Handbook of Research on

Uncovering New Methods for Ecosystem Management through Bioremediation

Shivom Singh and Kajal Srivastava

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Handbook of Research on Uncovering New Methods for Ecosystem Management through Bioremediation

Shivom Singh
ITM University Gwalior, India

Kajal Srivastava
G.B. Pant University of Agriculture and Technology, India

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Lignin is the second most abundant natural polymeric carbon source on earth after cellulose. It is a plant-originated polymer with three-dimensional network of dimethoxylated (syringyl), monomethoxylated (guaiacyl), and non-methoxylated (phydroxyphenyl) phenylpropanoid and acetylated units. The structural complexity and insolubility of lignin make it highly recalcitrant for degradation. Its biological degradation is critical to the global carbon cycle. Bioligninolysis involves application of microorganisms and their enzymes in degradation of lignin whichprovide environmental friendly technology for various industrial applications. As a major repository of aromatic chemical structures, lignin bears paramount significance for its removal from woody plants/lignocellulosic material, owing to potential application of bioligninolytic systems on commercial scale. This chapter provides an overview of microbial ligninolysis and its role in carbon cycling, various industrial process and pollution abatement for natural ecosystem management.

Bioremediation, a rapidly changing and growing area of environmental biotechnology employing microorganisms, presents a potentially more effective as well as economical clean-up technique than conventional approaches. The combination of several remediation techniques are considered to improve the remediation results especially in sites with complex contamination, as most traditional methods do not provide acceptable solutions for the removal of wastes from soils. The combination of electro kinetics with bioremediation, nanotechnology, biofilms, phytoremediation, chemical oxidation or electrical heating, presents interesting perspectives for the remediation. It is expected that the combination of these technologies will expand the dimensions of the remediation process to improve the remediation results, saving energy and time. Employment of new techniques is the need of the hour to carry forward this novel technology from its embryonic stage to all its developmental stages providing it with promising attributes to address some of the grave challenges faced by our environment today.

Section 2
Bioremediation through Plant and Its Interaction with Microbes

Global contamination of soil and water is a ruthless hitch. The negative effects of contaminants on the surroundings and on human health are miscellaneous and depend on the nature of the pollution. Methods for excavation and incineration to clean polluted sites resulted in the application of bioremediation techniques. In this section, we describe some general aspects of bioremediation tools and subsequently focus on the application of plant microbes interaction. These systems can be an interesting tool to further improve and develop bioremediation into a widely accepted technique.

Environmental restoration is a phenomenon required to keep the ecosystem intact, or enhance the rejuvenation of impaired environmental media; soil, water and air. Various methods of remediation exist, yet restoring the environment to the proximal or original state appear elusive to most methods. Interestingly, phytoremediation which is a biological process does not only restore environment in a greener way, but also can adopt diverse mechanisms such phytoextraction, phytodegradation, rhizodegrdation, phytostabilization and phytovolatization, to achieve the desired outcome. The chapter also unlined the merits and a few demerits of this principle, while the identification of sustainable plants and the mitigation of time constraints were the future directions mentioned for the projection of phytoremediation as the ideal approach for the restoration of the environment.

Vegetation filter is an emerging wastewater treatment option in which phytoremediation strategies are employed for municipal applications. Short rotation woody crops combine both treatment and reuse of effluent and operate on 'zero discharge' concept. This multifunctional system has become a viable alternative solution for wastewater treatment as well as biomass production by utilizing nutrient rich wastewater as cost efficient fertilizer. Fast growing species like Salix, Eucalyptus, and Populus with high water and nutrient requirements, highly selective heavy metal uptake and high evapotranspiration rate are generally preferred as vegetation filters for wastewater treatment. However, site-specific factors such as wastewater composition, climate, soil type, permeability, species or clonal characteristics must be taken into account when considering irrigation with municipal wastewater. This chapter discussses the prospects for vegetation filters to remediate contaminated water and soil and also facilitate recycling of valuable resources in society.

Pollution from petroleum, plant and animal origin oils, which are released via oil production and shipping operations, refineries, accidental spills, effluents of different industries such as hotels, restaurants, food processing, etc. is ubiquitous in the environment. This necessitates the need for cost effective and efficient remediation technologies. Dealing with the problem chemically and physically is known to generate secondary pollutants and incurs high cost. Expediting natural attenuation via stimulating pollutant degradation activity of residential microbial community and/or introducing competent microflora in to polluted sites has been identified as the most successful and cost effective technology and is termed bioremediation. Phytoremediation, an emerging branch of bioremediation, has also been recognized as a promising treatment technology. Chapter examines the extent of work carried out in in situ and ex situ bioremediation strategies to mitigate oil pollution, the validity of such practices in terms of efficiency of the process and the future research directives.

Section 3
Applied Bioremediation: Active and Passive Approaches

Applied bioremediation is gaining enormous reliability in the field of environmental management because of their eco-compatible nature. Active and passive approaches are offering plentiful opportunities of exploring bioremediation techniques for environmental clean-up. Employing novel and integrated strategies for the development of modern bioremediation processes is desperate need of the hour. These approaches will certainly add to the advancement of knowledge and will offer the necessary priceless resource and stimulus to the scientific field worldwide.

Pseudomonas putida showed maximum percent biosorption and bed saturation occurred at 20 minutes. Optimization results of Ni(II) and Cd(II) by Pseudomonas putida from the Design Expert software were obtained as bed height of 19.93 cm, initial metal ion concentration of 103.85 mg/L, and flow rate of 310.57 mL/h. The percent biosorption of Ni(II) and Cd(II) is 87.2% and 88.2% respectively. The predicted optimized parameters are in agreement with the experimental results. Experiments were carried out at established optimum conditions of bed height of 20.77 cm, flow rate of 309.09 mL/h, and initial metal ion concentration of 109.23 mg/L and results of biosorption of Ni(II) and Cd(II) were reproduced and they were in agreement with the predicted results. Based the experimental results, it was observed that the Pseudomonas putida was the best choice to remove Nickel and Cadmium ions from wastewater in a continuous column system.

The global environment is now facing a highly critical situation due to rapid urbanization and industrialization as well as increasing population in the limited natural resources. The population growth reflects the drastic changes of the life style of the people that created anthropogenic stress on the environment. There is requirement of highly developed environmental management systems and search of biotechnological technology to remove the contaminated materials and reestablish the natural resources Bioremediation is now considered as the most useful alternative method for eradicate the contaminated material from the nature for sustainable waste management. Now with recent advancement of the genetic approach multiplies the bioremediation process for protection of the natural environment by recycling the waste materials. This chapter covers detail notes on the use of most advanced technology to boost up the bioremediation process.

Pollution is the biggest menace to the living being in this planet today. Enzyme bioremediation is a "breakthrough technology" that holds the potential of pollutant eradication through exploiting the enzyme potential by using the various techniques. Enzyme biocatalysis is referred as white biotechnology and work by green chemistry concept. Moreover, developments in the design and application of enzyme cocktails, mutienzyme complexes, promiscuous enzymes and protein families (cupin and VOC superfamily) has recently emerged a new opportunity in bioremediation. The implementation of various enzyme modification approaches intended for potential bioremediation has been done by adopting enzyme immobilization using magnetic nanoparticles, designer enzymes generation through enzyme engineering, nano-technological advancement for single enzyme nanoparticle generations, electro-bioremediation and carbon nanotube construction. Hence, enzyme bioremediation have greater positive effects and propose significant promise to pollutant bioremediation. In conclusion, the enzymatic bioremediation open the new era of pollutant eradication for clean, safe and green environment.

Foreword

Bioremediation is a promising approach to improve environmental conditions by using naturally occurring microorganisms to degrade various types of wastes. This methodology has been successfully used to treat polluted soils, oily sludges, and groundwater contaminated by petroleum hydrocarbons, solvents, pesticides and other chemicals.

Remediation through biological methods has been shown to be an efficient and cost effective technique for the cleanup of contaminated sites and also to protect, maintain and sustain environmental quality. It is a rapidly advancing field and the technology has been applied successfully to remediate many contaminated sources. Chapters included in the book "Uncovering New Methods for Ecosystem Management through Bioremediation", anticipate that new insights into process optimization, validation, and impact on the ecosystem obtained by the advanced techniques will make bioremediation a more reliable and safer technology. The decontamination of natural resources is essential for the conservation of nature and environment using bioremediation process. Thus, there is an urgent need to study the effect of various microorganisms in combination against various pollutants for the conservation of natural resources and environment management.

This book provides valuable information and focus on the role of various biological agents used in bioremediation and their broad term application and acceptance. The provided chapters will be constructive for human value to better understand the feasibility of bioremediation and deals with the significance of bioremediation as it plays significant role in the restoration of degraded land which is a vital conservation effort for sustainable development and environmental management. Bioremediation strategies helped us in developing solutions to environmental problems by developing and promoting technologies that protect and perk up the environment, advancing scientific and engineering information to support regulatory and policy decisions; and providing the technical support and information transfer to ensure implementation of environmental, regulations and strategies at the national, state, and community levels. In this new age, bioremediation is considered as one of the safer, cleaner, cost effective and environmental friendly technology for decontaminating sites which are contaminated with wide range of pollutants. This book is a good step in that direction and will be helpful for the management of contaminated ecosystem.

Uma Melkania
G.B. Pant University of Agriculture and Technology, India

Preface

"Uncovering New Methods for Ecosystem Management through Bioremediation," is a maiden venture that owes its birth to our experience and deep rooted concern about the understanding of the bioremediation, its purpose, design and prospective.

With the rising population of the world and daily life demands supplied through industries and modern industrialized agricultural systems, the need for preservation of ecosystems is increasingly revealed. The repeated occurrence of the calamities such as wars, earthquakes, and tsunamis are additional reasons that necessitate further attention to the cleaning of the polluted and/disrupted ecosystems. One of the most economical and stable approaches to cope with this vital task is the use of the techniques developed through progresses in the field of bioremediation. Bioremediation as a branch of environmental biotechnology takes advantage of various living organisms including bacteria, fungi, algae, and plants in order to remediate the contaminated ecosystems (Bhatnagar and Kumari, 2013). It is accomplished either *in situ* or *ex situ* remediation. *In situ* remediation efforts focus on treating the contaminant at the polluted site. *Ex situ* remediation refers to the treatment of contaminated water or soil at an offsite location (Shukla, et al., 2010). In such cases, soil and groundwater from the contaminated site are transported to a place (like a bioreactor), where conditions are favourable for biological degradation.

This technique is one of the safer, cleaner, cost effective and eco-friendly tool for decontaminating the contaminated environment. The use of organisms to remove or neutralize pollutants from a contaminated site for efficient management is one of the reliable and better perspectives. In other words we can say "restoration ecology" is a promising approach for improving environmental conditions. Different techniques are involved for the management of such pollution free ecosystem. A continuous search for the new biological forms is required to regulate increasing pollution and environmental problems faced by man residing in an area. The objective of this book is to conduct a comprehensive review on various sources of bioremediation agents and their limitations in treating pollutants present in the environment.

The process of bioremediation involves the use of various microorganisms or plants to treat environment contaminated with organic molecules which can be living or non-living, natural or genetically engineered. These toxic heavy metals molecules are otherwise very difficult to break or mitigate by transforming them into innocuous products (Li and Li, 2011). Currently, a wide range of microorganisms (mentioned above) and plants are being studied for use in bioremediation processes. Some of these microorganisms have already been employed as biosorbents of various pollutants (Machado, et al., 2008). Biological agents have proved their capacity for remediation however; their long term and large scale use needs the application of genetic tools.

Bioremediation is considered to be very safe and helpful technology as it relies on microbes that occur naturally in the soil and pose no threat to environment and the people living in that area. The process of bioremediation can be easily carried out on site without causing a major disruption of normal activities and threats to human and environment during transportation. Bioremediation is less expensive than other

technologies that are used for clean-up of hazardous waste (Bhatnagar and Kumari, 2013). As natural resources are foremost assets to humans their contamination resulted in long term effects of pollution (noise and radiation), global warming, ozone depletion and greenhouse gases. The decontamination of these natural resources is necessary for the conservation of nature and environment using bioremediation process. Thus, there is an vital need to study the effect of various microorganisms in combination against various pollutants for the conservation of natural resources and environment management.

THE PERSPECTIVES

Advances in science and technology, since industrial revolution has also increasingly enabled humans to exploit natural resources and cause damage to the environment. The ideal solution for pollution abatement is Bioremediation, the most effective innovative technology to come along that uses biological systems for treatment of contaminants. Although, this novel and recent technology is a multidisciplinary approach, its central thrust depends on microbiology (Dua, et al., 2002). This technology includes biostimulation (stimulating viable native microbial population), bioaugmentation (artificial introduction of viable population), bioaccumulation (live cells), biosorption (dead microbial biomass), phytoremediation (plants) and rhizoremediation (plant and microbe interaction).

Bioremediation is an interdisciplinary technology involving microbiology, engineering, ecology, geology, and chemistry. Microbes are the primary stimulant in the bioremediation of contaminated environments (Iwamoto and Nasu, 2001). However, current knowledge of biological contribution to the effect of bioremediation and its impact on the ecosystem is limited. Usually the contaminated sites are treated with traditional methods like physical, chemical and thermal processes resembling excavation and transportation. Billions of dollars are expected to be used to clean up all sites polluted with polycyclic aromatic hydrocarbon (PAHs) in coming decades (McIntyre, 2003; Roseberg, 1993). Metal contamination in India is mainly due to industrial activities and it is estimated that about $3 billion are needed to remediate the metal contaminated sites alone in USA (Glass, 2000). In the US alone, reinstatement of all contaminated sites will cost approximately $ 1.7 trillion (Irene, 2003). The bioremediation technology is cost effective, eco-friendly and alternative to conventional treatments, which rely on incinerations, volatilization or immobilization of the pollutants. The conventional treatment technologies simply transfer the pollutants, creating a new waste such as incineration residues and not eliminate the problem (Shukla, et al., 2010).

This technology has the ability to rejuvenate the contaminated environments effectively. However, rapid advances in the last few years have helped us in the understanding of process of bioremediation. The use of culture independent molecular techniques has definitely helped us to understand the microbial community dynamics, structure and assisted in providing the insight in to details of bioremediation which has surely facilitated to make the technology safer and reliable (Thomas, 2008). In this context, bioremediation in relation to process optimization, validation and its impact on the ecosystem can be performed and by judicious use of the models that can predict the activity of microorganisms that are involved in bioremediation with existing geochemical and hydrological models, transformation of bioremediation from a mere practice into a science is now a reality (Shukla, et al., 2010). With the exciting new development in this field and focus on interdisciplinary research and using it on gaining the fundamental knowledge necessary to overcome the obstacles facing current technologies and also with respect to ethical, legal, and social issues involved this technology will go a long way in cleaning the environment in near future.

ORGANIZATION OF THE BOOK

This book has been designed as a resource book and will be a useful text that could be used for an introductory course in life sciences for graduate, post graduate and research students in professional courses in Indian and overseas Universities. It is very safe and helpful technology as it relies on microbes and plants that occur naturally in the soil and pose no threat to environment and the people living in that area, hence, this skill helps not only researchers, environmentalist, scientists and policy makers, but even also helpful for common man, farmers etc. Teachers will find it handy as it serves as a one-stop resource on environment related concepts. This book contain 20 chapters, which will be helpful to the readers to grasp and asses the different angle of bioremediation for environmental management. Each chapter begins with an introductory overview that previews the chapter's contents. It will be a useful tool for biologists, chemists and engineers interested in this area with information complementary to their own fields and thus, belong on the shelf of everybody active in the study of bioremediation. Therefore, this book will be quite supportive to the audience of scientific and non-scientific field.

Section 1: Microbial Bioremediation: Process and Technologies

Chapter 1 identifies microbe associated phytoremediation technologies for management of oil sludge. The chapter sets the scene for discussions presented by various authors. In particular the chapter identifies the environmental contamination due to petroleum compounds as a serious global issue and discusses about combined eco-friendly technology of plant and microbe(s) to treat oil sludge for its better management. In recent years, microbial assisted phytoremediation treatment technologies are being studied since these are reliable and cost effective for field applications.

Chapter 2 establishes a comprehensive model using *Trichoderma* spp. that may be helpful in bioremediation. The authors of this chapter contend that, microbes are suitable agents of bioremediation as they have unique ability to adapt and survive in changing environments. *Trichoderma* spp. are ubiquitous soil fungi, other than biocontrol properties they share a very unique phenomenon of soil bioremediation. In this context, bioremediation of soil cover restoring of soil microbiota is of particular importance.

Chapter 3 reviews the bioremediation of pesticides under the influence of bacteria and fungi. The authors examine the role played by fungi and bacteria to degrade the pesticides in environment. The study also identifies the future research problems in this regard that need to be experimented. The overall aim of the chapter is that importance be paid to cleaning up pesticides from environment by bacteria and fungi, as it will be a big business for the foreseeable future around the world. Inventions such as bioremediation offer promising prospects for business developments and for environmental health and save taxpayer money.

Chapter 4 gave an approach for bioremediation by engineering microbes for heavy metal tolerance. The authors presented it as an attractive technology for removal or recovery of metal ions from the environment. Engineered microbes for heavy metal tolerance and customized bio-adsorption for recovery of toxic metal ions have proved as indispensable tools in the area of applied biotechnology. They further suggest that, in near future these novel systems/technologies for specific metal tolerance, which are spatially regulated, might revolutionize future strategies for bioremediation.

Chapter 5 overviews the mechanism of arsenic pollution in various environment, microbial diversity in such environment, correlation of their activities to the biogeochemistry of Arsenic (As) and finally application of microbes as a bioremediation tool for Arsenic detoxification and bioremediation. The authors

argue that As pollution in drinking water and soils poses a threat to over 100 million people worldwide, making it one of the largest environmental catastrophe, particularly in Bangladesh and West Bengal, where, more than one-third of the population are at risk. Hence, authors believe that the development of research in this field can provide the key to handle this toxic and ubiquitous metalloid.

Chapter 6 presents an overview of microbial ligninolysis and carbon cycling in natural ecosystems. The authors through light on novel lignin-degrading microbes and their enzymes that combine with advanced technological tools and can contribute enormously towards more efficient and environmentally sound utilization of renewable lingo-cellulosic feed stocks for sustainable production of materials and energy. The authors also elaborate importance and mechanism of microbial ligninolysis.

Chapter 7 addresses harnessing potential of indigenous microorganisms. The author contends that the combination of several remediation techniques may improve the remediation results, especially in sites with complex contamination, including recalcitrant organic compounds and inorganic contaminants. The authors also focused on latest developments and mechanism in the field of bioremediation.

Section 2: Bioremediation through Plant and Its Interaction with Microbes

Chapter 8 analyses restoration of environment through phytoremediation and mention as the ideal approach for the restoration of the environment. The authors unlined the mechanism and principle of phytoremediation and also discuss phytoextraction, phytodegradation, rhizodegrdation, Phytostabilization and phytovolatization, to achieve the desired outcome.

Chapter 9 addresses short rotation woody crops as vegetation filters and their potential application in the treatment of municipal wastewater. The emphasis has been placed on emerging holistic approaches to remediate contaminated water and soil through vegetation filter and also facilitate recycling of valuable resources in society. The authors also presented conceptual land treatment system for wastewater purification.

Chapter 10 analyses bioremediation of oil contaminated soil and water. The authors examine the extent of work carried out in, *in- situ* and *ex situ* bioremediation strategies to mitigate oil pollution, the validity of such practices in terms of efficiency of the process and the future research directives. The chapter also discusses new approaches related to waste reduction and elimination of industrial pollution, and lead to a more sustainable future.

Chapter 11 unlined the potential application of plant-microbe interaction for restoration of degraded ecosystems. The authors aim to emphasize on potential application of microbes and plants to attenuate the organic and inorganic contaminants from the contaminated sites as well as eco-restoration of mine degraded/jhom lands by way of biodegradation and phytoremediation technologies. The chapter also defines rhizosphere microorganisms as a critical link between plants and soil.

Chapter 12 addresses microbial activity during bioremediation of contaminated soil and water. The authors discussed genetically altered species for certain persistent contaminants, employing combinations of methods in order to enhance the performance and as an effective method, instead of using single microbe, groups of microbes called microbial consortia can be applied to eliminate resistant contaminants. Future success on bioremediation depends on utilization of new technologies and further research.

Section 3: Applied Bioremediation: Active and Passive Approaches

Chapter 13 is an attempt to highlight different approaches of converting waste into vermicopost and the importance of vermicomposting as compared to synthetic fertilizers. The author contends that for the development of vermicomposting processes to succeed at commercial scale, it is necessary to link different disciplines such as microbial ecology, biochemistry and microbial physiology, organic farming together with biochemical and bioprocess engineering. In short, the key to successful vermicomposting resides in continuing to develop the scientific and engineering work that provides the real bases for both the vermicomposting and its evaluation; and simultaneously in explaining and justifying the valid reasons which allow scientists and engineers to actually use these technologies for the welfare and safety of a public which is more and more concerned about the environment and its protection.

Chapter 14 include different treatments for wool and silk fabric before dyeing with natural dyes by eco-friendly methods for production of smart textile having antimicrobial activity against bacteria and fungi and this treatment include nano materials or by using natural compounds in order to increase the value of textiles. The author discusses the application of antimicrobial agents in the development in the textiles as chitosan, quaternary ammonium salt and neem. The use of antimicrobial textiles may significantly reduce the risk of infections especially when they are used in close contact with the patients or in the immediate and non-immediate surroundings.

Chapter 15 study is the optimization of process parameters in biosorption of Ni(II) and Cd(II) ions by *Pseudomonas putida* using Response Surface Methodology (RSM) in a Packed bed bioreactor. The experimental data were also tested with theoretical models to find the best fit model. The authors also elucidates RSM as an efficient approach for predictive model building and optimization of Ni(II) and Cd(II) ions using *Pseudomonas putida* and from the packed bed bioreactor studies, they noticed that the maximum biosorption yields were obtained in packed bed bioreactor at optimum conditions, the packed bed bioreactor would be a good choice for the removal of Ni(II) and Cd(II) from wastewater using *Pseudomonas putida.*

Chapter 16 covers detail notes on the use of most advanced technology to boost up the bioremediation process. The author's present the recent advancement of the genetic approach that multiplies the bioremediation process for protection of the natural environment by recycling the waste materials. The chapter also defines bioinformatics approach in bioremediation along with new methodologies like: molecular biosensor, metarouter, etc.

Chapter 17 addresses new prospects for environmental cleaning by enzymes and referred enzyme biocatalysis as white biotechnology that work on green chemistry concept. The author highlight various enzyme modification approaches intended for potential bioremediation, which has been done by adopting enzyme immobilization using magnetic nanoparticles, designer enzymes generation through enzyme engineering, nano-technological advancement for single enzyme nanoparticle generations, electrobioremediation and carbon nanotube construction. Author also emphasis on global implementation of enzyme bioremediation, as it may open the new era of pollutant eradication for safe, clean and green environment.

Chapter 18 fill in the knowledge and experience gap among researchers and technology developers, readers and the people involved in electrostatic processes applied to agriculture and food processing. It will also introduce the engineering aspects of design and development of electrostatic spraying nozzle for agricultural applications. The authors link several research areas together to provide an integrated summary of the knowledge relevant to air-assisted electrostatic spraying and electrostatically assisted

atomization of electrically conductive liquid especially an attention has been given to pesticide spraying. The emphasis of the review leans towards explanation of physics and description of experimental work, interactions between space charge gradient and electric field produced which in turn can generate instability throughout the bulk of the continuum. It also discussed the interactions between finely divided charged particulate matters and naturally occurring ions present in the atmosphere, leads to neutralization of the charged droplets and hence deteriorates the performance of the spraying system.

Chapter 19 addresses bioremediation via nanoparticle. The authors describe that, nanoparticles can also limits the use of pesticides by biosynthesizing the nanoparticles by native microbes that is emerging as a new technology for mankind to protect their crop. They also point out that, the nascent field of green nanotechnology needs to be bloomed up to make the earth more green and clean with the rapid advancement of eco-friendly microbial synthesis procedures.

The chapters are written by specialized authors in their fields and represent the latest and most complete synthesis of this subject area. The state of the science described here represents pioneer work that focuses on the new and exciting field of bioremediation. The book contains elements from all scientific and engineering disciplines known globally and lays a strong foundation in the subject that will serve to connect knowledge developed during last two centuries. The book is encyclopedic in scope and presents various types of techniques used to clean up wastes in contaminated environments. The book covers aspects related to degradative fungi, biochemistry, enzymology, reactor engineering, genetic engineering, ecology of biodegradation, and practical applications. The knowledge flows broadly from fundamental to practical aspects, making it useful to learn and apply bioremediation holistically.

Shivom Singh
ITM University Gwalior, India

Kajal Srivastava
G.B. Pant University of Agriculture and Technology, India

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Shivom Singh
ITM University Gwalior, India

Kajal Srivastava
G. B. Pant University of Agriculture and Technology, India

Section 1

Microbial Bioremediation: Process and Technologies

Microbial biodegradation of pollutants has intensified in current years as mankind strives to discover sustainable ways to clean up degraded environment. The exclusion of a wide range of pollutants and wastes from the environment is an ultimate requirement to promote a sustainable expansion of our society with low environmental impact. Biological processes play a foremost role in the subtraction of contaminants and they take advantage of the astonishing catabolic versatility of microorganisms to degrade or convert hazardous compounds.

Chapter 1
Microbe Associated Phytoremediation Technology for Management of Oil Sludge: Phytoremediation for Oil Sludge Management

Anil Kumar
Devi Ahilya University, India

Monika Chandrabhan Dhote
Devi Ahilya University, India

ABSTRACT

Environmental contamination due to petroleum compounds is a serious global issue. Oil /petroleum refineries produce huge amount of oil sludge during drilling, storage, transport, refining which spoil soil and ground water resources. Such activities release different compounds viz. alkane, mono- polyaromatic hydrocarbons (PAH), asphaltene, resins and heavy metals. Due to physico-chemical properties, PAHs are one of most targeted compounds as they are highly persistent, carcinogenic, and have mutagenic effects on ecosystem. Such problems of PAHs drag researcher's attention to find some reliable and cost effective solution for oil sludge disposal management. Since last few decades, extensive research work has been carried out on various methods for treatment of oil sludge. In recent years, microbial assisted phytoremediation treatment technologies are being studied since these are reliable and cost effective for field applications. Here, we have discussed about combined eco-friendly technology of plant and microbe(s) to treat oil sludge for its better management.

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INTRODUCTION

The demand of oil/petroleum products is increasing day by day due to rapid global industrialization which resulted in pollution load on the environment. These industries release toxic compounds in the form of oil sludge during different upstream and downstream processes viz. drilling, storage, transportation and refining. This oily waste poses a crucial environmental pollutant and creates problem of ground water contamination due to improper disposal/discharge on nearby lands of refineries. The oily sludge is recalcitrant residue which contains hydrocarbons compounds of different classes, viscous liquids, solid particles, heavy metals, asphaltene, resins etc. The total fractions of oily sludge are total petroleum hydrocarbon compounds (TPHs) classified in four main groups including aliphatics, aromatics, nitrogen sulphur oxygen (NSO) containing compounds, and asphaltenes (Hu, Li, & Guangming, 2013). In complex oily sludge, aliphatics and aromatic hydrocarbons usually contribute up to 75% of petroleum hydrocarbons which are comprised of alkanes, cycloalkanes, benzene, toluene, xylenes, naphthalene, phenols, and various polycyclic aromatic hydrocarbons (PAHs) (Ward, Singh, & Hamme, 2003). Among NSO fraction, nitrogen (N) contents are less than 3% whereas sulphur (S) contents can be in the range of 0.3-10% and the oxygen (O) contents are usually less than 4.8% (Kriipsalu, Marques, & Maastik, 2008). However, this group of hydrocarbon compounds is characterized on the basis of physico- chemical properties like water solubility and structural complexity which decides their toxicity on the environment. The lower molecular weight, simple aliphatic compounds are degraded in natural environment by volatization, photooxidation/ natural attenuation etc. However, higher molecular weight aromatic compounds which include PAHs are difficult to degrade due to low aqueous solubility and limited bioavailability. They exert harmful and toxic effect on the environment and have been classified as hazardous chemicals due to carcinogenic and mutagenic nature. Degradation of such compounds is on top priority work and needs most safe and environmental friendly treatment method. During last decade, much research has been carried out on treatment technology for oily sludge management which includes land farming, incineration, solidification/stabilization, solvent extraction, ultrasonic treatment, pyrolysis, photocatalysis, chemical treatment, and biodegradation (Hu et al., 2013; Mater et al., 2006). But no single method is capable for removing / degrading total components of oily sludge.

Bioremediation provides a reasonably viable solution for remediation of sites contaminated with petroleum/oil hydrocarbons (Shukla et al., 2011; Gojgic-Cvijovic et al*.,* 2012; Rahman, Banat, Thahira, Thayumanavan, & Lakshmanaperumalsamy, 2002). Basic microbial metabolism of contaminants involves aerobic reaction(s) that acts on a wider range of hydrocarbon compounds, carry out more difficult degradation reactions and transforms pollutants into more simple molecules than those of plants (Cunningham, Berti, & Hung, 1995; Frick, Farrell, & Germida, 1999). Microorganisms surviving on contaminated sites have the degradation potential as they are flexible in nature and get adapted quickly to contaminants on polluted sites. However, the degradation efficiency is decided by the environment key factors like contaminant concentration, bioavailability, and catabolic strength of micro-flora, nutrients requirement, moisture level and geographical situations. The limitation of low bioavailability of these hydrophobic organic pollutants can be overcome by applying microbial biosurfactant. Microbes produce surfactants or release enzymes in the presence of contaminants which play much important role in pollutants detoxification process (Rahman et al., 2002). Biodegradation pathways of aromatic compounds proceed through certain catabolic enzymes viz. catechol dioxygenase which plays a central role in degradation mechanism. This enzyme attacks on aromatic rings either on meta- or ortho- side. The gene(s) encoding for catechol dioxygenase or other monooxygenases can be used as indicator of PAH/alkane

metabolism. The detection of catechol dioxygenase activity or its encoding gene in bacterial genome at an early stage of degradation can be used as a bio-monitoring tool for remediation.

In-spite of the eco-friendly nature of bioremediation technology, it is insufficient for remediation of complex compounds of oil sludge and needs support of other technologies. The diverse class of hydrocarbons and complexity of oil sludge compounds require application of another compatible methods. In this regard, phytoremediation is an emerging green technology that can become a promising solution for decontaminating hydrocarbon-polluted soils. The phytoremediation can complement degradation for better and fast remediation of toxic hydrocarbon compounds (Shukla et al., 2011; Gunther, Dornberger, & Fritsche, 1996; Yi, & Crowley, 2007; Muratova, Dmitrieva, Panchenko, & Turkovskava, 2008). Plants enhance the removal of PAHs from contaminating soils by various processes, such as influence on the microbial community, structure and functional diversity (Jennifer, John, Hung, & Jack, 2005; Siciliano, Germida, Banks, & Greer, 2003; Child et al., 2007; Ryan, & Miya, 2001) and release of root exudates which improve physical and chemical conditions of soil and also oxygen diffusion by their roots by providing channels for air flow (Khan et al., 2001). Plant root exudates contain water soluble, insoluble, and volatile compounds which include sugars, amino acids, organic acids, fatty acids, sterols, growth factors, nucleotides, flavanones, enzymes, and other compounds (Shimp et al., 1993; Schnoor, Licht, McCutcheon, Wolfe, & Carreira, 1995). In case of plant-microbe interaction system, root exudates play an important role in aerating oil sludge contaminated soil by link between plants and microbes that leads to the rhizosphere effect. Due to these effects, microbial populations and activity in the rhizosphere can be increased which results in improved biodegradation of organic contaminants. The compounds and composition of root exudates varies with age and type of plant. Lastly, the success of biodegradation of oil sludge depends upon effective, functional rhizosphere development that includes selection of suitable plant species and bacterial cultures. The root derived exudates stimulate the catabolic gene in contaminated soil and accelerate degradation process. The combined technology of plant-microbe interaction system overcomes limitations of the existing remediation technologies and easy for field application

Extensive work has been done on biodegradation/phytodegradation of lower range hydrocarbon compounds of oil sludge or low molecular weight two to three rings PAHs. Much limited literature is available on rhizodegradation of higher rings PAHs which are more persistent because of their low bioavailability, and strong adsorption onto the soil particulate matter. However, grasses have been proved to be more effective due to their fibrous root systems, resulting in large root length and surface area per unit volume of surface soil. The fibrous roots provide a larger surface for colonization by soil microorganisms than a tap root and allow for better interaction between the soil microbial community and the contaminants. Taking into account the striking morphological features viz. massive, finely structured, deep-growing root system of *Vetiver* grass, and its adaptability to a wide range of endemic and climatic conditions throughout the tropics and subtropics, it has been used for phytoremediation studies (Greenfield, 2002).

The biodegradation of complex mixture of hydrocarbons is practically impossible by using single bacterial species and usually requires action of multi-potential bio-degraders (microbial consortium) for complete mineralization oil hydrocarbons (Mishra, Ramesh, Kuhad, & Lal, 2001). There are many known consortia of micro-organisms which are capable of degrading mineral oil hydrocarbons under laboratory or field conditions (Mishra et al., 2001; Ratajczak, Geibdorfer, & Hillen, 1998). Keeping in view this aspect of biodegradation, the present chapter has been focused on cost effective microbial assisted phytoremediation technology for oil sludge management.

HYDROCARBONS CONTAMINATION

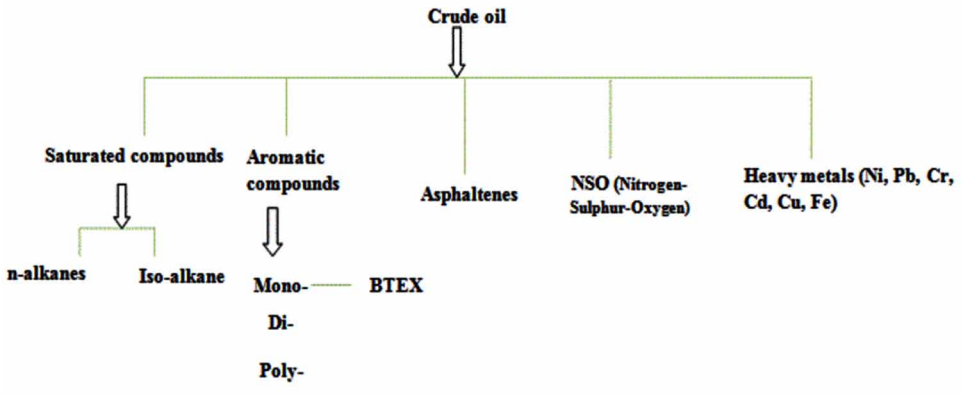
In recent era, oil spillage/ PAHs contamination is serious issue and a major challenge for environmental researchers. Indian refineries generate about 50,000 Tons/annum of oil sludge containing 30-40% oil, which is valued at about Indian Rupees 55 million (Ramaswamy, Kar, & De, 2007). India's crude oil production during 2010-11 was 37.7 million metric ton which is 11.9% higher than 33.69 million metric tons (MMT) produced during 2009-10. The production was slightly low in 2012-13, as it was 37.862 MMT, which is about 0.60% lower than the production of 38.090 MMT during 2011-12. India, USA, Germany, France, Italy, UK, Japan, China, Russia and South Korea together constitute 68.6% of total oil consumed in the world. India is the sixth largest energy consumer in the world. In India, Gujarat produces 55.10% of the onshore crude oil production and 32.3% of the gas production (Udiwal, & Patel, 2010). The petroleum oil may be considered as the principal source of energy for human; however, simultaneously it is an important environmental pollutant also.

Oil sludge is a complex mixture of hydrocarbons which is composed of Total Petroleum Hydrocarbons (TPHs), phenols, heavy metals viz. zinc, nickel, lead, chromium, cadmium, manganese and copper and treated as hazardous waste (Hu et al., 2013). The composition of oil sludge and concentration of the hydrocarbons varies with source and type of crude oil used for refining process, geographical location of refinery. TPH fraction of the oil sludge comprises aliphatic and cyclo- alkanes, aromatics, NSO (Nitrogen, sulphur, and oxygen) compounds and hydrophobic substances like asphaltenes and resins which are recalcitrant in nature. The oily sludge composition is shown in Figure 1.

The next class of hydrocarbons is aromatic compounds which are differentiated on the basis of number of benzene rings. Mono-aromatics, namely benzene, toluene, ethylbenzene, and xylene are collectively known as BTEX compounds which are important constituents in gasoline. The fusion of two or more benzene rings results in the creation of polycyclic aromatic hydrocarbons.

The PAHs constitute a large and diverse class of organic compounds which may contain simple two rings compound namely naphthalene to complex structured higher rings compounds having four, five, six or seven rings including high molecular weight Benzo perylene - (6 rings) compounds. Due to low aqueous solubility, high adsorption coefficient, limited volatility and recalcitrance towards natural

Figure 1. Saturated aliphatic hydrocarbons (n-alkanes) from C-4 to C-40 are the dominating constituents of most crude oil.



degradation, PAHs are relatively stable constituents of crude oil. Due to the resonance energy of the aromatic ring and the inertness of C-H and C-C bonds these are highly resistant compounds. These properties allow PAHs to accumulate in environment for longer time and eventually exert toxic effects. The hydrophobicity (Octanol/water coefficient, K_{ow}) of PAHs increases with increase in the number of aromatic rings and limit their bioavailability and microbial degradability. They have high potential for bio-magnification through trophic transfers and are known as suspected carcinogens due to lipophilic nature and are linked to other health problems. Therefore, due to its complex nature and presence of priority pollutants, the oil sludge is considered as potentially dangerous waste product (Marin, Hernandez, & Garcia, 2005) and hence, the huge amount of oil sludge generated during refining needs to be managed properly through safe and inexpensive way. Since last few decades, a number of traditional methods are available for detoxification of oil sludge and its components. The remediation technologies include physico-chemical and biological means but the success of these technologies is doubtful due to their non-ecological mode. The available traditional *ex situ* treatment methods are expensive and lead to contamination of surrounding areas during transportation and therefore, some long term *in situ* remediation solution is required to clean the contaminated sites. The oil contaminated lands/sites require remediation through cost effective and non-destructive/ eco-friendly ways. There is essential need to deliver some eco-friendly technology for management of such pollution problems.

Environmental Problems Associated with Higher Rings PAH Compounds in Oil Sludge

The oil waste generated from refineries is extremely complicated and contains water, oil emulsion, and impurities of suspended solids. It mainly consists of total organic carbon (TOC), chlorides, sulphates, nitrites, phenols and ammonia (Kriipsalu, Marques, Nammari, & Hogland, 2007; Saikia, Bora, & Dutta, 2003). Apart from this, it also contains solids (10-12%), water (30-50%) and oil (30-50%) and is classified as hazardous waste. Sludge produced in the oil industry can have up to 80% oil and 40% solids and is composed of various portions of alkanes (methane, ethane, and propane), mono-aromatic (benzene, toluene, ethyl benzene and xylene), polycyclic aromatic hydrocarbons (naphthalene, phenanthrene, anthracene, chrysene), paraffins, phenols, NSO compounds and asphaltene (Gojgic- Cvijoc et al., 2012; Mishra et al,. 2001). The hydrocarbons in the sludge penetrate from the top soil into the subsoil slowly, presenting a direct risk of contamination to subsoil and groundwater (Wetzel, Banks, & Schwab, 1997). On the other hand, the light hydrocarbons in the oil sludge vaporize, leaving behind a layer of oil-containing dust of soil which blows upwards to pollute the air. Therefore, the oil sludge should be treated properly to prevent harm to environment. The structural complexity of total petroleum hydrocarbons (TPHs) presents a challenge both in the understanding of their chemical behaviour and in remediation pattern because they consist of hundreds of compounds. These are considered persistent hazardous pollutants, and include compounds that can bio-concentrate and bio-accumulate in food chains, and are acutely toxic. Some compounds such as benzene and benzo[a]pyrene are recognized mutagens and carcinogens, Environmental Protection Agency (EPA, 2006). Among these TPHs, particularly high molecular weights PAHs have drawn attention due to their high persistency and non degradability in environment.

Higher PAHs tend to adsorb onto soil particulate matter and therefore neither are much bio-available, nor are translocated effectively in plants. The lower molecular mass (Mm) PAHs are lesser hydrophobic (log K, 3-5) and more water soluble than the higher Mm PAHs. Therefore, these are fairly biodegradable under aerobic conditions (Olson, Wong, Leigh, & Fletcher, 2003; Juhasz, & Naidu, 2000). Higher

molecular weight PAHs (log $K_{ow} > 5$) are much lesser bio-available and undergo very slow aerobic biodegradation. Biodegradation of two to three rings PAH compounds is well documented (Samantha, Singh, & Jain, 2002) but limited reports are available on more than four-rings (higher molecular weight) PAHs in oil sludge. Four rings PAHs include fluoranthene, pyrene, benzo[a] anthracene and chrysene. Among these, fluoranthene and pyrene are characterized by an identical molecular weight and a similar solubility in water. Contrarily, chrysene and benzo(a) anthracene are highly complex in structure with low solubility. Hence, there is need to understand the availability and biodegradation of such lesser explored PAHs (like chrysene and benzo (a) anthracene) in oil sludge. US Environmental Protection Agency has classified 16 PAH compounds as toxic priority pollutants (Liu, Han, Pan, & Riley, 2001) and few are listed in Table 1 with their physico-chemical properties.

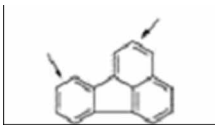
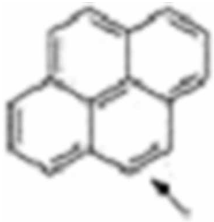
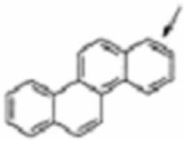
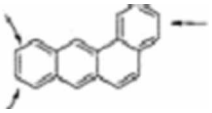
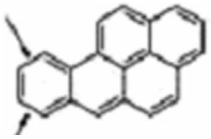
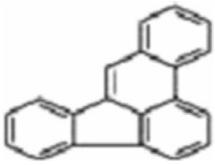
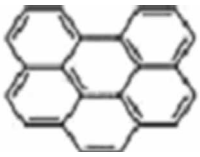
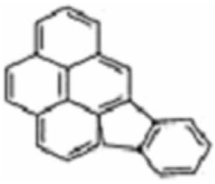
Toxicity of PAHs on the Environment

The PAHs are relatively non-volatile with low solubility in water and have a tendency to adsorb on particulate matter and form complexes. The highly complex PAHs are potentially carcinogenic and potent immune-toxicants (Propst, Lochmiller, Qualls, & McBee, 1999; European Commission, 2002), and are also dangerous to on and off-site environment due to their lipophilic nature. The toxicity increases with increase in the number of rings and molecular weight of compounds. PAHs can cause cancer by covalently binding to DNA and interfering with accurate replication, eventually leading to mutation and tumor initiation (European Commission, 2002; IARC, 2006). Levin et al., (1978) suggested that metabolites of chrysene are more potent carcinogen than the parent compound. Analyses carried out by Krishnamurthi, Saravana, & Chakrabarti, (2007) and a study by IARC, 2006 showed the presence of PAHs in petrochemical and integrated sludge extract, which was found to cause geno-toxicity, chromosomal abnormalities etc.

Hence, simply dumping these oily wastes or burning them with no previous treatment have serious environmental consequences and present a great risk to both ecosystems and human health (Baheri & Meysami, 2001). Therefore, the toxicity level of PAHs in the environment needs to be minimized with some eco-friendly technology. Although, various traditional remediation technologies are available for cleaning of hydrocarbon/oil sludge but these are not cost effective and eco-friendly. Oil industries need some long term, sustainable *in situ* remediation solution for waste management.

Since last few decades, biological based treatment technologies like bio-augmentation, biosparging, biostimulation, land farming, phytoremediation are promising solutions for oil waste management. Among these technologies, microbial assisted phytoremediation technology is emerging as safe and cost effective mode of treatment of refinery oil waste. The intrinsic capability of plant and microbe (catabolic enzymes, biosurfactant production, antioxidant enzymes, root exudates) has tremendous potential for detoxification of hydrocarbon pollutants by enhancing their aqueous solubility. Microorganisms of contaminated sites have dynamic property of biosurfactant production and active catabolic enzyme system which improve bioavailability of hydrophobic compounds. It is well known fact that bacterial genetic system is flexible which quickly adapts to environmental changes and distributes genetic information to each other on changing new environment (Bollag, 1992). Such selective microorganisms function more effectively on plant rhizosphere than applied individually. Subsequently the released plant root exudates enhance metabolic activity of soil microbes. Such efficient plant microbe interaction system combined technology is responsible for improved degradation of oil and its component *in situ* environment. This advanced approach is more sustainable, safe and economical than other available treatment technologies.

Table 1. Physico-chemical properties of PAH compounds

PAH Compound	Molecular Weight	Melting Point (^{0}C)	Water Solubility Mg/Lit of Water	Log K_{ow}	Structure
Fluoranthene	202.3	110	0.26	4.63	
Pyrene	202.3	156	0.14	4.47	
Chrysene	228.3	255	$1.52*10^{-3}$ to $2.21*10^{-3}$	5.30	
Benzo (a) anthracene	228.3	158	$1.72*10^{-3}$	5.30	
Benzo (a) pyrene	252.3	179	$1.83*10^{-3}$	5.74	
Benzo(b) fluranthene	252.3	168	$1.21*10^{-3}$	5.74	
Benzo(ghi)perylene	276.3	273	$2.61*10^{-4}$	6.20	
Indeno(1,2,3-c,d pyrene)	276.3	163	Insoluble	6.20	

MAJOR POLLUTION CONTRIBUTORS

Petroleum/oil industries are the major source of fuel (energy) and major pollution contributors. These industries generate oily waste during refining, storage, transportation process of crude oil that overburdens the environment with toxic compounds. The oil wastes disposed on the dumping sites of refineries without following any regulatory guidelines contain range of hydrocarbon compounds including simple structured alkanes to complicated PAH compounds. Additionally this waste also contains recalcitrant asphaltene, resins and toxic heavy metals. These unmanaged disposal leads to soil and ground water contamination due to leaching of oil compounds. Due to properties like low water solubility and bioavailability, most of PAHs are persisting in environment for longer time and are difficult to degrade. They enter in ecosystem by engrossing with biological processes and damage up to molecular levels due to carcinogenic and mutagenic activities (Wickliffe et al., 2014). Some mono-aromatic hydrocarbon compounds viz. BTEX also showed hazardous nature on high exposure. The available physico- chemical remediation technologies are not sufficient as these are expensive and not eco-friendly. Hence, researchers are now relying on biological based solutions to clean oil contaminated sites and to manage the oil waste (Simarro, González, Bautista, & Molina, 2013). Microorganisms of contaminated sites are excellent source for detoxification of organic pollutants. These are powered by potential catabolic enzymes that effectively attack on the pollutants and convert them to harmless/ lesser harmful end products. There are number of reports available on bioremediation studies carried out with bacterial and fungal strains (Simarro et al., 2013). Some studies have focused on development and application of microbial formulation for cleaning oil contaminated sites (Mishra et al., 2001). However, there is need for further research as contaminants and their complexity varies with environmental conditions. Exploration of genetic potential of microbes by metagenomic approach may also extend the knowledge and undiscovered ecological data of contaminated sites.

The application of selective microorganisms or their formulations will not solve the problem of contaminated /disposal sites individually. Generally, soil quality gets deteriorated with time due to long time exposure to contaminant, and hence the applied remediation technology simultaneously should support soil to resume its properties (productivity and fertility). In such cases combination of microbial techniques with plant based approaches are appropriate options for effective remediation of hydrocarbon polluted sites and restoration of soil quality. The plant releases root exudates in rhizosphere which enhance the removal of toxic compounds and help in sustaining vegetation on the contaminated sites. The result oriented rhizodegradation technology is socially and scientifically accepted for degradation of organic compounds than any other treatment methods.

Bioremediation Approach

Bioremediation is application of biological agents (microbes, plants) for detoxification/transformation of organic pollutants. Bioremediation of high molecular weight PAHs is difficult but not impractical because microbes found in natural environment have a very broad virtual ability to utilize all naturally occurring compounds as their source of carbon and energy (Volkering, Breure, Sterkenburg, & van Andel, 1992). This is due to their catabolic enzyme system which is stimulated under stressed conditions (Kanaly & Harayama, 2000). This key property of microorganisms plays an important role in the bioremediation of oil pollutants. These biodegrades either occur naturally in the contaminated sites or can be introduced into the site. Since few decades, attempts are being made to find suitable microbes

for detoxification of PAH compounds and to some extent research has been done in this area. A number of bacterial species are known to degrade PAHs and most of them are isolated from contaminated soil or sediments. *Pseudomonas aeruginosa*, *Pseudomonas fluorescens*, *Mycobacterium sp*., *Haemophilus sp., Rhodococcus sp., Paenibacillus sp.* are some of the commonly studied PAH-degrading bacteria. Walter, Beyer, Klein, & Rehm, (1991) reported that *Rhodococcus sp*. strain UW1 isolated from contaminated soil utilized pyrene and chrysene as the sole sources of carbon and energy. Rehmann, Hertkorn, & Kettrup, (2001) reported fluoranthene utilization and degradation pathway by *Mycobacterium* sp. strain KR20 in an incubation study of 10 days. Juhasz, Britz, & Stanley, (1997) reported degradation of fluoranthene, pyrene, benz[a] anthracene and dibenz[a,h] anthracene by *Burkholderia cepacia* with-in 10 days. Nwanna, George, & Olusoji, (2006) reported that *Acinetobacter anitratus*, *Alcaligenes faecalis*, *Acinetobacter mallei* and *Micrococcus varians* could grow efficiently when chrysene was used as the sole carbon source. Simarro et al. (2013) reported that *Pseudomonas* genus plays an important role in remediation of creosote contaminated soil. A degradation study by Okparanma, Ayotamuno, & Araka, (2009) indicated that *Pseudomonas* naturally degraded the 3 and 4-rings PAHs more efficiently than *Bacillus sp.* Lignolytic fungi too have the property for PAH degradation. *Phanerochaete chrysosporium*, *Bjerkandera adusta*, and *Pleurotus ostreatus* are the common PAH-degrading fungi (Haritash & Kaushik, 2009; Ogbo & Okhuoya, 2008; Eggen & Majcherzykb, 1998). Microbial degradation at some stage gets stimulated by addition of nutrients (NPK) which accelerate action of microbes and ultimately degradation of toxic compounds. Recently, Jasmine, & Mukherji (2014) reported that synergistic action of surfactant addition, nutrient addition and bio augmentation strategy are better for higher removal of TPHs of oily sludge.

Role of Microbial Consortium in Oil Sludge/ PAH Contaminated Sites Remediation

A number of previous studies have been reported on the role of multi-bacterial formulation in bioremediation of oil sludge and oil contaminated soils (Mukherjee & Bordoloi, 2011; Mohamed, Al-Dousary, Hamzah, & Fuchs, 2006; Rahman et al., 2002). Surkhoh et al., (1995) observed a sequential change in the composition of the oil-degrading bacteria over a period of time in sand samples that were contaminated with oil. Venkateswaran & Harayama, (1995) reported similar observations in sequential enrichments in the medium containing residual crude oil. Rahman et al. (2002) prepared bacterial consortium for degradation of crude oil and suggested that individual cultures showed lesser growth and degradation as compared to consortium. Several bacterial strains of inter-generic or intra-generic groups have been tested and reported for remediation of oil sludge (Samantha et al., 2002). Microorganisms can degrade hydrocarbons in a broad range of habitats and under both aerobic and anaerobic conditions (Al Turki, 2009). Some of these species are capable of degrading aliphatic hydrocarbons whereas others can degrade aromatic molecules. However, few are able to degrade both classes of molecules (White & Alexander, 1996). The complete degradation of PAHs may require a community of organisms that sequentially exchange and then transform excreted metabolites as the molecule is gradually broken down (Al Turki, 2009). Cameotra & Singh, (2008) reported that application of nutrients and crude bio-surfactants with bacterial consortia individually resulted in lesser TPH degradation (91-95%) whereas when nutrients and bio-surfactants were added together 98% TPH degradation was achieved.

Bacterial Degradation of Poly-Aromatic Compounds

The bacterial dioxygenases cleave the aromatic ring either at meta- or ortho- position but all microorganisms do not degrade organic contaminants on similar pattern. Some organisms release dioxygenase enzyme which incorporates two oxygen atom (Eweis, Ergas, Chang, & Schroeder, 1998; Pothuluri, Evans, Heinze, & Cerniglia, 1994), while other microorganisms produce mono-oxygenase that incorporates one atom of oxygen (Sutherland, 1992) converting the contaminants to simpler compounds. Catechol dioxygenase shows inherent ability of the bacteria for degradation of aromatic compounds (Cenci, Caldini, & Boari, 1999). The aerobic biodegradation reactions of aromatic compounds by mono-oxygenase and dioxygenase are presented in Figure 2 and Figure 3. Indigenous microorganisms in the soil can degrade a wide range of constituents, but when the concentration of the contaminant is high, there is a need to bio-augment or to enrich the soil for efficient degradation (Simarro et al., 2013; Mishra et al., 2001; Macnaughton et al., 1999).

Role of Bio-Molecules in Oil Sludge/PAH Contaminated Sites Remediation

Biosurfactants are important biological agents secreted by bacteria and fungi. The amphiphilic molecule of bio-surfactant works like a detergent with hydrophilic head and lipophilic tail that emulsifies the hydrophobic compounds by forming micelles. The compounds get solubilized in the core of the micelle

Figure 2. Degradation of aromatic compounds by bacterial monooxygenases
(Source: Arora, Srivastava, & Singh, 2010)

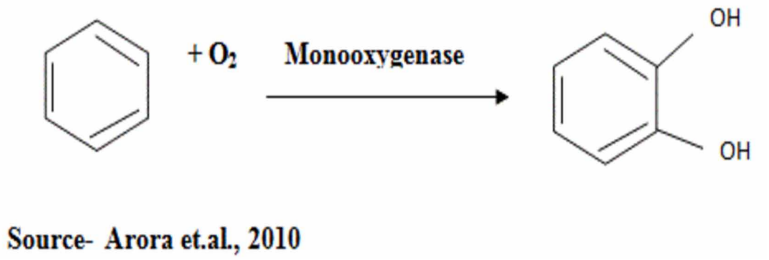
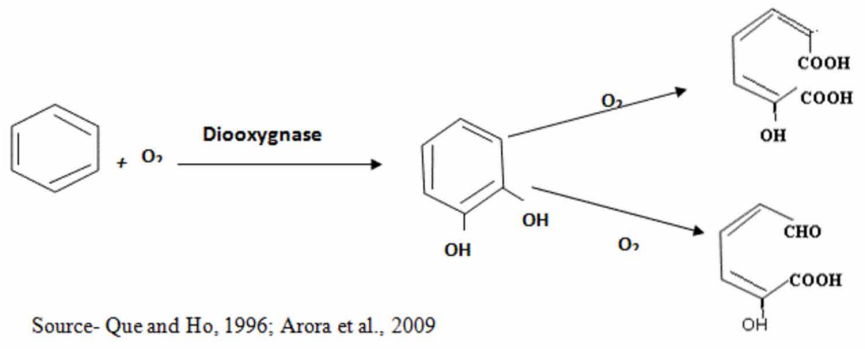


Figure 3. Degradation of aromatic compounds by bacterial dioxygenases
(Source: Que and Ho, 1996; Arora, Kumar, Chauhan, Raghava, & Jain,2009)



which leads to transfer of PAHs from solid to liquid phase (Al Turki, 2009; Banat, Makkar, & Cameotra, 2000; Volkering, Breure, & Rulkens,1997). Biosurfactants help in dispersing the hydrophobic compounds, and expand the surface area for the growth of microorganisms (Selberg, Budashova, & Tenno, 2007).

The microbial property of bio-surfactant production in the presence of PAH compounds can be effectively used for speedy bioremediation of oil-contaminated sites (Kumar, Leon, Materano, & Ilzins, 2007; Ron, & Rosenberg, 2002; Boonchan, Britz, & Stanley, 2000; Aronstein, Calvillo, & Alexander, 1991). Reports are available on production of bio-surfactants in the presence of low molecular weight PAHs (Shin, Kima, & Yeonghee, 2006; Calvo, Toledo, & Lopez, 2004). Bayoumi, (2009) reported role of six bio-surfactants producing strains (*Bacillus firmus, Bulkholderia. cepacia*, *Pseudomonas alcaligenes*, *Micrococcus lylae*, *Bacillus subtilis*, *Bacillus licheniformis*) for utilization and degradation of crude oil and certain higher PAHs.

Phytoremediation/Rhizoremediation Approach For Oil Sludge/PAH Detoxification

In-situ microbial degradation is a slow process that is affected by many environmental factors and needs support of other environmental friendly remediation technologies such as phytoremediation. The non-destructive, *in-situ* rhizoremediation technology relies on synergistic relationship between plants and their root-associated microbial communities (Kuiper, Lagerdijk, Bloemberg, & Lugtenberg, 2004; Tao et al., 2012; Kechavarzi, Pettersson, Leeds-Harrison, Ritchie, & Ledin, 2007; Khoramnejadian, Matinfar, & Khoramnejadian, 2013; Martin, George, Price Ryan, & Tibbett, 2014). Previous reports have confirmed that rhizospheric degradation is more enhanced process for oil sludge /PAHs detoxification than non vegetated bioremediation system (Agamuthu, Abioye, & Aziz, 2010; Diab, & Sandouka, 2010; Muratova et al., 2008; Parrish, Banks, & Schwab, 2005; Paquin, Ogoshi, Campbell, & Li, 2002; Pradhan, Conrad, Paterek, & Srivastava, 1998). Johnson, Maguirea, Anderson, & McGrath, (2004) found that the dissipation of chrysene is enhanced in a mixed clover/ryegrass when supplemented with rhizobial inoculum. A number of reports confirmed distribution and abundance of hydrocarbon utilizing microorganisms present in oil contaminated environments as compared to bulk soil (Gerhardt, Huang, Xiao-Dong, Glick, & Greenberg, 2009; Marc, Jordi, Maria, & Anna, 2005; Zucchi et al., 2003). Sun et al., (2011) observed 12.3% and 10.4% removal of 4-rings PAHs from contaminated soil when planted with alfalfa and tall fescue plant, respectively; whereas, intercropping resulted in improved degradation of 30.9% in 7 months. A pot study conducted by Teng et al., (2011) also found that alfalfa can play a significant role in the dissipation of PAHs in contaminated soil on inoculation with *Rhizobium meliloti,* which enhanced PAH degradation and microbial metabolism in soil. The rhizosphere soil of *Cyperus conglomeratus* plant was found to have remarkable efficiency to degrade the carcinogenic PAHs especially benzo(a)pyrene, (90.3%), chrysene (86.9%), benzo(a)flouranthene (84.1%) and indeno (1,2,3-c,d) pyrene (82.2%) (Diab, & Sandouka, 2010). Cofield, Schwab, & Banks, (2007) demonstrated that the 4-rings and 5-rings PAHs were degraded better in the planted soil (48.6% and 46.1%, respectively) than the 2-3 and 6-rings PAHs (3%, 25.8% and 6.1%, respectively). After one year study, Rezek, Wiesche, Mackova, Zadrazil, & Macek, (2008) estimated 50% reduction of total PAHs in the rhizosphere soil of ryegrass. Lee, Lee, Lee, & Kim, (2008) also demonstrated the effect of rhizosphere of grasses and legumes for PAH compounds dissipation than in the unplanted soil. A pot study conducted for higher PAHs by Campbell, Paquin, Awaya, & Li, (2002) on benzo[a]pyrene and chrysene contaminated soil with industrial hemp (*Cannabis sativa*) showed a very high tolerance to the contaminants and significant reduction of both the

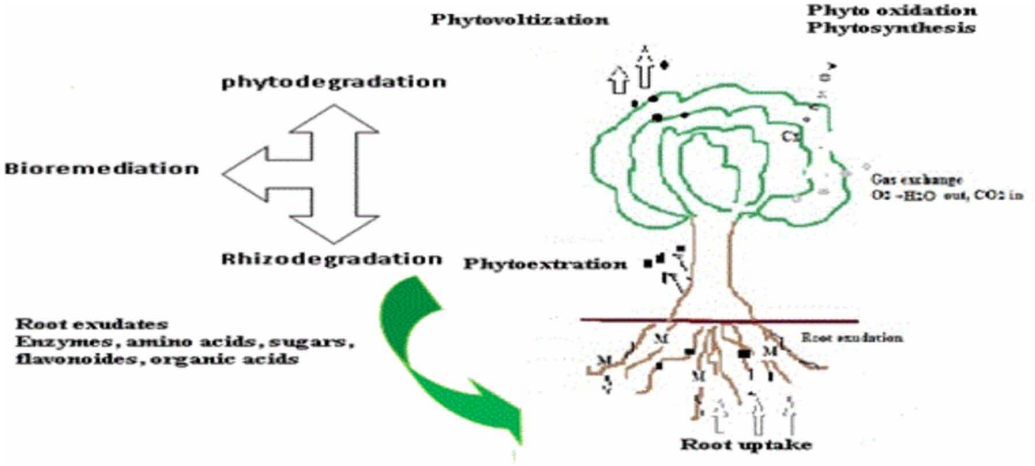
PAHs. Reilley, Banks, & Schwab, (1996) indicated that grasses and legumes enhanced the removal of PAHs from contaminated soils and such observations were also supported by Johnson et al., (2004). They found that improved remediation of chrysene got enhanced by mixed grass-legume systems, together with microbial inoculants. Olson et al., (2007) suggested that there is clear inverse correlation between PAH molecular mass and dissipation rate. Rhizospheric degradation of PAHs, specifically 4-rings compounds, is dependent on the selection of plant and their associated root microorganisms (microbial number and activity), level of contaminants (White, Wolf, Thoma, & Reynolds, 2006; Rentz, Alvarezb Pedro, & Schnoor, 2005) and many soil parameters like carbon: nitrogen ratio, aeration, pH, temperature, salinity etc. Previous studies have proven that rhamnolipids produced by *Pseudomonas* strains can enhance the uptake of PAHs by ryegrass roots and the degradation of PAHs by alfalfa (Zhang et al., 2010; Zhu, & Zhang, 2008). Arbabi, Simin, & Chimezie, (2009) carried out biodegradation of phenantherene (100, 500 and 1000 mg/kg in clayey-sand soil) by inoculating two bacterial consortium (BMTRS; Bacterial Mix of Tehran Refinery Site and BMBOZ (Bacterial Mix of Bushehr Oil Zone) and reported maximum 85% degradation by BMRTS consortium in solid phase reactor study.

Grass species are excellent plants to be used for phytoremediation due to their extensive fibrous root systems, which allow for more interaction between rhizophere microbial community and the contaminant (Hutchinson, Schwab, & Banks, 2003). Their roots can penetrate up-to a depth of 3 meters and exhibit inherent genetic diversity therefore can get established even under adverse conditions. A pilot scale phytoremediation experiment conducted by Moreira et al., (2011) with *Rhizophora mangle L* showed higher removal of TPHs from contaminated mangrove after 3 months study. Brandt, Merkl, Schultze-Kraft, Infante, & Broil, (2006) conducted a greenhouse study to determine the tolerance of *Vetiver zizanioides* in heavy crude oil contaminated soil and reported that it can tolerate 5% (w/w) petroleum concentration in the soil. Li, Luo, Song, Wu, & Christie, (2006) also suggested potential of *Vetiveria zizanioides* for degradation of benzo [a] pyrene (5-ring PAH). Muratova et al., (2008) estimated the oil sludge degradation in the root zone of mixed crop plants, grasses and legumes, among which the ryegrass accelerated clean up most effectively by degrading the entire main contaminant fraction in the oil sludge. Binet, Portal, & Leyval, (2000) showed that degradation or dissipation of 8 PAHs in the rhizosphere of ryegrass was more effective as compared to phyto-extraction, which indicated that dissipation is mainly attributed to biotransformation or biodegradation. Qixing, Zhang, Zhineng, & Weitao (2011) documented different phytoremediation mechanism of weed plants for removal of hydrocarbon compounds. In some cases, if the contaminated sites are deficient in effective number of bio-degraders, it can be bio-augmented with selective strains to improve degradation of hydrocarbon compounds (Yousaf, Andria, Reichenauer, Smalla, & Sessitsch, 2010; Germaine, Keogh, Ryan, & Dowling, 2009).

Functions of Root Exudates in Plant-Microbe Interaction System for Cleaning Oil Sludge/PAH Contaminated Sites

A representative rhizodegradation process has been predicted in Figure 4. The root exudates released in rhizospheric soil play fundamental role in degradation mechanism. The root exudates act as source of carbon, energy, nutrients, enzymes and sometimes oxygen to soil microbial populations in the rhizosphere (Phillips, Greer, Farrell, & Germida. 2012; Frick et al., 1999). The exudation of nutrition by plant roots creates nutrient enriched environment by stimulating microbial activity and number. Plant root exudates contain sugars, organic acids, amino acids as main components in addition to mucigel secreted root cell. Yoshitomi & Shann, (2001) reported that soil microorganisms exposed to root exu-

Figure 4. Rhizodegradation mechanism



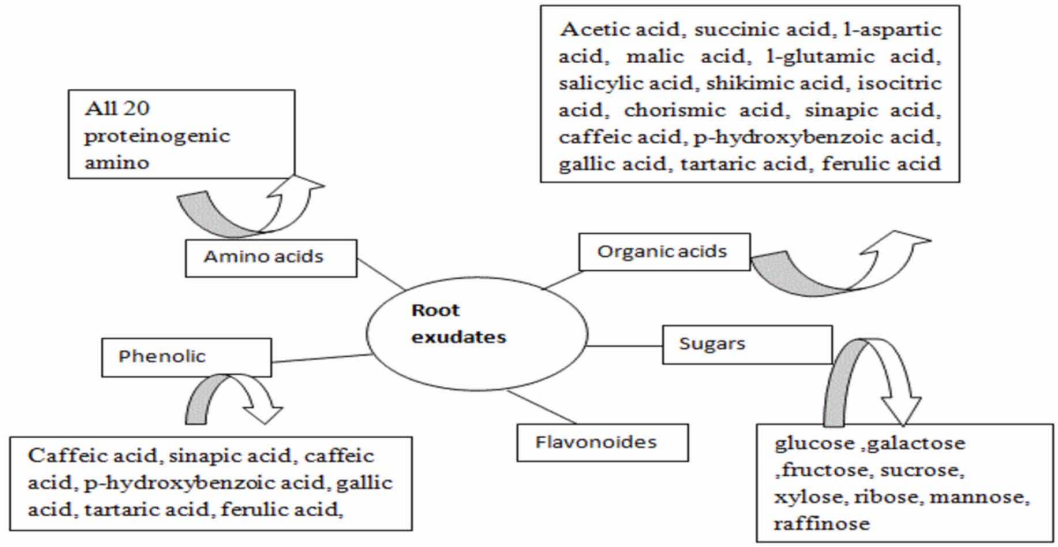
dates are perhaps able to grow better on root derived substances than a simple compound like glucose. The higher availability of organic matter in the rhizosphere has been reported to increase the size of the heterotrophic microbial community (4 to 100-fold) and the concentration of PAH degrader populations (4.5 to 15 fold) as compared to bulk soil. Therefore, the degradation of complex compounds, such as PAHs, may be enhanced by the presence of root exudates because of increased interaction between microbes, nutrients, and contaminants (Parrish et al., 2006; Rentz et al., 2005; Binet et al., 2000; Reilley et al., 1996). Rhizosphere soil supports a higher population and biomass due to increased availability of nutrients in the form of root exudates. Root exudates mediate both positive and negative interactions in the rhizosphere where positive interactions include symbiotic associations with beneficial microbes, such as *Mycorrhizae, Rhizobium* and plant growth-promoting rhizobacteria (PGPR). Whereas, negative interactions include association with parasitic plants, pathogenic microbes and invertebrate herbivores in soil (Badri & Vivanco, 2009).

Yoshitomi & Shann, (2001) observed that root exudates of corn (*Zea mays*) stimulated pyrene mineralization and also the growth of surrounding microbial community. Mueller & Shann (2007) also reported that amendment of bulk soil with root extracts significantly increased pyrene mineralization initially but not constantly. It depends upon nutrient status of the soil. A greenhouse study conducted by Parrish et al., (2006) reported 65-70% degradation of PAHs in rhizosphere of sweet clover than in tall fescue plant but did not find any changes in the presence of treatment of death and decay of root exudates. Miya & Firestone, (2000) observed that root exudates significantly enhanced phenanthrene degradation in rhizosphere soils, either by increasing contaminant bioavailability and/or increasing microbial population size and activity. Reilley et al., (1996) reported that pyrene degradation rate increased in rhizosphere than in non-rhizospheric soil. Aprill & Sims, (1990) reported that chrysene, benzo(a)anthracene, benzo(a) pyrene and dibenzo(a)anthracene had greater disappearance in vegetated soils than in non-vegetated soils. Degradation of benzo[a] pyrene, a higher rings PAH was achieved by Li et al., (2006) in rhizosphere of *Vetiver* plant while its roots also contributed in enhancing microbial number and biomass. As discussed above, combined rhizospheric potential is responsible for removal of toxic PAH compounds which could also be supported by catabolic genetic system of microorganisms of rhizosphere. Siciliano et al., (2003) observed a higher frequency of catabolic genes in tall fescue rhizosphere than in bulk soils, and suggested

that gene transfer or another mechanism of selection exists in the rhizospheric soil. Liste & Prutz, (2006) studied the expression of PAH degrading effective bacterial culture in rhizosphere of PAH contaminated soil and suggested that dioxygenase expressing bacteria were most numerous in contaminated rhizosphere of oat, mustard and cress. Rentz et al., (2005) observed that root exudates stimulated the expression of degradative enzymes of rhizospheric microorganisms and accelerated degradation mechanisms. A few organic acids of root exudates (salicylic acid) act as inducers for expression of catabolic gene which improves the efficacy of indigenous soil microorganisms for PAH-degradation (Singer, Crowley, & Thompson, 2003; Colbert et al.. 1993). Chen & Aitken, (1999) successfully used salicylate to stimulate mineralization of PAHs in soil. Kamath, Schnoor, & Alvarez, (2004) studied role of root exudates from hybrid poplars, willow, kou, milo, osage orange, mulberry, switch grass and potential root-derived substrates (e.g., sugars, carboxylic acids, amino acids, and phenolics) on the expression of nahG, one of the genes responsible for naphthalene dioxygenase transcription in *Pseudomonas fluorescens* HK44 strain. Yi & Crowley, (2007) observed that pyrene and benzo[a]pyrene degradation by Gram positive bacteria was stimulated by non-aromatic plant compounds, such as linoleic acid.

Apart from the functions of plant roots as organs for nutrient uptake, roots are able to release a wide range of organic and inorganic compounds into the root environment (Segura, Rodríguez-Conde, Ramos, & Ramos, 2009; Neumann & Römheld, 2000). The released root exudates contain organic acids, phenolics, enzymes, plant growth regulators, sterols and vitamin proteins that affect the enzyme systems of the microbes already living in the soil (Kang, Kim, Yun, & Chang, 2010). Different root exudates components are presented in Figure 5.

Figure 5. Root exudates components



SOLUTIONS AND RECOMMENDATIONS

The global civilization brings out plenty of adverse changes on environment including contamination of natural resources due to improper disposal of industrial waste. Before disposal of toxic wastes like oil sludge, industries/refineries should follow proper disposal guidelines and treat it with eco-friendly technologies. The microbe facilitated phytoremediation technology is one of safe and cost effective option for treatment of all components of oil sludge. The *in situ* remediation technology has been confirmed for its potential in the field conditions but still there is need for further research to clearly understand plant-microbe interaction mechanism with respect to environmental factors. The catabolic and microbial community of rhizosphere has to be explored with more advanced molecular methods. The study of detailed metabolic pathways associated with plant system may clearly explain insight of plant-microbe interaction mechanism. The role(s) of root exudates has to be studied properly with regard to PAHs degradation in field environment.

Advantages of Bioremediation/Rhizodegradation

Bioremediation involves application of indigenous microorganisms with selective plant species which have following advantages over other available physico- chemical methods:

- Usually more cost effective because it is an inherently naturally driven process which leads to complete destruction of pollution on treatment site only.
- *In situ* technology which requires minimal site disruption and releases lesser contaminants than *ex situ* methods or other physico- chemical technologies
- Operational requirements may be lower than on-site incineration, solidification, or soil washing, resulting in potentially fewer mechanical problems and lower costs
- Minimize transport cost and treat large volume of soil at once
- Development of green belt at contaminated site.

FUTURE RESEARCH AND DIRECTIONS

The more focused fundamental research is required in emerging area of rhizo-degradation which will provide new directions to understand plant, microbes and soil interaction mechanism under *in situ* environment. Additionally, the application of advanced culture independent metagenomic approaches may explore the involved microbial and catabolic community of oil contaminated sites. Such studies of rhizospheric soil may explore new insights regarding molecular mechanism of communities and pathways involved in hydrocarbon degradation. Further studies on catabolic gene expression in rhizospheric soil will explore the usefulness of this approach in field conditions. Generally, grasses and legumes are widely reported for phytoremediation of hydrocarbon compounds but more fundamental studies are required to select the best plant-microbe combinations with respect to environmental surroundings and nature of pollutants. These are some emerging areas of rhizoremediation technology that have not been widely explored.

CONCLUSION

The microbe associated phytoremediation technology is suitable option for treatment of oil sludge as compared to other available remediation technologies. The combined rhizo-remediation strategy is result oriented, safe and easy for field application. The microbial products viz. bio-surfactants, catabolic enzymes (dioxygenases) play vital role in enhancing efficiency of degradation process which is accelerated by root exudates of plant sources. The root exudates facilitate soil microbial activity and their catabolic enzyme system in *in situ* environment. The toxic hydrocarbon pollutants can be safely treated on site using rhizo-remediation approach without any natural destruction. Additionally, the application of vegetation on contaminated sites will promote in developing green belt near refineries dumping sites and manage oil waste disposal problem.

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KEY TERMS AND DEFINITIONS

Biosurfactant: Biomolecule of microbial origin having surfactant property.
HMW: Higher molecular weight- higher molecular weight hydrocarbon compounds.
PAH: Polycyclic aromatic hydrocarbon –Aromatic compounds containing two or more benzene rings.
Rhizodegradation: Degradation takes place under plant root sphere.
TPH: Total petroleum hydrocarbons- present in different fractions in oil.

Chapter 2
Role of Micro-Organisms in Bioremediation: A Comprehensive Model Using *Trichoderma* spp.

Ashish Kumar
Jawaharlal Nehru Krishi Vishwa Vidyalaya, India

Mansee Govil
G. B. Pant University of Agriculture and Technology, India

Shivom Singh
ITM University Gwalior, India

K. K. Sharma
VPKAS, India

S. K. Tripathi
Jawaharlal Nehru Krishi Vishwa Vidyalaya, India

R.K. Tiwari
Jawaharlal Nehru Krishi Vishwa Vidyalaya, India

A. N. Tripathi
ICAR Central Research Institute for Jute and Allied Fibres, India

Saurabh Singh
Jawaharlal Nehru Krishi Vishwa Vidyalaya, India

ABSTRACT

The astonishing metabolic abilities of the microbes should be harnessed to obtain new breakthroughs in evolution of degradation pathways and development of newer strategies for bioremediation and biotransformation process. Trichoderma species are important biological control agents used in plant disease management. Other than biocontrol properties they share a very unique phenomenon of soil bioremediation. In this context, bioremediation of soil cover restoring of soil microbiota is of particular importance. Introduction of microorganisms to soil is one of the most promising current approaches to improving soil production both in agriculture and forestry. The co-culture use of different species/strains of Trichoderma has already been reported in higher and quicker ways of solid waste decomposition than the use of a single species/strain. By virtue of the ability of Trichoderma spp. to decompose organic matter, they are free-living in soil as saprophytes. However, these species also have the capability to

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live on other fungi, and the ability to colonize plant roots and rhizosphere. In this chapter, the role of different micro-organisms including fungi and bacteria in bioremediation has been discussed. Further, it has been elaborated that how biocontrol agent Trichoderma spp. can be utilized in bioremediation and how it plays significant role in this process of bioremediation.

INTRODUCTION

The current scenario of environmental pollution posses serious threat to health of human beings, livestock, wildlife and eventually whole ecosystem through faulty production, distribution, use, disposal or accidental spills of many chemicals that are mandatory in practiced agriculture. There is an impending need to rejuvenate the polluted system through renewed practices and awareness of the deleterious effects which such unscrupulous use of chemicals is causing to all life on earth. However, it is beyond surmountable limits to calculate the corresponding cost incurred for restoring the contaminated ecosystem to healthy and acceptable level. In view of the fact, microbial bioremediation program offers one such option, which is more cost effective as compared to the traditional methods of physical and chemical remediation of the contaminated sites. The microbial bioremediation is based on the principal of immobilization or transformation of the contaminants to useful products which are no longer hazardous to human health and environment. The government, industry and also the public should be sensitized for implementation of bioremediation program on a larger and extensive scale so as to speed up the process of restoring the damaged environment.

In nature the degradation or detoxification of the harmful chemicals accumulated in the soil, ground water and waste water takes place gradually over long periods of time. The term bioremediation applies to technologies that aid to accelerate the above natural processes. However, the use of microorganisms, predominantly bacteria, for bioremediation or transformation of hazardous contaminants dates back to 600 B.C. Several pre-medieval civilizations such as the Romans and others used to treat their wastewater for detoxification through bioremediation. In present times bioremediation is being used on commercial scale for almost last 30 years (Ramnayar, 2005). Bioremediation is a technique that involves management of waste through use of organisms to neutralize pollutants in a pre-contaminated site. It is a "treatment that uses naturally occurring organisms to break down hazardous substances into less toxic or non-toxic substances".

In 1972 a sun oil pipeline spill occurred in Ambler, Pennsylvania wherein the first commercial use of bioremediation system was initiated to clean up the site (National Research Council, 1993). Since then, bioremediation has become a well-developed way of cleaning up different contaminants. Information on 240 cases of bioremediation involving treatment of contaminated soil or groundwater in the United States were reported in a survey prepared by the Environmental Protection Agency in 1992 (Alexander, 1999). Over the decades urbanization and modernization of the twentieth century has led to unprecedented increase in population, resulting in rising anthropogenic activities such as industrialization and destruction of the valuable biodiversity causing serious ecological imbalances. This has not only increased accumulation of conventional solid and liquid waste pollutants to critical levels but also produced a wide range of previously unknown contaminants (Ramnayar, 2005). Majority of the contaminants that enter into the ecosystems are in the form of chemicals and exert serious health hazards on man, animal life, plant life and microbes and lead to several ecological problems (Bower, Rittman, & McCarty, 1984). Different scientific approaches have been employed to develop some feasible clean-up process, only a few of them have turned out to be of routine application value

(Crawford & Crawford, 1998). The criteria for choice of an effective technology depend upon the social, political, and geographical conditions. Among all the techniques deployed till date, bioremediation has evolved to be the most promising one. The success of bioremediation is because of it being very economical and biologically and environmentally safe as it actually causes the organic contaminants to become transformed and fully mineralized (Salval, 2003).

Bioremediation aims to stimulate microorganisms already present in the soil by providing them nutrients and other chemicals that will enable them to destroy the contaminants. The bioremediation systems in modus operand today rely on microorganisms that are native to the contaminated sites and encourage them to work by supplying them with the optimum levels of nutrients and other chemicals essential to boost up their metabolism. Thus, the capabilities of the resident microbes limit the efficacy of most of today's bioremediation systems. However, it is regardless of whether the microbes are native or newly introduced to the site concerned. An understanding of how the microbes destroy contaminants is primary in understanding the process of bioremediation and accordingly developing an efficient system of bioremediation for decontamination of the affected site. The types of microbial processes that are employed in the cleanup dictate the kind of nutritional supplements that must be provided by the bioremediation system. Furthermore, the by-products of microbial processes provide indication that the bioremediation is successful.

Several biological agents, such as bacteria, fungi, or green plants, are well known since long time to remove or neutralize contaminants in polluted soil or water. Bacteria and fungi work by breaking down contaminants such as petroleum into less harmful substances that can be easily assimilated. Green plants can also be used to provide aeration to polluted soil and thus stimulate microbial action in turn. Contaminants such as salts and metals can also be absorbed into the tissues of plants, which are later harvested and disposed off. Such use of green plants to decontaminate polluted soil or water is called "Phytoremediation", which is a kind of bioremediation. Bioremediation can be practised either *in-situ* or *ex-situ*. The *in-situ* bioremediation involves treating the contaminated material at the site whereas the *ex-situ* bioremediation involves the removal of the contaminated material to be treated elsewhere from the actual site. Some common examples of bioremediation related technologies are phytoremediation, bioventing, bioleaching, landfarming, bioreactor, composting, bioaugmentation, rhizofiltration, and biostimulation.

DIFFERENT KINDS OF CONTAMINANTS

Man has exploited naturally occurring petroleum hydrocarbons and their derivatives for a wide range of purposes ranging from fuelling engines to manufacturing chemicals. Furthermore, the representative types of petroleum hydrocarbons and the derivatives like gasoline, fuel, oil, polycyclic aromatic hydrocarbons (PAHs), creosote, ethers, alcohols, ketones, and esters also have a broad range of industrial applications. As an example, PAHs are released during refining of crude oil and also during manufacturing of petroleum products such as plastics. Creosote is used in wood preservatives, ethers, esters, and ketones are key components in several chemicals which are the base of different perfumes, anaesthetics, paints and lacquers, or even insecticides.

Bioremediation procedures have been successfully employed to remove contaminants like gasoline, fuel oil, alcohols, ketones, and esters from contaminated sites. Substantial efforts have been made, in particular, for biodegradation and bioremediation research of gasoline. Gasoline components benzene,

toluene, ethylbenzene, and xylene which are together known as BTEX are relatively easy to bioremediate for several reasons stated as under:

- Relatively greater solubility compared to other common contaminants.
- Their capability to be used as the primary electron donor for many bacteria widely distributed in nature.
- Their ability to be rapidly degraded relative to other contaminants.
- The bacteria that degrade BTEX grow readily in presence of oxygen.

Halogenated aliphatic compounds are built from straight chains of carbon and hydrogen when varying numbers of hydrogen atoms are replaced by halogen atoms. Manufacturing and service industries ranging from automobile manufacturing to dry cleaning widely uses halogenated aliphatics as they are effective solvents and degreasers. Some highly chlorinated representatives of this class, such as tetrachloroethene, are known to be completely resistant to attack by aerobic microbes but recent evidence has shown that special classes of anaerobic organisms can completely dechlorinate tetrachloroethene to the relatively nontoxic compound ethene, which is readily decomposed by aerobic microbes. Another potential contaminant is a volatile chlorinated organic compound, Tri-chloro ethylene (TCE), that has been widely used as an organic solvent and degreasing agent and disseminates over large areas in the subsurface at contaminated site (Table 1).

ROLE OF MICROORGANISMS (AEROBIC/ANAEROBIC) IN BIOREMEDIATION

The oldest inhabitants surviving the upheavals in the history of evolution of the earth are the microbes. The present day problems of pollution can be most cost-effectively tackled by employing these simple organisms owing to their versatility and adaptive nature to the always changing environmental conditions (Stolz, 2001).

Like human beings, many microorganisms, utilizes molecular oxygen (O_2) as the electron acceptor. The process, in which the organic compounds are destroyed with the aid of O_2, is called as aerobic respiration. However, many microorganisms can exist without oxygen and the process is called as anaerobic respiration. In aerobic respiration, part of the carbon in the contaminants is oxidized to

Table 1. Different kinds of contaminants with their degradable nature

S. No.	Hardly Degradable	Moderately Degradable	Readily Degradable
1	Venyl chloride	Nitrobenzene	Diesel fuel
2	Tri-chloro ethylene (TCE)	Pentachlorophenol	Isopropyl alcohol
3	Polychlorinated biphenyls (PCBs)	Coal tars	Toluene
4	1,1,1-trichloro-2,2-di(4-chlorophenyl) ethane (DDT)	Creosotes	Gasoline
5	Per-chloro ethylene (PCE)	Lubricating oils	Jet fuel
6	-	Crude oil	Benzene
7	-	-	Methanol

carbon dioxide (CO_2) using O_2 while the rest of the carbon used to produce new cell mass. In this process, the O_2 gets reduced and produce water. Thus, the major by-products of aerobic respiration include carbon dioxide, water, and an augmented population of microorganisms. However, in anaerobic respiration, nitrate (NO_3^-), sulfate (SO_4^{2-}), metals such as iron (Fe^{3+}) and manganese (Mn^{4+}), or even CO_2 play the role of oxygen by accepting electrons from the degraded contaminant. Therefore it can be seen that anaerobic respiration uses inorganic chemicals as electron acceptors. The by-products of anaerobic respiration may also include nitrogen gas (N_2), hydrogen sulfide (H_2S), reduced forms of metals, and methane (CH_4), depending on the electron acceptor. These by-products are in addition to the new cell matter which is produced as a result of respiration process.

In order to grow and divide, microorganisms need to take in nutrients from their environment. These nutrients are metabolized by the microbes, which means that the substances the microbe takes up are broken down and utilized for anabolic processes and the unwanted and transformed substances are excreted in from of different substances. In bioremediation, the substances that are excreted by microbes are less harmful than the original pollutant. In nutshell, the microbes eat the pollutants and clean up the area.

The efficacy of microorganisms having a diversity of metabolic activities makes them applicable in wide ranging fields. This coupled with advances in the technology has lead to successful demonstrations of the usefulness of microorganisms in the ever-expanding bioremediation field. The success of application of environmental biotechnology as a successful remediation tool depends on the possibilities, which can help in triggering or enhancing specific activity of either indigenous or introduced microorganisms. To achieve optimal results of bioremediation the challenge is to enhance the activity of these microorganisms and develop means so as to bring the contaminant into direct contact with the organisms for proper functioning of the system.

- Three primary components for bioremediation system are:
- Presence of a potential contaminant in large amount exceeding that of allowed limits,
- An electron acceptor, and
- Presence of microorganisms which are capable of degrading the specific contaminant present at the site.

The overall goal in bioremediation is to stimulate microbes with nutrients and other chemicals which will either enhance or allow them in their native state to destroy the contaminants. The bioremediation systems in action today respond on microorganisms local to the contaminated sites, encouraging them to work by providing them with the optimum levels of nutrients and other chemicals necessary for their metabolism. Thus, bioremediation systems in today's scenario are limited by the capabilities of the local microorganisms. However, researchers are currently looking into ways to improve contaminated sites with non-native microorganisms including genetically engineered microbes, especially fitted to degrade or decompose the contaminants of concern at that particular site. It is possible that this process, known as bioaugmentation, could expand the variety of promises for future bioremediation system.

Microbes are capable of producing diverse metabolic enzymes. These enzymes can be involved directly in destruction or safe removal of environmental contaminants. On the other side, they can be indirectly involved in transformation of the contaminants to a safer intermediate and this can be self-sustaining and inexpensive (Watanabe, Futamata, & Harayama, 2002). This process of bioreme-

diation includes detoxification, where the waste is made less toxic and mineralization and thus the waste materials are converted into inorganic compounds like carbon dioxide, water and methane (Alexander, 1994). Hence, the interest of researchers is greatly increasing in indigenous diversity of microorganism for their capability of degrading different pollutants under different environmental conditions (Greene, Kay, Jaber, Stehmeier, & Voordouw, 2000).

In the process of bioremediation, microorganisms acquire energy by catalyzing energy-producing chemical reactions which involve breaking chemical bonds and transferring electrons away from the pollutant. Such type of chemical reaction is known as oxidation-reduction reaction. In such process of losing electron, the organic contaminant is oxidized and correspondingly, the chemical which gains the electrons is reduced. Here, the contaminant functions as electron donor, while the electron recipient is called the electron acceptor. The energy gained from these electron transfers is then invested, along with some electrons and carbon from the pollutant, to produce more number of cells. These two materials (the electron donor and acceptor) are necessarily required for cell growth and are completely called the primary substrates.

A diversity of bacteria including methanotrophs, selected methanogens, and species of Pseudomonas (P. cepacia, P. mendocina and P. putida), capable of degrading aromatic compounds can also degrade TCE and other chlorinated aliphatic compounds. In the environment methanotrophic bacteria have a ubiquitous distribution. The use of natural gas or methane with other nutrients can be used to stimulate the bioremediation activities through methane monooxygenase. It is an efficient option, which is inexpensive, and safe to manipulate the environment to accelerate bioremediation.

Most abundant bacterial strains with high biodegradation potential belong to the group *Pseudomonas* and *Cycloclasticus* and were reported early in the history of bioremediation (Watanabe, 2001). Another group of extensively used bacteria are *Thiobacilli* which are utilized by man for mining and bioremediation purposes. Other bacteria such as *Ralstonia metallidurans* and *Deinococcus radiodurans* have been seen to tolerate high levels of toxic metals and radioactivity respectively and thus can be used for the aforesaid purpose.

Another bacterium which can be used in multiple applications is *Rhodobacter. It* fixes carbon and nitrogen from the air to make biodegradable plastics and has also been used to produce fertilizers. A very interesting application of this organism is that it has even been used to make a yellow pigment which is fed to hens for making their eggs more enticing to children (Miltonsaier, 2005). The field of genetic engineering holds great potential to create organisms specifically designed for bioremediation (Lovley, 2003).

Many fungi have also been successfully exploited for bioremediation on industrial and commercial scale. Bioremediation through the use of fungi to decontaminate the area is termed as mycoremediation. The term mycoremediation refers specifically to the use of fungal mycelia in bioremediation systems. One of the primary roles of fungi in the ecosystem is decomposition, which is performed by the mycelium. The mycelium secretes extracellular enzymes and acids that break down the two main building blocks of plant fibers i.e. lignin and cellulose of the substrate. These are organic compounds composed of long chains of carbon and hydrogen and are structurally similar to many organic pollutants. The key to mycoremediation lies in finding out the right fungal species to target a specific pollutant.

In the above context, *Trichoderma* has been proven to be very effective example of biological plant disease management especially the soil born diseases. It is a free-living fungus which is common in soil and root ecosystems and is also highly interactive in root, soil and foliar environments. It reduces growth,

survival or infections caused by pathogens through different mechanisms like competition, antibiosis, mycoparasitism, hyphal interactions and also enzyme secretion.

Trichoderma strains play an important role in the bioremediation of soil that are heavily contaminated with pesticides and herbicides. They have the ability to degrade a wide range of insecticides including organochlorines, organophosphates and carbonates, etc. Different kinds of contaminants degraded by various microorganisms have been listed in Table 2.

FACTORS AFFECTING BIOREMEDIATION

A complex system of several limiting factors controls the optimization of bioremediation processes. The major factors affecting the success of process include: the existence of a microbial population that is capable of degrading the pollutants present; the availability of contaminants to the already present or

Table 2. Microorganisms associated with bioremediation of different contaminants

S. No.	Type of Contaminant	Associated Microorganisms	Reference
1	Marine petroleum hydrocarbon	*Pseudomonas* spp. *Cycloclasticus* spp.	Grossman et al., 2000
2	Anaerobic petroleum hydrocarbon	*Syntrophus* spp. *Methanosaeta* spp. *Methanospirillum* spp. *Desulfotomaculum* spp. *Geobacter* spp.	Dojka, Hugenholtz, Haack, & Pace, 1998; Rabus et al., 1999
3	Polycyclic aromatic hydrocarbon (PAHs)	*Burkholderias* spp. *Sphingoinonas* spp. *Mycobacterium* spp.	Grosser, Friedrich, Ward, & Inskeep, 2000
4	Crude oil	*Pseudomonas* spp. *Achormobacter* spp. *Vibrio* *Flavobacter* *Brevibacterium* spp. *Flavo bacterium* spp. *Norcadia* spp.	Savage, Diaz, & Golueke, 1985
5	Hexadacane	*Acinobacter* spp. *Trichosporon pullulans* *Candida petrophilium* *Pseudomonas aeruginosa*	Savage et al., 1985
6	Paraffins	*Trichosporon pullulans*	Savage et al., 1985
7	Jet Fuels	*Hormodendrum* spp. *Cladosporium* spp.	Savage et al., 1985
8	Napthalene	*Pseudomonas* spp.	Savage et al., 1985
9	Benzene	*Pseudomonas putida*	Savage et al., 1985
10	Kerosene	*Torulopsis* spp. *Candida tropicalis* *Corynebacterium hydrocarbonclastus*	Savage et al., 1985
11	Metal	*Ralstonia eutropha* *Ralstonia metallidurans* *Deinococcus radiodurans*	Valls, Atrian, de Lorenzo, & Fernandez, 2000

artificially inoculated microbial population; the environment factors i.e. type of soil, temperature, pH, the presence of oxygen or other electron acceptors, and nutrients.

Microorganisms, some beneficial and others detrimental, are present in almost every environmental condition which may be unsuitable for growth of any other organism and can be isolated from everywhere. Microbes have the inherent capability to adapt and grow in any adverse conditions ranging from subzero temperatures to extreme heat, desert conditions, in water, with an excess of oxygen, and in anaerobic conditions, with the presence of hazardous compounds or on any waste stream. The main requirements are an energy source and a carbon source for the survival of these organisms. Owing to this property of microbes and their interaction capacity with other biological systems, these can be used to degrade or remediate environmental hazards. We can subdivide these microorganisms into the following groups:

Aerobic

The term aerobic is committed for any process that takes place in the presence of oxygen. The present context implies the aerobic nature of respiration of the microbes. Some examples of aerobic bacteria that have been recognized for their degradative abilities are *Pseudomonas*, *Alcaligenes*, *Sphingomonas*, *Rhodococcus* and *Mycobacterium*. Reports have been obtained before that these microbes degrade pesticides and hydrocarbons and can be used for bioremediation process. These bacteria seem to use the contaminant as the sole source of carbon and energy thus degrading the pollutant in turn.

Anaerobic

A biological process occurring in the absence of oxygen is termed as anaerobic. Anaerobic bacteria are not as frequently used as aerobic bacteria for obvious reasons being presence of oxygen as limiting factor in most domains. However, there is an increasing interest in use of anaerobic bacteria for bioremediation of polychlorinated biphenyls (PCBs) in river sediments, dechlorination of the solvent trichloroethylene (TCE), and chloroform.

Ligninolytic Fungi

A diverse range of persistent or toxic environmental pollutants can be effectively degraded by some fungi such as the white rot fungus *Phanaerochaete chrysosporium*. Common substrates used in growing of such fungus include straw, saw dust, or corn cobs. Methylotrophs are aerobic bacteria that grow by utilizing methane for carbon and energy source. The initial enzyme in the pathway for aerobic degradation is methane monooxygenase. It has a broad substrate range and is active against a wide range of compounds also including the chlorinated aliphatic compounds like trichloroethylene and 1, 2-dichloroethane.

Environmental Factors

Among nutrients carbon is the most basic element of living forms which is needed in greater quantities than other elements and constitutes about 95% of the weight in addition to hydrogen, oxygen, and nitrogen. Phosphorous and sulfur contribute up to 70% of the remainder. The nutritional requirement of carbon to nitrogen ratio is 10:1, and carbon to phosphorous is 30:1.

Soil Factors

The type of bioremediation depends on the concentration of soil contaminants.

High Concentrations of Contaminants

It is considered high when it is roughly 5% or more. In such case, as an initial step the soil is agitated in a purifying water solution containing interface active agent and then separated from the oils. Thereafter, bioremediation process is started to further clean the soil efficiently. At the experimental stage, bioremediation alone has been found to be efficient enough to turn contaminated soil into soil suited for agriculture and other life oriented processes. However, work is still continuing to make this process even more efficient and effective.

Low Concentrations of Contaminants

For soils that have low concentrations of contaminants bioremediation alone can take care for removal or inactivation of the pollutants. Soil containing two per cent heavy oils, can be purified over a period of approximately 6 months to a year, but at a concentration of 0.8 per cent, the errand can be completed in only about one to two months. Such an environmental friendly method makes recycling and reuse of soil possible without much effort. Depending upon the different factors, the requisite conditions for microbial activity has been given in Table 3.

Table 3. Factors affecting microbial bioremediation activity

S. No.	Factor	Optimum Condition	Requisite Condition for Microbial Activity
Biotic			
1	Microorganisms	Aerobic/anaerobic	Aerobic/anaerobic
2	Biological process of microrganisms	Anabolism/catabolism	Anabolism/catabolism
Abiotic			
1	Soil type	Clay/silt	Low clay or silt content
2	Soil moisture availability	25-85% water holding capacity	25-28% of water holding capacity
3	Oxygen	>0.2 mg/L DO, >10% air-filled pore space for aerobic degradation	Aerobic, minimum air-filled pore space of 10%
4	Redox potential	Eh > 50 mill volts	
5	Nutrients availability (Carbon, Nitrogen and Phosphorus)	C:N:P= 120:10:1 molar ratio	N and P for microbial growth
6	pH	6.5-8.0	5.5 to 8.5
7	Temperature	20-30 ºC	15-45ºC
8	Contaminants	Hydrocarbon 5-10% of dry weight of soil	Not too toxic
9	Heavy metals	700ppm	Total content 2000ppm

ESTIMATION OF BIOREMEDIATION

The process of bioremediation can be estimated indirectly by measuring the oxidation reduction potential or redox potential in soil and groundwater. Together with this measurement of pH, temperature, oxygen content, electron acceptor/donor concentrations, and concentration of breakdown products (e.g. carbon dioxide) also act as indicators of the success of bioremediation and are employed to monitor the level of remediation performed. The biological breakdown rate (decreasing) as function of the redox potential has been given in Table 4.

