni *et al*. (2008)
Stenotrophomonas maltophilia BE11-1	K1: serine-metallo; K2: serine; K3: disulfide reductase	K1: EDTA, PMSF, SDS, Fe^{3+}; K2: PMSF, SDS, Fe^{3+}; K3: Fe^{3+}, Cu^{2+}, Mn^{2+}, Zn^{2+}	K1: Na^{2+}, Tween 20; K2: Ca^{2+}, Na^{+}, DTT, Tween 20; K3: EDTA, Na^{2+}, DTT, Triton X-100, Tween 20, DMSO	Fang *et al*. (2013)

[a]CMB, *p*-Chloromercuribenzoic acid; DFP, diisopropyl fluorophosphates; DMSO, dimethyl sulfoxide; DTT, dithiothreitol; EDAC, 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide; EDTA, ethylenediaminetetraacetic acid; EGTA, ethylene glycol tetraacetic acid; IAA, iodoacetamide; MCE, β-mercaptoethanol; PHEN, 1,10-phenanthroline; PMSF, phenylmethanesulfonyl fluoride; SDS, sodium dodecyl sulfate.

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Farag and Hassan, 2004; Xie *et al*., 2010; Han *et al*., 2012), human and animal hairs (Desai *et al*., 2010; Chen *et al*., 2011; Gurav and Jadhav, 2013; Jaoudai *et al*., 2013), nail, hoof and horn (Friedrich and Kern, 2003; Mohorčič *et al*., 2007; Błyskal, 2009; Tiwary and Gupta, 2010) and azokeratin (Kim, 2007; Bach *et al*., 2011). Other substrates that are susceptible to keratinase degradation include: (i) collagen (Farag and Hassan, 2004; Bernal *et al*., 2006a; Fang *et al*., 2013); (ii) elastin (Bressollier *et al*., 1999; Brandelli *et al*., 2010); (iii) gelatine (Lopes *et al*., 2008; Tork *et al*., 2013); (iv) albumin and haemoglobin (Lopes *et al*., 2008; Benkiar *et al*., 2013); and (v) fibrin (Tiwary and Gupta, 2010; Tork *et al*., 2013).

In addition to substrates listed above, keratinase is also able to degrade unusual recalcitrant animal proteins such as prions (Langeveld *et al*., 2003; Tsiroulnikov *et al*., 2004; Suzuki *et al*., 2006). Prions are fatal neurodegenerative transmissible agents causing several incurable illnesses in humans and animals. Prion diseases are caused by the structural conversion of the cellular prion protein, PrP^{C}, into its misfolded oligomeric form, known as PrP^{Sc} (Abskharon *et al*., 2014). The normal prion protein PrP^{C} consists of approximately 45% α-helix and only 3% β-sheet, but the abnormal conformer PrP^{SC} consists of approximately 30% α-helix and 45% β-sheet (Pan *et al*., 1993).This structure shares a high degree of similarity with feather keratin. The feather keratin molecule contains a 32-residue segment that is believed to form the framework of the filament that has a helical structure with four repeating units per turn; each repeating unit consists of a pair of twisted β-sheets related by a perpendicular diad (Fraser and Parry, 2007).

The substrate specificity of keratinases is strongly influenced by the chemical properties of their substrates. As keratin is composed of 50–60% hydrophobic and aromatic amino acids (Gradišar *et al*., 2005; Brandelli *et al*., 2010), keratinases appear to cleave preferentially hydrophobic and aromatic amino acid residues at the P1 position (Gradišar *et al*., 2005; Silveira *et al*., 2009; Brandelli *et al*., 2010; Gupta *et al*., 2013a). Hydrolysis studies using oxidized insulin B as the substrate showed that phenylalanine, valine, tyrosine and leucine were selectively cleaved by keratinases of *Thermoanaerobacter* sp. (Kublanov *et al*., 2009a), *B. pumilus* KS12 (Rajput *et al*., 2010), *Nesterenkonnia* sp. AL20 (Bakhtiar *et al*., 2005), *Pseudomonas aeruginosa* KP1 and KP 2 (Sharma and Gupta, 2010a, b) and *Streptomyces* sp. (Tsiroulnikov *et al*., 2004). Studies using synthetic amino acid *p*-nitroanilide (*p*NA), *p*-nitrophile ester (*O*N*p*) or 7-amino-4-methylcoumarin (AMC) as substrates (Table 38.4) demonstrated the substrate specificity of these keratinases. The residues in the P2 and P3 positions also play a role in the substrate specificity. *N*-Succinyl-Ala-Ala-Phe-*p*Na was susceptible to degradation by keratinases of *B. pumilus*, but not its analogue *N*-succinyl-Gly-Gly-Phe-*p*Na (Rajput *et al*., 2010). Macedo *et al*. (2008) showed that the keratinase KerS14 of *B.subtilus* preferred to cleave Arg at the P1 position, small amino acid residues at the P2 position, and Gln or Glu at the P3 position. Keratinases also seemed to prefer utilization of longer substrates which may be indicative that the presence of amino acids further along the cleavage site residue is important to the substrate specificity. This is probably due to the availability of additional active sites (Böckle *et al*., 1995; Bressollier *et al*., 1999; Mitsuiki *et al*., 2004).

38.2.5 Mechanism of keratinolysis

Over the years, a number of hypotheses have been proposed to explain the mechanism of keratin degradation by microbial keratinases (Korniłłowicz-Kowalska and Bohacz, 2011). Broadly speaking, it is agreed that keratin degradation encompasses two main stages: (i) deamination and (ii) keratinolysis (Kunert, 1976, 1989; Kaul and Sumbali, 1997). Deamination creates an alkaline environment for optimal enzymatic reaction by the alkaline proteases (Kunert, 1989, 1992, 2000; Kaul and Sumbali, 1997). The complex mechanism of keratinolysis that follows involves the cooperative action of sulfitolytic and proteolytic enzymes (Yamamura *et al*., 2002). Rahayu *et al*. (2012) noted the degradation activities on natural keratin substrates by purified keratinase from *Bacillus* sp. MTS was enhanced by the purified disulfide reductase, compared with activity of each enzyme alone. This is further supported by the observations of Fang *et al*. (2013) in which three kertinolytic enzymes (a serine protease, serine-metalloprotease

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Table 38.4. Substrate specificity of some keratinases using synthetic substrate.

Microbial source	Substrate[a]	References
Bacillus licheniformis	↓	Rozs *et al*. (2001)
B. licheniformis PWD-1	N-succinyl-Ala-Ala-Pro-Phe-*p*NA	Evans *et al*. (2000)
Bacillus pumilus KS12		Rajput *et al*. (2010)
B. pumilus A1		Fakhfakh-Zouari *et al*. (2010b)
Paecilomyces marquandii and *Doratomyces microsporus*		Gradišar *et al*. (2005)
Pseudomonas aeruginosa		Lin *et al*. (2009)
Streptomyces fradiaevar k11		Li *et al*. (2007)
Thermoanaerobacter sp.		Kublanov *et al*. (2009a)
Trichophyton vanbreuseghemii		Moallaei *et al*. (2006)
B. pumilus KS12	↓	Rajput *et al*. (2010)
	N-succinyl-Ala-Ala-Pro-Leu-*p*NA	
B. licheniformis	↓	Rozs *et al*. (2001)
B. pumilus KS12	N-succinyl-Ala-Ala-Ala—*p*NA	Rajput *et al*. (2010)
Lysobacter sp. AL10	↓	Allpress *et al*. (2002)
Microbacterium sp. kr10	CBz-Phe-*p*Na	Thys and Brandelli (2006)
B. licheniformis	↓	Rozs *et al*. (2001)
	Bz-Phe-Val-Arg-*p*Na	
Bacillus subtilis	↓	Macedo *et al*. (2008)
	Bz-Ile-Glu-Gly-Arg-*p*Na	
Streptomyces pactum DSM 40530	↓	Böckle *et al*. (1995)
	CBz-Phe-*o*Np	
Nesterenkonia sp. AL20	↓	Bakhtiar *et al*. (2005)
	N-succinyl-Leu-Leu-Val-Tyr-AMC	
Chryseobacterium sp.	↓	Silveira *et al*. (2009)
	L-Leu-AMC	

[a]↓, Indicates the cleavage P1 position. CBz, Carboxylbenzoyl group; Bz, benzoyl group; AMC, 7-amido-4-methylcoumarin.

and disulfide reductase) were isolated from *S. maltophilia* BBE11-1 and none of these enzymes showed keratinolytic activity independently.

During sulfitolysis, disulfide bonds between polypeptide keratin chains are cleaved and thiol groups liberated. Kunert (1972) showed that, in the presence of sulfite, disulfide bonds of the keratin substrate are directly cleaved to cysteine and S-sulfocysteine. Sulfitolysis changes the conformation of keratin and exposes more active sites, making them accessible for further digestion by alkaline protease and resulting in the release of soluble peptides and amino acids (Kunert, 1992; Böckle *et al*., 1995; Yamamura *et al*., 2002; Gradišar *et al*., 2005; Cao *et al*., 2008; Monod, 2008).

It is believed that keratin degradation in keratinolytic fungi also includes an additional mechanical step involving the frond mycelia in dermatophytes (Kanbe and Tanaka, 1982) and boring hyphae in non-dermatophytes that penetrate the substrate surface (Kanbe *et al*., 1986). The keratinolytic activities of dermatophytes are higher than the non-dermatophytes (Filipello Marchisio, 2000). Among non-dermatophytic fungi, soft keratin degraders are likely to produce thin boring hyphae and hard keratin degraders tend to form swollen boring hyphae (Korniłłowicz-Kowalska and Bohacz, 2011).

In prokaryotic cells, sulfitolysis can be achieved by the production of disulfide reductases, release of sulfite and thiosulfate (Kunert, 1989; Ramnani *et al*., 2005) or a cell-bound redox system (Brandelli *et al*., 2010; Sharma and Gupta, 2010a). Disulfide reductases produced by a number of microbes have been shown to effectively reduce the disulfide bonds, specifically: *Streptomyces pactum* (Böckle *et al*., 1995), *Vibrio* Kr 2 (Sangali and Brandelli, 2000), *Stenotrophomonas* D-1 and *S. maltophilia* BBE11-1 sp. (Yamamura *et al*., 2002; Fang *et al*., 2013), *Bacillus* sp. MTS (Rahayu *et al*., 2012) and *B. halodurans* PPKS-2 (Prakash *et al*., 2010a). It is noted that purified keratinases are generally less effective in hydrolysing native

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keratin, probably due to the removal of disulfide-bond reduction components during the purification process (Nam *et al.*, 2002; Cao *et al.*, 2008; Brandelli *et al.*, 2010). A suitable redox environment may be necessary for effective degradation of keratin. The presence of reducing agents (Böckle *et al.*, 1995; Gradišar *et al.*, 2005; Thys and Brandelli, 2006; Cao *et al.*, 2008) or a cell-bound redox system (Ramnani *et al.*, 2005; Ramnani and Gupta, 2007; Moreira-Gasparin *et al.*, 2009) stimulates keratin hydrolysis by purified keratinase. In a cell-bound redox system, the bacterial cells probably provide a continuous supply of reductant (e.g. sulfite) to break disulfide bridges (Ramnani *et al.*, 2005; Sharma and Gupta, 2010a).