USE OF *TRICHODERMA* SPP. IN BIOREMEDIATION

Trichoderma found naturally inhabiting in the rhizosphere is an antagonist genera that competes with other microorganisms for nutrients and space. It has been known that some species of this genus are able to produce certain enzymes which bring about break down of toxic substances in the surrounding soil area (Sene, Converti, Ribeiro, & Cássia, 2010). In the processs, *Trichoderma* aids to clean xenobiotic contaminants like pesticides, organochlorine and organophosphorus compounds (Argumedo, Alarcón, Ferrera, & Peña, 2009). *Trichoderma* species have been shown to degrade Organochlorine pesticides like DDT, dieldrin and endosulfan which are classified as highly toxic and persistent in ecosystems (Llado, Jiménez, Viñas, & Solanas, 2009). In light of this ability of the fungus *Trichoderma* it is suggestible that it could be used as a potential bioremediation tool for cure of soils contaminated with agrochemicals.

Owing to the capability of efficient substrate utilization through secretion of antibiotic metabolites and enzymes *Trichoderma* spp. are highly successful colonizers of their habitats. This inherent property makes them capable of dealing with diverse environments such as compost, agricultural soils, rhizosphere and waste material. Therefore, different *Trichoderma* strains are being utilized in sundry ways in agriculture, biotechnology, bioremediation, and waste management through biological methods without chemical intervention so as to reduce environmental pollution and foster beneficial aspects of the same (Schuster & Schmoll, 2010). The following text aims to throw some light on the bioremediation aspects using *Trichoderma* spp.:

Table 4. Reactions involved in biological breakdown process

S. No.	Process	Reaction Involved
I	Aerobic reaction	$O_2 + 4e^- + 4H^+ \rightarrow 2H_2O$
II	Anaerobic reaction	
1.	Denitrification	$2NO_3^- + 10e^- + 12H^+ \rightarrow N_2 + 6H_2O$
2.	Manganese IV reduction	$MnO_2 + 2e^- + 4H^+ \rightarrow Mn^{2+} + 2H_2O$
3.	Iron III reduction	$Fe(OH)_3 + e^- + 3H^+ \rightarrow Fe^{2+} + 3H_2O$
4.	Sulphate reduction	$SO_4^{2-} + 8e^- + 10\ H^+ \rightarrow H_2S + 4H_2O$
5.	Fermentation	$2CH_2O \rightarrow CO_2 + CH_4$

Pesticide Degradation

Pesticides have posed as a major source of contamination due to their over-use in agricultural practices. Improper handling and the persistent nature of some of them have led to accumulation of the pesticide residues leading to negative impacts on soil, water, and groundwater and eventually in food chains. The environment and human health is at threat due to pesticides which can bioaccumulate and biomagnify in living organisms or even arrive by diffusion and / or advection at different trophic levels. (Cooper, Laws, Goldman, & Narotsky, 2007).

Insecticide Degradation

T. harzianum degrades the insecticide endosulfan (1,4,5,6,7,7-hexachloro-5-norbornene-2,3-dimethanol cyclic sulfi te) under the a variety of nutrient media all through its different stages of growth (Katayama & Matsumura, 1993). The process of degradation of endosulfan is catalyzed by the endosulfan oxidation process which degrades endosulphan into endosulfan sulphate which was further hydrolysed to endosulfan diol. Artificial inoculation of nicotinamide adenine dinucleotide phosphate (NADPH) can enhance this endosulfan metabolism and the formation of the endosulfan sulfate can be catalyzed by the oxidase, a major oxidative enzyme in *T. harzianum*.

T. viride has the capacity to degrade the chlorpyrifos, an organophosphorous insecticide, in liquid culture (Al-Mihanna, Salama, & Abdalla, 1998). The degradation of chlorpyrifos has been proved more competent by mixed populations of different microbes than by single culture of fungi. Heptachlor can be converted to hepta- chlorepoxide by the ability of *Trichoderma.*

Biodegradation of Oxamyl Pesticides

Different strains of fungi like strains *T. harzianum*, *T. viride*, *Aspergillus niger*, *Fusarium oxysporum* and *Penicillium cyclopium* are capable for biodegradation of oxamyl pesticides. *Trichoderma* spp. utilizes oxamyl as a source of carbon and nitrogen. The fungus possesses different kinds of enzymes, which acts on amide and ester bond present in oxamyl structure. It has been reported that within 10 days of incubation *T. viride* degrades oxamyl upto 82.05%. However, *T. harzianum* can degrade upto 72.5% within same incubation period. This indicated the better potential of *T. viride* in oxamyl bioremediation. (Afify Aemmr, Abo-El-Seoud, Ibrahim, & Kassem, 2013).

Herbicide Degradation

The atrazine herbicide was the most widely applied herbicide worldwide during 2006 being applied in agricultural soils @ 29-34 million kg of active ingredient per year (Joo, Choi, & Hodgson, 2010). Atrazine, which is chemically $C_8 H_{14} ClN_5$, is a pre-emergent herbicide having systemic action. It inhibits photosynthesis and is considered toxic in the short and medium term. Furthermore, it is also not easily degradable also. Research has shown that this product is causing serious and detrimental environmental changes. In terms of human health it can act as endocrine breaker (DE), and cause teratogenic effects, and is also found to be affecting reproductive function in vertebrates (Cooper et al., 2007).

Concerned scientists have found fungi and bacteria that exhibit the capability to transform atrazine molecule in to products such as Hydroxyatrazine (HA), Desethylatrazine (DEA) and Desisopropylatrazine DIA through alkylation and chlorination reactions as result of the degradation process of these microbes (Govantes, Porrúa, García, & Santero, 2009). It has been reported that *Trichoderma* spp. can resist atrazine concentrations of 10,000 mg L-1 and grows exponentially in atrazine- contaminated soil. The growth rate of the range of 10^5-10^6 colony forming unit (CFU) per gram in 15 days was exhibited by the fungus during the experiment. *Trichoderma* was seen using atrazine as the only carbon and nitrogen source, while the control showed a decrease of 10^0-10^3 CFU per gram in the same period of time. The *Trichoderma* strain can degrade 89% of the atrazine in 40 days @ 10^4 - 10^5 CFU per ml of *Trichoderma* applied to sterilized and non- sterilized soil contaminated with atrazine @ 500 mg per Kg of soil. This also proved the viability and cultivability of *Trichoderma* in atrazine contaminated soil thus attenuating its scope in bioremediation of the same. Similarly fluometuron, a herbicide can also be degraded by *T. viride* and the biodegradation rate could be obtained upto 85% (Romeh, 2006).

Nitrosoglyphosate [N-(Phosphonomethyl) glycine, $C_3H_8NO_5P$] is a broad spectrum herbicide and widely used all over the world for elimination of annual and perennial weeds in different farming systems of agriculture. Variety of environmental and health problems are associated with over use of this herbicide. This is also toxic to soil organisms and its application for long duration increases susceptibility for various plant diseases. It has been reported that *T. viride* strain FPR3 can grow well when phosphorous was provided as sole source of nutrition in the culture media through the application of glyphosate. It was also noticed that total phosphorus concentration was continuously decreasing which shows utilization of phosphorus by *Trichoderma* and this has the capacity of degradation of glyphosate. Hence, treatment of soil with selective *Trichoderma* strains could be useful in bioremediation of glyphosate where this herbicide is extensively being used (Arfarita et al., 2013).

Biodegradation of Aromatic and Aliphatic Hydrocarbon

Various anthropogenic activities lead to accumulation of toxic polycyclic aromatic hydrocarbons (PAHs) in the environment. *Trichoderma* spp. has been identified as a potential agent in biodegration of some of these aromatic hydrocarbons like pyrene (Wang, Gong, Li, Zhang, & Hu, 2008) and phenanthrene (Hadibarata, Tachibana, & Itoh, 2007). However, it has been reported that in the process of bioremediation of phenanthrene using *Trichoderma* sp. S019 afforded 1-hydroxy-2-naphthoic acid, salicylaldehyde, salicylic acid and catechol as intermediate (Hadibarata et al., 2007).

Various strains of *Trichoderma* like *T. asperellum* strain TUB F-1067 (SA4), *T. asperellum* strain Tr48 (SA5) and *T. asperellum* strain TUB F-756 (SA6) have potential to degrade n-alkanes such as tridecane, tetradecane, pentadecane, hexadecane, heptadecane, octadecane and crude Omani oil (Elshafie, AlKindi, Al-Busaidi, Bakheit, & Albahry, 2007). Some other important aliphatic hydrocarbons like n-eicosane are also degraded by *Trichoderma* spp and produce nonadecanoic acid, n-octadecane, hexadecanoic acid, oleic acid and stearic acid (Hadibarata et al., 2007).

Biodegradation of Phenolic Compounds

T. atroviride produces the extracellular laccase which have the ability to transform phenolic compounds. The purified laccase from *T. atroviride* has its activity towards different substrates of laccase like 2-2, azinobis- (3-ethylbenzthiazoline-6-sulphonate) (ABTS), dimothoxyphenol (2,6-DMP), syringaldazine

and hydraquinone.The laccase, purified from *T. atroviride*, is a monomeric protein with an apparent molecular mass of 80kDa and isoelectric point of 3.5. This has its optimum activity at 3 and 5 pH for ABTS and 2,6-DMP respectively. In the process of bioremediation, the laccase is capable to oxidize the aromatic compounds present in industrial and agricultural waste water as catechol and o-cresol (Chakroun, Mechichi, Martinez, Dhouib, & Sayadi, 2010).

Bio-Conversion of Coal

T. atroviride are capable of solubilizing low-rank coal (LRC) by its ligninolytic system (Monkemann, Holker, & Hofer, 1997). Partly inducible heat-sensitive agent having strong hydrolytic properties is secreted by this fungus which aids in the solubilization of Rhenish lignite (Holker, Ludwig, Scheel, & Hofer, 1999). An unusual esterase also seems to be produced by this fungus for the cleavage of ester bonds in the presence of lignite. Certain alkaline substances and chelators are produced by *T. atroviride* that help in the dissolution of coal humic materials. Hard coal is modified and solubilized without the formation of tarlike products in the presence of *Trichoderma* spp.

Palm Oil Mill Effluent Degradation

Palm oil mill effluent (POME) contains a high organic load and is a major source of pollution which needs to be treated before disposal into any water body. *T. viride* has been reported to play a major role in the treatment of POME (Karim & Kamil, 1989).

Bioremediation of Aflatoxins

Aflatoxins are biologically active secondary metabolites derived from polyketide and produced by different species of *Aspergillus* like *A. flavus* and *A. parasiticus*. *T. viride* has been known to bioremediated the aflatoxins B and G extensively with high efficiency. It has been reported that *T. viride* as a result of bioremediation of aflatoxin produces aspergillic acid as well as different degradation products like dicotylphthalate, methyl jasmonate, butabarbitol and cyclopentanione. In this process, production of cyclopentanctione indicates the cleavage of cyclopentane ring of aflatoxins. Detailed analysis of aflatoxin B and G bioremediation using *T. viride* by mass specrometery revealed the presence of benzene fused with furan moiety as a dominant product. Besides this, 3-methyl-butenyl (Molecular weight of 146), tinuvin and benzene with other essential oil compounds like limonene and jasmonate are also produced which are less toxic than the original form of contaminant (El-Sheikh, Mahdy, & El-Aaser, 2007).

Heavy Metal Mobility, Adsorption, and Bioremediation

Heavy metals pollution in environment is a threat of possible overall disaster across the world. There are several cleaning methods including bioremediation using microorganisms. *Trichoderma* can remove and concentrate the various ions, such as Pb, Cd, Cu, Zn, and Ni, and sorption was widely recognised as the main mechanism of uptake (Kacprzak & Malina, 2005; Yazdani, Yap, Abdullah, & Tan, 2009; Srivastava et al., 2011). Significant enhancement has been achieved in Bioaccumulation coefficients (= concentration of the metal in dried plants divided by the initial soil content of the same element) for Cd, Cr, Cu, Ni, and Zn by the addition of *Trichoderma*. *Trichoderma* are able to release the chelating

compounds of organic acids creating the acidification of environment, which further helps in increased mobility of heavy metals. (Barea, Pozo, Azc´on, & Azc´on-Aguilar, 2005; Ledin, 2000; Wang and Chen, 2009; De Freitas Lima et al., 2011). With this view, addition of *Trichoderma* in to the soil causes the mobilisation of Cd, Pb, and Zn and results in increased leaching of heavy metals into the soil solution (Malgorzata et al., 2014).

Chromium-VI is one of the most important pollutant coming from the leather processing industries as they perform chrome tanning of the leather. Its toxicity results in skin ulceration, allegic contact dermatitis and ultimately carcinogenicity. The biosorption studies have shown significant chromium uptake by *Trichoderma* spp. (Shukla & Vankar, 2014). In a recent study by Mohsenzadeh & Shahrokhi, 2014, three *Trichoderma* species including *T. asperellum*, *T. harzianum* and *T. tomentosum* were used under different culture conditions in Cd-polluted media for two months and it was observed that all three species were able to remove the Cd from the media but upto different levels. Among the three species, *T. asperellum* depicted maximum efficiency in removal of Cd i.e. 10.75 mg/g, at fungal dry weight.

Cyanide Degradation

The one of the most common corrosive pollutant on the earth are cyanide compounds and they are found either as organic or inorganic cyanide. *Trichoderma* spp. have the capacity to degrade these cyanide compound but after alteration only. It has been reported that a mutant of *T. koningii* constructed by restriction enzyme mediated integration have a high capacity of cyanide degradation (Zhou, Liu, Chen, Xu, & Chen, 2007).

ADVANTAGES OF BIOREMEDIATION

Today, bioremediation is being used worldwide at a number of sites, including Europe, though with a varying degrees of success. Techniques are being further modified and improved through greater knowledge and experience gained from practice of the technique. In view of all the benefits that the technique offers, there is no doubt that bioremediation has great potential for dealing with contamination. The sundry advantages offered by Bioremediation are as following:

- Bioremediation makes use of a natural process which makes it acceptable by the public as a harmless waste treatment process for contaminated material such as soil without any side effects, that may cause any further or long term damage to the site. Microbes serve as agents of nature itself to degrade the contaminants and the residues from the treatment are usually harmless products such as carbon dioxide, water, and cell biomass.
- Theoretically, bioremediation is the complete solution for the successful destruction of a wide variety of contaminants. It offers to easily transform many compounds that are legally considered to be hazardous into harmless products. This further eliminates the need of treatment and disposal of contaminated material in future and thus puts off a major liability associated with it.
- Bioremediation eases off the task of transferring contaminants from one environmental medium to another, for example, from land to water or air by providing the complete destruction of target pollutants at the problem site itself.

- Bioremediation can be carried out on site, along with the ongoing activities without causing a major disruption of normalcy. This also eliminates the need for transport of the waste away from the site. The potential threats to human health and environment during transportation are also washed off.
- Bioremediation is more cost-effective as compared to other technologies that are used for cleanup of hazardous waste.
- This technology is miraculous in terms that it does not use any dangerous chemicals and is yet very effective. It uses the fertilizers commonly used on agricultural setups and home or public gardens and parks. These nutrients are added to the site to make the microbes grow which in turn degrade the pollutants. Bioremediation changes the harmful chemicals into water and harmless gases and the harmful chemicals are thus completely destroyed.

LIMITATIONS OF BIOREMEDIATION

- Bioremediation is possible only in case of those compounds that are biodegradable as not all compounds are susceptible to rapid and complete degradation.
- There is a rising concern that some of the products of biodegradation may be more persistent or toxic than the parent compound. Biological processes are often highly specific and it is difficult to exactly extrapolate from bench and pilot-scale studies to full-scale field operations. It is difficult to set up a proper coordination between the important site factors required for success including the presence of metabolically capable microbial populations, suitable environmental growth conditions, and appropriate levels of nutrients and contaminants.
- Further research is needed to develop and engineer bioremediation technologies that are suitable for sites polluted with complex mixtures of contaminants which are not evenly dispersed in the environment. Contaminants may be present in variable states of matter as solids, liquids, and gases and bioremediation needs to address the issue in all forms.
- Bioremediation process is often time consuming and takes longer than other viable treatment options, such as excavation and removal of soil or incineration.
- There is lack of some regulatory certainty regarding acceptable performance criteria for bioremediation.

CONCLUSION AND FUTURE PERSPECTIVES

It is a limitation that not all contaminants are easily treated by bioremediation using microorganisms alone as such. For example, heavy metals such as cadmium and lead are not readily absorbed or assimilated by microorganisms. One experiment has recently thrown light on a new aspect that suggests that fish bones have some success absorbing lead from contaminated soil. Bone char is another viable option which has also been shown to bring about bioremediation of small amounts of cadmium, copper, and zinc. However, attention needs to be drawn towards the fact that the assimilation of metals such as mercury into the food chain may worsen matters further still. Another relatively less touched upon process of phytoremediation can be brought in to focus here in view of its useful due to its unique feature in these circumstances. This technique of Phytoremediation employs natural plants or transgenic plants

which are able to bioaccumulate these toxins in their above-ground parts, which are then harvested for removal. Later on the heavy metals in the harvested biomass may be further concentrated by incineration or even recycled for industrial reuse. The elimination of a wide range of pollutants and wastes from the environment to step towards a hazard free and safe for future generation environment requires increasing our understanding on the subject and sensitization of the masses for the relative importance of the issue. Knowledge of different pathways and regulatory networks to carbon flux in particular environments and for particular compounds, can certainly accelerate the development of new bioremediation technologies and biotransformation processes. The new emerging era of bioremediation is yet to evolve fully and need special focus in research oriented programmes and field trials to bring out specific technologies of bioremediation suitable for a particular type of contamination present even in the most complex form possible. Genetic engineering approaches can prove highly beneficial in this direction to obtain the optimal and desired outputs. The use of genetic engineering to create organisms specifically designed for bioremediation has great potential. An upcoming example in this context which needs citation here is of the bacterium *Deinococcus radiodurans* which is the most radio resistant organism known so far has been modified to consume and digest toluene and ionic mercury from highly radioactive nuclear waste. More such innovative initiative need to be taken in this field so as to sound the horn of a new beginning where all the pollution which at present is outside the domain of bioremediation should also be successfully tackled naturally using bioremediation methods.

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KEY TERMS AND DEFINITIONS

Bioaccumulation: Bioaccumulation refers to the accumulation of substances, such as pesticides, or other chemicals in an organism. This occurs when an organism absorbs a toxic substance at a rate greater than that at which the substance is lost.

Biodegradation: Process by which organic compounds are broken down by living organisms.

Contaminant/Pollutant: Any substance that is either produced naturally or by humans, which is found in a place where it should not be, or existing at concentration above the allowable limit in a given area; such as in water, air or soil.

Environment: It is the sum total of every living thing and natural forces that make up the surroundings and influences the ability to live on earth.

Heavy Metals: These are metals that are relatively high in density or atomic weight. They are often metalloids of environmental concern, and have properties of metallic substances at room temperature.

Microorganism: Living organism (such as bacteria, fungi, viruses) too small to be seen with naked eye but visible under a microscope.

Pesticide Degradation: It is he process by which a pesticide is transformed into a benign substance that is environmentally compatible with the site to which it was applied.

Phytoremediation: A treatment process that tackles environmental problems via the use of plants without the need to excavate the contaminant material.

Toxins: Toxins can be small molecules, peptides, or proteins that are capable of causing disease on contact with or absorption by body tissues interacting with biological macromolecules such as enzymes or cellular receptors.

Trichoderma: *Trichoderma* is a genus of fungi that is present in all soils, where they are the most prevalent culturable fungi. Many species in this genus can be characterized as opportunistic a virulent plant symbionts.

Chapter 3
Bioremediation of Pesticides under the Influence of Bacteria and Fungi

Mamta
Jiwaji University, India

Rayavarapu Jaganadha Rao
Jiwaji University, India

Khursheed Ahmad Wani
ITM University Gwalior, India

ABSTRACT

The demand and development of chemicals, pesticides, fertilizers, and pharmaceuticals is increasing constantly posing a potential threat to the environment. The presence of pesticides and their impact makes their removal and detoxification a more urgent need. Bioremediation technologies have been successfully used and are gaining more and more importance with increased acceptance of eco-friendly remediation solutions among the scientific community. Bioremediation by fungi and bacteria is considered a better option for making environment free from pesticides, as chemical and physical methods are not only costly but also not very effective. However, the complex nature of pesticides is an obstacle to degrade the pesticides, so more versatile and robust microorganisms need to be identified which can produce the desired result in a very cost-effective manner. This study examines the role played by fungi and bacteria in degradation of the pesticides in environment and also identify the future research problems in this regard that need to be experimented.

INTRODUCTION

Rapid industrialization, urbanization and population growth has deteriorated the environment condition and is considered a threat for different kinds of ecosystems in many ways. Currently, land, water, air resources have become contaminated due to various toxic organo pollutants, viz., herbicides, insecticides,

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pesticides, plasticizers, coloring dyes, agrochemicals, solvents, pharmaceuticals, hydraulics, heavy metals, fire extinguishers, halogenated compounds, hazardous metal ions, etc. Human health and agricultural sustainability is seriously affected by the synthetic pesticides produced during the last 10 decades. The process of solidification and evaporation has exposed the environmental ecosystems to different types of pesticides and most of the pesticides are present in concentrations that are toxic to not only humans but to soil, water, marine and estuarine ecology. Pesticides traces have been found even in the samples of rain, fog and bark of trees as well.

Among the most persistent and globally distributed organic pollutants are hexachlorocyclohexane (HCH) and dichlorodiphenyltrichloroethane (DDT), hexachlorobenzene (HCB), heptachlor, aldrin, chlordane, dieldrin, endrin and mirex. Most of the organic compounds are synthetic and recalcitrant to photolytic, chemical, and biological degradation and due to their volatile nature these pesticides move long distances that results their distribution across the earth, including remote and isolated areas such as the Polar Regions (Allen-Gil et al., 1997). The pesticides can be transported in the vapour phase, surface runoff and leaching. The process of evaporation takes place due to tropical warm temperature and hence it is argued that trace of pesticides may be found in the atmosphere in areas where the temperature is high as compared to colder places. The concentration of pesticides will be highest near the point of release and decline with distance. However, mobile organo chlorine compounds that have their tendency to partition for easy movement are the exceptions (James, 2000). HCH isomers, DDT and its metabolites that are persistent organic compounds are the predominant chemical contaminants found along the Indian coast and were reported in major rivers (Rajendran & Subramanian, 1997; Zhou, Zhu, Yang, & Chen, 2006; Leong, Tan, & Mustafa, 2007; Imo, Sheikh, Hirosawa, Oomori, & Tamaki, 2007; Ma, Ran, Gong, & Zou, 2007; Ize-Iyamu, Asia, & Egwakhide, 2007; Kannel, Lee, Kanel, Khan, & Lee, 2007; Poolpak, Pokethitiyook, Kruatrachue, Arjarasirikoon, & Thanwaniwat, 2008; Doong, Lee, Lee, Sun, & Wu, 2008; Kaushik, Sharma, Jain, Dawra, & Kaushik, 2008). Organochlorine pesticide (OCPs) residues are important potential component of chemical pollutants used extensively for agriculture and sanitation purposes in India. These Pesticides enter the soil and ground water by direct treatment or being washed off from plant surfaces during rainfall. Depending on the phenotype and density of the plant type, it is estimated that an average of 35-50% of the plant protection material is deposited on soil immediately after spraying. The behavior of pesticides in soil and ground water involves persistence, movement and metabolism. The water solubility and binding capacity of organic and in organic constituents is an important factor for function of residues.

Due to the adverse effects of pesticides, especially DDT and HCH there use in agriculture has been banned in most countries. However it has not completely eliminated the residues of these compounds and their metabolites from the environment (Bhatnagar et al., 1992; Bhattacharaya, Sarkar, & Mukherjee, 2003). On the other hand India is still one of the major producer and consumer of organochlorine pesticides, particularly dichlorodiphenyltrichloroethane (DDT) and hexachlorocyclohexanes (HCHs) for agriculture and public health programs although it is banned. The consumption of insecticide in agriculture has been increased more than 100% from 1971 to 1994-95. For instance, insecticide consumption in India, which was to the tune of 22013 tons, has increased to 51755 tons by 1994-95 (www.indiastat.com). The Indian pesticide industry with 82,000 MT productions for 2005-06 is ranked second in Asia (behind China) and twelfth on global market. According to Green Peace report, India is producing 90,000 metric tons of pesticides as the largest industry in the whole of Asia. India as most of the rivers pass through agricultural fields, they are subjected to contamination with different pesticides used for crop protection (http://www.greenpeaceindia.org/nopesti.htm). It is estimated that about 25 tons of

organochlorines produced have been already transported to the sea (Mohapatra & Saha, 2000), due to higher levels of HCH that were reported to occur in Indian marine water.

Bioremediation is an effective and economical way to remove organic pollutants from water and soil by using microorganisms Bioremediation can be used effectively to reduce toxicity, path and volume of contaminants to that levels which are safe to human health and ecosystem (Nyer &Duffin, 1997; Patel, Patel, & Kalia, 2006). Microbes are used widely in bioremediation process as they are present everywhere, easily manipulated, high adaptive capacity and are cheaply produced (Genter, 1996; Jadhav & Govindwar, 2006).

The natural biological activity is an option that offers the possibility to destroy harmful contaminants, a process utilized by bioremediation. Bioremediation thus is defined as the process whereby organic wastes are biologically degraded under controlled conditions to harmless or non-poisonous state, or to levels below permissible limits established by regulatory authorities. The process of Bioremediation involves naturally occurring bacteria and fungi or plants to degrade or detoxify that are substances hazardous to human health and/or the environment (Vidali, 2001). The microorganisms may be indigenous to a contaminated area or they may be isolated from elsewhere and brought to the contaminated site for bioremediation. Contaminant compounds are transformed by living organisms through the reactions that take place as a part of the metabolic processes of living organisms that is useful to convert contaminated compounds into harmless substances. The process of bioaugmentation is used to enhance degradation process at the waste site. Microbes are amongst the most versatile biological systems that can adapt under extreme stress conditions and may be isolated from almost any environmental conditions. The versatile nature and adaptability of the microbes makes them favorite available resource in the present date for remediation of hazardous waste. Based on the nature and growth behavior, microbial systems are classified as aerobic, anaerobic and facultative anaerobic organisms. Bioremediation can be effective only where environmental conditions permit microbial growth and activity, its use often involves the manipulation of environmental parameters to allow microbial growth and degradation to proceed at a faster rate and to achieve effective bioremediation microorganisms must enzymatically attack the pollutants and convert them to harmless products. These strategies are mainly covered under two schemes as *in situ* and *ex situ* bioremediation. The selection of the method depends up on the nature and degree of saturation of specific class of contaminant or pollutant and level of toxicity. Bioventing, *in-situ* biodegradation, biosparging, bioaugmentation are the sub-classes of *in situ* bioremediation whereas landfarming composting biopiles bioreactors are sub-classes of *ex situ* bioremediation.

Pesticides are used widely in controlling crop pest to minimize losses of agricultural products and control insect vectors to prevent the outbreak of human and animal epidemics. Food storage have resulted increased use of the pesticide and herbicides in agriculture. India is a largest consumer of pesticide amongst the South Asian countries and the rate of pesticide consumption for crop protection accounts for 3% of the world consumption. High pesticide concentration in soil adversely affects human health by a process of biomagnification and causes serious environmental pollution. The variety of pesticides in India the most commonly used pesticides include organophosphates, organochlorins, neonicotiniods etc., are increasingly produced and released to the environment at a growing rate, which may pollute the soil, water and air due to non judicious applications (Yao, et al., 2007). There is now overwhelming evidence that some of these chemicals do pose potential risk to humans and other life forms and unwanted side effects to the environment (Jeyaratnam, 1985; Igbedioh, 1991; Forget, 1993). Human population is exposed to pesticides and the cause potentially serious health effects, though a disproportionate burden is shouldered by the people of developing countries and by high risk groups in each country. About 1

million deaths and chronic illnesses are reported per year due to pesticide poisoning world-wide. Ideally a pesticide should be lethal to the targeted pests, but not to non target species, including man. It is unfortunate that the scenario is not so, hence the controversy of use and abuse of pesticides has surfaced. The unchecked use of these chemicals, under the adage, "if little is good, a lot more will be better" has played great destruction with human and other life forms (ICMR, 2001).

Microorganisms play a noteworthy role in the transformation and degradation of pesticides. Even the most persistent and long lasting pesticides can be metabolized to some extent by different microbial cultures, either by utilization of the cultures or compounds as sources of energy or as a source of nutrients, or by cometabolism with other substrates supporting microbial growth (Castillo, Wiren-Lehr, Scheunert, & Torstensson, 2001).

MECHANISMS OF BIOREMEDIATION

Intrinsic Bioremediation

A natural process that utilizes the services of natural microflora and environmental conditions that reduce the pollutant toxicity to desirable level. The processes such as dilution, volatilization, biodegradation, adsorption, and chemical reactions with subsurface materials are allowed to reduce contaminant concentrations to acceptable levels during the process of intrinsic bioremediation. This process takes place naturally without any intervention from human beings. The process is suitable as it does not require to transfer of remediation wastes; less intrusive (as few surface structures are required) and may be applied to all or part of a given site, depending on site conditions and cleanup objectives and may be used in conjunction with, other active remedial measures and that will also reduce the burden of additional cost.

Biostimulation

The process of biostimulation involves the introduction of suitable like fertilizer and growth supplements to activate the indigenous microbes present at the active sites in soil or ground water. The biostimulation process that can be achieved *in situ* or *ex situ* may be activated with small amount of pollutant as well by switching on the operons for bioremediation enzymes. This involves injection of specific nutrients at the site (soil/ground water) to stimulate the activity of indigenous microorganisms. Fertilizers and growth supplements are common stimulants. Presence of small amount of pollutant can also act as stimulant by turning on the operons for bioremediation enzymes. Biostimulation can be done *in situ* or *ex situ*.

Bioventing

This process involves venting of oxygen through soil to stimulate growth of natural or introduced microorganisms. This technology stimulates the natural *in situ* biodegradation of any aerobically degradable compounds in soil by providing oxygen to existing soil microorganisms to activate the process. This technique shows considerable promise of stabilizing or removing inorganic from soil as it can induce changes in the valence state of inorganic and cause adsorption, uptake, accumulation, and concentration of inorganic in micro or macroorganisms. Extremely low moisture content or low permeability soils negatively affect the bioventing performance that may limit the effectiveness of the process.

Bioaugmentation

This technique involves the addition of microorganisms that can degrade the pollutant at different stages of the process. The microbe may be naturally exotic/acclimatized/genetically engineered The microorganisms from the remediation site are collected, separately cultured, and returned to the site as a means of rapidly increasing the microorganism population at the site. In some situations different microorganisms may be added at different stages of the remediation process because the contaminants change in abundance as the degradation proceeds. The introduction of microorganism will depend on the type and abundance of the pollutant generated during degradation process.

Composting

Composting is aerobic, thermophilic treatment process in which contaminated material is mixed with a bulking agent (compost rich in bioremediation microorganisms). Typically, thermophilic conditions (54 to 65°C) must be maintained to properly compost soil contaminated with hazardous organic contaminants and in most cases, this is achieved by the use of indigenous microorganisms. Soils are excavated and mixed with bulking agents and organic amendments, such as wood chips, animal, and vegetative wastes etc. to enhance the porosity of the mixture to be decomposed. Maximum degradation efficiency is achieved through maintaining aeration and moisture as necessary, and closely monitoring moisture content, and temperature. Basically three different process designs are used in composting:

- **Aerated Static Pile Composting:** Where compost is formed into piles and aerated with blowers or vacuum pumps.
- **Mechanically Agitated In-Vessel Composting:** Where compost is placed in a reactor vessel, mixed and aerated.
- **Windrow Composting:** Where compost is placed in long piles known as windrows and periodically mixed with mobile equipment. Windrow composting is usually considered to be the most cost-effective composting alternative but it may also have the highest fugitive emissions.

Land-Farming

It is a solid phase treatment system for contaminated soil where tilling and soil amendment techniques are used to encourage the growth of beneficial microorganisms in contaminated area. Different conditions that are controlled during land farming are:

- **Moisture Content:** Usually by irrigation or spraying
- **Aeration:** By tilling the soil with a predetermined frequency
- **pH:** By adding agricultural lime
- **Other Amendments:** Such as soil bulking agents, nutrients, etc.
- Land-farming may be done *in situ* or in a treatment cell and has been successfully used to remove large petroleum spills, wood-preserving wastes (PCP and creosote), coke wastes, and certain pesticides in the soil. The large requirement of space, proper management of leachates and prevention of volatile gases are some of the limitations associated with land-farming.

Bio-Filters

Use of microbial stripping columns (containing microorganism enriched compost/soil) is to treat organic gases (volatile organic compounds).

Bioreactors

Biodegradation of contaminants is facilitated in a large tank or reactor. Bioreactors can be used to treat liquid effluents/slurries or contaminated solid waste/soil.

DEGRADATION MECHANISM OF PESTICIDES

The metabolic cooperation in microbial community under natural environmental conditions is a well coordinated process (Abraham, Nogales, Golyshin, Pieper, & Timmis, 2002). The microorganisms interact both physically and chemically to change the structure of the pesticides that need to be degraded (Briceño, Palma, & Duran, 2007). The fungi render pesticides nontoxic generally by biotransformation by introducing minor structural changes to the molecule. The bacteria biotransform pesticide further when released into the environment (Diez, 2010). The production of extracellular enzymes that act on a broad array of organic compounds is the key that is utilized by fungi to degrade different types of pesticides. Some of these enzymes are involved in lignin degradation, such as lignin peroxidase, manganese peroxidase, laccase and oxidases (Bass & Field, 2011). It is widely argued that enzyme catalyzed degradation process is not only eco friendly, but is also a cost effective method, as enzymes are central to the biology of many pesticides (Riya & Jagatpati, 2012) and are involved *via* intrinsic detoxification pathway to degrade the pesticides by microorganism of soil and water of soil and water.

Several enzymes catalyze metabolic reactions that are used by the fungi to degrade the pesticides in the environment are hydrolysis, oxidation, addition of an oxygen to a double bound, oxidation of an amino group (NH_2) to a nitro group, addition of a hydroxyl group to a benzene ring, dehalogenation, reduction of a nitro group (NO_2) to an amino group, replacement of a sulfur with an oxygen, metabolism of side chains, ring cleavage. However, the biodegradation process is dependent on both accessibility and bioavailability of microorganisms to detoxify or transform the pollutant molecule (Trigo & Valencia, 2009). The carbon-carbon and carbon-oxygen bonds that result in the depolymerisation of lignin, and the subsequent degradation of aromatic and aliphatic fragments has great complexity, with enzymes involved in the cleavage of a variety of pesticides (Hammell, 1997). It is advocated that degradation of a number of polymeric dyes, including Remazol brilliant blue and Poly R-478 correlates well with ligninolytic potential (Glenn & Gold, 1983; Freitag &Morrell, 1992), and has been used as a presumptive test to screen fungi for their potential abilities to degrade lignin and xenobiotics (Field, de Jong, Costa, & de Bont, 1992). However, it is unclear how this process degrades those pesticides that do not undergo depolymerisation by such type of reactions. As persistent insecticides lindane and DDT, ligninolytic peroxidases have no involvement in degradation by *Phanerochaete chryosporium* (Kohler, Jager, Wilershausen, & Graf, 1988, Mougin, Pericaud, Malosse, Laugero, & Asther, 1996).

The three phase process of pesticide metabolism involves the initial properties of a parent compound that are changed through oxidation, reduction, or hydrolysis to generally produce a more water-soluble and usually a less toxic product than the parent molecule. The conjugation of a pesticide or pesticide

metabolite to a sugar or amino acid, which increases the solubility of water and reduces toxicity compared with the parent pesticide, is the second stage of this process. The third phase involves the conversion of Phase II metabolites into secondary conjugates, which are also non-toxic is the final stage of this pathway. In these processes fungi and bacteria are involved producing intracellular or extra cellular enzymes including hydrolytic enzymes, peroxidases, oxygenases, etc (Paul, Pandey, Pandey, & Jain, 2005; Van Eerd, Hoagland, Zablotowicz, & Hall, 2003).

ROLE OF FUNGI IN BIODEGRADATION

Fungi represent the promising group of microbes that are economically important and help in decaying different types of pollutants through biological degradation mechanism. The ability of both yeasts and moulds, to degrade and change a wide variety of hazardous chemicals has aroused interest among researchers in using them in bioremediation (Alexander, 1999; Kaczorek & Olszanowski, 2005). Mycodegradation is a process of degradation of pollutants by fungi (Singh & Thakur, 2006). Because of the ubiquitous nature of fungi (Paul, et al., 2005), their vast numbers and large biomass in the form of hypae, wider diversity and capabilities in their catalytic mechanisms, and their ability survive under extreme environmental conditions the search for pollutant-degrading microorganisms have become curiosity in human race. Fungi can mineralize xenobiotic compounds to CO_2 and H_2O through their highly oxidative and non-specific ligninolytic enzyme system, which is one of the reasons for the decolorization and degradation of a wide range of dyes (Fu & Viraraghavan, 2001; Wesemberg, Kyriakides, & Agathos, 2003). Eukaryotic fungi have shown diverse metabolic potential resulting in metabolites similar to those produced from mammalian metabolism (Zhang et al., 1996; El-Sayed, Halim, Zaghloul, Dunbar, & McChesney, 2000; Cha, Doerge, & Cerniglia, 2001; Park, Liu, Lee, Hur, & Kim, 2002; Park et al., 2003).Among eukaryotes, white rot fungi are unique for having evolved nonspecific methods for the degradation of lignin; curiously they do not use lignin as a carbon source for their growth (Kirk, Connors, & Zeikus, 1976).

Bioremediation technologies using fungus have achieved great momentum during the last few years and is emerging a potent tool to treat and convert pollutants into harmless substances. Fungi from the natural source can be screened out as an effective tool for biodegradation of toxic organic chemicals. *Phenerochaete* and other related fungi that have ability to attack on wood possess a powerful extracellular enzyme, the peroxidase that acts on the broad range of chemical compounds. *Phenerochaete chrysosporium* is having the ability to degrade variety of chemicals including polychlorinated biphenyl (PCB), pesticides, and polycyclic aromatic hydrocarbons (PAH). Endosulfan is a chlorinated pesticide which is banned even though it is in use for agriculture in many countries. The fungal isolate *Aspergillus niger* was tested for endosulfan (widely used in India for the protection of cotton, tea, sugarcane and vegetables) degradation and it was observed that the culture could tolerate 400 mg ml^{-1} of technical grade endosulfan, complete degradation of endosulfan was observed after 12 days of incubation (Bhalerao & Puranik, 2007). *Phenerochaete chrysosporium* which is white rot fungi is well known for xenobiotic metabolism and it was reported to have the ability to degrade isoproturon (Castillo et al., 2001). Besides these, different fungal species including *Trametes sp.* and *Polyporus sp.* have the capacity to degrade variety of chemicals including pesticides. Pesticide degradation was also reported using *Aspergillus flavus, A. fumigates, A. sydowii, A. terreus, Fusarium oxysporum* and *Penicillium chrysogenum* (Hasan, 1999).

Many fungi have been tested for their ability to degrade endosulfan, including *Aspergillus niger, Aspergillus terreus, Cladosporium oxysporum, Mucor thermohyalospora, Fusarium ventricosum, Phanerochaete chrysosporium, Trichoderma harzianum* (Bhalerao & Puranik, 2009). It is argued that white-rot fungi are able to degrade different types of environmental pollutants to carbon dioxide, including a number of chlorinated pollutants such as DDT, Lindane, chlordane (1,2,4,5,6,7,8,8- octachloro- 3a,4,7,7a- tetrahydro-4-7, methanoindan) polychlorinated biphenyls, 2,3,7,8- TCDD (2,3,7,8-tetrachlorodibenzo-p-dioxin) and 3,4 dichloroaniline (Arisoy, 1998). The commonly studied white rot fungus *Phanerochaete chrysosporium* has been shown to degrade and mineralize a wide variety of industrial and agricultural pollutants. Enzymes involved in degradation of pollutants in *P. chrysosporium* are found to be lignin peroxidases (LiP), and manganese-dependent peroxidases (MnP), have also been shown to facilitate both reductive and lipid peroxidation-mediated degradation of environmental contaminants. *P. chrysosporium* is capable of degrading several chlorinated xenobiotics under conditions which do not favour the production of LiP and MnP. *P. Chrysosporium* having the ability of degrading several chlorinated xenobiotics under conditions which do not favor the production of lignin peroxidises (LiP) and manganese-dependent peroxidises (MnP) (Kullman & Matsumura, 1996). Treatment with lignin-degrading enzymes or basidiomycetous fungi, lignin peroxidase, manganese-dependent peroxidase and laccases has been used widely. Basidiomycetous fungi, ascomycetous and hyphomycetous fungi isolated from marine environments are reported for having capabilities of degradation of effluent from textile industries. *Verticillium sp.* and *Brassica chinensis* are reported for degradation of chlorpyrifos in culture medium ranging from 1 to 100 mg/L. Methods of *in situ* bioremediation by different species of *Verticillium* are also developed and achieved wonderful results. Some of the well known fungi like *Penicillium chrysogenum, Scedosporium apiospermum, Penicillium digitatum and Fusarium solani* are also reported for degradation capabilities of Polychlorinated biphenyls (PCB). These fungi show the involvement of non ligninolytic enzymes for degradation of PCBs. Hydrocarbon degrading fungi are also studied for degradation of contamination in soil (Meysami & Baheri, 2003).

The chlorinated insecticides, including DDT, aldrin, dieldrin, heptachlor, endrin, chlordane, and endosulfan, are of major environmental concern. *Trametes versicolor, Aspergillus species, Phanerochaete chrysosporium,* White-rot fungus, *Coriolus versicolor* etc. are capable of degradation of chlorinated pesticides. The organophosphorus insecticides which are moderately persistent in nature includes chlorpyrifos, malathion, parathion etc. *Alternaria alternata, Cephalosporium sp., Cladosporium cladosporioides, Cladorrhinum brunnescens, Fusarium sp., Rhizoctonia solani,* and *Trichoderma viride*, allows the degradation of chlorpyrifos in liquid culture (Singh, 2008). A fungus capable of utilizing carbofuran as one and only carbon and energy source was characterized and identified as being a member of the genus *Gliocladium* (Slaoui, Ouhssine, Berny, & Elyachioui, 2007). Microorganisms responsible for bio-degradation of hydrocarbons are capable to utilize pesticide like K-othrin, dichlorvos and carbofuran as source of carbon and energy (Odokuma & Akubuenyi, 2008). Thus, environmental contaminants are a big threat for environment but recent reports of capability of diversified fungi to utilize contaminants in different biochemical processes of primary and secondary processes provide some hopes for development of *in situ* bioremediation process to get successful results. It's also reported that *P. chrysosporium* is capable of degrading several chlorinated xenobiotics under conditions which do not favour the production of LiP and MnP (Kullman & Matsumura, 1996). Degradation of endosulfan was reported by *Aspergillus niger, Trichoderma harzianum*, *Phanerochaete chrysosporium,* and *Mucor thermohyalospora* MTCC-1384 (Siddique, Okeke, Arshad, & Frankenberger, 2003). Compared to most degrading enzymes of bacteria which have narrow substrate specificity, the ligninolytic enzymes of these fungi are very nonspecific and

extracellular. Therefore, white rot fungi can degrade various insoluble organic pollutants simultaneously (Han, Choi, & Song, 2004). *Phanerochaete chrysosporium* has emerged as a model system for studying the fungal degradation of xenobiotics (Childress, Bennett, Connick, & Daigle, 1998).

Fungal degradation of chlorpyrifos was reported by *Verticillium sp.* and its use in bioremediation of contaminated soil (Fang et al., 2008). A fungal strain capable of utilizing chlorpyrifos as sole carbon and energy sources from soil and degradation of chlorpyrifos in pure cultures and on vegetables by this fungal strain and its cell-free extract is also reported. This strain was identified as an unknown species of *Verticillium*. It opens a new research direction for development of novel bioremediation process (Yu et al., 2006). In the process of biodegradation by *P. chrysosporium* the chlorinated pyridinyl ring of chlorpyrifos undergoes cleavage and make the less toxic. But the chlorpyrifos degradation proves more efficient by mixed populations than by pure cultures of fungi. Mixed population of fungi, such as *Alternaria alternata, Cephalosporium sp., Cladosporium cladosporioides, Cladorrhinum brunnescens, Fusarium sp., Rhizoctonia solani,* and *Trichoderma viride,* allow the degradation of chlorpyrifos in liquid culture more efficiently (Singh, 2008). The role of fungi in the detoxification of herbicides has been known for many years. *P. chrysosporium* can mineralize 2,4-D and mixtures of 2,4-D and 2,4,5-T. *Penicillium sp.* utilize 2,4-D as a carbon source. Prior to ring cleavage a strain of *Aspergillus niger* dechlorinates 2,4-D . Species of *Penicillium Pullularia* and *Fusarium solani* utilize most of Acylanilides (propanil, alachlor, butachlor, propachlor, metolachlor, karsil, dicryl, and others) as the sole sources of carbon and energy. Phenylureas are one of the most prominent and diversified groups of herbicides. Major phenylureas of environmental concern are linuron, diuron, chlortoluron, and isoproturon. Fungi such as *Aspergillus niger, Geotrichum candidum, Trichoderma viride,* and *Cladosporium sp.* are efficiently and effectively capable in biodegradation of phenylureas (Singh, 2008). Organosulfur fungicides include the dithiocarbamates which are actively degraded by *Pythium ultimatum*, Rhizoctonia solani and Stereum hirsutum a white-rot fungi. Organophosphorus fungicides like pyrazophos, etc. are also reported to be degraded by *Pyricularia oryzae*. Fungicides with aromatic and heterocyclic character like quintozene and benzimidazoles are reported to be degraded by *Rhizoctonia solani, Coriolus versicolor* and *Stereum hirsutum* (Singh, 2008). The compound (s) and parameters studied as well as the fungal species employed, if>5 species are tested the number of species is given along with the most efficient Diuron degrading species reported (Table 1).

ROLE OF BACTERIA IN BIODEGRADATION

Bacteria use natural organics such as proteins, carbohydrates, and others as their source of carbon and energy. Many of the toxic and persistent compounds of environmental concern are naturally occurring relatives of these organics. For other xenobiotics, repeated exposure has resulted in the adaptation and evolution of bacteria capable of metabolizing these man-made compounds (Zhang & Bennett, 2005).

Degradation strategies exhibited by microorganisms include co-metabolism the biotransformation of a molecule coincidental to the normal metabolic functions of the microbe; catabolism- the utilization of the molecule as a nutritive or energy source; and extracellular enzymes (phosphatases, amidases and laccases) -secreted into soil, which act on the molecule as a substrate. Three basic types of reactions can occur which can be microbially mediated are: degradation, conjugation, and rearrangements. Complete degradation and breakdown of a chemical and pollutant in the soil to carbon dioxide and water involves many different types of reactions. Microorganisms are key players in determining the environmental fate

Table 1. Overview of reported studies on fungal degradation of Diuron and related studies. Listed are the compound (s) and parameters studied as well as the fungal species employed. If>5 species are tested the number of species is given along with the most efficient Diuron degrading species reported

Compound	Fungi	Studied	References
9 phenylurea herbicides incl. Diuron	*Rhizoctonia solani* (b)	Transformation	(Weinberger and Bollag, 1972)
Diuron, Linuron, Monolinuron, Monuron & Buturon.	*Cunninghamella echinulatan Thaxter* (z)	Transformation, Metabolites	(Tillmanns, Wallnöfer, Engelhardt, Olie, & Hutzinger, 1978)
Diuron, chlortoluron & Isoproturon	*Rhizoctonia solani*(b) 90 species	Degradation (screening), biomass	(Vroumsia, Steiman, SeigleMurandi, BenoitGuyod, & Khadrani, 1996)
Diuron, Chlortoluron & Isoproturon	*Bjerkandera adusta* (b) 100 species	Degradation(Screening)	(Khadrani, Seigle-Murandi, Steiman, & Vroumsia, 1999)
10 phenylurea herbicides incl. Diuron	*Botrytis cinerea* (a) 8 species	Transformation	(Berger,1998)
Diuron	*Phanerochaete chrysosporium* (b)	Degradation, ligninolytic enzymes	(Fratila-Apachitei, Hirst, Siebel, & Gijzen, 1999)
Diuron	*Cunninghamella elegans* (z) *Mortirella isabellina* (z) *Beauveria bassiana* (a)	Degradation, metabolites, ecotoxicity	(Tixier, et al., 2000)
Diuron	*Beauveria bassina*(a) *Cunninghamella elegans* (z), *Aspergillus Niger*(a) *Mortierella isabellina* (z)	Metabolite degradation, ecotoxicity	(Tixier, Sancelme, Bonnemoy, Cuer, & Veschambre, 2001)
Diuron,Metalaxyl,Atrazine & Terbuthylazine	*Coriolus versicolor* (b) 9 species	Degradation, ligninolytic potential	(Bending, Friloux, & Walker, 2002)
Isoproturon	*Mortierella sp.*(z), *Mucor sp.* (z), *Alternaria sp.* (a), *Phoma cf. Eupyrena* (a), *Basidiomycete* strain Grl77(b)	Degradation, metabolites	(Rønhede et al., 2005)
Isoproturon	*Cunninghamella elegans* (z), 15 species	Degradation, metabolites	(Hangler, Jensen, Rønhede, & Sørensen, 2007)
Diuron, chlortoluron, Isoproturon & Linuron	*Mortierella* sp.(z)	Degradation, metabolites	(Badawi, et al., 2009)
Diuron	5 *Morteilla sp.Strains* (z)	Degradation,metabolites,phyl ogenetic relationship, nutrient effects, biomass	(Ellegaard-Jensen, Aamand, Kragelund, Johnsen, & Rosendahl, 2013)

(z) zygomycete, (a) ascomycete, (b) basidiomycete

of novel compounds because they can be used as carbon and energy sources by microorganisms (Singh & Walker, 2006). Attention has focused on the isolation of bacteria that play a role in the degradation of two types of compounds due to their widespread environmental problems viz. the petroleum hydrocarbons; and chlorinated compounds including the pesticides. Following the discovery of the insecticidal properties of DDT in the late 1930s, its subsequent use and the awareness of its environmental persistence, more than 300 bacterial strains have been shown to degrade DDT (Zhang & Bennett, 2005). In the similar way, discovery of toxic effect of other pesticides result in discovery of their degrading organisms. However, due to the efforts of researchers, the detailed pathway of microbial biodegradation of about 900

chemical species is available so far (Gomez, Pazos, Guijarro, Lorenzo, & Valencia, 2007). *Pseudomonas aeruginosa, Clavibacter michiganense, Arthrobacter atrocyaneus, Bacillus megaterium, Pseudomonas mendocina, Agrobacterium radiobacter* and other *Pseudomonas* species have been reported to degrade Monocrotophos in solutions and soils (Bhalerao & Puranik, 2009). Numerous bacterial species are reported for biodegradation of different pesticides as, *Pseudomonas stutzeri* strain S1 for beta-cyfluthrin degradation (Saikia et al., 2005), Leifsonia strain PC-21 and *Pseudomonas sp.* 1G for imidacloprid degradation (Anhalt, Moorman, & Koskinen, 2007; Pandey, Dorrian, Russell, & Oakeshott, 2009). The complete biodegradation of the pesticide involves the oxidation of the parent compound resulting in to the carbon dioxide, water and energy to the microbes for their growth and metabolism. Degradation of various types of toxic or non toxic chemical compounds by the microbes is mediated through the enzymes either intracellular or extracellular. Persistence of the particular pesticide in the soil is due to the absence of the microbial systems that bears the pesticide degrading enzymes in that target soil. In such cases, where natural microbial community of target soil can't be able to degrade or detoxify pesticides, the external addition of microorganisms which have pesticide degrading capacity is recommended (Singh, 2008). Degradation of the pesticides by the microbes not only depends upon the enzyme systems but also on the different environmental conditions such as, temperature, pH, water potentials and available nutrients. Some of the pesticides are readily degraded by the microbes however; some are recalcitrant in nature (Richins, Kaneva, Mulchandani, & Chen, 1997; Mulchandani, Kaneva, & Chen, 1999). Many pesticide degrading genes are reported to be harbored by the plasmid DNA (Laemmli, Leveau, Zehnder, & Vandermeer, 2000; Chung & Jong, 1998). The plasmid encoding genes that are responsible for degradation are known as the catabolite plasmids and several bacterial species viz, *Pseudomonas, Flavobacterium, Alcaligenes, Acinitobacter, Klebsiella, Moraxella, Rhodococcus* and *Arthrobacter* containing catabolite plasmids have been already identified (Sayler, Hooper, Layton, & King, 1990). The bacterium *Raoultella* sp. X1, is reported for co-metabolic degradation of organophosphorus compound dimethoate (Liang et al., 2009). Similary, biodegradation of dimethoate with detailed biochemical pathway is also demonstrated using *Paracoccus* sp. strain Lgjj-3 (Li et al., 2010). The biodegradation of organophosphorus compounds has been extensively studied and various enzymes systems involved in the biodegradation are also well known (Singh, 2008). Besides organophosphorus compounds, the neonicotinoids are also degraded by bacterial species, *Pseudomonas sp.* 1G is reported for the biotransformation of nenonicotinic compound imidacloprid besides organophosphorus compounds (Pandey et al., 2009) and *Stenotrophomonas maltophilia* CGMCC 1.1788 for biodegradation of acetamiprid (Chen, Dai, Ding, Yuan, & Ni, 2008). Certain rhizospheric organisms were also reported to be involved in degradation of pesticide contaminants. Evidence from degradation studies in a rhizosphere system suggests that a diverse and synergistic microbial community, rather than a single microorganism, will enhance degradation of xenobiotics. Organisms found in rhizosphere of bioremediation sites are *Bacillus sp*., ectomycorrhizal fungi and *Trichoderma harzianum* (Singh, Walker, Morgan, & Wright, 2003).

Bacteria capable to uptake and degrade various insecticides are isolated from various sources (Singh, Walker, Morgan, & Wright, 2004; Saier, 2005; McGuinness & Dowling, 2009). Some of the widely used insecticides like Carbofuran and DDT are degraded by bacteria like *Alcaligenes, Pseudomonas, Rhodococcus* and *Flavobacterium* (Aislabie, & Lloyd-Jones, 1995). A widely available insecticide alpha- endosulfan, beta-endosulfan is degraded by single bacteria like *Klebsiella oxytoca, Bacillus* Sp, *Pandoraea* Sp., *Micrococcus* Sp. and by mixed bacterial co-culture (Bhalerao & Puranik, 2007). *Flavobacterium* Sp, *Pseudomonas diminuta, Pseudomonas putida, Enterobacter* Strain B-14 were isolated from chlorpyrifos contaminated sites and showed degradation capacity for chlorpyrifos (Singh et

al., 2004). Several phenylurea herbicides and their metabolites have been detected as contaminants of groundwater, lakes, seawater, rivers and streams in different parts of the world. Bacillus sphaericus has the capacity to degrade certain herbicides at moderate efficiency (Sorensen, Bending, Jacobsen, Walker, & Aamand, 2003) in such ecosystems.

Fungicides like triticonazole were reported as very less susceptible to bacterial degradation but according to Beigel, Charnay, & Barriuso (1999) triticonazole is degraded in natural soil by some organisms, probably bacteria. *B. Sphaericus, N. Catarrhalis, P. aeruginosa* are able to degrade different fungicides in soil under laboratory conditions (Engelhardt, Wallnofer, & Plapp, 1973). The initial dose of Chloropyrifos (CP), Fenitrothion (FT) and Parathion (PT) was degraded upto 58.9%, 70.5% and 82.5%, respectively within 14 days by *Serratia marcescens* at concentration of 50 mg/l when grown in municipal solid waste. It is an indicator that autochthonous microflora in soil ecosystem is characterized by a degradation potential of organophosphorus pesticides. The degradation of OPP varied with respect to the type of soil and time period. The initial dose of OPP in sandy soil was removed after 42 days. However, the addition of sterile soils in this medium increased the disappearance rate of insecticides. The ability of *S. marcescens* to degrade OPP included this microorganism in the list of biodegradative microorganisms.

Studies with green been coffee evidenced that green been coffee could be used as a nutrient source and support for bacterial growth in pesticide degradation. Among the bacterial species *Pseudomonas aeruginosa, P. putida, Stenotrophomonas maltophilia, Flavimonas oryzihabitans,* and *Morganella morganii. P. aeruginosa and F. oryzihabitans* are also rapidly degraded organophosphate and fenamiphos (FEN). *P. putida* with a P-O-C linkage unexpectedly degraded the carbamates oxamyl and carbofuran the first wild-type bacterial strain able to degrade both OPs and carbamates and exhibited high bioremediation potential against spillage-level concentrations of aged residues of FEN and its oxidized derivatives.

Soil samples were taken from different agricultural fields and analyzed for organochlorine pesticide residues by gas chromatography. The results showed that some common organochlorine pesticides DDT, DDD, DDE, HCH and Aldrin. c-HCH was detected as 47.35 ppb whereas, the concentrations of a-HCH, b-HCH, p,p -DDE, o,p-DDT were 38.81, 1.79, 7.10 and 13.30 ppb, respectively, were present in the soil. Two *Pseudomonas* strains isolated from agricultural soil were found to possess c-hexachlorocyclohexane degrading ability when the isolates were grown in a mineral salt medium containing c-HCH as the sole source of carbon (Nawab, Aleem, & Malik, 2003).

CONCLUSION

The uses of microorganisms to remediate different sites that are contaminated with pesticides have gained momentum in recent past. It is now considered an easy option to clean different ecosystems without altering the ecology of that particular ecosystem. However, bioremediation is dependent on an interdisciplinary approach that makes it complex and less successful. The successful bioremediation methods of pesticides depend on the right consortium of microbes in right place with right environmental conditions to degrade different kinds of pesticides. The ability to achieve the required clean up targets keeping the cost into consideration and residual contamination remaining after the process of bioremediation is a greatest challenge before the scientific community, as funding for this kind of basic research is diminishing. On the other hand, Universities are not offering any course related to this field that may expertise researches to accept the challenges. Keeping the challenges of bioremediation aside

there is a tremendous market opportunities for bioremediation of pesticides in developed and developing countries. Cleaning up pesticides from environment by bacteria and fungi will be a big business for the foreseeable future around the world. Inventions such as bioremediation offer promising prospects for business developments and for environmental health. Bacterial strains and fungi need to be identified that will degrade the new pesticides under different environmental conditions in a cost effective manner.

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KEY TERMS AND DEFINITIONS

Bioremediation: Bioremediation or bioaugmentation is the process of using naturally occurring microbes to digest and convert unwanted waste material into harmless substances. Our bioremediation products use friendly bacteria to digest and covert FOG into carbon dioxide and water.

Biosparging: Is an i*n-situ* remediation technology that uses indigenous microorganisms to biodegrade organic constituents in the saturated zone. In biosparging, air (or oxygen) and nutrients (if needed) are injected into the saturated zone to increase the biological activity of the indigenous microorganisms.

Cometabolism: It occurs when an organism is using one compound for growth and gratuitously oxidizes a second compound that is resistant to being utilized as a nutrient and energy source by the primary organism, but the oxidation products are available for use by other microbial populations.

Depolymerization: Is the process of converting a polymer into a monomer or a mixture of monomers. All polymers depolymerize at high temperatures, a process driven by an increase in entropy.

Leaching: Is the movement of contaminants, such as water-soluble pesticides or fertilizers, carried by water downward through permeable soils. Generally speaking, most pesticides adsorb to soil particles (especially clay), become immobile, and do not leach.

Operon: In genetics, an operon is a functioning unit of genomic DNA containing a cluster of genes under the control of a single promoter.

Pesticide: A pesticide is any substance used to kill, repel, or control certain forms of plant or animal life that are considered to be pests.

Xenobiotic: A xenobiotic is a foreign chemical substance found within an organism that is not normally naturally produced by or expected to be present within that organism. It can also cover substances which are present in much higher concentrations than are usual.

Chapter 4
Engineering of Microbes for Heavy Metal Tolerance:
An Approach for Bio remediation Technology

Megha D. Bhatt
GSFC AgroTech Ltd.,Gujarat State Fertilizers & Chemicals Ltd., India

Deepesh Bhatt
SRICEAS, M.T.B. College Campus, India

ABSTRACT

Use of microorganisms and their enzymes to degrade heavy metal contaminants from the environment, is termed as bioremediation. This chapter majorly deals with heavy metals, their toxicity and their ill effects upon the environment. It depicts how microbes can help to combat the side effect of heavy metal toxicity by stimulating their natural defensive mechanism. In spite of their natural defensive system against metal pollution, still there is an urgent need of utilizing advanced molecular tools to further exaggerate their resistance ability for bioremediation. Earlier accumulation of heavy metals was done through overproduction of various metal binding proteins located in the cytoplasm. Recently cell surface engineering of microbes appears an attractive technology for removal or recovery of metal ions from the environment. To expedite the degradation of pollutant, a number of different molecular tools have been established for improving the microbial strains at molecular and genetic level. Microbial engineering thus, seems a promising approach which elucidates the effect of biotechnological processes used for decontaminating the polluted environment and in the future, humans and animals might gain from these organisms in remediating environmental contamination. However, these genetic modifications should be stable and harmless towards the nature as well as for the microbes itself and any genetic alterations must always ensure the actual pros and cons behind it.

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INTRODUCTION

Environmental pollution caused by organic and inorganic pollutants has constantly increased in parallel with growing population, rapid industrialization and increasing urbanization especially in developing countries (Hettige, Huq, Pargal, & Wheeler,1996). Heavy metals are the main constituent of these toxic pollutants. The rapid depletion in the environmental health provoked scientists to develop technologies and strategies for the removal and sequestration of these hazardous contaminants. A potential resolution of this problem could be bioremediation, which makes use of microbial system for the removal and transformation of the xenobiotic compounds and various heavy metals (Siddique et al., 2005; Hussain, Arshad, Saleem, & Khalid, 2007). Although, bioremediation involves multidisciplinary approaches but it solely depends upon the nature of microbial system (Shukla, Singh, & Sharma, 2010). Xenobiotics degrading microorganisms such as (*Pseudomonas, Burkholderia, Sphingomonas, Ralstonia, Comamonas, Achromobacter, Alcaligenes, Rhodococcus, Dehalococcoides*) have properties to accumulate or detoxify heavy metal pollutants namely Cd, Hg, Pb, Zn, U, etc. (Daly, 2000; Lloyd, Lovley, & Macaskie, 2003). These organisms accumulate heavy metals in a metabolic dependent manner as these metals act as a catalytic component of biochemical and enzymatic reactions occurring inside their system (Holm, Kennepohl, Solomon, 1996; Eide, 1998). This property is because of the net negative charge present in bacteria and the cationic charge present in many toxic metals. Bacterial surface majorly contains carboxyl, phosphoryl, hydroxyl and amino functional groups. Upon pH variation these groups usually deprotonate leading to a net negative charge on these functional groups; this property makes them functionally capable of metal adsorption. Moreover the high surface area to volume ratio of bacteria allows them to accumulate metal ions in an amount greater than their own weight (Beveridge, 1989).

This property instigated the idea that whether the genetic engineering of microorganisms may be possible to over accumulate or adsorb these metal ions on the bacterial cell surface. This may facilitate the cleanup of toxic metal ion from the environmental and industrial effluents. Recently bioremediation using engineered organisms has become a fascinating technique that has gained importance as an eco-friendly and efficient strategy (Shukla et al., 2010; Liu, Zhang, Chen, & Sun, 2011). There are still many more challenges ahead to use these engineered bacteria as a catalyst in bioremediation technology, taking into consideration, a virtually uncontrolled condition. In this regard recombinant DNA technology can be an effective tool for optimization of bioremediation, if we target the anaerobic and unculturable bacteria through metagenomic technology (Cases & De Lorenzo, 2002). We can explore genes for heavy metal removal from the nature irrespective of their host. Recent developments in bioremediation technology, which includes the utilization of metabolic engineering, whole-transcriptome profiling, protein engineering and proteomics are also considered significant for removal of toxic contaminant such as heavy metals (Thomas, 2008).

Another aspect is the recovery of metal ions by microorganisms using cell surface engineering, seems as an effective approach for developing customized bio-adsorbants. The cell surface display of known metal-binding proteins/peptides and the molecular design of novel metal-binding proteins/peptides have been performed using a cell surface engineering approach (Kuroda & Ueda, 2011). The cell surface engineering technology utilizes the property of a metal binding protein or peptide by fusing it with the cell surface protein in order to express the protein on the cell surface. These cell surface proteins have an intrinsic property to attach across the cell surface. Therefore over expression of various metal ions binding proteins, such as metallothioneins or cysteine rich peptides and glutathione, in bacteria may prove a promising strategy for remediation through microbial-based bio-adsorbent.

HEAVY METALS AND THEIR EFFECTS

Steady accumulation of heavy metal in the environment is a major threat which is mounting with an exponential rate. Heavy industrialization is leading to the accumulation of heavy metals like chromium, mercury, uranium, selenium, zinc, arsenic, cadmium, gold, silver, copper and nickel. Among them lead, arsenic, mercury, cadmium and aluminum prove most vulnerable to our ecosystem. Most heavy metals are transition elements of d orbital. This d orbital provides heavy metal cations having a tendency to form complex compounds. Therefore, these cations play an important role in biochemical reactions. Some heavy metals at higher concentration, form non-specific complexes inside the cell which leads to toxicity.

Heavy industrialization results in the concomitant release of toxic particles in air, water, soil that has an adverse effect for humans and plants. Human activity including automobile usage, coal combustion and rock mining, excessive use of pesticides, fertilizers and minerals further exacerbate the situation and toxic particle penetrate through soil and leach out to enter the aquatic environment. Reaching aquatic ecosystem it adversely affects the micro flora and other marine life by entering into their vital organs. Thereby, it impedes a number of vital physiological processes viz. respiration, metabolic activity and reproduction process. Larger fish that feed on smaller ones generally contain higher amounts of toxic chemicals due to bio-accumulation and bio-amplification. As compared to smaller fishes, these larger fishes when consumed by human's leads to hazardous implications such as multiple sclerosis, Alzheimer's disease, Parkinson's disease and more recently observed Japanese "Minamata disease" (American Fisheries Society [AFS], 2010).

Daily need household items like nail paints, emulsion paints, cosmetics, foils, cookware items are another common source of heavy metal exposure. Lead and aluminum are the most common heavy metals found in cosmetics, but other harmful chemicals such as petroleum, silicone and various industrial parabens may frequently exist in beauty products. Lead pipes fitted and lead derived paints in old houses were a potential source of water contamination earlier, which is now strictly barred for their usage. Juvenile generation, pregnant women and developing fetus are more prone towards the ill effect of these heavy metals as their developing organs are extremely vulnerable to the negative effects of heavy metals.

Heavy metals such as cadmium and lead are non-essential elements for plants. If over accumulated, may hinder the solute transport, thus cause perturbation in regular metabolism and relocation of essential elements that is essential process in plants. There is a temporal effect of these heavy metals in plants as it affects differently in various growth stages of a plant. For example in the early stage, cadmium inhibits photosynthesis and growth then inhibits the reproductive organ differentiation and finally disturbs the solute transport and nutrient mobilization in rice plants (Wang, 1996). In order to cope with these deleterious effects plants react by increasing the production of various antioxidant enzymes, chelating agents and essential peptides.

Higher organisms and plants respond to the presence of heavy metals with the production of cysteine-rich peptides such as glutathione (GSH) (Singhal, Andersen, & Meister, 1997), phytochelatins (PCs), and metallothioneins (MTs), that bind metal ions and sequester them in biologically inactive forms (Hamer, 1986; Stillman, Shaw, & Suzuki, 1992). Apart from this several microbes are known to counter the deleterious effect of these heavy metal ions by cell surface based adsorption.

MICROBES HELPING IN BIOREMEDIATION: GENERAL ATTRIBUTES

Microorganisms those help in bioremediation processes are majorly chemolithoautotrophic in nature and are able to use ferrous ion or reduced inorganic sulfur (or both) as an electron donor. These are acidophile in nature as the main byproduct of sulfur oxidation is sulfuric acid, so the pH range in which they grow is usually 1.5- 2.0. This acidophilic nature is not restricted up to sulfur oxidizing bacteria rather it implies on iron oxidizing bacteria too. They grow best in highly aerated conditions, as they are able to use electron acceptor other than oxygen. They can also fix the atmospheric CO_2 but the efficiency differs with different organisms. Bacteria, which are not able to fix atmospheric CO_2 in a high level, usually require either increased levels of CO_2 or a little amount of yeast extract. Bio-mining bacteria's are usually resistant to various heavy metals with different tolerance level. These general attributes of the bio-mining bacteria explains their growth in inorganic environment created by aeration in a suspension of iron or sulfur containing minerals in water. The metal acts as an electron donor, air acts as a carbon source (CO_2) and the preferred electron acceptor (O_2) and water is the medium for the growth. Some organisms are also reported to fix the atmospheric nitrogen in the rare condition.

HEAVY METAL: TOXICITY, TOLERANCE, AND RESISTANCE IN BACTERIA

To have any physiological or toxic effect, heavy metal has to enter inside the cell. At first sight, these divalent metal ions such as Mn^{2+}, Co^{2+}, Ni^{2+}, Cu^{2+}, Zn^{2+} and Fe^{2+}seem structurally very similar to the cell having a double positive charge and an ionic diameter between 138 pm to 160 pm (Weast, 1984) but is not always the case. Chromate having four tetrahedral oxygen atoms and two negative charge, that differ mostly in size and resembles that of sulfate. The same is true for arsenates and phosphates. So, a different uptake system in the organisms is present which has to bind tightly these metals in order to differentiate minor changes found in structurally similar metals.

Generally cells utilize two strategies for uptake of these heavy metals: one is non-specific and fast and another is very specific and comparatively slow. First uptake system is expressed constitutively and utilizes multiple substrates. It binds the substrate non-specifically and uses chemiosmotic gradient generated across the cell membrane of the bacteria. While the second one is very specific in binding the substrate and utilizes the energy resulted through ATP hydrolysis. Since, they are the expensive systems for the cell, so they are inducible in nature and are utilized by the cell in specific times, during starvation and in special metabolic condition (Nies & Silver, 1995). In gram-negative bacteria (Smith, Thompson, & Maguire, 1995; Tao, Snavely, Farr, & Maguire, 1995), archaea (Smith, Gottlieb, Kucharski, & Maguire, 1998) and baker's yeast (MacDiarmid & Gardner, 1998), fast and unspecific CorA (metal inorganic transport MIT family) magnesium uptake system is utilized for accumulation of Ni^{2+}, Co^{2+}, Zn^{2+}, and Mn^{2+} while, arsenate and chromate is transported by the fast pit (phosphate inorganic transport) system and fast sulfate-uptake system respectively (Nies & Silver, 1995). In spite of these systems, there are inducible P-type ATPase for magnesium uptake, ATP-binding cassette (ABC) transporters for Mn^{2+}, Zn^{2+} and Ni^{2+}. Slow and specific chemiosmotic transporters the Hox-N family for Ni^{2+} and Co^{2+}, and ABC transporters for sulfate and phosphate are also found in bacteria.

When a cell receives signals for the high concentration of heavy metal accumulated by unspecific system the specific transporters continuously opens in response to that signal. This accumulation of heavy metal is because these unspecific transporters majorly are expressed constitutively in the cell.

So this 'open gate' is the main reason behind the toxicity of heavy metals (Nies & Silver, 1995). After entering inside the cell, these heavy metals, especially of the high molecular weight interact with thiol group (SH) and by binding with SH group it inhibits many important functional enzymes. Other heavy metal cations may interact with further ions thereby inhibiting there physiological activity. In gram negative bacteria, heavy metal cation binds with glutathione (GSH) and makes the complex namely bisglutathionato complex which further reacts with molecular oxygen to form oxidized bisglutathione (GS-SG), the metal cation and hydrogen peroxide (H_2O_2) (Kachur, Koch, & Biaglow, 1998). As soon as the complex formation takes place, it reduces in NADPH dependent manner and the free metal ions are again ready to take up the two glutathione molecule. In this manner these heavy metal ions lead to oxidative stress inside the cell.

The tendency of the heavy metal to form complexes and cause oxidative stress due to open gate, forced the cell to evolve new metal resistance strategies. Since, the heavy metals cannot be transformed or degraded inside the cell which needs to develop new strategies for detoxification. First is to diminish these metal ions by efflux system (Nies & Silver, 1995); the second one is to reduce these ions to a less toxic form and the last is to differentiate these ions into complexes by thiol-containing compounds. For any metal, cell utilizes a combination of two or three of the above mentioned strategies. For heavy metal reduction the redox potential of that metal should lie between hydrogen/proton coupling and oxygen/hydrogen coupling potential. If any metal does not lie between above mentioned redox potential, then they have to follow between the complex and the efflux or both. Complex formation is a very expensive method as compared to efflux system so the latter is preferred and thus it can be concluded that heavy metal metabolism majorly employs upon transport metabolism (Nies, 1999). However, all these strategies are somehow limiting and therefore can't be employed for a concentration that exceeds a tolerance limit for the organism or plant.

Here is the classic example of heavy metal tolerance is *P.fluorescence* which can tolerate a high concentration of aluminum (Auger et al., 2013). After entering in the cell Al interfere with the Fe homeostasis and the enzyme which depends upon the catalytic activity of this metal are easily inactivated. Specially, the enzymes of the tricarboxylic acid (TCA) cycle and electron transport system (ETS) which depend upon Fe-S cluster for their activity and thus becomes inactivated (Lemire, Mailloux, Auger, Whalen, & Appanna, 2010; Yamamoto, Kobayashi, Devi, Rikiishi, & Matsumoto, 2002). These include aconitase (ACN), fumarase (FUM), succinate dehydrogenase (SDH), complex I and complex IV respectively (Lemire et al., 2010; Middaugh et al., 2005). In bacteria, the absence of the iron results the energy deficiency by disabling the aerobic production of ATP (Yamamoto et al., 2002). To compensate this loss, bacteria have to generate the energy via substrate level phosphorylation. The two ways of substrate level phosphorylation in the bacteria are: glycolysis and modified TCA cycle (Singh et al., 2009). In modified TCA cycle, bacteria have to express the iso-citrate lyase (ICL), which is further changed into succinate and glyoxylate (Middaugh et al., 2005). In a similar study the expression of iso-citrate lyase was recorded to increase five times than in normal condition (Lemire et al., 2010). In spite of decreased activity of aconitase (ACN), the flow of metabolite remains as such in the cell because of up-regulation of NADP-dependent isocitrate dehydrogenase (ICDH-NADP) (Middaugh et al., 2005). Working together with ICL are the enzymes acylatingglyoxylate dehydrogenase (AGODH), succinyl-CoA synthetase (SCS) and oxylate-CoA transferase (OCT) (Singh et al., 2009). After deactivation of oxidative phosphorylation, SCS produces ATP by substrate level phosphorylation and fulfills the demand of energy in the organism (Bochud-Allemann & Schneider, 2002). Completion of TCA cycle is done by switching an iron-dependent isoforms of fumarase (FUM A) to one which does not require a Fe-S cluster for catalytic activity (FUM

C). If this would have not occurred in the cell the TCA cycle would have been stopped (Chenier et al., 2008). In this way, natural resistance and tolerance towards heavy metals in bacteria paved the path for their genetic engineering to make more efficient strain in order strengthen the bioremediation process as a powerful tool.

GENETIC ENGINEERING FOR IMPROVED BIOREMEDIATION

There are majorly three techniques employed under genetic engineering based enhancement and engineering of microbes which are majorly as follows:

Manipulating Bacteria and Transgenic Plants

Genetic engineering of naturally resistant bacteria is pivotal in elaborating efficient biotechnological processes aimed at decontaminating metallic pollutants. One strategy for developing a superior strain is combining the catabolic segment more than one organism within a single host, in this way we can complete the metabolic pathway of xenobiotic compounds and inhibit the formation of toxic end products which are otherwise co-metabolized. A successful implementation of this technique has been done for the degradation of highly toxic trihalo-propanes, for which neutralization has not yet been possible (Bosma, Kruizinga, De Bruin, Poelarends, & Janssen, 1999).

A similar technique of combining complementary metabolic activity has been utilized for the microorganisms capable of degrading Polychlorinated biphenyl (PCB) by combining an oxidative pathway for (chloro)-biphenyl transformation (encoded by the *bph* genes) into (chloro)-benzoate with achlorobenzoate degradative pathway. Conjugative mating is used for developing hybrid strains to incorporate the bph gene into chlorobenzoate degraders (Reineke, 1998), which degrade the chlorobenzote through a degradative pathway with chlorocatechols as an intermediate product in the process. In *Comamonas testosteroni* strain VP44, degradation of biphenyl along with co-metabolism of chlorinated biphenyls takes place. By cloning and expressing the genes encoding enzymes for *ortho* and *para*-dechlorination of chlorobenzoates, strains capable of growing onchlorinated biphenyl and completely dechlorinating 2- and 4-chlorobiphenyl were obtained (Hrywna, Tsoi, Maltseva, Quensen, & Tiedje, 1999). There are many more toxic metals which are hazardous and toxic to the environment and are non-degradable but genetic engineering made it possible to transform or degrade them to a simpler form. Chromium (Cr) is a carcinogenic and toxic metal, found generally in industrial waste water. In order to remove chromium, genetically engineered bacteria namely *Ralston metallidurans* is used (Srivastava, Jha, & Mall, 2010). Cadmium (Cd) can be removed from waste water with the help of recombinant *Caulobacter* spp. strain JS4022/p723-6H (Patel, Zhang, & Michael, 2010). Over expression of *ArsM* gene in plants resulted in the removal of Arsenic from contaminated soils, which was possible earlier only through volatilization (Jackson, Seaman, & Bertsch, 2006; Chen, Zhu, & Hong, 2008; Williams, Lei, & Sun, 2009). Nickel (Ni) is possibly the most recalcitrant pollutant and can be accumulated by the engineered *E. coli* SE5000 strain from an aqueous solution (Fulkerson, Garner, & Mobley, 1998). These various examples further corroborate the fact that genetically engineered (GE) microbes are capable of enhancing the bioremediation of heavy metals from contaminated sites in the environment.

The combination of catabolic fragments can be introduced not only in bacteria, but also plant can be an appropriate recipient. It is a well-documented fact that plants efficiently accumulate metal ions

for bioremediation and can also accept the genetic fragment from bacterial origin. Mercury is the most toxic heavy metal which can be released into the environment. Cloning and expression of *Mer*A gene in the bacteria can remove the mercury from contaminated water and soil (Chen & Wilson, 1997).When a bacterial mercuric reductase gene was expressed in poplar plants these were able to germinate and survive normally in toxic levels of mercury and also transported the soil bound mercury out of the soil (Rugh, Senecoff, Meagher, & Merkle, 1998). Similar was the case observed with model plant *Arabidopsis thaliana* when the organomercurial lyase gene was expressed in *Arabidopsis thaliana* plants, they were able to grow normally in the presence of toxic organomercurial by transforming it into ionic mercury (Bizily, Rugh, Summers, & Meagher, 1999).

It was reported that transgenic cauliflower, after expressing the *CUP1* gene from yeast, showed a 16 fold increased accumulation of cadmium (Chatthai, Kaukinen, & Tranbarger, 1997; Sriprang & murooka, 2006). Similarly the accumulation of agro chemicals can also be prevented by utilizing the same property of transgenic plants (Macek, Kotrba, & Svatos, 2007). Thus transgenic plants having modified gene of bacterial origin, can be a viable alternative for efficient bioremediation.

Optimization of Enzymes Involved in Bioremediation

At the present time, with the help of detailed information of structure and function of the enzymes involved in bioremediation, it is possible to manipulate them for their better catalytic activity. Site directed mutagenesis is a popular approach used for improving enzyme function if the crystal structure and detailed characterization is available. Besides this, many DNA shuffling techniques like (random fragmentation of a population of mutant genes in a certain family followed by random reassembly) are developed, by which we can create chimeric proteins and protein variants with improved functionality.

In xenobiotic degradation, Haloalkane dehalogenases were among the first enzymes, which can be optimized for its better catalytic activity (Schanstra et al., 1996; Holloway, Knoke, Trevors, & Lee, 1998). A variant of enzyme muconate cycloisomerase (involved in the degradation of natural aromatic compounds) was created in order to increase the specificity constant for chloromuconate (Vollmer et al., 1998). Using this system, variants of cytochrome P450 with high activity against naphthalene was identified (Joo, Lin, & Arnold, 1999). In this way, such techniques will provide the base for successful interventions into environmental processes and, thus, will lead to further optimized strategies for bioremediation.

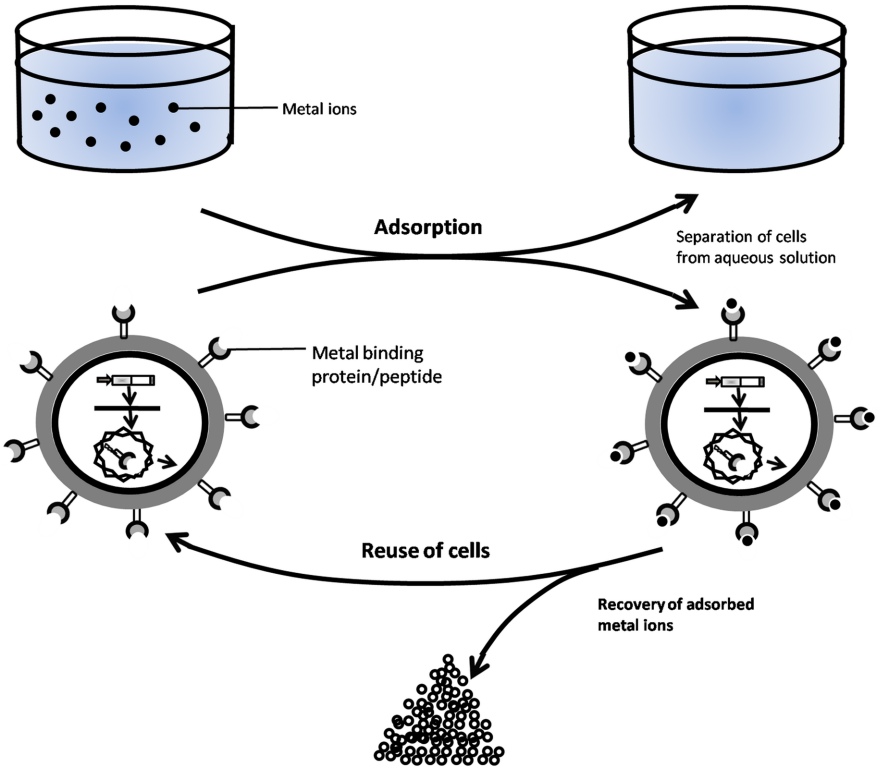
Adsorption of Heavy Metal through Microbial Cell Surface Engineering

Some microbes have an innate ability to adsorb the heavy metals. Bacteria, having a high surface to volume ratio can easily adsorb the metal even if present in a low concentration because it leads into a large area that can interact with metal ion in the surrounding environment. *Saccharomyces cerevisiae* is extensively investigated for heavy metal ion adsorption in aqueous solutions, in studies done by various researchers (Volesky & May-Phillips 1995; Vasudevan, Padmavathy, & Dhingra, 2002). Genetic engineering extensively enhanced the ability of these microorganisms to adsorb the heavy metal ions. Main focus of all these studies was to increase the ability of microorganisms to adsorb the metal ions through intracellular detoxification in a metabolic dependent manner (Perego & Howell 1997; Clemens, 2001; Hall, 2002). However enhanced adsorption of metal ions through metabolic independent manner with the help of cell surface engineering, now have become an emerging technology (Anonymous, 1997;

Ueda & Tanaka 2000a, b). It has many advantages over the previous methods. The time taken to adsorb a metal ion is less as compared to intracellular adsorption and the adsorbed metal can also be recovered easily without breaking the cell (Figure 1). In addition to this, the adsorption ability remains maintained as long as the metal binding molecule will adhere to the cell, no matter cell is alive or dead (Kuroda, Shibasaki, Ueda, & Tanaka, 2001). This technology majorly utilizes the protein expression and protein trafficking property present in a cell. If the molecular information of the cell surface protein is available we can express the heterologous protein at the cell surface by fusing it with the cell surface protein or a similar variant. This technology makes use of the cell surface architecture of both Gram-negative and Gram-positive bacteria.

Till date, various cell surface-engineered microorganisms with novel functions have been designed those have wide applications such as bioconversion using whole cell as biocatalyst, live vaccine development, bioadsorption, biosensor, and library screening. (Georgiou, Poetschke, Stathopoulos, & Francisco, 1993; Anonymous, 1997; Ueda & Tanaka, 2000a, b; Samuelson, Gunneriusson, Nygren, & Stahl, 2002; Lee, Choi, & Xu, 2003; Kondo & Ueda, 2004). Cell surface engineering have successfully generated microorganisms, those accumulate enhanced metal-binding proteins/peptides such as metallothioneins, phytochelatins, metallo-regulatory protein of bacterial mercury resistance operon, hexahistidine, histidine-rich peptide (Gly-His-His-Pro-His-Gly;HP), and cysteine-rich peptide (Gly-Cys-Gly-Cys-Pro-Cys-Gly-Cys-Gly; CP) and in turn these engineered microorganisms showed enhanced rate of bioremediation towards these toxic metals (Sousa, Kotrba, Ruml, Cebolla, & De Lorenzo, 1998; Kotrba, Doleckova,

Figure 1. A technique for adsorption and recovery of metals on microbial cell surface
(Permission acquired from: Kuroda & Ueda, 2010).



De Lorenzo, & Ruml, 1999; Bae, Chen, Mulchandani, & Mehra, 2000; Bae, Wu, Kostal, Mulchandani, & Chen, 2003; Samuelson, Wernerus, Svedberg, & Stahl, 2000; Kuroda et al., 2001; Kuroda & Ueda 2003; Saleem et al., 2008). It has also been reported that microorganisms exhibited increased tolerance towards toxic metal after cell surface surface-display of metal-binding proteins/peptides (Kuroda et al., 2001; Kuroda & Ueda, 2003, 2006). These results indicated that cell surface engineering could be an effective strategy for microbial strain improvement, thereby developing effective metal tolerant cells.

Designing of Metal Binding Proteins/Peptides for Display

Construction of bio-adsorbent for targeting specific metals is a challenging task. For development of bio-molecules, a series of 20 amino acids is required. It is advantageous to have different combinations of amino acid for the alteration of proteins/peptides. Majorly for the expression of any protein within a cell, its absolute quantification and purification is necessary, but in cell surface display based system, there is no need for such exercises (Georgiou et al., 1997; Wittrup 2001; Ueda, 2004). After Surface engineering, cells are considered as microparticles covered with proteins, so screening of these proteins/peptides with desired functions through the available libraries of proteins becomes easy. This system is also utilized in preparation of random peptide libraries (Matsui, Kuroda, & Ueda, 2009). The engineered cells having a property to bind the rare and hazardous metals were then screened from these random peptide libraries (Brown 1997; Mejare, Ljung, & Bulow, 1998; Kjaergaard, Schembri, & Klemm, 2001; Dong et al., 2006). Till now various metal binding proteins such as Glutathione (GSH), GSH-related phytochelatins(PCs), cysteine-rich metallothioneins (MTs) and synthetic phyto-chelatins (ECn), have been used for the cell surface engineering.

Yeast: A Model Organism for Cell Surface Designing

Being a eukaryote, yeast is **g**enerally **r**egarded **a**s **s**afe (GRAS), for its application in various fields. It has a number of qualities which make it a suitable host for protein engineering (Boder & Wittrup, 2000). Cell surface engineering is majorly done in baker's yeast (*Saccharomyces cerevisiae*), which is a model organism for surface engineering. A major advantage with yeast is that it allows complex processes such as glycosylation and folding of the expressed heterologous proteins. A major advantage is its rigid structure which is a prerequisite for surface engineering (Kondo & Ueda, 2004). In surface engineering, yeast cell has two kinds of mannoproteins: Sodium dodecyl sulfate (SDS) extractable mannoprotein and another one is glucanase extractable mannoprotein. These proteins contain putative glycosyl phosphatidylinositol (GPI) attachment signal at the C-termini (Van Der Vaart et al., 1997) and generally they are rich in serine or threonin. For the covalent attachment of proteins with the cell wall, this GPI anchor is needed. Proteins, which need to be anchored in the cell wall, are fused with mannoproteins and are covalently bonded with GPI anchor. The process of surface engineering in yeast is generally GPI anchor dependent (Lee et al., 2003).

Clean Gene Techniques for Bacterial Engineering

Instability of the cloned gene, when present on the plasmid or the inheritance of marker gene, is the main problem in recombinant DNA technology. This problem can be solved by using mini-transposon element by stable integration of foreign genes in host (De Lorenzo, Herrero, Sanchez, & Timmis, 1998).

In a relative study, transposons were used for highly stable integration of oxygenase gene in the chromosome of some recombinant strains (Panke, De Lorenzo, Kaiser, Witholt, & Wubbolts, 1999). Now a day, tellurite-resistance determinants are being used as non- antibiotic markers in place of antibiotic markers (Sanchez-Romero, Diaz-Orejas, & De Lorenzo, 1998). A major prerequisite now is to develop marker free recombinant strains those are mostly free of non-essential tags that are normally inherited through cloning. This results in the development of natural and viable strains that exclusively bear the DNA encoding for the phenotype of our choice (Panke, Sanchez-Romero, & De Lorenzo, 1998).

APPLICATIONS OF METAL RESISTANT BACTERIA

Metal resistant bacteria are known to detoxify heavy metals from their own cytoplasm but not from the outer environment, in term they are known as selfish bacteria (Nies, 2000). Let us take the example of metal resistant bacteria *Ralstonia* sp CH34. This bacterium can be used for the precipitation of heavy metal and degradation of xenobiotic compounds by using its metabolic waste but cannot be used directly for heavy metal remediation. Generally, heavy metals can be precipitated in the presence of carbonate ions. When this bacterium is grown in presence of organic acids such as lactate, gluconate or acetate, due to uptake of protons, pH increase and as a result carbonate is produced. Due to this increase in pH value, the solubility of carbonate is also increased and the process of metal detoxification is thus achieved. For example, if we grow CH34 in 3 g/l of sodium acetate then 100 mM carbonate is produced which is sufficient to precipitate up to a few mili molar (mM) concentration of heavy metals. Moreover, heavy metal cations namely (cd^{2+}, Cu^{2+}, Pb^{2+}, Ni^{2+} and Zn^{2+}) have a solubility product of carbonate in the range of 5.2 (Weast, 1984). This technique is successfully implemented in a tubular membrane reactor to precipitate heavy metal from metal contaminated waste water (Van Roy, Peys, Dresselaers, & Diels, 1997).

Many bacteria are also reported to have metal resistant capabilities along with plant growth promotion properties. Many researchers have demonstrated that heavy metal resistant and plant growth promoting endophytic bacteria can be isolated from the plants grown in the metal contaminated sites and can be used as a bioinoculants for the effective phytoremediation. These endophytic bacteria protect the plants from inhibitory concentration of heavy metal and promote the plant growth by producing IAA or ACC deaminase. However, a further understanding is still required to study the intimate relationship between plant growth promoting endophytic bacteria and plant, for an efficient phytoremediation of metal contaminated soil.

CONCLUSION

Today, bioremediation is primarily explored for purifying metal contaminated waste. Engineered microbes for heavy metal tolerance and customized bio-adsorption for recovery of toxic metal ions have proved as indispensable tools in the area of applied biotechnology. Having various applications, such as fusion of anchoring motif to the cell surface, termed as cell surface display, live vaccine development, peptide library screening and bioconversion, using whole cell biocatalyst. It is evident that these techniques are still in a juvenile phase and, thus, to fully exploit the natural diversity for biodegradation bioremediation purpose a detailed knowledge of protein translocation pathways is required. Cross talk between the xenobiotic and the microorganism, taking into consideration the biochemical activities and the survival

rate of that microorganism, will provide the ground for utilizing their positive involvement towards the environment. This might assist in formulating strategies for crafting superior biocatalyst and also enhancing the reproducibility of a microorganism that is to repeatedly perform in a similar manner under varying environmental conditions. As more and more scientific opinions are evaluated that explains difficulties in displaying multi-meric protein complexes, there are chances that further commercial applications of cell surface display will appear. In near future novel systems for specific metal tolerance, which are spatially regulated might revolutionize future strategies for bioremediation.

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KEY TERMS AND DEFINATIONS

Adsorption: The process by which molecules of a substance, such as a gas or a liquid, collect on the surface of another substance, such as a solid. The molecules are attracted to the surface but do not enter the solid's minute spaces as in absorption.

Bioremediation: The use of either naturally occurring or deliberately introduced microorganisms to consume and break down environmental pollutants, in order to clean a polluted site.

Clean Gene Technology: Clean-gene technology is a safe way to genetically modify crops. This means that crops do not carry what are called 'selectable marker genes', such as genes resistant to antibiotics or herbicides.

Genetic Engineering: Genetic engineering is the deliberate, controlled manipulation of the genes in an organism with the intent of making that organism better in some way. This is usually done independently of the natural reproductive process. The result is a so-called genetically modified organism (GMO).

Heavy Metal Tolerance: A biochemical and physiological adaptation to heavy metals (i.e. metals, e.g. copper and zinc, that have a density greater than 5 g/cm^3) shown by plant species or genotypes: such plants may therefore be found growing successfully on soils contaminated by metals, where other species or genotypes would fail.

Microbial Cell Surface Display: Cell-surface display allows peptides and proteins to be displayed on the surface of microbial cells by fusing them with the anchoring motifs. The protein to be displayed - the passenger protein - can be fused to an anchoring motif - the carrier protein - by N-terminal fusion, C-terminal fusion or sandwich fusion.

Xenobiotic Degradation: A xenobiotic is a chemical substance which is foreign to biosphere. Its chemical or microbial removal from nature is called Degradation.

Chapter 5
Arsenic Pollution in the Environment:
Role of Microbes in Its Bioremediation

Munawar Sultana
University of Dhaka, Bangladesh

Santonu Kumar Sanyal
University of Dhaka, Bangladesh

Mohammed Anwar Hossain
University of Dhaka, Bangladesh

ABSTRACT

Arsenic (As) pollution in drinking water and soils poses a threat to over 100 million people worldwide, making it one of the largest environmental catastrophes particularly in Bangladesh and West Bengal-where more than one-third of the population are at risk. Microbial As metabolism and mobilization in aqua system is relatively a recent issue.The presence of the arsenic oxidation, reduction, and extrusion genes (aioA, arrA, arsB, and acr3) are explored within microorganisms retrieved from As-contaminated environments. However, the nature of microbiome involved within a certain As transformation environment is still an area of research, specifically how microbial redox transformations occur, that can be exploited to mitigate the longstanding problem. The present chapter overviews the mechanism of As pollution in various environment, microbial diversity in such environment, correlation of their activities to the biogeochemistry of As and finally application of microbes as a bioremediation tool for As detoxification and bioremediation.

INTRODUCTION

Arsenic (As) is chemically classified as a metalloid, having properties of both a metal and a non-metal. The word 'Arsenic' is derived from the Greek word *arsenikon*, meaning 'potent'. The metalloid arsenic (As) was first discovered in 1250 by the German alchemist Albertus Magnus via heating of soap with orpiment (arsenic trisulfide) (Matschullat, 2000) and later it was classified as a member of group V of

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the periodic table of elements. The element occurs in the environment in different oxidation states and forms various species, e.g. As(V), As(III), As(0) and As (-III). As cannot be easily destroyed and can only be converted into different forms or transformed into insoluble compounds in combination with other elements, such as iron. It is widely distributed in the nature and is commonly associated with the ores of copper, lead, gold, sulfur and iron (C. Palache, 1951).

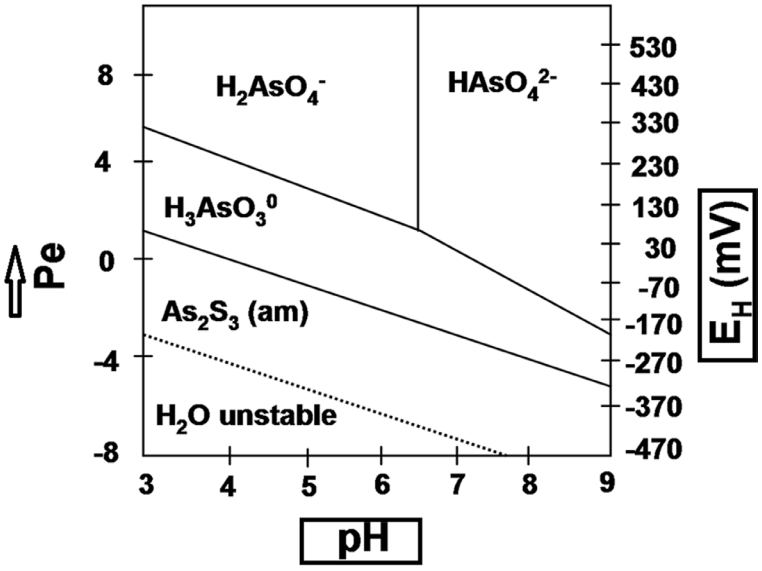
The predominant forms of As in soils and aquifers are inorganic arsenate [As(V)] and arsenite [As(III)], with the later being more mobile and toxic. Arsenic (As) is introduced into soil and groundwater during weathering of rocks and minerals, followed by subsequent leaching and runoff. Himalayan-derived sediment is the source of groundwater As contamination in large areas of south and southeast Asia. In Bangladesh and West Bengal (India), approximately 60 to 100 million people rely on drinking water containing As in excess of the World Health Organization standard of 10 µg/L (international) and 50 µg/L (Bangladesh). Consequently, people and livestock are being exposed to As via contamination of drinking water and consumption of food grown in As-contaminated soil or irrigated with As-enriched water (Banejad & Olyaie, 2011). So, arsenic distribution and toxicology in the environment is a serious concern, with millions of individuals being affected by As toxicities. Arsenic is known to have mutagenic and genotoxic effects on humans. Chronic exposure to As can cause a wide variety of adverse health effects including dermatological diseases, skin and internal cancers (Choong, Chuah, Robiah, Gregory Koay, & Azni, 2007). The following segments of this manuscript will illustrate the arsenic nature and toxicity, its severity and especially microbes mediated arsenic detoxification mechanisms, to explore the potential role of microbes in arsenic bioremediation.

SPECIATION AND TRANSFORMATION OF ARSENIC

Arsenic exists in the environment mainly in four oxidation states, As(-3) (arsine), As(0) (native or elemental arsenic), As(+3) (arsenite) and As(+5) (arsenate). The first two forms are relatively rare, whereas arsenite and arsenate are the two main forms occurring in aquatic environments (Lièvremont, Bertin, & Lett, 2009). Both biological and chemical parameters especially the redox potential (Eh) and the pH are important for the stability, speciation and distribution of arsenite [As(III)] and arsenate [As(V)] in the environment. As(V) is predicted to be thermodynamically stable form at Eh values > ca. -100 mV at pH 8.0, and > 300 mV at pH 4.0. Below those redox potentials, As(III) is the thermodynamically stable oxidation state, present either as $H_3AsO_3^0$ species, as As-S complexes (e.g. $H_2As_3S_6^-$) or as As(III) solid phases such as As_2S_3(Inskeep, McDermott, & Fendorf, 2002) (Figure 1). Due to various pK_a values of arsenate (H_3AsO_4) (pK_{a1} = 2.19, pK_{a2} = 6.94 and pK_{a3} = 11.5), the $H_2AsO^-_4$ form predominates in oxidizing environment with pH between 2.5 and ca. 7.0, whereas the $HAsO_4^{2-}$ is the predominant form of As(V) between pH 7.0 and 11.0. For arsenite, the lowest pK_a value is equal to 9.22. So, $H_3AsO_3^0$ is the predominant form of As(III) in slightly reductive environments with a pH level below 9.2 (Figure 1)

As(V) is a stronger oxidant (with potential of +130 mV for As(V)/ As(III) pair) than sulfate (-220 mV for sulfate/ sulfide), but weaker than nitrate (NO_3^-/NO_2^-: +440 mV) or oxygen (O_2/H_2O: + 818 mV) (Oremland, Saltikov, Wolfe-Simon, & Stolz, 2009). Arsenite has a greater hydrologic mobility and toxicity than As(V). Arsenate tends to adsorb to more mineral surfaces than As(III), thereby making it less mobile than As(III) in aqueous environments (Oremland et al., 2009).

Figure 1. pE (voltage potential or Eh)-pH diagram for the As-S-H_2O system at 25°C (As_2S_3 = amorphous orpiment)
(Source: Inskeep et al, 2002).

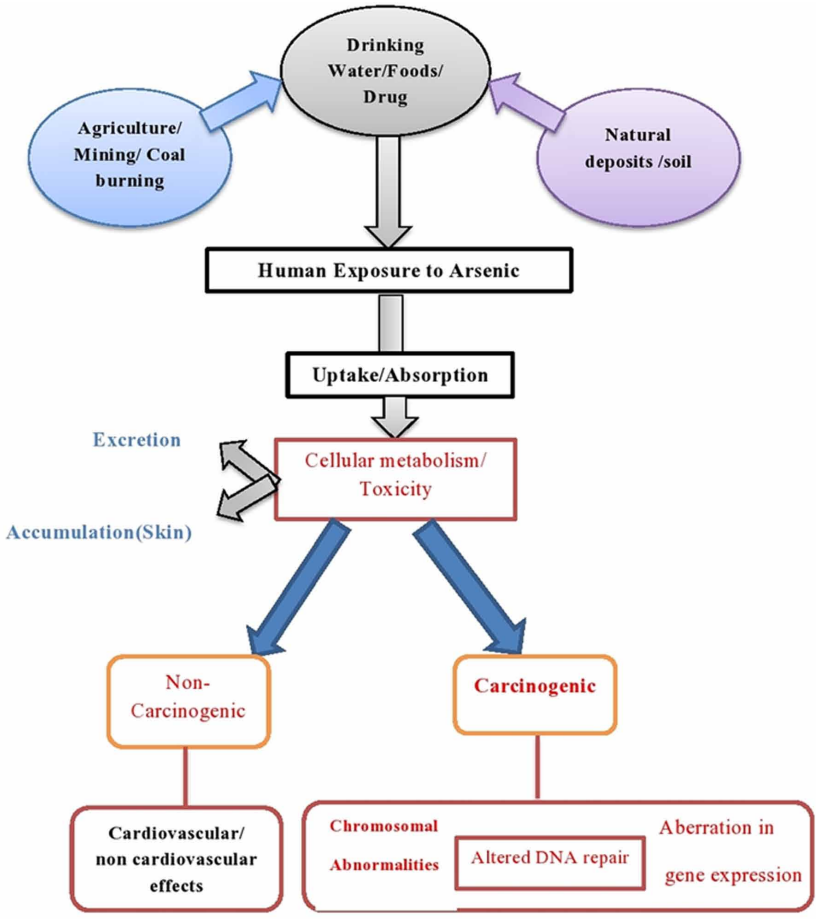


TOXICOLOGICAL ASPECT OF ARSENIC

The toxicology of arsenic is a complex phenomenon. The acute arsenic poisoning, requiring prompt medical attention, usually occurs through ingestion of contaminated food or drinking of arsenic contaminated water. The major early manifestation due to acute arsenic poisoning includes burning and dryness of the mouth and throat, dysphasia, colicky abnormal pain, projectile vomiting, profuse diarrhea, and hematuria. The muscular cramps, facial edema and cardiac abnormalities, shock can develop rapidly as a result of dehydration (Choong et al., 2007). Arsenic, which is found in several different chemical forms and oxidation states, causes acute and chronic adverse health effects, including cancer (Figure 2).The metabolism of arsenic has an important role in its toxicity. The metabolism involves reduction to a trivalent state and oxidative methylation to a pentavalent state. The trivalent arsenicals, including those methylated, have more potent toxic properties than the pentavalent arsenicals. In the trivalent state, inorganic and organic (methylated) arsenic may react with critical thiols in proteins and inhibit their activity. Regarding cancer, potential mechanisms include genotoxicity, altered DNA methylation, oxidative stress, altered cell proliferation, co-carcinogenesis, and tumor promotion (Hughes, 2002) (Figure 2).

Arsenate is a structural analogue of phosphate and competes with that essential ion in many enzymatic reactions, but its esters hydrolyze more rapidly than phosphate esters (Westheimer, 1987). That way, it short circuits oxidative phosphorylation (Gresser, 1981) and deprives cells of their energy supply. On the other hand, trivalent arsenic has a very high affinity for thiol groups and may therefore inhibit many enzymes that rely on thiol groups in critical positions. Behaving like a soft metal, arsenite also leads to the degradation of Fe-S clusters in proteins. Due to the protein inhibition and the higher bioavailability in aqueous environments at neutral or acidic pH, arsenite is considered to be more toxic and dangerous than arsenate. In humans, arsenic is used in the treatment of some forms of leukemia, but generally the

Figure 2. Sources of human exposure to arsenic and various modes of arsenic toxicity
(Source: Nriagu & Frankenberger, 2002).



exposure to this toxic metalloid results in the development of several diseases, including arsenicosis, malignancies, skin damages, gastrointestinal illnesses, and neuropathy (Neubauer, 1947). The major intake pathways are the consumption of contaminated drinking water or crops grown on contaminated soil, especially rice (Zhao, McGrath, & Meharg, 2010). The general problems of arsenic toxicities are:

- Skin itching to sun rays, burning and watering of eyes, weight loss, loss of appetite, weakness, lethargy and easily fatigued limited the physical activities and working capacities.
- Chronic respiratory complaints including chronic cough with or without expectoration.
- Gastrointestinal symptoms of anorexia, nausea, dyspepsia, altered taste, pain in abdomen, enlarged liver and spleen, and ascites (collection of fluid in abdomen)
- Moderate to severe anemia.
- Less commonly, conjunctival congestion and leg edema.

SEVERITY OF ARSENIC CONTAMINATION IN THE ENVIRONMENT

Arsenic contamination of the environment originating from both anthropogenic and natural sources is currently a global problem (Nriagu et al., 2007). The health of tens of millions of people world-wide is at risk of drinking As-contaminated well water. Natural occurrence of As within the subsurface aquifers is mostly reported, rather than being derived from identifiable point sources of pollution (Oremland & Stolz, 2005).

GROUNDWATER ARSENIC CONTAMINATION

Arsenic contamination in water, especially groundwater, has been recognized as a major problem of catastrophic proportions (Figure 3).Before 2000, there were five major incidents of arsenic contamination in groundwater in Asian countries: Bangladesh, West Bengal, India, and sites in China. Between 2000 and 2005, arsenic-related groundwater problems have emerged in different Asian countries, including new sites in China, Mongolia, Nepal, Cambodia, Myanmar, Afghanistan, DPR Korea, and Pakistan (Mukherjee et al., 2006) (Table 1).

Figure 3. Schematic representation of impact of Arsenic contamination in ground water
(Source: Md. Anwarul Abedin, 2013)

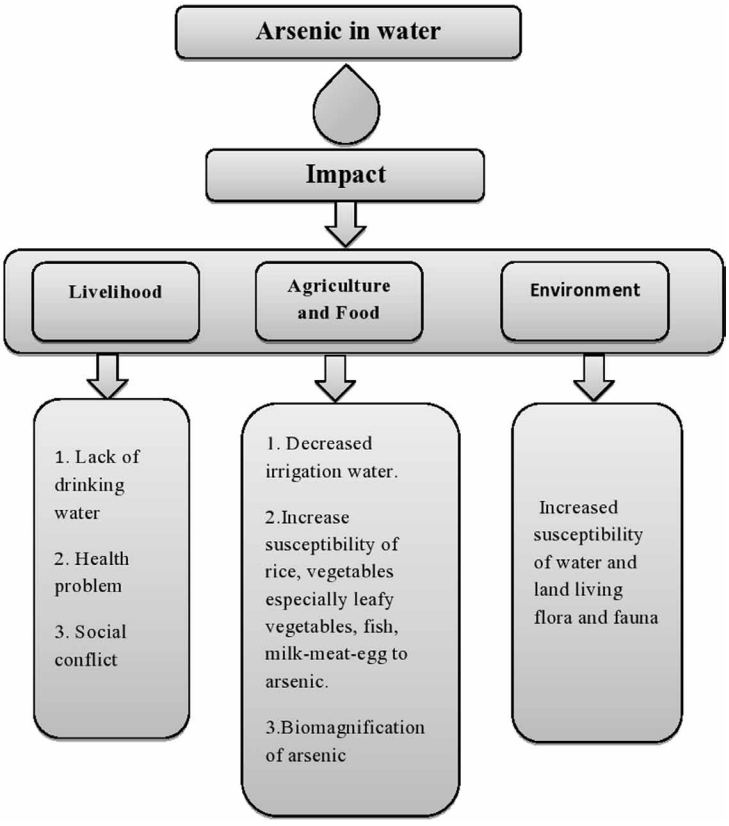


Table 1. Global Arsenic Contamination in groundwater

Country/ Region	Potential Exposed Population	Concentration (µg/Liter)	Environmental Conditions
Bangladesh	30,000,000	<1 to 2,500	Natural; alluvial/deltaic sediments with high phosphate, organics
West Bengal, India	6,000,000	<10 to 3,200	Similar to Bangladesh
Vietnam	>1,000,000	1 to 3,050	Natural; alluvial sediments
Thailand	15,000	1 to >5,000	Anthropogenic; mining and dredged alluvium
Taiwan	100,000 to, 200,000	10 to 1,820	Natural; Costal zones, black shale's
Inner Mongolia	100,000 to, 600,000	<1 to 2,400	Natural; alluvial and lake sediments; high alkalinity
Xinjiang, shanxi	>500	40 to 750	Natural; alluvial sediments
Argentina	2,000,000	<1 to 9,900	Natural; loess and volcanic rocks, thermal springs, high alkalinity
Chile	400,000	100 to 1,000	Natural and anthropogenic; volcanogenic sediments; closed basin lakes, thermal springs, mining.
Bolivia	50,000	-	Natural ; similar to Chile
Brazil	-	0.4 to 350	Gold mining
Mexico	400,000	8 to 620	Natural and anthropogenic; volcanic sediments, mining
Germany	-	<10 to 150	Natural ;mineralized sandstone
Hungary/Romania	400,000	<2 to 176	Natural, anthropogenic and organics
Spain	>50,000	<1 to 100	Natural; alluvial sediments
Greece	150,000	-	Natural and anthropogenic; thermal springs and mining
United Kingdom	-	< 1 to 80	Mining
USA and Canada	-	< 1 to > 100,000	Natural and anthropogenic; mining, pesticides. As_20_3 stockpiles, thermal springs, alluvial closed basin lakes

(Source: Nordstrom, 2002).

In a number of areas worldwide, oxidation and dissolution of arsenianpyrite, $Fe(AsS)_2$, and arsenopyrite, FeAsS, are additional processes that lead to high concentrations of dissolved arsenic (Nordstrom, 2002).Arsenic mass poisoning in groundwater in Bangladesh surpasses any incident seen before. Approximately 3.58 million people out of a total of 17.92 million are exposed to drinking water containing arsenic levels > 0.20 mg/l with a high risk of health hazard (Anawar, Akai, Mostofa, Safiullah, & Tareq, 2002). Thousands of patient having arsenicosis, suffering from various skin lesions, gangrene in leg, skin, lung, bladder, liver, and renal cancer have been identified. A big portion of the total population is highly vulnerable to various internal cancers.

IMPACT ON IRRIGATION WATER AND SOIL

Irrigation water with high levels of As may result in food chain contamination and loss of crop yield. Long-term use of As-contaminated irrigation water could result in As accumulation in the soil. As rich irrigation water can enrich the As level in agricultural soil up to five times than the normal soil (Ahsan,

DelValls, & Blasco, 2009). If absorbed by the crops, this may add substantially to the dietary As intake, thus posing additional human health risks. Over time, As accumulation in the soil could reach soil concentrations toxic to crops, thus reducing yields (Heikens, 2006). Also, irrigating with As-enriched water causes accumulation of As into edible parts of crops. Studies have reported that the transfer of As from water to soil might have both immediate and long term impact on crop irrigation such as for irrigating paddy soils (Ahsan & Del Valls, 2011). It was predicted that soil arsenic levels could be raised by 1 µg/g per annum due to irrigation with As-contaminated water (Meharg & Rahman, 2003). A study in Bangladesh in 2006 (Huq, Joardar, Parvin, Correll, & Naidu, 2006) revealed that vegetables can accumulate substantially-elevated amounts of arsenic and more than 150 mg/kg of arsenic has been found to be accumulated in arum (local *kochu*) vegetable.

ARSENIC SEVERITY: BANGLADESH PERSPECTIVE

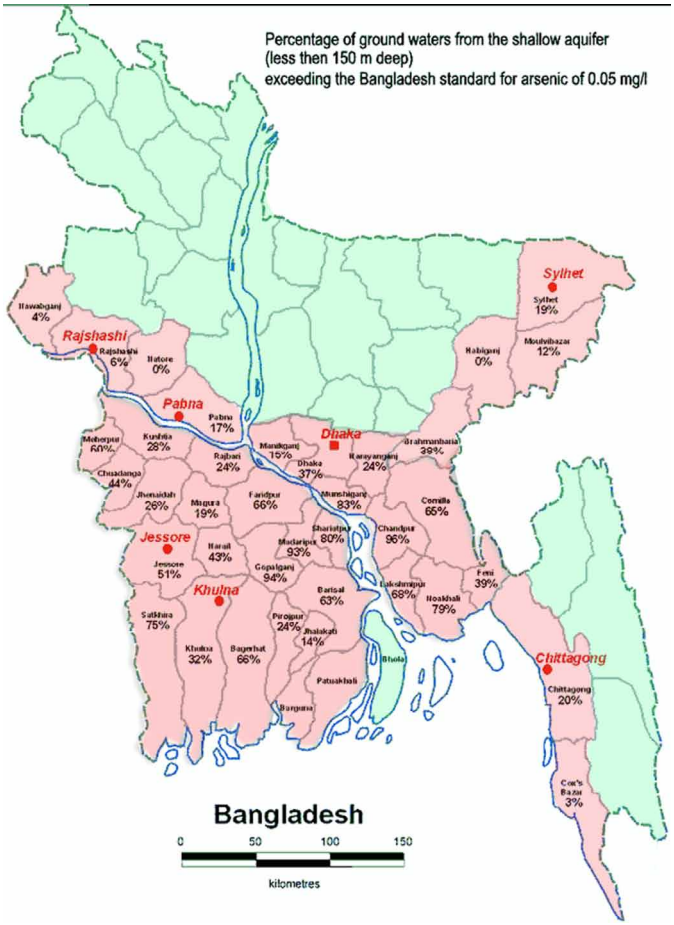
The most serious scenario of the world's recognized groundwater As problems occurs in Bangladesh. The region has been the subject of intensive water testing, hydrogeological and epidemiological investigation, patient identification, treatment and mitigation effort since groundwater As problem was first recognized by the national government and others in 1993. A random national survey (2007) of As in groundwater by Bangladesh government using laboratory data for 3208 groundwater samples from the shallow Holocene aquifer (<150 m depth), reported that 27% of the samples had As concentrations greater than the national standard for As in drinking water of 50 µg/L; 46% exceeded 10 µg/L (Figure 4).

More recent data from the Bangladesh Arsenic Mitigation and Water Supply Program (BAMWSP, 2005) showed that of almost 5 million boreholes tested nationally using field-test kits, some 30%, had As concentrations greater than 50 µg/L. Each dataset produced for Bangladesh groundwater demonstrates a very variable distribution of As regionally across the country, with the greatest proportion of exceed in groundwater's from the south and south-east (Figure 4). As many as a million water wells drilled into Ganges alluvial deposits in Bangladesh and West Bengal are at risk of As contamination and need periodic As assessment. Alarming situation in Bangladesh is that the irrigation of soil with As contaminated groundwater is threatening the quality of the human food supply, which could affect millions more people. Prolonged irrigation with contaminated water may result in increased As in rice and vegetables from Bangladesh (Heikens, 2006).

GEOCHEMICAL BASIS OF ARSENIC DISSEMINATION INTO GROUND WATER

The high-As in groundwater's of Bangladesh and West Bengal region are mainly from aquifers of Holocene age which comprise unconsolidated grey micaceous sands, silts and clays deposited as alluvial and deltaic sediments associated with the Ganges, Brahmaputra and Meghna rivers. The sediments are derived from the upland Himalayan catchments and from basement complexes of the northern and western parts of West Bengal. Many studies have observed that the highest concentrations of As in the shallow Holocene aquifer of Bangladesh occur at depths typically around 15-50 m (Harvey,2003; Kinniburgh & British Geological Survey, 2001; Klump, 2006),Concentrations of As in excess of 1000 µg/L have been found in some shallow groundwater from the region, although these are relatively rare.

Figure 4. A map of smoothed groundwater As distributions in Bangladesh
(Source: Kinniburgh & British Geological Survey, 2001)



The oxidation of arsenopyrite or ferrous hydroxides rich in arsenic present in the Bengal Delta sediments may be responsible for the release of arsenic oxides in solution to the groundwater. The subsequent migration of this arsenic contaminated groundwater through these deltaic sediments may be one of the major causes of arsenic pollution in Bangladesh. Arsenopyrite and ferrous hydroxides would be stable in the reducing environment below the groundwater level. If the groundwater level were lowered by increased irrigation during the dry season and the sediments exposed to the oxygen of the atmosphere these arsenic rich minerals would oxidize releasing arsenic. The behavior of As can be conceptualized in terms of 3 distinct redox zones (Nickson et al., 1998):

- Shallow, oxidizing zone with dissolved O_2, in which Fe (III) oxide and hydroxides are stable and As is adsorbed.
- Intermediate, moderately reducing zone without O_2, in which Fe (III) oxide and hydroxides undergo reductive dissolution and As is released. In this zone, dissolved concentrations of Fe^{2+} and As can be high (Nickson et al., 1998). However, concentrations of Fe^{2+} can be controlled by precipitation of minerals like siderite and vivianite.

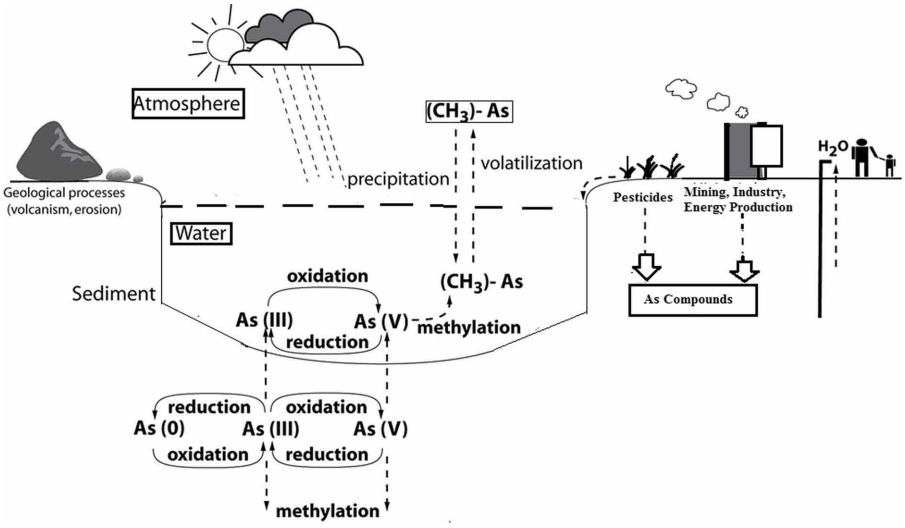
- Deep reducing zone, where SO_4 is reduced with resulting formation of H_2S. In this zone, As can co-precipitate in secondary sulfides like As-rich pyrite. However, if concentration of dissolved SO_4 and, thus, generated H_2S is low, then there is no precipitation of secondary sulfides.

Atmospheric arsenic is the result of wind erosion process, volcanic emissions, sea spray, forest fires and the process of volatilization occurring in cold climates, mainly as the result of biomethylation (Cullen & Reimer, 1989).Arsenic compounds occur naturally at significant levels especially in marine foodstuffs, for example prawns contain levels approaching 200 ppm. Marine organisms convert arsenic (mainly arsenate in seawater) into small organic compounds such as methylarsonic acid [$CH_3AsO(OH)_2$] or dimethylarsinic acid [$(CH_3)_2AsO(OH)$] (Mukhopadhyay, Rosen, Phung, & Silver, 2002) or can convert into organic storage form (arsenobetaine and arsenolipids) which is later released into the environment (Figure 5).

MICROBES AND ARSENIC

One does not normally associate arsenic with life, but it is now apparent that various types of microorganisms gain energy for growth from this toxic element (Oremland, 2003).These organisms are taxonomically diverse and metabolically versatile. Microorganisms and their enzymes are actively involved in the biogeochemical cycling of arsenic in the environment affecting the relative proportion of various arsenic oxidation states. Specifically, microorganisms are involved in arsenic oxidation, reduction and methylation reactions. Diverse microorganisms metabolize both inorganic and organic forms of arsenic, and their activities are part of a robust biogeochemical cycle. Arsenic can be biomethylated by a wide variety of bacteria mainly as a detoxification mechanism. Volatile (methylarsine, dimethylarsine and

Figure 5. Global arsenic geocycle and effects from anthropogenic sources
(Adapted from Mukhopadhyay et al., 2002)



trimethylarsine) and non-volatile arsenic compounds are formed by biomethylation. Volatile arsenic compounds can be produced aerobically or anaerobically, aerobically by bacteria such as *Staphylococcus aureus* and *Escherichia coli,* and anaerobically by *Methanobacterium, Pseudomonas* and *Alcaligenes.*

Such reactions are either involved in microbial energy metabolism or they serve as a resistance mechanism to protect from As toxicity.Arsenite [As(III)], can be oxidized to arsenate or As(V)] by chemoautotrophic arsenite-oxidizing bacteria (CAOs). Bacterial arsenite oxidation has been known for decades since 1918 when Green first isolated arsenite oxidizing bacteria from cattle dipping baths) (Green, 1918). The chemoautotrophic arsenite oxidation was first reported for *Pseudomonas arsenitoxidans*(Ilyaletdinov AN, 1981). CAOs use oxygen or, in some cases, nitrate as their terminal electron acceptor during the fixation of inorganic carbon (CO_2) into cell material. There are also heterotrophic arsenite oxidizers (HAOs), but they need organic carbon as their source of energy and cell material (Table 2).

Some bacteria can oxidize As (III) under anaerobic conditions in the presence of light, known as phototroph and growing under anoxygenic conditions. These bacteria use As(III) as an electron donor for maintain their photolithoautotrophy. For an example a photosynthetic bacterium closely related to *Ectothiorhodospira shaposhnikovii* can grow anaerobically in the light with As(III) (Stolz, Basu, & Oremland, 2010).

On the reductive side microbes that use As(V) as an electron acceptor in anaerobic respiration. These prokaryotes oxidize a variety of organic (e.g. lactate, acetate, formate and aromatics), or inorganic (hydrogen and sulfide) electron donors, resulting in the production of As(III). We refer to these prokaryotes as dissimilatory arsenate-respiring prokaryotes and their As(V) reductase as Arr. Many microbes reduce As(V) to As(III) as a means of resistance. These arsenate-resistant microbes (ARMs) do not gain energy from the process, but use it as a means of coping with high arsenic in their environment. Arsenate that has entered the microbe's cytoplasm is converted to As(III) through a process mediated by a small polypeptide (ArsC) and expelled out of the cell by an As(III)-specific transporter (ArsB)(Oremland & Stolz, 2005). Although the arsenite oxidases of CAOs and HAOs have notable similarities, the arsenate reductases of DARPs and ARMs are very different (Mukhopadhyay et al., 2002) .

Several studies have shown that bacterial metabolic routes such as arsenic oxidation and reduction play an important role in arsenic speciation in the environment and affect its bioavailability. However, knowledge available about arsenic-resistant bacteria is limited to their growth in vitro (Tsai, Singh, & Chen, 2009).Diversity of arsenic-metabolizing prokaryotes has been reported in several studies that have explored environmental genomic approaches such as meta-genomic, meta-transciptomic and meta-proteomics(Bertin, Médigue, & Normand, 2008).The application of molecular techniques to study microbial populations at contaminated sites without the need for culturing has led to the discovery of unique and previously unrecognized microorganisms as well as complex microbial diversity in contaminated soil and water which shows an exciting opportunity for bioremediation strategies. Investigations of bacterial communities in several microcosm studies have reflected the microbial involvement in arsenic pollution. Anaerobic incubations of sediments from Bangladesh and West Bengal (India) with electron donors have increased the release of As along with a shift in bacterial communities towards the Fe (III) reducing *Geobacteriaceae*. Also, molecular analyses with arsenic- contaminated Cambodian sediments in microcosm experiments have detected the presence of *arrA* gene encoding arsenate reductase when supplied with acetate. More recent studies on in-situ bacterial communities in As-contaminated tube wells in Bangladesh have indicated a different picture. Instead of Fe (III) or As (V) reducing bacteria, the study found only As tolerant bacteria such as *Comamonadaceae*, *Acidovorax*, *Acinetobacter*, and *Hydrogenophaga* in highly As-contaminated shallow aquifers and a number of aerobic bacterial popu-

Table 2. Reported Microorganisms in As (III) oxidation and As(V) Reduction

Microorganisms	Mechanism	Reference
Escherichia coli *Staphylococcus aureus* *Staphylococcus xylosis*	As(V) reduction under anaerobic conditions	(Tamaki, & Frankenberger Jr.,1992)
Geospirillum barnseii(strain SES-3)	As(V) reduction using lactate as the electron donor	(Laverman, 1995)
Anabaena oscillaroides	As(V) reduction to As(III)	(McLaren, & Kim, 1995)
Chrysiogenes arsenatis	As(V)reduction using acetate as the electron donor	(Macy,Santini, Pauling, O'Neill, & Sly, 2000)
Desulfotomaculum auripigmentum	Dissimilatory As(V) reduction using lactate as the electron donor	(Newman et al., 1997)
Bacillus arsenicoselenatis(strain E1H)	Dissimilatory reduction of As(V) to As(III)with the concomitant oxidation of lactate to acetate plus CO2	(Blum, 1998)
Sulfurospirillum Barnesii *Sulfurospirillum arsenophilum*	As(V) reduction using lactate, pyruvate or hydrogen and acetate as the electron donor	(Stolz et al., 1999)
Pyrobaculum arsenaticum *Pyrobaculum aerophilum*	As(V) reduction using hydrogen as the electron donor	(Huber, 2000)
Desulfomicrobium strain Ben-RB *Desulfovibrio* strain Ben-RA	As(V) reduction using lactate as the electron donor, oxidized lactate incompletely to acetate	(Macy etal., 2000)
*Pseudomonas arsenitoxidans*NT-26	chemolithoautrophic As(III) oxidation under oxic conditions	(Santini, Sly,Schnagl, & Macy, 2000)
Sulforospirillum barnesii	Dissimilatory As(V) reduction under anaerobic conditions	(Zobrist, Dowdle, Davis, & Oremland, 2000)
Thermus HR13	Heterotrophic As(III) oxidation under aerobic conditions and dissimilatory As(V) reduction under anaerobic conditions coupled with lactate oxidation	(Gihring & Banfield, 2001)
Thermus aquaticus *Thermus thermophilus*	Heterotrophic As(III) oxidation to As(V)	(Gihring & Banfield,2001)
Desulfitobacterium	Dissimilatory As(V) reduction using formate as the sole carbon source and electron donor	(Niggemyer, Spring, Stackebrandt, & Rosenzweig, 2001)
Termite isolate (strain TSA-1) *Rumen isolate* (strain BRA-1) *Hamster isolate* (strain HT-1)	Dissimilatory As(V) reduction using hydrogen as the electron donor	(Herbel, 2002)
Bacillus strain JMM- 4	As(V) reduction while the lactate is oxidized to CO2 via the intermediate, acetate	(Santini, Stolz, & Macy, 2002)
Azoarcus strain DAO1 *Sinorhizobium* strain DAO10	Anaerobic As(III) oxidation with inorganic C as the carbon source and nitrate as the electron acceptor	(Rhine, Phelps, & Young, 2006)

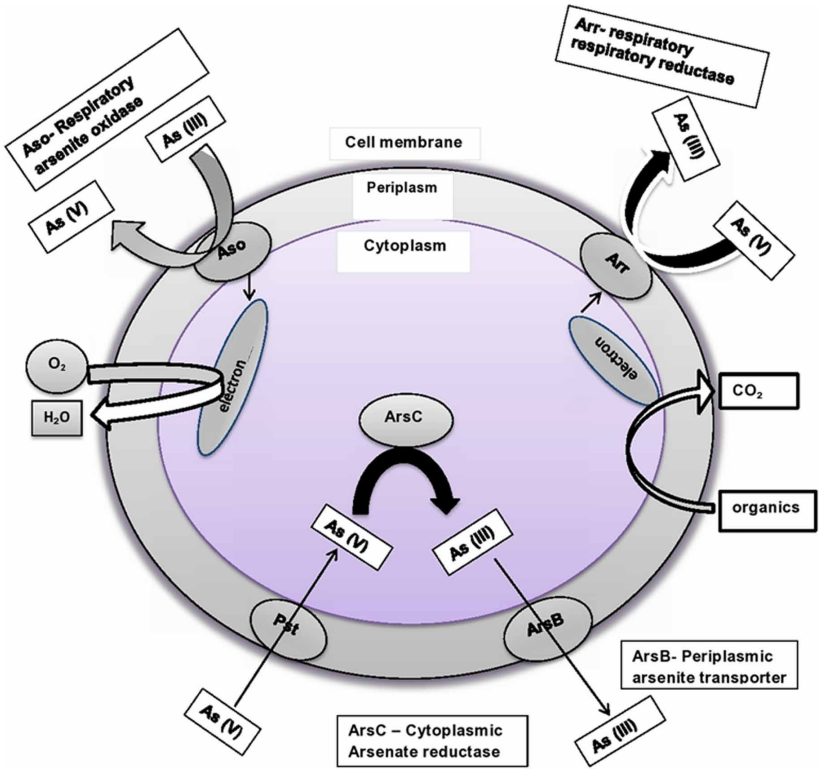
(Source: Wang & Zhao, 2009)

lation such as the genera *Aquabacterium*, *Limnobacter* and *Roseomonas* in the deep tube well region questioning the direct microbial involvement in As release (Sutton et al., 2009; Sultana, Härtig, Planer-Friedrich, Seifert, & Schlömann, 2011). They detected putative aerobic or denitrifying populations of *Pseudomonas, Elizabethkingia* and *Pantoea* in both shallow and deep aquifer region in Bangladesh. Therefore, it is still an area of research how microorganisms are involved in mediating As release in As-contaminated aquifers, how they are related to geology of a particular area and are influencing or are influenced by the geo-hydrological parameters in simultaneous derivation of the release process.

MICROBIOLOGICAL TRANSFORMATION OF ARSENIC

Many types of As transformations have been documented in a variety of microorganisms; those currently seen to lead arsenic speciation in nature are related to oxidations or reduction reactions (Oremland, 2003). These redox reactions are generally carried out by microorganisms either for detoxification or for energy generation to support cellular growth (Figure 6). In some cases, it can be even difficult to discriminate one from the other. The ubiquity of arsenic in the environment has led to the evolution in microbes of arsenic resistance mechanisms. The most common of these mechanisms is based on the presence of the arsenic resistance operon (*ars*), which codes for: (i) a regulatory protein, ArsR; (ii) an arsenite permease, ArsB; and (iii) an enzyme involved in arsenate reduction, ArsC (Mateos, Ordóñez, Letek, & Gil, 2006).

Figure 6. Genes involves in microbiological transformation of arsenic
(Source: Páez-Espino, Tamames, de Lorenzo, & Cánovas, 2009)



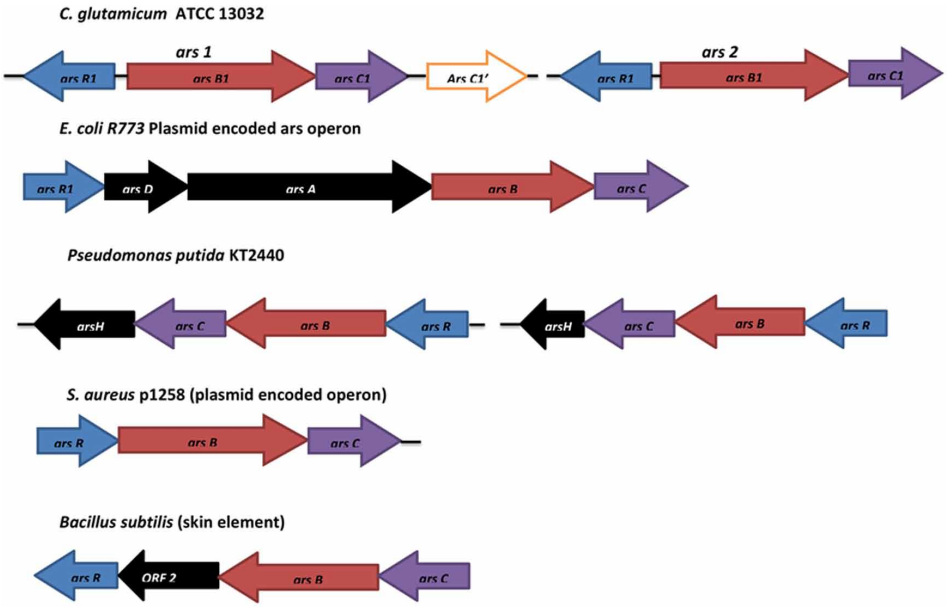
GENES INVOLVED IN ARSENIC RESISTANCE: THE ARS OPERON

Resistance to both arsenite and arsenate is widely found among both gram-negative and gram-positive bacteria. Usually, this is in the form of an *ars* operon consisting of a minimum of three co-transcribed genes, *arsR*(determining the regulatory repressor), *arsB* (encoding the membrane arsenite permease pump), and *arsC*(encoding an intracellular arsenate reductase) in the order *arsRBC*. Indeed, the *ars* operon occurs more widely in those bacterial genomes with over 1000 or 2000 genes. The arsenic resistance mechanism conferred by *ars* operon was often found on and originally described in plasmids, but chromosomal loci have been detected in more than 50 organisms including bacteria, archaea, yeasts and protoctists (Jackson & Dugas, 2003).

In *Escherichia coli* plasmid R733, the *ars* operon is composed of *arsA*, *arsB*, *arsC*, *arsD* and *arsR* whereas in *Staphylococcus* plasmid pI258, only three genes comprised the *ars* operon (Figure 7) (Silver & Phung, 1996). One of the most well-known As resistant bacteria (resistant to 12 mM As(III) and >400 mM As(V) (Mateos, Ordóñez, Letek, & Gil, 2006), *Corynebacterium glutamicum* was reported to have two complete *ars* operon referred to as *ars1* and *ars2*. The *ars1* consists of the typical three *arsR1B1C1*, with an extra *arsC1'*, located downstream from *arsC1* and two orphan genes (*arsB3* and *arsC4*) involved in arsenite permease and arsenate reductase, respectively, but sufficiently different from other similar counterparts (Figure 7). Both *ars1* and *ars2* operons are distantly located from each other on the chromosome (Ordóñez, Letek, Valbuena, Gil, & Mateos, 2005) and the regulatory protein gene *arsR* in both operons is expressed divergently from the rest of the *ars* genes (Mateos et al., 2006).

Ars operons vary in number and gene order among various species. Also, *ars* operons have been found in some arsenate-respiring bacteria, although arsenic resistance is not directly involved in arsenate respiration (Saltikov, Cifuentes, Venkateswaran, & Newman, 2003; Saltikov & Newman, 2003; Saltikov,

Figure 7. The genes involved in arsenic resistance in selected microorganisms
(Source: Mateos et al., 2006)



Wildman, Jr., & Newman, 2005). Many arsenite oxidizing bacteria also have an *ars* operon so as to both oxidize and reduce As (Kashyap, Botero, Franck, Hassett, & McDermott, 2006). In the yeast *Saccharomyces cerevisiae*, an As resistance gene cluster similar to that of bacteria have been found (Bobrowicz, Wysocki, Owsianik, Goffeau, & Ulaszewski,1997). The contiguous gene products are transcriptional regulator, ARR1/ACR1, arsenate reductase, ARR2/ACR2 and membrane efflux protein, ARR3/ACR3. In addition, there is an ABC ATPase, which also contributes to resistance to As(III) (Mukhopadhyay et al., 2002).

It has been argued that arsenite resistance is a very antique system that must have evolved early after the origin of life because of the wide range of toxic inorganic chemicals that were likely present at that time (Mukhopadhyay et al., 2002). The advent of arsenate-resistant microorganisms, however, is surely more recent; they probably evolved after the atmosphere became oxidizing, which created a pressure for the evolution of an arsenate reductase (ArsC) from a protein-tyrosine phosphatase. The ArsR regulatory protein and ArsB efflux pump protein provide resistance to arsenite, and the full (three elements) *ars* operon confers resistance to arsenite and arsenate (Figure 7).

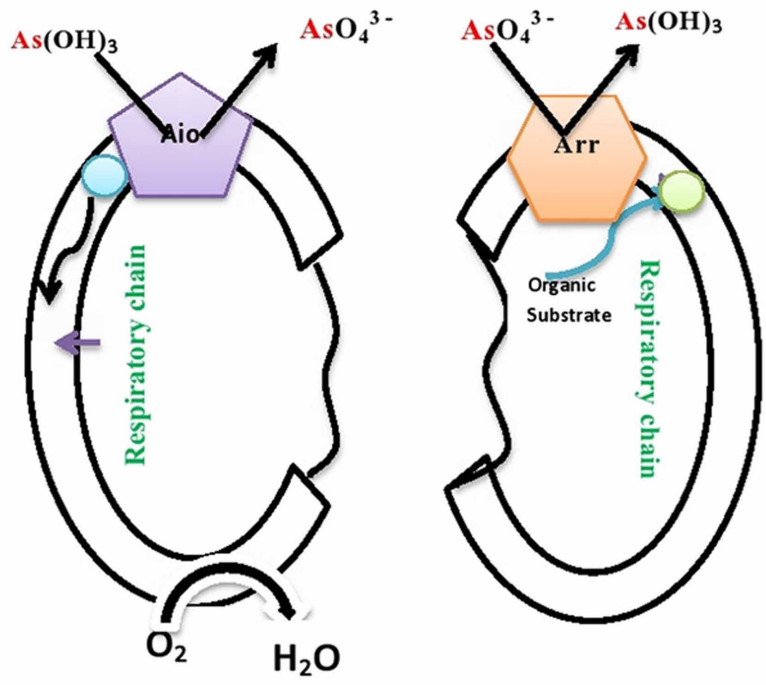
GENES INVOLVED IN ARSENITE OXIDATION AND REDUCTION

Arsenite [As(III)] is often the predominant species of arsenic found in source waters. As these waters flow down gradient and equilibrate with the atmosphere, As (III) is rapidly transformed to arsenate (AsV) via microbial oxidation (Hamamura, Mendo, Barroso, Iwata, &Tanabe, 2010). Arsenite-oxidizing bacteria are phylogenetically diverse (Battaglia-Brunet et al. 2006) but all perform arsenite oxidation by arsenite oxidase, an enzyme that belongs to the dimethylsulfoxide (DMSO) reductase family of the molybdopterin-containing proteins (Aio) (Lett, Muller, Lièvremont, Silver, & Santini, 2012), and a Fe-S Rieske protein (Sultana et al., 2012) (Figure 8).

The large catalytic subunit of arsenite oxidase (*aioA*) maintains a metal content core with molybdenum bound to pyranopterin cofactor. The catalytic pocket of (*aioA*) is comprised of unique, but highly conserved amino acid sequence IHNRPAYNSE with a non-coordinating alanine. Molybdenum is coordinated by four sulfur ligands in the oxidized form of the enzyme which is re-oxidized by a coordinated two-electron transfer (Stolz, Basu, Santini, & Oremland, 2006). A flat funnel-shaped cleft on the large subunit structure allows $As(OH)_3$ to enter (possibly coordinated by residues His195, Glu203, Arg419 and His423) and after oxidation, allows $HAsO_4^{2-}$ to exit the protein in the reverse direction (Anderson, Ellis, Kuhn, & Hille, 2001; Ellis, Conrads, Hille, & Kuhn, 2001). In case of arsenite detoxification, useful energy is not generated by this process (Mukhopadhyay et al., 2002). The diagnostic twin arginine (TAT) motif is found on the small subunit of Aio (AioB).The arsenite oxidase of heterotrophic and chemoautotrophic As(III) oxidizers are the same gene products, but one links to energy conservation while the others does not (Santini & vanden Hoven, 2004; vanden Hoven & Santini, 2004). The molecular similarity is well conserved amongst several species, and responds well to amplification with degenerate primers (Oremland et al., 2009; Quemeneur et al., 2008).

The primary enzyme involved in respiratory As(V) reduction is arsenate reductase (ARR). It is also a member of DMSO reductase family but significantly different from arsenite oxidase (Oremland et al., 2009) (Figure 8). It is a hetero-dimer periplasmic or membrane associated protein consisting of a larger molybdopterin subunit (ArrA) with a high potential iron-sulfur cluster [4Fe-4S] and the small subunit (ArrB) with at least three or possibly four [4Fe-4S] clusters (Simon Silver, 2005). The small

Figure 8. Representation of bacterial respiratory arsenite oxidase and respiratory arsenate reductase (Source: Simon Silver, 2005)



subunit is not homologous to Rieske polypeptide of small subunit of Aio and is twice the size of AioB. The catalytic pocket of the large subunit is comprised of highly conserved amino acid sequence (SHS-SICAEAE) with a cysteine residue coordinating the molybdenum in ArrA. Also, the TAT motif is found on the large subunit, ArrA.

ROAD TO MICROBIAL REMEDIATION OF ARSENIC

In recent decades following increasing environmental pollution by heavy metals, scientists attracted to biological remediation methods. In most cases of cleaning the contaminated ecosystems with chemical methods involves heavy costs and permanent damages (Table 3). Along with the rapid development of industries, arsenic contamination emerges as one of the world's most urgent environmental problems, especially for the developing countries. Therefore one appropriate method is using biological method. Application of microorganisms for heavy metals remediation such as As, is considered as a natural, stable and economical solution(Hadis, 2011). Microbial remediation of arsenic polluted environments is a key technique in practice, four aspects, i.e., the special adsorption of arsenic by micro-organisms, the transformation of arsenic speciation and the degradation and volatilization of arsenic compounds by micro-organisms, the effects to arsenic contamination of soil by the interactions between micro-organisms and plant roots and the molecular biological mechanism of bioremediation for arsenic.Bacteria have been reported to evolve numerous mechanisms, some of which can be exploited to reduce the toxicity of inorganic arsenic. These processes include oxidation of arsenic (III) into arsenic (V) making it less

mobile and less toxic because of its less bio-accumulation in host (Quemeneur et al., 2008), entrapment into bacteria. Although these strategies are still emerging, oxidation of arsenite by chemolithotrophic bacteria could be the most promising one. Therefore, it is necessary to know the genes responsible for such transformation within bacteria, as well as their growth and arsenite oxidation efficiency for bioremediation approach to pursue in a certain environment.

ENGINEERED MICROBES FOR ARSENIC BIOREMEDIATION

The use of engineered microbes as selective biosorbents is an attractive green technology for the low-cost and efficient removal of arsenic (Singh, DaSilva, Mulchandani, & Chen, 2008). Development of an arsenic accumulating microbe should comprise the ability of -firstly, modify the naturally existing

Table 3. Advantages and disadvantages of methods for the removal of arsenic compound

Method	Method in Detail	Advantages/Disadvantages
Physical approaches	1. Mixing both contaminated and uncontaminated soils 2. Washed with sulfuric acid, nitric acid, phosphoric acid, and hydrogen bromide 3.Immobilise soluble arsenites using cement 5. Emphasis on stabilisation/solidification (S/S) 5. Soil flushing using aqueous solutions using surfactants and cosolvents	High cost/usage to smaller-scale operations Chemicals usage/high cost/usage to smaller-scale operations Successfully used to stabilise As-rich sludges Treating As containing wastes in water Applied in the field, efficiency can vary from 0% to almost 100%
Chemical remediation approaches	1. Adsorption by using specific media, immobilization, modified coagulation along with filtration, precipitations, immobilizations, and complexation reactions 2. Formation of stable phases, for example, insoluble $FeAsO_4$ (and hydrous species of this compound such as scorodite, $FeAsO_4.2H_2O$) 3. Stabilization method using nanosized oxides and Fe(0) (particle size of 1 to 100 nm)	Economic but often displayed lower efficiencies (<90%) Use of selective stabilizing amendments is a challenging task Gained popularity/high success rate, but it could be expensive when remediating a large area
Physiochemical methods	Filtration or coagulation sedimentation, osmosis or electrodialysis, adsorptions, and chemical precipitations	Widely accepted in some places
Biological methods	Such as phytoremediation by using aquatic plants or microbial detoxification of arsenic	Widely accepted in some places
Intrinsic bioremediation	Degradation of arsenic by naturally occurring microorganisms	More suitable for remediation of soil with a low level of contaminants
Engineered bioremediation	Optimizing the environment conditions to promote the proliferation and activity of microorganisms	Favorable method used in high contaminated area
Microbial oxidation	Immobilization of As in the solid phase by using heterotrophic bacteria and chemoautotrophic bacteria to oxidize arsenite into a less toxic arsenate	Should be carried out in controlled environment and Required biological activity, and microbiological molecular analysis/involved adsorption or coprecipitation with Fe-oxyhydroxides.
Microbial Reduction	Reduction of arsenate into arsenite by microorganisms via dissimilatory reduction mechanism	Should be carried out in facultative anaerobe or strict anaerobe condition
Biomethylations	Biomethylations (by As(III) S-adenosylmethioninemethyltransferase)	Is a reliable biological process of removing arsenic from aquatic mediums

(Source: Wang & Zhao, 2009)

defense mechanisms and secondly,develop novel or hybrid pathways into one easily manipulated microorganism.Recent research demonstrated that arsenic could be removed through volatilization from the contaminated soil by bacteria which have *arsM* gene expressed and laboratory experiment showed that it is possible to use microorganisms expressing *arsM* as an inexpensive, efficient strategy for arsenic bioremediation from contaminated water and soil (Liu, Zhang, Chen, & Sun, 2011).

CASE STUDY

Microbial batch reactors to remove arsenic by oxidation of As(III) to As(V) and the use of bacterial arsenate reductase genes in transgenic plants for potential phytoremediation by intracellular sequestration after reduction from As(V) to As(III) were reported recently(Sandra Alvarado, Marcó P. Lué-Merú, Graterol Nelson, Anzalone Alvaro & Arroyo C. Jesús, 2008). A new study has identified bacterial strains capable of oxidizing toxic arsenic into a less toxic form, offering a feasible and affordable solution to the problem of arsenic in soil and water (Majumder, Bhattacharyya, Bhattacharyya, & Kole, 2013). Results of the study showed that selected bacterial isolates of *Geobacillus stearothermophilus* could completely oxidise 30 millimolars of toxic inorganic arsenite (Majumder et al., 2013) As (III) into less toxic arsenate As(V) within 24 hours,while both forms of arsenic occur in nature, the removal of As (III) from environmental systems is difficult due to its relatively higher solubility, whereas As (V) is poorly water-soluble and less bio-available.

Researchers isolated 12 strains of bacteria from arsenic contaminated soils in West Bengal and identified four of them as good arsenic oxidizers (Majumder et al., 2013). One of the strains AMO-10 performed the best, another strain AGH-02 was found effective in the bioremediation of soil. While the *G. stearothermophilus* has no known pathogenic properties, more tests are required before it can be declared safe for use in bioremediation.Oxidizing bacteria deployed in rice paddies can reduce arsenic uptake into rice grains (Meng et al., 2011).

In addition to using specific bacteria, Bangladeshi scientists (Rahman, Jalil, & Ali, 2013) have found, in cow dung, an efficient agent to treat the toxic sludge that is generated by the removal of naturally-occurring arsenic in groundwater. They found that microorganisms present in cow dung can volatilize arsenic in the sludge. We know that cow dung, constantly being produced by cattle in the rural areas of Bangladesh, retains nutrients that attract organisms capable of gasifying arsenic through methylation-a biochemical process of adding methyl groups. This group considers the use of cow dung for arsenic removal cost-effective because cow dung beds are common and readily available in the villages where groundwater extracted from shallow aquifers is the most important source of drinking water.

ARSENIC REMEDIATION TECHNIQUES: CONVENTIONAL VS BIOLOGICAL

Arsenic removal by water and wastewater treatment plants is generally accomplished with the application of some conventional treatment techniques. The most commonly used technologies include oxidation, co-precipitation and adsorption onto coagulated flocs, lime treatment, adsorption onto sorptive media, ion exchange resin and membrane techniques. The effective removal of arsenic from water requires a preliminary pre-oxidation step to transform As (III) to As (V). Currently, most of the **c**onventional treatment of arsenic contaminated water involves a pre-oxidation step oxidizing As (III) to As (V), and

subsequent removal of As (V) through adsorption onto adsorbents (Lièvremont et al., 2009; Oremland, 2003). Chemicals used in the oxidation of As (III) to As (V) can result in the formation of by-products which can be either harmful or difficult to remove from water(Ghurye & Clifford, 2004). The usage of these chemicals for the purpose of oxidation may not be very economical. Microbial oxidation of As (III) oxidation is not only considered an alternate strategy but also a cost effective treatment of arsenic contaminated water.

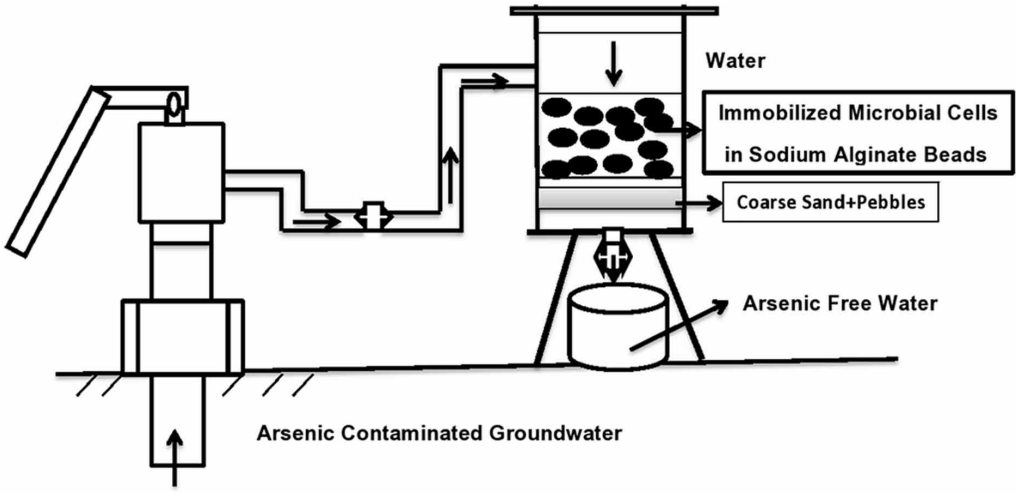
Over the years, microorganisms have evolved mechanisms to remediate both metal and metalloid contaminants from water and wastewater. This special ability of the microorganisms is usually demonstrated by changes in the redox states of the corresponding metals / metalloids or by adsorption onto its surface. The net result of both the processes leads to the reduction in the mobility of these contaminants in the environment (Lovley & Coates, 1997). Microorganisms have been found to have the capacity to concentrate or remediate the metals into forms that are further precipitated or volatilized from solution and hence less toxic or easily disposable. Thus, the microorganisms can only alter the speciation of the metal contaminants and convert them into less toxic form. The main two processes of metal form alterations, in which the microorganisms are involved, are the extracellular transformation and the metal uptake. The oxidation of arsenite had a special place in the extracellular transformation of this element.

The microbial influence on metal mobility can be applied for bioremediation purposes. The microorganisms can mobilize metals through autotrophic and heterotrophic leaching, chelation by microbial metabolites and siderophores, methylation (which can result in volatilization) (Gadd, 2004). For arsenic remediation, most important processes are the oxidation, biomethylation, biosorption, dissimilative arsenate-reduction or sulfate-reduction demonstrated a new insight to the arsenic removal process, based on the use of arsenic-oxidizing bacteria for the conversion of As[III] into As[V] followed of a chemical sorption of the arsenate in chabazite or kutnahorite (Figure 9). However, biological oxidation has recently gained increased importance and application due to the existence of certain advantages, over the conventional physicochemical treatment. The application of microbial oxidation was found to be an efficient treatment technique for removal of arsenic from initial concentrations between 60 and 80 µg/l to residual (effluent) arsenic concentrations lower than the limit of 10 µg /l (Zouboulis & Katsoyiannis, 2005).Although in Bangladesh some of the conventional strategies are adopted, due to lack of strain or lack of knowledge on specific genes, bioremediation has not been adopted or practiced. There is therefore, need of investigation of microorganisms thriving in As polluted environment, their diversity analysis, diversity of related genes as well as knowledge on microbial transformation mechanisms.

CONCLUSION AND FUTURE PERSPECTIVES

Concerned efforts have been made over the past decade to isolate and characterize organisms capable of generating energy from oxidation/reductions with oxyanions of arsenic. These efforts have been aided by the identification of their ecological environments where they are involved in biogeochemical cycling processes. Diverse microorganisms possess As detoxification systems in which As is oxidized to the less toxic form or reduced for subsequent excretion.Despite its toxicity, arsenic is readily used by various organisms to fuel their energy metabolism as an electron donor (in the case of arsenite) or as an electron acceptor (in the case of arsenate).Bioremediation of arsenic contaminated soils and groundwater shows a great potential for future development due to its environmental compatibility and possible cost-effectiveness. Biological methods involving microbial activities can be used efficiently to treat arsenic

Figure 9. A proposed microbiological detoxification system of arsenic containing tubewell water
(Source: Cavalca, Corsini, Zaccheo, Andreoni, & Muyzer, 2013)



contaminated soils and groundwater. This approach may be comprised of a two step detoxification process for arsenic contaminated groundwater. The first step of the process could be As(III) bio-oxidation performed in a 1st column reactor filled with immobilized arsenite oxidizing bacteria and the 2nd step could be the adsorption of As(V) in a second column filled with mineral adsorbent (Cavalca et al., 2013) Genetic engineering strategies may be applied to improve the arsenic tolerance and accumulation capacities of microorganisms. Although the microbiological study of As is almost a century old, we still have only a limited understanding of the ancient processes and complexities by which prokaryotes utilize or tolerate As. It is ourbelief that the development of research in this field can provide the key to handle this toxic and ubiquitous metalloid.

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KEY TERMS AND DEFINITIONS

Arsenic Metabolism: Arsenic metabolism is the set of life-sustaining chemical transformations of As within the cells of living organisms.

Bacterial Community: The composition and interaction of bacterial populations and the abundance of its members.

Bioremediation: Bioremediation is a waste management system that involves the use of organisms to remove or neutralize pollutants from a contaminated site.

Environmental Pollution: Environmental pollution is the introduction of contaminants into the natural environment that causes adverse change.

Metalloid: A metalloid is a chemical element with properties in between, or that are a mixture of, those of metals and nonmetals.

Microbial Detoxification: Microbial detoxification is the neutralization of the toxic substances by using various novel enzymes.

Toxicology: Toxicology studies the harmful effects of chemical, biological and physical agents in biological systems that establish the extent of damage in living organisms.

Chapter 6
Microbial Ligninolysis: Avenue for Natural Ecosystem Management

Rashmi Paliwal
G.B. Pant University of Agriculture and Technology, India

Krishna Giri
Rain Forest Research Institute, India

J.P.N Rai
G. B. Pant University of Agriculture and Technology, India

ABSTRACT

Lignin is the second most abundant natural polymeric carbon source on earth after cellulose. It is a plant-originated polymer with three-dimensional network of dimethoxylated (syringyl), monomethoxylated (guaiacyl), and non-methoxylated (phydroxyphenyl) phenylpropanoid and acetylated units. The structural complexity and insolubility of lignin make it highly recalcitrant for degradation. Its biological degradation is critical to the global carbon cycle. Bioligninolysis involves application of microorganisms and their enzymes in degradation of lignin whichprovide environmental friendly technology for various industrial applications. As a major repository of aromatic chemical structures, lignin bears paramount significance for its removal from woody plants/lignocellulosic material, owing to potential application of bioligninolytic systems on commercial scale. This chapter provides an overview of microbial ligninolysis and its role in carbon cycling, various industrial process and pollution abatement for natural ecosystem management.

INTRODUCTION

Lignin is the most important renewable source of organic carbon on earth and represents nearly 30% of the carbon sequestered in plant materials annually (Boerjan et al., 2003). About 438–425 million years ago (even before the appearance of vascular water-conducting cells), lignin like moieties were present in the earliest terrestrial life (Rogers & Campbell, 2004). Lignin as a major repository of aromatic chemi-

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cal structure has a central point for its removal owing to potential applications of ligninolytic systems in industries, such as bioethanol production and manufacture of cellulose-based chemicals and materials, pulping, bleaching, and treating the wastes (Mester & Tien, 2000). Pulp and paper industries are the primary user of wood and non-wood products for production of paper. In paper making industries lignin as a coloured material must be removed from pulp as it makes mechanical pulp fibers stiff and turn newsprints yellow. During the chemical and mechanical pulping process almost 90-95% dissolved lignin generated as wastewater, which is also known as black liquor. Whereas, in bioethanol production from lignocellulosic biomass, the lignin barrier limits the accessibility to fermentable sugars (derived from cellulose and hemicelluloses). Conventionally disruption of this barrier is achieved through mechanical and chemical methods, including high-temperature and strong chemical reagents. During which, chemicals react with lignin and other components of the pulp, results in formation of chlorinated organics that further contribute colour to wastewater (D'Souza et al., 2006).

A controlled biocatalytic breakdown process can represent lignin as a major renewable source of aromatic and phenolic bio-products, which would be valuable raw materials for the food and flavour industry, and for fine chemicals and materials synthesis such as *t*-cinnamic acid, ferulic acid, vanillic acid, vanillin acid and gallic acid (Raj et al., 2007). In the future, biorefinery processes will extract first high-value chemicals present in the biomass, such as fragrances, flavoring agents, food-related products, high-value nutraceuticals, and other fine chemicals. Later, plant polysaccharides and lignin will be processed into feedstocks for bio-derivate materials, bulk chemicals, and fuels (Ragauskas et al., 2006). Lignin content in plant biomass is major factor to determine the rate of litter decomposition in different ecosystems. Lignin degradation involves both biochemical and physical processes and regulated by various biotic and abiotic factors. Bioligninolysis involves multiple biochemical reactions, such as cleavage of intermonomeric linkages, demethylation, hydroxylations, side chain modifications and aromatic ring fission followed by dissimilation of aliphatic metabolites that have to take place simultaneously (Paliwal et al., 2012). Insolubility of lignin and lack of its sterioregularity contribute difficulty in its degradation. Although much work has been done to understand the process of biological lignin degradation, still many aspects of enzymatic degradation of lignin need to explore that could offer a solution to major problem of waste disposal and ecosystem impairment caused by industries.

BACKGROUND

Lignin plays a significant role in terms of protecting microbial access to labile carbon compounds in plants and provides structural integrity to the cell walls of woody plants. Wood and vascular tissues generally contain 20–30 g/kg of lignin, while on earth a proportion of lignin has been estimated equivalent to 3×10^{11} metric tons (Kirk & Farrell, 1987; Whittaker & Likens, 1975; Rahman et al., 2013). In lignocellulosic material lignin contributes 10-20% by weight and 40% by energy. Because of high energy content lignin can be consider as potential renewable resource of chemicals and fuels (Wang et al., 2013). Bioethanol has been produced since 1970's via extraction of sucrose. The second generation biofuels have attracted considerable attention over first generation (bioethanol) due to utilization of non-food part of plant crop for bioenergy production by conversion of plant lignocelluloses into bifuels. Therefore, lignin degradation is an important and crucial step not only in biomass conversion but also in nutrient cycling and carbon balance in terrestrial ecosystem. Degradation of lignin in nature can be achieved by two basic processes such as chemical (photolysis) and biological (bioligninolysis or micro-

bial ligninolysis). The term bioligninolysis here involves exclusively microorganism and their product (enzymes) for depolymerization of lignin. The importance and mechanism of microbial ligninolysis in nature along with the lignin chemistry have been discussed in the forthcoming paragraphs.

Chemistry of Lignin

Lignin is product of polymerization of phenylpropanoid units and associated with cellulose and hemicellulose in the secondary cell walls of vascular elements in higher plants. Lignin especially in woody plants, accounts for enormous reservoir of organic carbon in the biosphere. Lignin deposition occurs after the completion of cell growth and when the three layers of secondary cell walls, the outer (S1), middle (S2) and inner (S3), are assembled during thickening of the secondary cell wall (Baucher et al., 1998; Boerjan et al., 2003). Lignin as an integral cell wall constituent provides strength and resistance to the plants as well as a protective matrix surrounding the cellulose microfibrils of plant cell walls. Moreover, lignin participates in water transport in plants and constructs a barrier against microbial decomposition by protecting the readily absorbable polysaccharides (Monties & Fukushima, 2001).

Biosynthetically, lignin arises from three alcohol precursors: p-hydroxycinnamyl (coumaryl) alcohol, which gives rise to p-hydroxyphenyl units (H); 4-hydroxy-3-methoxycinnamyl (coniferyl) alcohol, the guaiacyl units (G); and 3,5-dimethoxy-4-hydroxycinnamyl (sinapyl) alcohol, the syringyl units (S) (Figure 1) (Wei et al., 2009) and in some cases acetylated forms (del Rio et al., 2004; Ralph et al., 2004; Martínez et al., 2008). The G:S:H ratio varies from species to species in plants. Free radical copolymerization of these alcohols produces the heterogeneous, optically inactive, cross-linked, and highly poly disperse polymer. Most gymnosperm (softwood) lignins contain primarily guaiacyl units; whereas, angiosperm (hardwood) lignins contain approximately equal amounts of guaiacyl and syringyl units and grass lignins are guaiacyl type containing high proportion of p-hydroxyphenyl units (Wong, 2009).

Chemically, lignin consisting phenylpropanoid units linked by various covalent bonds (e.g. aryl-ether, aryl-aryl, and carbon-carbon bonds) (Brunow, 2001). The most abundant linkage is ether-type bond and next common are carbon-to-carbon type linkage. Major linkages in lignin monomer units and their percent distribution in woods are given in Figure 2.

Figure 1. Structures of Monomeric Units (H,G and S) of Lignin.

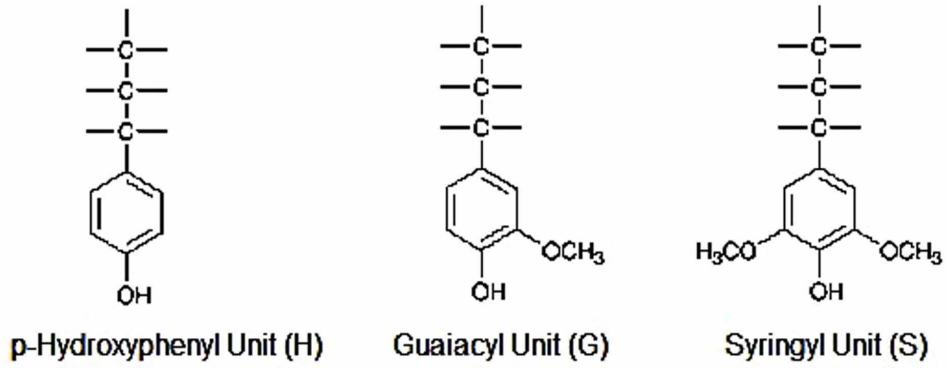
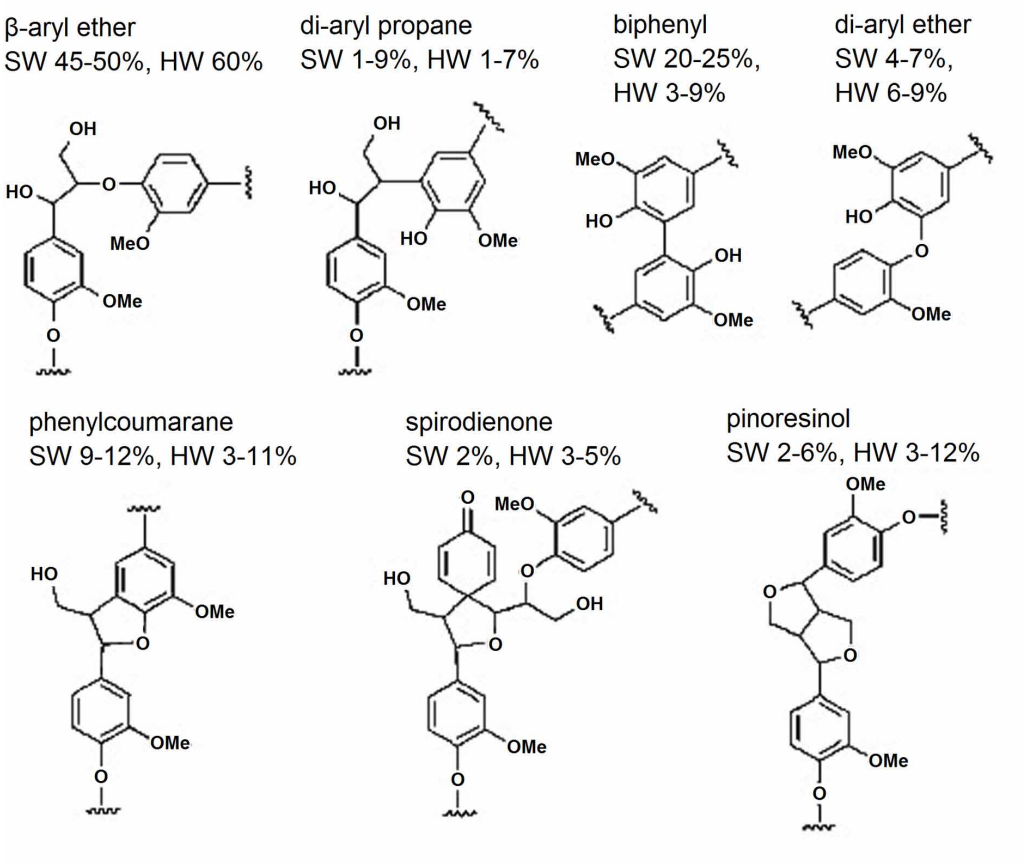


Figure 2. Structures of chemical linkages found in lignin. SW, softwood; HW, hardwood *(Source: Bugg et al., 2011)* .



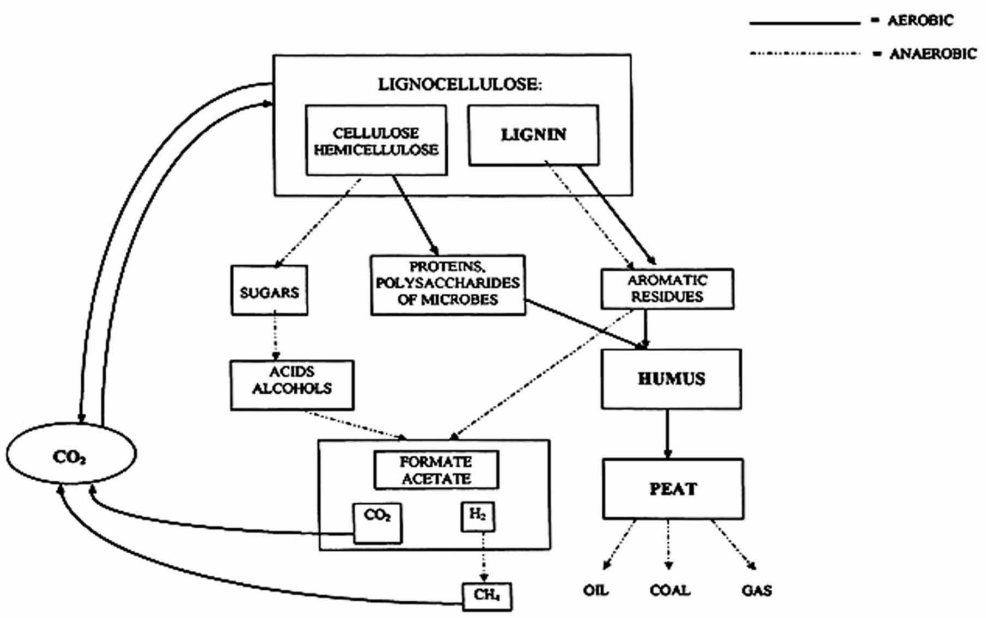
The Earth's Carbon Cycle

Most of the renewable carbon fixed either in lignin or other cell wall constituents such as, cellulose and hemicelluloses. Lignin plays a key role in the terrestrial and oceanic carbon cycles, as it limits the microbial accessibility to these more labile cell-wall polysaccharides (Austin & Ballaré, 2010). In forest ecosystem lignin represent approximate 20% of plant litter input into soil (Gleixner et al., 2001; Thévenot et al., 2010). In certain environment due to recalcitrant nature, lignin results in the formation of deposits of lignite and coals, which are the major forms of fossilized organic matter on earth. Because of unique chemical structure lignin stores more solar energy, therefore accounts for more fuel value in wood than cellulose (Zeikus, 1981). But most significant value of lignin is that it forms a protective sheath around the cellulose and the hemicelluloses in woody plants. This sheath must be disrupted before the polysaccharides are accessible to enzymatic attack. Since only few organisms can disrupt the recalcitrant structure of lignin, its degradation is considered as the rate-limiting steps in carbon cycling. In nature, the lignin sheath is disrupted mechanically by insects and marine borers, and biochemically by microorganism *viz.* fungi and bacteria. However, some author emphasized that it was endosymbiotic microorganisms that are responsible for lignin degradation in some insects (Bernhard-Reversat & Schwartz, 1997). Hence,

biochemical processes are by far the most important in the recycling of most of the earth's carbon by lignin decomposition (Figure 3).

Effect of increasing levels of global CO_2 on plants lignin content, rate of decomposition and composition of microbes has always been under debate. Being hard-to-decay lignin biodegradation is not an environmental conditions dependent process alone, but the degrading capacity of microbial population and lignin content also affects the process (Waldrop et al., 2000). A few investigators have suggested that elevated CO_2 concentration increases the lignin content in some plant species but with little or no effect on degradation process in short term (O'Neill & Norby, 1996; Norby et al., 2001; Henry et al., 2005). However, several workers reported that the biological decomposition is negatively correlated with the increasing concentration of lignin (Meentemeyer, 1978; Austin & Ballaré, 2010). The microbial diversity and biomass shifts in response to the global climate warming which is a direct consequence of increased CO_2 concentration. In the warming conditions composition of microorganisms may either remain steady or even increase, which would accelerate earth's carbon cycling (Belay-Tedla et al., 2009; Schindlbacher et al., 2011; Wang et al., 2012). The composition of the microbial community determines the rate and extent of lignin degradation. In this context role of microbes in recalcitrant carbon (lignin) degradation possess predominate significance especially to restore the deteriorated ecosystem by the way of enhancing recycling of carbon adhered in lignin. Many microorganisms are capable of utilizing labile carbon of plant as energy sources, however, only a restricted group of organisms have evolved with the ability to degrade lignin. White rot fungi (WRF) belongs to basidiomycetes possess the unique ability to efficiently metabolize lignin to CO_2 in order to achieve access to the carbohydrate polymers of plants and use them as carbon and energy sources. These wood-decay fungi are common inhabitants of plants litter and fallen trees. The most widely studied white rot fungi is *Phanerochaete chrysosporium.*

Figure 3. Lignin Degradation and Global Carbon Cycle
(Brown, 1985; Colberg, 1988; Tuomela et al., 2000)



Microbial Ligninolysis

As the removal of lignin barrier enables the subsequent utilization of plants labile carbon by other microorganisms, microbial breakdown of lignin proves to be a key step for closing the global carbon cycle. Due to highly recalcitrant complex random structure that lacks the regular hydrolysable bonds, lignin restrict the metabolization process by most of the microorganisms. To overcome this restriction and handling of intact lignin molecules, microbes have developed a unique strategy. Most of the organisms that are known to degrade lignin belong to fungi and, to a less extent, certain actinomycetes and bacteria, whose contributions have been discussed here.

Fungal Ligninolysis

A number of microorganisms, bacteria and filamentous fungi, are able to degrade lignocellulosic components to various extents. Of the more than one million species of fungi known, only few have the capability to degrade wood. These wood-rotting fungi belong to the phyla basidiomycota and ascomycota. They are generally grouped into soft-rot, brown-rot and white-rot fungi as per the characteristics of the wood being degraded and to the aspect of the resulting lignocellulosic residues. Some fungi along with their lignin degrading enzymes are listed in Table 1.

Soft-Rot Fungi (SRF)

Fungi taxonomically classified under phyla ascomycetes and deuteromycetes such as *Chaetomium globosum, Ustulina deusta, Alternaria alternata*, *Thielavia terrestris, Paelomyces spp.* etc. are known to cause soft-rot in wood (Daniel, 1994; Martinez et al., 2005). Soft-rot decay is usually characterized by formation of chains of cavities within the cell wall. It involves specialized microhyphae approximately of 0.3–0.4 mm in thickness, which passes through the secondary wall forming cavities along the cellulose microfibrillar structures and has spiral orientation that changes in different cell walls known as Type I attack, while others may leave a relative intact middle lamella by eroding the secondary wall completely called Type II attack (Buswell, 1991; Blanchette, 2003; Hamed, 2013). SRF show preference for wood polysaccharides *viz.,* cellulose and hemicellulose, but weakly affect lignin. Because of this limited action of SRF affected wood results in soft consistency when placed in wet environments and in dry environments, wood becomes brown and crumbly (Eriksson et al., 1990).

Brown-Rot Fungi (BRF)

BRF approximately comprise 10% of all wood-decaying fungi and primarily attack softwoods. Brown-rot is found in coniferous ecosystem, where the resulting residues form a large proportion of the humus material. BRF selectively utilize celluloses and hemicelluloses in wood, which rapidly weak its strength properties. During the advanced decay stages, the degraded wood converted to a residue of amorphous, break into cubical pieces and crumbles easily into brown powder that mainly composed of chemically modified lignin by reaction of dealkylation, demethoxylation and demethylation (Blanchette, 1995; Eaton and Hale, 1993). BRF mainly include basidiomycota *viz., Fomitopsis palustris, Gloeophyllum trabeum, Lenzites trabea, Poria cocos, Postia placenta* and *Serpula lacrymans*, and some species of the genera *Daedalea, Piptoporus*, *Pycnoporellus, Neolentinus* and *Paxillus* and, to a lesser extent, ascomycota

Table 1. Ligninolytic Enzymes of Fungal Origin.

Classes	Fungi	Enzymes	References
Basidiomycetes	*Phanerochaete chrysosporium*	LiP MnP Glyoxal oxidase (GLOX)	Gold and Alice,1993; Pointek, et al., 2001; Erden et al., 2009 Hofrichter, 2002 Martínez, et al., 2009
	P. chrysosporium	Mn independent Peroxidase	Wyatt and Broda,1995; Ruiz-Dueñas and Martínez, 2009
	P. sordid	MnP	Rüttimann, et al., 1994
	Pleurotus ostreatus *P. ostreatus D1*	LAC and MnP LAC	Eichlerová, et al., 2000; Kamitsuji, et al., 2004 Pozdniakova, et al., 2006
	Pleurotus sp., P. eryngii, and P. ostreatus	VP	Ruiz-Dueñas, et al., 2009; Ruiz-Dueñas, et al., 1999; Camarero, et al., 1999; Cohen,, et al., 2001
	Pleurotus saborcaju	Aryl alcohol oxidase (AAO)	Martínez, et al., 2009
	Heterobasidiun annosum	LAC and MnP	Johannson, et al., 1999; Hatakka, 1994; Maijala, et al., 2003
	Ceriporiopsis aneirina,	LAC and MnP	Tomšovskýa,et al., 2009
	C. resinascens	LAC and MnP	Tomšovskýa, et al., 2009
	C. subvermispora	LAC and MnP	Lobos, et al., 2001
	Dichomitus albidofuscus	LAC and MnP	Tomšovskýa,et al., 2009
	Trametes versicolor	LiP and MnP	Johansson, and Nyman, 1993; Johansson, et al., 2002)
	Trametes villosa	LAC	Li, et al., 1999
	Trametes sp. strain AH28	LAC	Xiao, et al., 2003
	Trametes pubescens	LAC	Galhaup, and Haltrich, 2001; Galhaup, et al., 2002; Shleev, et al., 2007
	Panus tigrinus 8/18	VP	Lisov, et al., 2007
	Bjerkandera sp. BOS55 *Bjerkandera adusta* *Bjerkandera sp. (B33/3)* *Bjerkandera fumosa*	VP	Mester, and Field, 1998; Palma, et al., 2000 Heinfling, et al., 1998; Wang, et al., 2003 Moreira, et al., 2001 Rodakiewicz-Nowak, et al., 2006
	Cerrena unicolor strain 137	LAC	Michniewicz, et al., 2006
	Physisporinus rivulo	MnP	Hakala, et al., 2006
	Coniophora puteana	LAC	Lee, et al., 2004
	Polyporus sp.	LAC	Sinegani, et al., 2006
	Polyporus ostreiformi	MnP and LiP	Dey, et al., 1994
	Cyathus bulleri	LiP and Chatechol Oxidase	Gupta, et al., 2001; Salony, and Bisaria, 2006
	Cyathus africanus, C. striatus and C. stercoreus	LAC, LiP and Chatechol Oxidase	Gupta, et al., 2001
Ascomycetes	*Melanocarpus albomyces* *Chaetomium thermophile* *Magnaporthe grisea* *Myrothecium verrucaria 24G-4* *Neurospora crassa* *Coniochaeta ligniaria NRRL 30616*	LAC MnP and LiP	Hakulinen, et al., 2006 Ishigami, and Yamada, 1986; Chefetz, et al., 1998 Iyer and Chattoo, 2003 Sulistyaningdyah, et al., 2004; Germann, et al., 1988 Lopez, et al., 2007
	Paraconiothyrium variabile	LAC, LiP, MnP	Gao, et al., 2011
	Fusarium proliferatum	LAC and AAO	Regalado, et al., 1999
	Penicillium chrysogenum	LAC	Rodríguez, et al., 1996
	Paecilomyces inflatus	LAC	Kluczek-Turpeinen, et al., 2003

Source: Paliwal, et al., 2012

including *Aspergillus niger, Fusarium oxysporum* and *Fusarium merismoides* (Martinez et al., 2005; Sanchez, 2009). In the early stages of decay process brown rot fungi colonize the wood tissues via rays, from where the hyphae penetrate into the axial wood structure (Daniel, 2003). Some recent reports suggested, low molecular weight agents (non-enzymatic) produced by the BRF are responsible for early stages cell wall depolymerization through the production of free radical species (Goodell, 2003). The BRF are preferentially depolymerize the S_2 layer of the secondary wall, while affecting the S_3 layer at the late phase of decay, probably owing to reduced density and lower lignin content in S_2 layer. By contrast, highly lignified primary wall and middle lamella generally resist attack of BRF.

White-Rot Fungi (WRF)

White rot fungi are widely known and extensively researched wood-rotting organisms with the ability to depolymerize all the major wood components such as cellulose, hemicelluloses and lignin. In advanced stages of white-rot decay the rotten wood characterized by a moist, soft and of spongy consistency, with a strength loss (Martinez et al., 2005). In some cases, WRF completely degrade wood to give residues that make unstable components of forest soils (Ryvarden, 1991). The common feature of white-rot fungi is their extensive degradation capability for lignin resulting in a bleached appearance of the rotten wood. However, they do not use lignin as carbon sources for their growth they have develop complex extracellular enzymetic system to degrade lignin. Thus, lignin degradation is essentially a secondary metabolic process, not required for the main growth processes (Paliwal et al., 2012). This unique property of WRF if harnessed properly during pretreatment, can lead to energy reductions during mechanical pulping, beneficial for biodegradation of recalcitrant biopolymers and bioremediation of xenobiotic compounds, and/or increase the efficiency of bioconversion (Skyba et al., 2013). WRF comprise numerous fungi, mostly basidiomycota, including *Coriolus versicolor*, *Dichomitus squalens*, *Lentinus edodes*, *Phlebia radiata*, *Panus tigrinus*, *Pleurotus ostreatus* and *Pycnoporus cinnabarinus*, and a few species of ascomycota such as *Xylaria spp*.

Bacterial Ligninolysis

The bacterial depolymerization of lignin is both limited and slower than that of fungi. There are several reports on bacterial ligninolysis; however, their potential and molecular mechanism is still need to be explored for their effective application on commercial scale. Pure culture studies on bacterial delignification are virtually absent because bacteria cannot grow effectively on cellulose and lignin together (Singh 2006; Paliwal et al., 2012). Some species of the genera *Cellulomonas, Bacillus* and *Pseudomonas*, and some actinomycetes have been reported to degrade lignocellulosic components of wood to some extent (Eriksson et al., 1990). However, very few species are able to degrade lignin and it seems that wood-colonizing bacteria work in synergy with lignolytic fungi (Blanchette & Shaw, 1978). Bacteria can metabolize the low-molecular-weight components produced from lignin degradation by fungi (Crawford et. al., 1983; Paliwal et al., 2012). Various species of bacteria have been found to possess ligninolytic properties where *Sphingomonas sp.* (Masai et. al., 2003) belong to α-proteobacteria, *Pseudomonas sp.* (Delalibera et. al., 2007) to γ-proteobacteria and *Rhodococcus, Nocardia and Streptomyces sp.* (Zimmermann, 1990; Crawford et al., 1983; Bugg et. al., 2011) to actinomycetes. Bacterial isolates *viz. Azotobacter, Bacillus megatarium and Serratia marcescens,* procured from compost and soil are shown to decolorize or solublize industrial lignin (Perestelo et. al., 1989; Morii et. al., 1995). Bacteria offers

Table 2. Bacteria with lignin degradation activity fall into three classes.

Bacteria	References
Actinomycetes	Ramachandra, et, al., 1988
Streptomyces viridosporus T7A	Yang, et. al., 2009
Streptomyces strain F-6 and	Zimmermann, 1990
Streptomyces strain F-7	Ahmad, et. al., 2010
Nocardia autotrophica	Masai, et. al., 2007
Rhodococcus sp.	Ahmad, et. al., 2010
Streptomyces coelicolor	Ghodake, et. al., 2009
Rhodococcus jostii RHA1	
Rhodococcus erythropolisa	
Arthrobacter globiformis	
α-Proteobacteria	
Sphingobium sp. SYK-6	
γ-Proteobacteria	
Pseudomonas putida mt-2 and	
Acinetobacter sp.	
Acinetobacter calcoaceticus NCIM 2890	

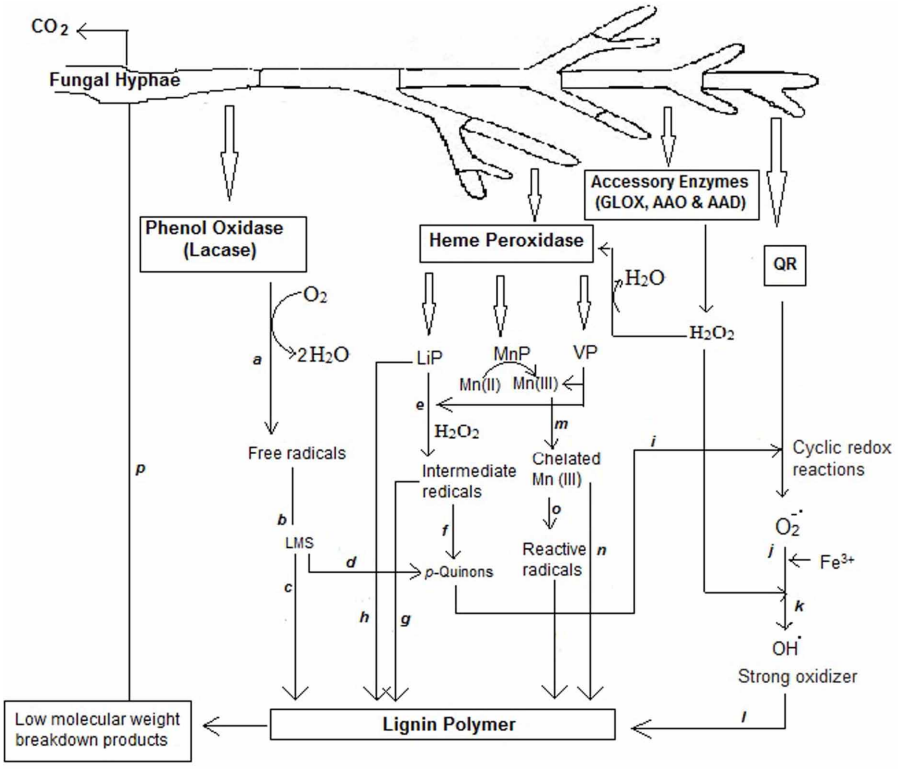
some unique class of enzyme for lignin degradation such as, laccases, glutathione S-transferases, ring cleaving dioxygenases, monooxygenases and phenol oxidases (Paliwal et al. 2012). The enzymology of bacterial lignin depolymerization is not well studied, yet there are certain species that use extracellular peroxidases for lignin degradation. Some bacterial having lignin degrading ability listed in Table 2 falls into three classes actinomycetes, α-protobacteria and γ-protobacteria.

Enzymes

WRF being the most potential lignin degraders in nature and have been studied extensively for their capacity to produce lignin degrading enzymes. After the discovery of laccase in 1883, there has been exceptional curiosity to look for the possible application of ligninolytic enzymes commercially in various fields (Maciel et al., 2010, Paliwal et al., 2012). Several workers have surveyed the extracellular ligninolytic enzymes. For example, Kirk and Farrell (1987) defined the lignin degrading mechanism of wood-rotting fungi as "enzymatic combustion" (Paliwal et al., 2012). Extracellular oxidative enzymes include an array of oxidases and peroxidases, on the bases of their preferred substrates oxidized and the presence of key substrate binding amino acid (aa) residues (Morgenstern et al., 2010). These include mainly lignin peroxidase (LiP; EC 1.11.1.14), manganese peroxidase (MnP; EC 1.11.1.13), versatile peroxidases (VP; EC 1.11.1.16), and phenol oxidases also known as laccase (LAC; EC 1.10.3.2) (Hatakka, 1994, Pointing, 2001; Mai et al., 2004; Kersten & Cullen, 2007; Paliwal et al., 2012). In general, due to haem pocket architecture the peroxidases (LiP, MnP, and VP) have high redox potential that facilitates oxidation of non-phenolic aromatic rings. A brief schematic mechanism of lignin depolymerization by ligninolytic enzyme system of WRF is presented in figure 4. Different ligninolytic and accessory enzymes on the basis of their cofactor, mediator and mode of action have been categorized in Table 3. Enzymes and their mode of actions are discuses below (Paliwal et al., 2012).

1. **Lignin Peroxidase (LiP):** It requires hydrogen peroxide and veratryl alcohol as mediator for lignin degradation. General interaction with substrate consist two steps:

Figure 4. A Schematic presentation of lignin biodegradation process in white rot fungi. (a) Substrate oxidation via reduction of dioxygen into 2 molecules of water leading to formation of free radicals. (b) Free radicals act as intermediate substrates for the enzymes (LMS). (c) Formation of mediators that can leave the enzymatic site and undergo non enzymatic reactions of oxidative polymerization or depolymerization of lignin polymer. (d) LMS involve in ligninolysis. (e) Formation of intermediate radicals, such as phenoxy radicals and veratryl alcohol radical cations via muli-step electron transfer reaction. (f) C_α-C_β breakdown yielding p-quinones. (g) repolymerization. (h) Direct oxidation of non-phenolic aromatic substrates by LiP. (i) Oxygen activation in redox cycling reactions involving QR. (j) Reduction of the ferric iron (k) Reoxidation of reduced ferric iron by reduction of H_2O_2 and release of hydroxyl free radical ($OH^{\cdot}$). (l) $OH^{\cdot}$ initiates the attack on lignin. (m) Oxidation of Mn(II) to Mn(III) which is then chelated by oxalate or other chelators. (n) Chelated Mn(III) complex acts as a reactive low molecular weight, diffusible redox-mediator for oxidation of phenolic substrate. (o) Formation of reactive radicals in the presence of a second mediator (such as acetic acid radicals, peroxyl radicals, superoxide and formate radicals) for oxidation of non phenolic substrate. (p) Finally the low molecular weight compounds are subsequently taken up by fungal hyphae and converted to CO_2. Abbreviations: LMS – Laccase Mediate System; LiP - Lignin Peroxidase; MnP - Mangenese Peroxidase; VP - Versetile Peroxidase; GLOX – Glyoxal Peroxidase; AAO – Aryl Alcohol Oxidase; AAD – Aryl Alcohol Dehyrogenase; QR – Quinon Reductase; $OH^{\cdot}$ - Free Hydroxyl Radical.
(Source: Paliwal et al., 2012)



a. **Step I:** Hydrogen peroxide (H_2O_2) activates the haem cofactor and oxidizes the resting ferric enzyme [Fe(III)] by $2e^-$ to produce compound I (an intermediate) that exists as a ferry iron porphyrin radical cation (LiP – I).

Native (ferric) peroxidase + H_2O_2 → [Fe(IV)=O·$^+$] + H_2O

LiP – I

b. **Step II:** LiP – I oxidizes aromatic substrates such as veratryl alcohol (VA) by $1e^-$ to give Compound II ([Fe(IV)=O, LiP-II]) and an aromatic cation radical (VA·$^+$). LiP-II again oxidizes aromatic substrates by $1e^-$ to return the enzyme in resting state. However, in some cases LiP-I can also return to the native enzyme by a direct $2e^-$ reduction (Wong et al., 2009; Paliwal, et al., 2012).

[Fe(IV)= O·$^+$] + VA → [Fe(IV)= O] + VA·$^+$

LiP – I LiP – II

[Fe(IV)=O] + VA → Native (ferric) peroxidase + VA·$^+$

LiP – II

Following these two steps, LiP oxidizes the phenolic units of lignin polymer. In phenolic dimeric substrates LiP cleaved C_α-C_β linckage of propyl side chain via hydroxylation and oxidation. Whereas, oxidation of non-phenolic substrate involve aromatic ring cleavage (C_α-C_β) and demithylation reactions.

Table 3. Enzymes involved in the degradation of lignin and their main reactions.

Enzyme Activity (Abbreviation)	Cofactor	Substrate, "Mediator"	Main Effect or Reaction
Lignin peroxidase (LiP)	H_2O_2	Veratryl alcohol	Aromatic ring oxidized to cation radical
Manganese peroxidase (MnP)	H_2O_2	Mn, organic acid as chelator, thiols, unsaturated lipids	Mn^{2+} oxidized to Mn^{3+}; further oxidation of phenolic compounds to phenoxyl radicals
Versatile Peroxidase (Hybrid peroxidases)	H_2O_2	Same or similar compounds as LiP and MnP	Same effect on aromatic and phenolic compounds as LiP and MnP
Laccase (LAC)	O_2	Mediators (hydroxybenzatriazole, ABTS)	Phenol are oxidized to phenoxyl radicals; mediator radicals
Glyoxal oxidase (GLOX)		glyoxal, methyl gloxal	Glyoxal oxidized to glyoxylic acid; H_2O_2 production
Aryl alcohol oxidase (AAO)		Aromatic alcohols (anisyl, veratryl alcohol)	Aromatic alcohol oxidized to aldehydes; H_2O_2 production
Other H_2O_2 producing enzymes		Many organic compounds	O_2 reduced to H_2O_2

(Source: Hatakka, 2001)

2. **Manganese Peroxidase (MnP):** MnPs generates Mn^{3+}, which acts as a diffusible oxidizer on phenolic or non-phenolic lignin units by lipid peroxidation reactions (Bugg et al., 2011).
3. **Laccase (LAC):** LAC requires atmospheric oxygen as electron acceptor to oxidize phenols, polyphenols, aromatic amines, and a range of non-phenolic substrates (Mai et al., 2004).
4. **Versatile Peroxidases (VP):** VPs act in a bifunctional fashion similar to MnP generating Mn (III) and LiP oxidizing both phenolic and non-phenolic aromatic compounds (Bugg et al., 2011).