In addition to sulfur-containing amino acids, sulfite is also produced by dermatophytes from environmental cysteine, a process that is governed by the key enzyme cysteine dioxygenase Cdo1, which is then secreted by the sulfite efflux pump Ssu1. As keratin is rich in cysteine, the mechanism of cysteine conversion and sulfite efflux may also play a role in keratin degradation (Grumbt *et al.*, 2013). Kasperova *et al.* (2013) also suggested that Cdo is a virulence factor, crucial for keratin degradation, as it is involved in the oxidation of cysteine to cysteine sulfinic acid during disulfide bridges cleavage.

38.3 Sources of Microbial Keratinases

Keratinolytic degraders can be found in diverse groups of microorganisms: from fungi, actinomycetes to bacteria. The origin and substrates of a number of notable keratinase producers are listed in Tables 38.5–38.8. These microorganisms are frequently isolated from keratin-rich environments such as soil and wastewater associated with the poultry industry and tannery wastes.

Dematophytic fungi are among the most recognized keratin degraders. Their virulence and pathogenicity have been linked to their ability to degrade both soft and hard keratin (Brouta *et al.*, 2002; Monod *et al.*, 2002; Giudice *et al.*, 2012). However, due to the potential risks of infection, biotechnological applications of these fungi have not been widely explored (Błyskal, 2009; Brandelli *et al.*, 2010). A comprehensive review of nearly 300 fungi species (both pathogenic and non-pathogenic) has been published by Błyskal (2009) detailing their ability to degrade different keratinous substrates. The number of strains that were able to utilize the keratinous substrates were: hair >> wool > feather > textile > hedgehog spine > nail > human plantar callus > hoof > horn (Błyskal, 2009). A number of keratinases produced by non-pathogenic fungi have been isolated and characterized (Table 38.5); these enzymes showed promising potential applications for a number of industries (see section 38.5 'Established Applications of Keratinases'). *Aspergillus* (Farag and Hassan, 2004; Kim, 2007; Anitha and Palnivelu, 2013; Mazotto *et al.*, 2013), *Coprinopsis* (Al-Musallam *et al.*, 2013), *Doratomyces* (Friedrich *et al.*, 2005), *Paecilomyces* (Gradišar *et al.*, 2005; Mohorčič *et al.*, 2007; Veselá and Friedrich, 2009), *Penicillium* (El-Gendy, 2010) and *Purpureocillium* (Cavello *et al.*, 2012) are the most common non-pathogenic fungi that produced keratinolytic activities.

Actinomycetes are also known to be a rich source of keratinase (Table 38.6). A number of mesophilic *Streptomyces* (Böckle *et al.*, 1995; Letourneau *et al.*, 1998; Bressollier *et al.*, 1999; Szabo *et al.*, 2000; Gushterova *et al.*, 2005; Tatineni *et al.*, 2008; Jaouadi *et al.*, 2010; Xie *et al.*, 2010) and thermophilic *Streptomyces* spp. (Chitte *et al.*, 1999; Ignatova *et al.*, 1999; Gushterova *et al.*, 2005, 2012; Syed *et al.*, 2009; Vasileva-Tonkova *et al.*, 2009a) produced keratinases that break down keratin. Another promising keratinase was isolated from *Nocardiopsis* sp. strain TOA-1 and has been demonstrated to degrade synthetic keratin substrate (Mitsuiki *et al.*, 2004), as well as scrapie prion (Mitsuiki *et al.*, 2006).

A number of Gram-positive and Gram-negative bacteria are also found to be important keratinase producers (Tables 38.7 and 38.8). From the Gram-positive category, members of the *Bacillus* genus are the most prominent and prolific of the keratin degraders. In particular, keratinases from *B. licheniformis* are capable of degrading feathers (Ichida *et al.*, 2001; Langeveld *et al.*, 2003; Fakhfakh *et al.*, 2009; Okoroma *et al.*, 2012), wool and animal hide (Desai *et al.*, 2010; Tiwary and Gupta, 2010) and PrP^{SC} prion (Langeveld *et al.*, 2003; Yoshioka *et al.*, 2007; Okoroma *et al.*, 2013). From the Gram-negative category, keratinases produced

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Table 38.5. Notable fungal keratinase producers, their origins, properties of the keratinases and their substrates.

Microorganism	Origin	Protease type	Molecular weight (kDa)	Optimal pH (range)	Optimal temperature (°C) (range)	Substrate	References
Aspergillus flavus K-03	Soil	Serine	31	8 (7–10)	45 (30–70)	Azokeratin, azocasein	Kim (2007)
Aspergillus niger	Mutated laboratory strains: 3T5B8, 9D40, 9D80 and 11D40	3T5B8 and 9D80: serine, 9D40 and 11D40: metallo	All strains: 60 and 9D80 also produced a band at 130	5	–	Casein, bovine serum albumin (BSA), ovalbumin, feather meal, feather keratin, human hair, sheep's wool	Mazotto *et al*. (2013)
Aspergillus parasiticus	Soil	Serine	36	7	50	Keratin	Anitha and Palanivelu (2013)
Aspergillus oryzae	Marine sediment	Metallo	60	Immobilized: 7.0–7.4, free enzyme: 8	Immobilized: 60, free enzyme: 50	BSA and casein keratin, chicken feathers, collagen, duck feathers, sheep's wool	Farag and Hassan (2004)
Candida parapsilosis	Feather waste	Serine	60	–	–	Native feather	Vermelho *et al*. (2010)
Doratomyces microspores MZKI B-399	Type strain	Serine	30	8–9	50	Porcine skin, skin keratins, nail keratins, hair keratins	Friedrich and Kern (2003), Friedrich *et al*. (2005)
Paecilomyces marquandii MZKI B-639	Type strain	Serine	33	8 (6–11)	60–65	Human nail plates and clippings	Gradišar *et al*. (2005), Mohorčič *et al*. (2007)
Penicillium spp. Morsy1	Marine soft coral *Dendronephthya hemprichii*	Metallo (two types I and II)	I: 19, II: 40	I: 7–8, II: 10–11	I: 50, II: 60–65	Poultry waste	El-Gendy (2010)

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Purpureocillium lilacinum (formerly *Paecilomyces lilacinus*)	Soil	Serine	37	6 (4–9)	60 (20–65)	Hair waste, blood	Cavello *et al*. (2012, 2013)
Scopulariopsis brevicaulis	–	Serine (two types KI and KII)	KI: 40–45, KII: 24–29	KI: 40, KII: 35	7.8	Human hair	Malviya *et al*. (1992)
S. brevicaulis	Marine sponge	–	28	7–7.5 (4–11)	50 (30–80)	Soluble keratin from chicken feathers	Sankar *et al*. (2014)
Trichoderma atroviride F6	Decayed feather	Serine	21	8–9 (4–11)	50–60 (26–70)	Casein, gelatin, BSA, feather, synthetic substrates	Cao *et al*. (2008)
Trichopyton sp. HA-2	Soil	Serine	34	8	35	Chicken feathers	Anbu *et al*. (2008)

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Table 38.6. Notable actinomycete keratinase producers, their origins, properties of the keratinases and their substrates.

Microorganism	Origin	Protease type	Molecular weight (kDa)	Optimal pH (range)	Optimal temperature (°C) (range)	Substrate	References
Actinomadura keratinilytica strain Cpt29	Poultry compost	Serine	29.2	10 (3–10)	70	Keratin, keratin azure	Habbeche *et al*. (2014)
Nocardiopsis sp. strain TOA-1	Tile-joint	–	19.1	12.5	60	Synthetic substrate	Mitsuiki *et al*. (2004, 2006)
Steptomyces pactum DSM 40530	Type strain	Serine	30	7–10	40–75	Keratin azure, feather meal and chicken feather	Böckle *et al*. (1995)
Streptomyces AB1	Soil	Serine	30	11.5	75	Keratin azure	Jaouadi *et al*. (2010)
Streptomyces albidoflavus	Soil (hen house)	Serine	18	40–70	6–9.5	Fibrous keratin, collagen, soluble keratin, gelatin, elastin, orcein	Bressollier *et al*. (1999)
Streptomyces gulbargensis	Soil	Serine-metallo	46	(7–9)	45 (30–60)	Feather meal	Syed *et al*. (2009)
Streptomyces S.K$_{1-02}$	Naturally degraded feather	Serine-metallo				Casein, keratin	Letourneau *et al*. (1998)
Streptomyces sp.	Soil	Four different serine proteases (I–IV)	I: 25, II: 50, III: 34, IV: 19	(7.5–10)	III: 50 (40–55), IV: 60–84	Keratin, azure, human hair, cock feather, and collagen	Chao *et al*. (2007), Xie *et al*. (2010)
Streptomyces sp.7	Soil from slaughterhouse	Serine-metallo	44	11	45	Keratin azure	Tatineni *et al*. (2008)
Thermoactinomyces candidus	Degrading sheep's wool	Serine	30	8.6	70	Native keratins	Ignatova *et al*. (1999)

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Table 38.7. Notable Gram-positive bacterial keratinase producers, their origins, properties of the keratinases and their substrates.

Microorganism	Origin	Protease type	Molecular weight (kDa)	Optimal pH (range)	Optimal temperature (°C) (range)	Substrate	References
Bacillus sp.	Soil from slaughterhouse and poultry farm	–	32	8	37	Azokeratin	Deivasigamani and Alagappan (2008)
Bacillus sp. 50-3	Faeces of agamid lizard *Calotes versicolor*	Serine-metallo	–	10	60	Azokeratin	Zhang *et al*. (2009)
Bacillus sp. P7	Fish intestine	Serine	–	9 (8–12)	55	Feather keratin	Corrêa *et al*. (2010)
Bacillus sp. SCB-3	Soybean waste	Metallo	134	7	40	–	Lee *et al*. (2002)
Bacillus cereus	Wool samples	Metallo	45.6	7	45	Azocasein, azocoll, keratin azure and wool	Sousa *et al*. (2007)
Bacillus circulans	Slaughterhouse wastewater	Serine	32	12.5	85	Keratin, casein, albumin, haemoglobin	Benkiar *et al*. (2013)
Bacillus halodurans PPKS-2	Rice mill effluents	Serine, disulfide reductase	30, 66	11 (7–13)	60–70	Hair from goat hide	Prakash *et al*. (2010a)
Bacillus licheni-formis ER-15	Soil	Serine	Dimeric 58 (30 + 28)	11 (7–12)	70 (30–80)	Feather, haemoglobin, hooves, fibrin and meat protein, buffalo hide	Tiwary and Gupta (2010)
B. licheniformis KI8102	Soil from poultry farm	–	32	7.5	50	Human hair, bovine hair, wool	Desai *et al*. (2010)
B. licheniformis MSK103		Serine	26	9–10	60–70	Prion-infected bovine brain homogenate	Yoshioka *et al*. (2007)
B. licheniformis N22	Primary effluent and poultry waste	–	28	8.5 (7–10)	50	Melanized feather	Okoroma *et al*. (2012, 2013)
B. licheniformis PWD-1	Type strain		33	7.5	50	Feather keratin, azokeratin	Lin *et al*. (1992), Langeveld *et al*. (2003)
Bacillus pseudofir-mus FA30-01			27.5	8.8–10.3 (5.1–11.5)	60 (30–80)	Azokeratin	Kojima *et al*. (2006)

Continued

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Table 38.7. Continued.