FACTORS REGULATING MICROBIAL LIGNINOLYSIS

Plant biomass degradation is a key step in nutrient recycling and humus building in the natural ecosystems. Lignin degradation can regulate the decomposition of whole litter. Successive decomposition of plant biomass is regulated by the combined effects of physico-chemical characteristics of the biomass, environmental conditions, and the activities of degraders. Some important factors are discussed below:

- **Community Structure:** The community structure and activity of decomposers in soil organic layers, changes throughout the degradation process as the quality of the substrate changes. Degradation of plant biomass is usually initiated by generalist primary colonizers such as fungi and bacteria. These colonizers utilize simple sugars, oligosaccharides, and other low molecular weight compounds. After this initial flush of microbial activity, less competitive secondary colonizers involved in the degradation of more recalcitrant polymers such as lignocellulose complexes (Cox et al., 2001). Both bacteria and fungi play typical roles in decomposition processes in natural ecosystems. They compete for simple plant-derived substrates and have developed antagonistic strategies. While, for more recalcitrant substrates they developed both competitive and mutualistic strategies. Bacteria produce growth factors for fungi and increase accessibility of substrates to fungi producing their own cellulases and pectinases (Boer et al., 2005).
- **Nutrients Availability:** Certain nutrient conditions such as nitrogen and carbon levels are also important to regulate the microbial ligninolysis. High levels of nitrogen in substrate may suppress the lignin degradation. Addition of small amount of nitrogen in cultures promotes the ligninolysis by certain microorganisms (Berg & McClaugherty, 2003). Presence of carbon source is very important to promote the microbial growth and degradation process. Lignin degradation is an energy demanding process pertaining to its recalcitrant nature microorganism cannot utilize lignin as direct carbon source. In the early stages of biomass decomposition, the major carbon components in litter are easily available cellulose and hemicelluloses, which are higher in concentration and supplies alternative carbon sources to the lignin-degrading organisms.
- **Environmental Conditions:** Environmental temperature and moisture conditions are additional stronger factors influencing the seasonal changes in microbial communities (Berg & McClaugherty, 2003). Lignin being recalcitrant polymer and major component of plant biomass has great importance in soil humus building and also in carbon sequestration in woody perennial vegetation. Elevated atmospheric carbon dioxide (CO_2) concentration and global climate change are major human induced limiting factors that may affect the microbial activity and plant litter chemistry. Although this has always been a matter of discussion that in which direction will plants and microbial activities will response to these factors. Cotrufo and Ineson (1995) conducted a laboratory microcosm study with birch and Sitka spruce at different CO_2 (350 and 600 ppm), and under two

nutrient regimes. They reported slightly lower mass loss in birch roots grown under elevated CO_2 concentration. That may be due to changes in litter chemistry, as the authors reported an increase in C/N ratio in roots grown under CO_2 enrichment (Berg & McClaugherty, 2003). In an another field study Cotrufo and Ineson (1996) found the lignin content of birch leaf litter increased from 17.7 to 28.7% when the atmospheric CO_2 concentration during growth was increase from 350 to 600 ppm. Franck et al. (1997) hypothesized that elevated atmospheric CO_2 would cause lower-quality litter production by plants that would not be easily decompose and release nutrients more slowly. Therefore microbial degradation of plant biomass is an important process for ecosystems functions such as release of nutrients, building stable humus, and at the same time storage of nutrients.

FUTURE ASPECT OF MICROBIAL LIGNINOLYSIS

For better understanding the process of microbial ligninolysis, knowledge concerning the molecular biology of the microbial ligninolytic system has advanced considerably in the recent past. In this context *Phanerochaete chrysosporium* has been most thoroughly studied. In order to get a better strain with high enzyme activity, there are several choices like, selection of a high enzyme-producing strain from hundreds of wild type isolates and/or generation of a mutant strain through mutagen treatment. Apart from this, heterologous expression of the enzymes with protein engineering paves way for the cost-effective creation of more robust and active enzymes. In this context efforts have been made to produce ligninolytic enzymes through heterologous expression systems (Paliwal et al., 2012). For example, Timofeevski et al. (1999), introduced a tryptophan residue analogous to the essential one in LiP to *P. chrysosporium* MnP by site-directed mutagenesis (single mutation, S168W), and created MnP with LiP activity, while full MnP activity was maintained.

Another tool to achieve easy delignification of plant biomass is biotechnological manipulation with lignin content of plant material. From the industrial perspective of wood utilization, lignin has a negative impact on pulping process. Native sources of ligninolytic enzymes cannot meet the market demand due to their low yields, incompatibility of the standard industrial fermentation processes with the requirement of substrate specificities and application conditions *viz.* pH, temperature, and reaction media required for the growth of many microorganisms. Lack of efficient microbial expression systems further becomes a bottleneck in the industrial application of these biocatalysts (Paliwal et al., 2012). Biotechnological alteration in secondary cell wall structure resulting from manipulation of genes involved in lignin biosynthesis is seen as a route to improve the utilization of plant cell wall polysaccharides in various agricultural and industrial processes. The objectives of these efforts are to reducing the energy demand by enhancing cell wall digestibility in ruminants and easier delignification in the papermaking process (Marita et al., 1999). Many researchers have achieved this goal by overexpression, downregulation, or suppression of genes involved in lignin biosynthesis and by plant transformation technology in industrially important plants (Paliwal et al., 2012). However, change in secondary cell wall structure could also result in altered growth, feeding behavior herbivores, lower pathogen and pest resistance, affect plant interactions with soil organisms, nutrient transformations and other adhered environmental problems. Therefore, it has been suggested that all these techniques need more investigation and practice in the field successfully from the controlled laboratory conditions for their sustainable application.

CONCLUSION

In past decade, several approaches have been made to screen the microorganisms leading to the discovery of new enzymes for lignin degradation. Exploration of enzymatic system of fungi is currently useful to understand the synergistic behavior of different enzymes involved in lignin degradation pathways. It is now clear that lignin degradation is a multi-enzymatic process involving the major oxidative enzymes (i.e. peroxidases and laccases) and numerous auxiliary enzymes to help and achieve the depolymerisation. Though the role of fungal ligninolytic system has been well established, study of bacterial lignin degradation has also become increasingly important. It is well understood that lignin has significant role in several ecological process ranging from carbon sequestration, nutrient cycling, litter decomposition and humus formation. Several workers had come up with their view about the impact of global carbon change on microbial ligninolysis and plant biochemistry, but still this area need further to be explore. Although manipulation of lignin content in plants via genetic engineering may have immense potential to combat with the problems related to the recalcitrant nature of lignin, but low lignin content could also result in altered ecological processes. Therefore, it is suggested that the benefits of transgenic trees should be compared with other alternatives such as improved biocatalysts for lignin degradation. Several industrial sectors such as, textile and wood pulp bleaching, food additives production, organic synthesis, medical, pharmaceutical, cosmetics, nanotechnology applications and industrial wastes bioremediation, have now come up with the application of lignin degrading enzymes. For this, novel lignin-degrading microbes and their enzymes combine with advanced technological tools can contribute enormously towards more efficient and environmentally sound utilization of renewable lignocellulosic feedstocks for sustainable production of materials and energy.

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KEY TERMS AND DEFINITIONS

Bioligninolysis: Breaking of polymeric structure of lignin exclusively by mean of microbes and their product (enzymes).

Biopulping: Fungal pretreatment of wood chips for the production of mechanical or chemical pulps.

Depolymerization: Process of converting a polymer such as lignin into a monomer or a mixture of monomer units.

Enzymatic Combustion: Enzyme catalyzed burning of biopolymer molecules such as lignin.

Enzymes: Biological molecules that catalyze biological reactions.

Heterologous Expression: A gene/gene fragment/protein is experimentally put into a host organism that does not naturally make or express that gene or gene fragment or protein.

Lignin: Polymer of aromatic alcohols and an integrated part of secondary cell walls of plant.

Recalcitrant Compound/Molecule: In environment any compound or molecule that persists in nature for long time and resist degradation.

Sustainable Development: Development that meet the needs of the present without compromising the ability of future generations to meet their own needs.

Synergistic Behavior: In biology when two or more agents (organisms or enzymes) perform an activity together to enhanced the results.

Chapter 7
Soil Bioremediation: Harnessing Potential of Indigenous Microorganisms

Ruqeya Nazir Shiekh
University of Kashmir, India

Fayaz Shah
University of Kashmir, India

Sajad Bhat
University of Kashmir, India

Faroz Ahmad Ahanger
Centre of Research for Development, University of Kashmir, India

ABSTRACT

Bioremediation, a rapidly changing and growing area of environmental biotechnology employing microorganisms, presents a potentially more effective as well as economical clean-up technique than conventional approaches. The combination of several remediation techniques are considered to improve the remediation results especially in sites with complex contamination, as most traditional methods do not provide acceptable solutions for the removal of wastes from soils. The combination of electro kinetics with bioremediation, nanotechnology, biofilms, phytoremediation, chemical oxidation or electrical heating, presents interesting perspectives for the remediation. It is expected that the combination of these technologies will expand the dimensions of the remediation process to improve the remediation results, saving energy and time. Employment of new techniques is the need of the hour to carry forward this novel technology from its embryonic stage to all its developmental stages providing it with promising attributes to address some of the grave challenges faced by our environment today.

INTRODUCTION

The quality of life on mother Earth depends to a large extent on the overall quality of the environment. In early times, there was a belief that there was an unlimited abundance of land and resources; today, however, the resources in the world show our carelessness and negligence in using them. The problems associated with contaminated sites now assume increasing visibility in many countries. Contaminated lands generally result from past industrial activities when awareness of the health and environmental ef-

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fects connected with the production, use, and disposal of hazardous substances were less well recognized than today. The problem is worldwide, and the estimated number of contaminated sites is on rise. It is now widely recognized that contaminated land is a potential threat to human health, and its continual discovery over recent years has led to international efforts to remedy many of these sites, either as a response to the risk of adverse health or environmental effects caused by contamination or to enable the site to be redeveloped for use.

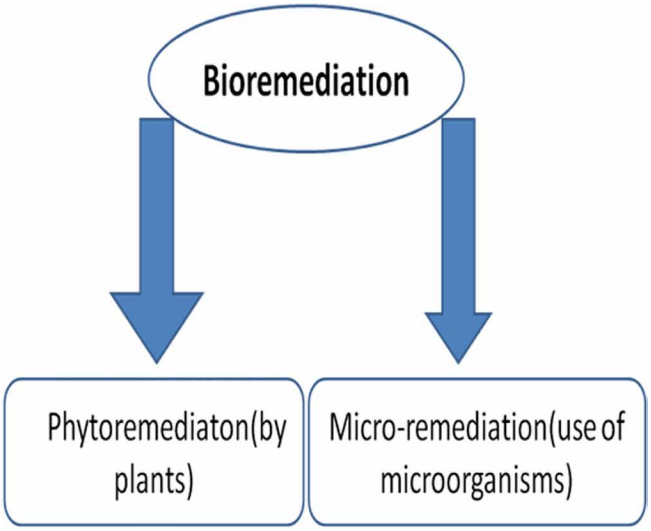
The conventional techniques used for remediation have been to dig up contaminated soil and remove it to a landfill, or to cap and contain the contaminated areas of a site. These methods are non economical and old. Through such methods, the contaminants are transported elsewhere and may create significant risks in the excavation, handling, and transport of hazardous material. Additionally, it is very difficult and more expensive to find new landfill sites for the final disposal of the material. The cap and contain method presents temporary solution since the contamination remains on site, requiring monitoring and maintenance of the isolation barriers long into the future, with all the associated costs and potential liability.

A promising approach than these traditional methods is to completely destroy the pollutants if possible, or at least to transform them to innocuous substances. Some technologies that have been used are high-temperature incineration and various types of chemical decomposition (e.g., base-catalyzed dechlorination, UV oxidation). They can be very effective at reducing levels of a range of contaminants, but have several drawbacks, principally their technological complexity, the cost for small-scale application, and the lack of public acceptance, especially for incineration that may increase the exposure to contaminants for both the workers at the site and nearby residents.

Bioremediation is an option that offers the possibility to destroy or render harmless various contaminants using natural biological activity. As such, it uses relatively low-cost, low-technology techniques, which generally have a high public acceptance and can often be carried out on site. Sustainable ecosystems can be designed to eliminate environmental toxins and reduce pathogen loads through the direct and indirect consequences of plant and microbial activities. Contamination of soils, groundwater, sediments, surface water, and air with hazardous and toxic chemicals is one of the major problems facing the world today. Nature has its own way of cleaning the environment by removing xenobiotics to maintain a perfect balance but in this era of industrialization the rate of xenobiotic discharges has crossed the tolerance limit of the nature. Therefore, there is a need to find out the method of remediating xenobiotics from the environment. Microbial remediation of xenobiotics has proved the effectiveness and low cost technology but there are several limitations in using microbes. It has long been recognized that microorganisms have distinct and unique roles in the detoxification of polluted soil environments and, in recent years, this process has been termed bioremediation.

Bioremediation is a soft bioengineering technique to clean up contaminated lands/sites using microbes (bacteria or fungus), plants (terrestrial and aquatic) and earthworms. It is also a technique to stabilize the eroded lands and prevent soil erosion. Bioremediation works carried out by the microorganisms are called ‘micro-remediation’ while those performed by plants are called ‘phyto-remediation’ (Figure.1). Earthworms have also been found to perform some environmental cleaning jobs and are termed as ‘vermi-remediation’. Recent advancements have also proven successful via the addition of matched microbe strains to the medium to enhance the resident microbe population’s ability to break down contaminants. Microorganisms used to perform the function of bioremediation are known as bioremediators. The objectives of the article are to provide the historical background in the field of bioremediation and environmental management by exploiting potential of indigenous microorganisms. The chapter also deals with the recent developments in the field of bioremediation.

Figure 1. Bioremediation Types



Principle of Bioremediation

Recent studies in molecular biology and ecology offers numerous opportunities for more efficient biological processes. Notable accomplishments of these studies include the cleanup of polluted water and land areas. Bioremediation is defined as the process whereby organic wastes are biologically degraded under controlled conditions to an innocuous state, or to levels below concentration limits established by regulatory authorities (Mueller 1996). By definition, bioremediation is the use of living organisms, primarily microorganisms, to degrade the environmental contaminants into less toxic forms. It uses naturally occurring bacteria and fungi or plants to degrade or detoxify substances hazardous to human health and/or the environment. The microorganisms may be indigenous to a contaminated area or they may be isolated from elsewhere and brought to the contaminated site. Contaminant compounds are transformed by living organisms through reactions that take place as a part of their metabolic processes.

For bioremediation to be effective, microorganisms must enzymatically attack the pollutants and convert them to harmless one (Vidali, 2001). As bioremediation can be effective only where environmental conditions permit microbial growth and activity, its application often involves the manipulation of environmental parameters to allow microbial growth and degradation to proceed at a faster rate.

Bioremediation techniques are typically more economical than traditional methods such as incineration, and some pollutants can be treated on site, thus reducing exposure risks for cleanup personnel, or potentially wider exposure as a result of transportation accidents. Like other technologies, bioremediation has its limitations. Some contaminants, such as chlorinated organic or high aromatic hydrocarbons, are resistant to microbial attack. They are degraded either slowly or not at all, hence it is not easy to predict the rates of clean-up for a bioremediation exercise; there are no rules to predict if a contaminant can be degraded. Yet bioremediation is based on natural attenuation, scientific community considers it more convenient than traditional ones.

Conditions for Bioremediation

The accomplishment of bioremediation processes is a complex system of many factors. These factors include: the existence of a microbial population capable of degrading the pollutants; the availability of contaminants to the microbial population; the environment factors (type of soil, temperature, pH, the presence of oxygen or other electron acceptors, and nutrients) (Figure.2).

Strategies of Bioremediation

Bioremediation utilizes the natural role of microorganisms in transformation, mineralization or complexation by directing those capabilities towards organic and inorganic environmental pollutants. The primary technique that has been used in bioremediation to enhance natural detoxification of contaminated environments is stimulation of the activity of indigenous microorganisms by the addition of nutrients, regulation of redox conditions, and optimization of pH conditions, etc. Strategies of bioremediation are studied under following headings (Figure 3).

EX SITU BIOREMEDIATION

This technique involves the excavation or removal of contaminated soil from ground. Ex situ bioremediation involves following techniques.

Land farming is a simple technique in which contaminated soil is excavated and spread over a prepared bed and periodically tilled until pollutants are degraded. The goal is to stimulate indigenous biodegradative microorganisms and facilitate their aerobic degradation of contaminants. In general, the practice is limited to the treatment of superficial 10–35 cm of soil.

Composting is a technique that involves combining contaminated soil with nonhazardous organic amendants such as manure or agricultural wastes. The presence of these organic materials supports the development of a rich microbial population and elevated temperature characteristic of composting.

Figure 2. Bioremediation requirements

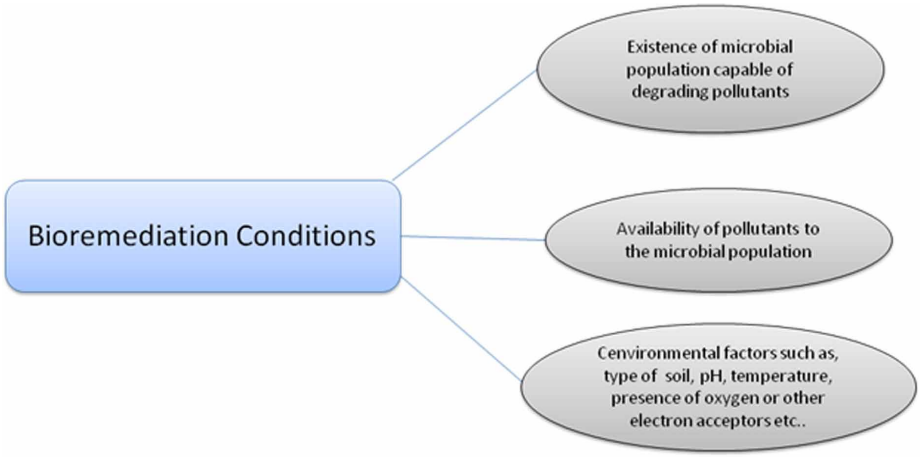
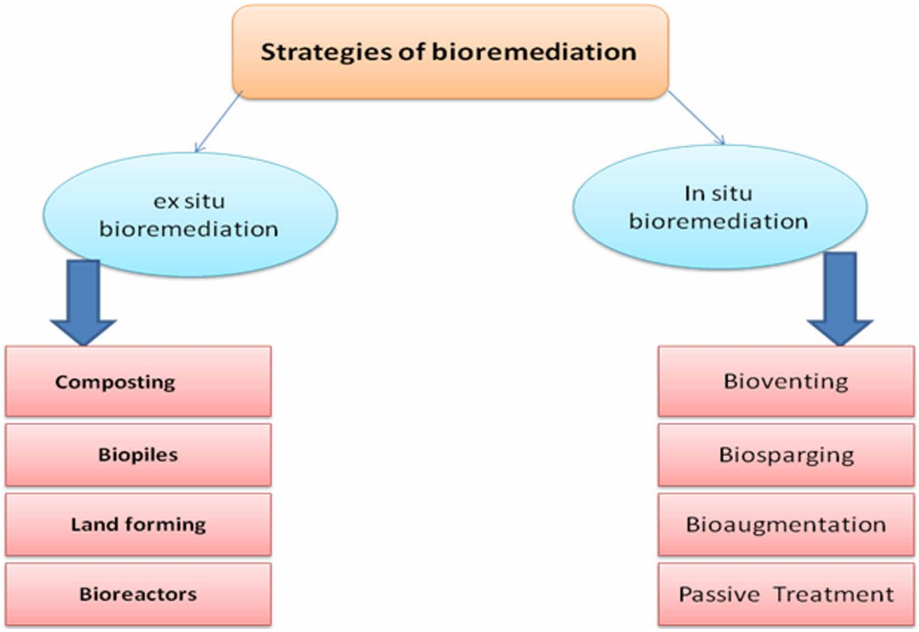


Figure 3. Strategies of Bioremediation



Biopiles are a hybrid of landfarming and composting. Essentially, engineered cells are constructed as aerated composted piles. Typically used for treatment of surface contamination with petroleum hydrocarbons they are a refined version of land farming that tend to control physical losses of the contaminants by leaching and volatilization.

Bioreactors. Slurry reactors or aqueous reactors are used for *ex situ* treatment of contaminated soil and water pumped up from a contaminated plume. Bioremediation in reactors involves the processing of contaminated solid material (soil, sediment, sludge) or water through an engineered containment system. A slurry bioreactor may be defined as a containment vessel and apparatus used to create a three-phase (solid, liquid, and gas) mixing condition to increase the bioremediation rate of soil bound and water-soluble pollutants as a water slurry of the contaminated soil and biomass (usually indigenous microorganisms) capable of degrading target contaminants. In general, the rate and extent of biodegradation are greater in a bioreactor system than *in situ* or in solid-phase systems because the contained environment is more manageable and hence more controllable and predictable.

IN SITU BIOREMEDIATION

In situ bioremediation does not require excavation or removing soil in order to perform remediation. The technique involves enhancing microbial activity in contaminated soil by providing the necessary nutrients, electron acceptors, moisture etc. The use of microbial inocula, cell-free enzymes, and plants for in situ bioremediation is still mostly experimental. To date, most in situ applications have been performed by the petroleum industry to clean up hydrocarbon spills and gasoline tank leaks. These techniques are generally the most desirable options due to lower cost and fewer disturbances since they provide

the treatment in place avoiding excavation and transport of contaminants. In situ remediation includes techniques such as bioventing, biosparging, bioslurping and phytoremediation along with physical, chemical, and thermal processes.

Bioventing involves supplying air and nutrients through wells to contaminated soil to stimulate the indigenous bacteria. Bioventing employs low air flow rates and provides only the amount of oxygen necessary for the biodegradation while minimizing volatilization and release of contaminants to the atmosphere. It works for simple hydrocarbons and can be used where the contamination is deep under the surface.

Bioventing is the only in situ bioremediation technique that allows for the treatment of unsaturated soil. Bioventing is not effective if the water table is within several feet of the surface. (Van Deuren, et al. 2002). Due to the pressure gradient in the soil, atmospheric oxygen flows into the subsurface. This oxygen starts an aerobic contaminant decomposition process. In many cases it is necessary to add nitrogen salts as an additive by sprinkling a nutrient solution on top of the soil or by injecting them into the soil above the contaminated soil zone (Held and Dörr, 2000).

Sufficient airflow is very important in the design of a bioventing system. The geometry of the exfiltration wells and the need for active or passive air injections are two particular design concerns. If a high concentration of pollutants exists, clogging of the soil pores may occur. In this case, pulsed soil vapor extraction is needed. Low permeability will also hinder Bioventing. If the soil vapors are volatile, they must be treated at the surface with an activated carbon filter or a biofilter. Bioventing is effective in removing petroleum hydrocarbons, aromatic hydrocarbons, and non-volatile hydraulic oils (Held and Dörr, 2000).

Biosparging. Biosparging involves the injection of air under pressure below the water table to increase groundwater oxygen concentrations and enhance the rate of biological degradation of contaminants by naturally occurring bacteria. Biosparging increases the mixing in the saturated zone and thereby increases the contact between soil and groundwater. It is used in both saturated and unsaturated soil zones. The technique was developed to reduce the consumption of energy. The injection of air into the aquifer results in small channels for the air to move to the unsaturated soil zone. Biosparging results in volatile contaminants being transported to the unsaturated zone, therefore soil vapor extraction is usually used to extract the volatile vapors and then treat them at the surface (Held and Dörr, 2000). In order for biosparging to be effective, the sparge points must be installed below the contamination zone because air always flows upward. The upflow of air will form an influence cone. In order to effectively remove contaminants from the soil using biosparging, the soil should be relatively homogeneous throughout the contamination zone.

Bioaugmentation. Bioremediation frequently involves the addition of microorganisms indigenous or exogenous to the contaminated sites. Bioslurping is a unique in situ treatment technique in that it also treats free product phases floating on top of the groundwater. This technique applies a vacuum to extract, soil vapor, water, and free product from the subsurface. Each of those products is separated and then treated. This technique is cost effective because only a small amount of groundwater and soil vapor are pumped at a time, therefore the treatment plant used to treat the vapor and free product can be small.

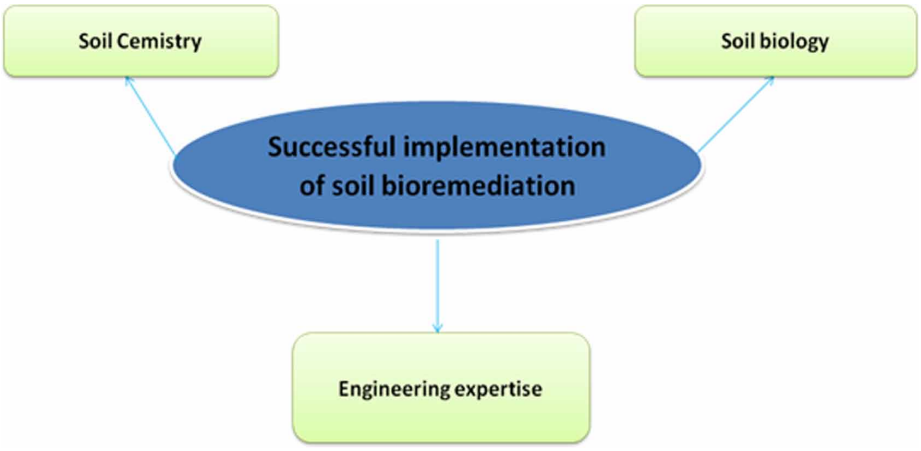
SOIL BIOREMEDIATION

Although "soil bioremediation" is a relatively new term, it describes a phenomenon that has existed since the beginning of life. A wide variety of naturally occurring toxic and recalcitrant organic compounds exist on earth, and many are naturally mineralized. The formation of most organic matter begins with plants capable of harnessing energy of sunlight. This organic matter serves as an important energy source for entire food chains. In a terrestrial ecosystem, the wastes produced by this food chain end up in the soil. Soil organic matter is recycled by diverse soil organisms including bacteria, fungi, actinomycetes, protozoa, earthworms, and insects. Microorganisms are ultimately responsible for mineralizing most organic matter to carbon dioxide. Residual organic matter that is not readily mineralized can be incorporated into humus. By studying these naturally occurring systems of soil bioremediation, researchers should be better able to apply these systems to the clean-up of manmade pollutants. The use of biological systems to bring about the timely remediation or detoxification of man-made pollutants is the focus of soil bioremediation. The successful implementation of soil bioremediation requires interdisciplinary cooperation among soil biology, soil chemistry, and engineering experts (Figure 4)

Microbial Populations for Bioremediation and Their Activity in Soil

Microorganisms are ubiquitous, inhabiting even the most hostile and extreme environments. Their ability to transform virtually all forms of organic material (natural or synthetic) makes them attractive agents of bioremediation. In nature, most organic matter is aerobically mineralized, using oxygen as the final electron acceptor. This degradation is usually a stepwise process involving different microorganisms in concert, or in succession, to bring about the mineralization of organic matter. The same is often true for the mineralization of xenobiotics. Slater and Lovatt showed that mixed communities of microorganisms may be more efficient at mineralizing some pollutants, such as chlorinated aromatic hydrocarbons and alkylbenzene sulfonates, than individual species. Sometimes pollutants cannot be directly assimilated by the microbes that oxidize them (co-metabolism), but may instead be further transformed by other populations. These commensal relationships can significantly enhance the mineralization of recalcitrant pollutants and prevent the accumulation of toxic intermediates.

Figure 4. Soil Bioremediation



In contrast to the oxygen-dependent metabolism characteristic of aerated portions of soil, other electron acceptors are involved in microbial degradation in anaerobic environments. These reduced environments provide optimal conditions for denitrifying, methanogenic, or sulfate-reducing microorganisms. Although longer incubation times are often required, the ability of anaerobic microorganisms to remove chlorine from environmentally persistent chemicals, such as PCBs (polychlorinated biphenyls), PCE (perchloroethylene), and DDT may be particularly useful for in situ bioremediation of groundwater. Some compounds, like benzene and related compounds can be completely mineralized to carbon dioxide or transformed to cell mass under anaerobic conditions. The ability of microorganisms to mineralize pollutants can also be increased through genetic alterations. Gene transfer in bacteria occurs naturally through conjugation, transduction, or transformation (figure.5).

Since numerous types of pollutants are to be encountered in a contaminated site, diverse types of microorganisms are likely to be required for effective mediation (Watanabe et al. 2001). The first patent for a biological remediation agent was registered in 1974, being a strain of *Pseudomonas putida* (Prescott et al. 2002) that was able to degrade petroleum. In 1991, about 70 microbial genera were reported to degrade petroleum compounds (U.S Congress, 1991) and almost an equal number has been added to the list in the successive two decades (Glazer and Nikaido, 2007).

Potential Microorganisms

- **Aerobic:** Examples of aerobic bacteria recognized for their degradative abilities are *Pseudomonas, Alcaligenes, Sphingomonas, Rhodococcus,* and *Mycobacterium.* These microbes have often been reported to degrade pesticides and hydrocarbons, both alkanes and polyaromatic compounds. Many of these bacteria use the contaminant as the sole source of carbon and energy.
- **Anaerobic:** Anaerobic bacteria are not as frequently used as aerobic bacteria. There is an increasing interest in anaerobic bacteria used for bioremediation of polychlorinated biphenyls (PCBs) in river sediments, dechlorination of the solvent trichloroethylene (TCE) and chloroform.
- **Ligninolytic Fungi:** Fungi such as the white rot fungus *Phanaerochaete chrysosporium* have the ability to degrade an extremely diverse range of persistent or toxic environmental pollutants. Common substrates used include straw, saw dust, or corn cobs.
- **Methylotrophs:** Aerobic bacteria that grow utilizing methane for carbon and energy. The initial enzyme in the pathway for aerobic degradation, methane monooxygenase, has a broad substrate range and is active against a wide range of compounds, including the chlorinated aliphatic trichloroethylene and 1, 2-dichloroethane. Tables.1 and 2 gives the general description of major microornisms involved in bioremediation and the chemical compounds degraded by them.

Some bacteria are mobile and exhibit a chemotactic response, sensing the contaminant and moving accordingly. Microbes such as fungi grow in a filamentous form toward the contaminant. Many different types of organisms such as plants can be used for bioremediation but microorganisms show the greatest potential. Microorganisms primarily bacteria and fungi are nature's original recyclers. Their capability to transform natural and synthetic chemicals into sources of energy and raw materials for their own growth suggests that expensive chemical or physical remediation processes might be replaced with biological processes that are lower in cost and more environmentally friendly. Therefore, microorganisms represent a promising, largely untapped resource for new environmental biotechnologies. Research continues to

Table 1. Naturally Occurring Bacteria Capable of Destroying Some Hazardous Wastes and Chemicals by Biodegradation

Organisms	Chemicals Degraded
Actinomycetes	Raw rubber
Closteridium	Lindane
Arthrobacter & Bacillus	Endrin
Alcaligenes spp. & Pseudomonas spp.	PCBs, halogenated hydrocarbons and alkylbenzene sulphonates, PCBs, organophosphates, benzene, anthracene, phenolic compounds, 2,4 D, DDT and 2,4,5-trichlorophenoxyacetic acid etc.
Trichoderma & Pseudomonas	Malathion
Flavobacterium spp.	Organophosphates
Cunniughamela elegans & Candida tropicalis	PCBs (Polychlorinated Biphenyls) & PAHs (Polycyclic Aromatic Hydrocarbons

Table 2. Naturally Occurring Fungus Capable of Destroying Some Hazardous Wastes and Chemicals by Biodegradation

Organism	Chemicals Degraded
Zylerion xylestrix	Pesticides / Herbicides (Aldrin, dieldrin, parathion and malathion)
P. sordida & Trametes hirsute	DDT, DDE, PCBs, 4,5,6-trichlorophenol, 2,4,6-trichlorophenol, dichlorphenol, and chlordane
Yeast (Saccharomyces)	DDT
Mucor	Dieldrin
Phanerochaete chrysoporium	Halocarbons such as lindane, pentachlorophenol,

verify the bioremediation potential of microorganisms. For instance, a recent addition to the growing list of bacteria that can reduce metals is *Geobacter metallireducens,* which removes uranium, a radioactive waste from drainage waters in mining operations and from contaminated groundwater. Even dead microbial cells can be useful in bioremediation technologies.

These discoveries suggest that further exploration of microbial diversity is likely to lead to the discovery of many more organisms with unique properties useful in bioremediation (U.S. EPA Seminars 1996). Application of microorganisms is not limited to one field of study of bioremediation, it has an extensive use; Petroleum, its products and oils constitute hydrocarbons and if present in the environment causes pollution. Oil slicks caused by oil tankers and petrol leakage into the marine environment are now a constantly occurring phenomenon. Several microorganisms can utilize oil as a source of food, and many of them produce potent surface active compounds that can emulsify oil in water and facilitate its removal. Unlike chemical surfactants, the microbial emulsifier is nontoxic and biodegradable. The microorganisms capable of degrading petroleum include pseudomonads, various corynebacteria, mycobacteria and some yeast (Mueller, 1996). Apart from degrading hydrocarbons, microbes also have the ability to remove industrial wastes, reduce the toxic heavy metals to a much less toxic soluble form. For instance, plants like locoweed remove large amounts of the toxic element selenium. The selenium is

stored in plant tissues where it poses no harm until and unless the plant is eaten. Many algae and bacteria produce secretions that attract metals that are toxic in high levels. The metals are in effect removed from the food chain by being bound to the secretions. Degradation of dyes is also brought about by some anaerobic bacteria and fungi (Colberg, 1995).

SOIL CONTAMINANTS AND BIOREMEDIATION

The rapidly growing industrialization along with an increasing population has resulted in the accumulation of a wide variety of chemicals. Thus, the frequency and widespread use of manmade chemicals has led to a remarkable effort to implement new technologies to eliminate or atleast reduce these contaminants from the environment. Commonly used pollution treatment methods (e.g. land-filling, recycling, pyrolysis and incineration) for the remediation of contaminated sites have also had adverse effects on the environment, which lead to formation of toxic intermediates (Debarati et al. 2005). Various man-made materials have been dumped at contaminated sites. As a result, many sites contain a complex mixture of contaminants, including petroleum products, organic solvents, metals, acids, bases, and radionuclides. Over a very long period of time, natural degradation activities would eventually destroy some of these contaminants.

PESTICIDES AND THEIR REMEDIATION

Pesticide can be defined as any substance or mixture of substances intended for preventing, destroying, repelling or mitigating any pest (USEPA, 2006). Pesticides can be subdivided into categories of herbicides, insecticides, fungicides, virucides, and others according to the targeted pest.

Pesticides are toxic to many organisms and pose a threat to the safety of environment as a whole and soil on particular. Many insecticides function by impeding normal nervous system functions. Organochlorine and Organophosphous compounds stimulate the nervous system and cause tremors, irritability and convulsions (Vaccari et al, 2006). Chronic exposure to organophosphates can cause destruction of nerve fibers (neuropathy) as well as muscle tissue damage (myopathy) (Vaccari et al. 2006). Organochloride insecticides have been found to accumulate in human adipose tissue. Prolonged exposure to Organochloride insecticides also causes convulsions, a hyperexcitable state of the brain, and cardiac arrhythmiatic symptoms. Organochlorine pesticides create a distinct issue for environmental engineers and scientists due to biomagnification.

The problem of environmental contamination by pesticides goes beyond the locality where it is used. The agricultural pesticides that are exhaustively applied to the land surface travel long distances and can move downward until reaching the water table at detectable concentrations, reaching aquatic environments at significantly longer distances. Therefore, the fate of pesticides is often uncertain; they can contaminate other areas that are distant from where they were originally used. Thus, decontaminating pesticide-polluted areas is a very complex task (Gavrilescu, 2005)

Microbial Degradation of Organochloride Pesticides

The fate of pesticides in the environment is determined by both biotic and abiotic factors. The rate at which different pesticides are biodegraded varies widely. Some pesticides such as DDT and dieldrin have proven to be recalcitrant. Consequently, they remain in the environment for a long time and accumulate into food chains for decades after their application to the soil (Kannan et al. 1994). Most of the studies involving the biodegradation of organochlorine pesticides are done in pure cultures. The culture is usually isolated from a soil sample, generally contaminated with organochlorine pesticides. The strains are characterized and tested with different concentrations of the pesticide studied. DDT-metabolizing microbes have been isolated from a range of habitats, including animal feces, soil, sewage, activated sludge, and marine and freshwater sediments (Johnsen, 1976; Lal & Saxena, 1982; Rochkind-Dubinsky et al. 1987). The degradation of organochlorine pesticides by pure cultures has been proven to occur *in situ*. Nature magazine published one of the pioneer works. Matsumura et al. (1968) were able to evidence the breakdown of dieldrin in the soil by a *Pseudomonas* sp. The bacteria strain was isolated from a soil sample from the dieldrin factory yards of Shell Chemical Biodegradation of DDT residues largely involves co-metabolism, that is, it requires the presence of an alternative carbon source, in which microorganisms growing at the expense of a substrate are able to transform DDT residues without deriving any nutrient or energy for growth from the process (Bollag & Liu, 1990). Under reducing conditions, reductive dechlorination is the major mechanism for the microbial conversion of both the *o,p'*-DDT and *p,p'*-DDT isomers of DDT to DDD (Fries et al., 1969). The reaction involves the substitution of aliphatic chlorine for a hydrogen atom. Among microorganisms, bacteria comprise the major group involved in organochlorine degradation, especially soil habitants belonging to genera *Bacillus, Pseudomonas, Arthrobacter* and *Micrococcus* (Langlois et al. 1970). In order to predict some of the factors that influence the capacity of biodegradation of DDT by a *Sphingobacterium* sp. Fang et al. (2010) studied the biodegradation at different temperatures, pHs, concentrations of DDT. Results of the experience showed that the degradation rates were proportional to the concentrations of *p,p'*-DDT, *o,p'*-DDT, *p,p'*-DDD and *p,p'*-DDE ranging from 1 to 50 mg.L-1. The ability of *Sphingobacterium* sp. to degrade DDTs was somewhat inhibited by DDTs at the level as high as 50 mg.L-1. According to the authors, this may be due to the fact that DDTs at high concentration are toxic to *Sphingobacterium* sp. and inhibit degradation. The experiment was also tested for different pHs, it was tested for pH 5, 7 and 9. The results indicated that a neutral condition is favorable for the degradation of DDT by *Sphingobacterium* sp., whereas higher or lower pH inhibits degradation. The influence of the temperature on the biodegradation was investigated by performing the experiments at temperatures of 20, 30 and 40^{0}C. The results indicated that the optimum temperature for the biodegradation of DDTs by a *Sphingobacterium* sp. in pure culture was at 30^{0}C.

BIOREMEDIATION OF PETROLEUM HYDROCARBON CONTAMINANTS IN SOIL

Soil contamination with oil spills is the major global concern today. Soil contaminated with Petroleum is a serious hazard to human health and also causes organic pollution of ground water which limits its use, causes economic loss, environmental problems, and decreases the agricultural productivity of the soil. The concern stems primarily from health risks, from direct contact with the contaminated soil, vapors from the contaminants, and from secondary contamination of water supplies within and underlying the

soil. The toxicity of petroleum hydrocarbons to microorganisms, plants, animals and humans is well established. The toxic effects of hydrocarbons on terrestrial higher plants and their use as weed killers have been ascribed to the oil dissolving the lipid portion of the cytoplasmic membrane, thus allowing cell contents to escape (Currier and Peoples, 1954). The most noticeable sources of contamination are releases from manufacturing and refining installations, oil-tanker spills and accidents during transportation of the oil. Crude oils are transported long distance either on land pipeline or on water in tankers and both of which are prone to oil spill and accidents. A great part of the oil pollution problem results from the fact that the major oil-producing countries are not the major oil consumers. It follows that massive movements of petroleum have to be made from areas of high production to those of high consumption.

Crude oil is a composite mixture of thousands of different chemical compounds. As the composition of each type of oil is unique, there are different ways to deal with them through microbes and flora. Bioremediation can occur naturally or can be encourage with addition of microbes and fertilizers. The microbes present in the soil first recognize the oil and its constituent by biosurfactants and bio emulsifiers, and then they attach themselves and use the hydrocarbon present in the petroleum as a source of energy and carbon. The low solubility and adsorption of high molecular weight hydrocarbons limit their availability to microorganisms. The addition of biosurfactants enhances the solubility and removal of these contaminants, improving oil biodegradation rates. The constituents of oil differ distinctly in volatility, volubility, and susceptibility to biodegradation. Some compounds are easily degraded, some resist degradation and some are non-biodegradable. The biodegradation of different petroleum compounds occurs simultaneously but at different rates because different species of microbes preferentially attack different compounds. This leads to the successive disappearance of individual components of petroleum over time.

HEAVY METAL CONTAMINATION IN SOILS AND BIOREMEDIATION

Bioavailability of Metals in Soils

Heavy metals exist both in bioavailable and non-bioavailable forms. Their mobility depends on two factors: (i) the metallic element that precipitates as positively charged ions (cations) and (ii) the one, which makes up negatively charged component of salt. Physico-chemical properties of soils, such as cation exchange capacity (CEC), organic matter, clay minerals and hydrous metal oxides, pH and buffering capacity, redox potential and extent of aeration, water content and temperature, together with root exudates and microbial activities determines the metal availability in soils . The toxicity of metals within soils with high CEC is generally low even at high total metal concentrations. Under oxidized and aerobic conditions, metals are usually found in soluble cationic forms while in reduced or anaerobic conditions, as sulphide or carbonate precipitates. At low soil pH, the metal bioavailability increases due to its free ionic species, while at high soil pH it decreases due to insoluble metal mineral phosphate and carbonate formation. The mobility and bioavailability of certain metals in soils is usually in the order: Zn > Cu > Cd > Ni. However, the concentration of heavy metals within all components of the ecosystems varies considerably. Coexistence and persistence of metals in soils as multiple contaminants facilitate the entry and accumulation of these pollutants into food webs and ultimately into the human diets. Contamination of agricultural soils with heavy metals (both by single or combination of metals) has thus become a global threat to the sustainability of the agro-ecosystems and therefore, is receiving considerable attention from

the environmentalists. Therefore, assessment of heavy metal bioavailability helps to evaluate the impact of metals on soil microbes and in predicting the application of bioremediation technologies that could be used to clean up metals from the polluted soils.

Microbial Bioremediation of Heavy Metals in Soil

Metals play important role in the life processes of microbes. Some metals such as chromium (Cr), calcium (Ca), magnesium (Mg), manganese (Mn), copper (Cu), sodium (Na), nickel (Ni) and zinc (Zn) are essential as micronutrients for various metabolic functions and for redox functions. Other metals have no biological role e.g. cadmium (Cd), lead (Pb), mercury (Hg), aluminum (Al), gold (Au) and silver (Ag). They are non-essential and potentially toxic to soil microbes. Some of them e.g. Cd2+, Ag2+, Hg2+ tend to bind the SH groups of enzymes and inhibit their activity (Turpeinen, 2002). Soil contamination by heavy metals may repress or even kill parts of the microbial community in soil. Interaction of metals with cellular proteins/enzymes is more commonly implicated in causing toxicity than interaction with membranes. Binding affects the structure and function of proteins and enzymes.

THE PROCESS AND MECHANISM OF MICROBIAL REMEDIATION OF HEAVY METALS

Microbial remediation of toxic metals occurs in two ways:

1. Direct reduction by the activity of the bacterial enzyme 'metal reductase'. It is applied for groundwater decontamination, using bioreactors (pump & treat) and also for soils after excavation (pulping or heaping and inoculation with appropriate microbial consortium). These techniques are *ex-situ* methods, and very expensive and has low metal extraction efficiencies.
2. Indirect reduction by biologically produced hydrogen sulfide (H2S) by sulfate reducing bacteria to reduce and precipitate the metals. This is an *in-situ* method, and an environmentally sound & inexpensive alternative to pump & treat (for contaminated groundwater) or excavate & treat (for contaminated soils). Microbial growth is induced in sub-surface zones by injecting substrates. The migrating metals are intercepted and immobilized by precipitation with biologically produced H2S.

There are at least three major microbial processes that influence the bioremediation of metals and these are:

- **Biosorption and Bioaccumulation:** Biosorption is sequestration of the positively charged heavy metal ions (cations) to the negatively charged microbial cell membranes and polysachharides secreted in most of the bacteria on the outer surfaces through slime and capsule formation. From the surface the metals are transported into the cell cytoplasm through the cell membrane with the aid of transporter proteins and get bioaccumulated.
- **Biologically Catalysed Immobilization:** Inside the microbial cells, metal ions gets fixed to Iron (Fe)-Oxides and into organic colloids and becomes immobilised. This is achieved by enzymatic reduction by microbes (described below).

- **Biologically Catalysed Solubilization:** Metal reducing bacteria enzymatically reduce and also under appropriate conditions, solubilize oxide minerals. Such dissolution reaction have been shown to release cadmium (Cd), nickel (Ni) and zinc (Zn) into solution during reduction of goethite (a form of Fe-oxide) by anaerobic bacterium *Closteridium* spp.

Microorganisms do not actually biodegrade inorganic metals, but changes (bio-transform) their oxidation state. This can lead to an increase in solubility (and subsequent removal by leaching), or precipitation and reduction in bioavailability. Metallic residues (heavy metals) may be converted into 'metal-organic combinations' that have less bioavailability (to pass into human food chain) than the 'metal-mineral combinations' of the heavy metals. Microbes transform the oxidation states of several toxic metals and increase their bioavailability in the rhizosphere (root zone) thus facilitating their absorption and removal by hyper-accumulating plants by phytoremediation (Figure 5).

Many divalent metal cations like Mn2+, Fe2+ and Zn2+ are very similar in structure. Also, the structure of oxyanions, such as chromate, resembles that of sulphate. Evolution has endowed microorganisms with effective mechanisms to distinguish between similar metal ions and between toxic and non-toxic metals. Microbes have solved this problem by developing two types of uptake mechanisms and systems for metal ions:

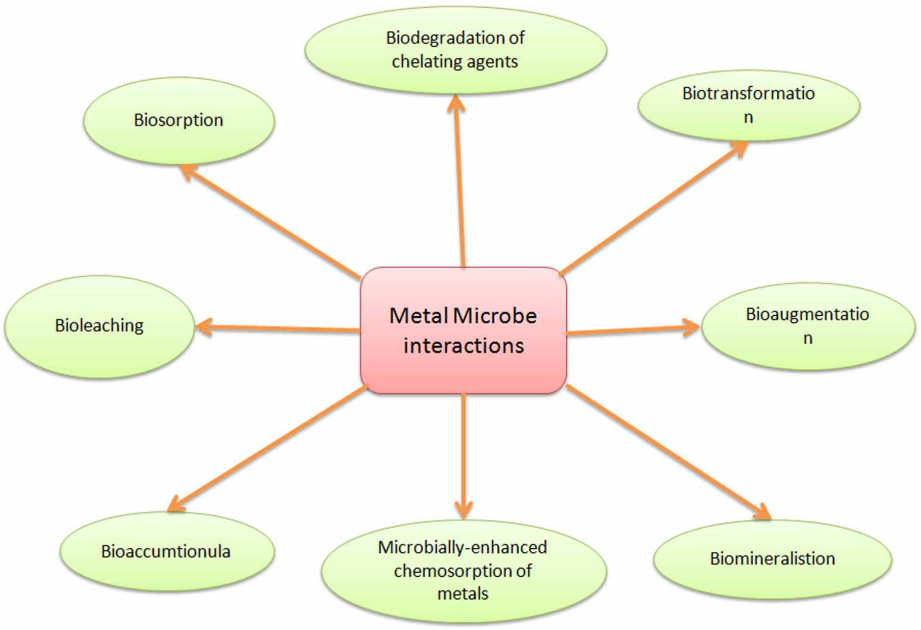
1. Selective, substrate-specific uptake system that are slow and require considerable cellular energy (ATP) and is only produced by the cell in times of need;
2. Substrate-non-specific rapid system, that transport metals using a chemiosmotic gradient across the cytoplasmic membrane of the bacteria rather than using ATP (Nies, 1999).

Even highly evolved, substrate-specific uptake mechanisms may not prevent entry of toxic metals in cells. Once inside, metal cations can interact with various cellular components including cell membranes, proteins and nucleic acids. Incompletely filled d-orbital's allow metals to form complex compounds with organic ligands, such as the proteins, nucleic acids & cell wall materials of micro-organisms. The ability of microbial cell surfaces to form complex with metals lies in their net negative charge at normal growth pH. The outer membrane of Gram negative bacteria effectively complexes metals including magnesium (Mg), nickel (Ni), strontium (Sr), manganese (Mn), lead (Pb), iron (Fe), sodium (Na) and calcium (Ca). In Gram –eve bacteria the, the net –eve charge results from the phosphates and carboxyl groups of lipopolysaccharide molecules, while the –eve charge in Gram positive bacteria results largely from teichoic acid. A more negative cell surface charge may more effectively attract and bind toxic metal cations. Toxic metals readily bind to sulfhydryl group of proteins (Nies, 1999; Sandrin & Hoffman, 2006).

BIOREMEDIATION THROUGH RHIZOSPHERE TECHNOLOGY

Soil bioremediation research has, for the most part, focused on the role of microorganisms. It has been shown, however, that plants may also play an important role in the direct and indirect removal of pollutants. Plants can physically remove pollutants from soil by absorbing or translocating them into plant tissue. There, metabolic processes may transform or mineralize pollutants. Plants can also concentrate organic or inorganic pollutants in harvestable portions of the plant; pollutants are then removed by removing the plants. Symbiotic relationships with mycorrhizal fungi may aid in these processes. Plants

Figure 5. Metal microbe interaction



can also indirectly remove pollutants by increasing the biological activity in soil through: (a) rhizosphere interactions; (b) relationships with nitrogen-fixing bacteria; (c) the contribution of dead plant material (leaf litter, etc.); and (d) the provision of suitable habitat for the many other organisms that inhabit the soil. Humification, the formation of a structureless organic polymer consisting largely of persistent organic substrates, occurs during the microbial decomposition of organic material in soil. It has been found that toxic pollutants can be rendered inert and, thus, be detoxified by their covalent binding to humus. Therefore, it appears feasible to effectively detoxify pollutants on a large scale by enhancing natural humification processes or by increasing free radical reactions through the use of oxidoreductive enzymes.

Genetic Engineering and Indigenous Micro-Organisms

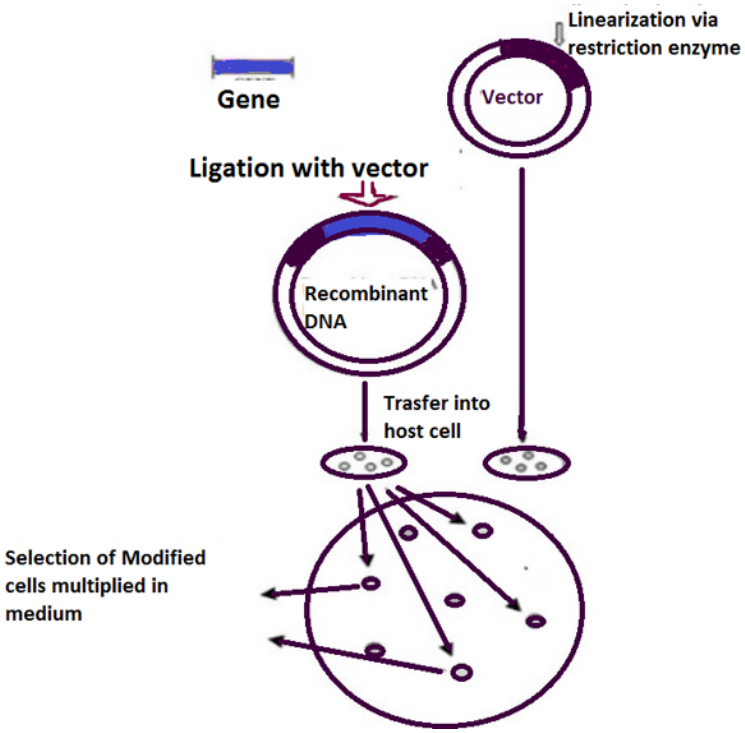
Nature plays its part in environmental cleaning by removing toxic substances to maintain a perfect balance but the recent industrialization has crossed the balanced limit of nature. Therefore, there is a increasing demand of new methods of remediating xenobiotics from the environment. Microbial remediation of xenobiotics/Contaminants has proved the effectiveness and low cost technology but there are several limitations in using microbes. Thus, genetic engineering approaches are used to construct novel microbial strains (Genetically engineered microorganisms) that possess unique features compared to the wild type and broad spectrum of catabolic potential for bioremediation of xenobiotics. Recent advances in the molecular biology field have been applied to microorganisms in order to produce novel strains with desirable properties for the bioremediation processes In 1970, the first GEMs called “superbug” was constructed to degrade oil by the transfer of plasmids which could utilize a number of toxic organic chemicals like octane, hexane, xylene, camphor and naphthalene.

Genetic engineering is a modern technology, which allows designing microorganisms capable of degrading specific contaminants. New artificial combination of genes is formed that do not exist together in nature. The most often techniques used include engineering with single genes or operons, pathway construction and alternations of the sequences of existing genes. It is completed in following steps

The first step involves selection of suitable gene/s. Second step, the DNA fragment/gene of interest to be cloned is introduced into a vector and then into host cells. The modified organisms are called recombinant cells. Third step involves production of multiple gene copies and selection of cells containing recombinant DNA. The final step includes screening for clones with desired DNA inserts and biological properties. The basic stages are shown in Figure 6.

Inspite of several pros of use of GMO in bioremediation, the instability of the inserted genetic material makes their use limited in the environment. Major reasons for this affair are, first, the GMOs efficiency depends on their ability to carry the inserted DNA in a stable manner; second, the transfer of genetic material to the indigenous organisms is perceived to be a negative attribute. These factors have incentivized the study of survival, competition and persistence of GMOs in the environment, as well as the potential risks involved in their use. Besides the significant advances that have already been made with regards to the development and utilization of GMOs for bioremediation of contaminants in the environment, many more challenges still remain.

Figure 6. Steps involved in Genetic Engineering



Latest Developments in the Field

Nanoremediation

Nanotechnology involves the design, characterization, production and application of structures/particles by controlling their size and shape at nanoscale. Nanotechnology has the potential to provide some beneficial replacements for current practices of site remediation. It is often described as an emerging technology which is truly capable of revolutionizing our approaches to common problems. An important challenge, however, in nanotechnology is to engineer particles with ideal optical and electronic properties by limiting their size and shape. Nanotechnology is also used as an environmental technology to protect the environment through pollution prevention and treatment, and cleanup of long-term problems such as hazardous waste sites. Nanotechnology is an emerging science that already shows promise in improving various life aspects ranging from medicine to industrial materials.

Nanoremediation is the use of nanoparticles for environmental remediation. Environmental nanotechnology is considered to play a key role in the shaping of current environmental engineering and science. The nanoscale has stimulated the development and use of noval and cost effective technologies for remediation, pollution monitoring pollution detection and remediation of pollutants. The nanostructured materials are used as biosensors for monitoring and detection of different compounds. The use of nanoparticals may have advantage over conventional method due the much larger surface area of nanoparticles on a mass basis. The unique structure and electronic properties of some nanoparticles are adsorbent of pollutants.

Nanoremediation is being explored to treat, soil, wastewater, sediment, or other contaminants. Nanoremediation is being explored in advanced countries predominantly in the United States for environmental cleanup. Some nanoremediation methods, particularly the use of nano zero-valent iron for groundwater cleanup, have been deployed at full-scale cleanup sites. Other methods remain in research phases. During nanoremediation, a nanoparticle agent must be brought into contact with the target contaminant under conditions that allow a detoxifying or immobilizing reaction. This process typically involves a pump-and-treat process or *in situ* application.The nanopstructure material developed for detection of pollution monitoring and remediation is highlighted in Tables 3 and 4.

Nanotechnology has great potential in performing chemical and physical processes that are useful in toxic chemical remediation. It is observed that otherwise recalcitrant species found in the environment are neutralized at unprecedented rates and efficiencies when treated with specialized nanoparticles such as nanoscale zero-valent iron (NZVI).

BIOFILMS

Recently biofilms have become a focus of interests for the researchers in the field of bioremediation of xenobiotic compounds. The term ‘biofilm’ was given and described by Costerton et al., in 1978. Biofilms are clusters of microbial cells that are attached to a number of different surfaces such as soil particles, biotic surfaces including roots, fungal hyphae and decomposing organic material. It can be defined as an aggregation of bacteria, algae, fungi and protozoa enclosed in a matrix consisting of a mixture of polymeric compounds, primarily polysaccharides generally referred as extracellular polymeric substances (EPS). In addition, proteins and DNA also contribute in EPS formation. Biofilms functionally contain

Table 3. Biogenic synthesis of gold nanoparticles by various microorganisms

S. No	Organism	Location	Range (nm)
Bacteria			
1	*Pseudomonas stutzeri*	Extracellular	200
2	*Morganella sp.*	Intracellular	20-30
3	*Klebsiella pneumonia*	Extracellular	5-32
4	*Plectonema boryanum*	Intracellular	1-10,1-100
5	*Lactobacillus strains*	Intracellular	
Fungi			
6	*Phaenerochaete chrysosoporium*	Extracellular	50-200
7	*Trichoderm asperellum*	Extracellular	13-18
8	*Aspergillus fumigates*	Extracellular	5-25
9	*Verticillium*	Intracellular	25

Table 4. Biogenic synthesis of gold nanoparticles by various microorganisms

S. No	Microorganism	Location	Range (nm)
Bacteria			
1	*Escherichia coli DH5_*	Intracellular	25-33
2	*Thermomonospora sp.*	Extracellular	8
3	*Rhodococcus sp.*	Intracellular	5-15
Fungi			
4	*Fusarium oxysporum*	Extracellular	20-40
Algae			
5	*Sargassum wightii*	Extracellular	8-12

numerous microorganisms like heterotrophs, sulfate reducers, methanogens, nitrifiers and denitrifiers which degrade different substrates, can be found at different points within the biofilm. The community structure within biofilms depends to a great extent on physicochemical microenvironment. Electro micrograph of biofilm is shown in Figure 7.

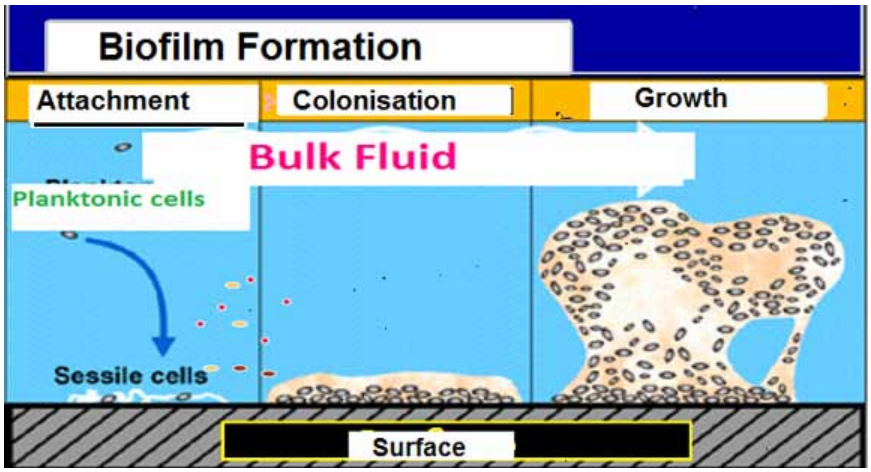
Biofilms give support to the high density of microbial biomass which facilitates the mineralization processes by maintaining optimal pH conditions, localized solute concentration and redox potential in the vicinity of the cells. This is achieved by the unique architecture of the biofilm and controlled circulation of fluids within it. The phenomenon of mass transport in biofilms is influenced by its structure which depends upon the local availability of the substrates. Solute transport in biofilm is driven by convective transport within pores and water channels and also by diffusion in the denser aggregates. Biofilms undergo dynamic changes during their transition from free living organisms to sessile biofilm cells, including the specific production of secondary metabolites and a significant increase in the resistance towards biological, chemical and physical assaults.

Figure 7. Electro micrograph of biofilm

Biofilm Formation

In most habitats, majority of microbial populations form biofilms on solid surfaces. Microorganisms in biofilms become highly differentiated from those in the planktonic state and often exhibit a developmental sequence, forming complex, multicellular structures which become surrounded by a network of water channels (Figure 8). At present, the consensus model in biofilm research proposes that microbial community development on surfaces is a stepwise process involving adhesion, growth, motility and extracellular polysaccharide production. Once an initial biofilm is established, cell to cell communication (i.e. quorum sensing) via extracellular signaling molecules regulates further modification and development of the biofilm. Recently *In vitro* model of bacterial biofilm formation on polyvinyl chloride biomaterial (PVC) has been reported. Bacterial biofilm formation on the surface of PVC material was found to be a dynamic process with maximal thickness being attained at 12–18 h. These biofilms became mature by 24 h.

Figure 8.
Source (Paul S)



BIOREMEDIATION OF POLLUTANTS USING BIOFILMS

Biofilm-mediated bioremediation presents a proficient and safer alternative to remediation with planktonic microorganisms because cells in a biofilm have a better chance of adaptation and survival as they are protected within the matrix. Owing to the close, mutually beneficial physical and physiological interactions among organisms in biofilms, the usage of xenobiotics is accelerated and consequently, biofilms are used in industrial plants to help in immobilization and degradation of pollutants. Bacterial biofilms can be the potential tools for bioremediation of PAHs. They are very common in environmental and clinical settings. Biofilm is a highly rigid structure resistant to a variety of environmental problems. Therefore, optimization of bioremediation processes as per the field conditions requires a thorough knowledge of biofilm structure, dynamics, and the microbiota interaction with pollutants and other environmental factors.

Biofilms occur frequently inside various engineered systems for wastewater treatment. These include traditional trickling filter systems, modified lagoons, and specialized supplementary systems for nutrient removal or treatment of specialized wastes. The major advantages of biofilm systems over suspension treatment is the high microbial density that can be achieved, leading to smaller treatment system footprints, and the inherent development of aerobic, anoxic and anaerobic zones which enable simultaneous biological nutrient removal. The intrinsic resistance of biofilm communities to changing environmental conditions creates the added advantage that biofilm-based treatment systems are more resilient to influent variation in toxicity and nutrient concentrations.

ELECTROREMEDIATION

Soil contamination is a critical issue as a threat to public health, food system and groundwater. Soil contamination is associated to industrial activities, mining exploitations and waste dumping. It is considered

Table 5. Biofilms in action

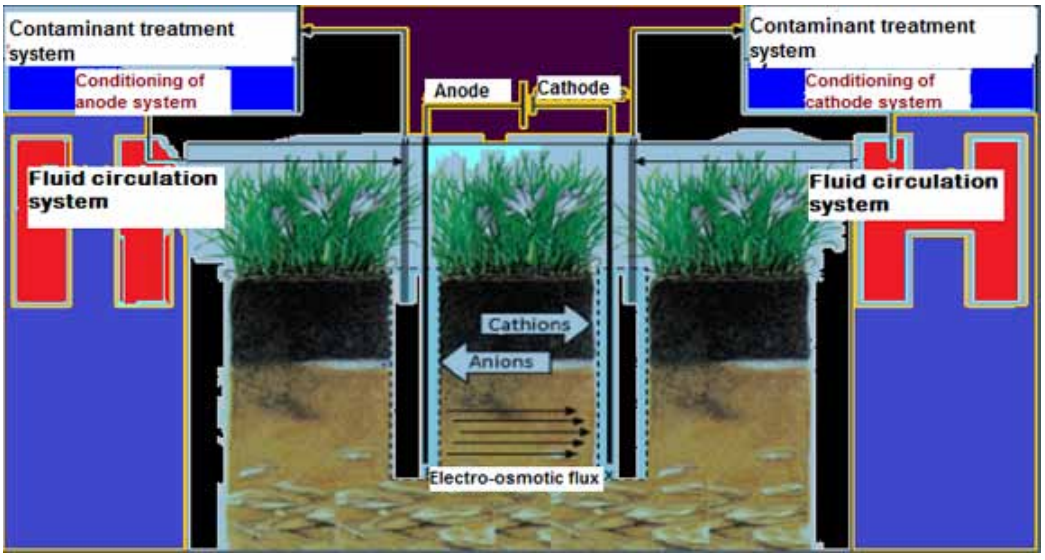
S. No	Pollutants	Biofilm Forming Cultures	Reference
1	Polychlorinated biphenyls	Anaerobes from sludge wastewater	Josephine et al
2	Hydrocarbon compunds	Bacteria from sewage water	Rafida et al
3	2,3,4,6-tetrachlorophenol and pentachlorophenol	*Pseudomonassp., Rhodococcus sp.*	Puhakka et al
4	dodecylbenzene solfonate sodium	*Stenotrophomonas maltophilia*	Hosseini et al
5	Polyethylene	*Aspergillus niger*	Mathur et al
6	Polyethylene	*Rhodococcus rubber(C208)*	Gilan et al [77]
7	Everzol Turquoise Blue G (Synthetic dye)	*Coriolus versicolor*	Kapdan and Kargi
8	Cr(VI), Fe(III)	*Escherichia coli*	Cristina et al [38]
9	Cr(VI), Cd(II)	*Escherichia coli*	Cristina et al [
10	Zn(II)	*Pseudomonas putida*	Brandy et al
11	Diesel	*Candida tropicalis*	Chandran &Das

a serious problem since it affects not only the environment, living organisms and human health, but also the economic activities associated with the use of soil, thereby making soil remediation a research hotspot in environmental science as well as one of the most challenging research fields.

Electrokinetic remediation is a green remediation technology developed in recent past, and has already been used for treatment of soils contaminated by heavy metals and organic pollutantss. Electrokinetic remediation may be defined as physical removal of contaminants through the application of low direct current. It is an environmental technique especially developed for the removal of contaminants in soil, sediments and sludge, although it can be applied to any solid porous material. The electric field induces the mobilization and transportation of contaminants through the porous matrix towards the electrodes, where they are collected, pumped out and treated. Main electrodes, anode and cathode, are inserted into the soil matrix, normally inside a chamber which is fill with water or the appropriate solution to enhance the removal of contaminants (Figure 8). Typically, a voltage drop of 1 VDC/cm is applied to the main electrodes.

Electroremediation occurs through following processes of Electromigration, Electro-osmosis and Electrophoresis. Electromigration is defined as the transportation of ions in solution in the interstitial fluid in the soil matrix towards the electrode of the opposite charge (Figure.9). Cations move toward the cathode (negative electrode), and anions move toward the anode (positive electrode). Electro-osmosis is the net flux of water or interstitial fluid induced by the electric field. Electro-osmosis is a complex transport mechanism that depends on the electric characteristics of the solid surface, the properties of the interstitial fluid and the interaction between the solid surface and the components in solution. The electro-osmotic flow transports out of the porous matrix any chemical species in solution. Soils and sediments are usually electronegative (solid particles are negatively charged), so the electro-osmotic flow moves toward the cathode. In the case of electropositive solid matrixes, the electroosmotic flow moves toward the anode. Detailed information about electro-osmosis can be found in literature.

Figure 9. Electroremediation
(Source: Claudio Cameselle)



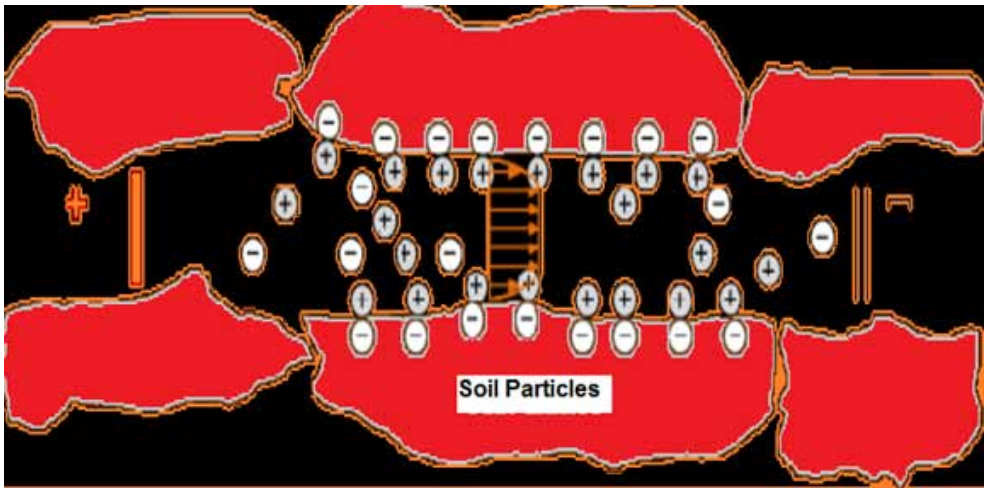
Electrophoresis is the transport of charged particles of colloidal size and bound contaminants due to the application of a low direct current or voltage gradient relative to the stationary pore fluid. Compared to ionic migration and electro-osmosis, mass transport by electrophoresis is negligible in low permeability soil systems. However, mass transport by electrophoresis may become significant in soil suspension systems and it is the mechanism for the transportation of colloids (including bacteria) and micelles (Figure 10).

FUTURE CONSIDERATIONS

The application of bioremediation technology to decontaminate polluted sites is still a developing science. The mechanisms driving microbial activity and the degradation pathways of specific pollutants need to be further elucidated before successful and better controlled site-specific treatments can occur. Recent advances in biotechnology are capable of modifying organisms at the molecular level for improved degradative performance. This approach has already contributed new tools for analysis and monitoring of complex environmental processes. Other techniques, like phytoremediation and application of immobilized cells and enzymes represent novel approaches that may help in treatment of hazardous wastes. A multidisciplinary research approach involving scientists and engineers is needed to provide new strategies for refining available bioremediation techniques. With the cooperation of soil biologists, chemists, and engineers, it should be possible to reduce pollutant concentrations at contaminated sites safely, economically and efficiently.

Environmental protection and pollution issues are frequently discussed worldwide as topics that need to be addressed sooner rather than later. Use of Nanotechnology, Electroremediation and biofilms can strive to provide and fundamentally restructure the technologies currently used in environmental detection, sensing, remediation and pollution removal. Some nanotechnology applications are near com-

Figure 10. Transport mechanism in electreremediation
(Source: Claudio Cameselle)



mercialization: nanosensors and nanoscale coatings to replace thicker, more wasteful polymercoating that prevent corrosion, nanosensors for dectection of aquatic toxins nanoparticles as novel photocatalyst for environmental cleanup.

Biofilm system is continuously drawing great attention for research. Based on the reports, it may be concluded that biofilms have the potentiality to remove heavy metals and other xenobiotic compounds from sites containing low levels of pollutants. Further research area needs to be extended on the focus of gene transfer within biofilms. Study of biofilm communities and gene transfer within biofilms would facilitate the development of better techniques for the bioremediation of polluted sites and wastewaters. The scientific knowledge accumulated in the recent past leads to conclusion that remediation of contaminated soils with organic contaminants is site specific. The results obtained in the remediation of a site cannot be assumed for other contaminated sites. This is due to the large influence of the physicochemical properties of the soil and its possible interactions with the organic contaminants. Recently, it has been considered that the combination of several remediation techniques may improve the remediation results, especially in sites with complex contamination, including recalcitrant organics compounds and inorganic contaminants. The combination of electrokinetics with bioremediation, phytoremediation, chemical oxidation or electrical heating, presents very interesting perspectives for the remediation of difficult sites. It is expected the combination of remediation technologies to improve the remediation results, saving energy and time.

Although our knowledge is not yet complete, it is time to initiate more comprehensive approaches to find common rationales in bioremediation. In some cases, for example, marine petroleum bioremediation, we have already found that similar bacterial populations occur even at geographically distant sites. Understanding the physiology and genetics of such populations may prove very useful to assess and improve bioremediation. Most importantly, we need to identify general aspects in certain types of bioremediation. For this purpose, I wish to propose the construction of a database that collects the results of molecular ecological assessments of contaminated and bioremediated sites. The database would provide bioremediation with ecological backgrounds and, in concert with currently available databases relevant to bioremediation, would facilitate the development of commonly applicable schemes for certain types of bioremediation

CONCLUSION

Bioremediation is an eco- friendly, cost-effective and natural technology targeted to remove heavy metals, radionuclides, xenobiotic compounds, organic waste, pesticides etc. from contaminated sites through biological means. Since this technology is used in in-situ conditions, it does not physically disturb the site unlike conventional methods i.e. chemical or mechanical methods. Bioremediation provides a technique for cleaning up pollution by enhancing the natural biodegradation processes. So by developing an understanding of microbial communities and their response to the natural environment and pollutants, expanding the knowledge of the genetics of the microbes to increase capabilities to degrade pollutants, conducting field trials of new bioremediation techniques which are cost effective, and dedicating sites which are set aside for long term research purpose, these opportunities offer potential for significant advances. There is no doubt that bioremediation is in the process of paving a way to greener pastures. New, emerging and innovative techniques like use of biofilms, nanopaticles or electreremediation is bound to speed up the environmental cleanup. Recently, it has been considered that the combination of

several remediation techniques may improve the remediation results, especially in sites with complex contamination, including recalcitrant organic compounds and inorganic contaminants. The combination of electrokinetics with bioremediation, phytoremediation, chemical oxidation or electrical heating, presents very interesting perspectives for the remediation of difficult sites. It is expected the combination of remediation technologies to improve the remediation results, saving energy and time. Regardless of which aspect of bioremediation that is used, this technology offers an efficient and cost effective way to treat contaminated ground water and soil. Its advantages generally outweigh the disadvantages, which is evident by the number of sites that choose to use this technology and its increasing popularity. Once again thanks to the bioremediation technology to clean up the polluted environment and therefore may be used as management tool.

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Section 2

Bioremediation through Plant and Its Interaction with Microbes

Global contamination of soil and water is a ruthless hitch. The negative effects of contaminants on the surroundings and on human health are miscellaneous and depend on the nature of the pollution. Methods for excavation and incineration to clean polluted sites resulted in the application of bioremediation techniques. In this section, we describe some general aspects of bioremediation tools and subsequently focus on the application of plant microbes interaction. These systems can be an interesting tool to further improve and develop bioremediation into a widely accepted technique.

Chapter 8
Restoration of Environment Through Phytoremediation

Arezoo Dadrasnia
University of Malaya, Malaysia

Chijioke U Emenike
University of Malaya, Malaysia

Salmah binti Ismail
University of Malaya, Malaysia

ABSTRACT

Environmental restoration is a phenomenon required to keep the ecosystem intact, or enhance the rejuvenation of impaired environmental media; soil, water and air. Various methods of remediation exist, yet restoring the environment to the proximal or original state appear elusive to most methods. Interestingly, phytoremediation which is a biological process does not only restore environment in a greener way, but also can adopt diverse mechanisms such phytoextraction, phytodegradation, rhizodegrdation, phytostabilization and phytovolatization, to achieve the desired outcome. The chapter also unlined the merits and a few demerits of this principle, while the identification of sustainable plants and the mitigation of time constraints were the future directions mentioned for the projection of phytoremediation as the ideal approach for the restoration of the environment.

INTRODUCTION

Environment is a confined and discrete space of integrated biotic and abiotic components. Complex interactions that are physically, biologically and chemically oriented keep the environment operational and intact. However, many anthropogenic activities adversely affect the natural state of the environment. Unfortunately, the wants of humans and the associated economic interests remain insatiable to the point that some direct environmental impairment is inevitable, but restoration of the affected/polluted environment is the subject of concern to many scientists. The removal of contaminants from the environment is a crucial approach towards returning any environmental medium to its natural/original state; hence

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the term "environmental restoration". While the introduction of contaminants or pollution of air, water and soil can be easy, rapid and persistent, the removal is often a daunting task. Many removal processes that are physically, chemically and biologically oriented exist, and had been discussed in terms of merits and demerits, but phytoremediation is becoming one of the most commonly practiced systems of environmental restoration. Hence, this chapter is designed to elucidate phytoremediation potentials as an option for the restoration of the environment.

PLANT-BASED REMEDIATION TECHNOLOGY

The 20th century is known as a time of increasing environmental knowledge preserve the global. Insufficient legislation and governmental action, led to the immense contamination of soil and groundwater at sites across the world. Since 1950, people observed a widespread of pollution in their environment and needs a considerable public attention. The proper disposal of hazardous waste became a priority for many private and public institutions. In the United State, billions of dollars invested in the restoration of contaminated lands. However, the lack of a suitable technology with reasonable cost has led to limit waste cleanups in polluted site and increase the pose a high risk to human health. Based on the source of pollutant, remediation technique is different. While, among different type of methods, phytotechnology inapplicable for all types. Phytoremediation is the biological form of remediation that significantly utilizes plants for such process from soil, water and air. "phyto" originally derived from green "phyton" (literally implying 'that which has grown") simply means "plants". Hence, the phenomenon is the use of plants to remove, transform, or translocate pollutants that are found in the environment, especially on the terrestrial and aquatic systems. When compared to other traditional soil remediation technologies like chemical processing that is often preceded by excavation, phytoremediation is viewed as a green alternative that is even cost-effective (Bell, Joly, Pitre, & Yergeau, 2014). Investigations have shown that phytoremediation had demonstrated significant improvement to effluents quality discharged from different mines and industries (Agamuthu & Dadrasnia, 2014; Mishra, Upadhyaya, Pandey, & Tripathi, 2008). In some cases, this remediation approach had been used to detoxify heavy metal contaminated waste water (Bennicelli, Stepniewska, Banach, Szajnocha, & Ostrowski, 2004; Bharti, & Banerjee, 2012), especially the removal of metal from coal mine effluent by separately using *Lemna minor* and *Azolla pinnata* which are macrophytes (Bharti & Banerjee, 2012). Anamika, Eapen, & Fulekar, (2008) reported the uptake of f cadmium, lead and zinc by *B. juncea* from growth solution after 21 days. Similar results were reported by Agamuthu & Dadrasnia, (2014) for uptake of Zinc by *Dracaena reflexa*. Kumar et al., (2013) suggested that those weeds growing in the natural polluted area have ability to adapted and accumulated the higher concentaration of these metals on their body. Therefore, these weeds have potential to use as phytoremediation purpose. They reported the high uptake of Pb and Cd by *Spinacia oleracea* and *Tridax procumbens*. Bramley-Alves, Wasley, King, Powell, & Robinson, (2014) conducted an experimental pot system for remediation of petroleum hydrocarbon from contaminated soil using *Poa foliosa* in clod climate. The results demonastarted the high tolerate of this plant in different levels of contamination and significantly reduction of organic compunds whitin 2 months. In addition, phytoremediation avail more benefits: biomass obtained from plants grown in a contaminated area can be used as biofuel after harvesting them. Also, there are situations where the plants will be allowed to continue growing on contaminated site, hence acting as potential species for the regrowth of the ecosystem, populating local biodiversity and enhancing atmospheric CO_2 fixation and restricting of the contaminated

soil. There are a number of air pollutatnts, for example acide rain, nitrogen oxides, ozone and sulfur dioxide; however, plants offer as an exallent tool for reduce the concentrations of these pollutants from polluted air. Also the characteristic and respond of plants has been used as an indicator of air pollution. Yang et al., (2010) reported the higher efficiency of *Chrysanthemum morifolium* on remove gaseous benzene-toluene. They suggested that phytofiltrarion is an effective way to remove organic compunds from indoor air pollution. Those clean up developed technologies to remediate of contaminants from ground water like DNT, TNT, heavy metal and organic compounds are advanced oxidation or granular carbon techniques. These ground water clean up method are expensive. In this regards phytotechnology can be as a potential for treatment. However, aquatic plants are used for treatment of wastewater to remove heavy metals, nutrients, leathate and organic compounds. *Elodea canadensis, Eichhornia crassipes, Ceratophyllum demersum* etc. has been used widely in phytotoxicology process. Aquatic plants are important in the control of water quality, metal bioavailability and nutrient cycling. Many plant enzymes are implicated during the phytodegradation and transfer contaminant compounds into less toxic forms such as lucosyltransferases, aromic dehalogenase, peroxygenases and phosphatase. Indeed, the transfer factor is an important parameter for determining the potential of phytodegradation (Souza, Dziedzic, Cubas, & Maranho, 2013; Matamoros, Nguyen, Arias, Salvadó, & Brix, 2012). In addition, remediation of radioactive contamination due to nuclear fuel testing, accidental spills and emission has been carried out using phytodegradation technology. The green plant has potential to uptake radionuclide from the environment (Fulekar, Singh, Thorat, Kaushik, & Eapen, 2010). Anamika, Eapen, & Fulekar, (2009) reported the bioaccumulation of ^{90}Sr and ^{137}Cs by *Catharanthus roseus* for a period of 15 days. He demonstrated that 70, 48 and 45% of ^{90}Sr and 73, 59 and 51% of ^{137}Cs were removed from the 3.7×10^2 $kBqL^{-1}$, 3.7×10^3 $kBqL^{-1}$ and 3.7×10^4 $kBqL^{-1}$ concentrations, respectively. Increased efficiency in environmental restoration can be achieved via phytoremediation because a synergistic interaction often exists between plants and the surrounding environment, especially microbes. Yet, the effectiveness of phytoremediation will not imply that it is devoid of demerits. Major concern linked to phytoremediation is "uncertainty": variation across plants that will survive a contaminated environment, efficiency depends on the concentration and composition of contaminants, and efficiency due to specific microbial interaction. In summary, phytotechnology can be used to remediate heavy metal, radioactive materials, and petroleum hydrocarbon. It might be because this method is very slow and takes time (some time more than 10 years), which makes it difficult to evaluate the early stage.

History of Phytoremediation

The basic idea of using plants to remove and clean of water and soil is quite old and has been employed for over 300 years. In 1948 some Italian researchers reported hyperaccumulation of Ni by *Alyssum bertolonii.* However, it was forgotten till 1977, when researchers in New Zealand observed same results. After onward, extensive studies have been conducted to determine plant abilities to remediate of contaminant and toxicities. Initially, researchers showed interest in phytoremediation studies, Chaney was the first scientist to report his results on 'hyperaccumulator plants potential. More recently, for treating radionuclide contaminated waters used aquatic plants. Now, numerous of labs in industry and academia are undertaking phytoremediation jobs on a large scale. Field studies of soil and groundwater plantations have been carried out to evaluate their effectiveness in remediation purpose. The following sections will be discus the feasibility of plants as a remedial technology for a polluted site.

Environment: Activities and Contamination

"Contaminated land" refers to land that has been contaminated by hazardous substances (such as arsenic, DDT, or oil) and may pose a risk to human health or the environment. Land contamination can occur as a result of poor environmental management and waste disposal practices, or accidental spills from industrial or commercial activities. In some cases, land was contaminated in the past by activities now known to be dangerous. Often these cases involve chemicals which have since been banned or are now subject to stricter controls. Common land uses which can cause contamination include service stations, cattle dips, tanneries, wood treatment sites, landfills, fuel storage, and refuse tips. These types of activities, identified as likely to cause land contamination, are listed as "notifiable activities".

On the other hand, in the past, the focus of water quality management was directed towards reducing pollutants in discharges of industrial process water and municipal sewage. Studies have shown that storm water runoff from industrial areas and urban typically contain significant quantities of the same general types of pollutants, including heavy metals, pesticides, herbicides and organic compounds. Efforts to improve water quality under the National Pollution Discharge Elimination System has broadened the scope of the program to include industrial and urban runoff plus runoff from landfills, mining and construction operations as well as transportation facilities such as airports.

Novel Environmental Biotechnology: Phytotechnology

Remediation of contaminated area can be achieved through chemical and physical methods such as incineration and disposal in landfill (Ayotamuno, Kogbara, & Agoro, 2009). The traditional treatment, physical and chemical methods may not remove and degrade the oil thoroughly. Hence, it is unavoidable to use a low cost method to remediate polluted soils, specifically in developing countries. Biological methods can be most effective in the removal of oil contamination from soil, where physical or chemical methods are not effective. In this way, biological processes are thought to be of low environmental risk and low cost but in some cases, longer time is required (Dadrasnia, Shahsavari, & Emenike, 2013; Jingchun, Xiaowei, Qing, & Rugang, 2009). Phytoremediation, a green technology, is quite a novel technique which uses plants to remediate contaminated sites such as soil, sediment, surface and groundwater (Kim, Choi, Sim, & Oh, 2005). Phytoremediation is relatively easy to implement and is cost-effective at minimal maintenance overheads, and as long as the impacted site can support plant growth, a remediation scheme can be used anywhere (Couto, Basto, & Vasconcelos, 2012). Phytoremediation appears effective, inexpensive and attractive and it is in contrast to other remediation methods (Wenzel, 2008). The view approach have a good image and is often, more cost effective than other techniques (Trapp & Karlson, 2001). Phytotechnology can be used to remediate heavy metals, radioactive materials, and petroleum hydrocarbon contaminations. Since this technique is slow and often takes longer time (some time more than 10 years), evaluating its potential at the early stage appear difficult. Some basic information on the potential application of phytoremediation is as follows; common and scientific name of plants, field or laboratory experiment, morphology and growth form of plant, evaluated potential of plant survival in high concentrations of pollutant, mechanism of phytoremediation, types of microorganism which are associated with the plants, age of plants at first exposure, availability of requirements for phytoremediation, contaminated storage sites of plants (i.e. root, steam, leaf or no storage), cultural information of plants and growth duration .

Phytoremediation is not applicable for all phytotoxic chemicals and where contaminants are in high concentrations or for specific chemicals (Andersen, 2006). Furthermore, phytoremediation is limited to contamination within the depth of the rhizosphere or the depth of influence from evapotranspiration, depending on the most important removal mechanisms in the specific phytoremediation application (Andersen, 2006). Major drawbacks of phytoremediation include the fact that the detoxification of organic pollutants is often slow and if decomposition is not complete, toxic compounds may accumulate in plant tissue and be released into the environment or enter food chains (Perelo, 2010).

Oil Spill Clean-Up Techniques and Role of Phytoremediation

Modern industrial society is built and ruled by petroleum hydrocarbons. Petroleum oils are essential to the current global networked economy, without it, our economic order would cease to function, bringing disaster to many populations (Dadrasnia & Salmah, 2014). However, oil spill as a result of industrial and human activities is a frequent occurrence in the world. These releases are significant since the potential hazard of a leaking underground storage tank is that the petroleum or hazardous waste can contaminate the groundwater supplies that serve as drinking water sources. The leakage of petroleum hydrocarbons from vehicles onto the road and washing of oil into the coastal environment has become a significant source of oil pollution. Diesel, kerosene and Gasoline are used as fuel for cars, ships, trucks and tractors. To appreciate the magnitude of unintended hydrocarbon release, let us look at some global statistics. In 2003, the world consumption of petroleum was over 63.5 million barrels per day (Jain et al., 2011). The Energy Information Administration (EIA) projects in the United States reported that, the world utilization of oil was 98 million barrels per day in 2006. The EIA estimate is that in 2030, the use of oil will reach to 118 million barrels/day (EIA, 2006). Sonawdekar, (2012) reported that the amount of natural crude oil spill was estimated to be 600,000 metric tons per year with a range of uncertainty of 200,000 metric tons per year. There have been many reports on oil spills worldwide. For instance, there was a crude oil spill of 0.04 mega tonnes into Prince William Sound, Alaska in 1989 (Swannell, Lee, & McDonagh, 1996). Furthermore, the Prestige oil spill occurred 209 km offshore and affected 1,900 km of shoreline in northern and northwestern Spain and western France, dumping 63,000 tonnes of fuel oil in 2002 (Fernández-Álvarez, Vila, Garrido-Fernández, Grifoll, & Lema, 2006). Some developing countries like Iran, which is the first oil-rich country in the Middle East region, started oil operations with current production capacities of over 4 million barrels/day of crude oil and 80,000 m^3/day of diesel fuel. There is up to 1.5×10^6 m^3 of soil contaminated around the Tehran refinery due to discharge of crude oil into the environment (Kebria, Khodadadi, Ganjidoust, Badkoubi, & Amoozegar, 2009). The awareness of this issue is increasing polluted sites in the world, leading to remediation a large number of sites in the near future. Also, the potential of remediation techniques will depend on the area where the spill has accrued. Recently, a diversity of biological techniques has been developed to increase the rate of degradation like phytoremediation. This technique is being evaluated for the remediation of polluted sites with oil. In this regard, phytoremediation can be an attractive option for remediation because this technique can be done *in situ*, and the cost is lower than other current methods. In addition, this technology minimizes the disturbance of the environment which might be happen during the remediation. Various plants, including grasses and legumes have been identified for their potential in this regard. They have high ability to survive and accumulate the petroleum hydrocarbons in contaminated sites.

Methods of Phytotreatment Application

In Situ Phytotreatment

In situ phytotreatment involves placement of plants in contaminated area which is in contact with polluted ground water for the purpose of restoration. In this approach, the contaminated material is not degrade prior to phytoremediation (Adadzi, 2010; Sun et al., 2011). After remediation, plants may be removed and harvested from the contaminated sites for recovery, if the mechanism of phytotreatment consist of accumulation and uptake of contaminant (Adadzi 2010; Auxiliadora & Fereres, 2003). A requirement of the *in situ* approach is that the contaminant must be physically accessible to the roots.

In Vivo Phytotreatment

For sites where the contaminants are not accessible to the plants, such as the contaminants in deep aquifers, an alternate method of applying phytoremediation is possible (Adadzi, 2010). In this approach, the contaminant is extracted using mechanical means, then it is transferred to a temporary treatment area where it can be exposed to plants selected for optimal phytoremediation (Adadzi, 2010). After treatment, the cleansed water or soil can be returned to its original location and the plants may be harvested for disposal if necessary (Adadzi, 2010). Generally, this approach is more expensive than the in-situ phytoremediation.

In Vitro Phytotreatment

This method is usually via components of live plants, like extracting enzymes. In theory, this approach could be applied in situ under some situations, e.g. applying plant extracts to a contaminated pond or wetland, or through the use of an enzyme impregnated porous barrier in a contaminated ground water plume (Adadzi, 2010). Theoretically, this approach is the most expensive method of phytoremediation because of the costs of preparing/extracting the plant enzymes; however, in some plants, such as tarragon, (*Artemisia dracunculas* var *satiya*), exudates are released under stress that could result in reduced production costs (adadzi, 2010; Susarla, Medina, & Mccutcheon, 2002).

MECHANISM OF PHYTOREMEDIATION

There are various mechanisms by which plants may remediate contaminated sites (Adadzi, 2010). Plants act as solar-driven pumping and filtering systems as they take up contaminants (mainly water soluble) through their roots and transport/translocate them through various plant tissues where they can be sequestered, volatilized or metabolized (Fulekar, 2010). Plants utilize different types of mechanisms for dealing with environmental pollutants in soil. The mechanisms of phytoremediation include biophysical and biochemical processes like adsorption, transport and translocation, as well as transformation and mineralization by plant enzymes (Pilon-Smits, 2005). Plants have been shown to be able to degrade halogenated compounds like trichloroethylene (TCE) by oxidative degradation pathways, including plant specific dehalogenases (Perelo, 2010). Dehalogenase activity was observed to be maintained after the plants were dead. Enzymes can become bound to the organic matrix of the sediment as where plants

die, they decay and are buried in the sediment, thus contributing to the dehalogenase activity observed in organic-rich sediments (Perelo, 2010). A variety of contaminant-degrading enzymes can be found in plants. These include peroxidases, dioxygenases, P450 monooxygenases, laccases, phosphatases, dehalogenases, nitrilases, and nitroreductases (Pilon-Smits, 2005). Phytoremediation is based upon the basic physiological mechanisms taking place in higher plants and associated microorganisms, such as transpiration, photosynthesis, metabolism, and mineral nutrition (Marmiroli, Marmiroli, & Maestri, 2006). Plants grow their roots in soils, sediments and water, and roots can take up organic compounds and inorganic substances; roots can stabilize and bind substances on their external surfaces when they interact with microorganisms in the rhizosphere (Marmiroli et al., 2006). Up taken substances may be transported, stored, converted, or accumulated in the different cells and tissues of the plant (Marmiroli et al., 2006). Finally, aerial parts of the plant may exchange gases with the atmosphere, allowing uptake or release of molecules (Marmiroli et al., 2006).

Phytoaccumulation/Phytoextraction

Phytoextraction involves the removal and subsequent storage of contaminants by the plant and is often applied to the exclusion and storage of metals that may undergo speciation in plants, but cannot be metabolized (Fulekar, 2010; Abhilash, Jamil, & Singh, 2009). It can also be explained to mean the ability of plants to take up contaminants into the roots and translocate them into the aboveground shoots or leaves. Once a chemical is taken up, the plant may store the chemical and/or its by-products in the plant biomass via lignification (covalent bonding of the chemical or its by-products into the lignin of the plant), sequester it into the cell vacuoles of aboveground tissues (as opposed to in root cells as part of phytosequestration, One characteristic that has been shown to correlate to uptake into a plant is log K_{ow} (octanol-water partition coefficient) (Yousaf, 2011). Specifically, organic chemicals having log K_{ow} values between 1 and 3.5 have been shown to enter into plants (Yousaf, 2011). Therefore, hydrophobic chemicals with log K_{ow} more than 3.5 are not sufficiently soluble in the transpiration stream or are bound so strongly to the surface of the roots that they could not be easily translocated into the plant xylem (Yousaf, 2011). On the other hand, chemicals with low log K_{ow} are not sorbet by roots, due to their high polarity.

Phytodegradation/Phytotransformation

Phytodegradation can be explained as a series of processes that plants utilize to metabolize the contaminants (metabolism within plant). Components of this mechanism are often utilized by plants exposed to herbicides and thus have been researched extensively (Abhilash et al., 2009). Specifically, phytodegradation, also called "phyto-transformation," refers to the uptake of contaminants with the subsequent breakdown, mineralization, or metabolization by the plant itself through various internal enzymatic reactions and metabolic processes. In the phytodegradation mechanism, plant enzymes are the main key in degrading contaminants such as metals, herbicides, and chlorinated solvents from soil, sediment and groundwater. For accruing phytodegradation, the compounds must be taken up by plants. One study identified 70 organic chemicals which could be taken up and accumulated by trees and plants (Feroz, Senthikumar, & Rao, 2012). However, phytodegradation can be limited by root depth. Generally, contaminant degradation due to enzymes produced by a plant can occur in an environment free of microorganisms (for example, an environment in which the microorganisms have been killed by high contaminant levels) (Feroz et al., 2012).

Phytostabilization

Phytostabilization is the use of plants to immobilize or make insoluble pollutants in contaminated sites by roots or within the root zone (rhizosphere). This mechanism prevents migration into ground water and reduces the mobility of contaminants. However, hydraulic control to prevent leachate migration can be achieved because of the large quantity of water transpired by plants. At a high level of concentration toxic effects may prevent plants from growing. Therefore, plants should be able to tolerate high levels of contaminants, have high production of root biomass with the ability to immobilize contaminants, and the ability to hold contaminants in the roots.

Rhizodegradation

Rhizodegradation can be described as the transformation of contaminants by resident microbes in the plant rhizosphere (i.e., the microbe-rich zone in intimate contact with the root vascular system) (Abhilash et al., 2009). The presence of plants on contaminated sites can drastically affect soil redox conditions and organic content (often through the secretion of organic acids from roots), as well as soil moisture (Abhilash et al., 2009; Fulekar, 2010). Rhizodegradation is also referred to as microbe-assisted phytoremediation or rhizoremediation (Wenzel, 2008). It is emerging as one of the most effective means by which plants can enhance the remediation of organic contaminants, particularly large recalcitrant compounds.

Phytovolatilization

Phytovolatilization is one of the main mechanisms which can accrue in the phytoremediation process. It is the uptake and transpiration of a contaminant by a plant, by the release of the contaminant or a modified form of the contaminant into the atmosphere from the plant through contaminant uptake, plant metabolism, and plant transpiration (Feroz et al., 2012). In the phytovolatilization process metabolic chemical compounds are released into the atmosphere through plant transpiration (Yousaf, 2011). Table 1, indicates a summary of the various phytoremediation processes (EPA, 2000).

Interaction Between Plants and Microorganisms

Microbe- plant interaction is particularly effective during the degradation of pollutants from soils (Gerhardt, Huang, Glick, & Greenberg, 2009). However, little is known about how these assistance are influenced by organic compounds. Since this efficiency of phytodegradation depends on the right establishment of plant microbe interactions (Nie et al., 2011). Indeed, interaction between bacteria and plant will affect plant growth either directly or indirectly. Plants, through their ‘rhizosphere’, could support the hydrocarbon-degrading microbes that assist in phytoremediation in the root zone (Nie et al., 2011). Then microbes can improve the soil nutrient availability to the plants. Petroleum hydrocarbon is identified as harmful not only for plant growth, but also to the microbe’s community. In order to better understand the interactions of petroleum hydrocarbons on microbe-plant there is a need to improve the feasibility and sustainability of phytoremediation.

Table 1. Phytoremediation overview

Mechanisms	Media	Contaminants	Plant Used	Results	References
Phytodegradation	Soil, Sediment,	Organic Compounds,	Algae, Stonewort,	Contaminant Destruction	White, Wolf, Thoma, & Reynolds, 2006
	Groundwater	Chlorinated Solvents Phenols, Herbicides,	Hybrid poplar, Bald, Cypress, Black willow		
Rhizodegradation	Soil	Crude Petroleum Oil	*Vicia faba*	47% of total petroleum	Diab, 2008
Phytoextraction	Soil	Aged PAHs	Rye grass	PAHs removal in 12 months Sweet clover was higher in the presence of plants 9% to 24% compared to 5% without plant	Parrish, Banks, & Schwab, 2004
Phytovolatilization	Groundwater soil, Sediments	Chlorinated Solvents	*Poplars*, *alfalfa,* black locust	Contaminant extraction from media and release and release to air	Singh & Lin 2009
Rhizodegradation	Soil	Petroleum hydrocarbons	*Carex exigua,*	70% loss of total petroleum	Euliss, Ho, Schwab, Rock, & Banks, 2008
			Panicum virgatum	Hydrocarbons was recorded after one year growth of these plants in contaminated soil.	