Microorganism	Origin	Protease type	Molecular weight (kDa)	Optimal pH (range)	Optimal temperature (°C) (range)	Substrate	References
Bacillus pumilus	Cow hide	Serine	65	8 (7.5–10)	35 (25–45)	Bovine hair	Kumar *et al*. (2008)
B. pumilus A1	Slaughterhouse wastewater		14	10	45	Feather	Fakhfakh-Zouari *et al*. (2010a, 2010b), Fakhfakh *et al*. (2013
B. pumilus SK12	Soil	Serine	45	10	60	Azo-casein, casein, gelatin, haemoglobin, elastin, feather keratin, fibrin, keratin azure, and α-keratin	Rajput *et al*. (2010)
Bacillus subtilis	Poultry waste	Three different serine proteases	54–100	9	50	Human hair; feathers	Mazotto *et al*. (2010), Villa *et al*. (2013)
B. subtilis NRC-3	Compost waste, soil	Metallo	32	7.5–8 (5–10)	40–50 (20–60)	Gelatine, casein, haemoglobin, albumin, collagen and fibrin	Tork *et al*. (2013)
B. subtilis RM-01	Soil	Serine	20.1	8 (5–8)	50 (25–55)	Chicken feather	Rai *et al*. (2009)
B. subtilis SLC	Soil	Serine	–	10 (2–12)	60	Keratin, gelatin, casein and haemoglobin	Cedrola *et al*. (2012)
B. subtilis 1271, *B. licheniformis* 1269 and *B. cereus* 1268	Agroindustrial residues from a poultry farm	Serine	*B. subtilis* and *B. licheniformis*: peptidases and keratinases in the 15–140 kDa range, *B. cereus*: keratinase: 200	10	40–50	Chicken feather	Mazotto *et al*. (2011)
Bacillus thuringiensis	Soil from feather dumping site	Metallo	–	10 (4–11)	50 (30–80)	Azokeratin	Sivakumar *et al*. (2013)
Brevibacillus	Soil	–	83.2	12.5	45	Goat skin	Rai and Mukherjee (2011)
Brevibacillus sp. strain AS-S10-II	Mutated strain	Serine	55	9–10 (5–11)	37 (25–55)	Chicken feather	Mukherjee *et al*. (2011)

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Brevibacillus brevis US575	Soil	Serine	29.1	8 (5–11)	40 (20–55)	Feather meal, chicken feather, rabit hair, goat hair, bovine hair	Jaouadi *et al*. (2013)
Clostridium sporogenes bv. *Pennavorans* bv. Nov.	Muds near the Solfatara volcano	–	28.7	8	55	Collagen, elastin and feather keratin	Ionata *et al*. (2008)
Keratinibaculum paraultunense gen. nov. sp. Nov KD-1 (anaerobic)	Grassy marchland	Serine		8.0–8.5 (6.0–10.5)	70	–	Huang *et al*. (2013)
Kocuria rosea	Soil	Serine	240	10 (8–11)	40 (10–60)	Keratin, collagen, gelatine and casein	Bertsch and Coello (2005), Bernal *et al*. (2006a)
Kytococcus sedentarius M17C (formerly *Micrococcus*)	Type strain	Serine (two types P1 and P2)	P1: 30, P2: 50	P1: 7.1, P2: 7.5	P1: 40, P2: 50	Natural, insoluble human callus	Longshaw *et al*. (2002)
Meiothermus sp. 140	Hot spring	Serine	76	8	70	Chicken feather, dove feather, duck feather, human hair, wool, and hog bristle	Kuo *et al*. (2012)
Microbacterium sp. kr10	Decomposed chicken feather	Metallo	42	7.5	50	Feather, casein, gelatin, keratin, BSA and haemoglobin	Thys and Brandelli (2006)
Nesternkonia sp. AL20	Soil	Serine	23	10 (4–11)	70 (40–80)	Casein	Gessesse *et al*. (2003)
Thermoanaerobacter keratinophilus (anaerobic)	Soil	Serine	135	Intracellular: 7, extracellular: 8	Intracellular:60, extracellular: 85	Native keratin	Riessen and Antranikian (2001)
Thermoanaerobacter sp. strain 1004-09 (anaerobic)	Hot spring	Serine	150	9.3	60	Albumin, gelatin, casein, α- and β-keratin	Kublanov *et al*. (2009a)

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Table 38.8. Notable Gram-negative bacterial keratinase producers, their origins, properties of the keratinases and their substrates.

Microorganism	Origin	Protease type	Molecular weight (kDa)	Optimal pH (range)	Optimal temperature (°C) (range)	Substrate	References
Chryseobacterium gleum	Type strain	Metallo	36	8	30	Leather	Chaudhari *et al*. (2013)
Chryseobacterium indologenes TKU014	Soil	Three different metallo proteases	40–56	5–11	30–50	Keratin	Wang *et al*. (2008)
Chryseobacterium L99 sp. nov.	–	Serine	33	8	40	Keratin azure	Lv *et al*. (2010)
Chryseobacterium sp. kr6	Feather	Metallo	64, 38, 20	8.5	50–60	Keratin azure	Brandelli (2005), Riffel *et al*. (2007), Silveira *et al*. (2010, 2012)
Chryseobacterium sp. RBT	Soil from poultry waste site			8.6	50	Chicken feathers, goat's hair	Gurav and Jadhav (2013)
Fervidobacterium pennavorans	Hot spring	Serine	130	10	80	Feathers	Friedrich and Antranikian (1996)
Fervidobacterium islandicum AW1	Geothermal hot spring	Serine	> 200 (97 subunits)	9	100	Soluble keratin; casein	Nam *et al*. (2002)
F. islandicum DSMZ 5733	Hot spring	Serine	76	8 (6–8.5)	80 (60–80)	Feather	Kluskens *et al*. (2002)
Lysobacter A03, *Arthrobacter* A08 and *Chryseobacterium* A17U psychrotolerant	Penguin feather	A03: serine, A08 and A17U: metallo	–	7–8.5	15–20	Feather	Pereira *et al*. (2014)
Lysobacter NCIMB 9497	Type strain	Metallo	148		50	Keratin azure	Allpress *et al*. (2002)
Paracocuss sp. WJ-98	Soil	Metallo	50	6.8 (6–8)		–	Lee *et al*. (2004)
Pseudomonas aeruginosa	Marine water	Metallo	34	8	60	Shrimp waste, bovine skin	Ghorbel-Bellaaj *et al*. (2012)
P. aeruginosa C11	Soil	Metallo	33	7.5 (5–10)	60	Feather, collagen, gelatin, casin	Han *et al*. (2012)
P. aeruginosa SK1	Soil	Serine	45	9	60	Feather, fibrin, inoculum and meat protein	Sharma and Gupta (2010a)
Serratia sp. HPC 1383	Tannery sludge	Serine	–	10	60	Feather	Khardenavis *et al*. (2009)

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Stenotrophomonas maltophilia BBE11-1	Poultry farm soil	Three keratinases isolated, K1, K2 and K3. K1: serine-metallo; K2: serine; K3: disulfide reductase	K1: 48, K2: 36, K3: 17	K1 and K2: 9 (7–11), K3: 8	K1: 40–60, K2 and K3: 40	Casein, BSA, feather, wool, collagen	Fang *et al*. (2013)
S. maltophilia L1	Decomposed poultry	Serine	35.2	7.8	40	Feather, hair, wool, horn	Cao *et al*. (2009)
Stenotrophomonas sp.	Deer fur	Serine and disulfide reductase	40 and 15	8 and 8	30	Casein, human hair, bovine hoof, collagen	Yamamura *et al*. (2002)
Xanthomonas maltophilia strain POA-1	–	Serine	36	9	60	Keratin	De Toni *et al*. (2002)

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by members of the genera *Chryseobacterium* or *Stenotrophomonas* have been widely studied and shown to degrade feather (Cao *et al.*, 2009; Jeong *et al.*, 2010; Chaudhari *et al.*, 2013; Gurav and Jadhav, 2013), animal hair (Cao *et al.*, 2009; Gurav and Jadhav, 2013), wool (Cao *et al.*, 2009; Fang *et al.*, 2013), hoof and horn (Yamamura *et al.*, 2002; Cao *et al.*, 2009). Some thermophilic anaerobic bacteria also demonstrated an ability to produce serine-type keratinases. *Fervidobacterium pennavorans* (Friedrich and Antranikian, 1996) and *F. islandicum* (Kluskens *et al.*, 2002; Nam *et al.*, 2002) were isolated from hot springs and produced keratinases that can degrade feathers efficiently. A novel new species of thermophilic anaerobic bacterium with keratinolytic activities, *Keratinibaculum paraultunense* gen. nov. sp. Nov KD-1, was isolated by Huang *et al.* (2013) from grassy marshland.

Other less common microbial sources that produce keratinases include several hyperthermophilic archaeons. *T. keratinophilus* (Riessen and Antranikian, 2001), *Thermoanaerobacter* sp. strains 1004-09 (Kublano *et al.*, 2009a) and VC13 (Tsiroulnikov *et al.*, 2004) are effective in hydrolysing both α- and β-keratins. In addition, *Thermococcus kodakarensis* produces keratinolytic proteases that degrade PrP^{SC} prion (Hirata *et al.*, 2013; Koga *et al.*, 2014) and *Desulfurococcus kamchatkensis* sp. Nov $1221n^{T}$ was able to utilize α-keratin (Kublanov *et al.*, 2009b). A small number of lichens including *Parmelia sulcata*, *Cladonia rangiferina* and *Lobaria pulmonaria* were also found to produce serine keratinases that could degrade hamster PrP^{TSC} prion (Johnson *et al.*, 2011).