PHYTOREMEDIATION AND AIR POLLUTION

While air pollution is prevalent in many societies, its clean up or remediation is one of the most difficult process, hence most people rely on nature to take care of it. However, it is interesting to note that some plants have the potential to reduce air pollutants through diverse means. For instance, nitrogen oxide (NO_2) is a popular air pollutant and is toxic. When NO_2 reacts photochemically with hydroxyl radicals, it forms photooxidants (ozone) (Yunus, Singh, & Iqbal, 1996). This implies that NO_2 can be nitrogen source alternative considering the fact that certain plants can assimilate the nitrogen in NO_2 to generate organic compounds which includes amino acids (Wellburn, 1990; Arimura, Takahashi, Goshima, & Morikawa, 1989). Plants with such potentials are either found in nature or obtained by genetic manipulations.

The ability to assimilate NO_2 varies among plant species and families. A significant case occurred where plants from 217 taxa were fumigated using ^{15}N contents via mass spectrometry. Parameters of interest were NO_2-RN which indicates the content of reduced nitrogen as obtained from the NO_2 in the fumigated plant leaves, and the "NO_2-affinity" which indicates the NO_2-derived reduced nitrogen as a percentage of the total reduced nitrogen (Morikawa, & Erkin, 2003). The result revealed that within 217 Texas, the NO_2-RN differed by a factor of 657 between the lowest (*Tillandsia ionantha* & *T.caput-medusae*; 0.01) and highest (*Eucalyptus viminalis*; 6.57). Similarly the NO_2-affinity differed about 85 fold between *Magnolia kobus* (highest at 12.7%) and *Codiaeum variegatum* (lowest at 0.15%). Other families that recorded high NO_2-RN and NO_2-affinity were Compositae, Myrtaceae, Solanaceae and Salicaceae (Morikawa, & Erkin, 2003). Furthermore, plants suitable for cleaning up air pollution and potential sinks for air pollutants can be produced through plant gene manipulations. Considering that a major portion of NO_2 absorbed by plants is assimilated via a primary nitrate assimilation pathway, it projects plants as having potential to clean up air pollution due to NO_2 emission. Nuclear-encoded genes

for nitrate reductase (NR), nitrite reductase (NiR) and glutamine synthetase (GS) have been evaluated for such activity. Plants with such potentials had been produced, especially transgenic *Arabidopsis*, *Pit tosporum tobia* and *Raphilolepis umbellate* (Erkin, Takahashi, & Morikawa, 2003; Kondo, Takahashi, & Morikawa, 2002; Takahashi, Sasaki, Ida, & Morikawa, 2001) that bear expression cassette of complementary DNA (cDNA) of NiR gene from spinach. It was found that increase in the NiR mRNA level increases the NO_2 assimilation ability of plants. Hence, the future production of transgenic NO_2-philic plants are feasible for phytoremediation of air polluted environment.

PHYTOREMEDIATION AND GROUNDWATER POLLUTION

Pollutants or contaminants can easily find their way into sub-surface or groundwater courses. Remediation of groundwater is still possible, especially with the use of phytoremediation approach. Usually, phytovolatization and phytodegradation are options used to address groundwater contamination.

The basic considerations for groundwater contamination are the depth of the groundwater and the contaminated zone. Under the in-situ condition, phytoremediation of groundwater is significantly limited to unconfined aquifers where the depths of the water tables are accessible/within the reach of plant roots and to the zone of contamination in the peripheral portion of the water table that is also within the reach of the plant roots. Basically, it is impossible for roots of the plants to grow through clean groundwater to a deeper zone of contamination. Whenever there is need to undertake in-situ phytoremediation of deeper contaminated water, it is necessary to determine whether the water table can be lowered by the plants or via pumping, or whether there is need to induce groundwater movement towards the root and such is achieved through modelling.

However, the seasonality and uncertainty of plants' rates of water uptake can hinder modelling. Hence, critical field measurements and conservative estimates of water uptake need to be executed along with proper monitoring of changes in the water table so as to confirm the results of modelling. In some situations where the plant roots cannot reach the depth of the groundwater, then extraction wells are used to pump the water from the subsurface before applying phytoremediation treatment. In general, a significant condition for containment of groundwater is that the rate of water uptake by plants should match the rate of groundwater flow into a phytoremediation area so as to prevent migration beyond the vegetation.

PHYTOREMEDIATION AND SOIL POLLUTION

Soil can be contaminated with different substances ranging from heavy metal to organics like pesticides. Among the remediation options for the restoration of polluted soil, phytoremediation is one of the most practised. In fact top on the list of phytoremediation effectiveness of soil is restoration of heavy metals polluted soils. For the success of phytoremediation of such contaminated soil (heavy metals), it is imperative to make use of plants that accumulate metal, or the one that can extract toxic metals from the soil such as Cd, Cr, Ni, Pb and Zn (Paz-Alberto & Sigua, 2013).

Some plants do not only accumulate metals in their roots, but also translocate the accumulated metals from the roots to other parts such as shoot and leaf. Even, some of the plants can accumulate very high concentrations of the metals in their shoot, and are known as "hyperaccumulators" (Huang & Cunninghan, 1996). Therefore, at the end of remediation, the metal-rich plant can be harvested and transported

out of the site without tedious/rigorous excavation, exorbitant disposal cost and loss of topsoil that is often associated with traditional remediation practices. Phytoremediation of contaminated soil is often influenced by climatic conditions and bioavailability of pollutant, especially in the case of certain heavy metals. The success of phytoremediation is enhanced when metal contaminants in both the soil and plant are mobilized. Therefore, two major amendment techniques that assist in the bioavailability of metals, especially Lead (Pb) are lowering the soil pH and adding synthetic chelates. A case of Pb removal from soil was done in the Philippines using *Vetiveria zizanioides L.* (vetiver grass), *Imperata clylindrica L.* (cogon grass) and *Paspalum conjugatum L.* (carabao grass) that were grown in soils containing different concentrations of Pb. The *V.zizanioides* recorded highest dry matter, and the highest survival. The other two; *I.cylindrica* and *P. conjugatum* showed similar trend in that order after *V.zizanioides*, hence the highest degree of Pb absorption was in the order of *V.zizanioides*, *I.cylindrica* and *P.conjugatum* just as the levels of Pb in the shoots and roots of the grasses did not vary significantly with the amount of Pb added to the soil. Therefore, it implies that increase in biomass of plant increases its Pb uptake potential. Also, extensive root system of plant enhances more contact with soil nutrients, and as such increase the potential to absorb Pb (Paz-Alberto & Sigua, 2013). Some other plant species have been found to accumulate heavy metals, even in the mining sites, hence they are seen as phytoremediation species especially; *Amaranthus spinosus L.*, *Eleusine indica L.*, *Alternathera sessili L.*, *Portuluca oleracea L.*, *Fimbristylis meliacea L.*, *Polygonum barbatum L.*, *Achyranthes aspera L.*, *Blumea sp.*, *Desmodium sp.* and *Muntingia calabura L.* (Wislocka, Krawozyk, Klink, & Morrison, 2006).

PHYTOREMEDIATION AND OIL SPILL POLLUTION

Oil spill is common in some environments considering the exploration activities typical of such places. Basically, crude oil is a mixture of compounds that vary in solubility, volatility and susceptibility to microbial degradation. As much as one can attest that aromatic hydrocarbons in crude oil can be degraded, yet the degree or rate at which such degradation can occur reduces with increase in condensed rings in its structure (Atlas, 1981). Due to the presence of an increased number of double covalent bonds and branching that characterize the asphaltic fraction of hydrocarbons, it is easier to degrade the aliphatic fraction while the aesthetic fraction remains resistant to microbial impact. Hence, in as much as anaerobic degradation of crude oil occurs, yet its slow and incomplete process is a concern.

Most oil spills occur on marsh wetland environments. Such environments have anoxic or hypoxic soils that have insufficient oxygen (Mitsch & Gosselink, 1993). Interestingly, plants in wetlands have the potential to enhance the remediation process of such environment via oxygen diffusion from the shoots to the root and even to the soil, where microbes in the soil utilize it for respiration (aerobic) (Schussler & Longstreth, 1996). Some fresh-marsh plant species had been investigated for restoration of oil spill polluted environment, especially *S.lancifolia*, *P.hemitomon*, *A.philoxeroides* and *P.australis*. The photosynthetic rates of *P.australis* and *A.philoxeroides* were depressed due to oiling. Similarly, the above- and belowground biomass of the plant speices were affected except for *S.lancifolia*. However, *S.lancifolia* and *P.hemitomon* were found to be more suitable for the revegetation of oiled fresh-marsh wetlands whenever the original vegetation fails to recover (Dowty et al., 2001).

Pro's AND Con's of Phytoremediation

Compared to other traditional methods phytoremediation as a new technology has many pros and cons. However the detailed advantages and disadvantages are presented in Table 2.

Coupling of phytoremediation of contaminated soil with soil amendments such as organic matter, compost, phosphate, fertilizers, Fe, Mn oxyhydroxides and clay minerals usually reduce the mobility of contaminants in soil.

Influence of Environmental Factors

A number of environmental factors affect the phytodegradation process. Water content in soil will affects plant and microbial growth and the availability of O_2 required for aerobic condition (Frick, Farrell, & Germida, 1999). Some other parameters such as, type of soil, age and type of plants, nutrients, toxicity of contaminates, water and oxygen availability, chemical properties of soil (pH, CEC), depth of contamination which is important in terms of where contaminants can be treated in the rhizosphere or by plant uptake are important considerations (Kamath, Rentz, Schnoor, & Alvarez, 2004). The inorganic mineral nutrients that are most often reported to limit the breakdown of petroleum hydrocarbons in soil are nitrogen and phosphorus (Gaskin & Bentham, 2010). In some cases, petroleum hydrocarbons are not readily desorbed, and are therefore not available for phytoremediation (Gaskin & Bentham, 2010).

Plant Selection Criteria

Plant selection is one of the important factors determining the success of the phytoremediation project (Team, 2001). After evaluating the conditions for plant growth at sites, the next stage is to choose the plant which can survive under the site conditions. A basic knowledge about the literature of plants can help to design a phytodegradation project. Some typical information which is needed about plants is the specific and common names, growth habit, and tolerance of plants in various conditions such as temperature, diseases and moisture. Native plants and crops can be evaluated as options to choose from the phytodegradation process due to their being suitable for the climatic conditions of the region (Reynoso-Cuevas, Gallegos-Martínez, Cruz-Sosa, & Gutiérrez-Rojas, 2008). Several types of plants have been

Table 2. Advantages and disadvantages of phytoremediation technology

S. No.	Advantages	Disadvantages
1.	Environmentally Friendly	Climate Dependent
2.	Many Mechanisms For Removal	Effectiveness Depends On Nature Of Chemicals
3.	Relatively Low Cost	Results Are Variable
4.	Easily Maintained And Implement	Limited To Site With Lower Contaminant Concentration
5.	High Public Acceptance	Effective Depth Limited By Plant Roots
6.	Reduced Dust Emission	Slower Than Mechanical Treatments
7.	Potential To Reduce Gas Emission	Phytotoxicity Limitations & Longer Time To Remediate

identified for their potential for use in the phytoremediation process. A comprehensive list of plants that has proved positive in phytoremediation of organic compounds is listed in Table 3. The most common plants are leguminous and grasses that have shown their potential in phytoremediation (Edwin-Wosu & Albert, 2010). Grasses are a suitable option to apply in phytoremediation due to the high root surface area (per m^3 of soil) which may penetrate into the soil (depth of up to 3 meter). There is a limitation of supplies available of nitrogen in oil polluted sites with leguminous plants due to the ability to fix N compared with other plants (Frick et al., 1999). Some characteristics of plants which make their suitable for remediation of hydrocarbon compounds are as follow:

- Plants high in phytotoxicity.
- Plants able to adapt to different climatic conditions and are able to be destroyed after remediation.
- Plants with the ability to transfer a high rate of oxygen to steam, root and leaf.
- Plants able to accumulate and absorb toxic substance (Muratova, Turkovskaya, Hübner, & Kuschk, 2003).

Costs of Phytoremediation

An estimate indicates that the general cost of phytoremediation for one hectare with a depth of 15 cm is about 2,500 to 15,000 USD which is based on the cost of 17 to 100 USD for each cubic meter. A recent estimate put the cost at approximately 300 USD per m^3 per year to phytoremediated a site contaminated with oil and organic compounds using deep-rooted plants and trees (Frick et al., 1999). There are various ways to reduce the cost of phytoremediation, for example, during the in-situ phytoremediation process, plants using solar energy as a source of energy helps to reduce the cost of phytoremediation. Maintaining a site for 10 years will help to spread the cost over a longer period.

Table 3. Plants used for phytoremediation of petroleum hydrocarbon

Plant Species	References
Carrot (*Daucus carota*)	(Wild & Jones, 1992)
Side oats grama (*Bouteloua curtipendula*)	(Aprill, & Sims, 1990)
Soybean (*Glycine max*)	(Dominguez-Rosado, & Pichtel, 2005)
Perennial ryegrass (*Lolium perenne L.)*	(Merini, Bobillo, Cuadrado, Corach, & Giulietti, 2009)
Rice (*oryza sativa L. Cv*.)	(Nwaogu, Agha, & Ihejirika, 2012)
Thuja orientalis	(Harekrushna, & Kumar, 2012)
Dracaena reflexa	(Dadrasnia, & Agamuthu, 2013)
Red clover (*Trifolium pretense L.*)	(Nwaogu et al., 2012)
Lettuce (*Latuca sativa*)	(Banks et al., 2003)
Tall fescue (*Festuca arundinacea*)	(Sharifi, Sadeghi, & Akbarpour, 2007)
Annual ryegrass (*Lolium multiflorum*)	(Sung, Munster, Rhykerd, Drew, & Corapcioglu, 2003)

SOLUTIONS AND RECOMMENDATIONS

Climate Dependence

Considering the fact that climate dependence is one of the limiting factors to effective phytoremediation, it then becomes necessary to understudy plant biodiversity across different geographical settings of the globe as relates to the importance for as an agent of remediation. When data inventory of the characteristics of different plant species is available, it will aid in identifying potential phytoremediator that may suit its environment on the grounds of climate tolerance.

Dependence of Effectiveness on the Nature of Chemicals

The nature of chemicals or pollutants of interest significantly influences the degree of phytoremediation effectiveness. Hence, a veritable way of bringing this bottleneck to control is to ensure detailed pre-characterization of polluted sites in comparison to the nature of pollutant/chemical when existing in its natural/homogeneous state. This will avail structural planning and effective choice of suitable phytoremediation plant.

Variations in Results

Variability of results from previously conducted environmental restoration via phytoremediation has caused many concerns amongst researchers. This has made replication of desired result difficult. Therefore, it is imperative to make an adequate comparison with past environmental issues before conducting phytoremediation in a given contaminated area. Such approach will minimize experimental bias and generate reliable results for future reference.

Limitation to Sites with Lower Contaminant Concentrations

Phytoremediation is best viewed to be efficient in lower contaminant affected zones because of the susceptibility of plants to the toxicity of pollutants. Therefore, for effective utilization of plants in environmental restoration the evaluation of the level of pollutant in the medium needs to be established. This will then be followed by drawing up plant selection criteria. Similarly, the introduction of more plants for every given area might enhance rapid biotransformation as plants interact with the pollutants.

FUTURE RESEARCH DIRECTIONS

Phytoremediation has carved a niche in the quest for using a biological approach to achieve restoration of contaminated and polluted environments. However, certain gaps and uncertainties within this remediation process have brought to the fore the need for further enquiries and detailed research works that is not only efficient but also sustainable. Therefore, research/studies on phytoremediation can be taken further in the following directions;

- Identifying more suitable plants that are inedible to humans and the majority of other macro-organisms like animals and birds, for phytoremediation of polluted sites. This will enhance sustainability because some remediation plants are either food for humans or other macro-organisms, hence the use can cause food shortage, or even distortion of food -chain and -web.
- Mitigating time constraints. The time factor has often been a deterrent to the adoption of biological approach for environmental remediations, and even pollution technologies such as wastewater treatment, and detoxifications of sewage sludge. Unlike most physico-chemical approaches, phytoremediation is time consuming. Therefore, future research should inculcate possible genetic manipulations that will ensure quick plant maturation while at the same time ensuring that such modifications/manipulations do not reduce the plant's bio –transformation, -translocation, and –conversion potentials.
- Mechanisms of phyto-removing needs further studying and based on that, prediction models can be improved. Meanwhile, depends on the species of plants the optimum concentration of pollutants are different; however, it should not be ignored for the technology promotion.

CONCLUSION

As earlier mentioned in this chapter, anthropogenic activities are the causes of environmental deterioration, and as such restoration becomes imperative if the colossal failure of the ecosystem is to be avoided. Discussing phytoremediation as an ideal instrument of environmental restoration was not simply meant to project the approach as the top-notch option; rather it was to buttress its viability and sustainability potential when considering its greener nature as compared to some other biological means.

While advocating for phytotechnology, one is expected to bring to the fore the outstanding benefits; green technology, relatively easy to implement anywhere as long as plant growth is possible. However, it is necessary to put into consideration a number of basic requirements when adopting a phytoremediation approach for the restoration/reclamation of an impaired environment; such as plant morphology and soil suitability, mechanism of phytoremediation, and age of the plant. Various methods explained, namely in-situ, in-vivo, and in-vitro applications are peculiar to the interests of concerned environmentalists engaged in the restoration exercise. Again, of significant importance is the interaction that exists between plants and its immediate environmental components, especially microbes. Studies have demonstrated that a robust plant-microbe interaction is pivotal to the efficiency of phytoremediation. This is highly attributed to the ability of microbes to enhance soil nutrient availability to the remediation plans. Finally, phytoremediation is environmentally friendly, hence the reason for the wide range of public acceptance. The relative low cost gives it an edge over other forms of remediation, especially when considering is accorded the fact that most anthropogenic activities are also cost-driven. Though complaints on its slower nature is a big issue, yet it possesses several mechanisms for pollutant removal; phytodegradation, phytoaccumulation, rhizodegradation, phytoextraction, phytostabilization and phytovolatilization. However, more scientific enquiry into options for time manipulation and generation of conventional plants that are sustainable will be the step in the right direction for the restoration of the environment through phytoremediation.

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KEY TERMS AND DEFINITIONS

Contaminant/Pollutant: Any substance that is either produced naturally or by humans, which is found in a place where it should not be, or existing at concentration above the allowable limit in a given area; such as in water, air or soil.

Environment: It is the sum total of every living thing and natural forces that make up the surroundings and influences the ability to live on earth.

Heavy Metals: These are metals that are relatively high in density or atomic weight. They are often metalloids of environmental conern, and have properties of metallic substances at room temperature.

Phytoremediation: A treatment process that tackles environmental problems via the use of plants without the need to excavate the contaminant material.

Phytotechnology: A protocol, procedure or approach that is based on the use of plants. It can be easily viewed as a branch of biotechnology that significantly utilizes plants in its processes.

Phytotoxicity: It is the degree of the toxic effect of compound/chemical/pollutants/contaminant on plant growth. This implies a condition where a given substance in the environment is harmful or lethal to plants.

Soil Remediation: A process of purifying and revitalizing contaminated or polluted soil. It may imply a complex process with the ultimate goal of bringing soil back to its original state, usable condition, and/or less risk state.

Chapter 9
Vegetation Filters: The Potential of Short Rotation Woody Crops for the Treatment of Municipal Wastewater

Mohini Singh
Central University of Haryana, India

R.K. Srivastava
G.B. Pant University of Agriculture and Technology, India

ABSTRACT

Vegetation filter is an emerging wastewater treatment option in which phytoremediation strategies are employed for municipal applications. Short rotation woody crops combine both treatment and reuse of effluent and operate on 'zero discharge' concept. This multifunctional system has become a viable alternative solution for wastewater treatment as well as biomass production by utilizing nutrient rich wastewater as cost efficient fertilizer. Fast growing species like Salix, Eucalyptus, and Populus with high water and nutrient requirements, highly selective heavy metal uptake and high evapotranspiration rate are generally preferred as vegetation filters for wastewater treatment. However, site-specific factors such as wastewater composition, climate, soil type, permeability, species or clonal characteristics must be taken into account when considering irrigation with municipal wastewater. This chapter discussses the prospects for vegetation filters to remediate contaminated water and soil and also facilitate recycling of valuable resources in society.

INTRODUCTION

Water quality management is a vital task for all countries. Wastewater can seriously deteriorate the quality of their receiving water bodies. As the freshwater sources are either becoming scarcer or polluted through human intervention, the wastewater reuse is becoming an important issue in the world with increasing demand for water for human consumption, agriculture and industrial purposes. The volume of wastewater generated will be increased in the future with increase in consumption and the treat-

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ment capacity will also be increased to keep pace with it. Table 1 shows the inconsistent water supply, wastewater generation and treatment scenario in India (Central Pollution Control Board [CPCB], 2009). Around 38,250 MLD of wastewater is generated by class I and class II cities in India (but, the installed sewage treatment capacity is merely 11786 MLD) which is estimated to grow 3.5 times to about 132 billion litres per day of wastewaters (with a potential to meet 4.5% of the total irrigation water demand) by year 2050 (Bhardwaj, 2005). Thus, it indicates that in coming years, there will be a twin edged problem to deal with reduced fresh water availability and increased wastewater generation due to increased population and industrialization.

Utilization of this wastewater as a resource is a high priority for the water managers of the country. Although various technological options are available for wastewater treatment, but most of the available technologies are often found to be unsuitable for applications in developing countries. Over the last few years there has been renewed concern over the application of natural treatment processes. Vegetation Filters (VF) is one such natural treatment system that combines both treatment and reuse of the effluent and operates according to the 'zero discharge' concept. This has several advantages such as low construction and operation costs, easy operation, less detention time, and negligible energy requirements. And, the wastewater gets purified through processes such as filtration, adsorption, chemical processes and biodegradation by combined action of environmental components such as soil matrix, microorganisms and plant uptake through roots.

This chapter presents one concept of multifunctional VF systems-the use of short rotation woody crops as vegetation filters for the treatment of nutrient-rich municipal wastewater. The land application of nutrient-rich municipal wastewater can lead to substantial yield increases and at the same time reduces the surface and groundwater pollution at relatively low costs. Thus, the concept is an attractive option for both farmers (lessen biomass production costs) and effluent treatment plant operators (lessen water treatment costs). The environmental benefits like carbon sequestration, soil erosion control, ground water recharge further inflates the benefits of vegetation filters. In this chapter, the prospects for vegetation filters are discussed from a multidisciplinary perspective based on current knowledge, including aspects such as treatment efficiency, vegetation function and biomass yield response.

Table 1. Trends of water supply, wastewater generation and treatment facility available in Class-I and Class-II cities of India (Source: CPCB, 2009)

Year	Water Supply (MLD)		Sewage Generation (MLD)		Treatment (MLD)	
	Class-I	Class-II	Class-I	Class-II	Class-I	Class-II
1978-79	8638	1533	7007	1226	2756	67
1989-90	15191	1622	12145	1280	2485	27
1994-95	20607	1936	16662	1650	4037	62
2003-04	29782	3035	23826	2428	6955	89
2008-09	44769	3324	35558	2696	11553	233

BACKGROUND OF TREATMENT SYSTEM

Natural treatment systems are one of the appropriate alternatives for the biological treatment of wastewater. The system involves the natural environmental components like vegetation, soil, microorganisms etc for treatment of wastewater. The general characteristics of natural systems include:

- Natural systems are aimed at recycling of nutrients, water and energy.
- Natural systems use aerobic and/or anaerobic microbiological processes to remove COD without the need for energy input.
- The oxygen for aerobic microbiological processes in natural systems is supplied by photosynthesis (algae, plants) or natural reaeration.

Among various natural treatment methods, land-based systems are considered to be one of the best wastewater treatment processes as they are capable of achieving comparable nutrient removal levels for a considerably low cost, provided land is available at reasonable prices. It is gaining momentum owing to the fact that it serves two objectives: (a) waste disposal; (b) recycling of waste components. The land treatment is one alternative where wastewater is disposed onto land that is designed, constructed and operated to treat wastewater through the use of crops, irrigation methods, ground and surface water monitoring to specific water quality limits. It involves the controlled application of wastewater to the land at rates compatible with the natural physical, chemical and biological processes that occur in the soil. These systems can often be the most cost-effective option in terms of both construction and operation and are therefore, frequently being used in small communities and rural areas. Based on wastewater discharge point and rate of wastewater application, they can be categorized into slow rate (SR) and rapid infiltration (RI), overland flow (OF) systems. The SR and RI land treatment systems depend on the infiltration and percolation capacity of the soil matrix for movement of applied wastewater. In slow rate systems, wastewater is applied to the vegetation either by sprinkling or by surface techniques. The manurial ingredients present in wastewater are utilized as nutrients for vegetative growth, and the treatment takes place physically, chemically and biologically as the wastewater percolates through the soil matrix. However, in RI system the wastewater is applied to the land at higher rates by spreading in basins. The system is most suitable for highly permeable soils with good natural drainage and it is devoid of any form of vegetation. OF land treatment system is essentially a biological treatment process which utilizes sheet flow of the applied wastewater on the surface of gentle slope.

Characteristics of Municipal Wastewater

Municipal wastewater is mainly comprised of water (99.9%) together with relatively small concentrations of suspended and dissolved organic and inorganic solids. Among the organic substances present in sewage are carbohydrates, lignin, fats, soaps, synthetic detergents, proteins and their decomposition products, as well as various natural and synthetic organic chemicals from the process industries. Depending on the concentration of various nutrients, organics and heavy metals present in it, municipal wastewater is categorized into low, medium and high strength wastewater (Table 2 & 3).

Municipal wastewater also contains a variety of inorganic constituents from domestic and industrial sources, including a number of potentially toxic elements such as mercury, arsenic, lead, zinc, cadmium, chromium, copper etc. Even if toxic materials are not present in concentrations likely to affect humans,

Table 2. Typical chemical composition of municipal wastewater (Source: Henze,Harremoës, la Cour Jansen., & Arvin, 2002)

Parameter	Units	High	Medium	Low
COD total	mg l^{-1}	1,200	750	500
COD soluble	mg l^{-1}	480	300	200
COD suspended	mg l^{-1}	720	450	300
BOD	mg l^{-1}	560	350	230
VFA (as acetate)	mg l^{-1}	80	30	10
N total	mg l^{-1}	100	60	30
Ammonia-N	mg l^{-1}	75	45	20
P total	mg l^{-1}	25	15	6
Ortho-P	mg l^{-1}	15	10	4
TSS	mg l^{-1}	600	400	250
VSS	mg l^{-1}	480	320	200

Table 3. Typical content of metals in municipal wastewater in mg/m^3 (Source: Henze et al., 2002)

Metal	High	Medium	Low
Aluminium	1,000	600	350
Cadmium	4	2	1
Chromium	40	25	10
Copper	100	70	30
Lead	80	60	25
Mercury	3	2	1
Nickel	40	25	10
Silver	10	7	3
Zinc	300	200	100

they might well be at phytotoxic levels, which would limit their use on plants. Pathogenic viruses, bacteria, protozoa and helminths may be present in raw municipal wastewater. Pathogenic bacteria will be present in wastewater at much lower levels than the coliform group of bacteria, which are much easier to identify and enumerate (as total coliforms/100ml). *Escherichia coli* are the most widely adopted indicator of faecal pollution and they can also be isolated and identified fairly simply, with their numbers usually being given in the form of faecal coliforms (FC)/100 ml of wastewater.

PHYTOREMEDIATION WITH SRWC

Vegetation filter is a plant-based natural treatment system which principally involves phytoremediation strategies for treatment of wastewater through fast-growing woody trees (*Salix*, *Populus*). The vegetation filter system can be regarded as biological reactor that trap and treat wastewater through various ecological processes, most important of which are:

- Stabilisation and retention of suspended matter and other nutrients in the effluent by the soil particles via constant exchanges with the soil solution.
- Decomposition of organic matter by the soil fauna (macro- and micro-organisms, bacteria, fungi).
- Absorption, by the willow roots, of nutrients (supplied in directly assimilable form by the effluent or produced by organic matter decomposition) and water supplied by the effluent.

Looking at the importance of woody trees, Thawale, Juwarkar, & Singh (2006) suggested 5-R concept of Vegetation Filters:

- Renovation of wastewater: the purification of wastewater when it moves through the vegetation and soil filters
- Reuse of wastewater: reuse of nutrient energy of wastewater into biomass
- Return of nutrients to land: the nutrients if not used by plants can replenish the minerals excavated from the earth resource
- Recharge of ground water table: return of the renovated wastewater to ground water table for future use
- Restoration of environment quality: eco-restoration of degraded land to preserve the environmental quality.

The use of SWRC as vegetation filters in phytoremediation could represent a simple, environmental-friendly alternative as well as a potentially cost-efficient alternative to conventional methods as suggested by Białowiec, Wojnowska, & Agopsowicz, 2007; Perttu & Kowalik, 1997; Aronsson, Heinsoo, Perttu, & Hasselgren, 2002 ; Licht & Isebrands, 2005. The basic strategy is to utilize high water and nutrient uptake capacity of fast-growing, intensively planted trees and and the filtration potential and microbial activity of the soil, to remove potential pollutants from wastewater (Mitch & Gosselink, 2000). It deals with two pressing concerns:

- The Pollution of watercourses by nitrogen and phosphorus (nutrients) from agricultural fertilisers and effluent from sewage treatment works.
- Legally binding commitment between countries to reduce its greenhouse gas emissions by a given amount by year 2012 under the Kyoto Protocol.

It also provides additional benefit by reducing the fertilizer input cost to commercial plantation for enhanced biomass production through the fertigation with wastewater (Pandey & Srivastava, 2012). Moreover, being non-edible in nature, the risk of contamination of food chain is further reduced which give it more social acceptability. In addition, short rotation woody crops fix carbon dioxide from the atmosphere and store carbon both above and belowground as biomass. The harvested portions of the trees, on the other hand displace other products that are made from non-renewable fossil fuels. One of the ways to decrease greenhouse emissions in the future is to plant fast growing woody crops on unproductive land thereby sequestering carbon and displacing fossil fuels by harvesting woody biomass for bioenergy, or by storing carbon in long-lived woody products. The primary aim of the technique is:

- To save natural water bodies from discharge of untreated wastewater by reusing them for SRP irrigation and fertigation
- To enhance efficiency in SRP biomass production up to 3 times by reusing domestic wastewater for fertigation
- To use the available nutrients contained in domestic wastewater can substitute the application of chemical fertilisers in SRPs and also to reduce the demand of fresh water for irrigation.
- To contribute to soil improvement on marginal land by enrichment of organic matters by prolongs fertigation with wastewater.

For many years now, pollutant removal efficiency of vegetation filter has been demonstrated in several countries by field lysimeter and full-scale experiments (Table 4). High Rate Transpiration System (HRTS) envisaged difference in the pollutant load removal in experiments done with and without vegetation. The system planted with *Dendrocalamus strictus* and *Casurina equisitifolia* showed 60.7-76.2% of total nitrogen, 17.7-70.3% of total phosphorus and 80-94.3% BOD removal whereas the one without vegetation removed 25-32% total nitrogen, 20-28% phosphorus and 68-79% BOD have been reported (Thawale et al., 2006). Some studies report tree plantations as a tertiary treatment to remove N from reclaimed water (Licht & Isebrands, 2005; Aronsson & Perttu, 2001).

Hasselgren (1998) reported that the wastewater irrigation greatly increased the removal rate of N and P in the willow-soil system was higher than conventional nitrification/denitrification and phosphate chemical precipitation treatment processes. Rockwood, Carter, Ma, Tu, & Alker, (2001) reported that *Eucalyptus grandis* irrigated with reclaimed water remediated by short-rotation woody crops (SRWCs) can yield about 13 dry Mg ha^{-1} $year^{-1}$, and extract over 300 kg of nitrate N ha^{-1} $year^{-1}$. Langholtz, Carter, Rockwood, Alavalapati, & Green, (2005) have recently studied an optimization of coppicing species (*Eucalyptus grandis*) used for phytoremediation purposes. In Florida, several species have shown potential for nutrient, metal and hydrocarbon remediation (Rockwood et al., 2001, 2004).

The proportion of nutrient recovery greatly depends on the specific plant ability for nutrient uptake, accumulation in biomass, nutrient load in wastewater, climatic conditions and harvesting intervals, etc (Woodard et al., 2002; Adeli, Varco, & Rowe, 2003). Tzanakakisa, Paranychianakisb, & Angelakis, (2009) evaluated the nutrient trapping capacity of different species and found out that efficiency of *Eucalyptus camaldulensis* was much more than *Poplar nigra.* Recovery of up to 650 and 100 kg ha^{-1} of N and P respectively, has been reported for annual and woody species and thus enhance the pollutant removal rate in land treatment systems (Geber, 2000; Woodard et al., 2002). The removal of pollutant load also varies with the length of the vegetation filter. wastewater after a passage of few meters in cropped and planted soil brings reduction in nitrates and bod which are major pollutants in domestic wastewater (Bouwer, 1985). Doyle, Stanton, & Wolf, (1977) have reported an increase in soluble phosphorus and

Table 4. Percent removals for some pollutants in studies on the effectiveness of land treatment system for wastewater treatment

Reference	% Removal								
	TKN	NH_4^+	TP	SP	FC	TC	SS	BOD	COD
1	94	-	85	-	99.9	99.9	-	-	-
2	42.6	-	-	-	-	-	-	94.1	-
3	41.7	-	41.3	-	-	-	70.3	65.7	58.7
4	-	-	-	-	-	-	98.8	99.7	99.1
5	21	-	17	-	-	-	-	17	16
6	90	94	-	-	99.9	99.1	-	-	89
7	73.2	-	43.6	-	-	-	-	92.1	-
8	85-95	-	95-96	-	-	-	-	91-98	-

1: Tzanakakis et al., 2003; 2: Perttu & Kowalik, 1997; 3: Taebi & Droste, 2008; 4: Pazoki, Abdoli, Karbassi, Mehrdadi, & Yaghmaeian, 2014; 5: Pandey & Srivastava, 2012; 6: Tzanakakis, Paranychianakis, & Angelakis, 2007; 7: Thawale et al., 2006; 8: Hasselgren, 1998.

nitrate removal efficiency with the increase in the length of the vegetation filter strip. with an increase in length of the filter strip from 0.5m to 4m, the dissolved phosphorus and nitrate reduction from dairy waste varied from 9%-64% and 0-68% respectively.

Heavy Metal Uptake by SRWC

Heavy metals in wastewater effluents show large variations depending on their concentration in the water supply and the type of effluent. Heavy metal concentrations potentially harmful to environment and public health are found in industrial effluent, agricultural drainage, landfill leachate, and in some cases in municipal wastewater. Vegetation can play an important role in the management of heavy metals. When wastewater contains relatively high concentrations of trace elements, plant species able to withstand these levels should be used to maintain the treatment efficiency of the system. The physiological mechanisms inducing tolerance to heavy metals are: (a) exclusion from entering roots; (b) preferential accumulation in roots of shoots (c) rendering of heavy metals to non-toxic forms through chemical binding, and (d) compartmentation in the vacuoles.

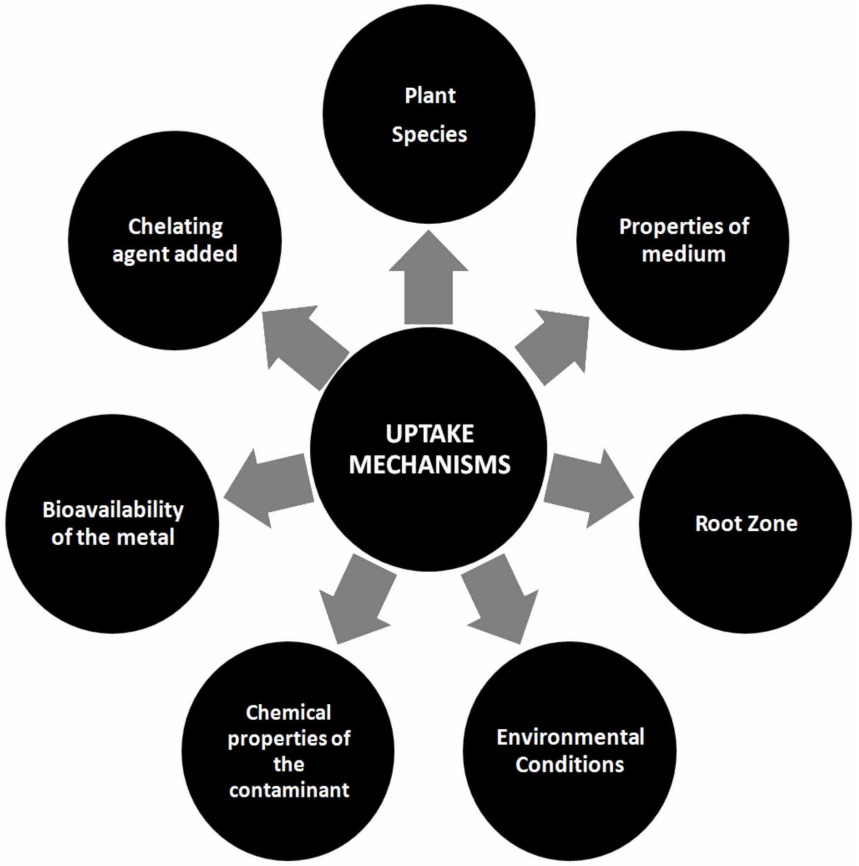
Similarly, the main purpose of SRWC, in addition to high biomass productivity, is a pronounced capacity of heavy metal uptake. Figure 1 shows various factors affecting the uptake of heavy metals by plant species. Greger (1999) evaluated various Salix spp. clones for cadmium uptake and found that some could remove five times more cadmium than the known phytoaccumulators *Thlaspi caerulescens* and *Alyssum murale.* Moffat, Armstrong, & Ockleston, (2001) suggested that Poplar trees irrigated with municipal wastewater and amended with biosoilds display an ability to remove cadmium at greater rates than it was applied on soil. It can be inferred that ideal plant species for SRWC used for the wastewater treatment with relatively high concentration of trace elements are those that can accumulate large amount of metals in their tissues, without posing a risk for wildlife or livestock animals and produce a high amount of biomass.

In addition, vegetation may affect the risks of metals through its effects on the availability and phytovolatilization rate. Plants can modify the chemical composition of the rhizosphere by excreting various substances known as “root exudates”. For e.g. Eucalyptus species reduces soil pH, thus increasing the availability of metals that may prove detrimental to plant growth and the activity of soil microbial community. Also, complex interrelationships between plant species and the structure of microbial community may strongly affect their availability. Plants release chelators that increase the availability of metals for plant uptake. Additionally, the release of enzymes in the rhizosphere may reduce the availability of metals such as copper(II), selenium(II), and chromium(VI), by converting them into non-available forms. Volatilization by plants can substantially contribute to the removal of some heavy metals.

Mechanism of Purification

Tree-based phytoremediation of wastewater is based on the high uptake capacity of trees and biodegradative potential of soils (Singh, 2011). It is gaining momentum owing to the fact that it serves two objectives: (a) waste disposal; (b) recycling of waste components. In the disposal of wastewater through land irrigation the soil and plants act as living treatment filters that trap and treat wastewater through various mechanisms and allow the remaining wastewater to drain through soil (Figure 2).

Figure 1. Factors affecting the uptake of heavy metals



The soil and its associated ecosystem components act as physico-biochemical reactors capable of treating or stabilizing pollutants. The wastewater, while passing through the soil matrix, provides filtration on the soil surface leading to the removal of coarse particles through degradation, adsorption, precipitation and utilization by plants. Degradation of soluble organic pollutants in the soil profile by microbial action, and mixing and aeration extended by macro soil habitant (earthworms and macrofauna) represents the waste treatment process occurring in the aeration tank. The suspended solids and bacterial biomass removal through adsorption, ion exchange and precipitation with hydroxides and carbonate indicate reaction processes occurring in secondary clarifier. Thus, phytoremediation the primary, secondary and tertiary treatment could be achieved, all in a single operation, with recycling and reuse benefits of wastewater and nutrients for biomass production (Witherow & Bledsoe, 1986).

Various processes involved in wastewater purification are:

- Phytoextraction: using metalt-accumulating plants from soil by transporting and concentrating them in the harvestable parts of roots and aboveground shoots.
- Phytodegradation: using plants and associated microorganisms to degrade metals and other pollutants.

Figure 2. Conceptual land treatment system for wastewater purification

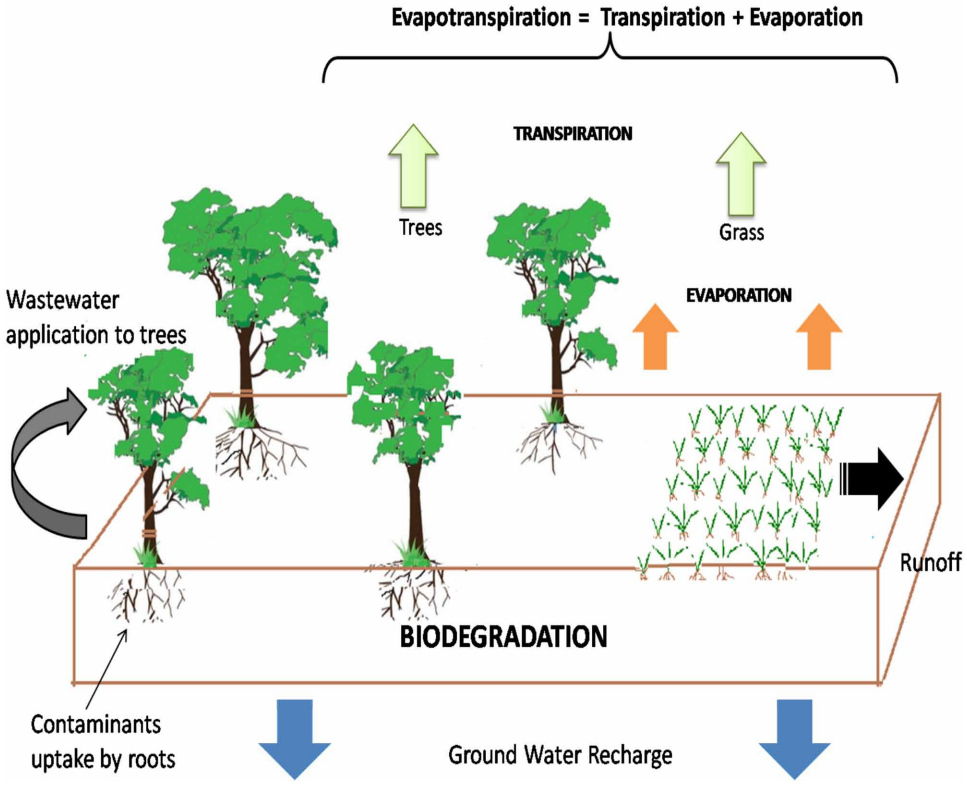
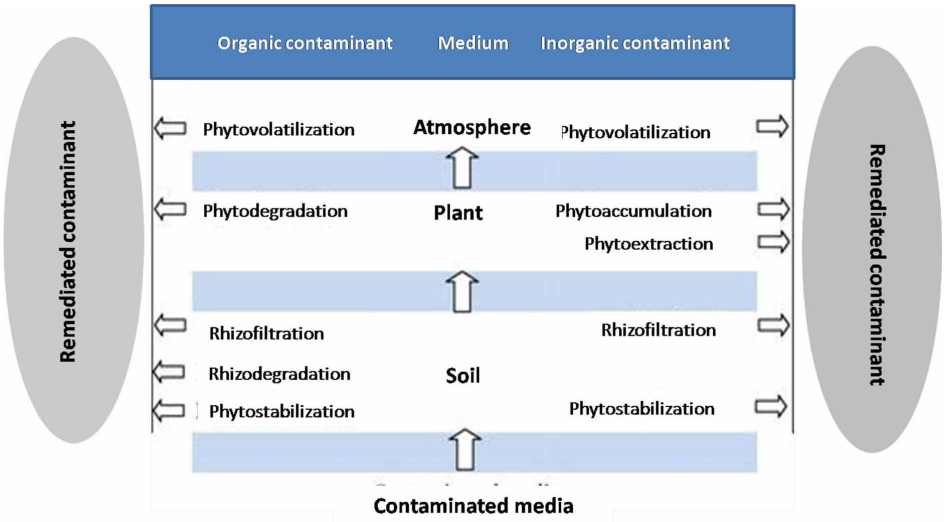


Figure 3. Pollutants removal by trees through various mechanisms



- Rhizofiltration: using plant roots to absorb and adsorb toxic metals and organics from polluted effluent.
- Phytostabilization: using plants to decrease the bioavailability of metals
- Phytovolatilization: using plants to volatilize pollutants from the environment.

SELECTION OF TREE SPECIES FOR PHYTOREMEDIATION

Vegetation plays a significant role in the treatment efficiency of vegetation filters, affecting the hydraulic load, nutrient removal, and biomass production. Thus, the primary criteria when selecting vegetation are (a) water requirements, (b) the potential for nutrient uptake, (c) salt tolerance, (d) trace elements uptake and/or tolerance, and (e) biomass production. Detailed knowledge of the ability of vegetation to remove a wide spectrum of constituents, both inorganic and organic, is important when selecting the most suitable plant species based on the effluent composition. Plant species often differ in growth rate and therefore show a different ability for phytoremediation. The tree species that have been promoted in Short Rotation Forestry (SRF) and possess remarkable wastewater remediation and biomass generation potential are:

Eucalyptus

Eucalyptus is a native to Australia extending from 7^0 N to 43^0 S in Astro-Malayan latitudinal range. It belongs to family Myrtaceae comprising 500 known species and 138 recognized Eucalyptus varieties. The species is native to Australia but its extends in many countries of the world. Eucalyptus is regarded as multipurpose tree for diverse uses and is most preferential species for plantation purpose. In India, Eucalyptus was first introduced around 1790, when a number of species were planted in the State palace garden at Nandi Hill near Mysore. It can survive in all types of soils and climatic conditions even in adverse conditions like infertility and scanty rainfall. Eucalyptus species that are widely grown in India are *E. citriodora*, *E. globules*, *E. grandis* and *E. camaldulensis*. The calorific value and specific gravity of Eucalyptus range from 4700-4800 KCal Kg^{-1} and 0.6 gcm^{-3}, respectively.

Melia azedarach

The species belongs to the family Meliaceae having around 12 species of trees in genus Melia, four of which including *Melia azedarach* is found in India. Well drained soils with precipitation between 600 and 1000 mm per yr or more is suitable for growing of this shade intolerant tree. It has excellent coppicing ability and grows well in tropical and sub-tropical climate with the temperatures between -5 and 40ºC with an elevation range from 900 to 1700 m. It is a fast growing tree and highly valuable species due to its multipurpose importance and recognition as a species of agroforestry/social forestry/urban forestry. It is a moderate sized, deciduous tree with short trunk and spreading crown, reaching a height up to 20m and diameter of 50-80cm. Leaves are 23-60cm long bipinnate or occasionally tripinnate. The timber obtained from this tree is tough, moderately hard, durable and lustrous with specific gravity around 0.66 g cm^{-3} and calorific value of wood in range of 5043-5176 K Cal kg^{-1}. The species yield valuable timber as well as good fuel wood species; leaves are highly nutritious thus can be used as fodder.

Poplars

Populus deltoides has recently received increased attention as a renewable source of biomass for energy. It belongs to a family Salicaceae and it is native to Northern America. It is a medium-sized to large tree, 20-30m tall, 100 cm dbh. *P. deltoides* is moderately light in weight and has specific gravity of 0.37 g cm^{-3}. It has proved to be extremely well suited for biomass production because of its rapid juvenile growth, high photosynthetic capacity and large woody biomass production in a single growing season. Due to special features its fast growth, an extensive root system, simple techniques of propagation through vegetative cuttings, *P. deltoides* tree is a desirable model system for tree plant research. The specific gravity of stemwood ranges from 4300-4800 KCal kg^{-1}. It is extensively used in agroforestry as its nitrogen contribution to the soil could be as much as 43 kg in 3 years and 102 kg in 11 years (Balatinecz & Kretschmann, 2001). Additionally, poplars are a primary source for a wide range of wood products, i.e., timber, lumber, fodder and pulp.

Salix

Salix alba commonly known as white willow belongs to family Salicaceae which is having 300 species, most of them are found in Europe. The species is indigenous to Central and Southern Europe, North America and Asia. It is a medium-sized to large deciduous tree growing up to 10-30 m tall, with a trunk up to 1 m diameter and an irregular, often leaning crown. The tree prefers to grow in moist and fertile soils in cold and temperate region of northern hemisphere. The leaves are typically elongated 5-10 cm long and 1-1.5 cm wide. They are covered with very fine silky white hairs, particularly on the base. Almost all willows take roots very readily from cuttings. The wood have calorific value of 4132 KCal kg^{-1} and specific gravity of 0.45 with excellent coppicing ability. Willows are often planted on the borders of streams so their interlacing roots may protect the bank against the action of the water. Frequently, the roots are much larger than the stem which grows from them. Willow is grown for biomass or biofuel, in energy forestry systems, as a consequence of its high energy in-energy out ratio, large carbon mitigation potential and fast growth. According to Aylott et al. (2008) many large scale projects are already at commercial scale in Sweden to produce willow as an energy crop. Table 5 shows the use of various plant species in vegetation filter system worldwide.

Nicholas, Carnus, & Oliver, (1997) suggested that the fast initial growth-rate of SRF crops, as well as the coppicing abilities of SRF crops is beneficial in land treatment systems and biomass from SRF is suitable for energy conversion. These plants due to their high transpiration capacity serve as biopump to remove nutrients and other pollutants effectively from wastewater. This renders the system to consume more water and thus minimizes the leaching problem in groundwater. These species have been used for riparian plantings for centuries (Lowrance et al., 1984; Licht & Isebrands, 2005) and they are still the most common tree species used for wastewater filtration because of their rapid biomass production, which causes a high nutrient turnover and makes abundant use of water (Nixon, Stephens, Tyrrel, & Brierley, 2001; Mirck, Isebrands, Verwijst, & Ledin, 2005; Białowiec et al.; Guidi, Piccioni, & Bonari, 2008). These species are ideal for wastewater irrigation because of their ability to uptake nutrients and water in early years for higher biomass production (Ian, 2003). *Eucalyptus camaldulensis* is the best-suited species under municipal effluent irrigation (Stewart, Hopmanns, Flinn, & Hillman, 1990; Laclau,

Table 5. Species used for the treatment of various effluents in land treatment systems

Plant species	Wastewater type	Reference
Acacia nilotica (L.)	Municipal effluent	(Singh and Bhati, 2004)
Ailanthus excels	Municipal effluent	(Toky, Riddell-Black, Harris, Vasudevan, & Davies, 2011)
Arundo donax	Primary effluent	(Tzanakakis et al., 2009)
Axonopus affinis, Panicum maximum	Textile wastewater	(Sapari, 1996)
Casuarina equisetifolia	Municipal raw sewage	(Kumar and Reddy, 2010)
Dalbergia sissoo	Municipal effluent	(Singh and Bhati, 2005)
Eucalyptus botryoides	Meat processing effluent	(Guo et al., 2002)
Eucalyptus globulus	Secondary effluent, meat processing effluent	(Guo et al., 2002), (Duncan, Baker, & Wall, 1998)
Eucalyptus cyanophylla	Primary effluent	(Tzanakakis et al., 2003)
E. robusta	Sewage effluent	(Edraki, So, & Gardner, 2004)
E. botryoides	Meatwork effluent	(Guo, Sims, & Horne, 2006)
E. ovata	Meat processing effluent	(Guo et al., 2002)
E. camaldulensis	Primary effluent, stormwater pond	(Tzanakakis et al., 2003), (Rockwood, 1996)
E. camaldulensis	Mixed industrial effluents	(Bhati and Singh, 2003)
E. globulus	Meat processing effluent	(Guo and Sims, 2000)
Eucalyptus sp.	Stormwater	(Pisano and Rockwood, 1997)
Melia azedarach	Municipal effluent	(Toky et. al., 2011)
Pasture grass	Dairy processing effluent	(Sparling et al., 2004)
Populus sp. (hybrid poplar)	Domestic effluent	(Moffat et al., 2001)
Populus × *euramericana* (hybrid poplar)	Primary effluent, food processing wastewater	(Vermes, 1996)
P. robusta	Primary effluent, food processing wastewater	(Vermes, 1996)
Salix spp.	Municipal effluent	(Perttu, 1993)
S. babylonica, Amorpha fwticosa	Domestic sewage	(Zhou, Zhang, & Sun, 2006)
S. viminalis, S. dasyclados	Agricultural drainage water	(Elowson, 1999)
Salix spp.	Municipal wastewater	(Perttu and Kowalik, 1997)
Salix spp.	Domestic effluent	(Amofah, Mattsson, & Hedström, 2012)

Bouillet, & Ranger, 2000; Bhati & Singh, 2003). It has been reported by Juwarkar, Thawale, Juwarkar, & Singh, (2003) that plants such as bamboo (*Bambusa arundinacea*), acacia (*Acacia mangium*), neem (*Azadirachta indica*), shisham (*Dalbergia sissoo*) and *E. hybrid* can transpire water equivalent of 7 to 13 times the potential evapo-transpiration from the soil matrix alone. Thus, enables the disposal of 350-450 m^3 of wastewater per hectare of land area per day. Fonseca, Melfi, & Montes, (2007) reported that *Casuarina equisetifolia* plants can transpire water equivalent to 8-12 times the potential evapo-transpiration.

ADVANTAGES OF WASTEWATER IRRIGATION TO TREES

The higher economic gains and least risk of pathogens due to non-consumable nature promote the effective reuse of wastewater in forest and fuelwood plantation. The benefits further increase as they can utilize water round the year and are more acceptable to the public than crop irrigation, and hence, requiring less land for a given volume of effluent. Growing woodlots of fast growing species to utilize the water and nutrient available in municipal effluent is not only a strategy for increasing productivity of nutrient and water deficient drylands but also helps in eco-restoration, ground water recharge, controlling land degradation, fodder production after uptake from soil under land disposal of the effluent. Commercial plantation can also gain benefits from wastewater reuse in plantation as it reduces fertilizers cost required for higher biomass. Also, long-term storage of nitrogen and carbon in biomass helps in retaining elements in the ecosystem and gaining carbon credits.

FERTIGATION POTENTIAL OF WASTEWATER

Irrigation of plants with water containing nutrients is termed as fertigation, a contraction of two words fertilization and irrigation. The most common nutrient applied by fertigation is nitrogen. Fertigation increases nutrient absorption by plants, reduce the demand of fertilizer and chemicals and reduces leaching of chemical fertilizers to the ground water. The use of wastewater for irrigation is now a day's explored as it is generally rich in dissolved nutrients. The application of wastewater at rates which ensure a balance between nutrient input and plant uptake promote the optimal plant growth and limit the risks of pollution. Also, it reduces the fertilizer input cost to commercial plantation for enhanced biomass production through the fertigation with wastewater (Pandey & Srivastava, 2012. Municipal wastewater contains macro and micro elements that are closer to the nutrients needs of short rotation woody plants (Perttu, 1994) and thereby can be cheaply applied as a substitute to chemical fertilizers and as a result enhance the tree growth. It is estimated that $1000m^3$ of municipal wastewater used to irrigate one hectare can contribute 16-62 kg total nitrogen, 4-24 kg phosphorus, 2-69 kg potassium, 18-208 kg calcium, 9-110 kg magnesium, and 27-182 kg sodium (Qadir et al., 2007). In the light of the global phosphorus crisis, wastewater and excreta can be critical sources of phosphorus that can help in crop establishment throughout the growth period (Rosemarin, 2004). Similarly, nitrogen supplied through wastewater helps in crop establishment in early growth stages by mitigating the negative effects of excess salts if added through wastewater irrigation. Optimal level of potassium helps in crop maturity and quality. With the nitrogen content of an effluent at 10 to 30 mg l^{-1} and the phosphorus content at 4 to 10 mg l^{-1}, and assuming an average annual wastewater application rate of 8000 m^3ha^{-1}, the total annual input is 160 kg ha^{-1} N and 56 kg ha^{-1} P. A young plantation growing rapidly can take up 120 to 150 kg N per ha and about 12 kg P per ha per year; therefore, sufficient levels of these nutrients will be available for potential maximum growth (Commonwealth Scientific and Industrial Research Organisation [CSIRO], 1995). Municipal wastewater irrigation has been extensively studied for *Eucalyptus* sp., *Forsythia* sp., *Medicago arborea*, *Buddleia variabilis* and *N. oleander*. Growth of plants was significantly favored, owing to the beneficial effect of the nutrients present in the wastewater. Danso, Drechsel, Wiafe-Antwi, & Gyiele, (2002) therefore, stated that with low investments and quick returns, this lucrative practice can reduce the demand for chemical fertilizers and enables many farmers to leap over the poverty line.

ENHANCED BIOMASS PRODUCTION THROUGH FERTIGATION

The technology of biomass production by using municipal wastewater as fertigation has come up as a new concept for reuse of wastewater in which the nutrients present in wastewater are utilized by the entire plant system for their growth and development (Central Soil Salinity Research Institute [CSSRI], 1989). Municipal wastewater contains macro and micro elements that are closer to the nutrients needs of SRWC (Perttu, 1994) and thereby can be cheaply applied as a substitute to chemical fertilizers and as a result enhance the tree biomass. Nitrogen supplied through wastewater helps in crop establishment in early growth stages, phosphorus helps in crop establishment throughout the growth period and optimal level of potassium helps in crop maturity and quality.

Perttu & Kowalik (1997) as well as Labrecque, Teodorescu, & Daigle, (1997) envisaged 2-3 times higher yields in wastewater fertilised plot in comparison to unfertilised plots. Similarly, Hasselgren (1998) asserted biomass production after wastewater application to be up to three times as high as without wastewater application. Borjesson & Berndes (2006) have reported 30-100% increase in the total biomass for the trees irrigated with domestic wastewater than those irrigated with traditional rain-fed plantation in Southern and Central parts of Sweden. Mitchell, Stevens, & Watters, (1999) have reported average biomass production covering a wide range from 2.2-13.5 t dry matter per ha per year (DM ha^{-1} yr^{-1}) for alder, poplar and willow depending on location, climate, plant species, clones, etc. Some studies have shown higher biomass production in willow than in poplar plantations (Perttu, 1993). Heller, Keoleian, & Volk, (2003) suggested average production of 10 DM $ha^{-1}yr^{-1}$ in bioenergy plantation with wastewater irrigation. Moreover, Guo, Sims, & Horne, (2002) stated up to 76% increase in productivity resulting from wastewater application with 24 t DM $ha^{-1}yr^{-1}$ biomass yield for *E. globules.* Sims & Riddell-Black (1998) predicted a feasible productivity with wastewater use and optimal yield obtained was up to 20 t DM $ha^{-1}yr^{-1}$ for *Eucalyptus globules.*

SOIL RESPONSES TO WASTEWATER IRRIGATION

The use of municipal wastewater has been increasingly considered to be beneficial for biomass production, and due to its significant source of nutrients for the plants it can help to reduce the requirements for commercial fertilizers. However, under certain conditions, this type of water if not appropriately managed, can have negative impacts on soils, particularly soil salinity and sodicity. Among the potential risks associated are degradation of soil structure, decrease in soil hydraulic conductivity, runoff and soil erosion problems, soil compaction, soil contamination with faecal coliform and groundwater contamination as a result of high nitrogen concentration. However, the general conclusion for SRPs is that the soil impact is low and more or less independent of applied wastewater rates. Al-Jamal, Sammis, Mexal, Picchioni, & Zachritz, (2002) reported that over irrigation can, besides effects on plant performance and soil properties cause N leaching to the groundwater rather than affecting the soil properties.

Depending on effluent characteristics, the fluctuations in soil pH have been observed in wastewater irrigation trial. Effluent irrigation can significantly increase pH throughout the soil profile without adversely affecting the overall soil chemical properties and the nutrient accumulation in soil occur mainly in the upper 0.35 m. Pinto, Maheshwari, & Grewal, (2010) has also reported increase in soil pH in domestic wastewater irrigation trials. Reduction in the soil bulk density has been reported by various researchers with meatworks effluent irrigation (Guo & Sims, 2003), farm dairy effluent irrigation (Hawke &

Summers, 2003) and sewage irrigation (Mathan, 1994) due to increase in soil organic matter. Moreover, higher organic matter is also reported by Yadav, Goyal, Sharma, Dubey, & Minhas, (2002) in wastewater irrigated trial probably due to higher BOD load in wastewater and also due to higher plant growth and more litter fall and degradation. An increase in organic matter content, total N and/or C/N ratio following irrigation with Olive Mill effluent has been observed previously by Sapari, (1996). Treatment and reuse of textile wastewater by overland flow. Sierra, Marti, Garau, & Cruanas, (2007) and Mechri, Ben Mariem, Baham, Ben Elhadj, & Hammami, (2008) reported beneficial effect on soil fertility and yield. However, according to Brzezinska et al. (2006) short-term municipal wastewater irrigation had a weak effect of pre-treated municipal irrigation and could not stimulate the soil biological activity significantly.

SOIL ENZYMES AS INDICATOR OF SOIL HEALTH

Physical and chemical properties have been widely used to assess soil quality. However, these properties usually change slowly and, therefore, significant changes occur only over many years. By contrast, Ndiaye, Sandeno, McGrath, & Dick, (2000) suggested that soil microbial and enzymatic properties respond relatively quickly to small changes in soil conditions before it can be detected by other soil analysis. The main source of enzymes in soils are microorganisms that are considered sensible indicators when monitoring changes in soil status affected by different factors like agricultural management or pollution (Filip, 2002). It has been extensively reported that any change in soil status affected by wastewater application is reflected in soil enzyme activities (Filip, 2002; Brzezińska, Stępniewska, & Stępniewski, 2001;Speir, 2002). Zaman, Cameron, Di, & Inubushi, (2002) studied the influence of surface application of wastewater on enzyme activities in different soil depths and found that results are more pronounced in upper soil layers.

Porazinska et al. (2003) found that In addition to wastewater irrigation, plant species and their rhizosphere also significantly affect the composition of soil microbial community. The rhizosphere is basically, a zone of enhanced microbial activity because plants roots release 17% of the photosynthate captured, most of which is available to the microbial community (Nguyen, 2003), and subsequently number of microorganisms in this zone are abundant. This result in a different environment in the rhizosphere from that in bulk soil. Root exudates from different plants play an important role in stimulating the growth of unique bacterial and fungal populations in the vicinity of roots. As a result, microbial growth in the rhizosphere, as well as their activity, differs from one plant species to another depending on type of root exudates and plant debris. Specific interactions between plant and its rhizospheric effect on soil microbial communities have been the interest of investigators (Pandey & Palni, 2007). However, the rhizosphere studies conducted so far are largely based on short-duration species (Routt & Katznelson, 1961). Only a limited number of reports have focused on the course of processes mediated by soil microorganisms in SRWC plant-soil system (Brzezinska et al., 2006).

BIOMASS PRODUCTION AND ENERGY SECURITY

The Kyoto target set by Europe is an 8% reduction in annual greenhouse gas emissions by the first commitment period (2008-2012), compared to 1990. In this context, the European Commission set the target to increase renewable energy sources to reach 12% of the European gross energy consumption by 2010

(European Commission [EC], 1997). Biomass has always been a major source of energy for mankind and is presently estimated to contribute of the order 10-14% of the world's energy supply. Short rotation plantations can be expected to play a major role in the production of biomass for bioenergy. These woody species can be grown in short-rotation and intensively managed systems under wastewater irrigation to ensure high, consistent biomass yields. Biomass produced in short-rotation plantations can serve as a substitute for fossil fuels, reducing as such the emission of greenhouse gases to the atmosphere and helping to attain the greenhouse gas emission reduction target (EC, 1997; Perttu, 1998). Table 6 shows energy yields from different biomass species. Burning fossil fuels uses ''old'' biomass and converts it into ''new'' CO_2; which contributes to the ''greenhouse'' effect and depletes a non-renewable resource. However, burning of biomass from SRF plantations can be considered as a CO_2 neutral process, since the CO_2 liberated during the burning process will be sequestered during the next rotation period (International Energy Agency [IEA], 2002).

In general, the characteristics of the ideal energy crop are:

- high yield (maximum production of dry matter per hectare),
- low energy input to produce,
- low cost,
- composition with the least contaminants,
- low nutrient requirements.

SRWC can play an important role for our future energy generation from renewable biomass because it has a high net calorific value, on a dry basis, of 4.0-5.0 K Cal g^{-1}. The energy value of 20t of dry SRC would be equivalent to that of 8 ton of coal. Growing SRC as a fuel is very energy efficient, with a high energy and carbon balance.

ENVIRONMENTAL RISKS AND BENEFITS

In developing countries wastewater is discharged, often untreated, into streams, rivers, or lakes. The accumulation of nutrients and pollutants causes eutrophication, leading to the loss of aquatic life. This process results in an increased risk for those who use the polluted water for domestic use, including drinking. The untreated wastewater may also cause groundwater contamination. Wastewater irrigation sites fall into three categories: (1) Unrestricted access sites or simply unrestricted irrigation is used to irrigate vineyards, trees used for wood production for houses and furniture, pasture, horticultural crops,

Table 6. Energy yields from selected biomass source (Source: McKendry, 2002)

Biomass	Crop yield (dmt/ha/yr)	Calorific value (MJ/kg, dry)	Energy yield (GJ/ha)
Poplar	10-15	17.3	173-259
SRC willow	10-15	18.7	187-280
Switchgrass	8	17.4	139
Miscanthus	12-30	18.5	222-555

parks, and golf courses; (2) Restricted access sites or simply the restricted irrigation is used for irrigation in fenced or isolated woodlands or meadows or to grow food products, except for salad crops and vegetable that may be eaten uncooked; and (3) Agricultural sites-areas where nonhuman food crops are grown. When selecting a site for irrigation with wastewater, cost trade-off factors should be considered. These factors include the cost of the land and the cost of transporting the wastewater to the site. In addition, pumping treated wastewater to an upland site costs more than allowing gravity flow to a lowland site; and the cost of a stream crossing to reach a possible site may outweigh the higher land costs nearby. Restricted access and remote sites have lower treatment requirements, resulting in lower treatment costs.

Wastewater collection, its treatment, disposal of waste (biosolids), and use of treated water is a big challenge to environmental security due to the fact that there are so many variables and no two wastewater streams are identical. There will always be a need for continuous studies on wastewater availability, and treatment needs to be fine-tuned after determining the composition of the raw wastewater. The high cost of plant construction and operation in developing countries receives less attention from the service providers. Landscaping is needed to improve the plant's appearance, and safe working conditions for the employees should be ensured. The consideration of noise, odor, and the location of the plant are serious issues to be resolved before such a plant is designed and installed. To avoid odor associated with sepsis, adequate volumes of air are required to the basin for mixing.

CONCLUSION AND IMPLICATIONS FOR PRACTICE

The concept of linking SRF with the land treatment of municipal wastewater could in this way help in effective effluent disposal, biomass production and land restoration. However, for long-term application careful attention must be paid to site characteristics, nature of the waste stream, water use efficiency, water distribution and application methods, species selection and market potentials. Nutrient dynamics, buildup of salts in the soil and the effect of salinity on vegetation are also critical issues that should be pre-examined. The preferred approach, from a sustainable point of view, is to manage the wastewater in a carefully designed manner by distributing it at lower loading rates and large buffering capacities over a sufficiently large area of land and at acceptably long time intervals such that the nutrients can be effectively taken up by the crop and therefore recycled. This practice would reduce the risk of soil or water pollution. The additional capital and operational costs for distributing a given volume of wastewater over the greater land area required would be substantial, but with a land treatment system, an added advantage is that the value of the crop/biomass produced would compensate the treatment cost. Currently, there is insufficient knowledge of the capacity of SRF trees to take up water and nutrients from effluents and more information is currently being gleaned from experimental lysimeter studies. In the future, more intensively managed systems may be able to be designed and implemented with greater confidence of success as the understanding of the overall biological, chemical and physical system improves. The results will enable better designed, more sustainable vegetation filters with enhanced biomass production to be established in the future.

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KEY TERMS AND DEFINITIONS

Biomass: Biomass is biological material derived from living, or recently living organisms. It most often refers to plants or plant-based materials which are specifically called lignocellulosic biomass. As an energy source, biomass can either be used directly via combustion to produce heat, or indirectly after converting it to various forms of bio fuel.

Land Treatment System (LTS): Land treatment refers to the application of partially treated wastewater to the land that is designed, constructed and operated to treat wastewater through the use of crops, irrigation methods, ground and surface water monitoring to confirm to specific water quality limits. It involves the controlled application of wastewater to the land at rates compatible with the natural physical, chemical and biological processes that occur on and in the soil.

Nutrients: Nutrients are the nutritious components in foods that an organism utilizes to survive and grow. It refers to mainly nitrogen and phosphorus originating from agricultural and urban areas.

Short Rotation Woody Crops (SRWC): "Short Rotation Crops" means woody crops such as willows, poplars, Robinia and Eucalyptus with coppicing abilities as well as lignocellulosic crops such as reed canary grass, Miscanthus and switch grass.

Treatment Efficiency: The treatment efficiency of any natural treatment system is the basic indicator of its phytoremediation potential. It depends on the amount and composition of wastewater, type of plant species used, climatic and other conditions.

Vegetation Filters: Vegetation filter is a plant-based natural treatment system which principally involves phytoremediation strategies for treatment of wastewater through fast-growing woody (*e.g*. *Salix*, *Populus*) trees and/or herbaceous perennials (*e.g*. *Phragmites australis*). In a properly designed VFS, water flows evenly through the strip, slowing the runoff velocity and allowing contaminants to settle from the water.

Chapter 10
Bioremediation of Oil Contaminated Soil and Water:
In situ and Ex situ Strategies for Feasibility Assessment

Chandrika Malkanthi Nanayakkara
University of Colombo, Sri Lanka

Ayoma Witharana
University of Moratuwa, Sri Lanka

ABSTRACT

Pollution from petroleum, plant and animal origin oils, which are released via oil production and shipping operations, refineries, accidental spills, effluents of different industries such as hotels, restaurants, food processing, etc. is ubiquitous in the environment. This necessitates the need for cost effective and efficient remediation technologies. Dealing with the problem chemically and physically is known to generate secondary pollutants and incurs high cost. Expediting natural attenuation via stimulating pollutant degradation activity of residential microbial community and/or introducing competent microflora in to polluted sites has been identified as the most successful and cost effective technology and is termed bioremediation. Phytoremediation, an emerging branch of bioremediation, has also been recognized as a promising treatment technology. Chapter examines the extent of work carried out in in situ and ex situ bioremediation strategies to mitigate oil pollution, the validity of such practices in terms of efficiency of the process and the future research directives.

INTRODUCTION

Generally, microorganisms brake down natural compounds more contentedly in their inhabitant environment and as per nature's rule all natural compounds do decay sooner or later. In such a context bioremediation is the naturally available technique for getting rid of contaminants. When the rate of contamination exceeds the rate of natural degradation, pollution becomes evident making it a requisite

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to take measures to advance the natural process. This man's intervention over natural decay to increase the rate of microbial degradation is termed bioremediation. It uses microorganisms or plants to completely break down, sequester, reduce toxicity or detoxify substances hazardous to humans and/or the environment (Vidali, 2001). Microbes utilize the target contaminants as a source of energy by taking it through a series of oxidation-reduction reactions in order to make useable energy forms for metabolism. As a result, byproducts of metabolized contaminants are released back into the environment, which are usually in a non-or less toxic form than the original compound. Currently, not only microorganisms but the products of microbial origin such as surfactants and enzymes are also in use, bioremediation should be broadly defined in order to encompass organisms, their products and genes as well.

In the context of bioremediation of oil contaminated environments, new techniques are introduced and existing techniques are improved while providing greater contribution to the pool of knowledge and experiences. Different techniques of bioremediation have been used at a number of sites under diverse environmental conditions, with varying degrees of success. Oil contaminated environments can be treated either at the site of pollution itself (*in situ*) or taking them away from the site (*ex situ*). However, irrespective of the fact that whether the remediation technique is applied *in situ* or *ex situ,* bioremediation approaches for oil contaminated environments fall into three major categories viz. biostimulation, bioaugmentation and introduction of genetically modified microorganisms. Like any other technology, bioremediation also has its limitations; some contaminants may resist microbial degradation completely or degraded either slowly or not at all. Furthermore, being a microbe driven process bioremediation is highly dependent on site environmental conditions which permit efficient microbial growth and activity. Therefore, application of bioremediation often involves the manipulation of environmental parameters in such a way that they allow microbial growth and degradation to proceed at an adequately faster rate.

Therefore, in this chapter it is intended to essentially discuss: (i) Production, usage and pollution caused by petroleum and plant/animal origin oil; (ii) Bioremediation strategies available for petroleum oil contaminated environments; (iii) Factors affecting the bioremediation process; (iv) Limitations/drawbacks of the available technologies; (v) Monitoring of bioremediation applications (vi) Bioremediation of plant/animal oil contaminated environments (vii) Phytoremediation and finally (viii) Recommendations for future research directions and conclusion.

Oils and Fats: Production, Usage and Pollution

In a broad definition, oil refers to any greasy substance that is liquid at room temperature and insoluble in water. Oil can be categorized into two forms based on their origin viz. petroleum origin oil and plant or animal origin fats and oils. Petroleum origin oil generally refers to crude oil formed in deep earth rock strata due to the intense pressure and heat exerted on buried animals and plants over a long time period. Plant oil generally refers to vegetable and/or cooking oil that has been extracted from plants. Animal fats and oils are obtained from rendered tissues of livestock animals. In addition, dairy product industry produces large amounts of fat and oil polluted waters. Fat refers to any substance of plant or animal origin that is non-volatile, insoluble in water and usually solids at room temperatures. Fats and oils differ from each other only from their physical state at room temperature. Chemically, fats and oil are fundamentally the same primarily consisting of triglycerides. Plant origin oils predominantly contain fatty acid esters of the trihydroxy alcohol or glycerol (Kumar et al., 2012).

Despite the source/origin, oils are released into the environment via oil production and shipping operations, from refineries (Vasudevan & Rajaram, 2001), accidental spills (Cipnyte, Grigiškis, & Baškys, 2009), and from effluents of different industries such as hotel, restaurant, food processing, textile, metal, municipal wastewater, etc. (Chen, 2013). As reported by U.S. Energy Information Administration in 2012, the world petroleum oil production and consumption is led by Saudi Arabia and the USA, respectively. Saudi Arabia produced 11.73 million barrels per day and USA consumed 18.5 million barrels per day in 2012. It is reported that petroleum derived hydrocarbons including automotive lubricant oils are one of the most frequent contaminants of soil and water (Montagnolli, Lopes, & Bidoia, 2009). In Europe approximately 1.1 million tones/year of lubricant oil has an anonymous destination (Stempfel, Hostettle, & Gasser, 1993). Even though this entire amount is not released straight into the environment, it is said that there is a potential in leaking harmful volumes (Montagnolli et al., 2009). Soil acts as a depository for many pollutants including hydrocarbons, which is a concern as it has adverse impacts on human health and is aggravated by its persistence nature in the environment (Semple, Reid, & Fermor, 2001). As a consequence, these potentially toxic and persistent contaminating substances are tending to get dispersed all over the environment (Wild & Jones, 1995). Hydrocarbons with stable molecular structures exhibit high hydrophobicity and low solubility and hence show low tendency to detach from soil through leaching and volatilization (Towell et al., 2011).

Annual production of plant origin fats and oils in the world is approximately 2.5-3 million tons, from which a major portion amounting to 75% are derived from plants (Haba, Espuny, Busquets, & Manresa, 2000). According to the Food and Agriculture Organization (FAO, 2010), global vegetable oil consumption during the last few decades has increased annually by 2.2% and Malaysia and Indonesia are the major producers. These oils are mainly used for culinary and industrial applications throughout the world. In industrial applications, fats and oils are used to manufacture animal feeds, fatty acids, soaps, personnel care products, cosmetics, paints, emulsifiers, etc. The used fats and oils from food service establishments are considered to be one of the major constituent in wastewater (Gaur, Cai, Tuovinen, & Mancl, 2010). The U. S. Environmental Protection Agency reported (USEPA, 2004) that annually nearly 3-10 billion gallons of untreated wastewater is discharged from restaurants, homes, and industrial sources, which may have increased in several folds by now. Generally plant/animal oil can exist in wastewater in several forms such as dispersed or emulsified oil (Dumore & Mukhopadhyay, 2012). In aquatic environments, presence of oil at high concentrations may reduce oxygen transfer rate and increase chemical oxygen demand (Al-Darbi, Saeed, Islam, & Lee, 2005). Moreover, blockage of pipelines and sewers due to solidification of oil and grease and formation of microbial biofilms has become a serious problem (Ashley, Fraser, Burrows, & Blanksby, 2000).

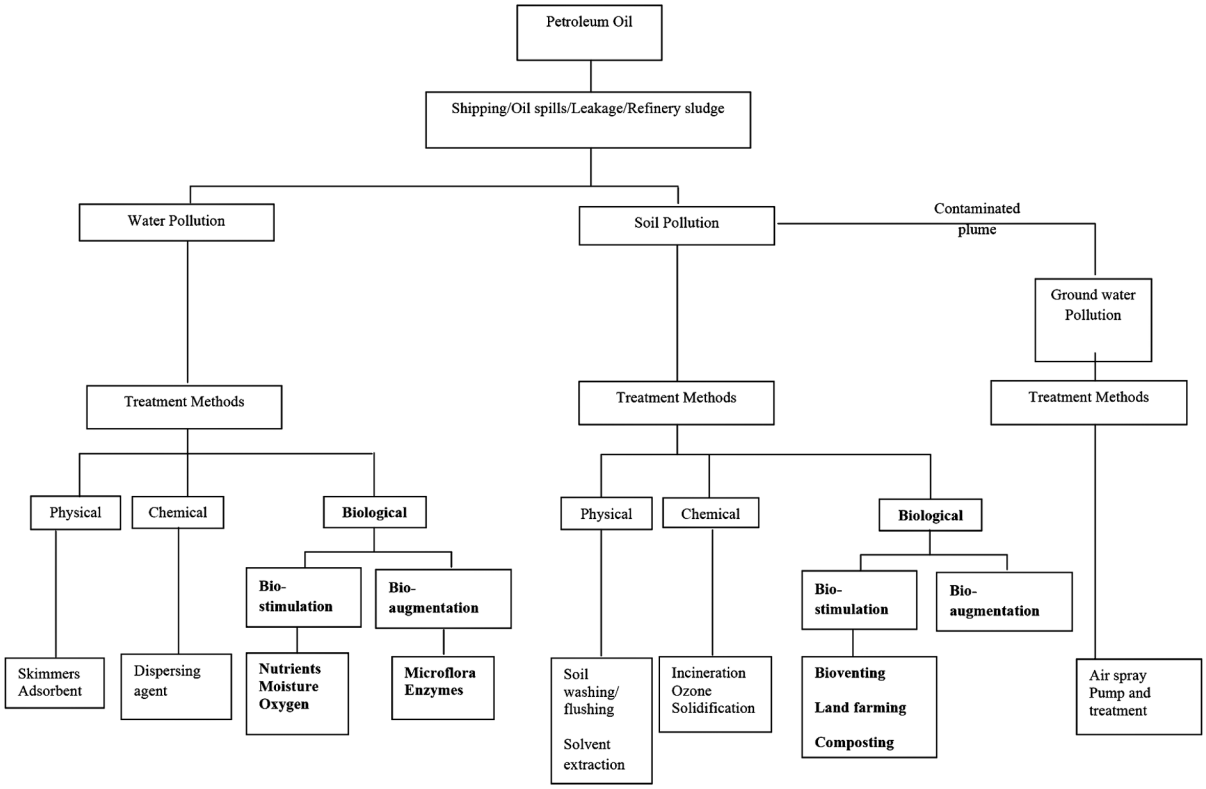
Methods of Treating Soil and Water Polluted/Contaminated by Oils and Fats

The nature and extent of the damage caused by oil pollution/contamination is well documented. Different clean-up technologies are currently being implemented all over the world to treat oil and fat polluted soil and water. However, any such clean-up technology should focus on the sensitivity of the environment and the nature and/or toxicity of oil. In aquatic environments, water immiscible phase can be physically removed by using boomers and skimmers or natural and synthetic sorbent materials can also be used. In environmentally sensitive areas chemical and biological treatments can be employed subsequent to mechanical treatments (USEPA, 2008).

In the case of treatment and/or clean-up of soil pollution caused by petroleum-based products, engineered/mechanical methods such as soil vapor extraction, soil washing and incineration are used (Figure 1). More advanced techniques are employed when the pollutants reach ground water table and aquifers. However, despite the condition of the contaminated site, some treatment technologies such as incineration, burial of sludge in secure landfills are costly. For instance, in the USA, 90% of site remediation costs are linked with petroleum hydrocarbons and estimated cost tends to be well above US $1 trillion (Maier & Soberon-Chavez, 2000). Conversely, bioremediation strategies are considered as cost-effective and environmentally friendly approaches reporting its employment in approximately 25% of petroleum-contaminated sites (Holden, La Montagne, Bruce, Miller, & Lindow, 2002). Petroleum hydrocarbon removals by active techniques such as addition of nutrients or bio-surfactants, or low cost methods using passive techniques such as natural attenuation are the commonly used approaches.

Wastewater having high fat, oil and grease (FOG) is released into environment via plant and/or animal based fat and oil manufacturing/using industries (Figure 2). Primary treatment of such waters involves physical separation of fats and oils from wastewater. Grease trap, a specially designed rectangular tank through which wastewater passes at a pre-determined rate, allows an adequate time period to pass by for rising of fat/oil particles to the water surface. Dissolved air flotation system (DAF) is used to separate oil droplets dispersed in water. In this method, air is dissolved into wastewater under pressure. When the pressure is released, dissolved air releases itself into the water column in the form of micro-bubbles, which aggregate at the water oil/fat interphase and separate the two phases from each other (Camma-

Figure 1. Technologies available for treatment of petroleum oil spills



rota & Freire, 2006). In chemical hydrolysis of oil and grease, i.e. treating wastewater with alkali, has two objectives: (i) neutralize the acidity of wastewaters (ii) maintain an alkaline pH which facilitates the degradation of fat in the subsequent feeding tank. Further, it increases the ratio of soluble COD to total COD with the simultaneous reduction of volatile solid content during anaerobic phase of digestion (Lefebvre, Paul, Mauret, Baptiste, & Capdeville, 1998). Although, these chemical treatments methods are successful in reducing fats and oils from wastewater, finally end up with formation of chemical sludge.

In biological treatment methods, various microorganisms and enzymes have been used for the treatment of wastewater containing oil and fat. In early methods, direct cultivation of lipophilic microbial flora in wastewater has been attempted (Anon, 1994; Wakelin, & Forster 1997). Currently, biological treatment methods detailing the use of microorganisms alone and in combination with their enzyme pools have been developed and patented (Santis-Navarro, Gea, Barrena, & Sánchez, 2011; Kumar et al., 2012). As the chemical and physical characteristics of petroleum origin oils and those of fats and oils of plant/animal origin differ, their treatment methodologies are also discussed separately in the subsequent text.

BIOREMEDIATION STRATEGIES

Pollutants added to soil are degraded by residential microflora albeit at a rate not on par with that of the addition of pollutants. This natural recovery or natural attenuation is primarily a no-action option which relies on the high versatility and ubiquitous nature of microorganisms. For remediation of remote or inaccessible areas or where the cleanup actions could cause more harm than the oil contaminants itself, microbial degradation is the most cost-effective and ecologically sound strategy. In other sites, when the rate of contamination exceeds the rate of degradation, expediting the natural attenuation is needed.

The three primary requirements needed to be fulfilled for the bioremediation to be successful are:

- The presence of a contaminant at a non-toxic concentration,
- The presence of a respiratory electron acceptor, and
- The existence of a microbial community that can degrade the contaminant in question (USEPA, 2006).

With these requirements being met, bioremediation can take place in any environment given the credential to the very high versatility and ubiquitous occurrence of microorganisms. All the other factors discussed in subsequent sections are secondary, affecting only the rate of degradation. In large oil spills the first response options are the physical and chemical methods, which seldom achieve the completion of the clan-up process (USEPA, 1999). Hence, bioremediation has gained momentum as one of the most promising secondary treatment options available especially after the reported success in cleaning up of the 1989 Exxon-Valdez spill. There are ample evidences for successful application of bioremediation techniques to treat contaminated soil and water, especially for petroleum oil, its derivatives and some chemical contaminants such as BTEX, PAHs, chlorinated phenols, and pesticides (Coulon et al., 2010). However, relatively little research has been conducted on biological remediation of soil and water polluted with plant/animal oil and fat.

BIOREMEDIATION OF PETROLEUM OIL CONTAMINATED ENVIRONMENTS

Bioremediation technologies can be broadly classified into two categories: *in situ* and *ex situ*. *In situ* treatment techniques are applied on contaminated material on site with minimal disturbance and are generally considered to be the most favoured option. This is due its cost effectiveness and low level of disturbance to the environment since the treatment does not involve excavation and transport of contaminated material (USEPA, 1999). However, *in situ* treatment has its limitations, i.e. due to low mixing and aeration, treatment is limited by the depth. In the soil environment this range spans usually from uppermost few centimeters to about 30 cm. Moreover, environmental conditions are hard to control for optimal biodegradation *in situ*. In e*x situ* technologies, the matrix is physically dislocated for treatment either by excavating (soil) or pumping (water) (Vidali, 2001). The decision on the treatment technique is based on the site characteristics, the volume to be treated and availability of a suitable technology. The bioremediation technologies currently in use, the benefits and limitations of the respective process and important factors to be considered in conducting the process are listed in Table 1.

Table 1. Bioremediation technologies at a glance

Technology	Example	Benefits	Limitations	Important Factors to Consider
In situ	• Biostimulation • Biosparging • Bioventing • Co-composting • Fertilization	• Cost effective • Organisms non-invasive • Less disturbance to the site	• Environmental limitations • May not be the most effective • Bioavailability • Diffusion and mass transfer problems • Long duration	• Efficiency of the indigenous microorganisms • Presence of other toxic compounds • Conducive environmental parameters • Soil type • Distribution of pollutants • Contaminant concentration
	Bioaugmentation	• Cost effective • Adds the most efficient degrading organism	• Organisms could be invasive • Bioavailability, • diffusion and mass transfer problems • Problems in surviving and establishing in a novel environment • Environmental limitations • Long duration	
Ex situ	• Land farming • Composting • Biopiles	• Cost effective • Encourages native microbial growth	• Site disturbances • Space requirement • Need to control abiotic loss	As above
	• Bioreactors • Slurry reactors • Aqueous reactors	• Rapid degradation • Optimized conditions • Enhanced mass transfer • Increased bioavailability	• Site disturbances • High capital and maintenance cost • Technical expertise • Limited capacity	• Toxicity of amendments • Toxic pollutants • High demand on energy • Costly maintenance
Addition of GMOs	• Plants • Microorganisms	• Greatly enhanced degradation capacity • Optimized organisms or their products • *Ex situ* or *in situ* applications	• Costly • Expert handling • Establishment and persistence in the environment for the duration of the process questionable. • Negative public perception	• Need for containment • Possibility of horizontal gene transfer • Emergence of antibiotic resistant microorganisms

Irrespective of the fact that whether the technology is applied *in situ* or *ex situ,* bioremediation approaches for oil spills fall into three major categories:

- Stimulating the growth and metabolism of locally available microbial community (Biostimulation);
- Introduction of microorganisms or consortia selected for the purpose (bioaugmentation). This also includes the use of microbial products such as enzymes and biosurfactants; and
- Introduction of genetically-modified microorganisms (GMMs), which are designed genomes to contain improved oil-degrading properties.

Most bioremediation systems for petroleum oil contaminated soil are run under aerobic conditions.

However, in selecting a technique the degree of saturation of the pollutant and the level of aeration of the matrix are the major factors to be kept in mind.

Biostimulation

Stimulation of native flora through the lifting of barriers limiting luxuriant growth is called biostimulation. Addition of decomposable carbon compounds, vitamins and other growth factors, increased aeration, pH control, etc. facilitate microbial growth and activity so that they can degrade the waste faster. As hydrocarbons have a high proportion of carbon, hydrocarbon contamination may put a high demand on available sources of other major inorganic nutrients limiting degradation (Sang-Hwan, Seokho, DaeYaeon, & Jeong- gyu, 2007). Hence lifting growth limitation by providing such nutrients is a necessity. The biggest advantage of biostimulation is that the main role is played by the native microflora. Natives are well-suited to the existing environmental conditions of the site and are spatially distributed throughout the matrix according to their own preferences. In this approach, facilitating microbial degradation while enhancing environmental factors and improving contaminant characteristics are important facts. Techniques employed for the stimulation of indigenous microorganisms through facilitating their growth and metabolism includes composting, bioventing, biosparging, land farming and biopiles.

Problems Associated with Biostimulation

Rosenberg et al., (1992) raised an important question regarding environmental safety of enriching oil contaminated sites with large quantities of nutrients in the form of fertilizers. Yet, rigorous monitoring of the Exxon-Valdez cleanup effort in 1989 did not substantiate this doubt on 'fertilizer toxicity' on sensitive marine species. The other concern is eutrophication of aquatic environments due to excessive addition of nutrients, which also proved foundationless (Atlas, 1995). On the other hand, long-term exposure of indigenous microorganisms to biodegradable waste could result in the selection and proliferation of an efficient degrader flora. This is because a shift in native microbial diversity towards an oil degrading community is inevitable in the presence of oil as the main carbon source. Although this leads to a noticeable shift in community structure (Evans et al., 2004), it does not necessarily mean a complete wipe out of the original flora. Moreover, microbes always occur in consortia where occurrence of complex interactions among the members of the community is a characteristic. As microbial degradation products can support the growth of other microbes in a particular matrix, the growth of non-degrading microbes is also supported ensuring their continued existence albeit at a lower density.

Bioaugmentation

When the native microflora is not in sufficient densities or inefficient in their activity, introduction of effective microorganisms at a higher density would be a favourable option. Once the oil is used up by these organisms they have no advantage over the native microorganisms and eventually, may decrease in numbers and disappear. The increased efficiency of the system is a direct result of the elevated density of bacterial cells, which accompanies an increased microbial growth and activity. Towell et al., 2011 investigated the microbial degradation of hexadecane, octacosane, phenanthrene and pyrene using ^{14}C-labelling. Although the observed ability of native organisms to mineralize ^{14}C-target hydrocarbons was low ($\geq$ 16%), the study clearly indicated the potential of indigenous microbial community to degrade target contaminants actively and extensively.

Concerns over Bioaugmentation

Bioaugmentation, enriches the existing microflora with the addition of specially selected microorganisms, either indigenous or exogenous to the site of contamination. The success of bioaugmentation programmes cannot always be anticipated as field conditions are far more diverse and dynamic than those in the laboratory and green house. Anyway, after the completion of the degradation process, slow conversion to a climax microbial diversity may take place although the same community structure may or may not be established. But neither the impact of added organisms on native microflora nor the establishment of an apex community after the degradation is accomplished have been assessed so far. On the other hand, augmented organisms may face difficulties in surviving and establishing in a fresh environment where competition from the native organisms and hostilities of the site environment confronting them. In such instances, without any doubt, native organisms would be the better performers (Towell et al., 2011). Based on the fact the added population having a very high cell density compared to those of natives, they may out-compete the natives. In this process, an irreversible change in the soil microbial community structure may occur (Bundy, Paton, & Campbell, 2004).

Use of Genetically Modified Organisms for Bioremediation

Development of genetically modified organisms (GMOs) primarily focused on plants due to its applicability in developing plants resistance for biotic and abiotic stresses. But, currently GMOs are produced for a multitude of purposes. Among all organisms, bacteria are the most manipulative organism for genetic engineering and hence have been modified genetically to express various characteristics. With respect to bioremediation, GMOs have been developed for: (1) monitoring the process and tracking the added bacteria; (2) measuring the presence and bioavailable concentration of contaminants; (3) designing organisms with improved efficiency or altered degradation capacity; and (4) measuring the toxicity level of the contaminant. Bacteria with genetic modifications introduced to fulfil various requirements are listed in Table 2.

Process Monitoring and Tracking the Persistence of Organism

Detecting the course of biodegradation is essential in order to ascertain the rate of degradation and determine the end point of the process. Measuring the rate of biodegradation in the field is extremely

Table 2. Genetically modified bacteria used in hydrocarbon bioremediation

Genetically Modified Bacterium	Purpose of Development	Gene Introduced	Reference
Pseudomonas fluorescens HK44	Tracking introduced organisms	*luxCDABE*	Sayler and Ripp 2000
Pseudomonas putida PaW340(pDH5)	Perchlorobenzoic acid in soil	pDH5 plasmid	Massa et al., 2009
Pseudomonas putida KT2442(pNF142::Tn*Mod*-OTc)	Improved degradation of naphthalene and tracking	pNF142::Tn*Mod*- OTc plasmid, *gfp*	Filonov et al., 2005
Pseudomonas putida PaW85	Petroleum	pWW0 plasmid	Jussila et al., 2007
Escherichia coli AtzA	Atrazin degradation	atrazine chlorohydrolase	Strong, McTavish, Sadowsky, & Wackett, 2000
Burkholderia cepacia L.S.2.4	Improved toluene degradation	pTOD plasmid	Barac et al., 2004
Burkholderia cepacia VM1468	Toluene degradation	pTOM-Bu61 plasmid	Taghavi et al., 2005
P. fluorescens F113rifpcb and *P. fluorescens* F113L 1180	Monitor rhizosphere community shifts and rhizoremediation	*Bph*	Liu et al., 2007
Comamonas sp. strain CNB-1	Rhizosphere competence and rhizoremediation	*Gfp*	Liu et al., 2007

difficult due to the fragmented nature of the contamination and the fact that there are other paths through which the contaminant can be lost from the system. Additionally, the contaminant is a complex mixture of compounds having different structures, toxicities, solubility levels, degradability characteristics and breakdown products (McMillen et al., 1995). Use of internal standards, for an example calculating the initial ratio of easily degradable components to that of slowly degrading compounds of the contaminant and comparing it with the same at a later stage of the degradation process has been suggested by Atlas (1995). These ratio-based techniques have provided researchers with a tool to observe biodegradation rates *in situ* (Atlas 1995). As these techniques involve are very tedious tasks, development of more biologically-oriented monitoring processes that can demonstrate the mitigation of biological effects and reduction of risks have been stressed by a number of authors (Stroo et al., 2000).

The development of microbial indicators for *in situ* detection of bioremediation is a revolutionary step made feasible by recent advances in genetic manipulation. Use of biosensors, especially bacterial biosensors, to monitor the presence of the contaminant and its bioavailability is the most favoured detection method at present. These biosensors have a genetic makeup consisting of promoters from genes of which the expression is induced by the presence of the pollutant of interest (Simpson et al., 1998). These promoters in turn induce the reporter genes to which it is fused to. The expression of those genes will result in the production of detectable proteins that can be easily monitored and quantified. The bioluminescent genes luc, lux and gfp together with its derivatives are the most commonly used reporter genes for this purpose (Ripp et al., 2000). The bacterium *Escherichia coli* K-12 strain carries the promoter of the *xylS* gene from *P. putida.* This promoter is induced by the presence of aromatic hydrocarbons in the immediate environment of the organism. The promoter has been fused to a promoterless luciferase operon

obtained either from firefly or the bacterium *Vibrio harveyi* (Ripp et al., 2000). When the expression of luciferase gene is induced by the presence of aromatic compounds, light is emitted as a result. The intensity of the emitted light can be quantified and it correlates with the amount of hydrocarbon present.

Although field level data regarding the use of GMOs are scanty, Ripp, et al. (2000) reported an intermediate-scale field release of a genetically engineered bacterium, *Pseudomonas fluorescens* HK44 to demonstrate the ecological fate and capabilities of the released organism in the monitoring and control of poly-aromatic hydrocarbon (PAH) degradation. The success of this bioindicator provided a real-time detection of contaminant bioavailability and delineated the biodegradation process.

Designing Organisms with Improved/Altered Degradation Capacity

Although the organism is competent in remediation, the speed with which it works needs to be increased in certain processes. In enzyme catalyzed metabolic pathways, the rate of the whole process is dependent on the step mediated by the enzyme having the lowest substrate use efficiency. Hence, identifying such enzymes or the regulatory step of the pathway is a crucial element in improving metabolic processes. This identification should be followed by experimental elevation of the activity of the rate-limiting enzyme/protein through detecting the increase in transcriptomics, or kinetic properties (Timmis, & Pieper, 1999). Being hydrophorbic most fractions of petroleum oil, have a low bioavailability making it difficult to be degraded. Using molecular tools it is possible to improve bioavailability by constructing bacteria to include the ability to produce surfactants. Coupling high degradative function with biosurfactant production may lift such restrictions in nature.

Equivalent environmental conditions cannot be found on a global scale and controlling such factors is not also easy. Hence, constructing genetically modified microorganisms (GMMs) that function well in extreme conditions is another possibility. Such a combined strategy of ecological, microbial, and environmental biotechnology can overcome the problems facing *in situ* bioremediation (Kalam, Amin, & Sidik, 2014).