38.4 Optimization of Keratinase Production

Production of keratinase from a commercial perspective requires an integrated approach that combines optimal fermentation conditions, operational optimization and effective downstream processing. Medium composition and culture conditions are the two important factors that affect the yield of an enzyme in a fermentation process. The keratin source usually serves as the sole carbon and nitrogen sources in a growth medium. The addition of separate carbon and nitrogen sources has been shown to increase enzyme production in some microorganisms (Ramnani and Gupta, 2004; Brandelli *et al.*, 2010), but suppress production in others (Brandelli and Riffel, 2005; Brandelli *et al.*, 2010). It is suggested that as each microorganism has its own optimal set of growth parameters, these conditions should be treated on a case-by-case basis (Cai and Zheng, 2009; Brandelli *et al.*, 2010). The most significant parameters that affect keratinase production can be investigated using a one-factor-at-a-time method. Optimization of the selected components can be achieved using a statistical approach such as employing the Plackett-Burman design and response surface methodology (RSM) to develop a mathematical model to identify the optimum conditions for higher keratinase production (Bernal *et al.*, 2006b; Tatineni *et al.*, 2007; Embaby *et al.*, 2010; Haddar *et al.*, 2010; Tiwary and Gupta, 2010; Pillai *et al.*, 2011; Rai and Mukherjee, 2011). Alternatively, the optimal components concentration can be deduced using a central composite design, followed by analysis using the RSM (Daroit *et al.*, 2011; Harde *et al.*, 2011; Bach *et al.*, 2012).

Investigations carried out on keratinase production methods have focused predominantly on submerged fermentations (SF; De Azeredo *et al.*, 2006; Brandelli *et al.*, 2010). However, the use of solid-state fermentation (SSF) has gained prominence as it has a number of advantages over SF, including: (i) lower production expense; (ii) smaller water and energy demand; (iii) less effluent production; and (iv) more stable products. Therefore,SSF technology holds a tremendous promise, especially in developing countries (Hölker and Lenz, 2005; Mukherjee *et al.*, 2008; Rai *et al.*, 2009). A number of researchers have demonstrated the potential of SSF, for example De Azeredo *et al.* (2006) reported higher keratinase activity in *Streptomyces* sp. 594 cultured in SSF than SF. Similarly, keratinolytic activity produced *by Aspergillus niger* strain 3T5B8 using SSF was found to be seven times higher than those recorded in SF (Mazotto *et al.*, 2013). Mukherjee *et al.* (2008) successfully produced keratinase from *B. subtilis* DM-04 using *Imperata cylindrica* grass and potato peelings (in a ratio of 1:1) as a low-cost medium. Likewise, Rai *et al.* (2009) obtained β-keratinase from *B. subtilis* strain RM-01 in SSF using a chicken-feather substrate; and Kumar *et al.* (2010) reported

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B. subtilis MTCC9102 was able to produce a significant amount of keratinase under optimized conditions in SSF using a horn-meal substrate. Da Gioppo *et al*. (2009) recorded comparable enzymatic activities from keratinase produced by *M. verrucaria* grown in SF and SSF using poultry feather powder and cassava bagasse as substrates. *Paenibacillus woosongensis* TKB2 cultured in SSF conditions using chicken feather as substrate, with rice straw (2:1), moistened with distilled water (1:5, w/v adjusted to pH 8.5) and fermented for 72 h, increased the production of a keratinase that can dehair goat hides within 14 h without the addition of lime (Paul *et al*., 2013a).

The use of immobilized microorganisms as well as purified enzymes has also been investigated. Prakash *et al*. (2010b) demonstrated that whole-cell immobilization was useful for continuous production of keratinase and feather degradation by *B. halodurans* PPKS-2. A number of materials have been employed to immobilize cell-free keratinase successfully including sintered glassbeads, chitin, chitosan beads, biotinylated acrylic beads and nanoparticles. Keratinase of *Aspergillus oryzae* immobilized on sintered glass beads showed a higher thermal stability at 70°C and longer half-life than the free enzyme (Farag and Hassen, 2004). Rajput and Gupta (2013) reported increased enzymatic stability at 70°C when the keratinase produced by *B. subtilis* immobilized on chitin by covalent cross-linking. Similarly, keratinase of *B. subtilis* immobilized on poly(ethylene glycol)-supported Fe_3O_4 super paramagnetic nanoparticles showed a fourfold increase in the enzymatic activity over the free enzyme; and enhanced thermal stability, storage stability and recyclability were also observed (Konwarh *et al*., 2009). The thermal stability of the keratinase from *Chryseobacterium* sp. kr6 immobilized on glutaraldehyde-activated chitosan beads also improved around twofold when compared with the free enzyme at 65°C, and the immobilized enzyme remained active after several uses (Silveira *et al*., 2012). *Aspergillus flavus* K-03 also displayed a higher level of heat stability and an increased tolerance towards alkaline pHs compared with the free keratinase and retained 48% of the original enzyme after 7 days of incubation (Kim, 2007). Improved thermal stability and pH tolerance was also observed in a fusion protein of keratinase and streptavidin immobilized on biotinylated acrylic beads, although its rate of reaction were lower than those of the free enzyme (Wang *et al*., 2003).

38.5 Established Applications of Keratinases

The ability of microbial keratinases to degrade keratin and other recalcitrant materials holds much biotechnological potential and has generated a significant amount of research interest in the last couple of decades. One of the earliest reviews on the biotechnological applications of keratinases, written by Onifade *et al*. (1998), documented the potential of these enzymes in producing livestock feeds. Subsequently, other potential biotechnological applications of keratinases have been identified. A number of excellent reviews have extensively examined the use of keratinases in the waste management industry, agroindustry, pharmaceutical and biomedical industries, leather and bioenergy industries (Thanikaivelan *et al*., 2004; Gupta and Ramnani, 2006; Karthikeyan *et al*., 2007; Brandelli, 2008, 2010; Korniłłowicz-Kowalska and Bohacz, 2011; Gupta *et al*., 2013b). In their review on the biotechnological applications and market potential, Gupta *et al*. (2013a) provided a detailed survey of keratinases applications, highlighted their uses and provided a list of commercial products involving the use of keratinases.

38.5.1 Waste management

A large number of keratinous wastes are generated every year mainly from poultry production and processing, as well as leather and textile industries (Suzuki *et al*., 2006; Korniłłowicz-Kowalska and Bohacz, 2011). Approximately 8.5 million t of poultry waste is produced worldwide annually; India contributes about 3.5 million t (Gupta *et al*., 2013a), the USA 1.8 million t and the UK 1.5 million t (Okoroma *et al*., 2012). Livestock and poultry farms and slaughterhouses also produce a significant number of keratinous wastes in the form of feather, bristles, hair, down, horns and hooves (Braikova *et al*., 2007; Korniłłowicz-Kowalska and Bohacz, 2011).

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Since the outbreak of bovine spongiform encephalopathy (BSE) in the UK, the European Union (EU) and the USA have imposed strict guidelines on the use of animal by-products. In the EU, animal by-products are grouped into three categories based on the level of risk in transmitting the pathogens and toxic substances. Only category 3 keratinous wastes can be processed and used for livestock, pet and fish food, and for composting (Lasekan *et al*., 2013).

Currently, the poultry industry manages their waste via a number of disposal methods. Carcass and feather wastes are generally rendered into bone, meat and feather meal and then burnt in cement kilns and disposed of in landfill sites (Cascarosa *et al*., 2012). Diseased mortalities are disposed of in disposal pits or incinerated (Nayaka and Vidyasagar, 2013). Composting has been championed as an environmentally friendly alternative to manage keratinous wastes (Ichida *et al*., 2001; Nayaka and Vidyasagar, 2013), where organic keratinous wastes are ultimately degraded and converted to inorganic nitrogen (ammonium and nitrate) and sulfurs (sulfates) that can be easily absorbed by plants. Nevertheless, the rate of degradation in compost may be slow due to the recalcitrant nature of keratins and their resistance to normal proteolytic enzymes. Within the compost, the succession is dominated by bacteria and actinomycetes during the first 2–4 weeks of composting; this is then gradually replaced by fungi. Cellulolytic meso- and thermophilic fungi are the first to emerge while keratinolytic strains are detected in the compost biomass at the sixth week of the process (Korniłłowicz-Kowalska and Bohacz, 2010). The growth of keratinolytic fungi is found to correlate with the mineralization of organic nitrogen and sulfur in the composted mass (Bohacz and Korniłłowicz-Kowalska, 2009). The addition of keratinase producing microorganisms as an inoculum could, in theory, accelerate and enhance the process. Ichida *et al*. (2001) showed that by adding *B. licheniformis* and a *Streptomyces* sp. isolated from the plumage of wild birds to compost bioreaction vessels, the bacteria-soaked feathers degraded more quickly and more completely than the controls. Nayaka and Vidyasagar (2013) also demonstrated that the addition of *Streptomyces albus* helped to enhance degradation of chicken feather compost and the release of valuable by-products acceptable in land-use applications. However, Tiquia *et al*. (2005) failed to observe significant changes in the rate of feather degradation when *B. licheneformis* (OWU 1411T) and *Streptomyces* sp. (OWU 1441) were co-composted with poultry litter and straw; the microbial community structure over time was found to be very similar in inoculated and uninoculated waste feather composts (Tiquia *et al*., 2005).

Under laboratory conditions, a number of microbial strains demonstrated their abilities to degrade feathers and other keratins. Chaudhari *et al*. (2013) observed the dissolution of whole chicken feathers in 72 h at 30°C by *C. gleum*. Thermophilc *B. licheniformis* strain N22 was able to degrade completely melanized feathers in 48 h in the absence of any reducing agent (Okoroma *et al*., 2012). Complete disintegration of intact feathers into soluble proteins was achieved within 5 days at 30°C by *Serratia* sp. HPC 1383 (Khardenavis *et al*., 2009) and in 3 days by *Streptomyces* AB1 (Jaouadi *et al*., 2010). *B. brevis* US575 was able to degrade a range of keratins including whole chicken feathers, rabbit fur and goat hair in 10 h at 37°C. These observations, among many others, suggest the potential of using keratinolytic microorganisms in keratinous waste management. The commercial products Versazyme® and Valkerase® manufactured by BioResource International (BRI) both contain keratinases from *B. licheniformis* and are marketed for recycling of keratin waste (Gupta *et al*., 2013a).

38.5.2 Agroindustry

In many ways, keratinous waste management is closely associated to its valorization; keratinous wastes are rich in protein and can be converted to valuable amino acids by hydrolysis, the resulting hydrolysate is a valuable agricultural resource.