Timmis & Pieper, (1999) discussed the design of improved biocatalysts in detail. Accordingly, the designing should involve different aspects of optimization: (i) creating entirely new metabolic pathways that were not present originally, (ii) expanding the range of utilizable substances of existing pathways enabling utilization of diverse compounds, (iii) avoiding substrate misrouting into routes that are unproductive or toxic or makes highly reactive intermediates, (iv) improving the substrate flux through pathways to avoid piling up of inhibitory intermediates and feedback inhibition, (v) increasing the stability of genes introduced for enhanced degradative activities, (vi) increasing the solubility and bioavailability of hydrophobic pollutants, and (vi) improving in general the relevant properties related to the process of degradation by the microorganisms in concern.

Measuring the Toxicity of the Contaminant

Lux marked biosensors have been developed to detect the toxicity of the contaminant in the matrix. This is an important process as bioremediation is a secondary treatment process utilized in sites that can support the microbial and plant growth. On the other hand, the same biosensor can be employed for process monitoring as survivability is increased with decreased toxicity due to biodegradation. Bundy et al., (2004) monitored the change in toxicity of paraffin and motor oil applied soils using two luminescent bacterial bioassays: *Vibrio fisheri* and *Pseudomonas putida*. Concomitant monitoring with phospholipid

fatty acid profiling was in agreement with the bioassay results showing that luminescent bacteria can be successfully applied to detect toxicity of the contaminant which will aid in selecting and implementing a suitable strategy.

Problems Associated with the use of Genetically Modified Organisms

Recently, genetically modified organisms (GMOs) have been the focus of many public activists casting shadows on the safe and healthy use of such organisms. Moreover, ethical and political issues impede the development of the field of study. Apart from such obstacles, the strategy itself is having an inherent set of problems, which are detailed below.

Survivability in the Environment

The GM microorganisms introduced to a novel environment where a diverse biological community is in a complex interactive mode, the GM organisms must compete with this native flora for its survival and establishment and the ways with which it needs to interact is poorly known. Additionally it is usually implanted in multiphasic, heterogeneous matrices. For an example, soil is an assemblage of solid, liquid and air phases where oils could be present as dissolved material in soil water and lipids, vapours in soil atmosphere and adsorbed onto soil particles and organic matter. Worsening the situation, it has to face a multitude of poorly controllable external parameters, some of which may impose considerable stress to the organism (Cases, & De Lorenzo, 2005). Therefore, it is needed to tailor-make an organism which is not only be able to efficiently degrade the contaminant, but also be better suited for competition with other biotic components and adapted to stressed environmental conditions (Table 3). Otherwise, a total failure may be resulted due to disappearance of the introduced organisms. This genetic drift of introduced organisms has been demonstrated by Rainey, & Travisano (1998). Work conducted on two common bacteria; *Escherichia coli* and *Pseudomonas*, has pointed out that the non-homogeneous nature of natural niches may exert a selection pressure on the organisms and chooses the best suited strains for the niche parameters. The result is the evolution of genetically distinct populations that diverged from the initial genetic makeup of the inoculated population. In view of all this, Cases, & De Lorenzo (2005) raised two important questions: "What determines the correct expression of a desired catabolic pathway in time and space? Is it possible to engineer and release GMOs with a high degree of biotechnological performance and predictability?" The answer lies in comprehending the general rules of microbial physiology and ecology and its alignment in the process involved. Additionally, the survival rate of introduced bacterial species could be improved by the use of strains that have a selective advantage over others, such as strains supported by plants: rhizosphere competent microflora. In this context, the possibility of transferring *P. putida* plasmid pWW0 carrying genetic makeup for petroleum hydrocarbon degradation into rhizosphere bacteria has been reported by Jussila, Zhao, Suominen, & Liodström, (2007).

Negative Public Perception

As GMOs are organisms with modified genetic capacity, public concern towards the use of such organisms is largely negative. Active microbial cells exchange their genetic material through conjugation, transduction, and transformation (Jussila et al., 2007). Transformation does occur in nature albeit at a lower frequency. Therefore, DNA interacts with other organisms in the environment while still within

Table 3. Environmental conditions affecting microbial degradation (Sources: Rawe, Krietemeyer, & Meagher-Hartzell,1993; Vidali, 2001; USEPA, 2006)

Parameter	Condition Required	Optimum Value for Oil Degradation
Soil moisture	25-28% of water holding capacity	30-90%
Soil pH	5.5-8.8	6.5 – 8.0
Oxygen	Aerobic; minimum air filled pore space of 10%	0.3 g oxygen for each gram of oil oxidized
Nutrient content	Carbon, nitrogen, phosphorus, d potassium	C, N, P, and K is 100:15:1:1
Temperature	15-45 ^{0}C	20-30 ^{0}C
Contaminant	Not too toxic	Hydrocarbon 5-10% of dry weight of soil
Heavy metals	Total content 2000 ppm	700 ppm
Soil type	Low clay or silt content	

a live cell or existing as free DNA liberated from dead material. Although such integration in nature needs timely fulfillment of a number of requirements for successful integration and expression, this mere thought of unintended genetic transformation seems to be scary for the general public.

Spread of Antibiotic Resistance

Antibiotic resistance is incorporated into the genetic element used in transformation as a marker gene that aids in selecting transformed cells. Usually, the antibiotic used as markers are the ones that are not used for therapeutic purposes. Also, the use of therapeutic antibiotics in sub lethal levels in animal feeds has increased the bacterial community fraction with resistivity to such antibiotics, which should be of more concern to the authorities.

Containment of Genetically Modified Organisms

Containment is the physical restriction of the GMO (the modified gene) within the site it was introduced. This is essential in preventing horizontal gene flow into the native flora. Most molecular approaches for the containment of genetically modified plants have been mainly focused on male and seed sterility and maternal inheritance (Daniell, 2002). Other containment strategies that can be used in restricting the gene flow between the GMO and the natives such as cleistogamy, apomixis, chemical induction/deletion of transgenes, transgenic mitigation, genome incompatibility, etc. have been identified. However, it should be noted that no strategy has proved broadly applicable to all crop species, and a combination of approaches may prove most effective for engineering the next generation of GM crops (Daniell, 2002).

Release of GMMs present a different perspective with respect to containment. Once they are released into the environment, microorganisms spread to the neighbouring sites with wind and water. Additionally, *in situ* monitoring is difficult making it impossible to be recalled or removed selectively. Hence, for containment of the modified genes, suicide functions can be inserted together with the gene/s of interest. Such a suicidal GEM could be designed to remain viable and active only under predetermined specific environmental conditions or to die if escaped from that location (Steidler et al., 2003). Alternatively, a GMM could be designed to survive until a specific environmental signal that would trigger its death occurred or was introduced (Bej, Saul, & Aislabie, 2000)

Obtaining Government Permission on Release of GMOs

The potential impact of GMOs on the environment and animal, plant or human health is very complex and has not been understood fully. Hence, risk assessment of GMOs follows a very stringent procedure necessitating long duration field trials with extensive data collection. Bioremediation is not a very profitable industry that attracts large investments. This leads to an overall aversion to GMO implementation in environmental systems. Researchers satisfy themselves by concentrating on optimizing and commercial development of naturally occurring microorganisms rather than developing GMOs which puts a very high demand on time and resources. The intended use in this case is cleaning up of already contaminated sites that need speedy recovery in which a removal of threats is anticipated. Therefore, gaining public trust is more likely with this kind of introduction of GMMs.

LIMITATIONS OF BIOREMEDIATION

Although bioremediation attracts attention as a low cost and greener alternative for oil spill cleanups, it has a set of inherent limitations. Understanding limitations may help to improve the process efficiency by addressing them in a more specific manner.

Treating Cold Environments

The cold ecosystems are adapted to harsh environmental conditions with unique biota. As these environments are very sensitive to environmental changes, the same level of pollution by a respective contaminant may exert a greater impact on cold ecosystems than on the other ecosystems (Snape, Riddele, Filler, & Williams, 2003). Temperature has a great impact on the rate of all metabolic reactions including degradation. Hence in colder environments biodegradation is a relatively slow process and the success of bioremediation depends on toxicity, adaptability to freeze-thaw processes, availability and concentration of oxygen and nutrients, and presence of electron acceptors (Si-Zhong et al., 2009). Low temperature increases viscosity of oil, decreases water solubility of medium molecular weight compounds, reduces evaporation of low molecular weight volatiles, and delays onset of biodegradation (Margesin, & Schinner, 2001). Additionally, low temperature affects the composition of microbial communities (Atlas, 1981), as well as the mass transfer of substrate and/or electron acceptors (Aislabie, Saul, & Foght, 2006). Apart from low temperature, the other crucial limitation for bioremediation failures in cold ecosystems is oxygen. This is a common constraint as oxygen is scarce in soil water and diffusion is partly or completely blocked due to low temperature. Therefore, oxygen transport which determines its distribution within the matrix is considered to be the rate-limiting step in bioremediation mediated by aerobes. It has been calculated that the mass of oxygen utilized in hydrocarbon remediation is to be around 0.3 g oxygen for each gram of oil oxidized (Atlas, 1981).

Although the microbial degradation activity proceeds at very low rates at sub-zero temperatures, many species face difficulties in withstanding freezing and thawing. At temperatures below -12 °C, bacteria cease growth and metabolism altogether due to the formation of intracellular ice crystals interrupting metabolism (Margesin, & Schinner 1999). On the other hand, low temperatures are not detrimental to organisms always. Elevated temperatures may facilitate volatilization and solubilization of some hydro-

carbons, enhancing their toxicity within the immediate environment of the organism which may delay the onset of degradation (Niehaus, Bertoldo, Kahler, & Antranikian, 1999). Less water solubility of hydrocarbons at lower temperatures decreases the potential toxicity of such compounds.

Limited Bioavailability of Hydrocarbons

Since oils are water immiscible, increasing its availability is an important step in the bioremediation process. Additionally, the added or indigenous microorganisms must come into contact with the hydrocarbons for the membrane-bound oxygenases to commence degradation. The bioavailability of oil in water can be highly elevated by emulsification (Ron, & Rosenberg, 2002). Hence additives are used to enhance the efficiency of extracting NLPLs. The most favoured characteristics of a good additive are: low toxicity, biodegradability and efficiency in extracting the pollutant (Mulligan, Yong, & Gibbs, 2001). Surface-active agents that reduce the surface tension of a water immiscible liquid, increasing its wetting properties and spreading can facilitate the biodegradation process by several folds. Such surface active molecules are called surfactants and those produced by organisms are called biosurfactants. A number of researches focused on introducing surfactants alone or microorganisms producing biosurfactants *in situ* reported varying degrees of success (deGusmão, Ruffino, & Sarubbo, 2010).

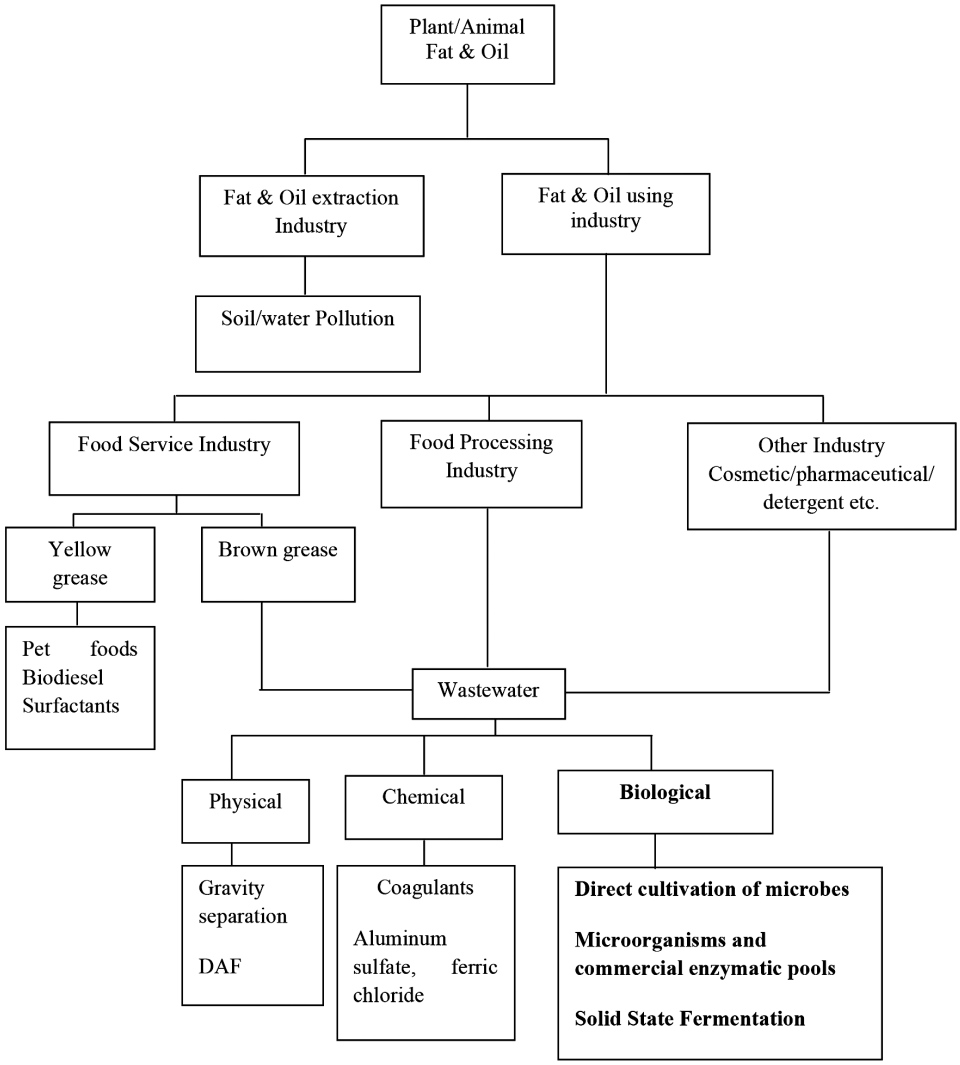
In general, a surfactant molecule contains a hydrophorbic portion that has an affinity towards water and a hydrophilic portion that shows affinity towards non-aqueous phase. This causes the molecules to gather at the interfaces between droplets of water and those of oil, or lipids, causing emulsification of oils (Mulligan et al., 2001). Although a large number of chemical surfactants of petrochemical origin are available, surfactants of biological origin are more favoured. The qualities that make the biosurfactant superior over the chemical surfactants are: low toxicity, better environmental compatibility; higher biodegradability, higher foaming; high selectivity and specific activity at extreme temperatures, pH, and salinity (deGusmão et al., 2010). Furthermore, biosurfactant producing microorganisms can be grown on cheap substrates lowering the cost of production.

Microbial bioremediation, irrespective of the technology needs nursing the microbes to support their growth. External intervention in times of need is a necessity. Yet, a carefully controlled balance is needed to be maintained in order to prevent further pollution due to the intervention. Using a natural source capable of supporting microbial growth *in situ* is one of the major requirements to reduce the cost of the process with simultaneous increase in effectiveness. Plant assisted microbial degradation which is termed as phytoremediation is the best alternative available currently.

BIOREMEDIATION OF PLANT/ANIMAL ORIGIN OIL CONTAMINATED ENVIRONMENTS

Plant/animal fats and oils are released into the environment either by spills or as waste/used oils. These fat and oils are primarily linked with oil extraction (palm, olive coconut etc.) and oil using industries such as food service, food processing, cosmetics, pharmaceutical, etc. (Figure 2). Bioremediation of fats and oils by means of lipophilic microbes in wastewater and/or enzymatic applications such as lipase has been in a concern of many scientists in past few decades. Hence, microbial lipases are in the focal point of oil bioremediation research and development of associated enzyme technologies.

Figure 2. Soil and water pollution caused by plant/animal fat and oil and treatment methods



Bioreactors

For biological treatment of high fat and oil containing wastewater, a single or mixed culture of lipolytic microbial flora are cultivated in bioreactors in the form of attached or suspended growth (Table 4). Meantime, reactor condition is enhanced to facilitate the best performance of microbes. For instance, Wakelin, & Forster (1998) treated two fast food restaurant wastewaters using a consortium of gram negative bacteria which had been isolated from grease trap residues from fast food restaurants and activated sludge. In this study a novel bioreactor, the weir tank reactor was employed, which achieved a high removal rate (84±96%) of fat, oil and grease. Immobilizing bacteria in porous material or provision of substrate for attachment proved to better in enhancing fat and oil degradation over suspended culturing (Keenan, & Sabelnikov, 2000).

Table 4. Bioaugmentation (direct cultivation of microbes) approaches to treat restaurant wastewater with high oil and grease contents

Type of Wastewater	Microorganisms	Reference
Restaurant wastewater	lipophilic yeasts (*Candida intermedia, Candida schatavii, Candida visuvanathii, Candida fluvatilis, Candida pseudolambica)*	Anon, 1994
Restaurant wastewater	Pure and mix cultures of *Acinetobacter sp., Rhodococcus rubra, Nocardia amarae* and *Microthrix parvicella*	Wakelin & Forster, 1997
Restaurant wastewater	Pure culture of *Pseudomonas aeruginosa*	Dharmsthiti & Kuhasuntisuk, 1998
Restaurant Wastewater	Mixed culture of 15 bacterial isolates	Tano-Debrah, Fukuyama, Otonari, Taniguchi, & Ogura,1999
Restaurant Wastewater	Mixed culture composed of *P. aeruginosa, Acinetobacter calcoaceticus* (both lipase-producing bacteria) and *Bacillus* sp. (an amylase and protease producing bacterium)	Mongkolthanaruk, & Dharmsthiti, 2002

Although in reported literature it was able to achieve high removal efficiency, most of these investigations have been done under controlled laboratory conditions. Therefore, it is timely to design and conduct pilot scale investigations to make industrial applications realistic and effective.

Biological Formulations

Meanwhile, development of microorganisms and/or enzymes pools for the biological treatment of effluents with high fat and oil concentrations came into research platform (Table 5). Consequently, patented catalytic formulations and bio-additives having the ability to break down organic contaminants rapidly into usable forms have been introduced (Mendez et al., 2005). On the other hand, in economic point of view, use of such products in intensive industrial applications depends on the reduction of production costs (Sharma, 2012). Therefore, research for selection of highly productive strains, and development and optimization of the product to be traded are needed.

Solid State Fermentation

Use of microbial enzyme preparations obtained by solid-state fermentation (SSF) to treat food industry wastewater received great attention. As solid state fermentation systems are able to use cheap organic waste material as substrate, need for relatively small space in comparison with the product yield, high volumetric productivity, and direct applicability (Cammarota, & Freire, 2006), this has been tested on different industries having diverse and relatively high fat and oil contents in wastewater (Table 6).

Co-Digestion

In the recent past, investigations conducted based on bioremediation of waste/used oil have opened up new avenues for other applications such as for biofuel and biogas production in conjunction with co-digestion as energy recovery option. Waste cooking oil augmented with *Penicillium chrysogenum* yielded a high acid value of 26.92 mg/g, indicating the presence of free fatty acids which has a potential

Table 5. Use of microorganisms and/or commercial enzymetic pools (patented) for the treatment of effluents with high oil and grease contents

Enzymes/Product Name	Microorganisms	Reference
Hydrolases (proteases, amylases, lipases and cellulases)	*Bacillus subtilis*	Cail, Barford, & Lichacz, 1986
FR pat.2, 659.645	Lipase producing bacteria	Cammarota and Freire, 2006
Enzyme complex	*Aerobacter aerogenes, Bacillus subtilis, Cellulomonas biazotea, Nitrosomonas sp., Nitrobacter winogradskyi, Pseudomonas stutzeri*	Cammarota and Freire, 2006
Amerzyme-A-100	*Bacillus subtilis*, a protease and amylase producing bacterium, and *Aspergillus niger*, a lipase, cellulase and pectinase producing bacterium.	Mendez, Castro, Pereira, Furigo Jr., 2005
Hydrolases, lipase, protease and amylase	*Penicillium restrictum*	Cammarota et al., 2003
Lipolytic enzymes	Microbial consortium derived from a mixture of wastewater sludges	Santis-Navarro et al., 2011
Lipase	*Pseudomonas aeruginosa* LB-2	Parihar, 2012
Lipase	*Penicillium chrysogenum* SNP5	Kumar et al., 2012

Table 6. Environmental applications of hydrolases

Microorganism	Wastewater Source/ Characteristics	Reactor Type	Reference
P. restrictum	Dairy effluent Organic load: 4.0 kg COD/m^3	Up-flow anaerobic sludge blanket bioreactor (UASB)	Cammarota, Teixeira, & Freire, 2001
P. restrictum	Dairy effluent Oil and Grease: 250 mg/L.	Anaerobic reactor	Leal, Cammarota, Freire, & Sant'Anna Jr., 2002
P. restrictum	Dairy effluent Oil and Grease: 400, 600, 800 mg/L	Batch activated sludge systems	Jung, Cammarota, & Freire, 2002
P. restrictum	Dairy effluent Oil and Grease: 400–800 mg/L	Continuous activated sludge reactor	Rosa, 2004
P. restrictum	Poultry slaughterhouse Oil and grease: 150–1200 mg/L	Anaerobic reactor	Valladão, Freire, & Cammarota, 2007
P. restrictum	Dairy effluent 1200 mg of oil and grease/L	Anaerobic reactor	Rosa et al., 2009
P. restrictum	Poultry slaughterhouse 800 mg oil and grease (O&G)/L	Anaerobic reactor	Valladão, Torres, Freire, & Cammarota, 2011
P. simplicissimum	Fish processing plant wastewater: 1500 mg oil and grease (O&G)/L.	Up-flow anaerobic sludge blanket bioreactor (UASB)	Alexandre, Valente, Cammarota, & Freire, 2011

for biofuel production (Kumar et al., 2012). Attempts to increase methane yield from mesophilic anaerobic digestion of fats recorded a total fat removal over 88% with a methane yield of 58% (Fernández, Sánchez, & Font, 2005).

PHYTOREMEDIATION

Phytoremediation is a strategy where higher plants and/or their products are used to remove, stabilize and/or degrade soil and water contaminants (Wenzel, 2009). It has been the major focus of interest for the last two decades. Currently, the technology is used for treating many classes of contaminants in soil and fresh waters. Plants are selected based on the nature and concentration of the contaminant, growth rate of the plant, ability to produce a large spreading root system, ability to tolerate the presence of the contaminant in the immediate environment, rate and the extent of the degradation expected and the ability to adapt to the environmental conditions of the site (Wenzel, 2009). Plants and/or their products can be employed in several different ways as discussed below (Susarla, Medina, & McCutcheon, 2002).

As phytoremediation involves plant as well as associated microflora, employing phytoremediation for cleaning up of oil spills demands a better understanding of the complex interactions in the rhizosphere. The roots exude an array of organic carbon compounds which sustain a selected microflora community on and around the roots. This phenomenon, which is called the rhizosphere effect, is the cumulative effect of the metabolisms of rhizosphere microorganisms and plant roots. Some of the available information on the use of plants for phytoremediation is given in the Table 7.

Phytoremediation has a number of advantages over the other techniques of bioremediation. As per Glick (2003), the advantages are: (1) Preservation of the natural structure and texture of the soil; (2) No demand on energy as it is primarily derived from sunlight; (3) Ability to achieve high levels of biomass and genetic diversity in the soil; (4) Provision of substrates to induce co-metabolism; (5) Counteracting the downward flow of contaminant; (6) Low in cost; (7) Aesthetically appealing; (8) Ecologically less disturbing; and (9) Having the potential to be rapid. With all these bonuses, phytoremediation alone is not significantly faster than micro mediated bioremediation. Hence more research is needed in ascertaining the factors governing the establishment of an efficient plant microbiome.

FUTURE RESEARCH DIRECTIVES

Plant/Animal Oil Contamination Removal

In this section, the review of bioremediation of environments contaminated with plant/animal oil has covered various means of microfloral applications as well as their field level applications. Based on the above review, following future research directions can be recommended for industries of producing wastes with a high content of far and oil:

- Investigation of low-cost technologies for large-scale industrial applications.
- Combining bioremediation with genetic and metabolic engineering.
- Advance research on enzyme technology.
- Extensive and continual screening for efficient microbial strains from natural environments.

Table 7. Plant species tested for hydrocarbon degradation capacity

Plant Species	Matrix	Reference
Vicia faba L. (Broad bean)	Total petroleum hydrocarbon, diesel in soil	Yateem, Al-Sharrah & Bin-Haji, 2008; Kaimi, Mukaidani, & Tamaki, 2007
Lolium perenne L. (Rye grass)		
Vicia sativa L. (Common vetch)	Diesel in soil	Adams and Duncan, 2002
Glycine max (L.) Merr. (Soy bean)		Dominguez- Rosado & Pichtel, 2004
Phaseolus vulgaris L. (Common bean)		
Helianthus annuus L. (Sunflower)		
Brassica juncea (L.) Czern. (Indian mustard)		
Trifolium pratense L. and *T. repens* L. (Clover)		
Eichhornia crassipes (Mart.) Solms (Water hyacinth)	Petrolium hydrocarbons in water	Ndimele, 2010
Andropogon gerardii Vitman (Big Bluestem)	Total PAH in soil	Aprill, & Sims, 1990
Schizochyrium scoparius (Little Bluestem)		
Sorghastrum nutans (L.) Nash(Indian grass)		
Bouteloua curtipendula (Michx.) Torr. (Sideoats gramma)		
Bouteloua gracilis (Willd. ex Kunth) Lag. ex Griffiths (Blue gramma)		
Panicum virgatum L. (Switchgrass)	Total PAH	Aprill, & Sims 1990; Reilly, Banks, & Schwab, 1996
Buchloe dactyloides (Nutt.) Engelm. var. *Prairie* (Prairie buffalograss)	Naphthlene in soil	Qiu, Leland, Shah, Sorensen, & Kendall,1997
Buchloe dactyloides (Nutt.) Engelm. (Common buffalograss)	PAH	
Zoysia japonica Steud. var. *Meyer* (Meyer zoysiagrass)		
Panicumcoloratum var. *Verde* L. (Verde kleingrass)		
Medicago sativa L. (Alfala)	PAH in soil	Reilly et al., 1996
Festuca arundinaceae Schreb. (Tall fescue)		
Sorghum vulgare L. (Sudangrass)		

Phytoremediation

While acknowledging the limitations, overcoming challenges for better utilization of this technology need to be encouraged. There is a need to critically assess the reasons for failing the trials of extending phytoremediation results of laboratory and green house into the field. Gerhardt, Huang, Glick, & Greenberg, (2009) identified two reasons: (1) Imposing of significant stress by field environmental conditions on plants (2) Inability of the currently available methods of assessment to reflect the actual scenario. As this may undermine the efforts of a large number of bench work research and create negative perceptions, the efforts need to be dealt with caution. Also one should not forget the fact that, although the term phytoremediation describes the use of plants in bioremediation there is an integral role played by the plant associated microbiome: Phyllospheric, rhizospheric and endophytic microflora. Hence, any research effort to improve phytoremediation needs to focus on this inseparable plant-microbe association or plant microbiome.

Converging Phytoremediation with Microbial Remediation

Although plants with high biodegradative capacities are introduced into the hydrocarbon polluted sites, in the presence of the contaminant plant growth is generally reduced. Hence, attainment of a sufficient biomass for degradation to be completed within a reasonable time frame would not be possible. Therefore, mitigation of plant stresses in contaminated soil should be a priority if phytoremediation is to become an effective and viable remedial strategy. Plant stress could be of abiotic or biotic origin. Soil microorganisms can ameliorate most stress situations: mycorrhizae can alleviate water stress; plant growth promoting rhizobacteria (PGPR) produce a number of substances that enhance plant growth; biological control agents safeguard plants from pests and disease causing organisms; degraders mineralize toxic compounds, etc. So far, most experiments examining the mechanisms of plant-microbe interactions deal with only a single host-single microorganism interaction. The rhizosphere is an area of high microbial activity and contains billions of microbes including PGPR, pathogens and microfauna in a complex network of interactions (Badri, Weir, Daniel van der Lelie, & Vivanco, 2009). Hence, further studies should be directed towards unraveling these multiplex interactions at a molecular level in order to explore the feasibility of converging phytoremediation with microbial remediation.

Rhizosphere competency of contaminant degraders to efficiently colonize the growing roots is a fundamental requirement of successful rhizoremediation. Plant roots are a dynamic entity and plant-associated bacteria need to migrate from the bulk soil to the rhizosphere of growing root. Successful establishment leads to aggressive colonization of the rhizosphere and root surface (rhizoplane). Only the rhizosphere competent microorganisms can effectively colonize and utilize rhizodeposited compounds. Numerous bacterial traits such as recognition of chemical signals, reaching root, attachment to the root surface and colonization etc. are essential steps of the process. With the magnitude of organisms available with their diverse metabolic pathways, there is a great potential in manipulating rhizosphere for a better rhizoremediation strategy. Hence, rhizoremediation will continue to be the focus of laboratory and greenhouse experiments with extended field work to address problems that are currently being encountered.

Exploiting Endophytic Association

Apart from the naturally occurring rhizosphere microorganisms, plant inoculation with endophytic microorganisms may have a definite advantage. Endophytic bacteria have been defined as bacteria colonizing the internal tissues of plants without causing symptomatic infections or negative effects on their host (Badri et al., 2009). They reside in apoplasm or symplasm and are known to promote plant growth through nitrogen fixation, phytohormone production and antagonizing pathogens. Bacteria and fungi are found to be endophytic colonizers and most importantly the same genera are known to exist in association with rhizosphere as well (Ma, Prasad, Rajkumar, & Freitas, 2011). This suggests the existence of a microbial continuum connecting bulk soil to the interior of the plant body. Also, endophytic inoculations are more successful than rhizosphere inoculations as endophytes are not challenged by already existing microorganisms for the same niche and are protected from environmental stresses. Although attention has been paid in employing endophytes and plant growth promotion and plant protection, there is scanty information available on endophyte induced changes in plant function that could affect the hydrocarbon absorption, distribution and degradation in plants. In order to implement the endophyte assisted phytoremediation in the field level, intensive future research is needed on understanding the diversity and ecology of the organisms and their role in relieving plant stress under hydrocarbon pollution.

Development of Sensitive Process Monitoring Methods

Developments of new protocols or improving existing protocols for sampling, process monitoring, and interpretation of the data will make a considerable impact on demonstrating the effectiveness of phytoremediation. According to Gerhardt, et al., 2009, developing cost-effective methods that can differentiate between petrogenic and phytogenic carbon compounds could immensely increase the acceptability of this technology. This will also motivate regulatory authorities to change guidelines to make them applicable for remediation of organic contaminants. On the other hand, one impediment to the field monitoring is the inability to conclusively prove that contaminant removal is solely due to biodegradation and not a concerted effect of biological, physical and chemical processes (Balba et al., 1998). Hence, there is an urgent need to understand which information can be extrapolated across the scales, from laboratory to filed, and which does not (Balba et al., 1998).

Modeling Soil-Plant- Atmosphere Continuum

Due to the complexity of the soil-plant-atmosphere continuum, development of mathematical models may help in understanding the phytoremediation process better. Identifying and quantifying the contribution of the parties that involve in the process may help in the identification of areas to be emphasized in future research. The model PLANTX, developed by Trapp, & McFarlane (1995) describes: (i) the dynamic uptake from soil, solution and atmosphere, (ii) metabolism and accumulation of anthropogenic chemicals in plant organs (roots, stems, leaves and fruits). However, as of yet, the plant physiological, microbiological, hydrological, and environmental controls upon the application of phytoremediation are poorly understood (Ouyang, 2002).

Exploiting Co-metabolism

According to Cunningham, & Berti (1993), co-metabolism is the process by which a compound that cannot support microbial growth on its own is acted upon by microbes in the presence of another growth supporting substrate. Most of the hydrocarbons when provided alone could not be used as the carbon and energy source for microbial growth due to their recalcitrant nature. But when the microbes are being provided with a food substrate to support their growth and survival, they are capable of degrading pollutants. In co-metabolism, an enzyme responsible for the degradation of a natural substrate gratuitously transforms the pollutant. This can be affected naturally in plant assisted microbial remediation where microbes are supplied with rhizodeposited carbon compounds. This phenomenon can be successfully applied in the degradation of recalcitrant hydrocarbons.

Trouble Shooting in Scaling Up

The objective of the studies conducted under controlled conditions in the laboratory or greenhouse is to apply it under field conditions in mass scale. Unfortunately, this extension is often a complex process requiring carefully planned project implementation and management. Field condition carries unique problems often leading to failures. Understanding (i) additional mass transfer mechanisms and limitations

operating at field level; (ii) the presence of multiple phases, contaminants and competing microorganisms; (iii) spatial distribution differences; and (iv) edaphic factors which may hinder bacterial growth may pave the road to success (Sturman, Stewart, Cunningham, Bouwer, & Wolfram, 1995).

Development of Microbial Consortia

A variety of approaches involving designing new or improved biocatalysts: microorganisms and their products, for bioremediation have been extensively studied over the recent years. Understanding microbial ecology and physiology, flora-fauna-microbe interactions in an ecosystem is imperative in this regard. It is a well-known factor that microorganisms preferably live in biofilms rather than as planktons. In this context, a heterogeneous mixture of organisms lives in harmony depending on each other for various needs while keeping their population in check. Therefore, the simplest strategy is improving the biodegradative performance of a consortium through the addition of a 'specialist' organism, rather than concentrating on one key organism (Dams, Paton, & Killham, 2007). Although certain microorganisms such as *Sphingobium cholophenolicum* ATCC 39723 do possess the entire metabolic pathway for the degradation of a certain pollutant, majority of microorganisms does not (Dams et al., 2007). Genetic manipulation is the key to the production of microorganisms with complete degradative pathways. Complicating the scenario more, oils, especially crude oil is a mixture of alkanes, alkenes, aromactic and polyaromatic hydrocarbons. It is impossible for a single microorganism to possess the array of enzymes needed for the degradation of a number of different compounds. This necessitates the congregation of organisms having different capacities and occupying different niches to achieve a single goal. By studying the work conducted addressing various aspects of bioremediation so far, efforts should be taken to come up with a consortium of microorganisms in order to achieve greater reliability, predictability and efficiency in *in situ* bioremediation. However, GMOs for bioremediation has not been commercialized and is not widely sanctioned for release into the environment (Azad, Amin, & Sidik, 2014). In upcoming years cost-efficient, eco-friendly, safe methods that would result in a product with a marketable potential will hopefully evolve.

Overcoming Degradation Limiting Regulatory Processes within a Cell

Gene expression is controlled by other associated cell signals. Transcription of the genes involved in metabolic pathways is generally controlled by positively-acting regulatory proteins which are activated by the cellular signals indicating the detection of the respective metabolites. Therefore, uncoupling the expression of catabolic genes from the cellular signal induction by using artificial regulatory systems may allow considerable flexibility in process control. This is especially so in the case of non-energy yielding metabolism of a pollutant where cells may not allocate energy for biodegradation. Additionally, when gene expression induction requires an addition of a toxic exogenous inducer, the use of artificial regulatory systems or constitutive expression signals may be helpful (Timmis, & Pieper, 1999). Bioremediation rates become unnoticeable when the process proceeds and the level of contaminant become progressively low. Under such situations incorporation of starvation-induced promoters can uncouple gene expression from growth facilitating the continued catabolic activity in under nutrient limiting conditions.

The recent development of the "omics" technologies (genomics, proteomics, metabolomics) and the expected generalization of Systems Biology approaches (Pazos, Valencia, & de Lorenzo, 2003) may allow us to take a fresh look at bioremediation technologies. Systems Biology is about taking a holistic

approach towards complexity. A paradigm shift in bioremedial approaches can be expected with the development of computational and conceptual tools for solving complex biological scenarios that deals the query with a holistic perspective (Cases, & De Lorenzo, 2005). According to Cases, & De Lorenzo (2005), "In this context, the integration of data on the catalytic performance of microbial communities with information on the chemical fate of pollutants will offer a sound scientific basis to eco-engineering of interventions, which are still dominated by trial-and-error, experience-based approaches".

TACKLING REGULATORY ISSUES FOR THE RELEASE OF GMOS

One major obstacle that has to be dealt with caution in designing genetically modified organisms (GMOs) is to face the bureaucratic hurdles involved in obtaining permission for environmental release. These restrictions are at different levels in different parts of the world, certain nations showing more aversion to the technique without reasonable judgments. Yet, it can be expected that with the accumulation of more evidences on safe use such restriction will be eased or lifted in the future.

As the policies and guidelines are in order for greenhouse and field testings of genetically modified plants, it would be more attractive if the focus is shifted towards plants rather than microorganisms. This will help to overcome the legislative barriers pertaining to GMO release. In the development of plants as clean up biosystems, attention needs to be paid on the ability of the plant to selectively support the metabolism and survival of degrading bacteria in the rhizosphere, in addition to improving the ability of plants to take up, sequester or degrade hydrocarbons. Yet, the genetic modification of microorganisms to improve their performance in the rhizosphere holds great promise in bioremediation technologies and hence need not to be abandoned simply because of the restrictions imposed upon release of such organisms in to the environment.

Metagenomics provide an insight to the microbial consortia associated with roots enabling the tracking of responses to compounds released by plants. With the development of techniques such as stable isotope probing (SIP), a clear picture on the type of metabolic pathways in operation and the role of rhizosphere microorganisms in the cleanup process, the support the plant provide to the microorganisms in the form of carbon substrates etc. can be drawn (Prosser, Rangel-Castro, & Killham, 2006). Integration of this wealth of knowledge in the process improvement is the greatest challenge for the future bioremediation researcher. Development of Synthetic Biology successfully recreating the cells/genetic material from scratch can revolutionize biotechnology (Cases, & De Lorenzo, 2005). We are in the dawn of a new era in environmental biotechnology where the most fruitful interfaces will not focus only on microbiology and chemistry. Instead, focus is shifted to engineer complex systems and redesign biological components (Cases, & De Lorenzo, 2005). The success of such new approaches may ultimately make a difference in bioremediation approaches, waste reduction and elimination of industrial pollution, and will lead to a more sustainable future.

CONCLUSION

Oil pollution of soil and waters is an environmental and health hazard that demand immediate attention due to the possibility of large-scale spills to occur anytime in the future. This vulnerability in turn demands efficient and fast bioremediation of environmental pollution. Improved organisms, their genes

or gene products are the areas to be concentrated for devising effective solutions. With this intention, increased use of molecular tools is inevitable in designing more efficient organisms, monitoring the bioremediation process, detecting bioavailability of oils, and measuring toxicity of oils. Research programmes initiated for the development of a 'super bug' or a 'super consortium of bugs' may be futile if proper comprehension of microbial ecology and physiology is not possible. Use of plant-microbe association for bioremediation is a more attractive and aesthetically appealing alternative in the current focus on single species methodologies. Metagenomics, System Biology, and 'omics' may provide an insight to the microbial ecology and gene expression, which will enable us to adopt a holistic approach. With the development of Synthetic Biology creating life from scratch is not a dream anymore. Therefore, redesigning of biological systems is not far and may shed light on different bioremediation approaches.

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KEY TERMS AND DEFINITIONS

Bioaugmentation: Addition of an enriched degrading microbial inoculum to increase the microbial density of a targeted site.

Bioavailability: Free availability of a compound for a microorganism to work on or absorb.

Bioremediation: Use of living organisms, their genes or gene products to reclaim a contaminated site.

Biostimulation: Increasing the activity of residential microflora by providing factors limiting their growth and activity.

Genetically Modified Organism: An organism whose genetic makeup is modified by adding gene/genes of interest.

Hydrocarbons: Open chain or cyclic organic compounds containing carbon and hydrogen, mainly.

Microbiome: An ecological community of microorganisms sharing the plant/animal body by living within or colonizing surfaces.

Phytoremediation: Use of vegetation to extract, sequester and/or detoxify a pollutant.

Rhizosphere: The matrix volume influenced by the presence of a plant root.

Chapter 11
Potential Application of Plant–Microbe Interaction for Restoration of Degraded Ecosystems

Krishna Giri
Rain Forest Research Institute, India

Gaurav Mishra
Rain Forest Research Institute, India

Rashmi Paliwal
G.B. Pant University of Agriculture and Technology, India

Shailesh Pandey
Rain Forest Research Institute, India

J.P.N Rai
G.B. Pant University of Agriculture and Technology, India

Deep Chandra Suyal
G.B. Pant University of Agriculture and Technology, India

P.K. Verma
Forest Research Institute, India

ABSTRACT

Rapidly increasing human population, urbanization, industrialization, and mining activities have become the serious environmental issue of today's world. Conventional physico-chemical remediation methods are highly expensive and generate secondary waste. However, bioremediation of contaminated ecosystems using indigenous microbes and plants or amalgamation of both has been recognized as a cost effective and eco-friendly method for remediation as well as restoration of polluted or degraded ecosystems. Further, variety of pollutant attenuation mechanisms possessed by microbes and plants makes them more feasible for remediation of contaminated land and water over physico-chemical methods. Plants and microbes act cooperatively to improve the rates of biodegradation and biostabilization of environmental contaminants. This chapter aims to emphasize on potential application of microbes and plants to attenuate the organic and inorganic pollutants from the contaminated sites as well as eco-restoration of mine degraded and jhum lands by way of biodegradation and phytoremediation technologies.

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INTRODUCTION

Fast growing human civilization, industrialization, mineral mining, oil exploration, modern agricultural practices and related anthropogenic activities in the world has resulted elevated levels of toxic metal and xenobiotic pollutants (pesticides, pharmaceuticals petroleum hydrocarbons etc) in the environment (Bernhoft, 2012). Mineral mining, oil exploration and various metal processing industries has led to the dramatic increase in concentration of toxic heavy metals and metalloids such as iron, chromium, Nickel, cadmium mercury, lead, zinc, arsenic etc (Giri et al., 2014a); petroleum hydrocarbons (PHC), and polycyclic aromatic hydrocarbons (PAHs). However, intensive agriculture, and crop protection strategies led to the build up of variety of persistent organic pollutants such as insecticides, fungicides, herbicides, rodenticides, nematicides and other toxic organic compounds in the air, water and soil. In order to cater the demands of fast growing population, the rapid expansion of industries, food, health care, vehicles, etc. is necessary, but it is very difficult to maintain the quality of environment with all these new developments, which are unfavourable to the environment. The adverse effects of metals and pesticide toxicity have been well documented. These pollutants impose hazardous impacts on living organisms and ecosystem health (Bernhoft, 2012; Godt et al., 2006; Jomova et al., 2011; Patrick, 2006; Auger et al., 2013).

Therefore, remediation of these contaminants is becoming one of the serious environmental issues in the world (Chaudhry, Blom-Zandstra, Gupta, & Joner, 2005). The common remedial measures for restoration of contaminated environment include various Conventional physico-chemical methods. These technologies have several disadvantages such as high energy requirement or large chemical input that may cause generation of secondary wastes ; and all these disadvantages make a conventional treatment process very costly (Yang, He, & Wang, 2009). Phytoremediation has now emerged as a promising strategy for *in-situ* removal of many organic and inorganic contaminants (Macek, Mackova, & Kas, 2000; Pilon-Smits, 2005; Greenberg, 2010). Microbe-assisted phytoremediation, including rhizoremediation, appears to be effective for removal and/or degradation of contaminants from contaminated environment, particularly when used in conjunction with appropriate agronomic techniques (Singer, Thompson, & Bailey, 2004; Chaudhry et al., 2005; Huang, El-Alawi, Gurska, Glick, & Greenberg, 2005 ; Zhuang, Chen, Shim, & Bai, 2007). However, restoration of mine degraded and jhum land represents an indefinitely long-term commitment of ecosystem restoration process. Natural recovery in mine spoils/jhum land is a very slow process which may take many years of natural succession on a mine degraded land for the total nutrient pool recovery to the level of native forest soil. The first step in any restoration program is to protect the disturbed habitat and communities from being further wasted followed by to accelerate re-vegetation process for increasing biodiversity and stabilizing nutrient cycling. As a result of natural succession by planting desirable plant species on mine degraded ecosystems/jhum lands a self-sustaining ecosystem may be developed in a short period of time (Giri et al., 2014a). This chapter provides an overview of plant microbe interaction for restoration of degraded environment.

BACKGROUND: HEAVY METAL POLLUTION

Metals are found naturally in soil, water and sediments in background concentration and have been used by humans for thousands of years. Metals with atomic mass over 20 and specific gravity above 5 g cm^{-3}

are known as heavy metal. They can be metalloids that have toxic effect on biological components of an ecosystem even at low concentration. Metals in soil may range in different concentrations from less than one to as high as 100000 mg kg^{-1} (Pal & Rai, 2010). High concentration release of heavy metals into the environment due to human activities have adverse impact on ecosystem functioning. Although some metals *viz.,* Co, Cu, Fe, Mo, Mn, Zn and Ni are essential for cell as they are required for normal growth and metabolism for all life forms, while other (e.g. As, Cd, Hg, Pb, and Se) are toxic and/or non essential due to complex compound formation within the cell. Once introduced into the environment heavy metals cannot be degraded easily and persist indefinitely for longer period and pollute the ecosphere (Azcon, Peralvarez, Roldan, & Barea, 2010).

Rapid industrialization and consumerist life style has led to an unprecedented increase of such toxic substances in natural ecosystems. Although several long term health effects of heavy metals are well known for a long time, exposure to these toxic substances is continue and even increasing in some parts of the world, particularly in developing and/or less developed countries. Heavy metal pollution occurs both at the production level as well as the end use of products and run-off in industries (Table 1). They enter the human body through food, water and inhalation of polluted air, use of cosmetics, drugs, poor quality herbal formulations particularly 'Ayurvedic/Sidha bhasamas', (herbo-mineral preparations) and `Unani' formulations, and even items like toys which have paints containing lead (INSA, 2011).

Injudicious applications of synthetic fertilizers such as phosphate have deposited heavy metals in much higher concentrations on earth surface than natural background sources. Phosphate fertilizers show big source of cadmium. For example in Scandinavia, cadmium concentration in agriculture soil increases by 0.2% per year (Mohammed, Kapri, & Goel, 2011). In recent years the use of energy-saving CFL bulbs has increased enormously. According to a report CFL bulbs production has increased 500 million in 2010 from 19 million in 2002. These bulbs can prove to be a major health hazard as each contains 3-12 mg of mercury, with no system to recover these bulbs and safe disposal (INSA, 2011).

Table 1. Sources of heavy metals (Source: INSA, 2011)

Metal	Industry
Chromium (Cr)	Mining, industrial coolants, chromium salts manufacturing, leather tanning
Lead (Pb)	lead acid batteries, paints, E-waste, Smelting operations, coal- based thermal power plants, ceramics, bangle industry
Mercury (Hg)	Chlor-alkali plants, thermal power plants, fluorescent lamps, hospital waste (damaged thermometers, barometers, sphygmomanometers), electrical appliances etc.
Arsenic (As)	Geogenic/natural processes, smelting operations, thermal power plants, fuel burning
Copper (Cu)	Mining, electroplating, smelting operations
Vanadium (Va)	Spent catalyst, sulphuric acid plant
Nickel (Ni)	Smelting operations, thermal power plants, battery industry
Cadmium (Cd)	Zinc smelting, waste batteries, e-waste, paint sludge, incinerations & fuel combustion
Molybdenum (Mo)	Spent catalyst
Zinc (Zn)	Smelting, electroplating

Remedial Measures

Strategies for remedy of heavy metal pollution involve reducing the bioavailability, mobility and toxicity of heavy metals. This can be achieved by three complementary functions *viz.,* technological, management and regulatory.

- Technological methods involve development of the treatment system whereby pollution load in the waste or effluent is brought within the safe limits before discharge in the environment. Technology for remediation should be cost effective and environmentally sustainable.
- Management function is important to ensure that the right technologies are being adopted and also monitor the end results.
- Regulations ensure the safety and health of workers as well as the public in general by regulating the toxic metal levels in effluent release in the environment.

Conventional techniques such as thermal processes, physical separation, electrochemical methods, washing, stabilization/solidification and burial are too expensive, require high energy and may generate secondary pollutants that affect biological functioning of an ecosystem. Therefore, alternate techniques such as bioremediation, particularly plants microbe interaction is gaining much attention for heavy metal pollution and ecosystem restoration of disturbed environment. Bioremediation technologies are more acceptable and offer many advantages over conventional treatment methods, for example, cost effectiveness, high efficiency, minimizing the disposable sludge volume and it also offers the flexibility for desorption techniques for biomass regeneration and recovery of metals (Eapen & D'Souza, 2005). Plants based technology capable of extracting and accumulating significant level of heavy metal. Phytoremediation approaches with its subset (e.g. phytoextraction, phytovolatilisation and phytostabilisation) for heavy metal pollution abatement has been well documented in recent years. At present, more than 400 plant species of 45 families are known to accumulate heavy metals (Pal & Rai, 2010; Reeves & Baker, 2000; Guerinot & Salt, 2001). Several plant species e.g. *Alyssum bertolonii*, *Brassica juncea*, *Eichhornia crassipes*, and *Iberis intermedia* have been found to sequester various metals in their tissues (Robinson et al., 1997; Brooks, Chambers, Nicks, & Robinson, 1998; Anderson et al., 1999). Success of the process of absorption and transformation of heavy metals into plant system strictly depends on their solubility and complexity (Rungwa, Arpa, Sakulas, Harakuwe, & Timi, 2013). However, Plants have constitutive and adaptive mechanisms for extracting, accumulating and tolerating high concentrations of their rhizospheric contaminants. Plants have developed range of potential mechanism to tolerate and avoid toxic effect of high metal concentration, which are as follows:

- Immobilization of heavy metals in cell walls, preventing their contact with protoplasm. Plant cell wall acts as a cation exchanger and can hold variable quantities of metal (Rauser, 1999).
- Compartmentalization and formation of complexes with inorganic and organic acid, phenol derivatives and glycosides in the vacuole (Singh, Singh, & Gupta, 2010).
- Chelation in the cytoplasm by peptide ligands such as metallothioneins (MTs) and phytochelatins (PCs). MTs are cysteine-rich polypeptides. PCs are trace metal binding peptides play key role in metal tolerance. PCs protect plant enzymes from trace metal poisoning (Pal & Rai, 2010; Singh et al., 2010).

Microorganisms associated with plants root system also play significant role in plants mediated heavy metal remediation technologies. Such microbial community can be classified into two major group *viz.,* michorrhizal fungi and plant growth promoting rhizobacteria (PGPR). These microbes in rhizosphere provide a critical link between plant and soil. Michorrhizal fungi form major component of rhizosphere and show mutualistic association with most plants (Marques, Rangel, & Castro, 2009). Michorrhizal fungi such as, arbuscular mycorrhizal fungi (AMF) can benefits plant (Marques et al., 2009) in following ways:

- Improve nutrient absorption through extensive extra radical hyphal networks, which explore the soil, absorb nutrients, and translocate them to the roots.
- Modify root system resulting in a more extensive length and increased branching and therefore enhanced nutrient absorption capacity of roots.
- Changes the chemical composition of root exudates and influences soil pH thus quantitatively affecting the microbial populations in the rhizosphere.
- Improve soil structure.
- Regulate hormones.
- Tolerance and protection against biotic and abiotic stress such as soil-borne plant pathogens, insect herbivores, drought and high levels of heavy metals.

On the basis of relationship with plants, plant growth-promoting rhizobacteria (PGPR) communities can be divided into two groups (a) symbiotic bacteria and (b) free-living rhizobacteria.

These organisms are able to enhance plant growth through various mechanisms (Marque et al., 2009), such as:

- Allowing plants to develop longer roots during early stages of growth by reducing ethylene production.
- Nitrogen fixation.
- Specific enzymatic activity.
- Supply bioavailable phosphorous and other trace elements for plant uptake.
- Production of phytohormones such as auxins, cytokinins, and gibberellins.
- Produce antibiotic that protect plants from diseases.
- Increase plant tolerance against flooding, salt stress, and water deprivation.
- Produce siderophores (low molecular mass compounds, 400-1000 K dalton). Play key role in solubilizing unavailable forms of heavy metal bearing minerals by complexation reaction (Aafi, Brhada, Dary, Maltouf, & Pajuelo, 2012; Rajkumar, Sandhya, Prasad, & Freitas, 2012).

Different microorganisms apply different mechanisms for growth and metal tolerance in plants, so it can be beneficial to design the process of phytoremediation in combination with appropriate microbial consortium, which may include AMF and PGPR.

Analytical Techniques

Analysis of pollution level is an integral part of environmental management. In environmental samples, heavy metals can exist in a range of physicochemical forms such as, hydrated metal ions and inorganic and organic complexes. There are many analytical methods for analyzing the heavy metals in environment

such as, atomic absorption spectrometry (AAS), inductively coupled plasma atomic emission spectrometry (ICP/AES), inductively coupled plasma mass spectrometry (ICP/MS), X-ray fluorescence (XRF) and ion chromatography (IC). Most of these techniques required sample digestion before quantification of metal. The aim of digestion is to achieve a selective or complete extraction of metals from the samples. Mostly, the digestion procedures are based on the addition of inorganic acids such as, aqua regia, HNO_3-HF, $HFHNO_3$-H_2SO_4-$HClO_4$, HNO_3-$HClO_4$ in a closed vessel, which may be heated on different sources (Scancar, Milacic, & Horvat, 2000; Hseu et al., 2002 ; Jeneper & Hayao, 2005).

Atomic Absorption spectroscopy is based on absorption of radiation by atoms. Absorption results in the excitation of electrons of atoms which jump to the higher energy levels. The amount of energy absorbed in the form of photons by sample is measured by AAS. The energy required for an electron to leave an atom is known as ionization energy and is specific to each chemical element. Absorbance is directly proportional to the concentration of the analyte present in the sample (García & Báez, 2012).

Inductively coupled plasma atomic emission spectrometry (ICP/AES) is based on principle that atoms emit light when excited by plasma. Plasma is ionized gas with very high temperature range from 7000 to 10000 ˚K. Excited atom emits characteristic spectra (Wang, Jia, & Wu, 2003). Inductively coupled plasma mass spectrometry (ICP-MS) is a very powerful, highly sensitive and specific technique for the analysis of trace (ppb-ppm) and ultra-trace element and isotope. ICP-MS is composed of plasma (a high temperature i.e. 8000 ˚K ionization source), quadrupole mass spectrometer (MS) analyzer (sensitive rapid scan detector) and a distinctive interface. The detection of elements is done by their mass-to-charge ratio (*m*/z) and intensity of a specific peak in the mass spectrum is proportional to the amount of that isotope (element) in the original sample. ICP/AES and ICP/MS are the future techniques for heavy metal detection in environmental samples because of accuracy, rapid and multi element analysis (Tu, Wang, & Welch, 2010).

X-ray fluorescence is a non-destructive method for analyzing samples. Fluorescence involves emission of an X-ray photon after ionization of atom by a primary X-ray beam. When primary X-ray beam strikes a sample, it interacts with electron and knocks it out of its inner shell forming voids. These voids present an unstable condition of atom, which stabilized when the void promptly filled by outer shell electron and give off X-ray with specific wavelength. This characteristic X-ray is the measure of elemental composition of a sample (Meirer et al., 2010).

Recent Advances in Heavy Metal Bioremediation

The process of phytoremediation has gained much attention in last few years to explore molecular and biochemical pathways involve in heavy metal uptake, transport and storage in plants (Eapen & D'Souza, 2005). However, the process of phytoremediation is rather slow and an improved technique via biotechnological approach can overcome the problem. Genetic modifications in plants to enhance the efficiency of remediation technique require a deep insight into the complete mechanism of heavy metal extraction by plant.

Development of transgenic plants with increased metal selective organic acid, ligands and phytochilatins could have promising applications in heavy metal decontamination. It is well known that organic acids and peptide ligands form complexes with metals. For example, free histidine is found as metal chelator in xylem exudates of nickel (Ni) hyperaccumulators, therefore, by modifying histidine concentration in xylem exudates Ni accumulating capacity of plants can be improve. Cellular targeting manipulation specifically in metal transporters and vacuoles is important since the compartmentalization of heavy metals

is safe mechanism adopted by most plants without disturbing the cellular functions. Great successes have been achieved in the development of transgenic plants with enhanced heavy metal accumulating capacity but majority of genes have been transferred from other plants or organisms (Eapen & D'Souza, 2005).

To develop plant species better suited for phytoremediation of metal contaminated sites *Thlaspi caerulescens* has been used as source of genes by various workers (Gleba et al., 1999; Lombi, Zhao, Dunham, & McGrath, 2001). Brewer, Saunders, Angle, Chaney, & McIntosh, (1999), developed somatic hybrids between *T. caerulescens* and *Brassica napus*. The selected high biomass hybrids for Zn tolerance were found to capable of accumulating Zn level that would otherwise toxic to *B. napus*. In other study somatic hybrids from *T. caerulescens* and *B. juncea* were also able to remove significant amounts of Pb (Gleba et al., 1999). Transgenic *B. juncea* showed efficient affinity for Se uptake with enhanced Se tolerance than the wild species (Van Huysen, Terry, & Pilon-Smits, 2004). Transgenic *B. juncea* with Se tolerance was developed by transferring the selenocysteine methyltransferase (SMT) from the *A. bisulcatus* (Se hyperaccumulator). SMT transgenic plants of *B. juncea* accumulate 60% more Se than the wild-type when grown in a contaminated soil (Zhao & McGrath, 2009; Rascio & Navari-Izz, 2011). Transgenic plants have proved to be a promising biotechnological approach, but only few field studies have been performed till now (Zhao & McGrath, 2009; Rascio & Navari-Izzo, 2011). Application of mixed microorganisms with plant species can provide effective future measures for heavy metal decontamination. However, several obstacles need to overcome for commercial application of such treatment system (Hrynkiewicz, Dabrowska, Baum, Niedojadlo, & Leinweber, 2012) such as,

- Commercially cost-effective mass-production and formulation of microbial inoculums.
- Microbial inoculum should be relatively universal for various plants and soils and its effectiveness should be relatively easy to evaluate.
- Effectiveness of microbial consortium to function in natural conditions.
- Knowledge of possible interactions between plants and associated soil microorganisms in natural environment.

However, additional research is expected to overcome these problems (Rajkumar et al., 2012), for example

- Complete physiological and molecular characterization of several environmentally relevant microorganisms.
- Exploration of mechanism followed by microbial chelators-metal complex uptake in plants.
- Effects of factors influencing the solubility and plant availability of nutrients/heavy metals.
- Identification of signaling processes that occur between plant roots and microbes.
- Effect of manipulation in rhizosphere zone processes such as coinoculating ecologically diverse microorganisms on phytoremediation process.

Such knowledge may enable us to exploring the mechanism of metal-microbes-plant interactions and to improve the performance and use of beneficial microbes as inoculants for microbial assisted phytoremediation (Rajkumar et al., 2012).

PESTICIDE POLLUTION

Pesticides have long history since the emergence of agriculture and considered among the most serious environmental pollutant. Human beings are facing the problem of pests (including weeds, insects and pathogenic agents) causing considerable agricultural losses. If these pests are not controlled, they diminish the quality and quantity of crop production (Richardson, 1998). In ancient greece civilization, natural pesticides such as, some inorganic chemicals and compounds extracted from plants were earlier used as pesticides for example, pyrethrine extracted from *Chrysanthemum* flowers used to control the pest development during winter storage of crop. However, agricultural revolution in the 19th century has lead to the intensive and diversified use of the pesticides corresponding to compounds derived from minerals and plants. As an example, the development of Bouillie Bordelaise (Bordeaux mixture) in 1880, consisting of copper sulphate and lime allowed better control of cryptogamic diseases in Bordeaux and French vineyard. It is still in use for vineyard and fruit tree protection. Development and application of pesticides for the control of various insectivorous and herbivorous pests is considered as fundamental contributor to this "Green Revolution". The use of synthetic organic pesticides began during the early decades of 20th century and increased tremendously after the World War II, with the introduction of synthetic organic molecules such as DDT, aldrin (two insecticides) and the herbicide 2,4-D in the agricultural market. Due to their advantages of being effective and cheap, use of synthetic pesticides is continously increasing in the whole world. Globally, about 4.6 million tons of chemical pesticides are being sprayed every year and only 1% of sprayed pesticides is effective to crop rest 99% is released into the non-targeted environment (Zhang, Jiang, & Ou, 2011).

Although pesticide application ensures better yield in agricultural production, however, they are potent contaminent of soil and water resources and affect living beings through the food chain (Briceno, Palma, & Duran, 2007). Pesticide residues become persistent in the environment due to highly recalcitrant nature, intesive and continuous application, where they have often been detected beyond the permissible limits in different compartments of the environment as well as in food chain. In many parts of the world, particularly in developing countries, clean drinking water is a limited resource and, in this context, intensive agricultural production is a major environmental and health problem because pesticide residues accumulate in surface and under ground water (Rasmussen, Aamand, Rosenberg, Jacobsen, & Sorensen, 2005). Contamination with pesticides is not restricted only to developing countries but also in Europeon countries, where pesticide residues have often been detected in surface and ground water resources (Gooddy, Chilton, & Harrison, 2002). As a result, the use of pesticides in conventional agriculture has attracted much attention in recent years due to rising public and governmental concerns about their impact not only on environmental contamination but also on human and animal health.

Pesticide exposure to environment is dependent on various factors like production, formulation processing and application doses. A pesticide may enter in to the environment *via:*

- Direct intentional application in the soil to control pre emergent weeds, plant pathogens, soil insects /pests, and/or
- Indirect unintentional entry followed by foliar application for post emergent weeds and insects/ pests (Mathew, 2006).
- Certain portion of pesticides may undergo spillage from formulation plants during processing and waste disposal processes.

These recalcitrant compounds build up regularly in the environment and adversly deteriorate soil biology, as they are non-biodegradable slow degradable (Sacki & Toyota, 2004). Owing to low water solubility, pesticides have strong affinity for particulate matter and consequently enter in to water sediments (Giri, Rawat, Rawat, & Rai, 2014b). For instance lindane, the most commonly used isomer of HCH is known to accumulate in food chains, causing toxicity in wild/domestic animals and human beings. Apart from food contamination, human beings are exposed to lindane by inhalation, polluted water and dermal contact (Giri et al., 2014b).

Repeated applications of haloginated insecticide endosulfan causes its accumulation in the soil and water environment. Consequent upon accumulation, it is extremely toxic to aquatic fauna, while provoking chronic symptoms like testicular and prostate cance, breast cancer, sexual abnormality, genotoxicity and neurotoxicity in various mammalian species (Giri & Rai, 2012). Some of the major pesticides classes are described in Table 2 along with their health effects on human beings.

Table 2. Classification and characterization of some major pesticides (Source: Zacharia, 2011)

Pesticide Name	Trade Name	Type	Health Effects
Chlordane $C_{10}H_6C_{l8}$	Toxichlor, Niran, Octachlor, Synklor, Corodane	Organochlorine Insecticide	Suspected carcinogen, affect central nervous system, gastrointestinal tract and liver.
Chlorpyrifos $C_9H_{11}C_{l3}NO_3$	Dowco179, Dursban, Lorsban, Pyrinex, Killmaster	Insecticide	May affect the central nervous system and liver
DDT $C_{14}H_9C_{l5}$	Dicophane, Agritan, Gesapon, Gesapex, Citox, Detox, Anofex	Organochlorine Insecticide	Probable carcinogen, reproductive, liver and kidney problems, eye, nose, skin, throat irritant
Lindane $C_6H_6Cl_6$	Aficide, Agrocide, Benzene hexachloride, Bexol, Celanex	Organochlorine Insecticide	Suspected carcinogen, affects central nervous system, respiratory, reproductive systems
Pentachlorophenol C_6Cl_5OH	PCP, Dowside 7, Permacide, Permagard, Pentakil,	Organochlorine Fungicide	Possible carcinogen, eye, skin, nose, throat irritant, liver and kidney damage
Diaznon $C_{12}H_{21}N_2O_3PS$	Basudin, Dazzel, Gardentox, Royazol, Out, Nucidol	Organophosphate Insecticide	Eye and skin irritant, may cause gastrointestinal symptoms
Dichlorvos $C_4H_7Cl_2O_4P$	Unitox, Lindan, DDVP, Vapona, Nuvan, Cypona	Organophosphate Insecticide	Suspected carcinogen, can affect the central nervous system
Ethion $C_9H_{22}O_4P_2S_4$	Ethanox, Hylmox, Nialate, Rhodocide	Organophosphate Insecticide	Affect central nervous system, gastrointestinal system, chest, nose
Malation $C_{10}H_{19}O_6PS_2$	Chemathion, Malacide, Detmol, o,odimethyl-thiophosphate	Organophosphate Insecticide	Skin, eye, nose irritant, affects respiratory and central nervous system
Permethrin $C_{21}H_{20}Cl_2O_3$	Ambush, Ectban, Pounce, Nix Dragnet, Spartan	Pyrethroid Insectcide	Eye, skin, respiratory irritant, affect central nervous system
Rozol (Chlorophacinone) $C_{23}H_{15}ClO_3$	Amvac, Romix special, Mouce seed®	Rodenticide	Skin and eye irritant, may affect liver
Thymol $C_{10}H_{14}O$	6-isopropyl-mcresol	Fumigant	Skin and eye irritant

Bioremediation of Pesticides

Bioremediation is utilization of microorganisms for degradation of hazardous chemicals in soil, sediments, water, or other contaminated materials. Often these microorganisms metabolize the chemicals to produce carbon dioxide or methane, water and biomass. Alternatively, the contaminants enzymatically transformed to metabolites that are less/non toxic or environmentally innocuous. It should be noted that in some instances, the metabolites formed are more toxic than the parent compound. For example, perchloroethylene and trichloroethylene may degrade to vinyl chloride which is highly toxic in nature in contrast to the parent compound (Sacki & Toyota, 2004).

There are a number of possible pesticide degradation pathways in the soil, plants, animals and aquatic environment including chemical treatment, volatilization, photodecomposition and incineration. However, most of them are not applicable for the diffused contamination with low concentration because of being expensive, less efficient and environmental friendly. Thus, keeping in view the environmental concerns associated with pesticides/recalcitrant compounds, there is a need to develop safe, convenient and economically viable methods for its remediation. The most common breakdown process is performed by microorganisms particularly fungi and bacteria. In this context, several researchers have focused their attention to investigate the microbial biodegradation which has been reported as a primary mechanism of pesticide dissipation from the environment (Cox, Walker, & Welch, 1996; Pieuchot, PerrinGanier, Portal, & Schiavon, 1996). Although bioremediation is a more acceptable strategy for pesticide degradation, but complexity of the mechanisms has made it slow to emerge as an economically viable remediation method (Zhang & Quiao, 2002; Nerud, Baldrian, Gabriel, & Ogbeifun, 2003). It is noteworthy that processes of bioremediation have been developed extensively for taking care of sites heavily contaminated with organic pollutants, however, up to now, the sites diffusely contaminated are only monitored and natural attenuation is the process of interest leading to contaminant abatement.

Microbial biodegradation occurs mostly in the soil solution. Pesticide microbial biodegradation is carried out by soil microorganisms like bacteria fungi and actinomycetes possessing a large set of enzymes susceptible to transform these pesticides. It is the principal mechanism for diminishing the persistence of pesticides in soil environment (Arbeli & Fuentes, 2007). Soil serves as a potential habitat for diverse variety of microorganisms which have the ability to interact not only with other living components but also the physical elements including pesticides for the fulfilment of their energy requirement. When pesticides are applied in the soils, enzyme-driven biochemical reactions carried out by the indigenous soil microorganisms result in modification of the structure and toxicological properties of pesticides leading to their complete conversion into harmless inorganic end products (Hussain, Siddique, Arshad, & Saleem, 2009a). Enzyme system plays a central role in pesticide degradation. Four major enzymes involve in complete mineralization of pesticides (Ortiz-Hernández, Sánchez-Salinas, Dantán-González, & Castrejón-Godínez, 2013).

- Hydrolases, esterases and phosphotriesterases: Hydrolases represent a broad group of enzymes that catalyzes the hydrolysis of ureas, thioesters, ester, peptide, carbon-halide bonds. Esterases catalyze hydrolysis of carboxylic esters (carboxiesterases), amides (amidases), phosphate esters (phosphatases) bonds etc. Phosphotriesterases (PTEs) hydrolyse the phosphoester bonds i.e., P-S, P-O, P-F, P-NC in organophosphorus pesticides.

- Oxidoreductases: These represent a broad range of enzymes and catalyze the oxidation-reduction reaction by transferring electrons from one molecule to another. These enzymes utilize molecular oxygen as electron acceptor.
- Mixed function oxidases (MFOs): MFOs comprising of two enzymes, cytochrome P450 and NADPH-cytochrome P450 reductase. MFOs posses very high inspecificity and metabolize a wide variety of compounds for example, organophosphates, carbamates, pyrethroids, DDT, inhibitors of the chitin synthesis, juvenile hormone mimics, etc. MFOs catalyze the incorporation of molecular oxygen into the substrate, for this it requires nicotiamide-adenine dinucleotide phosphate (NADPH) and O_2.
- Glutathione S-transferases (GSTs):GSTs catalyze the conjugation reaction of hydrophobic components with the tripeptide glutathione to form a conjugate which can be metabolized or excreted.

Certain class of pesticides such as pyrethroids, organophosphates and some carbamates are less persistent and more susceptible to degradation. However, most organochlorines due to their highly persistence nature, resist biodegradation. Pesticides degradation by soil microbial communities has been reported by several researchers (Fenlon, Jones, & Semple, 2007; Hussain, Arshad, Saleem, & Khalid, 2007; Shi & Bending, 2007; Hussain, Sorensen, Devers-Lamrani, El-Sebai, & Martin-Laurent, 2009b; Sun et al., 2009) and it has been described as a primary mechanism of pesticide dissipation from the environment (Cox et al., 1996; Pieuchot et al., 1996). The efficiency of pesticide biodegradation varies considerably between different groups of the microorganisms and even between the different members belonging to the same group of microorganisms. Although a strong diversity of microbial species is found in the soil, however, the adaptability of these different degrading microbial species in the contaminated soils assures the continuity of biodegradation process. Microbial biodegradation of pesticides in the soil can be categorized into two principal types based on the mode and pathway of degradation i.e. metabolic and co-metabolic.

Metabolic Degradation

Metabolic pesticides degradation is carried out by soil microbial population harbouring specific catabolic enzymes allowing complete mineralization of target compound. A large number of pesticide degrading fungal and bacterial strains have been isolated and characterized from the soil environment (Hussain et al., 2009b). Although often metabolic biodegradation can leads to incomplete degradation resulting in the formation of metabolites (Turnbull, Ousley, Walker, Shaw, & Morgan, 2001; Hangler, Jensen, Ronhede, & Sorensen, 2007; Badawi et al., 2009). The enzymes required for metabolic degradation of pesticides are either harboured by a single microorganism or scattered in various microbial populations working as a cooperative consortium, jointly involved in the degradation of the pesticides (Fournier, Soulas, & Parekh, 1997). Complete metabolism of pesticides involves three important phases (Ortiz-Hernández et al., 2013).

Phase I: Involves oxidation, reduction, or hydrolysis of pesticide to a water-soluble and usually a less toxic product.

Phase II: In this phase conjugation of a pesticide or pesticide metabolite to a sugar or amino acid take place, this increases the water solubility and reduces toxicity of contaminant.

Phase III: Conjugation of phase II metabolites result in formation of non-toxic secondary conjugates.

Co-Metabolic Degradation

The co-metabolic degradation corresponds to the non specific degradation of xenobiotic molecule by microorganisms. In most of the cases, this is a non-inducible phenomenon occurring because of the presence of detoxifying enzymes able to degrade xenobiotics depicting homologies with their substrate. In this case, the target pesticides do not contribute to the growth of the degrading organisms (Novick & Alexander, 1985; Dalton & Stirling, 1982). For this reason, the degradation rate of pesticide in a given environment depends primarily on the size of microbial biomass and on the competitiveness of the degrading microbial population towards sources of energy and nutrients in the soil. In other words, pesticide degradation rate is dependent on size of the biomass (Fournier et al., 1997). In general, co-metabolism does not yield in extensive degradation of the molecule but rather causes incomplete transformation such as oxidation, hydroxylation, reduction, N-dealkylation or hydrolysis (Fournier et al., 1997) which may lead to the formation of metabolites that may prove even more toxic and recalcitrant than the parent compound (De Schrijver & De Mot, 1999).

Some compounds can only be partially metabolized by microbial populations and transformed into metabolites that may either accumulate in the environment or be metabolized further by other microbial species. These metabolic reactions do not provide benefit to the responsible organism because they do not gain either carbon or energy. These processes are typically fortuitous and occur because the responsible population produces one or more enzymes that are comparatively nonspecific and can react with structural analogues compounds of the "normal" substrate for enzyme(s). Co-metabolism is important for the degradation of many environmental contaminants particularly chlorinated pesticides solvents (e.g. trichloroethylene,), polychlorinated biphenyls, and many polyaromatic hydrocarbons (Fournier et al., 1997). Giri & Rai (2012), studied Biodegradation of endosulfan isomers in broth culture and soil microcosm by *Pseudomonas fluorescens*. After 15 days incubation, maximum 92.80% α and 79.35% β endosulfan isomers were degraded in shake flask culture at 20 mg/L concentration, followed by 50 and 100 mg/L, while the corresponding values in static condition were 69.15 and 51.39%, respectively.

Advantages and Limitations of Bioremediation

Intrinsic or engineered bioremediation processes offers several potential advantages that are attractive to site owners, regulatory agencies, and the public. These include:

- Lower cost than conventional technologies.
- Contaminants usually converted to innocuous products.
- Contaminants are destroyed, not simply transferred to different environmental media.
- Nonintrusive, potentially allowing for continued site use.
- Relative ease of implementation.

However, there are some limitations to bioremediation as well, such as:

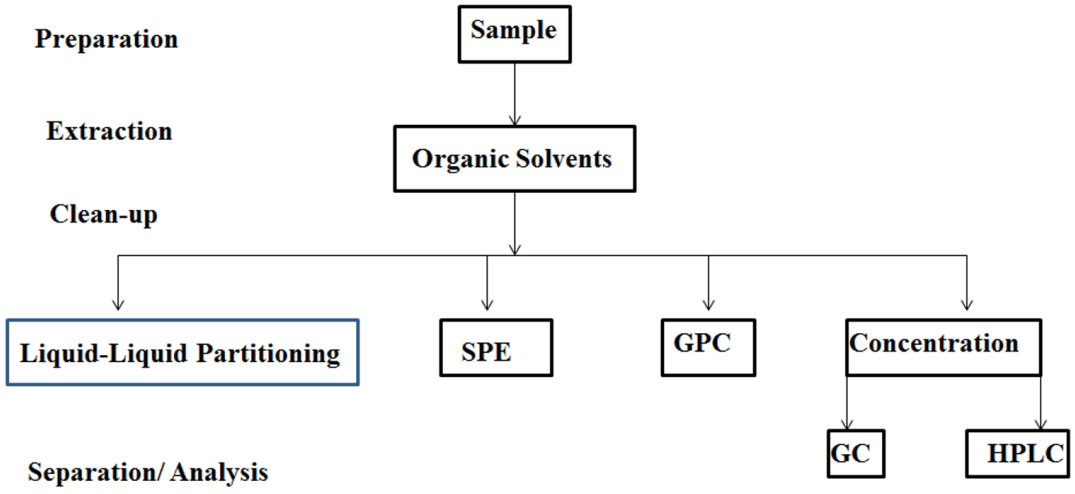
- Difficult to control the laboratory optimized conditions in the field.
- Amendments introduced into the environment to enhance bioremediation may cause other contamination problems.
- May not reduce concentration of contaminants to the required levels.

- Requires more time.
- May require more extensive monitoring.

Analytical Techniques

Development of new technologies and their implementation in the analysis of pesticides in environmental samples has greatly affected the way we perceive and use pesticides. In the 1940's, pesticides were perceived as miracle chemicals that gave tremendous gain in crop yields and they were used without adequate regard to health and the environment. At the time, thin layer chromatography (TLC) with semi-quantitative detection was the primary means of analysis. Gas liquid chromatography (GLC or GC) with packed columns became the method of choice as commercial instruments improved and selective quantitative detectors were developed in the late 1950's, to mid 1960's. By the time of publication of Rachel Carson's *Silent Spring* in 1962, GC was predominant method of pesticide analysis (Hawthorne, Yang, & Miller, 1994). When the environmental and ecological impacts of pesticides were come in to lime light, the perception of pesticides began to change. Laws that established regulatory controls on the use of pesticides and their presence in the environment required residue analysis using state-of-the-art instrumentation (Hopper, 1999). With the development of improved capillary columns for GC in the 1970's, tremendous gains in separation power were achieved and the capabilities of multi-residue methods improved accordingly. During the same time frame, high performance liquid chromatography (HPLC) was commercialized and its implementation in pesticide residue analysis permitted detection of many compounds that were not analysed easily. Through the complementary nature of GC and HPLC, a wide range of pesticides could be analysed and many environmentally safer pesticides were developed and registered using these sophisticated technologies (Richter et al., 1996). Presently potentially advanced and sophisticated pesticide analytical methods such Gas chromatography mass spectrometry (GC-MS) Liquid chromatography mass spectrometry (LC-MS), etc, have been developed and commercialized (Figure 1).

Figure 1. Pesticide residue analysis method in environmental samples (Source: Hawthorne et al. 1994)



Use of selective detectors and multiple analyses are common
(SPE= solid phase extraction,
GPC= gel permeation chromatography, GC= gas chromatography,
HPLC= high performance liquid chromatography)

Recent Advances in Pesticide Bioremediation

Pesticide degrading catabolic genes and their respective enzymes of microorganisms have been isolated and identified by several researchers. For example lindane (Kumari et al., 2002), endosulfan (Sutherland, Horne, Harcourt, Russel, & Oakeshott, 2002; Hussain et al., 2007), DDT (Barragan-Huerta et al., 2007) and monocrotophos degrading microbial genes and enzymes have been (Subhas & Singh, 2003; Das & Singh, 2006) isolated and identified. Genetic studies revealed that plasmids are the main place to harbour pesticide catabolising genes in microbial community. Sutherland et al. (2002) had reported *Esd* gene having sequence homology to monooygenase family which uses reduced flavin, provided by a separate flavin reductase enzyme, as co-substrates in *Mycobacterium smegmatis*. *Esd* catalyzes the oxygenation of β-endosulfan to endosulfan monoaldehyde to endosulfan hydroxyether. *Esd* did not degrade either α-endosulfan or the metabolites of endosulfan and endosulfan sulphate. Wier et al. (2006) have reported that *Ese* gene of *Arthrobacter* sp. encoding enzyme from monooxygenase family is capable of degrading both the isomers of endosulfan. After, understanding the gene of interest and enzyme involved, the Superbugs can be created to achieve the desired result at fast rate in short time frame. Lal, Dogra, Malhotra, Sharma, & Pal, (2006) has reviewed the degradation of HCH and distribution of *lin* gene in Sphingomonads. *S. indicum* B90A was found to contain two non-identical *lin*A genes (designated as *lin*A1 and *lin*A2). The *lin*A-encoded HCH dehydrochlorinase (*Lin*A) mediates the first two steps of dehydrochlorination of γ-HCH (Singh, 2008). Besides, genetically modified microbes are used to enhance the capability of pesticide degradation. However, genetically engineered technology for environment use is still controversial because an adverse genotype can be readily mobilized in the environment. For developing of a technology following points should be taken care (Singh, 2008):

- Heterogeneity of contaminant.
- Concentration of contaminant and its effect on biodegradative agent (microbe)
- Persistence and toxicity of contaminant
- Behaviour of contaminant in soil environment
- Conditions that favourthe microbial population.

The degradation of persistent chemical compounds by microorganisms in the natural environment has revealed a larger number of enzymatic reactions with high bioremediation potential (Finley, Broadbelt, & Hatzimanikatis, 2010). These biocatalysts can be obtained in quantities by recombinant DNA technology, expression of enzymes, or indigenous organisms, which are employed in the field for removing pesticides from polluted sites. The microorganisms contribute significantly for the removal of toxic pesticides used in agriculture and in the absence of enzymatic reactions many cultivable areas would be impracticable for agriculture (Abramowicz, 1995).

Although, significant advances have been made in understanding the roles of plant associated microbial pesticide degradation and application of these processes on pilot scale bioremediation (Joshi & Juwarkar, 2009; Li, Ye, & Wong, 2010; Shi et al., 2011). An exciting alternative to the use of plant-associated bacteria to degrade toxic organic compounds in soil is the use of recombinant DNA technology to generate transgenic plants expressing bacterial enzymes resulting in improved plant tolerance and metabolism of toxic organic compounds in soil. Transgenic plants have been produced for phytoremediation of both heavy metals and organic pollutants (Eapen, Singh, & D'Souza, 2007). Transgenic poplar plantlets expressing bacterial mercuric reductase were shown to germinate and grow in the presence of

toxic levels mercury. *Arabidopsis thaliana* was engineered to express a modified organomercurial lyase (Rugh, Senecoff, Meagher, & Merkle, 1998) and those transgenic plants grew vigorously on a wide range of concentrations of highly toxic organomercurials, probably by forming ionic mercury which should accumulate in the disposable plant tissues. The first report of genetically modified plant for the transformation of xenobiotic contaminants to nontoxic material was reported by French, Rosser, Davies, Nicklin, & Bruce, (1999). They previously reported that *Enterobacter cloacae* PB2 is capable of growth with trinitrotoluene (TNT) as a nitrogen source (Bhatiya & Malik, 2011).

ECOSYSTEM DEGRADATION

Ecosystem degradation resulting from resource extraction, land-use change, shifting cultivation, invasion by exotic species, forest fire and subsequent biodiversity loss alter the functions and services provided by forest ecosystems. Mineral mining exerts a long lasting impact on landscape, ecosystem and socio-cultural economic considerations. Mining and its subsequent activities have been found to degrade the land to a significant extent. Overburden removal from the coal field results in significant forest and top soil loss (Figure 2). Most of the mining wastes are inert solid materials and toxic in nature. These toxic substances are inherently present in the ore, e.g. heavy metals such as iron, mercury, arsenic, lead, zinc, cadmium, etc (Giri et al., 2014a). These heavy metals leach out of the stored waste piles and contaminate immediate environment. However, some toxic chemicals are also found in waste, as they are added intentionally during extraction and processing. The major environmental impacts due to coal mining are changes in soil stratification, reduced biotic diversity, and alteration of structure and functioning of ecosystems; these changes ultimately influence water and nutrient dynamics as well as trophic interactions (Giri et al., 2014b). Land degradation due to forest clearance, shifting cultivation and mining activities is the cumulative effect of air and water pollution, soil quality degradation and biodiversity loss (Giri et al., 2014a). This process works through a cycle known as land degradation cycle. The magnitude and impact of mining on environment varies from mineral to mineral and also depends on the potential of the surrounding environment to attenuate the negative effects of mining, geographical disposition of mineral deposits and size of mining operations. A list of minerals has been prepared by Department of Environment, which is supposed to have serious impact on environment. These minerals include coal, iron ore, zinc, lead, copper, gold, pyrite, manganese, bauxite, chromite, dolomite, limestone, apatite and rock phosphate, fireclay, silica sand, kaolin, barytes. Mineral production generates enormous quantities of waste/ overburden and tailings / slimes (Giri et al., 2014a).

Acid mine drainage is a serious environmental issue of coal/mineral mining activities. This occurs when sulphide ores are exposed to the atmosphere, which can be enhanced through mining and milling processes where oxidation reactions are initiated. Mining increases the exposed surface area of sulfur-bearing rocks allowing for excess acid generation beyond natural buffering capabilities found in host rock and water resources. Once acid drainage is created, metals are released into the surrounding environment, and become readily available to biological organisms. When fishes are exposed directly to metals and H^+ ions through their gills, impaired respiration may result chronic and acute toxicity. Fishes are also exposed indirectly to metals through ingestion of contaminated sediments and food materials. A common weathering product of sulfide oxidation is the formation of iron hydroxide ($Fe(OH)_3$), a red/orange coloured precipitate found in thousands of miles of streams affected by acid mine drainage. Iron hydrox-

Figure 2a. Open cast coal mining

Figure 2b. Acid mine drainage

Figure 2c. Coal dumping in Margherita Assam, India

Figure 2d. Coal dumping in Margherita Assam, India

ides and oxyhydroxides may physically coat the surface of stream sediments and streambeds destroying habitat, diminishing availability of clean gravels used for spawning, and reducing fish food items such as benthic macro invertebrates. Acid mine drainage, characterized by acidic metalliferous conditions in water, is responsible for physical, chemical, and biological degradation of aquatic ecosystems (Ashraf, Maah, & Yusoff, 2010). Acidic water adversely affects the soil environment by way of making the soil acidic and rich in inorganic component and poor in organic content. Deterioration of soil quality has severely affects the crop growth and yield in the area mainly due to high concentrations of hydrogen ions, which inactivate most enzyme systems, restrict respiration, and root uptake of salts and water by plants. It also leads to deficiency of nitrogen, phosphorous, calcium, magnesium, molybdenum and boron as well as iron and manganese toxicity. Solubilisation and transport of phosphorus from soil to the water environment due to acidity is an important issue associated with decreased agriculture productivity (Giri et al., 2014b). Open cast coal mining and other mineral mining activities resulted forest degradation, biodiversity loss and severe environmental pollution in mining areas. These mineral mining activities are being carried out in various parts of the country such as Madhya Pradesh, Jharkhand, Chhattisgarh, Orissa, Assam, Meghalaya, Arunachal Pradesh and Nagaland.

Shifting cultivation also called slash and burn agriculture is the clearing of forested land for raising or growing the crops until the soil nutrients are exhausted and/or the site is overtaken by weeds and then moving on to clear more forest. It has been often reported as the main cause of deforestation and land degradation (Barbier, Burgess, & Folke, 1994; Ross, 1996). Mostly all reports indicate shifting agriculture is responsible for about one half of tropical deforestation in the world (Figure 3 a & b). In India shifting cultivation/jhum cultivation is predominant in Northeast part of the country, particularly in Assam, Nagaland, Meghalaya and Mizoram. Shifting cultivation has been considered one of the major causes for ecological degradation and deforestation in the country, which has become a serious environmental issue.

ECO-RESTORATION OF DEGRADED ECOSYSTEMS

Ecological restoration cover a broad range of goals such as, amelioration of highly degraded abiotic conditions (toxic pollutant levels and the absence of topsoil on old mine sites), reinstatement or enhancement of key ecosystem functions (production, erosion control, water flow and quality) and re-establishment of target biotic community (rare species, native species, high diversity and eradication of invasive species). In terrestrial ecosystems, for effective and sustained achievement of any of these goals, maintenance/improvement of plant-soil interactions is very important. Soil conditions constrain plant performance and community composition (Grime, 2001; Pywell et al., 2003). Therefore, consideration of limitations imposed by soil quality is important for restoration of plant communities in terrestrial ecosystem. In contrast, plant composition can impact almost every aspect of soil structure and function. Ecological restoration is the practice of restoring ecosystems as performed by practitioners at specific project sites, whereas restoration ecology is the science upon which the practice is based (Eviner & Haukes, 2008).

Restoration ecology ideally provides clear concepts, models, methodologies and tools for practitioners in support of their practice. Sometimes the practitioner and the restoration ecologist are the same person the nexus of practice and theory. The field of restoration ecology is not limited to the direct service of restoration practice. Restoration ecologists can advance ecological theory by using restoration project sites as experimental areas. For example, information derived from project sites could be useful in re-

Figure 3a. Shifting Cultivation and Ecosystem Degradation in Mizoram

Figure 3b. Shifting Cultivation and Ecosystem Degradation in Mizoram

solving questions pertaining to assembly rules of biotic communities. Further, restored ecosystems can serve as references for set-aside areas designated for nature conservation. Ecological restoration is one of several activities that strive to alter the biota and physical conditions at a site. These activities include reclamation, rehabilitation, mitigation, ecological engineering and various kinds of resource management, including wildlife, fisheries and range management, agro forestry, and forestry. For all these activities microbial communities lies at the heart of plant–soil interactions and ultimately responsible for:

- Biogeochemical transformations in soil.
- Play a significant role in impacting soil structure.
- And have strong effects on plant growth and competitive dynamics.

Success in eco-restoration studies requires the presence of key microbial communities, particularly those that are obligate or facultative symbionts with plant roots. Plant seedlings grow substantially better when planted into a community with established mycorrhizal connections than in disturbed sites or in isolation Eviner & Haukes, 2008. In some cases, such as with pine trees, establishment requires simultaneous introduction of plants and ectomycorrhizal fungi if these root symbionts are not already present. Addition of symbiont inoculum can also facilitate restoration efforts when microbial communities have been disturbed or altered (Eviner & Haukes, 2008). For example mycorrhizal inoculations, have been shown to increase plant establishment and growth (Cuenca & Lovera, 1992); soil organic matter, nitrogen, aggregation (Requena, Perez-Solis, Azcon-Aguilar, Jeffries, & Barea, 2001), and alter succession by shifting competitive interactions between plants (Allen, Allen, Egerton-Warburton, Corkidi, & Gomez-Pompa, 2003). In addition, inhibiting microbial symbiont establishment can be used as a tool to reduce establishment and growth of undesirable species. For example, in a study, absence of arbuscular mycorrhizal fungi (AMF) and actinorhizal *Frankia,* native *oleaster* shrub growth decreased by 4-fold, whereas growth of an invasive leguminous shrub decreased by 5-fold in the absence of specific *Bradyrhizobium* strains (Parker, Malek, & Parker, 2006; Eviner & Haukes, 2008).

FUTURE RESEARCH DIRECTIONS

Isolation of various plants associated microbes and characterization of its beneficial metabolites/processes are time consuming, since it requires the analysis of more than thousands of isolates. Thus strong molecular research effort is required in order to find out specific biomarker associated with the beneficial microbes for efficient microbe assisted bioremediation. Although promising results have been reported under laboratory conditions, showing that inoculation of beneficial microbes particularly plant growth promoting bacteria and/or mycorrhizae may stimulate heavy phytoextraction or phytostabilization. Only a few studies have demonstrated the effectiveness of the microbial assisted plant bioremediation of pesticides and toxic metals in the field conditions (Brunetti, Farrag, Rovira, Nigro, & Senesi, 2011; Juwarkar & Jambhulkar, 2008; Wu, Wong, Shu, Khan, & Wong, 2011; Yang, Tu, Wang, Liao, & Yan, 2012). Emphasis should be placed when developing bioremediation systems using plant-associated bacteria, to choose wild type bacteria, or bacteria enhanced using natural gene transfer, to avoid the complications of national and international legislation restricting and monitoring the use of genetically modified microbes (GMMs). However, with a global political shift towards sustainable and green bioremedia-

tion technologies, the use of plant-associated bacteria to degrade toxic synthetic organic compounds in environmental soil may provide an efficient, economic, and sustainable green remediation technology for future environment (Bhatiya & Malik, 2011).

Much is still unknown about tolerances, degradative capacities and ecological interactions of organisms that have potential use in eco-restoration. However, it is clear, that plants and microbes act cooperatively to improve the rates of biodegradation of toxic contaminants and improve nutrient contents in degraded lands. In designing an eco-restoration program the oxidative capacity of a plant should be considered in terms of its action on the contaminant itself and for its potential to support rhizospheric microbes with the capacity to enhance biodegradation. Additional basic biological and ecological information in these areas will allow us to make better informed decisions on how to widen bottlenecks in bioremediation/ eco-restoration processes (Cohen, Yamasaki, & Mazzola, 2004).

CONCLUSION

Since plant-microbe association possess the capability of plant growth promotion and/or metal mobilization/immobilization in the soil. Therefore, it has been the matter of interest for bioremediation of toxic pollutants. Bioremediation is cost effective, faster than natural attenuation, high public acceptance and generates less secondary wastes and emerged as an integrated tool for environmental cleanup. The potential role of plants and associated rhizomicrobial population in facilitating microbial degradation for *in situ* bioremediation of surface soils contaminated with hazardous organic compounds is substantial. Further understandings of the critical factors which influence the plant-microbe-toxicant interaction in soils permit us real understanding of this approach for *in situ* bioremediation. To effectively restore an ecosystem or ecological community, it is often critical to consider multiple species, multiple functions, and their interactions. Furthermore, the restoration of self-maintaining systems is increasingly requiring the consideration of human-induced local- to global changes in the environment. Studies on plant–soil interactions vis-à-vis plant microbe interaction provide an important foundation for eco-restoration. In order to help managers with the challenge of designing successful restoration techniques at a specific site, we need to embrace the variability of ecological studies and develop frameworks to understand this variability. Bioremediation is not a Panacea to restore all the contaminated environmental sites, however, in comparison to other remediation processes i.e. incineration, thermal disposition, land farming etc. it has a better future in development of technology for removal of contaminants from actual site and restoration of degraded lands. With a global political shift towards sustainable and green technologies, the use of plant-associated microorganisms to degrade toxic synthetic organic and inorganic pollutants in environment provides an efficient, economic, and sustainable green remediation technology for future environment.

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KEY TERMS AND DEFINITIONS

Bioavailability: The fraction of contaminant actually available to microorganisms is said to be bioavailable.

Biodegradation: Biodegradation is a natural process, where the degradation of a xenobiotic chemical or pesticide by an organism is primarily a strategy for their own survival.

Bioremediation: Bioremediation is the use of living organisms such as microbes and plants for mitigation and wherever possible, complete elimination of the noxious effects caused by environmental pollutants.

Biosorption: Biosorption is a physiochemical process that occurs naturally in certain biomass which allows it to passively concentrate and bind contaminants onto its cellular structure.

Co-Metabolism: The co-metabolic degradation corresponds to the non specific degradation of xenobiotic molecule by microorganisms.

Ecological Restoration: Ecological restoration is the process of assisting the recovery of an ecosystem that has been degraded, damaged, or destroyed.

Environmental Pollution: Introduction of contaminants into the natural environment that cause adverse effects on living organisms and ecosystems.

Heavy: Metals: A heavy metal is a metallic element which has high density, specific gravity or atomic weight and usually toxic in nature.

Lower Metabolic Pathway: The organic pollutant degradation pathway involving cleavage of the aromatic ring structure is called lower metabolic pathway.

Metabolic Degradation: Metabolic biodegradation of the organic pollutants is carried out by the soil microbial populations harbouring specific catabolic enzymes leading to the complete mineralization of target compound.

Phytoextraction: Plant roots take up contaminants and store them in stems and leaves.

Phytoremediation: Phytoremediation is the process of removing/eliminating inorganic toxic metals and organic compounds using plants and trees from contaminated environment.

Phytostabilization: Plants are used to reduce the mobility and bioavailability of environmental pollutants.

Phytovolatilization: Contaminants taken up by the roots pass through the plants to the leaves and are volatized through stomata, where gas exchange occurs.

Upper Metabolic Pathway: The organic pollutant degradation pathway leading to formation of some key intermediates/secondary product is called upper metabolic pathway.

Xenobiotics: A synthetic organic compound such as drug, pesticide, or carcinogen that is foreign to a living organism is called xenobiotic compounds.

Chapter 12
Microbial Functional Activity in Bioremediation of Contaminated Soil and Water

Tarlan Sheikhavandi
Cukurova University, Turkey

ABSTRACT

Since the beginning of the industrialization, application of chemical compounds on lands and disposal of contaminants to soil and water systems have caused numerous sorts of alterations in environment, and therefore affected the inhabitant biodiversity. This chapter aims to provide an introduction to bioremediation, an innovative multidisciplinary technology which employs microorganisms in order to reduce, eliminate, contain or transform hazardous contaminants in soil, sediment or water. So far, microorganisms and plants have been utilized to breakdown or transform several contaminants into less toxic forms. Main focus of chapter will be on several bioremediation techniques, employing indigenous microorganisms to decompose biodegradable pollutants in order to stabilize or to transform the contaminants into non-hazardous by-products. Besides, it will elucidate several factors effecting bioremediation process, involving energy source as a dominant necessity of microbial activity. Undoubtedly, bioremediation offers a greener pathway of remediation in comparison with wide varieties of conventional and artificial treatments.

INTRODUCTION

Soil and water, as two main elements of the environment and interacting systems, play an important role in maintaining the environmental balance and preserving ecological stability. Soil, as the top layer of the earth, consisting of organic and inorganic fractions alongside various species of fauna and flora, undertake the supreme responsibility of embracing the biodiversity in order to protect and nourish. On the other hand, water, covering 71% of the earth's surface, including aquatic biodiversity, plays a fundamental role in dynamism preservation for both water body biodiversity and other forms of life all over the planet Earth. By all means, soil and water are two entangled founders of the planet. An inattentive

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interference in either of soil and water media can lead to disruption of favorable characteristics, and due to human activities, which have never been so considerably planned; a common intrusion aspect has been anthropogenic pollution. With the beginning of the industrialization era, utilization of chemical compounds on lands or disposal of contaminants directly or indirectly to soil and water systems has caused numerous sorts of alterations in soil and water properties and affected the inhabitant biodiversity. Hazardous compound entrance may result in a decrease in soil and water fauna and flora, leading to a balance loss. Besides, pollutants are capable of entering the food chain and eventually human body, causing serious health problems. Soil, as a capable and dynamic system with physical, chemical and biological properties, deriving from soil forming factors and processes, might be able to buffer the alteration to some extent, but water on the other hand, seems to be more vulnerable according to non-buffering medium which can be affected immediately after introduction of the pollutant. To reduce the negative effects and remediate contaminated soil and water numerous methods have been developed. By the mid-1980s, remediation technologies started to appear under the spot light, and in the late 80s, the technology could have been called a beneficial business even for those with few years of experience, meanwhile, an increase in competition forced companies to search for new cost-effective methods to survive (Havrank, 1998). These methods apply varieties of *in situ* and *ex situ* physical, chemical and biological techniques to generally remediate the medium by stabilization, transformation or elimination of the pollutants. Physical and chemical methods have been observed to be unable to completely abolish the contamination, and only transform the pollution in to other types. Among three applications, biological techniques, known as bioremediation, have been enhanced due to their low cost, safety and natural structure (Williams, 2001). This chapter will briefly define soil and water contamination, but the main focus will be on the bioremediation and recent technologies.