Animal feed and feed supplements

Feather waste contains large amounts of amino acids such as cysteine, glycine, arginine and phenylalanine (Onifade *et al*., 1998), but the waste has to be hydrolysed to release these valuable amino acids. The processing methods commonly employed to hydrolyse feather waste

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include thermal, chemical and enzymatic treatments (Papadopoulos, 1985). Thermohydrolysis involves heating feather waste at high temperature (80–140°C) and pressure (10–15 psi). The treatment is energy intensive, causes the destruction of essential amino acids such as methionine, lysine and tryptophan and creates an additional pollution burden (Papadopoulos, 1989; Wang and Parsons, 1997). It is thought that the loss in the nutritional value is brought about by the combined effects of the destruction of certain essential amino acids and the reduction in amino acids availability. The latter is caused by the formation of cross-linkages that reduced the rate of protein digestion, possibly by preventing enzyme penetration or by blocking the sites of enzyme attack (Papadopoulos, 1989). Physicochemical treatments incorporate organic solvents such as DMSO and dimethyl formamide (DMF), acid or alkali in the keratinous waste to facilitate disulfide-bond cleavage,which in turn encourages solubilization of keratin and the release of amino acids (Coward-Kelly *et al.*, 2006a; Korniłłowicz-Kowalska and Bohacz, 2011). However, the amino acid composition of these products is low in arginine, histidine, lysine, methionine and threonine; and, especially for hair waste, the composition compares poorly with the essential amino acid requirements for various monogastric domestic animals (Coward-Kelly *et al.*, 2006b). Digestion experiments carried out on young chicks also showed that sodium hydroxide added during thermal treatment may have a negative effect on the digestibility of the feed (Papadopoulos, 1989). The use of keratinases or keratinolytic microorganisms in the treatment of feather meal overcomes some of the limitations posed by thermal and chemical treatments. Keratinase PWD-1 is found to improve the digestibility of keratin and significantly enhance the growth of poultry (Odetallah *et al.*, 2003). The application of *K. rosea* in the production of feather meal has been shown to: (i) improve the digestibility of the fermented production; (ii) increase the lysine, histidine and methionine content; and (iii) boost the availability of these amino acids (Bertsch and Coello, 2005). The commercial product Versazyme®, produced by BBI and Cibenza DP100™ by Novus International, has been marketed as a protease feed additive that improves the nutritional value of feed (Gupta *et al.*, 2013a).

The nutritional value of animal feeds can also be enriched by the introduction of a hydrolysate supplement, produced by keratinolytic microorganisms (Gupta and Ramnani, 2006; Brandelli, 2008, 2010). Wool protein hydrolysate from *B. pumilus* A1 also presented a very high *in vitro* digestibility (97%) as compared with that of the untreated wool (3%; Fakhfakh *et al.*, 2013). Similarly, the feather protein hydrolysate of *B. pumilus* A1 presents a signicantly higher digestibility (98%) compared with that of the untreated feathers (2%) and it also possesses antioxidant activity, thus it may be useful as supplementary protein and antioxidants in animal feed formulations (Fakhfakh *et al.*, 2011). The alkaline keratinase produced by *Brevibacillus* sp. strain AS-S10-II converted feather-keratin to at least seven volatile amino acids (cystenine, valine, threonine, lysine, isoleucine, phenylalanine and methionine; Mukherjee *et al.*, 2011). Similarly, feather hydrolysate from *Vibrio* sp. strain kr2 (Grazziotin *et al.*, 2006) and *Streptomyces* sp. (Ramakrishnan *et al.*, 2011) were found to be effective in improving the nutritional value of feather meals. It has been suggested that since keratin is naturally low in some essential amino acids such as methionine and phenylalanine, the use of keratinolytic microbial cultures may further enrich the hydrolysate by the presence of microbial proteins and biomass (Grazziotin *et al.*, 2006; Vasileva-Tonkova *et al.*, 2009a, b; Brandelli *et al.*, 2010).

Fertilizers

Hydrolysates produced by keratinolytic microorganisms are also ideal as fertilizers or soil amendments due to their high nitrogen and amino acid contents (Vasileva-Tonkova *et al.*, 2009a; Brandelli *et al.*, 2010). Alkaline hydrolysed sheep's wool (Gousterova *et al.*, 2008) and thermally degraded wool waste (Nustorova *et al.*, 2006) have been shown to be beneficial to both plants and soil microbes as the hydrolysed product could be readily utilized by the soil microorganisms. Rice seeds treated with feather hydrolysate from *Bacillus* sp. AJ4 and AJ9 demonstrated a 30% increase in vigour index as well as improvement in feed conversion ratio and plant growth (Arasu *et al.*, 2009). Hydrolysates from bovine hooves and horns using *Paecilomyces marquandii* are also a good source of fertilizer

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as they contain large quantities of amino acids (except for proline and tryptophan) and they compared favourably with other fertilizers in promoting plant growth (Veselá and Friedrich, 2009). The filter-sterilized hydrolysate of *P. woosongensis* TKB2, using raw feather as the sole substrate, can promote the germination of seeds and growth of *Cicer arietinu* seedlings significantly; it also improves nodule formation and increases the soil fertility and can be exploited as a useful biological fertilizer (Paul *et al.*, 2013b).

38.5.3 Leather and textile industry

Leather processing involves three major processes: (i) pre-tanning (beamhouse operation) where hides or skins are cleaned using sodium sulfate (Na_2S) and lime; (ii) tanning where the leather materials are stabilized with chromium sulfate ($CrSO_4$), solvent and lime; and (iii) post-tanning and finishing where aesthetic value is added.

During the conventional lime-sulfide dehairing process, a large amount of Na_2S is involved and the waste generated by this operation causes serious environmental and waste disposal problems. Thanikaivelan *et al.* (2004) provided a detailed review on a number of biocatalysts that have used in: (i) cleansing and rehydration (soaking); (ii) removal of unwanted hair (dehairing); and (iii) removal of undesirable proteins (bating) and (iv) eliminating fat (degreasing). The use of keratinolytic microorganisms with good dehairing action has been hailed as a promising and viable alternative to chemical dehairing (Thanikaivelan *et al.*, 2004; Dettmer *et al.*, 2011, 2013). A histological study of porcine skin degradation by *Dormatomyces microsporus* revealed that keratinase first attacked the proteins in the frontiers between the stratum corneum and the rest of epidermis as well as along the border; this is followed by the attack on the epidermal layers beneath the stratum corneum and the outer sheath of hair roots (Friedrich *et al.*, 2005). *B. brevis* US575 has been shown to be effective in removing hair from rabbit, goat, sheep and bovine hides (Rai and Mukherjee, 2011; Jaouadi *et al.*, 2013) and *P. aeruginosa* A2, grown in shrimp shell powder, demonstrated a powerful dehairing capability on bovine hide (Ghorbel-Bellaaj *et al.*, 2012). Enzymatic depilation generally only requires small quantities of Na_2S and could be an eco-friendly alternative to the chemical process. Keratinases from *B. subtilis* S14 (Macedo *et al.*, 2005) and *Trichoderma harzianum* MH-20 (Ismail *et al.*, 2012) could even be applied in the absence of Na_2S. Thus the use of a keratinase-assisted tanning process can significantly reduce the impact of dehairing waste in the environment.

Keratinases produced by a number of *Bacillus* strains (Macedo *et al.*, 2005; Prakash *et al.*, 2010b; Cai *et al.*, 2011; Benkiar *et al.*, 2013), the *Brevibacillus* sp. AS-S10-II strain (Rai and Mukherhjee, 2011), *Microbacterium* sp. kr10 (Thys and Brandeli, 2006), *Aspergillus nodulans* (Gupta *et al.*, 2013a), *P. woosongensis* TKB2 (Paul *et al.*, 2013c) and *T. harzianum* MH-20 lack collagenolytic activities. These enzymes are of interest in the bating process as conventional bating enzymes containing collagenase causes physicochemical changes in the leather (Thanikaivelan *et al.*, 2004). Application of keratinases with low collagenolytic properties can breakdown keratin tissue in the follicle without affecting the tensile strength of the leather (Macedo *et al.*, 2005).

Keratin hydrolysates have also been applied successfully to the tanning and retanning processes. In the conventional chrome tanning process, a large amount of unused chromium (Cr) is discharged into the effluent causing a major pollution concern. The permissible level of Cr in the waste stream is less than 2 mg/l in most countries (Buljan, 1996), thus there is a need to improve the Cr uptake in the tanning process. The addition of keratin hydrolysate (2–3% w/w) from horn meal (using *B. subtilis*) helps to reduce the Cr level in the wastewater from 35% to 10% (Karthikeyan *et al.*, 2007). The low-molecular-weight keratin peptides present in the hydrolysates react with Cr to form a Cr–keratin complex which upon interacting with collagen in the leather enhances the uptake of Cr (Ramamurthy *et al.*, 1989). Keratin hydrolysates are used in the retanning process to improve the properties of leathers; they are used as a filling agent to enhance poor substance skin, grain smoothness and softness (Karthikeyan *et al.*, 2007).

Keratinases also have important applications in the textile industry. A number of microbial

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keratinases, including those from *B. licheniforms* (Liu *et al*., 2013), *B. cereus* (Sousa *et al*., 2007), *Chryseobacterium* L99 (Lv *et al*., 2010) and *Pseudomonas* sp. (Cai *et al*., 2011), are able to improve felt-shrink resistance and dyeing characteristics of wool and polyester-blended fabric with no loss of fibre weight. It is reported that keratinase – acting in combination with other enzymes such as cutinase, lipase and transglutaminase – can be used to further improve the wool processing (Gupta *et al*., 2013a).

38.5.4 Consumer products

A number of consumer products have been known to involve keratinases, from formulation of detergents to personal care products such as shampoo, cosmetics and acne treatment (Brandelli *et al*., 2010; Gupta *et al*., 2013b).

Detergent

The application of keratinases in the detergent industry has been most promising as many of these alkaline proteases are thermally stable at wash temperatures and are tolerant of surfactants (Rai *et al*., 2009; Prakash *et al*., 2010a; Rajput *et al*., 2010; Cavello *et al*., 2012). Table 38.3 presents a number of keratinases that are stimulated by the presence of surfactants and reducing agents which make them ideal candidates for detergent formulation, notably: *A. keratinilytica* Cpt29 (Habbeche *et al*., 2014), *A. parasiticus* (Anitha and Palanivelu, 2013), *Brevibacillus* sp. AS-S10-II (Mukherjee *et al*., 2011), *C. gleum* (Chaudhari *et al*., 2013) and *S. maltophilia* BE11-1 (Fang *et al*., 2013). Due to their substrate specificity, keratinases can clean within a short period of time without damaging the fibre strength and structure (Paul *et al*., 2014) and a number of keratinases are shown to be capable of hydrolysing keratinous materials that fix on soiled collars and cuffs (Gupta and Ramnani, 2006). The alkaline keratinase of *P. woosongensis* TKB2 is effective at removing blood stains from surgical garments and composite stains of blood, egg yolk and chocolate from conventional clothes in a short period without changing the texture of the cloth and cloth fibres (Paul *et al*., 2014). Similarly, keratinase of *B. thuringiensis* TS2 is also effective in the removal of blood and egg stains as well as depilation of goat hide (Sivakumar *et al*., 2013). Another application of keratinases in the detergent industry involves their uses in cleaning up drains that are clogged with keratinous waste and keratinous dirt associated with laundry (Itsune *et al*., 2002; Farag and Hasan, 2004; Brandelli, 2008). A commercial product, BioGuard Plus, is manufactured by RuShay Inc. and marketed for drain pipe and septic tank cleaning (Gupta *et al*., 2013a).