ROLE OF MICROORGANISMS IN BIOREMEDIATION

Microorganisms have been utilized by ancient Romans for waste water remediation since 600 B.C., and since 1972 the technique was considered as an effective remediation process (Pal, Patra, Reza, Wildi, & Pote, 2010). Later, varieties of microorganisms were figured out to be capable of enzymatically uranium reducers. These microorganisms included *Geobacter metallireducens* and *Shewanella putrefaciens*, which also maintain energy by combining the reduction with oxidation of acetate or H_2 (Lovley & Phillips, 1991). Subsequently, Lovely et al. (1992) utilized *Desulfovibbrio desulfuricans* in elimination of uranium from uranium-contaminated water. Following, same bacteria were employed alongside bicarbonate extraction to remediate the uranium-contaminated soils by reduction of the uranium content of the extract from U(VI) to U(IV) (Phillips, Landa, & Lovley, 1995). *Desulfovibro vulgaris* also proved to reduce Cr(VI) to Cr(III) by using c3 cytochrome in water remediation (Lovley & Phillips, 1994). Similar studies revealed that Fe(III)-reducing microorganisms can be used to exceed the organic pollutant degradation rate by stimulating Fe(III) availability (Lovley, Woodward, & Phillips, 1994). Attempts to verify heavy metal reducing microorganism resulted in determination of species, such as *T. selenatis* and *Salana multivorans*, reducing Selenit to elemental selenium (Macy, Michel, & Kirsch, 1989), *Thauera selenati*, reducing selenate to selenite (Macy et al., 1993), and a modification in bacterium *Deinococcus radiodurans* made the bacteria capable of absorbing and digesting toluene and ionic mercury from highly radioactive nuclear waste (Brim et al., 2000). Further molecular analysis of *Geobacter* proved that it was significantly enrolled in bioremediation of organic and metal pollutants in subsurface media.

Thenceforth, genomic research revealed information on common bioremediation species, such as *Geobacter*, function in various polluted media. Studies have also shown that *G. metallireducens* contains chemitaxis to Fe(II), which can be beneficial to guide it to Fe(III) oxides anaerobically (Childers, Ciufo, & Lovely, 2002). According to Jian et al. (2008), SRB can make a reduction in the concentrations of exchangeable Cd. The Cd-contaminated soil bioremediation efficiency of the method has been declared to be 70%. Ever since, the same bacteria have been used in treatment of As-contaminated water and the eliminated amount has reported to be enhanced from 10% to 47% for As (III) and from 39% to 92% for the As (V) (Teclu, Laing, & Wallis, 2009). Nevertheless, based on recent studies, several microorganisms, such as *Shewanella*, *Clostridium*, *Geobacter*, *Thermus*, *Phytobaculum*, and *Desulfosporosinus* seem to be capable of U(VI) reduction (Mitmunya & Chirwa, 2013). Evidentially containing neurotoxic properties, acrylamide is known to be degraded by several microorganisms, namely *Bacillus*, *Psedomonas*, *Rodococcus*, *Arthrobacter*, *Xanthomonas*, *Rhodopseudomonas*, *Rastonia*, *Geobacillus*, *Entrobacter*, and a filamentous fungal Aspergillus *oryzae* (Charoenpanich, 2013), and the immobilized organophosphate degrading enzyme A (OpdA) on nonwoven polyester fabrics has revealed to be effective in remediation of pesticide contaminated water (Gao, Truonga, Paul Caciolib, Butlerb, & Kyratzis, 2014).

SOIL AND WATER CONTAMINATION

Contamination and pollution which are generally used as the alteration in natural body of the environment by introduction of foreign substances in the medium or an elevation in undesirable elements, affecting the general properties and more significantly chemical characteristics of the body which are believed by some experts to be distinguished as the non-hazardous and hazardous appearance (Alloway & Ayers, 1997). These foreign substances, also called xenobiotic, can be caused by industrial activities and wastes, municipal (urban) wastes, agricultural and irrigation misuse of chemicals or nuclear power plant derivatives (Alloway, 1990). Soil contamination is defined as the existence of xenobiotic chemicals which are anthropogenic, organic or inorganic compounds in the media, or heightened concentration of pre-existed constituents in soil. On the other hand, water contamination refers to direct or indirect disposal of contaminant in to the water bodies as a result of inadequate treatments. Soil and water contamination can be categorized by the source of pollution and compound characteristics (Valentín, Nousiainen, & Mikkonen, 2013). Foreign substances can be classified by different aspects within a concept. Contaminants or pollutants can be categorized according to their chemical and physical characteristics, abundance or stability, influence on the ecosystem or their toxicity (Van der Perk, 2013). Known as one of the most important source of drinking water, groundwater has been contaminated with petroleum hydrocarbons leaking from underground storage tanks. These hazardous contaminants including benzene, toluene, ethylbenzene, and xylene (BTEX) are considered as organic pollutants, and Petroleum hydrocarbons (PHCs), polychlorinated biphenyls *(*PCBs), organic solvents, organochlorine pesticides (OCPs), persistent organic compounds (POPs), hormones, cosmetics, detergents are being generally categorized within waste water contaminants group (Leung et al., 2005; Tarradellas & Diercxsens, 1987). Aliphatic organic compounds (AOC) are more likely to be degraded than the aromatic compounds. volatile organic compounds (VOCs), known as typical water contaminants, contain halogenated solvents and petroleum products utilized by numerous industrial branches, such as paint, military and dry cleaning. These constituents are highly mobile and are able to be easily leached into groundwater in vicinity of contaminated sites. The EPA National Priority list contains of 11 VOCs, namely trichloroethylene, toluene, benzene,

chloroform, tetrachloroethylene, 1,1,1-trichloroethane, ethylbenzene, trans-1,2-dishloroethane, xylene, dichlomethane and vinyl chloride. Chlorine hydrocarbons, produced during the chlorination of municipal water, are known as carcinogens (Hodgson, 2010). Organochlorine Compounds such as DDT (1,1,1,-trichloro-2,2-di (4-chlorophenyl)ethane), aldrin, dieldrin, and chlordane, are among the main dangerous contaminants. DDT is known to be banned due to its bioaccumulation. In soils, pesticides may go through different pathways, ideally, these chemicals would be degraded by biological, chemical and photochemical processes. Degradation rate depends on the half-life of the component, which is due to soil characteristic, such as pH, temperature, humidity, biodiversity, and environmental conditions. Plant uptake, soil adsorption, groundwater depth, leaching, and vaporization would define their mobility and fate in time, therefore, they may also be transferred by wind, runoff, or leaching, and cause ground and surface water pollution; specifically in usage of high concentrated water-soluble pesticides. Sandy soils alongside heavy precipitation or irrigation result in less degradation and more wash down (Levine, 2007). Naturally occurring in fresh water in trace amounts, arsenic can be found in elevated concentration and cause water pollution. According to WHO and EPA, maximum legal concentration of "As" in drinking water is 50 μg L^{-1} and the recommended level has been given as 10 μg L^{-1}. On the other hand, seawater generally contains 0.001-0.008 mg L^{-1} arsenic. Arsenic concentration in uncontaminated water normally ranges between 1-10 μg L^{-1} (Mandal & Suzuki, 2002). Prominent contaminant heavy metals are; lead (Pb), chromium (Cr), arsenic (As), cadmium (Cd), nickel (Ni), zinc (Zn), copper (Cu), mercury (Hg), silver (Ag), and selenium (Se). Such metals can occur in soils in trace concentration according to parent material and forming processes. Following table presents information about concentration of some elements in soils.

Heavy metals behavior and fate in soil depend on soil and metals general characteristics. Heavy metal speciation in soil affects the mobility, availability, and their toxic impact. In some regions of the world, aside from the background concentration in some areas, radioactive contamination might be a great concern for surface and groundwater in vicinity of nuclear weapon producing areas. Radionuclides, such as uranium or radon, typically known for their unstable nucleus, are other soil and water contaminants which emit ionizing radiation (Hodgson, 2010).

Table 1. Some element concentrations in soils (Source: Maestri, Marmiroli, Visioli, & Marmiroli, 2010)

Element	Average Range in Soils (mg kg^{-1})
Mercury	<0.1
Selenium	1-2
Cadmium	1-2
Copper	2-60
Nickel	2-200
Chromium	5-1000
Lead	10-150
Zinc	25-200

HAZARD ASSESSMENT

Risk is a fundamental and dominant concept in toxicology and environmental health. The utilization of scientific methods and measurements in order to estimate health risk is the very basic of risk assessment in the field of environmental sciences. Environmental pollution can cause health risks for both human and ecosystem. Effects of various types of pollution have been the topic of many studies for decades (Chowdhury & Chandra, 1987; Fan, 1988). Chemicals, introduced to the environment, seldom endure their form, speciation, or position. Intrusion of such chemicals into the water, beside human health related issues, can have an impact on aquatic ecosystem productivity and species composition due to water quality degradation (Binkley et al., 1999). Released to the soil, compounds tend to be degraded by soil biodiversity and get detoxified accordingly. Persistent compounds maintain their existence and stay in soil and water as contaminants. Henceforth, toxicology, ecotoxicology and radioecology are known as different concepts to define the interactions between the environment, contaminants, their fate, and unfavorable effects. It has often occurred that there has been more than a single contaminant in system, so the toxicity of combination would be taken into consideration. Contaminants interactions, as synergistic or antagonistic, may enhance or decrease the toxicity of the hazardous effect on ecosystem, involving biodiversity and microbial population. Some chemicals are on the other hand being directly absorbed by plants and bacteria which subsequently will enter the food chain while others may approach the human and animal bodies by inhalation, drinking water and polluted flying dust. Pollutants bioaccumulation, as the accumulation of the pollutants in organs or tissues by time, is related to the biological half-life of the contaminant. Biomagnification characteristic of contaminants, which is used for magnifying concentration of pollutants in tissues, is another factor to be considered during the risk assessment process (Hodgson, 2010; Nordberg et al., 2007; Van der Perk, 2013). Most provided models for ground water risk assessment do not consider the gas phase transport procedure for solvents such as BTEXs and chlorinated solvents (Troldborg, 2010). Computer software such as GIS can be utilized to position data and interpolate maps of contaminated area (Dinev, Banov, & Nikova, 2008). Generally, risk assessment for all toxic compounds follows the same framework of; a) Exposure assessment, b), Dose-response relationship assessment c) Hazard identification, and d) characterization of the risk.

BIOREMEDIATION PRINCIPLES

Contamination, by any source or amount, affects the ecosystem and risks the biodiversity in both soil and water. Natural attenuation of contaminants which is a natural phenomenon offers a reduction in toxicity, mobility or bioavailability of the inner-media contamination. Natural attenuation procedures in soil and water can appear as biodegradation, chemical transformation, immobilization, volatilization, and dilution or dispersion. These processes performance depends on soil and water characteristics. Some dominant soil characteristics are; organic content, pH, mineralogy and hydraulic conductivity, and water characteristics like velocity, direction, pH, and oxygen content. To remediate the contaminated media variety of techniques has been provided; these methods would include *in situ* and *ex situ* physical, chemical and biological technologies which as a relatively cost-effective and natural technique, biological methods seem to be preferable. Biological technologies, named as bioremediation techniques, refer to the usage of biological mechanisms, living organisms or specific species of plants in non-sterile environment for elimination, extraction, transformation, and stabilization of the contaminants in order to secure the fauna

and flora. Bioremediation is a multidisciplinary approach which is generally based on microbiology. This method employs microorganisms such as yeast, fungi or bacteria in order to reduce, eliminate, contain or transform hazardous contaminants in soil, sediment or water. So far, microorganisms and plants were used to breakdown or transform several contaminants into less toxic forms. Bioremediation has been employed for treatment of solvents, explosives, PAHs, PCBs, metals, and radionuclides. Microorganisms, already presented in the contaminated medium, usually survive the unfavorable conditions of the contaminated media. Native microorganisms can act as the electron acceptors or can metabolize the organic pollutants to produce carbon, methane and water (Hazan, Benson, Metting, Faison, Palmisano, & Mccullough,, 2003; Singh & Tripathi, 2007). Bioremediation technology includes natural attenuation as intrinsic bioremediation and enhanced bioremediation as nutrient addition (biostimulation) or microorganism inoculation (bioaugmentation). Surface and ground water bioremediation techniques include bioaugmentation and enhanced bioremediation technologies (e.g. bioventing, biosparging, liquid delivery system, anaerobic bioremediation, phytoremediation etc.). Efficiency of bioremediation depends on enzymatic attack of pollutants by microorganisms in order to transform the components to less hazardous substances. Since environmental condition can affect the performance, the process often involves the manipulation of the environmental factors in order to achieve favorable growth of microorganisms and degradation rate. Bioremediation techniques assume to be cost-effective and more affordable than conventional methods. On the other hand, natural principle of the method makes it more acceptable by the public. As for other remediation techniques, bioremediation also has shortcomings such as the inability of microorganisms to eliminate chlorinated organic or high aromatic hydrocarbons (Vidali, 2001).

FACTORS AFFECTING BIOREMEDIATION

There are several factors effecting bioremediation process and efficiency. Soil environment is competitive for microorganisms, and those with the capability to adapt to the stresses will survive. The stresses include:

Biotic Stresses

Soil indigenous microbes generally compete with one another. This rivalry can be for substrate, nutrients or growth parameters. Moreover, microorganisms can harm neighboring organisms by producing allelopathic (inhibitory or toxic) secretions, such as antibiotics. Since several organisms have predatory or parasitic effect on surrounding biodiversity, the non-indigenous organisms may have a small chance of survival after introduction to the soil. This effect seems to be important while introducing organisms in order to biodegrade the contaminants (Pepper, Gerba, & Gentry, 2014).

Abiotic Environmental Factors

In addition to sunlight as an environmental necessity for phototrophic microorganisms, which is normally limited to the top layer, soil physical parameters, such as temperature and moisture also shift during the days and seasonally and hence offer unfavorable conditions for photosynthesizing microorganisms. Although soil microbial population seems to be unaffected by temperature fluctuations, microbial communities can differ in their preferable temperature. Due to buffering effect of soil on temperature, especially in subsurface, most soil organisms seem to fit in the mesophilic (prefer 20-45°C) category. Soil

texture and water availability affect the bioactivity as well; the optimum bioactivity appears to be at 0.1 atm, known as the transition between capillary and free water, and medium texture soils. The resistance to dehydration has been shown to be the highest in fungi, which is followed by actinomycetes and later with the bacteria (Pepper et al., 2014). According to Lauber et al. (2009) pH is the major determinant of bacterial diversity at continental scale while the vegetation and organic content of soils can be effective in regional or local scale. Undisturbed soil pH normally values from 6 to 8, which most organisms seem to have their optimum pH within this range, although there are several exceptions, such as *Thiobacillus spp*., which has the optimum pH of 2-3. Soil Redox potential (Eh) is another important factor in degradation process, ranging from +800 mV in oxidizing soil to -300mV in reducing environment. Bioactivity of soils is undoubtedly affected by carbon and nitrogen content as two major soil nutrients for microorganisms. Considering the low concentration of these elements in soils, most soil organisms exist in semi-starvation or dormant condition, except for rhizosphere which contains root exudates (Pepper et al., 2014).

Contaminant Biodegradability

Microorganisms of several species contain the capability to metabolize wide variety of organic contaminants due to evolutionary processes relate to enzymatic mechanisms. Regardless of abnormally stressed environment, every environment includes widespread microbial species which are able to metabolize natural compounds. Microorganisms are not capable of degrading some synthetic components as these constituents are recently introduced to the environment and their effective metabolisms have not evolved yet. Furthermore, some of these compounds, such as chlorofluorocarbons seem to be inherently persistent. Compounds which contain naturally occurring constituents are more probable to be metabolized in comparison with those with chemically distinctive structure. Synthetic chemicals containing carbon-chlorine or carbon-fluorine bonds are known to be metabolized slowly and in some cases not at all. Dominant soil and water contaminants such as polychlorinated biphenyls, chlorinated solvents, and chlorinated aromatic compounds are examples of such compounds which may need microbial consortia or co-metabolism in

Table 2. Optimum environmental conditions for biodegradation (Source: Vidali, 2001)

Environmental Factors/Soil Characteristics	Conditions Required for Microbial Activity	Optimum Condition for Organic Pollutant Degradation
Texture	Low clay or silt content	Sandy loam (Kristensen, Henriksen, Mortensen, Scow, & Moldrup, 2010)
Moisture	25-28% of water holding capacity	30-90%
Oxygen Content	Aerobic, minimum air filled pore space of 10%	10-40%
Temperature(°C)	20-45 (Pepper, et al., 2014)	20-45
Nutrient Content	N and P for optimum growth	C:N:P=100:10:1 (Dion, Nautiyal, & Rummel, 2014)
pH	5.5-8	6.5-8
Contaminant	Not too toxic	5 – 10% of dry weight of soil
Heavy Metal	Total content of 2000 mg kg^{-1}	700 mg kg^{-1}

order to be degraded. Degradation rate of mixture contaminants is not the same and depends on several factors, such as microbial diversity, nutrient availability, and etc. In highly contaminated areas, excessive concentrations of pollutants can create toxicity affecting the biodiversity, in such conditions physical, chemical or combination techniques can apply to reduce the contamination to initiate bioremediation (DEQ, 1998). Besides, there is an inadequacy in bioremediation technology when comes to measurable and non-measurable biochemical products, and inability to prevent unfavorable chemical conversions. By all means, bioremediation success lies upon the amenability of the xenobiotics to get transformed by organisms.

Microbial Species Cooperation for Enhanced Biodegradation

A single strain of bacteria is rarely capable of metabolizing most organic toxicants. Bioremediation performance depends on the microbial consortia and biodiversity. It has often been observed that different species cooperate in the stepwise or sequential decomposition of complex organic compounds, or switch genetic material in order to enhance degradation capabilities. Certain species may excrete beneficial material for other microorganisms, while others microorganisms excretion can inhibit other species activities. Certain interactions between microbial species, such as commensalism, syntrophism, and interspecies hydrogen transfer are assumed to be beneficial in bioremediation process. Commensalism happens where one population indirectly benefits from others remaining. Decomposition of compounds, such as PCBs, chlorinated solvents and some pesticides usually is possible by co-metabolic commensalism, or sequential degradation by different species. Syntrophism on the other hand, is an optional association between two or more microbial populations supplying one another nutritional needs. Under anaerobic conditions, collaboration of the microorganisms will be more essential in order to degrade certain organic contaminants in to nonhazardous products such as acetate, CO_2, CO_4, and H_2. Interspecies hydrogen transfer plays a dominant role in elimination of the fermentation products and increases the anaerobic mineralization (Alvarez & Illman, 2006). Utilized organisms are generally categorized into three groups including, autochthonous (indigenous), allochthonous (non-indigenous), genetically modified organisms (GMOs). Organisms, present in an environment, have the capability to function through effective mechanisms under optimized conditions, while non-indigenous organisms have to be isolated from one environment to be introduced to another. To be stabilized, these transients need to adjust to their new conditions afterwards. Genetically altered organisms are created to adapt, grow, and function under adverse conditions including severe contamination presence. Recent bioremediation approaches lay upon organism metabolisms or absorption of xenobiotics. Several microorganisms have shown the ability to eliminate certain contaminant through biodegradation by enzymatic mechanisms or absorption as storage in both *in situ* and *ex situ* applications. There have been several studies based on bioremediation effectiveness using one or combination of organisms, DNA technology, or combination of methods. Current pioneering developments in molecular and –omics technologies including molecular profiling, proteomics, metabolic engineering, ultrafast pyro-sequencing, microarrays, mass spectrometry, transcriptome and proteome analysis of the overall population alongside bioinformatics equipment can lead to a better understanding of indigenous microorganisms and result in enhancement of their degrading capabilities or capacities of recalcitrant pollutants including chlorinated aliphatic, and polychlorinated biphenyl. Genetic engineering techniques such as recombinant DNA technology can create microbes with desired characteristics in order to eliminate limiting factors, involving elevation in degradation rates or creation of new degradation pathways for certain bacteria strain. Several studies on

genetically altered organisms have been carried out in laboratory conditions, which eliminate the interference factor from the equation. Hence, application of such GMO-specific degradation or biodegradation in non-sterile conditions or various xenobiotic occurrences may cause complications and lead to invalid conclusions (Kulshreshtha, 2012). There are also non-scientific factors affecting bioremediation, such as regulations, research and technology, human resources, economic and liability. By all means, these factors may terminate bioremediation application in certain conditions. In cases of successful utilization of bioremediation these factors are positive. Depending on the remediation area or the conditions one or more of the above factors may be critical (Boopathy, 2000).

Microbes and Metals Interactions

Metals in soils can attend different "pools" that will determine their relative mobility and availability (Kumar et al., 2011; Lindsay, 1979);

1. Exchangeable/dissolved fraction
2. Bounded by mineral constituents
3. Bounded by organic components
4. Precipitation products as pure or combination
5. Take part in or fixed by crystalline phase of primary or secondary minerals

According to the source of introduction (geological or anthropogenic) metals can be associated with any of the pools above. Metals with strong bounds,called complexes, are assumed as less harmful due to their low probability of availability to plants and organisms in soils, however, the exchangeable, organically and inorganically bounded fractions are of high importance and are capable of affecting the lability, migration, and bioavailability of metals. Dissolved in soil solution or exchangeable fraction of adsorbed metals present high mobility and bioavailability, thus the fraction can indicate the environmental impact of metals in soils. Studies on microbe-metal interactions proffer better understanding of microorganism capabilities in heavy metal and radionuclides modification. Major techniques to alleviate heavy metal content are known as precipitation, ion exchange, adsorption onto activated carbon, membrane processes, and electrolytic techniques. Since such technologies seem not to be operational due to their cost and regulatory, more cost effective methods including metal sorbents such as microbial cells have been developed. Metal sorption mechanisms by microbial species, involving surface complexation, ion exchange, and microprecipitation, generally include varieties of active and passive mechanisms; Passive mechanisms contain extra cellular complexation and cell wall binding, where complextion (i.e. *Citrobacter spp*) occurs by the means of extra cellular polymer substances, such as polysaccharides, proteins, nucleic acid, and sidrophores, and cell wall binding (i.e. *Aspergillus spp*), uses ion exchange reactions, ligand complexation, or ligand destruction. On the other hand, active mechanisms involve the application of rhamnolipids (i.e. *P.aeruginosa*), intracellular accumulation (i.e. *Pseudomonas spp*), metallothionein (i.e. *Synechococcus spp*), precipitation (i.e. *Citrobacter spp*), oxidation/reduction (i.e. *T.ferroxidans*), efflux pump (i.e. *A.eutrophus*), and volatilization (i.e. *Fungal spp*) of contaminants. In order to utilize the microbial biomass in large-scale process immobilization of biomass seems to be unavoidable. Immobilization of biosorbents is accomplished by using inert agents, such as alginate, silica, polyacrylamide, polymethane, and polysulfone. Such immobilization has been utilized in bioreactor configurations, involving rotating and fixed systems which are going to be discussed later in this

chapter. In order to employ bio-components as an efficient filter material in radionuclide and heavy metal treatment process, pH consistency, sustainability, and the immobilization process is undeniably crucial. Methods, such as sol-gel technology provide the opportunity to of immobilization without activity or structure modification alongside producing a metal selective filter material. Major utilization of metal-microbe interaction is known to be in bioleaching of contaminated sites and mineralization of contaminated organic matter (Merroun, 2007; Rajendran & Muthukrishnan, 2003).

MICROBIAL REMEDIATION (MICRO-REMEDIATION)

Providing the favorable conditions for microbial growth, they can be used to biodegrade/biotransform several complex and hazardous organic chemicals into less toxic or harmless compounds. After the application of 'super bug' in oil spills remediation, various microbial techniques, for contaminated sites, have been provided. Microbial destruction of toxic organic contaminants is through bacterial and fungal degradation using anaerobic and aerobic pathways. There is an ongoing progress in establishment of microbial treatments for metal contaminated sites as well. Environmentalists consider microbes as 'eco-friendly nano-factories' to treat metal contaminated areas through biotechnological application of microbes, such as yeast, bacteria, algae, diatoms and actinomycetes. Several microbial processes have been suggested as the major pathways of metal bioremediation including biosorption and bioaccumulation, catalyzed immobilization, catalyzed solubilization, transformation and detoxification (Sinha, Valani, Sinha, Singh, & Herat, 2009). Accordingly, biosorption can be described as ability of several biomaterials to accumulate heavy metal or radionuclide species from solutions through metabolism related mechanisms or physiochemical technique. According to some studies, biosorption is considered an industrial waste water treatment technique in concentration range below 100 mg kg^{-1}, which surpasses conventional water treatment methods by low cost, efficiency, and possibility of recovery of both biosorbent and heavy metals (Fulekar, 2010). There are several differences between biosorption and bioaccumulation, such as rate of uptake, toxificant affinity, reusability, selectivity, maintenance, and etc. Biosorption process includes a solid phase (sorbent, biosorbent or biological material) and a liquid fraction (solvent or water) contaminated with metal ions (sorbates). The mechanism is complicated and is affected by several factors including status of biomass (living or non-living), metal-solution chemistry, biomaterial type, and chemistry of the environment. Living biomass mechanism follows two stages of passive and active biosorption. Passive mechanism, reversible equilibrium of adsorption-desorption, is non-metabolism dependent and is a result of metal binding mechanisms including complexation, electrostatic adsorption, ion exchange, and inorganic microprecipitation. Active mechanism which seems to proceed slower is defined as the penetration of metal ions through cell membrane into the cells. Non-living biosorbents generally use the passive mechanisms. The equilibrium of biosorption technique is generally explained by Langmuir and Freundlich models, which are used for isotherm adsorption equilibrium. Recently, application of some eco-friendly biosorbents, such as agricultural wastes, fungi, algae, bacteria, and yeast has been broadly suggested. Equally, both living and non-living microbial cells have been proved to be able to be used in biosorption technique; however disadvantage of living cells due to the need for nutrient, BOD and COD elevation and more attention, non-living cells seem more preferable (Sueem & Saral, 2014). Biosorption of heavy metals using macrofungi or mushroom in soil and waste water have also been recently studied. Results have revealed that mushrooms can significantly reduce Cd, Cu, Ni, and Cr concentration by biosorption (Sueem & Saral, 2014). Immobilization process of microbial

biomass is an important factor for a continuous industrial process. Immobilized cell systems provide the opportunity of better recovery. Due to biosorbents mechanism, kinetics, uptake capacity, and physical properties, several reactor configurations have been provided, including batch stirred tank reactor, continuous flow stirred tank reactor, fixed packed bed contactor, fluidized bed reactor, pulsating bed contractor, and multiple bed contact arrangement (Sueem & Saral, 2014). Catalyzed immobilization occurs inside microbial cells due to metal ion fixation by Iron oxides and into organic colloids. Bacteria enzymatically reduce the metal and under favorable circumstances solubilize oxide minerals which results in solubility, precipitation, or reduction in bioavailability of toxic metals. Metal detoxification by microbes occurs through resistance mechanisms of 'valence transformation' by extracellular chemical precipitation or volatilization (Sinha et al., 2009).

MYCOREMEDIATION (FUNGAL REMEDIATION)

Fungi have always been regulated and utilized by human for several purposes. Environmentally, fungi are known to be one of the major groups of plant decomposers which are capable of degrading plant polymers, such as cellulose, hemicellulose, and lignin. Fungi are known to contain biodegradation capabilities and also they have proved to be able to accumulate metals, specifically radionuclides. Degradation of Polyethylene by *Penicillium simplicissimum*,known as white-rot fungi, is an example of such capabilities. Laboratory results have provided information on fungi and various waste type relationship, and so far fungi have been employed in bioremediation of toxic compounds in soil, wastewater or sediments. Fungi are known to be effective in elimination of metals or decomposition and mineralization of organic compounds, such as phenols, petroleum hydrocarbons, chlorinated pesticides and many other hazardous contaminants. Accordingly, mycoremediation is the technique of utilizing fungi to clean up the polluted soil and sediment. On the other hand, the term mycotransformation can also refer to the process of biotransformation of compounds, waste, and wastewaters by such organisms. White-Rot Fungi which has been proven to contain lignin decomposing enzymes is capable of degrading vast variety of persistent organic pollutants. White-Rot fungi, such as *Phanerochaete chrysosporium*, *Pleurotus ostreatus*, *Trametes versicolor*, *Bjerkandera adusta*, *Lentinula edodes*, *Irpex lacteus* have been able to efficiently decompose and eliminate insoluble toxic compounds in comparison to other fungi or microbes. However, in order to achieve the optimum performance in mycoremediation comprehensive knowledge of fungi physiology, ecology, biology, enzymology, genetics, and many other principals would be necessary. In order to enhance the fungi performance in polluted sites different types of substrates are known to be employed. These substrates, such as wood chips, straw, peat, fish oil are applied during *in-situ* and *ex-situ* inoculations. Moreover, soil conditions may need alterations and amendments to be able to provide a better fungal habitat and encourage White-Rot fungi colonization, while the amendments themselves appear to be beneficial due to sorption of pollutants and decreasing the contamination. It has also been observed by several researchers that the addition of straw has improved mycotransformation by White-Rot fungi. Since fungi have been grown on substrates before inoculation, the carbon/nitrogen ratio in the substrates play an important role affecting performance of fungi. Such strategies also need cost associated calculations, disposal possibilities considerations, and amendment site availability. On the other hand, regulations of effective factors and contaminated site conditions have to be considered. It has also been suggested that the use of surfactants can enhance POP mycotransformation in both liquid culture and soil; it may also elevate PAH transformation by affecting the bioavailability. Soil pH, aeration, alkalinity, elemental concentrations, and bioavailability have great influence on the fungal degradation; for instance

reduction in pH or enhanced aeration is known to stimulate the fungal growth and degradation of toxic compounds such as atrazine and POPs in liquid cultures and soils. It has been evident that the complete degradation of POPs and PAHs by fungi in non-sterile soils never reaches more than 30% which is even less in aged soils due to the appearance of transformation products by several chemical, biological and enzymatic reactions. In cases of extreme toxic soil contamination, *ex situ* remediation techniques, such as a solvent wash before fungi treatment is more advisable. There have been several attempts to enhance the efficiency of the mycodegradation by means of molecular techniques which includes studies on the improvement of extracellular fungal enzymes excretions. These enzymes, including laccases, lignin and manganese peroxidase are known to be responsible for POP transformation (Gadd, 2001; Singh, 2006).

PLANT-ASSISTED BIOREMEDIATION (PHYTOREMEDIATION)

Phytoremediation, as a vast area of study, is based on principles of environmental biotechnology and bioengineering. It considers the living plant a solar driven pump capable of extracting, accumulating, degrading, and volatilizing dissolved toxic substances from soil, air, and water. While, bioremediation, as mentioned before, uses microorganism activities, phytoremediation aims to utilize higher plants in order to remediate polluted sites. Bioremediation is known to be highly effective in contaminated areas with organic pollutants as a cost-effective and environmentally friendly method. Microbial remediation process relies on degradation by microbes, whereas in heavy metal pollution there is no possibility of degradation. Studies have suggested that in heavy metal polluted sites, due to limited microbial potential for degradation, plant application to sequester specific metals in tissues would be a more preferable remediating mechanism. Hyperaccumulator plants which are primarily introduced for phytoremediation of polluted areas are known to posses limited capabilities to accumulate specific elements, and hence have to accumulate higher than 1000 μg/g of heavy metals, such as Ni, Cr, Pb, Cd, and etc. Since there is an undeniable connection between phytoremediation technology and bioremediation process, the term and cleaning pathways need to be comprehensively clarified. There are several phytoremediation techniques briefly given in table 3 (De Mello- Farias, Chaves & Lencina, 2011; Tangahu et al., 2011). Subsequently, a current topic in demand is plant-assisted bioremediation which generally refers to use of both plants and organisms in treating contaminated soils. Specific plant roots are known to excrete substances that are nutrients for microorganisms, bacteria, and fungi located in rhizosphere. These microorganisms are capable of degrading several organic compounds: For example fungi-assisted phytoremediation strategies have been revealed to be fairly effective in organic-metal contaminated soils (Shukla et al., 2010). Regardless of the vast background sturdies in metalliferous plants, recent research topics by microbiologists seem to lay on plant-associated bioremediation. There are known to be three typical mechanisms to enhance plant trace element uptake; (i) elevation of root surface area and hair production, (ii) cause an enhance in element availability, and (iii) increase in soluble movement of the element to plant. According to several researches metal-tolerant microorganisms are able to elevate plant establishment and growth despite the toxic element concentration. Bacteria with abilities to promote plant growth and fungi including arbescular mycorihzal fungi (AMF) have been revealed to attribute in varieties of efficiency enhancing mechanisms including stimulation in plant growth, phytohormones production, stress ethylene prevention, and plant nutritive status improvement (Sessitsch et al., 2013). The efficacy of phytoremediation can also be enhanced by surfactants including EDTA, EDDS and LAS due to increase in elemental uptake by plants. But application of such synthetic compounds (surfactants) at high concentrations can also cause toxicity for plants. Natural surfactants, such as rhamnolipid, surfactin,

Table 3. Phytoremediation techniques summary modified from (Source: De Sousa, 2007; Marmiroli, Marmiroli, & Maestri, 2006)

Technology	Mechanism Summary	Target Media	Target Contaminant
Phytoextraction	Contaminant take up into tissues	Soil, Sediment, Sludge	Inorganic contaminants
Phytostabilization	contamination Containment due to transformation or erosion prevention	Soil, Sediment, Sludge	Organic and Inorganic contaminants
Phytovolatilization	Sorption, translocation, or transpiration of the contaminants	Groundwater, Soil, Sediment, Sludge	Organic, Inorganic, Volatile Contaminants
Phytohydraulics	Hydrology controlling, containment or degradation	Ground water, Surface Water	Organic and Inorganic Contaminants
Phytodegradation	Decomposition within plant by enzymatic and photosynthetic activity	Soil, Sediment, Sludge, Ground Water, Surface Water	Organic Contaminants
Phytosequestration	Sequestering in rhizosphere using phytochemicals/on root by protein transport and cellular processes	Soil, Sediment	Inorganic Contaminants
Rhizodegradation	Enhanced biodegradation in rhizosphere by phytochemicals	Soil, Sediment	Organic Contaminants
Riparian Corrirdors	Contaminant destruction	Ground Water, Surface Water	Organic Contaminants
Rhizofiltration	Contaminant absorption by hydroponic plants	Ground Water, Waste Water	Inorganic Contaminants

saponin, and sophorolipids are also able to enhance phytoremediation efficiency in contaminated soils. Naturally occured surfactants have been suggested to be responsible in increasing contaminant mobilization and hence the plant uptake elevation (Pacwa-Plociniczk, Plaza, Piotrowska-Seget, & Cameotra, 2011). According to recent progress in the era of phytoremediation, transgenic plants are engineered to enhance removal abilities. Transgenic plants are assumed to be preferable in metabolizing herbicides and persistent contaminants. A genetic combination of fast growing, high biomass yielding, highly tolerant, easily cultivated, and hyperaccumulator of toxic metals in shoot can fulfill the purpose of phytoremediation. Symbiotic engineering, as a peculiar bioremediation system, provides both bacterium rhizobium and leguminous plants advantages by using several beneficial genes like *AtPCS* (encoding phytochelatin synthase), *MTL4* (metallothionein) and *IRTI* (iron regulated transporter gene) (Sinha et al., 2009).

EARTHWORMS ASSISTED BIOREMEDIATION/ VERMIREMEDIATION

Vermiculture, as an innovative technology, is known to be utilized for several environmental approaches involving soil remediation and land management. Earthworms have been revealed to be able to eliminate heavy metal, pesticides and lipophilic organic compounds, such as PAH from soil. Vermiremediation is a novel technology, providing low-cost and convenient site treatment. Several treatment methods have been considered including direct application of earthworms to the contaminated soil, co-application with

organic material, application as a part of feeding regime, and indirect use by application of digested materials (vermicompost). Generally earthworms can enhance the growth and activity of beneficial 'decomposer aerobic microbes' in soil due to aeration improvement and releasing chemical mediators. Earthworms contain millions of decomposer microbes and have shown to increase the number of bacteria and actinomycetes in the digested material to 1000 fold. Moreover, they excrete urine, intestinal mucus, glucose and other nutrients, such as N and P into the soil. Under desirable circumstances, earthworms and microorganisms perform symbiotically and synergistically to ameliorate and improve soil bioremediation. It has revealed that earthworms biodegrade organic pollutants, such as phthalate, phenanthrene, and fluoranthene indirectly by causing an elevation in microbial activities. Therefore vermiremediation can provide cost-effective and environmentally sustainable *in situ* treatment opportunity (Sinha et al., 2009; Ameh, Mohammed-Dabo, Ibrahim, & Ameh, 2013).

SURFACTANT ENHANCED BIOREMEDIATION

Hydrophobic organic chemicals (HOC) are known to be less soluble in groundwater. As a result, recovery by pump and treat technique is limited due to minimal bioavailability and availability to oxidative and reductive agents applied during the process. Hence, HOC's can stay in soil matrix more than expected. The adsorption of the contaminants by soil particles is normally assumed as the major factor affecting efficiency of the remediation process. Surfactants, known as active substances with a hydrophilic head and hydrophobic end, can be categorized into cationic, anionic and non-ionic groups possessing different characteristics. As mentioned before, these substances are generally used as cleaning agents during remediation procedure. Surfactants can provide certain biochemical and physiochemical properties to stimulate conditions of the soil or aquifer to a better adjusted microbial platform by enhancing the contaminant bioavailability. Chemically produced surfactants (synthetic surfactants) are available in low cost and have been widely used by remediation technologies. Among surfactants, biosurfactants stand out with beneficial characteristics, such as low toxicity, low critical micelle concentration (CMC), biodegradability, being eco-friendly, high selectivity, and adjustability to extreme environmental conditions. Naturally produced surfactants or biosurfactants are known to be produced extracellularly or as a part of the membrane by vast varieties of microorganisms including fungi, bacteria, and yeast. *Pseudomonas aeruginosa* is known to produce biosurfactants called rhamnolipids, which was previously mentioned in this chapter playing an important role during active mechanisms by microbes. Some other biosurfactant producing organisms are; *Bacillus subtilis, Nocardia amarea,* and *Saccharomyces lipolytica*. Biosurfactants, classified either by their chemical composition, molecular weight or microbial origin, can be synthesized by application of several microorganisms and carbon sources, such as hydrocarbons, carbohydrates, vegetable oils, and oil wastes. The production is affected by the medium and culture conditions. Another group of biosurfactants are phytogenic surfactants including saponins and lecithins, and humin acids. These compounds can be produced by decaying roots. Environmentally accepted biosurfactants have been utilized in remediation of inorganic contaminants, such as heavy metals, and organic contaminants, such as hydrocarbons. Some studies have suggested that biosurfactants are able to encourage degradation of adsorbed organic compounds by stimulating mass transport. Generally biosurfactants can facilitate desorption of soil contaminants as a part of biodegradation process or assist recovery process of an aqueous soil washing method (Bustamante & Diez, 2012).

NANO-BIOREMEDIATION

Nanotechnology, an emerging and novel trend, employs microfabrication approaches to study bio-systems and is widely used in fields of science from space science to deep oceanic studies. Nano materials (NMs) on the other hand, suggest advanced research challenge and represent the foundation of a current category of atomically engineered materials. Nanocrystals are used for materials in the range of 0.1-100 nanometer, and exhibit unique physical and chemical characteristics. Using microorganisms including bacteria, yeasts, fungi, algae and actinomycetes, nanotechnology helps to synthesize eco-friendly metal nanoparticles (NPs) to reduce toxic chemicals which are important in biomineralization, bioremediation, bioleaching, and microbial erosion. Application of NMs environmentally in treatment of contaminated water, waste, and soil has been considered to treat heavy metal pollution, uranium remediation, or hydrocarbon remediation in both large scale and portable applications in order to enhance the efficiency of techniques and decrease the cost of degradation. Metal NMs, oxide NMs, single-enzyme NPs, carbon nanotubes, and nanocomposites are examples of nanotechnology application in bioremediation. There have been several points rationalizing NMs application in bioremediation including elevation in surface area of the material in nanoscale or higher redox reactivity in comparison with contaminants. Regarding the undeniable importance of size and shape of applied material, NMs of any size and shape can be utilized in clean-up process. Due to possibilities of various forms of NPs and NMs limitations such as diffusion into contamination area can be eliminated. Considering the impeccable potential, the frequent appearance of such technology in sustainable development is highly predictable (Rajendran & Gunasekaran, 2007; Rizwan, Singh, Mitra, & Morve, 2014).

BIOREMEDIATION TECHNOLOGIES

According to the elimination of the pollutants and transportation of wastes for remediation, there is a fundamental division separating bioremediation methods into *in situ* and *ex situ* technologies. In bioremediation technology, a single technique usually is not capable of remediating recalcitrant compounds, which creates the need for combining the method with several physical and chemical treatment techniques in order to increase the efficiency in transforming recalcitrant contaminants. In some contaminated environments specific solutions such as employing microbial consortia instead of single strain can lead to a more favorable result. Determination of the suitable strategy within the range of bioremediation technology depends on contaminants biochemistry and bioavailability as well as the opportunity to optimize bioactivity. To design bioremediation projects data related to geology, hydrology, biology, chemistry, and environmental sciences would be necessary. Hydrogeology and biochemistry seems to provide adequate understanding of contaminated area, the living organisms, and their metabolisms related to biodegradation process. Some common bioremediation techniques are discussed in this section (Shukla, Singh, & Sharma, 2010).

In Situ Technologies

In site Bioremediation techniques depend on the presence of appropriate microorganisms. *In situ* treatment prevents destructions of the contaminated site during the remediation process. Generally applied in soil and groundwater, these methods concentrate on the existing organisms by optimizing conditions or

providing essential nutrients for them to grow and degrade contaminants. Most studies address optimizing pH, temperature, effluent dilution and aeration in order to apply the method in the field as well as the sterilized environment. Such techniques seem to be more profitable, eco-friendly and consequently preferable by public. However, these methods are affected by inconstant nature of environment from site to site as well as bioavailability of the contaminants in the media. Some of these methods are:

Intrinsic Bioremediation or Monitored Natural Attenuation (MNA)

The term intrinsic bioremediation, also known as natural attenuation, is generally defined as natural cleaning up of contaminated area. This passive remediation process relies on the natural degradation process happening in soil and groundwater from VOCs, SVOCs, fuel hydrocarbons, metals, and less effectively pesticides. This may include physical, chemical, and biological transformation, such as biodegradation, volatilization, oxidation-reduction, and sorption. Biodegradation, both aerobic and anaerobic, is the focus of natural attenuation responsible for pollutant elimination. During aerobic biodegradation dissolved oxygen (DO) plays a dominant role as the electron acceptor for the microbes, while in anaerobic decomposition nitrate, ferric iron, sulfate, and carbon dioxide (CO_2) are the major replacements as electron acceptors. Intrinsic bioremediation do not only transfer the pollutants to another phase or translocate within the environment, it also transforms the pollutants to harmless by-products, such as carbon dioxide and water. As a whole, the process is nonintrusive, natural, cost effective, and since it's not a mechanized remediation technique, there is no equipment limitations. In order to take natural attenuation as the only remediation technique, it needs to indicate adequate decomposition rate based on observable concentration reduction of the pollutants in groundwater flow and field scale, and microbiological laboratory data on biodegradation efficiency. In situations of inadequate natural potential, human interferences seems to be suggestible during the bioremediation process. Intrinsic bioremediation and biostimulation methods share the same process and research on one would affect the other, like a progress in stimulated bioremediation methods can lead to better understanding of intrinsic bioremediation (Bemmel, 2010; Kao & Prosser, 1999). Pertinence of MNA depends on adequate information on biogeohydrochemical characteristics of the contaminated site, and method is not assumed as an option due to unpredictability of the procedure. Natural attenuation is generally encouraged where risk analysis reveals no probabilities of contamination translocation or risk for living organisms in short and long term.

Biostimulation

Biostimulation, briefly, can be described as an *in situ* modification of the environment to stimulate existing microbial colonization and enhance contaminant biodegradation. This term is practically used for the addition of electron donors or accepters, nutrients (usually source of carbon, nitrogen and phosphorus), and the modifications of other environmental characteristics, such as humidity and oxygen content to provide the optimal conditions and subsequently encourage naturally occurring biodiversity. Amendments can be added via injection in either liquid or gaseous form. Accelerated mineralization of pollutants by nutrient addition will require the study of natural microbial diversity and requirements of their growth. Despite of natural microbial potential of polluted sites, sediments or wastewater for contaminant remediation, some factors such as inadequate electron accepters or donors, nutrient availability or the deficiency of metabolic pathways of degradation are able to terminate or delay the remediation. Nitrate can be considered as a replacement electron acceptor in absence of oxygen for several microor-

ganisms, such as Eubacteria which is capable of decomposing herbicides under denitrifying conditions. Later in nitrate depleted soils and sediments there is iron-reducing conditions. Different dinitroaniline herbicides, same as alachlor and atrazine seem to be transformed by iron reducing microbes (Xu, Stucki, Wu, Kostka, & Sims, 2001). Bromine and iodine atoms are other known to take part in removal of several herbicides by chlorine respiring bacteria, such as s *Desulfitobacterium chlororespirans* (Cupples, Sanford, & Sims, 2005). *In situ* biostimulation also provides a cost-effective and practical solution for several water contamination types. In such cases, enzymes and stimulants are utilized to speed up the degradation and eliminate the pollutants in water column using indigenous microbes. Application of some well-developed carbon complexes, enzymes, organic fertilizers, and soil conditioners is known to be highly effective in elimination of water and sediment contamination. This method is normally employed along with bioaugmentation under the enhanced bioremediation techniques. Although bioaugmentation seems to efficiently detoxify the polluted area with herbicides, there are results for the full-scale field failure of the method (Chu, ASCE, & Lo, 2003; Kanissery & Sims, 2011).

Bioaugmentation

The process of imposing new species to the local population is called bioaugmentation. Bioaugmentation can involve introducing either natural microorganisms or genetically altered variants that can biotransform (usually toxic metals) or biodegrade (toxic organics) (Sinha et al., 2009). Process needs a verification of indigenous microorganisms of the contaminated area to determine the possibility of biostimulation. This method is generally used for homogenous subsoil and groundwater with inadequate biological activity. Bioaugmentation cultures mostly include specific microbial selection. Microorganisms with metabolic machinery and adaptation capabilities can decompose various organic pollutants. Therefore, they can be utilized for *in site* remediation. Efficiency of the process depends on several variables, including bioavailability and speciation of the pollutants alongside environmental factors. This methods is commonly used in municipal wastewater treatment as an *ex situ* treatment method and mainly for organic compounds, including petroleum hydrocarbons, PAHs, VOCs, and SVOCs. Recent investigations have aimed to utilize genetically engineered microorganisms (GEMs) in bioaugmentation. Genetically altered microbes contain enhanced metabolic capabilities against hazardous organic contaminants. Moreover, there is still a controversy on application of such manipulated organisms due to their unnatural characteristic which would probably persist in the environment. Utilization of GEMs with capabilities to transform or degrade specific contaminant would be more reliable (Hazan et al., 2003; SES, 2012).

Bioventing

Bioventing is an *in situ* remediation method for the elimination of organic soil contaminants by utilizing the indigenous microorganisms. Basic bioventing system consists of a well and a blower. The blower pumps the air into the soil. This technique is used to biodegrade the constituents which are absorbed by soil; hence the capillary border and saturated zones are not affected. During the process oxygen, and nutrients if necessary, are imposed into the unsaturated zone to accelerate the local microorganism activity. Bioventing is similar to soil vapor extraction (SVE) with a difference in elimination process. Bioventing is an enhanced biodegradation process, while SVE is the elimination of components through volatilization. Effectiveness of the process depends on temperature of the area, permeability of the soil and volatile compounds concentration. This method is able to treat aerobically biodegradable contami-

nants like petroleum products, such as gasoline and diesel fuel, aromatic hydrocarbon, and non-volatile hydraulic oils. This technique is better suited for mid-weight simple petroleum products with less volatile characteristics which are located under the surface (EPA, 2004).

Biosparging

Biosparging is the process of utilizing indigenous microorganisms to biodegrade organic components existing below the water table in both saturated and unsaturated soil zones. It can also be defined as injection of air into the aquifer. Biosparging is mostly applied along with other techniques, such as SVE and biostimulation. This method generally uses 'under pressure oxygen' and nutrient (if necessary) injection to soil and groundwater to activate the degradation process or accelerate biological activity of native microorganisms. Biosparging is mostly beneficial in removal of PAHs, SVOCs, and medium-weight petroleum products, such as diesel fuel, lighter products like gasoline tends to volatilize and get eliminated rapidly. Heavier products like lubricated oil may need longer time to be biodegraded. Since biosparging can cause groundwater mounding and contamination spread, this method is not useful in presence of free products. For an effective biosparging, the air injection points with small diameters need to be placed below the polluted zone. Simplicity and low cost of such installations makes the system more flexible and preferable. Vapor migration needs to be controlled in case of existing basements, sewers or similar subsurface spaces, vapor extraction is followed to extract the volatile vapors, and otherwise there can be an accumulation of hazardous compound concentration. Groundwater of a confined aquifer cannot undergo this process since there is no unsaturated zone for the vapor to elude (Boopathy, 2000; Vidali, 2001; Williams, 2001). According to US Air Force Center for Engineering and the Environment (AFCEE), among bioremediation technologies, bioventing and biosparging are two most feasible technologies in remediation of underground storage tank leakages. However, biosparging is not proper to be used in areas with free phase contaminant due to the risk of spreading pollutants in water (Laitinen, 2006).

Bioslurping

Bioslurping is known as an *in situ* groundwater treatment that is generally used where there seems to be a floating phase of free contaminants, such as VOCs and SVOCs on the groundwater. This method seems to be a modification of bioventing as vacuum-enhanced free-product recovery remediation techniques. The process includes a vacuum-enhanced withdrawn of the soil vapor, water and free products. Later the compounds are drawn to a vapor-liquid separator, then an oil-water separator, and treated afterwards. There are two dominant factors affecting the fluid movement into a well which are hydraulic gradient and aquifer transmissivity. Bioslurping accelerates the fluid recovery by elevation in of the hydraulic gradient and aquifer transmissivity. Since there is a small amount of soil and groundwater vacuumed at a time, there is no need for large plants and subsequently, is cost effective . However, bioventing, biosparging, or biosurlping would only be beneficial in homogenous soils. In situations of an existing heterogeneous site, passive treatment or natural attenuation would be better suited (Williams, 2001). Bioslurping method effectiveness depends on geohydrological knowledge and accurate placement of the extraction point (Laitinen, 2006).

Ex Situ Technologies

These method demands disruption and excavation of contaminated soil or groundwater pumping in order to optimize the biodegradation process. These techniques sometimes include transportation, removal of contaminated material and utilization of mechanically engineered systems. Several examples of known methods are:

Land Farming

This surface remediation technology includes mechanical soil excavation and separation by sieving. The process contains spreading excavated soil in thin layer in order to enhance aerobic microbial degradation by aeration, nutrient addition, and moisture optimization. It has been clear that this method is highly effective in reducing concentration of nearly all the petroleum compounds, including adsorbed constituents, which are known to be existed at underground storage tank (UST) sites if the contaminated soil is shallow. Lighter compounds, such as gasoline which are more volatile, are more likely to be eliminated by evaporation during the processes, while other compounds tend to be biodegraded. Although, an emission control can also be essential in order to restrain emission of VOCs by simply preventing them to enter the atmosphere. Compounds with lower contain of lighter products tend to degrade to a larger extend than evaporate, while heavy and non-volatile petroleum compounds are dominantly biodegraded. However such products may need longer periods to decompose (EPA, 2004).

Composting

Composting, as a bioremediation technique, is defined as an aerobic process which relies on the biodegradation of organic materials by microorganisms. Process includes soil excavation and addition of organic material, such as wood chips, straw, manure, and vegetative waste to elevate the microbial activity via enhanced air movement. Consuming the bulking agents, microorganisms metabolically generate heat which results in temperature raise in compost pile. Composting process has been divided into four periods due to temperature alteration; mesophilic, thermophilic, cooling and maturing. These stages generally depend on the microbial population anatomy. An acceleration in microbial respiration leads to temperature raise which eventually causes a reduction in mesophilic microbial population and an increase in thermophilic microbial activities. In such environmental conditions with temperature of 45-60 °C, a large extend of decomposition and biomass formation can take place (Hazan et al., 2003; Semple, Reid, & Fermor, 2001).

Biopiles

Biopiles, occurred as either *in situ* or *ex situ* remediation process, is the combination of landfarming and composting methods. A basic biopile system is consisting of treatment bed, aeration, irrigation and collection systems. Several common names, such as biocells, bioheaps, biomounds, and compost piles are used for the process. Aerated composted piles are shaped out of the contaminated soil by heaping action. Piles can be up to 20 feet high and can also be covered to prevent runoff, evaporation and volatilization and enhance solar heating. This technique has been known as an effective method in reducing concentration of petroleum components which generally are located in underground storage tank (UST)

sites. Similar to landfarms, biopiles are above-ground systems. During the process aeration, nutrient addition and humidity improvement of the excavated soil is performed to promote aerobic microbial degradation and eliminate petroleum constituents. Most volatile components, such as gasoline, leave by evaporation during the aeration processes; hence it is important to control the volatile organic compounds emission. Mid-weight less volatile and heavy non-volatile components are more likely to be biodegraded by microbial respiration. However, high-weight non-volatile petroleum products may entail longer biodegradation period then mid-weight constituents. It usually takes 3 to 6 month to complete the treatment (EPA, 2004; Vidali, 2001).

Biofilters

This technique which is also known as soil bed reactor (SBR) has been originally developed in Germany to utilize the microbial mechanisms to control industrial gases. The technique refers to biological transformation or gas phase remediation. It has been claimed that the air contaminants could be biodegraded by specific bacteria. As a recent economical method, biofilteration includes the usage of naturally bioactive media, such as peat, compost, soil, and etc. Existing microorganisms in such media are known to be responsible for polluted air decomposition and elimination of contaminants And this has been the principal in developing soil biofilters. Biofilters consist of low clay and high organic carbon content soil packed in a bed with contaminated air flow to activate the biodegradation process. According to high microbial population, compost has become the media of choice for biofilters. In order to achieve the expected performance, some factors, such as nutrient content, pH maintenance, and moisture also need to be regulated. Wood chips addition would be helpful to keep the compost material at optimum humidity, while lime pallets can be utilized to control pH, fertilizers to supply nutrients, and carbon powder to adsorb contaminants. Finally, in order to prevent compaction biofilters are suggested to be shallow and shape regulations are assumed to be important (Govid, 2009).

Bioreactor

Bioreactors are *ex situ* slurry or aqueous stirred tanks which are often employed for the treatment of contaminated soil and water. The process is to treat the pumped up soil, sediment, sludge or water in a restraint system using aerated biodegradation or biotransformation. A variant definition of this engineered system is a restraint vessel and apparatus which generates a mixing environment of solid-liquid-gas in order to guarantee a permanent contact by generating water slurry of the contaminated soil and biomass. There might also be a pH control to finally optimize the condition and elevate the decomposition rate of water-soluble and soil-bound contaminants. Generally, biodegradation rate is known to be higher in bioreactors than *in situ* or solid-phase systems, because the process is more predictable and contained. In fixed bed reactors, there is also an addition of compost to enhance biodegradation rate. At the end of the treatment the slurry has to be dewatered and the water needs further treatments. The downside of the method is the need for pretreatments, such as excavation or contaminant extraction by washing or vacuuming from the soil or sediment in order to be placed in vessel which will relatively cause high cost (Hazan et al., 2003; Vidali, 2001; Williams, 2001).

Table 4. Summary of bioremediation techniques (Source: Vidali, 2001; Williams, 2001)

	Technology	Mechanism Summary	Target Media	Effectively Removed Contaminant Example
In situ	Natural attenuation	Biodegradation by indigenous microorganisms	Soil	Inorganic contaminants, PAHs, Heavy metals
	Biostimulation	Optimization of the environment for biodegradation	Soil, wastewater	Inorganic compounds, PAHs, Heavy metals
	Bioaugmentation	Imposing new species to the local population	Soil	Inorganic compounds, PAHs, Heavy metals
	Biosparging	Oxygen and nutrient (if necessary) injection	Soil, groundwater	PAH, non-chlorinated solvents
	Bioventing	Oxygen and nutrient (if necessary) injection	Unsaturated soils	PHCs, PAH, non-volatile hydraulic oils
	Bioslurping	Vacuum-enhanced free-product recovery	Groundwater, soil	Free product (Petroleum)
Ex situ	Land farming	Microbial degradation by aeration, nutrient addition and moisture optimization	Soil Surface	PAH, PCP
	Composting	Surface organic material application	Soil Surface	TPH,PAHs
	Biopiles	Combination of landfarming and composting	Soil Surface	BTEX, phenols, PAHs, TNT, RDX
	Biofilters	Gas phase remediation/Biological transformation	peat, compost, soil	Inorganic compounds, VOCs
	Bioreactors	Mixing environment of solid-liquid-gas	Soil, sediment, sludge, water	PAHs, PCBs

ADVANTAGES AND LIMITATIONS

Bioremediation, as a novel method of treatment, relies on the organisms which are capable of degradation due to their physiological and metabolic mechanisms. The technique offers several advantages over the conventional methods, however there are some limitations (Kumar, Bisht, Joshi, & Dhewa, 2011; Vidali, 2001).

Advantages

- Bioremediation seems to be more preferable as a waste treatment method or remediation technique for polluted material such as soil and water.
- The process includes biodegradation activities of microbes in contaminated media due to an acceleration of population which declines after decomposition process.
- Generally, by-products of such activities are non-hazardous and include carbon dioxide and water.
- The technique offers the opportunity to eliminate vast variety of hazardous contaminants with no further need of disposal or treatment.
- For several compounds, the complete decomposition occurs, so unlike several conventional methods, there would be no transformation or translocation of the contaminant to another media.

- *In situ* bioremediation techniques offer minimal physical destruction, inhibition of normal activities, or transportation of large amounts of hazardous waste off site,
- Theoretically the method seems to be more cost-effective than other remediation technologies.

Limitations

- Practically, additives which are applied during the bioremediation process might be considered as unpredictable and treacherous due to inhibition or encouragement of other species rather than the target microorganisms.
- Genetically altered microorganisms which are produced to decompose certain contaminant are probable to remain in the environment after the remediation, so the natural body will be changed.
- Bioremediation may take long periods to clean-up a contaminated area, it is labor-intensive, and can be highly costly.
- Bioremediation methods are limited to biodegradable compounds, and it is a matter of concern that the by-product of the decomposition would be more resistant and toxic.
- There also seems to be a wide range of factors affecting the performance of remediation, as mentioned before in this chapter, and taking control over such factors needs to be considered

CONCLUSION AND FUTURE PERSPECTIVES

Soil and water, as two intimately connected media, are assumed as two undeniable vital sources. The contamination in either media can influence the surrounding environment and affect human health. Contamination of organic or inorganic compounds derived from any source or containing any characteristics requires devoted effort of remediation. Among several offered technologies, bioremediation, known as a multidisciplinary technique, eco-friendly pathway, and more acceptable by public, is the method of remediation by means of microbial activities or other living organisms. There are several techniques, including utilization of vast varieties of microbes, fungi, bacteria, plants and technologies which are used to evaluate, detoxify, or eliminate the pollutants. In order to achieve foremost results, aside from the regulations, bioremediation demands dedicated investigation related to new species of microorganisms, isolation technologies, enzymes and additives. Genetic engineering approaches to increase degradation capabilities of certain species, discovering new technologies to enhance performance of such activities, long term research sites for field trials alongside devoted laboratory studies can provide suitable occasions for significant progress. Undoubtedly, bioremediation offers a greener pathway of remediation in comparison with wide varieties of conventional and artificial treatments. Generally advantages of bioremediation exceed the limitations which is also another aspect supporting its popularity. Furthermore, a promising era, molecular biological technologies (MBTs), provides new approaches to a more functional and undeviating evaluation of microbial community, characterizing natural attenuation, providing crucial data in order to optimize ongoing remediation process, future response of the site biodiversity to the treatment or additives and eventually supplementary verification of site closure than any conventional microbiological method (Koenigsberg, Hazan, & Peacock, 2005). Current revolutionary developments in molecular and "-omics" technologies, including molecular profiling, ultrafast pyro-sequencing, DNA microarrays, mass spectrometry, meta transcriptomics, and proteome analyses of certain colony accompanying bioinformatics equipment can provide elucidated

perception of aboriginal species and illuminate their bioremediation process. Bioinformatics technology has also been proved to be capable of identification and analysis of several cell constituents, such as gene and protein function, interaction and metabolism. It also provides faster analysis of cellular process which can reveal the cellular mechanisms to regulate microbial cells as treatment tools. There are highly sensitive techniques to detect the biodegradation responsible genes for several compounds. Study of existence and expression of such genes can result in higher treatment performance of bioremediation. Therefore, the modulation of conditions would be taken into consideration to provide optimal microbial environment for biodegradation. Genetic engineering, or in this case Recombinant DNA technology, can offer alterations of organisms characteristics to achieve desirable level of activity, such as enhanced degradation capabilities or generating new metabolic processes for resistant compounds. Since the experiments of such innovations are majorly laboratory-based, confront vast varieties of contaminants, and are restrained by several microbial and environmental interferences, invalid conclusions are highly probable. While such research areas are assumed to be high-priced and unpredictable, development of GMOs for bioremediation still seems skeptical. Withdrawal of such obstacles would improve promising techniques of bioremediation and eventually lead to the favorable results. Genetically altered species for certain persistent contaminants, employing combinations of methods in order to enhance the performance and as an effective method, instead of using single microbe, groups of microbes called microbial consortia can be applied to eliminate resistant contaminants. Future success on bioremediation depends on utilization of new technologies and further research (Kulshreshtha, 2012).

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KEY TERMS AND DEFINITIONS

Biodegradation: Process by which organic compounds are broken down by living organisms.

Biostimulation: Enhanced microbial activity.

DNA Microarrays: Highly effective platform in transcriptomics that provides determination of mRNA expression level of every gene in an organism.

DO: Measure of dissolved oxygen in water.

Eh: It refers to the chemical species tendency to acquire electron and hence be reduced.

Heterotrophic: An organism that cannot fix carbon and utilizes carbon for growth.

Lability: It refers to status of a transient chemical species constantly undergoing or likely to undergo change.

Microfabrication: The technique of fabrication of miniature structures of micrometer or smaller scales.

-omics: Referring to fields of study in biology ending in "-omics", including genomics, proteomics or metabolomics.

Speciation: The speciation of an element is the distribution of an element amongst defined chemical species in a system.

Transcriptomics: Study of the subset of transcribed genes in specific organisms.

Volatilization: Process of vaporizing a dissolved sample.

Section 3

Applied Bioremediation:
Active and Passive Approaches

Applied bioremediation is gaining enormous reliability in the field of environmental management because of their eco-compatible nature. Active and passive approaches are offering plentiful opportunities of exploring bioremediation techniques for environmental clean-up. Employing novel and integrated strategies for the development of modern bioremediation processes is desperate need of the hour. These approaches will certainly add to the advancement of knowledge and will offer the necessary priceless resource and stimulus to the scientific field worldwide.

Chapter 13
Biological Alchemy:
Gold from Garbage or Garbage into Gold

Mamta
Jiwaji University, India

Anil Dhar
Regional Sericulture Research Station, Jammu, India

Rayavarapu Jaganadha Rao
Jiwaji University, India

Khursheed Ahmad Wani
ITM University Gwalior, India

ABSTRACT

The story of garbage processing is changing globally and is being considered as a potential option in the hierarchy of integrated solid waste management that involves stabilization of organic material by the joint action of earthworms and microorganisms. Vermicomposting is an economically viable technique in which the job is done by certain species of earthworms that enhances the process of waste conversion and produces a better end product vermicompost. Vermicompost is highly nutritive fertilizer and more powerful growth promoter over the conventional compost. It is rich in nitrogen, phosphorus and potassium commonly referred as NPK, micronutrients, growth hormones and enzymes. Its commercialization is a good business opportunity and is emerging as an industry itself. The farmers need to raise the crops by organic farming that will reduce the cost and will decrease the impact on environment. The present chapter is an attempt to highlight different approaches of converting waste into vermicompost and the importance of vermicomposting as compared to synthetic fertilizers.

INTRODUCTION

Different types of inorganic and organic waste is a worldwide menace and it is becoming more and more difficult to manage this problem day by day due to rapid increase in population and industrialization which leads to decrease in land space and as well as changes in our life style (Singh et al., 2011). Nowadays most of the waste generated is either disposed of in an open dump in developing countries or in landfills in the developed ones. However, land filling and open dumping requires a lot of land and could also result in several environmental problems.

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A sustainable approach to handle this will be to treat and reprocess organic waste on-site, and to produce useful products. Composting is the most economical and sustainable option for organic waste management as it is easy to operate and can be conducted in contained space provided it is managed properly to produce a good quality produce (Thyagarajan et al., 2010). Composting is a natural process of organic waste treatment which is currently practiced with various modifications (Nair, Vanja, & Anda, 2006).

The composting of waste by earthworms is a simple biotechnological process, in which certain local species of earthworms are used to enhance the process of waste conversion and produce vermicompost (Nagavallemma et al., 2004). Vermicomposting of different types of solid wastes, prior to land application may be a sustainable waste management technique, as the vermicast and vermiwash obtained at the end of vermicomposting process is rich in plant nutrients and is devoid of pathogenic organism. Utilization of vermicompost produced from urban/municipal solid waste in agriculture will facilitate in growth of organic farming and countries economy by lowering the consumption of inorganic fertilizer and avoiding land degradation and soil toxicity problem. Vermicomposting of urban/MSW can be an excellent and best sustainable practice, as it will be helpful in recycling valuable plant nutrients (Singh et al., 2011). Process of vermicomposting differs from composting in many ways (Gandhi, Sangwan, Kapoor, & Dilbaghi, 1997). It is a process in which earthworms and microorganism need moderate temperature 10-32°C (not atmospheric temperature but temperature within the pile of moist organic material) which is known as mesophilic process. Earthworms, through a unique type of biological process, are capable of transforming garbage into 'gold' (Vermi, 2001; Tara Crescent 2003).

Vermicomposting involves the stabilization of organic solid waste through earthworm consumption that converts the waste into earthworm castings. Vermicomposting is the method of combined activity of microorganisms and earthworms. Vermicompost is one of the richest soil conditioners there and improves soil structure and increases its water holding capacity. It brings beneficial microbial activity to plants and provides essential nutrients, available over a long period of time. Plants that receive vermicompost are more productive and resistant to parasites and disease (Singh, 2009). It is proving to be highly nutritive 'organic fertilizer' and more powerful 'growth promoter' over the conventional composts and a 'protective' farm input (increasing the physical, chemical & nutritive value of soil by improving its microbial content, which restore its natural fertility) against the 'destructive' chemical fertilizers which has destroyed the soil properties and decrease its natural fertility over the years. It is rich in NKP (nitrogen 2-3%, potassium 1.85-2.25% and phosphorus 1.55-2.25%), micronutrients, and beneficial soil microbes and also contains 'plant growth hormones and enzymes (Katiyar, Jat, & Singh, 2013).

The earthworm choice for vermicomposting is the key step as it affects the rate of waste stabilization. The different type of earthworms can be used for waste management and sludge stabilization all over the world. The earthworm's species having the capability to colonize organic throw away naturally, high rates of organic matter consumption, digestion and assimilation, able to tolerate a wide range of environmental stress, having high reproductive rates by producing large number of cocoons having short hatching time, rapid growth and maturation rate of hatchlings to adults (Domínguez & Edwards, 2004) are suitable to be used in vermicomposting process. Earthworms sustain aerobic conditions in the waste mixture, ingest solids, and convert a share of the organic matter into biomass and respiration products (Benitez, Nogales, Elvira, Masciandro, & Ceccanti, 1999). Earthworms expels the residual partially stabilized matter as discrete material commonly known as vermicasting (Benitez et al., 1999). The amount turned over by earthworm depends on the availability of total suitable organic waste. If the soil physical conditions like temperature and moisture content are suitable the number of earthworms increases in the piles, until the food becomes a limiting factor. The smaller earthworms feeding on the

litter produces cast in form of almost entirely fragmented litter, whereas the larger earthworms consume large proportion of soil, and their casts have less organic matter.

Environmental degradation is a major threat confronting the world, and the violent use of chemical fertilizers contributes largely to the deterioration of the environment through depletion of fossil fuels, generation of carbon dioxide (CO_2) and contamination of water resources. It leads to the loss of soil fertility due to imbalanced use of fertilizers that has damaged agricultural productivity and causes soil microbial degradation. Now there is a growing realization that the adoption of ecological and sustainable farming practices can only reverse the declining trend in the global productivity and environmental protection (Wani & Lee, 1992; Wani, Rupela, & Lee, 1995; Aveyard 1988,).

Chemical fertilizers which ushered the 'green revolution' in the 1950-60's came as a 'mixed blessing' for mankind and soil health. It boosted food productivity, but at the cost of environment and society. It dramatically increased the 'quantity' of the food produced but decreased its 'nutritional quality' and also the 'soil fertility' over the years. It killed the native soil microorganisms which help in renewing natural fertility. Over the years it has worked like a 'slow poison' for the soil with serious 'withdrawal symptoms'. The excessive use of 'nitrogenous fertilizer' (urea) has also led to increase in the level of 'inorganic nitrogen' content in groundwater (through leaching effects) and in the human food with grave consequences for the human health.

In order to meet the needs of ever growing population widespread use of chemical pesticides became a necessity for the growth of high-yielding varieties of crops which was highly 'susceptible to pests and diseases'. Continued and long term application of chemical pesticides induced 'biological resistance' in crop pests and diseases and much higher doses are now required to eradicate them. Studies indicate that there is significant amount of 'residual pesticides' contaminating our food stuff long after they are taken away from farms for human consumption.

Adverse effects of agro-chemicals on the agricultural ecosystem (soil, flora, fauna & water bodies in farms) and also on the health of farmers using them and the society consuming the chemically grown food have now started to become more evident all over the world. According to World Health Organization (WHO) and United Nation Environment Program (UNEP) nearly 3 million people suffer from 'acute pesticide poisoning' and some 10 to 20 thousand people die every year from it in the developing countries (UNEP/GEMS, 1992). US scientists predict that up to 20,000 American people may die of cancer, each year, due to the low levels of 'residual pesticides' in the chemically grown food. Higher use of agro chemicals also adversely affecting their economy as the cost of agrochemicals has been rising all over the world. The only solution that may tackle the problem is organic farming.

Organic farming systems with the aid of various nutrients of biological origin such as compost are thought to be the answer for the 'food safety, farm security and environmental balance' in future. Among them 'composts' made from biodegradation of organics of MSW (municipal solid waste) which is being generated in huge amount every day all over the world are most important. The organic fraction of the MSW (about 70-80%) containing plenty of nitrogen (N), potash (K) and phosphorus (P) is a good source of macro and micronutrients for the soil. Composts also contain plenty of 'beneficial soil microbes' which help in 'soil regeneration' and 'fertility improvement' and protect them from degradation while also promoting growth in plants (De Brito Alvarez, Gagne, & Antoun, 1995; Weltzien, 1989). Composts also protect plants from pests and diseases.

The modern practice of agriculture and denudation of forests exert an impact on the soil as a habitat, affecting soil fauna and species diversity (Evans, Mc, & Guild, 1948). Earthworms, being one of the major inhabitants of the soil, are often exposed to these manmade hazards. The species that can tolerate

repeated soil disturbances and relatively limited supply of organic material are favored at the expense of those that cannot. Although agricultural practices affect earthworm population in general, sometimes they also favor the growth of certain species (Edwards & Lofty, 1972). However, large populations are recorded from uncultivated areas (Edwards & Lofty, 1977). Despite such limitations, earthworms still exist in their variety and diversity in variety of soil types.

Because of the predominance of the earthworms in some tropical and temperate soils, they increase their ecological importance. Many workers have worked on the various aspects of the ecophysiology of earthworms inhabiting different ecosystems (Block & Banage, 1968; Abrahamsen, 1972; Dash & Patra, 1977; Kale & Krishnamoorthy, 1978, 1981a). Much more investigation are required with regards to qualitative and quantitative composition of earthworms, their seasonal variations in different soil conditions prevailing in India and to device and utilize earthworm based biotechnologies of welfare of human beings.

Composts are aerobically decomposed products of organic wastes such as the cattle dung and animal droppings, farm, agriculture and forest wastes and the municipal solid wastes (MSW). Bombatkar (1996), called them as 'miracle' for plant growth. They supply required macro and micro nutrients to plant roots and stimulate growth; increase organic matter content of the soil including the 'humic substances' that affect nutrient accumulation and promote root growth (Canellas, Olivares, Okorokova, & Facanha, 2000; Siminis, Loulakis, Kefakis, Manios, & Manios, 1998). They in fact improve the total physical and chemical properties of the soil. Vermicompost and vermiwash also add useful micro-organisms to the soil and provide food for the existing soil micro-organisms and thus increase their biological properties and capacity of self-renewal of soil fertility (Ouédraogo, Mando, & Zombre, 2001; Shiralipour, Mc-Connell, & Smith, 1992). One ton of compost may contain 10 lbs of nitrogen (N), 5 lbs of phosphorus (P_2O_5) and 10 lbs of potash (K_2O). Compost made from poultry droppings contains highest nutrient level among all compost (Bombatkar, 1996).

Earthworms consume various organic wastes and reduce the volume by 40-60%. Each earthworm weighs about 0.5 to 0.6 gm, eats waste equivalent to its body weight and produces cast equivalent to about 50% of the waste it consumes in a day. These castings have been analyzed for chemical and biological properties. The moisture content of worm castings ranges between 32 and 66% and it is evident that vermicompost provides all nutrients in readily available form and also enhances uptake of nutrients by plants. Sreenivas, Muralidhar, & Rao (2000), studied the beneficial integrated effect of application of fertilizer and vermicompost on soil available Nitrogen (N) and uptake of ridge gourd (*Luffa acutangula*) at Rajendranagar, Andhra Pradesh, India. Soil nitrogen increased significantly with increasing levels of vermicompost and highest N uptake was obtained at 50% of the recommended fertilizer rate plus 10 t ha^{-1} vermicompost. Similarly, the uptake of N, phosphorus (P), potassium (K) and magnesium (Mg) by rice (*Oryza sativa*) plant was highest when fertilizer was applied in combination with vermicompost (Jadhav, Talashilkar, & Pawar, 1997).

Vermicompost is a nutritive 'organic fertilizer' rich in NKP (nitrogen 2-3%, potassium 1.85-2.25% and phosphorus 1.55-2.25%), micronutrients, and beneficial soil microbes like 'nitrogen-fixing bacteria' and 'mycorrhizal fungi' and are scientifically proving as 'miracle growth promoters and protectors'. Kale & Bano (1988) reported 7.37% nitrogen (N) and 19.58% phosphorus as P_2O_5 in vermicast. Moreover, Suhane, (2007) found that exchangeable potassium (K) was over 95% higher in vermicompost. A good amount of calcium (Ca), magnesium (Mg), zinc (Zn) and manganese (Mn) have also been reported. Additionally, vermicompost contain enzymes like amylase, lipase, cellulase and chitinase, which continuously break down organic matter in the soil (to release the nutrients and make it available

to the plant roots) even after they have been excreted by the earthworms. Annual application of proper amount of vermicompost also lead to significant increase in soil enzyme activities such as 'urease', 'phosphomonoesterase', 'phosphodiesterase' and 'arylsulphatase'. The soil treated with vermicompost has significantly more electrical conductivity (EC) and near neutral pH. Vermicompost has very 'high porosity', 'aeration', 'water holding capacity'and 'moisture content'. Vermicasts have a vast surface area, for providing strong absorbability and retention of nutrients by the compost. They appear to retain more nutrients for longer period of time. Study showed that soil mixed with vermicompost had significantly greater 'soil bulk density' and hence porous, lighter and never show compactness. Increase in porosity has been attributed to increase in number of pores in the range of 30-50 μm and 50-500 and decrease in number of pores greater than 500 μm.

In tropical countries like India high temperature conditions result in nutrient deficient soil. High inputs of chemical fertilizers also decrease soil organic matter to a large extent. In both the cases, a series of chain reactions take place leading to biological degradation of the soil. For such soils, vermi-conservation technology using burrowing earthworms like *Polypheritima elongata* and *Lumbricus terrestries,* appears to be appropriate rather than vermicomposting worms (Bhawalkar, 1993, 1994, 1996). It combines soil processing with waste processing at the same time (Two-in-one Package). Reclamation of soil may be achieved using this technology.

WASTE INTO VERMICOMPOST

The various industrial wastes which have been already vermicomposted and turned into nutrient rich manure include paper waste (Elvira, Sampedro, Benitez, & Nogales, 1998; Kaur, Singh, Vig, Dhaliwal, & Rup, 2010), textile industry sludge (Garg & Kaushik, 2005), guar gum industrial waste (Suthar, 2006), sugar industry wastes (Sen & Chandra, 2007), distillery sludge (Suthar & Singh, 2008), leather industry (Ravindran, Dinesh, Kennedy & Sekaran, 2008) and beverage industry sludge (Singh, Kaur, Vig, & Rup, 2010), agroindustrial sludge (Suthar, 2010), primary sewage sludge (Hait & Tare, 2011), tannery industries (Ravindran & Sekaran, 2011). A number of workers have tried to process poultry manure through vermicompostiong. The manure from poultries has high nutritive value and is sometimes used in animal feeds, therefore, its use in the production of earthworms for preparation of worm meal. Despite the deleterious effect of poultry manure on the earthworm *Perionyx excavates* (Kale & Krishnamoorthy, 1981b), and its failure as feed in mass production of the earthworm *Eudrilus eugeniae* (Graff, 1981), six different dietary formulations with poultry manure as base were tried in an attempt to establish the feasibility of use of poultry manure in mass culturing of the earthworm *Eudrilus eugeniae*, a prolific breeder, for worm meal. Worm meal has been recognized as a valuable source of protein in animal diet (Sabine, 1978; Guerrero, 1983). The nutritive value of a variety of worm meals and their use as the pig's diet, poultry and fish food has been demonstrated by various workers (Sabine 1978; Graff, 1981). Earthworm species employed for vermicomposting of solid organic wastes is shown in Table 1.

Investigation by (Lowe & Butt, 2002) highlighted the ability of *E. eugeniae* to partially detoxify the wastes and convert the toxic cassava peels in to valuable vermicompost. In general, an organic C loss has been observed during the vermicomposting process (Kale, Bano, & Krishnamoorty, 1982; Garg & Kaushik, 2005; Suthar, 2007). Earthworm transforms substrate conditions, which consequently affects the carbon losses from the substrates through microbial respiration in the form of CO_2 and even through mineralization of organic matter Table 2. The inoculation of worms in waste material considerably en-

Table 1. Earthworm species employed for vermicomposting of Solid Organic Wastes (SOW)

S.No.	Solid Organic Waste (SOW)	Species Employed	Reference
1	Potato peels	*Pheretima elongate*	(Munnoli, Arora & Sharma, 2000)
2	Press mud	*Pheretima elongate* *Eudrilus eugeniae, Eisenia fetida* *Megascolex megascolex*	(Singh, 1997) (Munnoli, 2007) (Munnoli and Bhosle, 2008)
3	Canteen waste	*Eisenia fetida*	(Kale, 1994)
4	Tomato skin seed	*Pheretima elongate*	(Singh, 1997)
5	Onion residue	*Eisenia fetida/Eudrilus eugeniae*	(White, 1996)
6	Sericulture waste	*Perionyx excavates*	(Gunthilingaraj and Ravignanam, 1996)
7	Sericulture waste	*Phanerochaete chrysosporium*	(Kallimani, 1998)
8	Board mill sludge	*Lumbricus terrestris*	(Butt, Nieminen, & Siren, 2005)
9	Sugar cane residues	*Pheretima elongate*	(Bhawalkar, 1995)
10	Gaur gum	*Eudrilus eugeniae*	(Bhawalkar,1995; Suthar 2006)
11	Agricultural residues	*Eudrilus eugeniae*	(Kale, 1994)
16	Sago waste	*Lampito mauritii*	(Rajesh, Yeom, Esakkiraj, Kumar, & Lee, 2008)
17	Sago waste	*Eisenia fetida*	(Subramanian, Sivrajan, & Sarvanapriya, 2010)
18	Onion waste	*Eudrilus eugeniae*	(Mishra, Singh, Upadhyay, & Singh, 2009)
19	Garlic waste	*Eisenia fetida*	(Mishra, et al., 2009)
20	Source separated from human feces	*Eisenia fetida*	(Yadav, Vinod, & Mansoor, 2010)
21	Paper mill sludge	*Eisenia fetida*	(Kaur et al., 2010)
22	Press mud, bagassi, sugar cane trash	*Drawida willsi*	(Kumar, Verma, Singh, Umesh, & Shweta, 2010)
23	Press mud	*Perionyx ceylanensis*	(Mani and Karmegam, 2010)
24	Kitchen waste, Garden waste, cow dung	*Eisenia fetida*	(Wani, et al., 2013)

Table 2. Rating of substrates for vermicomposting

S. No.	Types of Substrates	C:N Ratio	Suitability
1	Fish, scrap poultry manure, night soil, activated sludge, pig manure, sheep dropping, meat scraps, cotton seed meal and other oil seed residues	1-19	Most suitable due to high Nitrogen content
2	Garbage, sea weed, butter cup, amaranthus, lettuce, cabbage and vegetable waste which are fresh, green and succulent including wastes from food processing industries	19-27	Moderately suitable
3	Saw dust flax, waste straw, coir waste, etc. including all crop residues with high lignocelluloses content, high carbon and low moisture	27-208	Less suitable

hances the amount of nitrogen (N) due to earthworm mediated nitrogen mineralization of wastes. It also observed by different researchers that the earthworm also enhances the nitrogen levels of the substrate by adding their excretory products, mucous, body fluid, enzymes and even through the decaying tissues of dead worms in vermicomposting sub-system (Suthar, 2007).

The earlier investigators proved that earthworms prefer food with higher quality of fungus and calcium (Cooke & Luxton, 1980; Parthasarathi & Ranganathan, 2000) and higher content of sugar and nitrogen (Lee, 1985; Edwards & Bohlen, 1996). Kurien & Ramasay (2006) used taro (*Colocasia esculenta*) as feeding material for *Eudrilus eugeniae* and *Eisenia foetida* and showed that vermicasts were produced with steadily increasing output in all the reactors.

Shweta, Kumar, Sharma, & Sonal, (2006), vermicomposted bagasse, rice bran, flower waste, leaf litter, banana leaf, fruit waste, kitchen waste and saw dust in combination with cow dung and mixed dung alone showed that mixed dung was best substrate to increase the biomass and cocoon production. Wani, Mamta, & Rao, (2013) indicated that vermicomposting of different organic waste like garden waste, kitchen waste and cow dung not only produces a value added product (vermicomposting) but at the same time reduce the quantity of waste (Table 3). The vermicompost of cow dung, garden waste and kitchen waste in combination were used with brinjal plants under field conditions. Different treatments affected significantly the seed germination of test crops. Plant height, number of leaves and fruit weight was higher in the vermicompost treated field as compared to control and no disease incidence was observed in the fruits of vermicompost treated plot. The study revealed that vermicompost amendments affected brinjal crop differently and we recommend that vermicompost should be used by farming community instead of synthetic fertilizers while raising brinjal crops Mamta, Wani, & Rao (2012).

Kostecka (1999) found that flax seeds are very attractive to *E. foetida* and can be useful for quick reproduction. The great attractiveness was confirmed by rapid vermicomposting and accumulation of 43 percent of *E. foetida* in the bed and a considerable increase of earthworm number and biomass. Dominguez, Edwards, & Webster (2000) reported that maximum weight and highest growth rate were attained in the mixture with food waste whereas; smallest size and lowest growth rate was achieved in the mixture of sewage sludge with sawdust. Earthworms showed much higher reproductive rates in the paper and cardboard mixtures compared to sewage sludge alone. Vermicomposting using *Lumbricus rubellus* for 49 days was conducted after 21 days of pre-composting. Three different combinations of treatments were prepared with eight replicates for each treatment (T1) namely cow dung: kitchen waste in 30:70 ratios, cow dung: coffee grounds in 30:70 ratio (T2), and cow dung: kitchen waste: coffee grounds in 30:35:35 ratio (T3). The multiplication of earthworms in terms of numbers and weight were measured at the end of vermicomposting. Consequently, only T2 showed significant increase (from its initial stage) compared to other treatments. The presence of coffee grounds in T2 and T3 showed higher percentage

Table 3. Nutrient content and different physico-chemical parameters in garden waste, kitchen waste and cow dung

Waste	pH	MC	TOC	Humus	Nitrogen	Phosphorus	Potassium
Garden waste	6.3 ± 0.06^{a}	2.7 ± 0.10^{a}	11.7 ± 0.24^{a}	68.7 ± 0.91^{a}	1.02 ± 0.07^{a}	0.37 ± 0.03^{a}	0.60 ± 0.02^{a}
Kitchen waste	7.2 ± 0.04^{b}	3.1 ± 0.08^{b}	13.3 ± 0.31^{b}	63.9 ± 1.99^{b}	1.30 ± 0.02^{b}	0.50 ± 0.02^{b}	1.01 ± 0.18^{b}
Cow dung	8.1 ± 0.06^{c}	2.6 ± 0.04^{b}	18.4 ± 1.16^{b}	64.1 ± 0.94^{c}	1.97 ± 0.07^{c}	0.62 ± 0.03^{c}	0.88 ± 0.18^{c}

All values are in mean ± 1 S.E. Values bearing different superscripted alphabets differ from each other at $P < 0.05$ (based on Duncan's multiple range test).

of nutrient elements in vermicasts produced. The data reveal that coffee grounds can be decomposed through vermicomposting and to help to enhance the quality of compost produced rather than sole use of kitchen waste in vermicomposting (Adi & Noor, 2009).

Experimental studies on the agronomic impacts of earthworms & its vermicompost on crop plants all over the world is conclusively proving that their application in farm soil over subsequent years can lead to enhanced production of 'safe food', both in 'quantity & quality' without recourse to agro-chemicals. Several scientists working on vermiculture throughout the world have confirmed the positive role of earthworms and its metabolic products (vermicast) on crop growth and development. Important among them are Alam, Jahan, Ali, Ashraf, & Islam, (2007); Ansari (2008); Atiyeh, Arancon, Edwards, & Metzger, (2000) Atiyeh et al. (2000); Arancon et al. (2003) Arancon, Edwards, Bierman, Welch, & Metzger, (2004) Arancon, Edwards, & Bierman, (2006); Bhat & Khambata (1994); Bhatia (2000); Bhatia, Sinha, & Sharma (2000); Baker & Barrett (1994); Garg & Bhardwaj (2000); Krishnamoorthy & Vajranabhaiah (1986); Palanisamy (1996); Reddy (1988); Scheu (1987); Sharma (2001); Suhane (2007); Spain, Lavelle, & Mariotti (1992); Tomar, Bhatnagar, & Palta (1998); Valani (2009); Wilson & Carlile (1989) ; Webster (2005).

Studies on vegetable and cereal crops done in India at University of Rajasthan (1997-2001) and at Bihar Agriculture University (2007-2009) and in Australia at Griffith University (2007-2009), has also testified and strengthened the views of other workers. Application of vermicompost in potted and field crops displayed excellent growth performances in terms of height of plants, number of leaves, color and texture of leaves, appearance of fruiting structures as compared to chemical fertilizers and the organic compost. There is also less incidences of pest and disease attack and reduced demand of water for irrigation.

The post-harvest residues of some local crops, e.g. wheat (*Triticum aestivum*), millets (*Penniseum typhoides* and *Sorghum vulgare*), and pulse (*Vigna radiata*) were subjected to recycle through vermi-composting by using the epigeic earthworm *Eudrilus eugeniae* Kinberg, under controlled conditions. The crop residues were amended with animal dung; and three types of vermibeds were prepared: (i) millet straw (S. *vulgare* + *Pennisenum typhoides* in equal quantity) + sheep manure (1:2 ratio) (MS), (ii) pulse bran (*Vigna radiata*) + wheat straw (*Triticum aestivum*) + cow dung (1:1:2 ratio) (PWC), and (iii) mixed crop residues (mixing of all types crop residues, used in this study)+cow dung in 1:1 ratio (MCR + CD). The fourth treatment was cattle shed manure (CSM). At the end, ready vermicompost showed lower organic C content and higher concentrations of other important plant nutrients. Organic carbon (C) content decreased in the order: MCR+CD (27.6%) > PWC (22.8%)>CMS (22.6%) >MS (19.4%). The ready vermicompost obtained from MCR+CD vermibed showed the maximum increase (% of initial level) in content of total N (143.4%), available P (111.1%) and exchangeable K (100.0%). The end product showed reduction in C:N ration between the ranges of 60.7% (CSM) and 70.3% (MCR + CD), at the end of the vermicomposting process. The composting earthworm *E. eugeniae* exhibited the highest values of biological parameters: maximum mean individual biomass (1261.25±7.0 mg), biomass gain (955.84±11.03 mg), growth rate (10.62±0.10mgwt.worm^{-1} day^{-1}), cocoon numbers (87.67±6.51), and reproduction rate (0.66±0.01 cocoonsworm^{-1} day^{-1}) in CSM container, while MS vermibeds showed the least values of these parameters. During tests, the maximum mortality for *E. eugeniae* was recorded in MS (16.67±7.63%) followed by CSM> PWC>MCR+CD. Results indicated that the C:N ratio of the substrate drastically influenced the growth characteristics of *E. eugeniae*, and it showed the close relations with maximum individual biomass gain (R2 = 0.96), individual growth rate (R2 = 0.82), and reproduction rate (cocoonworm^{-1} day^{-1}) (R2 = 0.72), in different treatments. This study clearly indi-

cates that vermicomposting of crop residues and cattle shed wastes can not only produce a value added product (vermicomposting) but at the same time acts as best culture medium for large-scale production of earthworms.

A laboratory study was undertaken to examine the temporal changes in physico-chemical properties during vermicomposting of sago industry waste. The sago industry waste was mixed with cow dung, poultry manure at different proportions, and it kept for pre-treatment for 21 days and subsequently vermicomposted for a period of 45 days under shade. Earthworm species (*Eisenia foetida*) was introduced at the rate of 50 gm/kg of waste. The moisture content of substrate and temperature were monitored at regular intervals. The vermicomposts were sampled at the interval of every 15 days 0, 15, 30 and 45 days for the assessment of temporal changes in physicochemical properties. The data revealed that equal proportion of sago wastes; cow dung and poultry manure produced superior quality manure with desirable C: N ratio and higher nutritional status than other methods of composting. *E. foetida* is an earthworm suitable for composting organic wastes such as poultry manure with extreme pH and high temperature and sago waste with high organic carbon in a shorter period of time interval. This study suggests that the sago industry solid waste could be effectively converted into highly valuable manure that can be exploited to promote crop production.

Vermicomposting converts household waste into compost within the period of 30 days, reduces the C:N ratio and retains more N than the traditional methods of preparing composts (Gandhi et al., 1997). The C:N ratio of the raw olive cake, vermicomposted olive cake and manure were 42, 29 and 11, respectively. Both the raw olive cake and vermicomposted olive cake immobilized soil N.

ADVANTAGES OF VERMICOMPOST

High Levels of Bio-Available Nutrients for Plants

Vermicompost contains most nutrients in plant-available forms such as ‘nitrates’ (N), ‘phosphates’ (P), ‘soluble’ potassium (K), and magnesium (Mg) and ‘exchangeable’ phosphorus (P) and calcium’ (Ca) (Edwards, Domínguez, & Arancon, 2004; Edward & Burrows, 1988). Vermicomposts have large particulate surface areas that provide many micro-sites for microbial activities and for the strong retention of nutrients (Arancon et al., 2004; Arancon et al., 2006). Nutrient content and different physio-chemical parameters in garden waste, kitchen waste and cow dung as obtained by Wani et al., (2013) is shown in Table 3.

High Level of Beneficial Soil Microorganisms Promoting Plant Growth

Vermicompost are rich in ‘microbial populations & diversity’, particularly ‘fungi’, ‘bacteria’ and ‘actinomycetes’ (Brown, 1995; Chaoui, Zibilske, & Ohno, 2003; Scheu, 1987; Singh, 2009). Parle, 1963, reported bacterial count of 32 million per gram in fresh vermicast compared to 6-9 million per gram in the surrounding natural soil. Scheu, (1987) reported an increase of 90% in respiration rate in fresh vermicast indicating corresponding increase in the microbial population. Suhane, (2007) found that the total bacterial using Actinomycetes, Azotobacter using count was more than 1010 per gram of vermicompost.

Rich in Growth Hormones

Vermicompost further stimulates plant growth even when plants are already receiving 'optimal nutrition' as it has consistently improved seed germination, raise seedling growth and development and increased plant productivity much more than would be possible from the mere conversion of mineral nutrients into plant-available forms. Arancon, (2004) found that maximum benefit from vermicompost is obtained when it constitutes between 10 to 40% of the growing medium. Neilson, (1965); Tomati, Grappelli, & Galli (1988) have also reported that vermicompost contained growth promoting hormone 'auxins', 'cytokinins' and flowering hormone 'gibberellins' secreted by earthworms. Canellas et al., (2000) found that humic acids isolated from vermicompost enhanced root elongation and formation of lateral roots in maize roots. Pramanik, Ghosh, Ghosal, & Banik (2007) also reported that humic acids enhanced 'nutrient uptake' by the plants by increasing the permeability of root cell membrane, stimulating root growth and accelerating proliferation of 'root hairs'.

Vermicompost Is Free of Pathogens

Nair et al., (2006) indicated that vermicomposting leads to greater reduction of pathogens after 3 months of storage. Whereas, the samples which were subjected to only thermofilic composting, contained higher levels of pathogens even after 3 months.

Vermicompost Has No Toxic Chemicals

Several studies have found that earthworms effectively bioaccumulate or biodegrade several organic and inorganic chemicals including 'heavy metals', 'organochlorine pesticide' and 'polycyclic aromatic hydrocarbons' (PAHs) residues.

Vermicompost Protects Plants against Various Pests and Diseases

There has been considerable evidence in recent years regarding the ability of vermicompost to protect plants against various pests and diseases either by suppressing or repelling them or by inducing biological resistance in plants to fight them or by killing them through pesticidal action (Anonymous, 2001; Al-Dahmani, Abbasi, Miller, & Hoitink, 2003).

Induce Biological Resistance in Plants

Vermicompost contains some antibiotics and actinomycetes which help in increasing the 'power of biological resistance' among the crop plants against pest and diseases. Pesticide use was significantly reduced where earthworms and vermicompost were used in agriculture (Singh, 1993; Suhane, 2007).

Repel Crop Pests

There seems to be strong evidence that worm's varmicastings sometimes repel hard-bodied pests (Anonymous, 2001; Arancon, 2004). Edwards & Arancon, (2004) reports statistically significant decrease in arthropods (aphids, buds, mealy bug, spider mite) populations and subsequent reduction in plant dam-

age, of tomato, pepper and cabbage trials with 20% and 40% vermicompost additions. GEORG (2004), doing commercial vermicomposting in California, U.S., affirms that his product repels many different insects pests. There explanation is that this is due to production of enzymes 'chitinase' by worms which breaks down the chitin in the insect's exoskelton (Munroe, 2007).

Suppress Plant Disease

Edwards & Arancon (2004) found that use of vermicompost in crops inhibited the soil-born fungal diseases. They also found substantial suppression of plant-parasitic nematodes in field trials with pepper, tomatoes, strawberries and grapes. The high levels of agronomically beneficial microbial population in vermicompost protects plants by out-competing plant pathogens for available food resources, by starving them and also by blocking their access to plant roots by occupying all the available sites. Edwards & Arancon, (2004), reported the agronomic effects of small applications of commercially produced vermicompost, on attacks by fungus *Rhizoctonia* on radish and *Pythium* on cucumber, in the greenhouse, by *Verticillium* on strawberries and by *Phomposis* and *Sphaerotheca fulginae* on grapes at the field. In all these experiments vermicompost use suppressed the incidence of the disease significantly and also found that the ability of pathogen suppression disappeared when the vermicompost was disinfectd/sterilized, convincingly indicating that the biological mechanism of disease suppression involved was 'microbial antagonism. Szczech, Rondomanski, Brzeski, Smolinska, & Kotowski, (1993); Orlikowski, (1999); Rodriguez, Zavaleta, Sanchez, & Gonzalez, (2000); Zaller, (2006) also found that the aqueous extracts of vermicomposts depress soil-borne pathogens and pests, in their field experiment that only half as many plants of tomatoes sprayed with aqueous extract of vermicompost were infected with *Phytopthora infestans* (that cause 'late-blight' disease) as those of control ones.