Personal care products

Hair is comprised mainly of keratin protein (90%) and a small amount of lipid (1–9%). Keratin hydrolysates are efficient restorers in hair care processes, they contain active peptides that repair and condition the hair (Villa *et al*., 2013). Most keratin hydrolysates for hair care products are obtained from nails, horns and wool via chemical hydrolysis and hydrothermal methods (Barba *et al*., 2008). However, using microbial keratinases to obtain keratin hydrolysis is also gaining popularity (also see section 38.5.2 'Agroindustry'). Crude chicken feather hydrolysate produced by *S. maltophilia* is found to be protective to hair, as evidenced by the improved flexibility and strength for both normal and damaged hair (Cao *et al*., 2012). Villa *et al*. (2013) successfully formulated a mild shampoo and a rinse-off conditioner with the enzymatic hydrolysate which appeared to increase the brightness and softness of hair.

Keratinases also found applications in other personal care products (Gupta *et al*., 2013a) including: (i) cosmetic skin whitening and bleaching (Yang, 2012); (ii) exfoliation and removal of stratum corneum (Ding and Sun, 2009); (iii) removal of corns and calluses (Encarna and Elena, 2011); (iv) treatment of acne (Spyros, 2003) due to the build-up of sebum caused by blockage of hair-shafts by excess keratin; and (v) anti-dandruff shampoo (Selvam and Vishnupriya, 2012). Proteos Biotech produces two types of commercial products: (i) Keratoclean® Hydra PB and Pure100 Keratinase™, for the removal of corns and calluses; and (ii) Keratoclean® Sensitive PB and Keatopeel PB for the treatment of acne (Gupta *et al*., 2013a).

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38.5.5 Pharmaceutical industry

The two most common diseases affecting the nail unit are onychomycosis (fungal infections of the nail plate and/or nail bed) and psoriasis (an immune-mediated disease causing nail pitting and onycholysis detachment of the nail from the nail bed; Murdan, 2002). The nail plate consists mainly of 80% hard keratin and 20% soft keratin (Lynch *et al.*, 1986). In order to deliver an effective topical treatment for nail disease, it is necessary for the hard keratin of the nail plate to be weakened or compromised. A number of keratinolytic microorganisms are able to utilize keratin filaments and keratinous tissues as substrates, including: (i) native human foot skin by *Streptomyces* sp. (Xie *et al.*, 2010); (ii) native callus and extracted keratin polypeptides by *Kytococcus* (Longshaw *et al.*, 2002); and (iii) human nail plates and clippings by *P. marquandii* (Gradišas *et al.*, 2005; Mohorčič *et al.*, 2007). Using modified Franz diffusion cells and bovine hoof membranes as a model, Selvam and Vishnupriya (2012) demonstrated keratinases increase the permeability, partition coefficient and the drug reflux of the membrane. In addition, keratinase from *P. marquandii* has been demonstrated to enhance drug delivery by partially hydrolysing the nail plates (Gradišar *et al.*, 2005; Mohoričič *et al.*, 2007). Keratinases are effective instruments to hydrolyse the nail keratins as they cleave the disulfide linkage to increase the access of drug treatment, thus they can act as ungula enhancers (Gupta *et al.*, 2013b). Commercial products involving keratinases for the treatment of nail disorders include FixaFungus™ by FixaFungus and Kernail-Soft PB by Proteos Biotech (Gupta *et al.*, 2013a).

The ability of keratinases to hydrolyse keratin can also be applied in wound healing. In third-degree burns, the avascular nature of the wound eschar may prevent effective diffusion of systemic antimicrobial agents to the wound where the amount of microorganisms is usually very high (Manafi *et al.*, 2008). Enzymatic debridement of the wound will enhance penetration of the topically administered antibiotics and encourage wound healing (Krieger *et al.*, 2012). Martínez *et al.* (2013) developed a gel matrix from enrofloxacin and the keratinase produced by *Paecilomyces lilacinus* LPS #876, based on a cryogel of polyvinyl alcohol–pectin (PVA–P), for the treatment of wounds and eschars and to regulate the controlled release of antibiotics.

As dermatophytes are prolific keratinase producers, recombinant keratinases have been proposed by a number of researchers as potential candidates for the production of vaccines against dematophytes. A purified recombinant keratinolytic metalloprotease (r-MEP3) was tested as a subunit vaccine in experimentally infected guinea pigs in order to identify protective immunogens against *Microsporum canis* (Brouta *et al.*, 2003). Although the vaccination induced a strong antibody response, the protocol did not prevent fungal invasion or development of dermatophytic lesions (Vermout *et al.*, 2004). In another investigation, a recombinant keratinase (SUB3) was produced by expressing the virulence factor of *M. canis* in the *Pichia pstoris* expression system. It was found to be non-antigenic to guinea pigs; it elicited a specific lymphoproliferative response, but not a specific humoral immune response, suggesting SUB3 could be a tool for future vaccination trials in cats (Descamps *et al.*, 2003). Serine protease produced by *Dermatophilus congolensis* has also been cloned for inclusion in a vaccine to prevent lumpy wool disease (dermatophilosis) using degenerate primers and polymerase chain reaction (Mine and Carnegie, 1997). A novel subtilisin homologue, derived from *Penicillium citrinum*, with IgE antibody-binding properties has been identified and demonstrated to have a high degree of homology in the amino acid sequence with the allergen Tri r 2 in *Trichophyton*; this presents the potential of developing a vaccine against *Trichophyton* asthma (Woodfolk, 2005).

38.5.6 Prion decontamination

PrP^{Sc} has less α-helical content than PrP^{C} and is rich in β-sheet structure (Pan *et al.*, 1993). It is the cause of all neurodegenerative prion diseases (Colby and Prusiner, 2011). Infectious prion can be introduced to the environment via a number of routes including: (i) improper disposal of mortalities; (ii) shedding of biological materials; (iii) effluents from slaughterhouses and hospitals (Bartelt-Hunt and Bartz, 2013); and (iv) recycling waste products such as bonemeal of infected animals (Johnson *et al.*, 2011).

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Storage and disposal of these clinical and biological wastes is a major public health concern.

Incineration, thermal hydrolysis and alkaline hydrolysis are the common treatments employed to destroy prions. These methods are harsh and energy intensive, they cause irreversible damage to delicate medical instruments and prevent the capture of any recoverable materials (Okoroma *et al*., 2013). The ability of keratinases to degrade the β-keratin of feathers provides an environmentally friendly and sustainable alternative to degrade prion. Since the earliest report of enzymatic degradation of scrapie prion by Cho (1983), a number of studies have been carried out to explore the applications of microbial keratinases to treat and degrade prion from a number of microbial sources, including proteases from *Bacillus* sp. (Langeveld *et al*., 2003; Yoshioka *et al*., 2007; Okoroma *et al*., 2013), *Streptomyces* sp. (Hui *et al*., 2004; Tsiroulnikov *et al*., 2004), *T. kodakarensis* (Hirata *et al*., 2013), *Nocardiopsis* sp. TOA-1 (Mitsuiki *et al*., 2006), lichens (Johnson *et al*., 2011) and other thermophilic organisms such as *Thermoanaerobacter*, *Thermosipho* and *Thermococcus* sp. (Suzuki *et al*., 2006). The keratinase produced by *B. licheniformis* PWD-1 is able to degrade brain stem tissue from cattle infected with BSE and sheep infected with scrapie in the presence of detergent and at elevated temperature (> 100°C; Langeveld *et al*., 2003). *B. licheniformis* N22 can produce a keratinase that degrades scrapie prion to undetectable levels in the presence of a biosurfactant using Western blot analysis and cell culture assay within 10 min at 65°C (Okoroma *et al*., 2013). Similarly, keratinases from *Thermoanaerobacter* subsp. S290 and *Streptomyces* subsp. S6 have been shown to degrade brain homogenates of mice infected with the 6PB1 BSE strain (Tsiroulnikov *et al*., 2004). The keratinase E77 from *Streptomyces* sp. (Hui *et al*., 2004) and NAPase from *Nocardiopsis* (Mitsuiki *et al*., 2006) can degrade hamster brain homogenate containing scrapie prions. The enzymes extracted from *P. sulcata*, *C. rangiferina* and *L. pulmonaria* are able to reduce the prion protein from hamsters, mice and deer infected with transmissible spongiform encephalopathies (TSEs) (Johnson *et al*., 2011).

Three commercial keratinase-based enzymes are marketed for degradation of infectious prion proteins: (i) Versazyme® is manufactured by BRI; (ii) Pure100 Keratinase™ is produced by Proteos Biotech; and (iii) Prionzyme™ produced by Genencor International (Gupta *et al*., 2013a). Coll *et al*. (2007) measured the effectiveness of Versazyme® in degrading BSE prion in meat and bonemeal (MBM). They found that the enzyme catalysed the hydrolysis of MBM to improve the solubility of insoluble proteins, and it was more effective against bone than soft tissue particles. Prionzyme™ is currently the only effective enzyme-based decontamination technology that demonstrates significant removal of prion from medical and dental instruments (Gupta *et al*., 2013a).

Composting may also serve as a practical and economical means of disposing of specified risk materials or animal mortalities potentially infected with prion diseases. A thermophilic condition and alkaline environment is highly conducive for microbial keratinase activity (see section 38.2.1 'Optimal pH and temperature'). A number of studies have demonstrated biodegradation of prion protein using compost (Huang *et al*., 2007; Xu *et al*., 2013). In a field trial, Xu *et al*. (2014) also observed that composting reduced PrP^{TSE}, resulting in one 50% infectious dose (ID_{50}) remaining in every 5600 kg of final compost for land application.

38.6 Potential Applications of Keratinases

In addition to the established biotechnological applications, there are a number of potential applications that utilize the ability and stability of keratinase to hydrolyse keratin over a range of temperatures and pH values and in the presence of alkaline or reducing agents.

38.6.1 Biological control

The potential for keratinases to act as a biological control agent has been explored by several research groups recently. Keratinase produced by *S. maltophilia* R13 is effective against a number of fungal pathogens including *Fusarium solani*, *Fusarium oxysporum*, *Mucor* sp. and *A. niger* that cause diseases in valuable plants and crops (Jeong *et al*., 2010). Similarly, keratinase produced by *Thermoactinomyces* showed antifungal

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properties against these plant pathogens (Gousterova *et al.*, 2012). Yue *et al.* (2011) reported that the keratinase produced by *Bacillus* sp. 50-3 has the ability to work effectively against agricultural pests such as root-knot nematodes (*Meloidogyne incognita*).

In insects, the tracheae are found on the exoskeleton and each tracheal tube is lined with a thin strip of cuticle called the taenidia which reinforces the tracheae to maintain the structure of the tracheal walls. As insect tracheal taenidia contain a protein similar to the vertebrate keratins (Baccetti *et al.*, 1984), this protein may present a possible target for keratinase hydrolysis to control harmful insects such as mosquitoes that are the major vectors of a number of serious tropical diseases. The use of two recombinant baculoviruses containing the *ScathL* gene from *Sarcophaga cylindric* (vSynScathL) and the keratinase gene from *Aspergillus fumigatus* (vSynKerat) has been successful in destroying the larvae of an agricultural pest, *Spodoptera frugiperda*, by degrading extracellular matrix proteins and interfering with the phenoloxidase activity of the insect host (Gramkow *et al.*, 2010). Tangentially, keratinase hydrolysate can be used as a substrate for pesticide production. Poopathi and Abidha (2007) found that poultry waste is a low-cost and effective substrate to cultivate *Bacillus sphaericus* and *B. thuringiensis* serovar *israelensis* to produce mosquitocidal toxin.

38.6.2 Green energy

Conversion of keratinous waste into biofuel is a promising application to generate green energy that may address some of the global demand for energy. In a two-step formation process, keratinous waste was first hydrolysed by *B. lichenifomis* and the hydrolysate was subsequently utilized by*Thermococcus litoralis* to produce biohydrogen gas (Bálint *et al.*, 2005). In a comparison study, bacteria from *Thermoanaerobacterales* are found to be more efficient in substrate conversion than *Clostridiaceae* and *Enterobacteriaceae* (Rittmann and Herwig, 2012). Production of methane can also be achieved by combining the biological degradation of keratin-rich waste with keratinase in an anaerobic digester. Chicken feather waste pretreated with a recombinant *B. megaterium* strain showing keratinase activity prior to biogas production, was able to produce methane in the order of 0.35 Nm^3/kg dry feathers, corresponding to 80% of the theoretical value on proteins (Forgács *et al.*, 2011, 2013).

38.6.3 Silk degumming

Keratinases also hold potential for degumming silk. Natural raw silk is composed primarily of fibroin (62.5–67%) and sericin (22–25%) (Mahmoodi *et al.*, 2010). Sericin is a fibrous protein that binds the fibroin fibres together; it renders the raw silk harsh and stiff and reduces the effectiveness of dye uptake by the material. During the degumming process, sericin is hydrolysed and solubilized by degumming agents (Chopra and Gulrajani, 1994). A number of proteases have been examined for their ability to degum silk (Arami *et al.*, 2007), but some appeared to be only suitable for treating Murshidabad silk (Chopra and Gulrajani, 1994) and many appeared to be low in specificity towards sericin (Freddi *et al.*, 2003). The use of proteases combined with ultrasonic treatment is found to improve the effectiveness of the degumming process and improve the properties of silk yarn such as strength and elongation (Mahmoodi *et al.*, 2010). The application of a more substrate-specific enzyme, such as keratinase from *B. subtilus* (Cai *et al.*, 2008) that does not hydrolyse silk, may further improve the process.

38.6.4 Other applications

As keratinase is a specific type of alkaline protease, it may find applications in areas that are currently the domain of other alkaline proteases. For example, alkaline proteases of *B. pumilus* and *Staphylococcus auricularis* are able to inhibit biofilm formation by 86% and 50%, respectively, as well as recover 0.4013 g and 0.3823 g of silver (Ag) from 1 g of X-ray and photographic films, respectively (Bholay *et al.*, 2012). Alkaline proteases from *Aspergillus versicolor* (Choudhary, 2013) and *B. subtilis* ATCC 6633 (Nakiboglu *et al.*, 2001) also provide good Ag recovery from X-ray films. Other novel and emerging applications of keratinases include removal of cerumen (earwax), pearl bleaching and processing of edible bird's nests (Gupta *et al.*, 2013a).

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38.7 Conclusion

Keratinases are versatile and valuable enzymes that degrade keratins and similar recalcitrant proteins. Increased awareness of their biotechnological applications and potential has provided strong impetus to study this group of alkaline proteases. Diverse groups of microorganisms are able to produce keratinases and more are being discovered every year. Knowledge of their chemical and biochemical characteristics improves the understanding that is needed to fully explore their value. Of the more established biotechnological applications, keratinases have proven to be highly effective in management and valorization of keratinous wastes and nutritional improvement of animal feed. Keratinase hydrolysates offer eco-friendly alternatives to improve the dehairing, tanning and retanning processes and reduce damage to the environment caused by the chemical discharge of the leather industry. Within the laundry and pharmaceutical industries, keratinases are used in improved detergent formulations, prion decontamination, enhanced drug delivery and personal care products such as nail and acne treatments. Other biotechnological prospects for keratinases are continuously being explored and investigated. The use of keratinases as biological control agents is an exciting prospect for the agroindustry and the public health domain. The involvement of keratinases and their hydrolysates in bioenergy production may help to alleviate some of the global energy demand from unsustainable sources. Microbial keratinases also promise to improve silk degumming and recovery of valuable resources such as silver from X-ray films. Novel applications of keratinases continue to emerge as research advances. Further understanding of the molecular characteristics, enzyme kinetics and the use of recombinant technology may help to broaden the substrate specificity and the applications of this important group of enzymes.

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39 Philippine Fungal Diversity: Benefits and Threats to Food Security

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Abstract
Fungal diversity plays a vital role in sustaining human life, either benefiting or threatening food security. Benefits that derive from fungi range from food and medicine, to decomposition of organic matter, recycling of nutrients and as plant growth promoters, including biological control of fungal plant pathogens. There are generally fewer threats to mankind posed by fungi, such as being pathogens of agricultural crops and mycotoxin producers, than there are benefits to mankind. This chapter focuses on the beginnings and development of mycology as a science in the Philippines and presents the main repositories of fungal cultures in the Philippines. The four case studies generated from my research are highlighted, namely: (i) biological control of plant pathogens; (ii) microbial gifts for agriculture; (iii) breeding for resistance against cereal blast; and (iv) mycotoxins in maize (corn). In view of habitat loss and climate change threatening fungi, there is an urgent need to promote fungal conservation for a sustainable agriculture and healthy planet.

39.1 Introduction

Microorganisms, particularly fungi, are responsible for the outstanding breakthroughs in medicine and agriculture, including the threat they impose to food security and food safety and yet very little is known about the need to conserve microbial diversity (Colwell, 1997; Doyle *et al.*, 2005). Mycology for most people is unheard of unless one mentions the word ‘mushroom’ or ‘fungi’ and more often than not the study of fungi is considered under botany because fungi are classified as lower plants. Mycology is first encountered in basic biology courses but very few students would be interested to pursue this study as a major in college and as a career in life. The reasons are varied and mostly stem from a person’s interest in other fields of biology whose organisms are more visible and that can be easily appreciated for their beauty, such as animals and plants (Moore *et al.*, 2008; Minter, 2011). Emphasis has focused on the importance of conservation of these life forms, but conservation of fungi is lagging behind due to lack of appreciation of the role of fungi in the environment by other biologists and conservationists. After all, threats to the survival of fungi such as climate change, loss of habitat and pollution are the same as for the rest of living organisms. For each plant that becomes extinct, fungal species associated with it also disappear (Strobel, 2002).

Although some fungi are also major threats to food security, humanity has been the beneficiary of products derived from them including breakthroughs in modern medicine and the vital role they play in cycling of carbon and nutrients

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in the ecosystem, yet little is known about conserving thousands of these fungal species (Hawksworth, 1991). It is estimated that there are 1.5 million species of fungi but only 10% of these have been discovered (Hawksworth, 2001). This estimate is rather conservative as according to Blackwell (2011) there could be 5.1 million fungal species based on high-throughput sequencing methods. Despite declining research support, proper education and training of the next generation of specialists in the field of mycology is crucial if the viability and continuity of this neglected discipline is to be ensured. It is important to tell anyone regardless of age, background or occupation the nuts and bolts of mycology. The discipline of mycology should be a 'megascience' (Hawksworth, 2009). In this chapter, the historical development of mycology and the output of my research team in light of the importance of fungal diversity on food security in the Philippines are discussed.

39.2 The Genesis and Development of Philippine Mycology

Mycological expeditions in the Philippines have been documented as early as 1820 (Graff, 1916) but teaching and research on fungi formally began during the American colonial occupation through the founding of the University of the Philippines College of Agriculture in Los Baños, Laguna (Teodoro, 1937). Also, during the Spanish period there had been reports of fungal collection mostly conducted by European and American mycologists (Teodoro, 1937). Graff in 1916 compiled a bibliography and new species list of Philippine fungi from 1820 to 1915, comprising hundreds of newly described and first reports of fungal species. The most comprehensive fungal record in the Philippines was the classic book entitled, *An Enumeration of Philippine Fungi* published in 1937 by Dr Nicanor G. Teodoro, a noted Filipino pioneer plant pathologist. For further readings on the growth and development of Philippine mycology, the reader is referred to the review paper by Tadiosa (2012). In this review, the author stated that the fungal diversity in the Philippines is estimated at 3956 species and 818 genera.

A Philippine society solely devoted to fungi – the Mycological Society of the Philippines (MSP) – was organized on 19 October 1998. At the University of the Philippines Visayas in Iloilo, Dr Tricita Quimio, professor of mycology at the University of the Philippines Los Baños (UPLB) was appointed chairman of the organizing committee by a small group of mycologists attending a conference on tropical microbial diversity. She was then the Philippine representative to the International Mycological Association Committee for Asia (IMACA). The first scientific meeting was held on 8 April 1999 at UPLB with 167 charter members. Dr Kevin B. Hyde of the University of Hong Kong talked on 'Where are the missing fungi?' and Dr T.K. Tan of the National University of Singapore on 'Mycology in ASEAN: Where are we?'.

Despite the strong historical foundation of fungal research in the Philippines, there have only been a few recent developments in terms of fungal surveys to advance the field, probably because of the dearth of mycologists in the country. Some examples include work on wood-decaying fungi in Philippine dipterocarps (Tadiosa and Militante, 2006); macrofungi of Bazal-Baubo watersheds in Aurora province (Tadiosa *et al*., 2011) and Mt Makulot, Cuenca, Batangas (Tadiosa *et al*., 2007). Tadiosa and Briones (2013) reported 75 species belonging to 36 genera and 23 families of fungi of the Taal volcano protected landscape in Southern Luzon. Fifty-three species of entomopathogenic fungi belonging to 22 genera were reported on selected areas in the Philippines from 1998 to 2001 (Villacarlos and Meija, 2004). Fungi from bamboos were intensively studied by Cai *et al*. (2003) on freshwater and river ecosystems and Hyde *et al*. (2002) examined the vertical distribution of fungi on bamboo. Su *et al*. (2014) studied the distribution and diversity of marine fungi in Manila Bay. The prospects of using DNA-based methods such as the LSU (large subunit) and ITS (internal transcribed spacer), for identifying the forest fungi causing root and wood rot in the Philippines has just begun (Case *et al*., 2013).

39.3 Case study 1: Biological Control of Plant Pathogens

Our work on biocontrol started with *Trichoderma* as a means to manage sheath blight of rice caused by *Rhizoctonia solani* (Cumagun and Lapis, 1993; Cumagun and Ilag, 1998a, b). Our group is also the first to molecularly characterize

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Philippine *Trichoderma* strains (Cumagun *et al*., 2000). Of the many *Trichoderma* strains that have been isolated and screened for biocontrol of plant diseases in the Philippines, only a single strain has reached product commercialization (Cumagun, 2012).

We also evaluated another fungus, *Paecilomyces lilacinus* strain UP1, as a biological control agent of the root knot nematode *Meloidogyne incognita* attacking tomato under screenhouse conditions (Oclarit and Cumagun, 2009). *P. lilacinus* was formulated on rice substrate in powder form. Root weight, gall index rating, number of galls, egg masses and nematodes per one gram root sample were determined and the percentage reduction in gall number was computed. Root weight and gall index ratings were significantly higher in untreated plants than those with *P. lilacinus* and with the commercial fungicide Nemacur. Number of galls, nematodes and egg masses per one gram root sample were significantly reduced by the application of *P. lilacinus* at all levels and this was comparable with Nemacur. However, the egg mass count in plants treated with the lowest concentration of the biocontrol agent was not significantly different from the non-inoculated control. The percentage reduction in gall number was the highest at treatment with 7.92×10^6 spores/ml of *P. lilacinus* (Oclarit and Cumagun, 2009).

39.4 Case Study 2: Microbial Gifts for Agriculture

Our group has been involved in a diversity study of Cercosporoid fungi and other related genera in which we utilized a Lucid key to facilitate identification (Mahamuda Begum *et al*., 2012). We reported that the genus *Edenia*, an endophytic fungus isolated in Mexico, is shown to be a hyphomycete (order Pleosporales) forming a pyronellea-like synanamorph in culture (Crous *et al*., 2009). Our strain infecting candlebush (*Cassia alata*) was isolated from the Philippines. *Edenia* has been reported to possess allelochemical (Macías-Rubalcava *et al*., 2008), antiparasitic and anticancer activities (Martinez-Luis *et al*., 2011). Our local isolate may well serve as a potential biocontrol agent against the devastating Fusarium wilt in banana that affects the country's export industry.

The Philippines is one of the tropical megabiodiversity regions of the world (Australia State of the Environment Report, 2001). It is a hotbed for plant diseases but at the same time its rainforests are rich sources of wonder drugs and antibiotics beneficial for agriculture, medicine and commerce. In light of deforestation, the beneficial microbes are being destroyed and perhaps lost forever. To study fungal endophytes that harbour plants in the tropical rainforests is an exciting field of study especially when one engages in addressing the problem of unsustainable use of fungicides in the Philippine banana export industry to control two devastating fungal diseases: Panama wilt (*Fusarium oxysporum* f. sp. *cubense* or Foc) and sigatoka leaf disease (*Mycosphaerella* spp.). Tropical race 4 of Foc is spreading in many areas of Mindanao island (Molina *et al*., 2011). Aerial spraying with fungicide is used to protect against sigatoka, with spraying taking place on average 40 times per season. This causes fungicide resistance and also the fungicide mostly reaches non-target communities of organisms in areas up to 3.2 km around the target, according to the US Environmental Protection Agency (US EPA, 2015). The Philippine Department of Health opposed the practice of aerial spraying claiming that it poses health problems to around 200,000 people who live in four provinces. *Muscodor*, an endophytic fungus obtained from tropical rainforests, produces a mixture of volatile organic compounds which effectively inhibit and kill a wide range of pathogens (Mitchell *et al*., 2008). *Muscodor* is safe to humans and wildlife and thus can be applied by aerial spraying as a viable alternative to fungicides and the banned soil fumigant, methyl bromide. This technology is expected to: (i) ensure food security and safety; (ii) promote biodiversity conservation; (iii) protect the health of the citizens and the environment; and (iv) provide sustainable pest management solutions.

A list of commercialized fungal-based products developed by the National Institute of Microbiology and Biotechnology (BIOTECH) at UPLB is shown in Table 39.1 whereas Table 39.2 shows the repositories of fungal cultures and specimens in the Philippines. All microbial collections in Table 39.2 are members of the Philippine National Collection of Microorganisms (PNCM). PNCM serves as the national repository of microbial strains in the country. It offers

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Table 39.1. The National Institute of Microbiology and Biotechnology (BIOTECH) commercialized products derived from Philippine fungi.

Product name	Fungi	Uses
Brown Magic	Endomycorrhiza	Growth promoter of orchid seedlings and protects them from diseases
Bio Quick	*Trichoderma* sp.	Bio-organic fertilizer
Bio Green	*Trichodermas* sp.	Bio-organic fertilizer
Mycogroe	Ectomycorrhizal fungi	Biofertilizer
Mycovam	Vesicular arbuscular fungi	Soil-based biofertilizer for crops except crucifers and lowland rice
VAM root inoculant	Vesicular arbuscular fungi	Growth-promoting substances and disease control
Lipase	*Rhizopus* sp.	Hydrolyses coconut oil to produce high value β-monoglyceride
Pectinase	*Aspergillus* sp.	Juice and wine clarification, oil extraction from freshly grated coconut and essential oil extraction
Microbial rennet	*Rhizopus chinensis*	Milk coagulation for cheese production

Table 39.2. Major repositories of fungal cultures and specimens in the Philippines.

Name of culture collection	Description
University of the Philippines Los Baños (UPLB) Mycological Herbarium	A collection of about 11,000 ascomycetes, basidiomycetes and myxomycetes. It provides identification services of macrofungal specimens to students, university faculty and researchers
UPLB Microbial Culture Collection	A repository of microorganisms and provides cultures upon request for instruction, research and industrial purposes. It offers other services such as microbiological analysis of samples which includes microbial cultural counts, isolation and identification, water quality analysis, short-term training in basic microbiological techniques and other microbiologically related analyses
Philippine National Herbarium Collection (PNHC)	Fungi are just one part of the vast botanical collections of 180,000 specimens of the PNHC
Microbial Research and Service Laboratory (MRSL), University of the Philippines, Natural Sciences Research Institute Culture Collection (UPCC) in Diliman	MRSL is one of the four technical laboratories of the Natural Sciences Research Institute. MRSL houses the UPCC which maintains about 500 cultures of bacteria, fungi and yeasts in viable and pure form
University of Santo Tomas Collection of Microbial Strains (USTCMS)	The USTCMS offers a unique collection of indigenous, clinical and biotechnological microbial strains such as filamentous fungi and yeasts of terrestrial and marine origin, microalgae and *Streptomyces* along with bacteria

microbiological analyses, identification of isolates and preservation of cultures. PNCM is an accredited laboratory of the Food and Drug Administration – Department of Health (FDA-DOH) and identified as a National Reference Laboratory (NRL) for microbiology by the Bureau of Products Standard of the Department of Trade and Industry (DTI). It is also a member of the World Federation for Culture Collections. At the international level, the Asian Consortium for the Conservation and Sustainable Use of Microbial Resources (ACM) was founded in 2004 by 12 Asian countries (Cambodia, China, Indonesia, Japan, Korea, Laos, Malaysia, Mongolia, Myanmar, Philippines, Thailand and Vietnam) on the occasion of the 10th International Congress on Culture Collections in Tsukuba, Japan to strengthen the relationships among Asian countries, as

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well as encourage and facilitate international cooperation for biotechnology using microbiological resources in Asia. Currently, 22 organizations from 13 countries including India are members of ACM.

39.5 Case Study 3. Breeding for Resistance Against Cereal Blast

Magnaporthe is considered to be the world's most important pathogen of cereals and a causal agent of blast disease of approximately 100 grass species. These isolates are divided into host-specific subgroups such as *Oryza* isolates pathogenic to rice, *Setaria* isolates pathogenic to *Setaria* spp. including foxtail millet (*Setaria italica*), and *Eleusine* isolates pathogenic to *Eleusine* spp. including finger millet (*Eleusine coracana*) (Kato *et al.*, 2000). In the 1980s in Brazil, blast disease was reported for the first time in wheat, the most widely cultivated crop in the world. Its causal agent proved to be a new subgroup (*Triticum* isolates) of *Magnaporthe oryzae* which had spread to other countries in South America and became a major threat to wheat production. North America is currently under alert regarding the spread of this disease. Conquering blast disease in rice alone would be enough to feed 60 million people yearly (fungicides by foliar and seed treatment provide insufficient control of the disease, thus development of resistant cultivars remains an effective, economical and environmentally friendly way to manage the disease). An isolate from oat in the Kobe University collection was isolated in the 1980s in Brazil. An infection assay revealed that this isolate (*Avena* isolate) was an *Avena*-specific strain. Although this strain has not yet caused a serious epidemic of oat blast, there is a possibility that the next blast outbreak may occur on oat in South America. To develop a strategy to combat against oat blast, genetic analyses of its avirulence/virulence and identification of resistance genes are needed. In the present study, we mapped *PWT3* by molecular approach and demonstrated that it is an avirulence gene under the control of gene-for-gene interactions by successfully identifying its corresponding resistance gene *Rwt3* in wheat and barley cultivars. *Rwt3* is the third known wheat gene for resistance to *M. oryzae* and provides resistance to isolates such as from oats (Takabayashi *et al.*, 2002).

39.6 Case Study 4. Mycotoxins in Maize

Fusarium verticillioides is a major pathogen of maize (corn) worldwide and is the most frequently reported *Fusarium* species in maize causing seedling, stalk and ear rots. The fungus is also common in farm and storage samples. It reduces the kernel quality and nutritional value and is a potential threat to human and animal health as a result of mycotoxins called fumonisins that it produces. Pre- and postharvest grain losses range from 30% to 80% reduction. This study aims to evaluate *Fusarium* contamination and mycotoxin production in maize seeds in the main island of Luzon, the Philippines. *Fusarium* species producing toxins are always associated with cereals from warm tropical countries. This work suggests that mycotoxins pose a serious food problem in the Philippines but that there has been an extreme lack of research. For example, the Food and Agriculture Organization of the United Nations (FAO) website for the Philippines shows data that relate to only two maize fumonisins (FB-1, FB-2) which were found in > 50% maize samples (D'Mello and Macdonald, 1998; Placinta *et al.*, 1999). High diversity in the population of *F. verticillioides* exists in Philippine maize fields and almost all the isolates tested using molecular tools were fumonisin producers (Magculia and Cumagun, 2011). Similarly, high variation in aggressiveness was found in the population, suggesting the ability of the fungus to evolve in response to chemicals and resistant cultivars (Cumagun *et al.*, 2011). The project provided important information relevant to human or animal consumption of maize and generated useful results such as the report on the high fumonisin ability of the isolates from the northern part of Luzon which is a considered a major maize-growing area in the Philippines (Cumagun *et al.*, 2009). Improved farming and post-handling storage and processing practices for grains should be a priority concern so that postharvest grain losses and the risk of mycotoxin contamination could be significantly reduced.

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