NUTRITIONAL QUALITY OF VERMICOMPOST

The nutritional quality of vermicompost is determined primarily by the type of the substrate (raw materials) and species of earthworms used for composting, along with microbial inoculants, liming, aeration, humidity, pH and temperature. Cattle dung has been found to yield most nutritive vermicompost when composted by *Eisinea fetida*. Pramanik et al., (2007) found that application of lime at the rate of 5 gm/kg of substrate and 'microbial inoculation' by suitable 'cellulolytic', 'lignolytic' and 'N-fixing' strains of microbes not only enhance the rate of vermicomposting but also results into nutritionally better vermicompost with greater enzymatic (phosphatase and urease) activities. Kaushik & Garg (2004) found that inoculation with N-fixing bacteria significantly increased the 'nitrogen' (N) content of the vermicompost. Liming generally enhance earthworm activities as well as microbial population. Earthworms after ingesting microbes into its gut proliferate the population of microbes to several times in its excreta (vermicast). It is therefore advantageous to use beneficial microbial inoculants whose population is rapidly increased for rapid composting and also better compost quality. Pramanik et al., (2007) studied the vermicomposting of four substrates viz. cow dung, grass, aquatic weeds and municipal solid wastes (MSW) to know the 'nutritional status and enzymatic activities' of the resulting vermicomposts in terms of increase in total nitrogen (N), total phosphorus (P) and potassium (K), humic acid contents and phosphatase activity.

Total Nitrogen

Sinha, Sunil, Dalsukh, & Chauhan, (2009) found that cow dung recorded maximum increase in nitrogen (N) content (275%) followed by MSW (178%), grass (153%) and aquatic weed (146%) in their resulting vermicomposts over the initial values in their raw materials without liming and microbial inoculation. Application of lime without microbial inoculation, however, increased N content in the vermicompost from 3% to 12% over non-limed treatment, irrespective of substrates used.

Total Phosphorus and Potassium

Similarly, the vermicompost prepared from cow dung had the highest total phosphorus (12.70 mg/g) and total potassium (11.44 mg/g) over their initial substrate followed by those obtained from aquatic weeds, grasses and MSW. This was also irrespective of lime application and microbial inoculation. Among the microbes inoculated for vermicomposting, *Bacillus polymyxa* a free-living N-fixing bacterium was most effective in increasing total phosphorus (11-22%) in the vermicompost after liming.

Humic Acid

It was highest in vermicompost prepared from cow dung (0.7963 mg/g), followed by those from grasses (0.6147 mg/g), aquatic weeds (0.4724 mg/g) and MSW (0.3917 mg/g). And this was without liming and microbial inoculation. However, microbial inoculation again increased humic acid contents in vermicompost from 25% to 68% depending upon the substrate used. Inoculation by *Phanerochaete chrysoporium* recorded highest humic acid contents without liming as compared to other inoculants. But under limed condition, inoculation by *B. polymyxa* was most effective in increasing humic acid contents irrespective of substrates used for vermicomposting.

Phosphatase Activity

Vermicompost obtained from cow dung showed the highest 'acid phosphatase' (200.45 µg *p*-nitrophenol/g/h) activities followed by vermicompost from grasses (179.24 µg *p*-nitrophenol/g/h), aquatic weeds (174.27 µg *p*-nitrophenol/g/h) and MSW (64.38 µg *p*-nitrophenol/g/h). The 'alkaline phosphatase' activity was highest in vermicompost obtained from aquatic weeds (679.88 µg *p*-nitrophenol/g/h) followed by cow dung (658.03 µg *p*-nitrophenol/g/h), grasses (583.28 µg *p*-nitrophenol/g /h) and MSW (267.54 µg *p*nitrophenol/ g/h). This was irrespective of lime application and microbial inoculation. However, when inoculated by fungi all showed maximum phosphatase activities under both limed and non-limed conditions (Vinotha, Parthasarthi, & Rangnathan, 2000).

IMPROVED CROP GROWTH AND YIELD

Vermicompost plays a major role in improving growth and yield of different crops, vegetables, flower and fruit crops. The use of in soil vermicompost gave higher germination (93%) of mung bean (*Vigna radiata*) compared to the control (84%). Further, the growth and yield of mung bean was also significantly higher with vermicompost application. In the same way, in another pot experiment, fresh and dry

matter yields of cowpea (*Vigna unguiculata*) were higher when soil was amended with vermicompost than with biodigested slurry (Karmegam, Alagermalai, & Daniel, 1999; Karmegam & Daniel, 2000). The efficiency of vermicompost was evaluated in a field study by Desai, Sabale, & Raundal, (1999) and according to their application the vermicompost along with fertilizer N gave higher dry matter (16.2 g plant^{-1}) and grain yield (3.6 t ha^{-1}) of wheat (*Triticum aestivum*) and higher dry matter yield (0.66 g plant^{-1}) of the following coriander (*Coriandrum sativum*) crop in sequential cropping manner. Similarly, a positive response was obtained with the application of vermicompost to other field crops such as sorghum (*Sorghum bicolor*) (Patil & Sheelavantar 2000) and sunflower (*Helianthus annuus*) (Devi, Agarwal & Dayal, 1998; Devi and Agarwal, 1998).

Application of vermicompost at 5 t ha^{-1} significantly increased (5.8 t ha^{-1}) yield of tomato (*Lycopersicon esculentum*) in farmers' fields in Adarsha watershed, Kothapally, Andhra Pradesh in comparison to control (3.5 t ha^{-1}). Similarly, greenhouse studies at Ohio State University in Columbus, Ohio, USA have indicated that the vermicompost enhances transplant growth rate of vegetables. Application of vermicompost with a transplant grown without vermicompost had the highest amount of red marketable fruit at harvest and there were no symptoms of early blight lesions on the fruit at harvest. The yield of pea (*Pisum sativum*) was also higher with the application of vermicompost (10 t ha^{-1}) along with the recommended Nitrogen, Phosphorus and Potash than with these fertilizers alone (Reddy et al., 1998). Vadiraj, Siddagangaiah, & Potty, (1998) reported that application of vermicompost produced herbage yields of coriander cultivars that were comparable to those obtained with chemical fertilizers.

The fresh weight of flowers such as *Chrysanthemum chinensis* increased with the application of different levels of vermicompost. Also, the number of flowers per plant (26), flower diameter (6 cm) and yield (0.5 t ha^{-1}) were maximum with the application of 10 t ha^{-1} of vermicompost along with 50% of recommended dose of NPK fertilizer. However, the vase life of flowers (11 days) was high with the combined application of vermicompost at 15 t ha^{-1} and 50% of recommended dose of NPK fertilizer (Nethra, Jayaprasad & Kale, 1999).

ECONOMICALLY VIABLE TECHNIQUE

The new economic theory of development today is 'Environmental-Economics' which advocates for judicious balance between 'economy and ecology' in all developmental programs including agricultural development and amalgamation of 'economic development' programs with 'ecological conservation' strategies to usher in the era of sustainable development. The cost of production of vermicompost is simply insignificant as compared to chemical fertilizers. While vermicompost is produced from a 'cheap raw material' (community wastes including farm wastes) which is in plenty all over the world and is growing in quantity with the growing human population, the chemical fertilizers are obtained from 'petroleum products' which are not only very 'costly raw materials' but also a 'vanishing resource' on earth. While vermicompost can be produced 'on farms' by all farmers, big and small, the chemical fertilizers has to be produced in 'factories' at a high economic and environmental cost. This means vermicompost can be afforded by all farmers. The worms itself becomes an economically valuable products for the farmers to be sold to fishery, poultry, dairy and pharmaceutical industries.

Vermicompost production is also an 'economically productive' process as it 'reduces wastes' at source and consequently saves landfill space. Construction of engineered landfills incurs 20-25 million US dollars upfront before the first load of waste is dumped. Over the past 5 years the cost of landfill

disposal of waste has increased from $ 29 to $ 65 per ton of waste in Australia. Then, landfills have to be monitored for at least 30 years for emissions of GHG and toxic gases & leachate (Waste Juice) which also incur cost. During 2002-2003, waste management services within Australia cost $ 2458.2 million. Even in developing nations where there are no true landfills, dumping of wastes incurs high cost on local government.

Earthworms converts a product of 'negative' economic & environmental value i.e. 'waste' into a product of 'highly positive' economic and environmental values i.e. 'high nutrition content organic fertilizer' (brown gold) which improve soil fertility and enhance farm productivity to produce 'safe food' (green gold) in farms. Vermiculture can maintain the global 'human sustainability cycle-i.e. producing food in farms back from food & farm wastes. Vermicomposting is a self-regulated, self-improved and self-enhanced, very less or no-energy requiring zero-waste technology, easy to construct and maintain. It excels all other waste conversion technologies by the fact that it can utilize waste organics that otherwise cannot be utilized by others. It excels all other biological or mechanical technologies for production of 'bio-fertilizer' because it achieves 'greater utilization' than the rate of 'destruction' achieved by other technologies and the process becomes faster with time as the army of degrader worms and the decomposer microbes multiply in millions in short time (Sinha, Sunil, Agarwal, Asadi, & Carretero. 2002; Sinha, Nair, Bharambe, Swapnil, & Bapat, 2008). Earthworms involves about 100-1000 times higher 'value addition' in any medium (composting wastes or soil) wherever it is present (Appelhof, 1997; Appelhof, 2003).

Production of chemical fertilizers in industries is an 'environmentally damaging' process in its entire lifecycle, since harnessing of raw materials from the earth crust, to their processing in factories and their use in agriculture farms. It generates huge amount of toxic and hazardous wastes and pollutants at every stage of production and use. It also uses copious amount of energy in production process and emits huge volumes of greenhouse gases (GHG). It is an 'economically unproductive' process of development. Huge money has to be spent on infrastructure development for production of chemical fertilizers and in installations of equipments for pollution control, transport and then on safe disposal of hazardous waste in engineered landfills. Its application in farms pollutes the soil and water bodies and kills beneficial soil organisms with severe economic and environmental implications.

A matter of considerable economic and environmental significance is that the 'cost of food production' by vermiculture will be significantly low by more than 60-70% as compared to chemical fertilizers and the food produced will be a 'safe chemical-free food' for the society. It is a 'win-win' situation for both producers (farmers) and the consumers (feeders). The cost of production of vermicompost is simply insignificant as compared to chemical fertilizers. While the former is produced from 'human waste'-a raw material which is in plenty all over the world, the latter is obtained from 'petroleum products' which is a vanishing resource on earth. Vermicompost can be produced 'on-farm' at low-cost by simple devices, while the chemical fertilizers are high-tech & high-cost products made in factories (Munroe, 2007). As vermicompost also helps the crops to attain maturity and reproduce faster, it shortens the 'harvesting time' (Sinha, et al., 2009). This further cuts on the cost of production and also adds to the economy of farmers as they can grow more crops every year in the same farm plot.

Vermicompost Application Reduces Use of Chemical Pesticides and Cost

Continued application of chemical pesticides induced 'biological resistance' in crop pests and diseases and logrithamatically much higher doses are now required to eradicate them. There has been considerable evidence in recent years regarding the ability of vermicompost to protect plants against various pests and diseases either by suppressing or repelling them or by 'inducing biological resistance' in plants to fight them or by killing them through pesticidal action (Suhane, 2007).

Vermicompost Application Reduces Use of Water for Irrigation and Cost

Studies indicate that vermicompost is able to retain more soil moisture thus reducing the demand of water for irrigation by nearly 30-40%. (Sinha et al., 2009, Suhane, 2007; Suhane, Sinha, & Singh, 2008).

Better Growth and Higher Yield with Lower Amount of Vermicompost

Studies indicate that smaller amounts of vermicompost in fact promote better growth performances of crops. Subler, Clive, & Metzger, (1998) reported that in all growth trials the best growth responses were exhibited when the vermicompost constituted a relatively small proportion (10%-20%) of the total volume of the container medium. Valani, (2009) found that 200 gm of vermicompost applied in pot soils performed better growth in wheat crops than those with 400 gm & 500 gm of vermicompost. Singh, (1993) found that in the farm plots where vermicompost was applied in the 2nd, 3rd and 4th successive years, the growth & yield of wheat crops increased gradually over the years at the same rate of application of vermicompost i.e. at the rate of 20 Q/ha. In the 4th successive year the yield was 38.8 Q/ha which was very close to the yield (40.1 Q/ha) where vermicompost was applied at the rate of 25 Q/ha use of vermicompost in farm soil eventually leads to increase in the number of earthworm population in the farmland over a period of time as the baby worms grow out from their cocoons. It infers that slowly over the years, the earthworms build up the soil's physico-chemical and biological properties, the amount of vermicompost can be slowly reduced while maintaining the same yield. The yield per hectare may also increase further as the soil's natural fertility is restored and strengthened. In a study in Australia, Webster, (2005) found that vermicompost increased yield of 'cherries' for three years after 'single application'. Yield was much higher when the vermicompost was covered by 'mulch'. At the first harvest, trees with 5 and 20 mm vermicompost plus mulch yielded cherries of the value of $ 63.92 and $ 70.42 respectively. After three harvests, yield per tree were $ 110.73 and $ 142.21 respectively for the 5 mm and 20 mm vermicompost with mulch.

With vermicompost alone (without mulch), trees yielded cherries of $ 36.46 per tree with 20 mm vermicompost in the first harvest and after three harvest $ 40.48 per tree. Webster, (2005) also studied the agronomic impacts of compost in vineyards and found that the treated vines produced 23% more grapes due to 18% increase in number of bunch of grapes. The yield in grapes was worth additional $ 3,400/ha.

Chemical Fertilizers Are Needed to Maintain Growth and Yield

On the contrary, in chemical agriculture, the amount of chemicals used per hectare has been steadily increasing over the years to maintain the yield constant as the soil became 'addict'. Nearly 3- 4 times of agro-chemicals are now being used per hectare what was used in the 1960s. And the cost of chemical

fertilizers has also been steadily increasing since then. There is also significant loss of chemical fertilizer from the farm soil due to oxidation in sunlight. Suhane, (2007) calculated that upon application of 100 kg urea (N) in farm soil, 40-50 kg gets oxidised and escapes as 'ammonia' (NH_3) into the atmosphere, about 20-25 kg leaches underground which pollutes groundwater, while only 20-25 kg is available to be used up by the plants.

COMMERCIAL VERMICULTURE

Vermiculture is a growing and developing industry not only for managing waste and land very economically but also for promoting 'sustainable agriculture' by enhancing crop productivity both in quantity & quality at significantly low economic cost than the costly agrochemicals (Bogdanov, 1996). Earthworms not only converts 'waste' into 'wealth' it itself becomes a valuable asset as worm biomass. Large-scale production of nutrient rich 'vermicompost' (especially from the municipal solid wastes) with potential to replace chemical fertilizers and protein rich 'earthworms' can be a good business opportunity today with awareness growing about use of these products in agriculture and other allied industries (GEORG, 2004). MSW is growing in huge quantities in every country with growing population and there will be no dearth of raw materials for production of vermicompost. Vermiculture have also enhanced the lives of poor in India and have generated self-employment opportunities for the unemployed. It has become good source of livelihood for many. In several Indian villages NGO's are freely distributing cement tanks and 1000 worms and encouraging men & women to collect waste from villages and farmers, vermicompost them and sell both worms and vermicompost to the farmers. People are earning from Rupees 5 to 6 lakhs (Approx. $ 15-20 thousands) every year from sale of both worms and their vermicompost to the farmers. Mostly they use farm waste and also municipal solid wastes (MSW) collected from streets and waste dumpsites. It is estimated that one ton of earthworm biomass on an average contain approximately one million worms (Anonymous, 1980). One million worms doubling every two months can become 64 million worms at the end of the year. Considering that each adult worm (particularly *E. fetida*) consume waste organics equivalent to its own body weight everyday, 64 million worms (weighing 64 tons) would consume 64 tons of waste everyday and produce 30-32 tons of vermicompost per day at 40-50% conversion rate (Visvanathan, Trankler, Joseph, & Nagendran, 2005). In any vermiculture practice, earthworms biomass comes as a valuable by-product and they are good source of nutritive 'worm meal'. They are rich in proteins (65%) with 70-80% high quality essential amino acids 'lysine' and 'methionine' and are being used as feed material to promote 'fishery', 'dairy' and 'poultry' industry. They are also finding new use as a source of 'collagen' in the manufacture of pharmaceuticals and in the making of 'antibiotics' from the ceolomic fluid as it has anti-pathogenic properties.

Nonetheless, the importance of local (native) species should not be ignored and due attention to them must be given. Kale et al., (1982) tried South Indian variety, Oriental worm, *Perionyx excavatus* for composting of animal wastes under laboratory conditions. Similarly *Perionyx sansibarious* in Kerala and *P. pallus* in Maharastra have been tested for organic waste degradation and they were found to be highly satisfactory. Agrawal, (2009) has also shown that the local variety, *Perionyx cressiseptatus* can be employed for vermicomposting, particularly of high moisture content waste. Kale and Bano (1988) recommended a mixed culture of exotic worms with local species. Many species of epigeic earthworms were tested for mass cultivation over different parts of the world, including the tropical and temperate

regions. According to (Kale, 1993) three earthworm species, *Eisenia foetida, Edurilus eugeniae* and *Perionyx excavatus* come in the order of preference for their ability to degrade the wastes. They are very efficient and adaptable in cultures under semi natural conditions in India.

CONCLUSION

Although vermicomposting is being engineered into a novel and green technology, earthworms have been used routinely for the treatment and transformation of waste products for at least 100 years so far. The waste industry which is based on the exploitation of earthworms in now properly controlled and engineered systems also depends on the metabolic activities of earthworms which degrade the organic matter in vermicompost. Anthropogenic activities have caused widespread pollution of the natural environment. vermicomposting has grown into a green, attractive, highly beneficial and promising alternative to traditional physico-chemical techniques for the different types of wastes, as it can be more cost-effective and it can selectively degrade the wastes without damaging the site or its indigenous flora and fauna. However, vermicomposting technologies have had limited applications due to the constraints imposed by substrate and variability in environmental condition, and the limited biodegradative potential and viability of naturally occurring earthworms.

This review was not intended to address the much voluminous literature on vermicomposting, but rather to revisit the basic of vermicomposting and demonstrate that the application of vermicomposting in the fields of waste management. The application of diverse vermicomposting technologies must be based on sound and reliable scientific data obtained in both fundamental as well as research environmental laboratories. For the development of vermicomposting processes to succeed at commercial scale, it is necessary to link different disciplines such as microbial ecology, biochemistry and microbial physiology, organic farming together with biochemical and bioprocess engineering. In short, the key to successful vermicomposting resides in continuing to develop the scientific and engineering work that provides the real bases for both the vermicomposting and its evaluation; and simultaneously in explaining and justifying the valid reasons which allow scientists and engineers to actually use these technologies for the welfare and safety of a public which is more and more concerned about the environment and its protection.

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KEY TERMS AND DEFINITIONS

Anthropogenic: The term anthropogenic used by Russian geologist Alexey Pavlov denotes the influence of human beings on the environment However, the term was used in English by British ecologist Arthur Tansley in reference to human influences on climax plant communities.

Ecophysiology: The branch of physiology that deals with the physiological processes of organisms in relation to environment.

Garbage: All easily decomposable and putrefying organic (animal and vegetable) waste from preparation, handling, storage, and sale or serving of food refuse other than industrial-waste and effluents.

Organic Farming: Organic farming is a form of agriculture that relies on techniques such as crop rotation, green manure, compost, and biological pest control.

Vermiculite: Vermiculite is a hydrous, silicate mineral that is classified as a phyllosilicate and that expands greatly when heated. Exfoliation occurs when the mineral is heated sufficiently, and the effect is routinely produced in commercial furnaces. Vermiculite is formed by weathering or hydrothermal alteration of biotite or phlogopite.

Vermitea: Worm tea, usually referred to as vermicompost tea, or VCT, is a type of compost tea that is made by soaking vermicompost in oxygenated, de-chlorinated water. Worm leachate is the excess water that drips through the worm bin and picks up undigested material.

Vermiwash: Vermiwash is a liquid that is collected after the passage of water through a column of worm action and is very useful as a foliar spray. It is a collection of excretory products and mucus secretion of earthworms along with micronutrients from the soil organic molecules.

Waste: Waste and wastes implies unwanted or unusable materials. The term is often subjective (because waste to one person is not necessarily waste to another) and sometimes objectively inaccurate (for example, to send scrap metals to a landfill is to inaccurately classify them as waste, because they are recyclable.

Chapter 14
Green Strategy for Production of Antimicrobial Textiles

Nagia Farag Ali
National Research Center, Egypt

ABSTRACT

The article deals with the measurement of the antimicrobial activity for some natural dyes against various types of microbes as (Escherichia coli, Staphylococcus aureus and Pseudomons aeruginosa), Using nano materials for some metals or its oxides as titanium oxide for treatment of fabrics before dyeing, these materials were fixed on the fiber by chemical bonds to acquire new properties as antimicrobial activities against bacteria and fungi and also to protect from ultra violet rays. Using a traditional and microwave heating for extraction of dyes and dyeing methods because microwave heating is a more effective method than traditional heating. Other additional features are that, they are cheaper, more economical, eco-friendly, and produce a higher dye uptake as compared to conventional techniques, environmentally friendly pre-treatment by chitosan before dyeing in order to obtain dyed fabric with high quality and more protected against microbes. Application of antimicrobial agents in the development in the textiles as chitosan, qutenary ammonium salt and neem.

INTRODUCTION

A renewed international interest has arisen in natural dyes due to increased awareness of the environmental and health hazards associated with the synthesis, processing and use of synthetic dyes. Natural dyes comprise colorants that are obtained from animal or vegetable matter without any chemical processing (Ali & El-Mohamedy, 2011). During the last decade the use of natural dyes, has gained momentum due to increased demand for these dyes by the food, pharmaceutical, cosmetic as well as the textile coloration industry. Textile processing industry is one of the major environmental polluters. In order to process a ton of textile, one might have to use as much as 230 to 270 tons of water. The effluent generated by this much water would pollute the environment as it contains a heavy load of chemicals including dyes used during textile processing. There are two main ways to limit the environmental impact of textile processing. One is to construct sufficiently large and highly effective effluent treatment plants, and the other way is to make use of dyes and chemicals that are environment friendly.

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Bacteria and fungus, either pathogenic or not, are normally found on human skin, nasal cavities, and other areas, such as in the genital area. Microbial shedding from our body contributes to microorganism spreading into a textile material either directly in clothes or on surrounding textiles. Recent studies strongly support that contamination of textiles in clinical settings may contribute to the dispersal of pathogens to the air which then settle down and infect the immediate and non-immediate environment. It is one of the most probably causes of hospital infections. Typically, pathogenic microorganisms like *Klebsiella pnuemoniae, Pseudomonas aeuroginosa, Staphylococcus epidermidis, Staphylococcus aureus* and *Candida albicans* have been found on textiles.

In addition, microorganism proliferation can cause malodors, stains and damage of mechanical properties of the component fibers that could cause a product to be less effective in its intended use. Additionally, may promote skin contaminations, inflammation and in sensitive people, a topic dermatitis. Fortunately, the use of antimicrobial textiles may significantly reduce the risk of infections especially when they are used in close contact with the patients or in the immediate and non-immediate surroundings.

BACKGROUND OF TEXTILE INDUSTRIES

Textile industry continuously searches for new technologies in order to accomplish the consumer's demands. Especially in recent years, new developments allowed the production of functional and smart textiles which are capable of sensing changes in environmental conditions or body functions and responding to these changes. Likewise, consumers' attitude towards hygiene and active lifestyle has created a rapidly increasing market for a wide range of textile products finished with antimicrobial properties, which in turn has stimulated intensive research and development. As a consequence, the number of biofunctional textiles with an antimicrobial activity has increased considerably over the last few years (Haug et al., 2006). Application is nowadays extended to underwear, sportswear, home furnishing and protective clothing in areas with high risk of infection by pathogens (hospitals, schools and hotels); and because they are able to absorb substances from the skin and can release therapeutic compounds to the skin, they find applications for prevention, as surgical lab coats, or therapy, as wound dressings (Goodarzian & Ekrami, 2010). Thus, biomedical products will perhaps be the largest application of antimicrobial textiles. Microorganisms and textiles are an excellent substrate for bacterial growth and microbial proliferation under appropriate moisture, nutrients and temperature conditions. In a clinical setting, they can be an important source of bacteria that may contaminate the patients and clinician personnel (Ali & El-Mohamedy, 2011).

Over the last 5 years the global fibers market has moved further into a global commodity market. This change is redefining and accelerating global trade patterns at all levels of the high value chain. The development of special textiles is the consequence of merging fundamentals scientific and technical knowledge, as there is a quest for high performance textiles. Thus, constant and continued endeavors offered scientists jointly ventured with material technologies had made dreams into reality. These special textiles totally provide the potential for providing new technology. Over all world textiles, challenging a continued growth of hi-tech fibers in various fields. These fibers have high tenacity, high strength to weight ratio which are the prerequisites characteristics of industrial textiles. These find applications in every walk of life including Space, Ocean, composites, aircrafts, defense automobile and many more.

Present chapter deals with these special fibers and explores the wealth of their properties and application.

Natural dyes are not only used to impart color to an infinite variety of materials such as textiles, paper, wood etc. but also they are widely used in cosmetic, food and pharmaceutical industry. They have wide range of medicinal importance in pharmaceutical industry. Medicinal importance of some important natural dye yielding plants is discussed below along with their chemistry of pigments. Almost any organic material will produce a color when boiled in a dye-bath, but only certain plants will yield a color that will act as a dye. The plants given in are a selection of plants that have stood the test of time, and are used widely and traditionally by natural dyers (Ali & EL Mohamedy, 2010).

PROBLEMS RELATED TO THE TEXTILE INDUSTRY AS FOLLOWS

The health hazards associated with synthetic dyes have led to revival of natural dyes. The natural dyed materials have good resistance to mutt invasion. Some of its contacts are anti-allergic and proved to be safe for body contact. Majority of the naturally dyed materials are non-toxic. Natural dyes cover all the dyes, which are derived from plants (the textile industry must go towards developing of new technologies to reduce the energy and water consumption. The use of microwave in textile wet processing is one way for this purpose. The advantages of microwaves, which it is use much less liquid, they can exhaust or save dyes and leave no waste of liquid dye compared to conventional methods. Microwave dyeing has other advantages such as less power consumption, easy production of desired shades, and quick dyeing

Microbial damage of fabrics is a common problem in many parts of the world and microbial contaminated fabrics in hospitals are known to be the major source of cross infection, so all textile materials used in hospitals should prevent or minimize the transmission of infection diseases.

The damage of mechanical properties of textile can caused by microorganism proliferation and also malodors, stains and that could cause a product to be less effective in its intended use. Additionally, may promote skin contaminations, inflammation and in sensitive people, atopic dermatitis. Fortunately, the use of antimicrobial textiles may significantly reduce the risk of infections especially when they are used in close contact with the patients or in the immediate and non-immediate surroundings (Ali & EL Mohamedy, 2010).

ANTIMICROBIAL AGENTS FOR TEXTLES

Antimicrobial agents are natural or synthetic compounds that inhibit the growth (bacteriostatic or fungistatic) because they can be protein, lipid synthesis or enzyme inhibitors, all of which are essential for cell survival; or kill (biocidal) the microorganisms by damage in the cell wall. Almost all antimicrobial synthetic agents in use on textiles are biocides (Ali & EL Mohamedy, 2010).

Synthetic Compounds

Several antimicrobial agents have been tested in textiles: Quaternary ammonium compounds, silver, polyhexamethylene biguanides (PHMB) and triclosan even in an industrial scale. They have powerful bactericidal activity, as indicated by the MIC value, and also different application methods, effectiveness on fibers depending on chemical composition, and side-effects. However, the majority have a reduce spectrum of microbial inhibition and may cause skin irritation, ecotoxicity and bacteria resistance .

Moreover, the biocide can gradually lose activity during the use and launderings of the textile. Thus, (Haug et al., 2006)great amounts of these biocides are applied to the textiles to control the bacterial growth efficiently and to keep its durability. In addition, despite the fact that synthetic antimicrobial agents used in textiles can be effective against a wide range of microorganisms, wearing these textiles in a continuous manner can lead to sensitization and bacteria resistance(Ali & El-Mohamedy 2011).

Natural Compounds

To minimize the above mentioned risks associated with the application of antimicrobial agents, there is a great demand for antimicrobial textiles based on non-toxic and ecofriendly bioactive compounds. Due to the relatively lower incidence of adverse reactions of natural products in comparison with synthetic pharmaceuticals, they can be exploited as an attractive ecofriendly alternative for textile applications. Natural bioactive compounds have been widely reported as antimicrobial agents for textiles in a finishing setting. However, commercial applications were not reported yet, except for the case of chitosan. Typically, chitosan and plant extracts are the most explored. Yet, there are several major challenges regarding extraction, isolation of the bioactive compounds, application and durability. Nevertheless, due to their ecofriendly nature and non-toxic properties they are still promising candidates as antimicrobial agents for textiles (Goodarzian & Ekrami, 2010).

Natural dyes are mostly eco-friendly, biodegradable, less toxic, and less allergenic as compared to synthetic dyes. Most of the natural dyes are safe and some even have curative effect e.g., curcumin in turmeric has antibacterial properties (Sachan & Kapoor, 2007). However, in spite of the merits of natural dyes as compared to the synthetic ones, the use of the former is still not widespread due to non-availability of standard shade cards and standard application procedures. Natural dyes are derived from naturally occurring sources such as plants (e.g., indigo and saffron); insects (e.g., cochineal beetles and lac scale insects); animals (e.g., some species of mollusks or shellfish); and minerals (e.g., ferrous sulfate, ochre, and clay) without any chemical treatment. A spectrum of beautiful natural colors ranging from yellow to black exists in the above sources (Ali & EL Mohamedy, 2010).

These colors are exhibited by various organic and inorganic molecules (pigments) and their mixtures are due to the absorption of light in the visible region of 400-800 nm. This absorption of light depends on the structure or constituents of the coloring pigment/ molecules contain various chromophores present in the dye yielding plant to display the plethora of colors. The current preference for naturally derived colorants is due to their healthfulness and excellent performance. Several synthetic colorants have been banned because they cause allergy-like symptoms or are carcinogens. Nowadays, natural dyes are commonly used in the cosmetic industry due to no side effects, UV protection and anti-aging properties(Ali & El-Mohamedy 2011).

Many common natural dyes are reported as potent antimicrobial agents owing to the presence of a large amount of tannins. Several other sources of plant dyes rich in naphthoquinones such as lawsone from *Lawsonia interims* L. (henna), juglone from walnut and lapachol from alkanet are reported to exhibit antibacterial and antifungal activity. This is clear evidence that some natural dyes by themselves have medicinal properties. Some of its contacts are anti-allergic and proved to be safe for body contact. Majority of the naturally dyed materials are non-toxic. Natural dyes find use in the coloration of textiles, foods, drugs, and cosmetics. Small quantities of dyes are also used in coloration of paper, leather, shoe polish, wood, cane, candles, etc. In the earlier days, dyes were derived only from natural sources. Natural dyes, when used by them have many limitations of fastness and brilliancy of shade. However, when

used along with metallic mordents they produce bright and fast colors. Natural dyes can be broken down into two categories such as substantive and adjective (non-substantive). Substantive dyes are chemically bounded to the fiber without the aid of any color fixing agents (mordents) viz., indigo, turmeric. Majority of the natural dyes being non-substantive are used in conjunction with mordents(Ali, Hussain & Nawaz, 2008). Mordant forms a complex between fiber and dye, which is insoluble in water and thus gives a fast color. Some of the adjective natural dyes are madder, logwood, catechu, lac, cochineal, kermes, beetroot, marigold, rose and many more to list. Trend of natural dyes is restored as the result of the awareness of some synthetic azo dyes. As they are reduced or hydrolyzed, they release some aryl amine compounds carcinogenic or allergic to human being. Nowadays, people consciously concern their health and global environment, so they require safe and eco-friendly products According to the demand; various studies on natural dyes have been conducted (Cristea & Vilarem, 2006).

The advantage of natural dyes is eco-friendly, i.e., they do not create any environmental problems at the stage of production or use and maintains ecological balance. These natural dyes can be used for coloring food, cosmetics, and clothing for children. Unlike the synthetic dyes, which are carcinogenic, these dyes are very eco-friendly and hence can be used in specialty applications where non-toxicity is a must. The exploration of natural dyes can be environmentally and economically viable. Aim of the study is to show the feasibility of providing high-quality natural dyes extracted from plants, insects,and fungi (Asem, 2011), thus improving our environment and giving opportunities to the fabric industry to catch up with the current consumer trends towards more aesthetic fabrics and natural products. Some natural dyes extracted from desert plants used for dyeing cotton, wool, and silk fabrics after optimizing the dyeing conditions as temperature, pH, concentration and time. Microbial damage of fabrics is a common problem in many parts of the world and microbial contaminated fabrics in hospitals are known to be the major source of cross infection, so all textile materials used in hospitals should prevent or minimize the transmission of infection diseases (Nagia & El-Mohamedy 2007).

A wide number of antimicrobial compounds are known as quaternary ammonium salts which bind microorganisms to their cell membrane and disrupt the lipo-polysacride structure resulting in the breakdown of the cell

APPLICATIONS

Natural dyes can produce color on cotton, wool, silk, etc. without any chemical processing of the dyes. Color index lists 32 natural reds, 28 natural yellows, 6 natural orange, 12 natural brown, 5 natural green, 3 natural blue and 6 natural black. However, these dyes were continuously neglected because of the synthetic dyes (Samanta & Agarwal, 2009). Some of the well known ancient dyes include madder, a red dye made from the roots of the *Rubia tinctorum*, blue indigo from the leaves of *Indigofera tinctoria*, yellow from the stigmas of the saffron plant, and dogwood, an extract of pulp of the dogwood tree (Ali et al., 2008). Today, dyeing is a complex, specialized science. Nearly all dyestuffs are now produced from synthetic compounds. This means that costs have been greatly reduced and certain application and wear characteristics have been greatly enhanced. But many practitioners of the craft of natural dying (i.e. using naturally occurring sources of dye) maintain that natural dyes have a far superior aesthetic quality which is much more pleasing to the eye. On the other hand, many commercial practitioners feel that natural dyes are non-viable on grounds of both quality and economics.

Natural Dyes Obtained from Plants

There are more than 450 plants that can yield dyes. In addition to their dye-yielding characteristics, some of these plants also possess medicinal value. Natural dyes are environment friendly for example, turmeric, the brightest of naturally occurring yellow dyes is a powerful antiseptic which revitalizes the skin, while indigo gives a cooling sensation (Jothi, 2008). Many of the plants used for dye extraction are classified as medicinal and some of these have recently been shown to possess antimicrobial activity. Many natural dyestuff and stains were obtained mainly from plants and dominated as sources of natural dyes, producing different colors like red, yellow, blue, black, brown and a combination of these. Almost all parts of the plants like root, bark, leaf, fruit, wood, seed, flower, etc. produce dyes. It is interesting to note that over 2000 pigments are synthesized by various parts of plants, of which only about 150 have been commercially exploit (Pigi, Del Caro, Pinna, & Agabbio, 2003).

EXTRACTION OF NATURAL DYES FROM PLANTS

Conventional Extraction

It was carried out for 100 ml boiled distilled water using varying amounts of the dye (2-12%) for different time intervals (20-120 min). After filtration and certain dilution, the optical density of the dye liquor at 535 nm was measured (Zhang & Laursen, 2005).

Ultrasonic Extraction

It was carried out as described above in 100 ml distilled water using varying amounts of the dye materials (2-12%) at different temperatures (50-80 ^{o}C) using different sonic powers (100-500W) and for different time intervals (20-120 min). After filtration and dilution, the optical density of the dye liquor at 535 nm was measured (Shokry, El-Khatib, & Ali, 2010, Tiwari, Singh, Mishra, & Shrivastava, 2010)

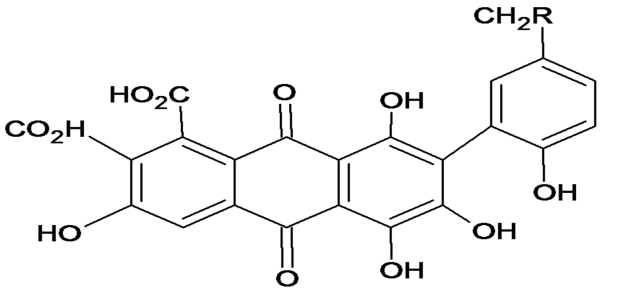
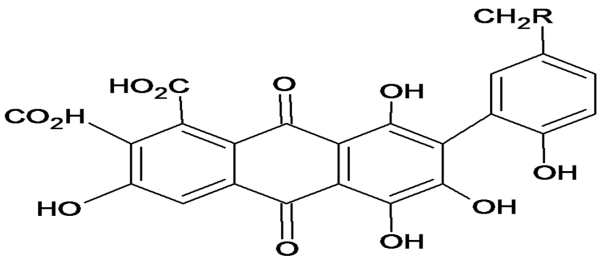
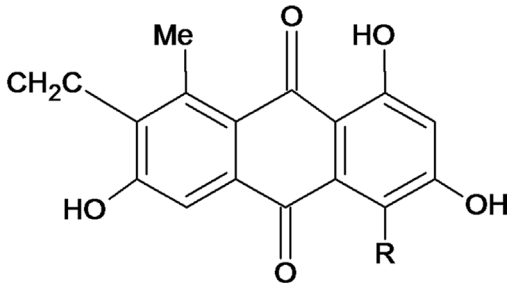
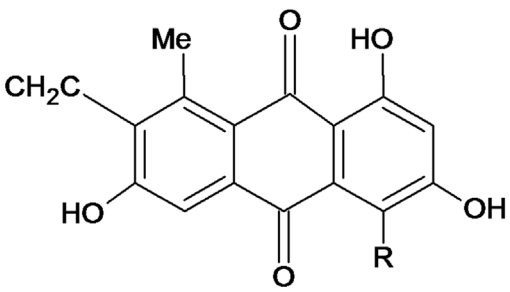
Microwave Extraction

Microwave extraction was carried out in 100ml distilled water using varying amounts of the dried stigmas of flowers (0.1- 1.5%). After filtration and dilution, the optical density of the dye liquor was measured at λmax440 nm. The best concentration at higher optical density of the dye liquor was carried out for different time periods (1-6 minutes).

Treatment with Chitosan

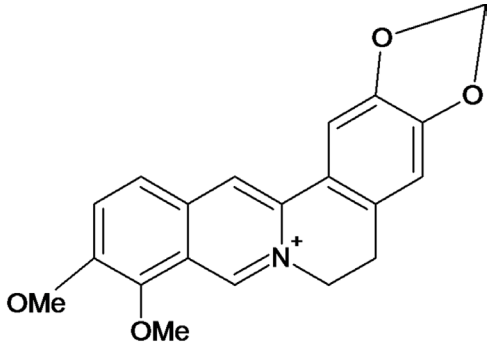
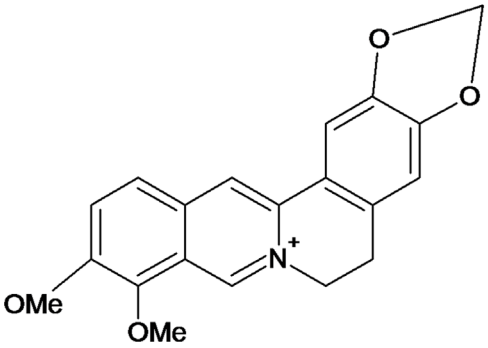
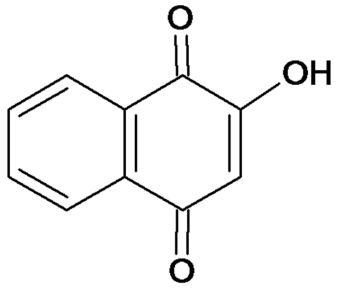
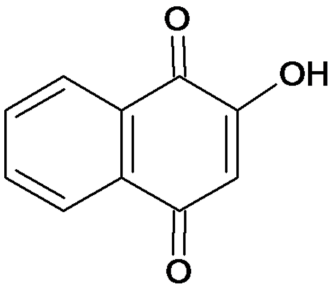
Chitosan (low, medium and high molecular weights) solutions were freshly prepared by dissolving different concentrations of chitosan (0.5, 0.75, 1.0, 1.5 and 2.0 g/l) in distilled water containing acetic acid (4g/l). The cotton and silk fabrics were immersed in these solutions at a 20:1 liquor ratio. The fabric samples were then squeezed into a wet pick of 100%. The padded samples were dried at 100^{o}C for 3 minutes, followed by curing at 150^{o}C for 3 minutes, then washed in distilled water and finally dried at ambient conditions.

Table 1. Some natural dyes, their names, sources, Chemical composition

No	Name of Natural Dyes	Source of Plant or Insect	Chemical Composition
1	Powdered madder	Roots	  R=H_1 flavokermesic acid R= OH, Kermesic acid
2	Berberine	Leaves	  R=CH_2OH, laccaic acid B R= $CH_2NHCOMe$, laccaic acid A B

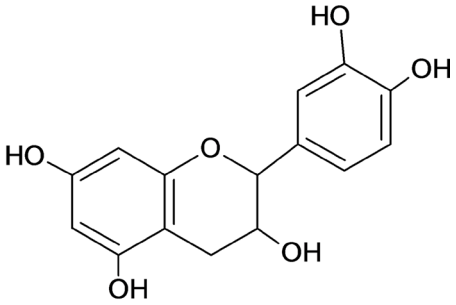
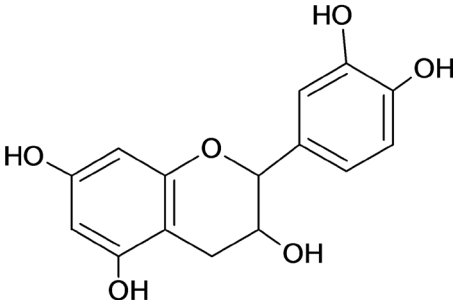
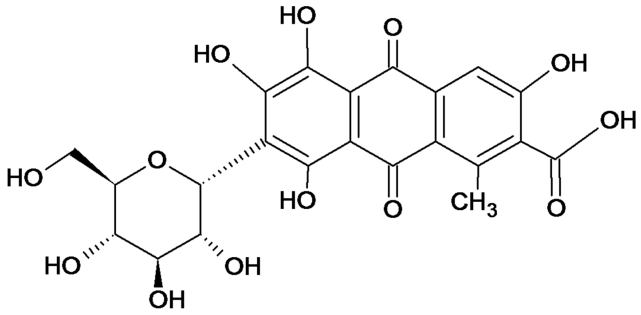
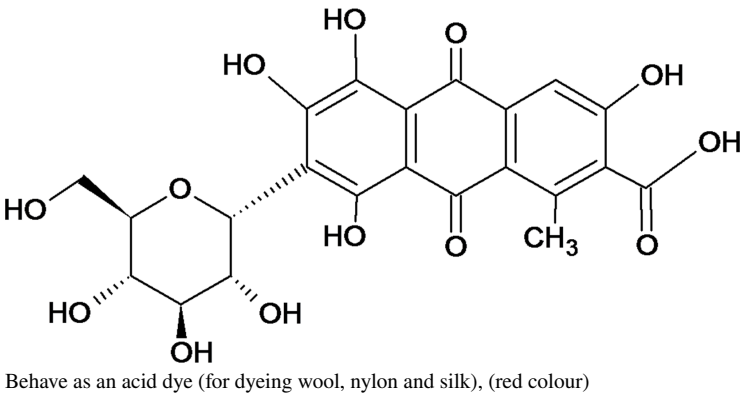
continued on following page

Table 1. Continued

No	Name of Natural Dyes	Source of Plant or Insect	Chemical Composition
3	Heena	Leaves	  Behave as basic dye for dyeing acrylic (yellow colour)
4	Cutch	Bark	  Behave as an acid dye (for dyeing wool, nylon and silk), (yellow & brown colour)

continued on following page

Table 1. Continued

No	Name of Natural Dyes	Source of Plant or Insect	Chemical Composition
5	Cocheanel	Insect	  Behave as an acid dye (for dyeing wool, nylon and silk), (orange colour)
			  Behave as an acid dye (for dyeing wool, nylon and silk), (red colour)

PRODUCTION OF TEXTILE NATURAL DYES FROM PLANTS

Tumeric (*Curcuma Longa*)

Turmeric (*Curcuma longa*) is a plant native to south India and Indonesia (Hana & Yanga. 2005). It is also cultivated in China and the whole of South East Asia. It is also called "Haldi". Its tuberous rhizomes have been used as a condiment, a colorant and an aromatic stimulant since antiquity. Turmeric consists of various molecular constituents, including three gold colors alkaloidal curcuminoid, curcumidesmethoxy curcumin and bisdemethoxy curcumin (Adeel, Ali, Bhatti, & Zsila, 2009). The curcuminoid content responsible for color depends upon the turmeric variety and within a variety on the maturity at harvest. It may be present to the extent of 4 to 8% in turmeric harvesting at the right maturity being an important factor for color and aroma (Saima, Ali, Hussain, & Nawaz, 2008). Some isomeric forms of curcumin are displayed below.

Curcumin has anti-inflammatory, antifungal and anti-tumorous. It is also widely used as food colorant. It is called C.I Natural Yellow 3, Curcumin from *Curcuma longa* has antioxidant, anti-inflammatory, anti cancer and hepatoprotective. The pharmacological activities of cucrcumnoids are due to unique molecular structure. The phenolic yellow curry pigment curcumin used in the Alzheimer's disease (Hussein, Barakat, Merfort, & Nawwar, 2007). It involves amyloid (Abeta) accumulation, oxidative damage and inflammation potent. It has anti-inflammatory effects in arthritis, Finally it has anti-platelet, anti viral, anti fungal, anti bacterial effects and powerful antiseptic agent (Goodarzian & Ekrami, 2010).

Henna (*Lawsonia Interims*)

Better color yield is obtained in case of alkaline extraction of colorant from henna leaves than aqueous extraction of colorants from henna leaves in alkaline medium. Required quantities (1%, 5%, 10% and 20% on weight of fibers) of powdered henna leaves are taken in an aqueous alkaline solution of Na_2CO_3 (pH 8.5-9) using M:L (material to liquor) ratio 1:20 and heated at 80-85 ^oC for one hour with occasional stirring, then are cooled and filtered through a clean cotton cloth. The solution is reddish orange in color. The remaining residue is percolated with Na_2CO_3 until all of the color is extracted and then filtered. The filtrate is made neutral (pH 7) using HCl and used for dyeing wool.

Saffron (*Crocus Sativus*)

It is commonly known as crocus, it consists of dried stigmas and upper parts of styles of plant *Crocus sativus* Linn. It is a widely used as natural dye in food and cosmetic industry. Saffron is used in folk medicine as an antispasmodic, eupeptic, gingival sedative, anti catarrhal, nerve sedative, carminative, diaphoretic, expectorant, stimulant, stomachic, aphrodisiac and emmenagogue. Its active constituents have anticonvulsant, antidepressant, anti-inflammatory and antitumor properties, radical scavenger as well as learning and memory improving properties and promote the diffusivity of oxygen in different tissues. *Crocus sativus* has been shown to have antidepressant effects; two active ingredients are crocin and safranal (Miquel, Bernd, Sempere, Díaz-Alperi, & Ramírez, 2002) showed that saffron extract and its constituents; crocin, safranal and picrocrocin inhibit the growth of human cancer cells (Hella cells)

in vitro. Crocin analogs isolated from saffron significantly increased the blood flow in the retina and choroid as well as facilitated retinal function recovery and it could be used to treat ischemic retinopathy and/or age-related macular degeneration. Picrocrocin and safranal in patients with coronary artery disease. Indicates the potential of saffron as an antioxidant. Antiparkinsonian effect of Crocetin, which is an important ingredient of saffron, may be helpful in preventing Perkinsonism.

In a dye bath containing different concentrations (1- 15 g/l) of saffron dye with a liquor ratio 1:100, the silk fabric was dyed by conventional heating at different path pHs (2-8) for different time periods (10-60 minutes) and at the boiling temperature. For comparison, the same dyeing condition was made for microwave heating for different concentrations of saffron dye (1- 15 g/l) and time periods (1-6 minutes). Thus, in a dye bath containing (10 g/l) saffron dye with a liquor ratio 1:100 at pH 3. The dyed samples were rinsed by warm water and then cold water, washed in a bath containing 5g/l non-ionic detergent at 50ºC for 30 minutes, then rinsed and dried in shade at room temperature (Nagia & El-Mohamedy 2007).

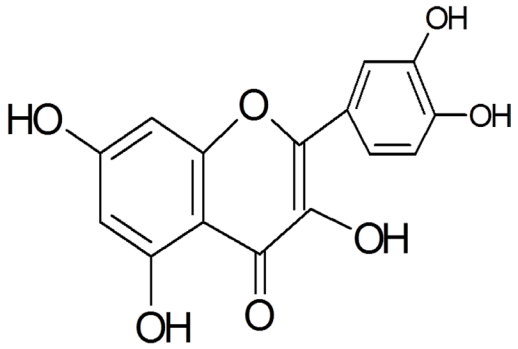
Golden Shower *(Cassia Fistula)*

Cassia fistula tree is native of tropical climate area and it is widely present in Asian countries such as Pakistan, India, and Bangladesh etc. Coloring matters of *Cassia fistula* bark has ample natural tannins, phlobaphenes and oxyanthraquinone substances, which probably consist of emodin and chrysophanic acid; along with fistuacacidin, barbalion and rhein. The main coloring component of stem bark of cassia fistula is 1, 8-dihydroxi- 6-methoxi- 3-methyl anthraquinone. The ethanolic bark extract of Amaltas is good source of natural dye. A butanol extract of the powdered stem bark contained tannins.

Onion *(Allium Cepa)*

Onion has shown antibacterial, salmonella typhimunium mutagencity was reduced in hamburger when onion were added Growth of oral pathogenic bacteria, including *sptococcus mutans*, *streptococcus subinus, porphromonas gingivalis*. The outer driest papery skin of onion also is good source of natural dye, it yield the dye CI “natural yellow 10”. The suitability of onion peel which is nutrient, consumed daily and useless for any purpose could be used as natural dye for wool fabric.

Figure 1. Onion dye structure



PRODUCTION OF TEXTILE NATURAL DYES FROM INSECTS

Lac Insect (*Laccifer Lacca*)

Lac dye is described as the scarlet pigment present in the live, pre-emergent insects (*Laccifer lacca*) which develops a resinous cocoon, known as stick lac on the twinges of over 160 host trees. Kermes (*Kermes ilicis*) is a scale insect produces red crimson dye. The insect kermes is the parasite of oak plant native to Mediterranean and Middle East region. Kermes was the first animal dye discovered when alternatives for dyeing wool and silk were unavailable. Cochineal is a deep crimson dye extracted from female cochineal insects (*Coccus ilicis*). The coloring matter of cochineal dye is carminic acid, hence can be popularly used on protein fibers such as silk and wool.

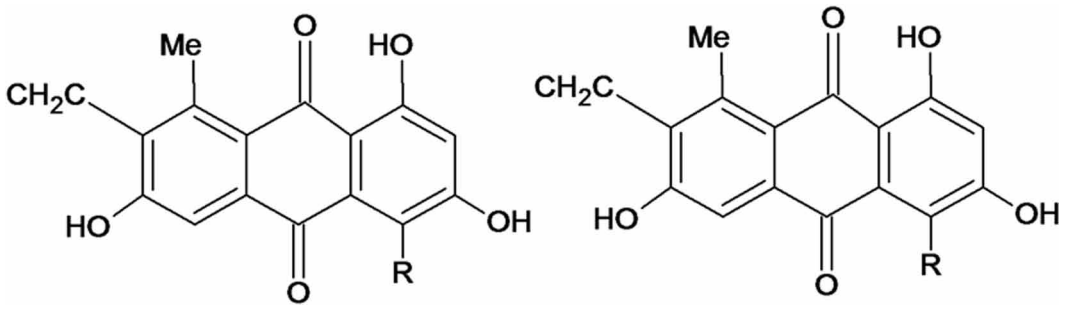
Lac Dye Extraction

Lac dye can be obtained by extracting stick lac with water and sodium carbonate solution followed by precipitating with lime. Stick lac was crushed into pieces, wetted with water and kept overnight. The wet pieces of stick lac were churned. The water soluble dye driven out during churning. The solution was filtered, and the extracted dye was found to be 50 to 98 percent pure. Indian Lac research Institute (2006) presented a note on lac dye entitled "Lac dye-A potential material for proteinous fibers." Lac dye is an important by- product of lac industry; when stick lac is crushed and washed with water, the water soluble dye nearly 1.0 per cent dissolves and is thrown off as a lac effluent. The waste effluents of lac processing units when treated with mineral acids, the lac effluent precipitates out as a solid sludge which is filtered. The filtrate is treated with calcium carbonate to obtain calcium salt of lac dye. The calcium salt is converted into sodium salt which is then passed through cation exchange resin column. The dilute solution is concentrated to give pure lac dye crystals which are washed with cold water and sun dried.

ECOFRIENDLY SUN PROTECTIVE CLOTHING

Although most people view sun protective as their first line of defense against the sun, what you wear can really play a major role in safeguarding your skin. Scientific articles about sun protection found that donning sun-protective clothes and scaling back sun exposure beats out sunscreen as a skin cancer

Figure 2. Colouring component of Lac extract



prevention strategy. And, if you choose eco-friendly garments-those that are certified organic, made from renewable materials, and unbleached-you can double your green impact: keep petrochemical-based sunscreens from entering the environment while donning clothes that come with little environmental cost.

You may have all the sun protection you need right in your closet without buying a single stitch more-after all, the ultimate in green think is to not buy new when possible. Garments vary widely in their ability to hold off the sun, or in their Ultraviolet Protection Factor (UPF) number. UPF indicates how much of the sun's UVA and UVB radiation is absorbed by the fabric; a UPF of 50 allows only 2 percent of the UV rays to get through the fabric. To assess your wardrobe here are some rules of thumb:

A tighter weave keeps out more sun: Probably the most important factor lies in the weave of the fabric; the tighter the weave, the greater the sun protection because fewer ultraviolet (both UVA and UVB) rays can pass through the fabric to your skin (Schmidt & Zimniewska, 2006).

NANOTECHNOLOGY: A NEW STRATEGY TO DEVELOP NON-TOXIC ANTIMICROBIAL TEXTILES

Antimicrobial Textiles

Among the various semiconductor photocatalysts, TiO2 has proved to be the most suitable catalyst for widespread environmental application because of its biological and chemical inertness, strong oxidizing power, non-toxicity and long-term stability against photo and chemical corrosion. Commercial Degussa P25 TiO2 (surface area = 50 m2/g) has been widely used as a catalyst in photodegradation of dyes. However, the photocatalytic activity of TiO2 must be further enhanced from the point of view of practical use and commerce.

Nanophotocatalysis using nanostructured semiconductors constitute one of the emerging technologies due to its high catalytic efficiency. A number of methods for preparing Nano TiO2 have been reported (e.g. ultrasonic technique, sol-gel process and hydrothermal process). Khataee, Vatanpur, Amani & Ghadim, (2009) reported 100% color removal efficiency of Acid Blue 9 dye with concentration of 20 mg/L at pH = 6.3, catalyst dose = 150 mg/L and contact time = 150 min using UV/Nano-TiO2. Complete color removal of Acid Blue 25 with concentration of 50 mg/L was reported by Niyaz & Arami, (2006) using Nano-TiO2 with 600 mg/L H2O2 dose after 140 min. Decolorization efficiency of 96% within 120 minutes of Acid Orange 20 was reported by Theodora, Nikolaos, Xekoukoulotakis, Ioannis, & Dionissios, (2007) at catalyst concentration of 250 mg/L. The photocatalytic activity of Nano-TiO2 was reported to be higher than micron TiO2 (Degussa P25) in the degradation of active brilliant red X-3B (Mao, Li, Dang, & Zhang, 2005), and methylene blue (Wang, Mao, & Lin, 2006).

Reactive dyes are a class of dyes with high application rate in the textile industry due to their reactivity with fibers and their colour stability. Under typical reactive dyeing conditions (pH > 10, temperature > 65°C and salt:60-100 g/L) as much as 20-50 percent of the initial mass of the reactive dye remains in the spent reactive dye bath in the hydrolyzed form which has no affinity for the fiber (Sreedhar & Kotaiah, 2005). For this reason, they are also one of the dyes most widely reported in the literature (Neppolian, Kanel, Choi, Shankar, & Murugesan, 2003). It has been extensively demonstrated that the photochemical properties of TiO2 are strongly dependent on its crystal structure and morphology as well as grain size (Yin *et al*., 2008). This work characterized the decolorization of reactive dyes using nano-TiO2 in suspension. Solar radiation was also employed in the systems studied. The use of solar energy to start

the photochemical degradation of several organic pollutants, such as textile effluents, has been widely reported. Although solar energy is a free, renewable and environmentally friendly energy source, it is not widely used in tropical countries like India.

Chitosan is a deacetylated derivate of chitin, non toxic, resistant to microorganisms, biodegradable and biocompatible. The antimicrobial activity of chitosan is influenced by several factors such as the type of chitosan, the degree of deacetylation (Hussein, Jain, Panwan, Gupta, & Khare, 2005). Molecular weight and other physical and chemical factors such as pH, ionic strength and addition of non-aqueous solvents (Mangoni et al., 2008). Chitosan can be considered an antimicrobial agent for textile finishing. However, its application in textile materials is effective against a wide range of microorganisms only at high concentrations, which causes a decrease of the air permeability on fabrics and turns the fabric very inflexible. Another disadvantage is the low durability after application (Gao & Cranston 2008, Kramer et al., 2006).

Sericin is a natural macromolecular protein derived from silkworm *Bombyx mori* which constitutes 25-30% of the silk protein. It is a bimolecular of great value since it has antibacterial properties, UV resistance, resists oxidation and has hydrating properties (Joshi, Ali, & Purwar, 2009). It has several applications, such as moisturizing agent in shampoos and creams, and is also an important biomaterial for various applications including textiles. Although the application of sericin as an antibacterial agent for textiles has not been reported yet, it has been found evidence of such a potential application (Joshi, Ali, & Rajendran, 2007).

Neem (*Azadirachta indica*) is an evergreen tree of India, which belongs to the plant family *Meliaceae*. This is recognized as one of the most promising sources of compounds with antimicrobial and medicinal properties. The active ingredients of neem are found in all parts of tree. The extract of neem has been widely used in pesticide formulations that due to their pest repellent properties have the potential to inhibit the growth of Gram-positive and Gram-negative bacteria. At present, little has been reported of its use in textiles as an antimicrobial agent. Few studies concerning application of seed and bark extracts to cotton and cotton/polyester blends have been reported. Textile industry continuously searches for new technologies in order to accomplish the consumer's demands. Especially in recent years, new developments allowed the production of functional and smart textiles which are capable of sensing changes in environmental conditions or body functions and responding to these changes (Williams, HaloSource, & Cho, 2005). Likewise, consumers' attitude towards hygiene and active lifestyle has created a rapidly increasing market for a wide range of textile products finished with antimicrobial properties, which in turn has stimulated intensive research and development As a consequence; the number of biofunctional textiles with an antimicrobial activity has increased considerably over the last few years (Kruse & Kristensen, 2008).

Application is nowadays extended to underwear, sportswear, home furnishing and protective clothing in areas with high risk of infection by pathogens (hospitals, schools and hotels); and because they are able to absorb substances from the skin and can release therapeutic compounds to the skin, they find applications for prevention, as surgical lab coats, or therapy, as wound dressings. Thus, biomedical products will perhaps be the largest application of antimicrobial textiles (Purwar & Joshi, 2004).

Microorganisms and Textiles

Textiles are an excellent substrate for bacterial growth and microbial proliferation under appropriate moisture, nutrients and temperature conditions. In a clinical setting, they can be an important source of

bacteria that may contaminate the patients and clinician personnel. Bacteria and fungus, either pathogenic or not, are normally found on human skin, nasal cavities, and other areas, such as in the genital area. Microbial shedding from our body contributes to microorganism spreading into a textile material either directly in clothes or on surrounding textiles (Madigan, Martinko, & Parker, 2006). Recent studies strongly support that contamination of textiles in clinical settings may contribute to the dispersal of pathogens to the air which then settle down and infect the immediate and non-immediate environment. It is one of the most probably causes of hospital infections. Typically, pathogenic microorganisms like *Klebsiella pnuemoniae*, *Pseudomonas aeuroginosa*, *Staphylococcus epidermidis*, *Staphylococcus aureus* and *Candida albicans* have been found on textiles. In addition, microorganism proliferation can cause malodors, stains and damage of mechanical properties of the component fibers that could cause a product to be less effective in its intended use. Additionally, may promote skin Contamination, inflammation and in sensitive people, a topic dermatitis. Fortunately, the use of antimicrobial textiles may significantly reduce the risk of infections especially when they are used in close contact with the patients or in the immediate and non-immediate surroundings.

SOLUTION AND RECOMMENDATION

To minimize the risks associated with the application of antimicrobial agents, there is a great demand for antimicrobial textiles based on non-toxic and ecofriendly bioactive compounds. Due to the relatively lower incidence of adverse reactions of natural products in comparison with synthetic pharmaceuticals, they can be exploited as an attractive ecofriendly alternative for textile applications. Natural bioactive compounds have been widely reported as antimicrobial agents for textiles in a finishing setting. Microbial damage of fabrics is a common problem in many parts of the world and microbial contaminated fabrics in hospitals are known to be the major source of cross infection, so all textile materials used in hospitals should prevent or minimize the transmission of infection diseases. A wide number of antimicrobial compounds are known as quaternary ammonium salts which bind microorganisms to their cell membrane and disrupt the lipo-polysacride structure resulting in the breakdown of the cell.

New developments allowed the production of functional and smart textiles which are capable of sensing changes in environmental conditions or body functions and responding to these changes through dyeing fabrics natural dyes using ultrasonic method In recent decades ultrasonic has established an important place in different industrial processes and has started to revolutionize environmental protection. This technique has been studied and used for a variety of applications in liquids, dispersions and polymers. Microorganism proliferation can cause malodors, stains and damage of mechanical properties of the component fibers that could cause a product to be less effective in its intended use. Additionally, may promote skin contaminations, inflammation and in sensitive people, atopic dermatitis. Fortunately, the use of antimicrobial textiles may significantly reduce the risk of infections especially when they are used in close contact with the patients or in the immediate and non-immediate surroundings.

Antimicrobial agents are natural or synthetic compounds that inhibit the growth (bacteriostatic or fungistatic) because they can be protein, lipid synthesis or enzyme inhibitors, all of which are essential for cell survival; or kill (biocidal) the microorganisms by damage in the cell wall. Almost all antimicrobial synthetic agents in use on textiles are biocides. The production of functional and smart textiles which are capable of sensing changes in environmental conditions or body functions and responding to

these changes through dyeing fabrics with natural dyes. In recent decades using the ultrasonic method has established an important place in different industrial processes and has started to revolutionize environmental protection.

FUTURE RESEARCH DIRECTIONS

Textile industry is not only considered as one of the oldest industrial sectors, but it is also one of the first global industries which are the major supplier of employments and materials, chemicals, finishing agents and dyes. Today the textile industry is focusing on the development of innovative textile for the market and the society needs such as protective clothing for working personnel's in medical centers, hospitals, biomaterials, army, firemen, food industry and restaurants in addition to other future industrial opportunity related to textile industry such as automotive, aircraft, air and liquid filtration, construction, agriculture and transport. Textile industry and private sectors and small private producers of textiles are in need of finishing agents and dyes either natural or synthetic which are produced from local eco-friendly raw materials. These are important items for the textile industry dyes and auxiliary industries in addition to carpets industry.

CONCLUSION

Natural dyes is used as a substitute of synthetic dyes for dyeing different kinds of textiles to obtain antimicrobial and smart textiles. The use of antimicrobial textiles may significantly reduce the risk of infections especially when they are used in close contact with the SKIN or in the immediate and non-immediate surroundings. Dyeing with natural dyes extracted from desert plants, insects and fungi to overcome environmental pollution, natural dyes have antimicrobial activity against various types of microbes as *(Escherichia coli, Staphylococcus aureus and Pseudomons aeruginosa)*, Using nano materials for some metals or its oxides as titanium oxide for treatment of fabrics before dyeing, to acquire new properties as antimicrobial activities against bacteria and fungi and also to protect textiles from ultra violet rays. Using a traditional and microwave heating for extraction of dyes and dyeing methods because microwave heating is a more effective method than traditional heating. Other additional features about microwaves are that they are cheaper, more economical (saving time and energy), eco-friendly, and produce a higher dye uptake as compared to conventional techniques, Environmentally friendly pre-treatment by chitosan before dyeing in order to obtain dyed fabric with high quality and more protected against microbes. Application of antimicrobial agents in the development in the textiles as chitosan, qutenary ammonium salt and neem.

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KEY TERMS AND DEFINITIONS

Antimicrobial Textiles: Antimicrobial fabrics and textiles are fiber-based substrates to which antimicrobial agents have been applied at the surface, or incorporated into the fibers, rendering a product that kills or inhibits the growth of microorganisms.

Chitosan: Chitosan is a linear polysaccharide composed of randomly distributed β-linked D-glucosamine and N-acetyl-D-glucosamine. It is made by treating shrimp and other crustacean shells with the alkali sodium hydroxide. It is biopolymer.

Green Production: Green production is a business strategy that focuses on profitability through environmentally friendly operating processes.

Microwave: An electromagnetic wave with a wavelength in the range 0.001-0.3 m, shorter than that of a normal radio wave but longer than those of infrared radiation.

Nano Materials: A material having particles or constituents of nanoscale dimensions, or one that is produced by nanotechnology.

Natural Compounds: A natural product is a chemical compound or substance produced by a living organism that is, found in nature.

Natural Dyes: Natural dyes are dyes or colorants derived from plants, invertebrates, or minerals. The majority of natural dyes are vegetable dyes from plant sources: roots, berries, bark, leaves, and wood and other organic sources such as fungi and lichens.

Chapter 15
Optimization Studies on Biosorption of Ni(ii) and Cd(ii) from Wastewater in a Packed Bed Bioreactor

K. Narasimhulu
National Institute of Technology, India

Y. Pydi Setty
National Institute of Technology, India

ABSTRACT

This chapter refers to the study of the biosorption of Ni(II) and Cd(II) in packed bed bioreactor by Pseudomonas putida. The conventional treatment methods of Nickel and Cadmium were elaborated and compared with biosorption. The methods for optimization of process conditions for biosorption of Ni(II) and Cd(II) in packed bed bioreactor by Pseudomonas putida were explained. The optimum conditioned were determined to be flow rate of 300 mL/h, initial metal ion concentration of 100 mg/L and bed height of 20 cm with weight of biosorbent of 12 g, and it was found that the Agar immobilized Pseudomonas putida showed maximum percent biosorption and bed saturation occurred at 20 minutes. Optimization results of Ni(II) and Cd(II) by Pseudomonas putida from the Design Expert software were obtained as bed height of 19.93 cm, initial metal ion concentration of 103.85 mg/L, and flow rate of 310.57 mL/h. The percent biosorption of Ni(II) and Cd(II) is 87.2% and 88.2% respectively. The predicted optimized parameters are in agreement with the experimental results. Experiments were carried out at established optimum conditions of bed height of 20.77 cm, flow rate of 309.09 mL/h, and initial metal ion concentration of 109.23 mg/L and results of biosorption of Ni(II) and Cd(II) were reproduced and they were in agreement with the predicted results. Based the experimental results, it was observed that the Pseudomonas putida was the best choice to remove Nickel and Cadmium ions from wastewater in a continuous column system.

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INTRODUCTION

Heavy metal pollution has become one of the most serious environmental problems today. India is rich in water resources, having a network of many rivers and vast alluvial basins to hold plenty of ground water. India is also blessed with snow-capped peaks in the Himalayan range which can meet a variety of water requirements of the country. However, with the rapid increase in the population of the country and the need to meet the increasing demands of irrigation, domestic and industrial consumption of water, the available water resources in many parts of the country are getting depleted and the water quality has deteriorated. In India, water pollution comes from three main sources: Domestic sewage, industrial effluents and run-off from agriculture (Brar, Malhi, Singh, Arora, & Gill, 2000). Earth's surface comprising of 70% water is the most valuable natural resource existing on our planet. Without this invaluable compound, the life on the earth would not exist. Although this fact is widely recognized, pollution of water is a common problem being faced today. Heavy metal pollution occurs directly by effluent outfalls from industries, refineries and waste treatment plants and indirectly by the contaminants that enter the water supply from soils/ground water systems and from the atmosphere via rain water (Vijayaraghavan & Yun, 2008). Modern industry is, to a large degree, responsible for contamination of the environment. Lakes, rivers and oceans are being overwhelmed with many toxic contaminants. Among toxic substances reaching hazardous levels are heavy metal ions (Vieira & Volesky, 2000). Heavy metals belong to the group of contaminants, which comes under the inorganic division. The characteristics of heavy metals are described as (Wang & Chen, 2006):

- Toxicity that can last for a long time in nature.
- Transformation of low toxic heavy metals to more toxic form in certain environmental conditions.
- Bioaccumulation and bioaugmentation of heavy metals by food chain that could damage normal physiological activity and endanger human life.
- Heavy metals cannot be degraded including biotreatment.
- Heavy metals are very toxic even at low concentration (1.0-10 mg/L).

TREATMENT TECHNOLOGIES FOR HEAVY METALS REMOVAL

Heavy metals contamination is becoming a great concern to the government policies and also for having awareness about environment. Several heavy metals removal technologies including chemical precipitation, ion exchange, reverse osmosis, electrodialysis, ultrafiltration and phytoremediation are commonly used in industries (Ahalya, Ramachandra, & Kanamadi, 2003). However, these technologies are becoming uneconomical and unfavourable to remove heavy metals from industrial wastewaters. Description of these treatment technologies are presented in table 1 which also gives the disadvantages associated with each process.

With increasing environmental attention and legal constraints on discharge of effluents, a need of cost effective technology is essential (Alluri et al., 2007). Recently, more focus is given on using microbial biomass as a biosorbent to sequester metal ions from contaminated effluent (Alluri et al., 2007). In recent years, biosorption has been studied for the removal of metal ions, especially at the concentrations ranging from 1-100 mg/L, due to its lower cost and higher effectiveness than conventional methods such as chemical precipitation and ion exchange (Kapoor & Viraraghavan, 1995). A better understand-

Table 1. Treatment methods used in heavy metals removal (Source: Rakhshaee, Khosravi, & Ganji, 2006; Sannasi, Kader, Ismail, & Salmijah, 2006)

Treatment Method	Process Description	Disadvantages
Chemical precipitation	Precipitation of metal ions from contaminated water involves the conversion of soluble heavy metal salts to insoluble salts that will precipitate.	Large amount of sludge produced during the process will cause a disposal problem
Ultrafiltration	Pressure driven membranes are used for the removal of metal ions	Generation of sludge causes disposal problem
Reverse osmosis	Metal ions are separated by a semi-permeable membrane at a pressure greater than osmotic pressure caused by the dissolved solids	The process is expensive
Ion exchange	Metal ions from dilute solutions are exchanged with ions held by the charged membranes	High cost, Partial removal for certain ions
Electrodialysis	Metal ions are separated through the semipermeable ion selective membranes. An electrical potential between the two electrodes causes a separation of cations and anions thus cells of concentrated and dilute salts are formed.	Metal hydroxides formed lead to clogging of the membrane
Phytoremediation	Uses certain plants to clean up soil, sediment and contaminated water with metal ions	The process takes a long time to remove metal ions, Regeneration of the plant is difficult

ing of biosorption mechanism is important to the design and optimization of the biosorption process (Wang & Chen, 2006). The mechanisms involved in metal ion biosorption are complicated; however, five mechanisms involved in metal ion biosorption have been identified: (i) Physical Adsorption, (ii) transport across the cell membrane, (iii) ion exchange, (iv) complexation and (v) precipitation (Veglio & Biolchini, 1997). Volesky, (2001) further classified metal ion biosorption into three classes: chemisorption (ion exchange, complexation, coordination, chelation), physical adsorption and microprecipitation. The mechanism may vary among metal ions and biosorbents (Mehta & Guar, 2005). Biosorption is a metabolic-independent process and the sequestration of metal ions by cells can take place through adsorption, ion exchange, coordination, complexation etc. by the presence of functional groups on the biomass such as carboxyl, hydroxyl, amine and phosphate, which exhibit affinity to metal ions (Palmieri, Garcia Jr, & Melnikov, 2000). In the development of metal ion biosorption, Tsezos, (2001) described that biosorption was brought to the foreground of scientific interests as a potential basis for the recovery of radionuclide metal ions from wastewater during 1970s. In the early 1980s, more research was conducted, mainly focusing on finding specific microbial biomass and hastening this technology into application. However, due to some unsuccessful commercial applications in the market, research in biosorption got shifted back to the fundamental principles rather than advancing towards the biosorption process design of the 1990s onwards. This regression in research was based on a lack of understanding of the biosorption mechanism, which hindered adequate assessment of the process performance and limitations and the expected widespread application of this technology (Kratochvil &Volesky, 1998).

The major advantages of biosorption process over conventional technologies include (Kratochvil & Volesky, 1998; Ahalya et al., 2003):

- Non living cells are less sensitive to metal ion concentrations.
- Process is less expensive and more efficient.

- Supply of nutrients is not required.
- Sludge production can be minimized.
- Biosorbent can be regenerated.
- Metal ions recovery is possible.
- Process can be operated at ambient conditions of pH and temperature.

Chemical Precipitation

It is effective and by far the most widely used process in industry (Ku & Jung, 2001) because it is relatively simple and inexpensive to operate. In precipitation processes, chemicals react with heavy metal ions to form insoluble precipitates. The precipitates formed can be separated from the water by sedimentation or filtration. The treated water is then decanted and appropriately discharged or reused. The conventional chemical precipitation processes include hydroxide precipitation and sulfide precipitation.

Ni(II) can be removed by precipitation as hydroxide at pH ranging from 10.0 to 11.0 (solubility: 0.12 ppm). It can also be removed by precipitation as sulfate or carbonate. The presence of cyanide may interfere with nickel precipitation. However, Cd(II) can be removed by precipitation as hydroxide at pH ranging from 8.0 (solubility: 1 ppm) to 11.0 (solubility: 0.05 ppm). It can also be removed by precipitation as sulfide. The effluent concentration is 0.05 ppm. Cd(II) can also be removed by co-precipitation at pH of 6.5 with $FeCl_3$ where $Fe(OH)_3$ floc is formed. The effluent concentration is 0.008 ppm. Cd(II) can also be removed by precipitation as carbonate. The pH required in this case is between 7.5 and 8.5. The effluent concentration is comparable to that obtained through hydroxide precipitation at high pH. Cyanides interfere with any of these processes and must be removed prior to cadmium precipitation. The most widely used chemical precipitation technique is hydroxide precipitation due to its relative simplicity, low cost and ease of pH control (Huisman, Schouten, & Schultz, 2006). The solubilities of the various metal hydroxides are minimized in the pH range of 8.0-11.0. The metal hydroxides can be removed by flocculation and sedimentation. A variety of hydroxides have been used to precipitate metals from wastewater, based on the low cost and ease of handling, lime is the preferred choice of base used in hydroxide precipitation at industrial settings (Baltpurvins, Burns, Lawrance, & Stuart, 1997). The concentrations of Cr (VI), Cu(II), Ni(II) and Cd(II) in effluents can be reduced from initial concentration of 100.0 mg/L to 0.08, 0.14, 0.03 and 0.45 mg/L, respectively. In hydroxide precipitation process, the addition of coagulants such as alum, iron salts, and organic polymers can enhance the removal of heavy metals from wastewater. Although widely used, hydroxide precipitation also has some limitations. Firstly, hydroxide precipitation generates large volumes of relatively low density sludge, which can present dewatering and disposal problems (Kongsricharoern & Polprasert, 1995). Secondly, some metal hydroxides are amphoteric, and the mixed metals create a problem using hydroxide precipitation since the ideal pH for one metal may put another metal back into solution. Thirdly, when complex agents are in the wastewater, they will inhibit metal hydroxide precipitation.

Ion Exchange Process

Ion-exchange processes have been widely used to remove heavy metals from wastewater due to their many advantages, such as high treatment capacity, high removal efficiency and fast kinetics (Kang et al., 2004). Ion-exchange resin, either synthetic or natural solid resin, has the specific ability to exchange its cations with the metals in the wastewater. Among the materials used in ion-exchange processes, synthetic

resins are commonly preferred as they are effective to remove the heavy metals from the solution (Alyüz & Veli, 2009). The most common cation exchangers are strongly acidic resins with sulfonic acid groups ($-SO_3H$) and weakly acidic resins with carboxylic acid groups (-COOH). Hydrogen ions in the sulfonic group or carboxylic group of the resin can serve as exchangeable ions with metal cations.

The uptake of heavy metal ions by ion-exchange resins is affected by certain variables such as pH, temperature, initial metal ion concentration and contact time (Gode &Pehlivan, 2006). Ionic charge also plays an important role in ion-exchange process. Besides synthetic resins, natural zeolites and naturally occurring silicate minerals have been widely used to remove heavy metal ions from aqueous solutions due to their low cost and high abundance. Many researchers have demonstrated that zeolites exhibit good cation-exchange capacities for heavy metal ions under different experimental conditions (Motsi, Rowson, & Simmons, 2009).

Ultrafiltration

Ultrafiltration (UF) is a membrane technique working at low transmembrane pressures for the removal of dissolved and colloidal materials. Since the pore sizes of UF membranes are larger than dissolved metal ions in the form of hydrated ions or as low molecular weight complexes, these ions would pass easily through UF membranes. To obtain high removal efficiency of metal ions, the micellar enhanced ultrafiltration (MEUF) and polymer enhanced ultrafiltration (PEUF) were proposed. MEUF was first introduced for the removal of dissolved organic compounds and multivalent metal ions from aqueous streams (Landaburu-Aguirre, García, Pongrácz, & Keiski, 2009). This separation technique is based on the addition of surfactants to wastewater. When the concentration of surfactants in aqueous solutions is beyond the critical micelle concentration (CMC), the surfactant molecules will aggregate into micelles that can bind metal ions to form large metal-surfactant structures. The micelle containing metal ions can be retained by a UF membrane with pore sizes smaller than micelle sizes, whereas the untrapped species readily pass through the UF membrane. To obtain the highest retentions, surfactants of electric charge opposite to that of the ions to be removed have to be used. Sodium dodecyl sulfate (SDS), an anionic surfactant, is often selected for the effective removal of heavy metal ions in MEUF. Metal removal efficiency by MEUF depends on the characteristics and concentrations of the metals and surfactants, solution pH, ionic strength, and parameters related to membrane operation. Landaburu-Aguirre et al., (2009) investigated the removal of zinc from synthetic wastewater by MEUF using SDS. Sampera, Rodríguez, De la Rubia, & Prats, (2009) used MEUF to remove Cr(VI), Cu(II), Ni(II) and Cd(II) from synthetic wastewater using two anionic surfactants: SDS and linear alkylbenzene sulfonate (LAS) in a lab-scale membrane system. The molar concentration ratio of the surfactant to metal is higher than 5.0 in all the experiments. When the initial SDS concentration was below the CMC, metal retention higher than 90% was unexpectedly obtained, except for Ni(II). Moreover, it was shown that complete removal of metal ions, except for Ni(II), could be achieved at an LAS concentration below CMC. The retentate is the concentrated solution of surfactants and heavy metals retained by membrane. Since the surfactant may account for a large portion of operating costs, it is essential to recover and reuse the surfactant as economically as feasible. And if the surfactant and heavy metals are not disposed, they will cause secondary pollution. PEUF uses water-soluble polymer to complex metallic ions and form a macromolecular complex, having a higher molecular weight than the molecular weight cut off of the membrane. The macromolecular complex will be retained when they are pumped through UF membrane. After that, retentate can be treated in order to recover metallic ions and to reuse polymeric agent.

Reverse Osmosis

The reverse osmosis (RO) process uses a semi-permeable membrane, allowing the fluid that is being purified to pass through it, while rejecting the contaminants. RO is one of the techniques able to remove a wide range of dissolved species from water. It accounts for more than 20% of the world's desalination capacity. RO is an increasingly popular wastewater treatment option in chemical and environmental engineering. Cu(II) and Ni(II) ions were successfully removed by the RO process and the rejection efficiency of the two ions increased up to 99.5% using Na_2EDTA (Mohsen-Nia, Montazeri, & Modarress, 2007). Dialynas & Diamadopoulos, (2009) applied a pilot-scale membrane bioreactor system in combination with RO and they found heavy metal removal efficiencies were very high. The major drawback of RO is the high power consumption due to the pumping pressures and the restoration of the membranes.

Nanofiltration

The nano filtration (NF) technique is mainly used for the removal of two valued ions and the larger mono valued ions such as heavy metals. NF is a promising technology for the rejection of heavy metal ions such as nickel (Murthy & Chaudhari, 2008) from wastewater. NF process benefits from ease of operation, reliability and comparatively low energy consumption as well as high efficiency of pollutant removal. They found that an increase of pH and a decrease of operating temperature and feed concentration led to higher removal for both membranes. Among the parameters affecting the rejection, feed concentration plays a key role for the production of a permeate stream. Furthermore, Murthy & Chaudhari, (2008) focused on the removal of heavy metal ions using NF membrane. They reported about the application of a thin-film composite polyamide NF membrane for the rejection of nickel ions from aqueous wastewater (Murthy & Chaudhari, 2008). The maximum observed rejection of nickel is found to be 98% and 92% for an initial feed concentration of 5 and 250 mg/L, respectively. They investigated the binary heavy metals (cadmium and nickel) separation capability of a commercial NF membrane from aqueous solutions (Murthy & Chaudhari, 2009). The maximum observed solute rejection of Ni(II) and Cd(II) ions is 98.94% and 82.69%, respectively, for an initial feed concentration of 5 mg/L.

Electrodialysis

Electrodialysis (ED) is another membrane process for the separation of ions across charged membranes from one solution to another using an electric field as the driving force. In most ED processes, ion-exchange membranes are used. The membranes are actually of two basic types: cation-exchange and anion-exchange membranes. This process has been widely used for the production of drinking and process water from brackish water and seawater, treatment of industrial effluents, recovery of useful materials from effluents and salt production (Sadrzadeh, Mohammadi, Ivakpour, & Kasiri, 2009). ED has also proven a promising method in heavy metal wastewater treatment. Natraj & Spurr, (2007) performed a new working system to investigate the removal of hexavalent chromium ions using a built ED pilot plant comprising a set of ion-exchange membranes. Results were satisfactory in meeting the maximum contamination level of 0.1 mg/L for chromium.

Coagulation and Flocculation

Coagulation and flocculation followed by sedimentation and filtration is also employed to remove heavy metal ions from wastewaters. Coagulation is the destabilization of colloids by neutralizing the forces that keep them apart. Many coagulants are widely used in the conventional wastewater treatment processes such as aluminum, ferrous sulfate and ferric chloride, resulting in the effective removal of wastewater particulates and impurities by charge neutralization of particles and by enmeshment of the impurities on the formed amorphous metal hydroxide precipitates. El Samrani et al., (2008) investigated the removal of heavy metal ions by coagulation of combined sewer overflow with two commercial coagulants, a ferric chloride and polyaluminium chloride (PAC). Coagulation is one of the most important methods for wastewater treatment, but the main objects of coagulation are only the hydrophobic colloids and suspended particles. In order to remove both soluble heavy metal and insoluble substances efficiently by coagulation, sodium xanthogenate group was grafted to polyethyleneimine. This new kind of coagulant was an amphoteric polyelectrolyte. When the pH of water sample is lower, the colloidal substances with negative charges can be coagulated by it, but the cationic Ni(II) ion cannot be removed very well. When the pH of water sample is higher, the turbidity removal decreases, and the Ni(II) removal increases. Flocculation is the action of polymers to form bridges between the flocs and bind the particles into large agglomerates or clumps. Once suspended particles are flocculated into larger particles, they can usually be removed or separated by filtration, straining or floatation. Today many kinds of flocculants, such as PAC, polyferric sulfate (PFS) and polyacrylamide (PAM), are widely used in the treatment of wastewater, however, it is nearly impracticable to remove heavy metal very well from wastewater directly by these current flocculants. Macromolecule heavy metal flocculant is a new kind of flocculant. Chang, Zhang, & Wang, (2009) prepared a macromolecule heavy metal flocculant mercaptoacetyl chitosan by reacting chitosan with mercaptoacetic acid. They reported that this new flocculant could not only remove turbidity, but also remove heavy metals in wastewater.

BIOSORPTION

Biosorption can be defined as the removal of metal or metalloid species, compounds and particulates from solution by biological material (Gadd, 1993). Large quantities of metals can be accumulated by a variety of processes dependent and independent on metabolism. Both living and dead biomass as well as cellular products such as polysaccharides can be used for metal removal. Heavy metal pollution is one of the most important environmental problems today. Various industries produce and discharge wastes containing different heavy metals into the environment, such as mining and smelting of metalliferous, surface finishing industry, energy and fuel production, fertilizer and pesticide industry and application, metallurgy, iron and steel, electroplating, electrolysis, electro-osmosis, leatherworking, photography, electric appliance manufacturing, metal surface treating, aerospace and atomic energy installation etc. Thus, metal as a kind of resource is becoming shortage and also brings about serious environmental pollution, threatening human health and ecosystem. Three kinds of heavy metals are of concern, including toxic metals (such as Cr(VI), Cu(II), Ni(II), Cd(II), etc.), precious metals (such as Pd, Pt, Ag, Au, Ru etc.) and radionuclides (such as U, Th, Ra, Am, etc.) as reported by Wang & Chen, (2006). Methods for removing metal ions from aqueous solution mainly consist of physical, chemical and biological technologies. Conventional methods for removing metal ions from aqueous solution have been suggested, such

as chemical precipitation, filtration, ion exchange, electrochemical treatment, membrane technologies, adsorption on activated carbon, evaporation etc. However, chemical precipitation and electrochemical treatment are ineffective, especially when metal ion concentration in aqueous solution is between 1 and 100 mg/L and also produce large quantity of sludge which is difficult to treat. Ion exchange process, membrane technologies and activated carbon adsorption process are extremely expensive when large amount of water is being treated and also when wastewater containing heavy metals in low concentration is treated. They cannot be used for large scale wastewater treatment.

In recent years, applying biotechnology (like biosorption process) in controlling and removing metal pollution has been paid much attention, and gradually became important in the field of metal pollution control because of its potential applications. Biosorption process utilizes various natural materials of biological origin, including bacteria, fungi, yeast, algae, etc for the removal of heavy metal ions from wastewater. These biosorbents possess metal-sequestering property and can be used to decrease the concentration of heavy metal ions in solution from ppm to ppb level. It can effectively sequester dissolved metal ions out of dilute complex solutions with high efficiency; therefore, it is an ideal process for the treatment of high volume and low concentration complex wastewaters (Wang & Chen, 2006). The capability of some living microorganisms to accumulate metallic elements has been observed at first from toxicological point of view (Volesky, 1990). The first major challenge for the biosorption field was to select the most promising types of biomass from an extremely large pool of readily available and inexpensive biomaterials (Kratochvil & Volesky, 1998). Although many biological materials can bind heavy metals, only those with sufficiently high metal-binding capacity and selectivity for heavy metals are suitable for use in a full-scale biosorption process. A large number of biomass types have been investigated for their metal binding capability under various conditions. Volesky & Holan, (1995) have presented an exhaustive list of microbes and their metal-binding capacities. The published work on testing and evaluating the performance of biosorbents offered a good basis for new and potentially feasible metal biosorbents. Öztürk, (2007) reported Nickel [Ni(II)] biosorption capacity (mg/g) of 45.90 using *Bacillus thuringiensis.* Selatnia, Bakhti, Madani, Kertous, & Mansouri, (2004) reported the Chromium [Cr(VI)] biosorption capacity (mg/g) of 32.60 Nickel [Ni(II)] biosorption capacity (mg/g) of 32.60 and the Cadmium [Cd(II)] biosorption capacity (mg/g) of 64.90 using *Streptomyces rimosus.* Lu, Shi, Wang, & Chang, (2006) reported the Copper [Cu(II)] biosorption capacity (mg/g) of 32.50 and Cadmium [Cd(II)] biosorption capacity (mg/g) of 46.20 using *Entero bacter sp.* Pagnanelli, Toro, & Veglio, (2002) have carried out a preliminary study on the use of olive mill residues as heavy metal sorbent material. The results revealed that copper was maximally adsorbed in the range of 5.0 to 13.5 mg/g under different operating conditions.

BIOSORBENTS

Fungal Biosorbents

Although fungi are a large and diverse group of eukaryotic microorganisms, three groups of fungi have major practical importance: the molds, yeast and mushrooms. Filamentous fungi and yeast have been observed in many instances to bind metallic elements. Fungi are ubiquitous in natural environments and important in industrial processes. A range of morphologies are found, from unicellular yeast to polymorphic and filamentous fungi, many of which have complex macroscopic fruiting bodies. Their

most important roles are as decomposers of organic materials, with concomitant nutrients cycling, as pathogens and symbionts of animals and plants, and as spoilage organisms of natural and synthetic materials, e.g. wood, paint, leather, food and fabrics. They are also utilized as producers of economically important substances, e.g. ethanol, citric acid, antibiotics, polysaccharides, enzymes and vitamins (Gadd, 1993). The importance of metallic ions to fungal and yeast metabolism has been known for a long time (Gadd, 1993). The presence of heavy metals affects the metabolic activities of fungal and yeast cultures and can affect commercial fermentation processes, which created interest in relating the behavior of fungi to the presence of heavy metals. The results from such studies led to a concept of using fungi and yeast for the removal of toxic metals (such as lead and cadmium) from wastewater and recovery of precious metals (such as gold and silver) from process waters (Kapoor & Viraraghavan, 1997). Both living and dead fungal cells possess a remarkable ability for taking up toxic and precious metals. In the field of biosorption, the molds and yeast are of interests and many investigations have been reported and reviewed. The molds are filamentous fungi. The yeasts are unicellular fungi and most of them are classified with the Ascomycetes. The most important commercial yeasts are the baker's and brewer's yeast, which are members of the genus *Saccharomyces*. The original habitats of yeast were undoubtedly fruits and fruit juices, but the commercial yeast of today are probably quite different from wild strains because they have been greatly improved through the years by careful selection and genetic manipulation of eukaryotic cells and they are thus excellent models for the study of many important problems in eukaryotic biology. Yeast cells are much larger than bacterial cells and can be distinguished microscopically from bacteria by their size and by the presence of internal cell structures, such as the nucleus (Madigan, Martinko, Parker, & Brock, 1997). Fungi and yeast are easy to grow, produce high yields of biomass and can be manipulated genetically and morphologically. The fungal organisms are widely used in a variety of large-scale industrial fermentation processes. For example, strains of *Aspergillus* are used in the production of ferrichrome, kojic acid, gallic acid, itaconic acid, citric acid and enzymes like amylases, glucose isomerase, pectinase, lipases and glucanases; while, *S. cerevisiae* is used in the food and beverage industries. The biomass can be cheaply and easily procured in substantial quantities. It is also a by-product from the established industrial fermentation processes. The use of biomass as an adsorbent for heavy-metal pollution control can generate revenue for industries and at the same time it eases the burden of disposal costs associated with the waste biomass produced. Alternatively, the biomass can also be grown using unsophisticated fermentation techniques and inexpensive growth media (Kapoor & Viraraghavan, 1995). It is not a priority from the economical point of view to use the waste biomass, but the fungal cultures are also amenable to genetic and morpholocial manipulations which may result in better raw biosorbents material (Volesky, 1990). Table 2 summarizes some of the important results of metal biosorption using fungal biomasses.

Marine Algae as Biosorbents

Algae are of special interest for use as biosorbents due to their high sorption capacity and their availability in practically unlimited quantities in the seas and oceans (Rincon, Gonzalez, Ballester, Blazquez, & Munoz, 2005). However, there are few publications on biosorption with algae as compared to those using other biomass materials (mainly fungi and bacteria). The topic is relatively novel, with exponential growth of interest throughout the scientific community in the last few years. According to the statistical review on biosorption, algae have been less used as biosorbent material than other kinds of biomass materials, especially fungi and bacteria (15.31% in the former case and 84.69% in the second) as reported by Romera,

Table 2. Biosorption by fungal biomass (mg/g) (Wang & Chen, 2009)

Metal Ion	Species of Fungi	References
Cd(II)	*Penicillium spp.* (living cells)	Kapoor & Viraraghavan (1997)
Cd(II)	*Aspergillus niger, Penicillium, Saccharomyces, Trichoderma, Rhizopus* (living cells)	Kapoor &Viraraghavan (1997)
Cr(VI)	*Aspergillus, Penicillium, Rhizopus, Saccharomyces, Trichoderma, Rhizopus* (living cells)	Kapoor &Viraraghavan (1997)

Gonzalez, Ballester, Blazquez, & Munoz, (2006). From the published literature, brown algae among the three groups of algae (red, green, brown algae) received the most attention. Higher uptake capacity has been found for brown algae than for red and green algae (Brinza, Dring, & Gavrilescu, 2007). The reason seems to be that they offer better sorption than red or green algae (Romera et al., 2006). Davis et al., (2003) have published a review on the biochemistry of heavy metal biosorption by brown algae. Brinza et al., (2007) reviewed some marine micro and macro algal species as biosorbents for heavy metal ions. The microalgae mentioned in the review include *Chlamydomonas reinhardtii, Chlorella salina, Chlorella sorokiniana, Chlorella vulgaris, Chlorella miniata, Chlorococcum sp, Cyclotella cryptica, Lyngbya taylorii, Phaeodactylum tricornutum, Porphyridium purpureum, Scenedesmus abundans, Scenedesmus quadricauda, Scenedesmus subspicatus, Spirogyra sp., Spirulina platensis, Stichococcus bacillaris and Stigeoclonium tenue*. Chojnacka, Chojnacki, & Górecka, (2005) reported the biosorption performance of Cr(III), Cd(II) and Cu(II) ions by blue–green algae namely *Spirulina sp.* Nayak, Lahiri, Mukhopadhyay, & Pal, (2003) studied the biosorption of heavy metals and toxic radionuclides by three genera of algae from different taxonomic groups. Thirty freeze-dried strains of algae were examined by Klimmek, Stan, Wilke, Bunke, & Buchholz, (2001) to adsorb cadmium, lead, nickel, and zinc from aqueous solution. They performed screening batch adsorption experiments with same initial concentration of each metal ion. The initial concentrations where the surface of *C. vulgaris* was saturated with the particular metal were selected for the screening. The maximum biosorption capacity (q_{max}) according to the Langmuir model was used to screen these 30 algae. A wide range of the values of q_{max} between the different strains of algae and between the four metals can be observed. The results were shown in Table 3, which offered the q_{max} for 30 algal strains (Klimmek et al., 2001).

Zhou, Huang, & Lin, (1998) investigated the sorption and desorption of Cu(II) and Cd(II) by macroalgae and microalgae. The experimental results showed that *S. platensis* had the highest capacity for Cd(II), followed by *Nannochloropsis oculata*, *P. tricornutum*, *Platymonas cordifolia* and *Chaetoceros minutissimus* among those five microalgae tested for the same metal ion. Lee, Park,Yang, Jeong, & Rhee,(2000) investigated the screening of Cr(VI) biosorbent from marine algae. They compared chromate adsorption capacity of 48 species of red, brown, or green marine algae from the east coast of Korea. A red marine algae, *Pachymeniopsis sp*. was identified as a highly chromate-selective biosorbent with high adsorption capacity of 225 mg/g. The metal uptake capacity by biosorbent in the mixed metal ion solution system decreased greatly in comparison with single component metal uptake system (Lee, Lim, & Kam, 2002). Batch equilibrium sorption experiments were used for screening the cost-effective marine algal biomass harvested from the Gulf of Persian.

Table 3. The maximum biosorption capacity (q_{max}) for algal strains (unit: mmol/g) (Klimmek et al., 2001)

Biomass	Cd (II)	Ni (II)
S. Hofmani	0.33	0.17
L. Taylorii	0.32	0.43
A. Densus	0.24	0.26
K. Spiculiformis	0.34	0.28
V. Dichotoma	0.28	0.37
C. Kessleri	0.24	0.12
M. Species	0.25	0.2
N. Parmeloides	0.23	0.22
S. Maxima	0.27	0.12
C. Vulgaris	0.29	0.31
G. Longicauda	0.27	0.2
R. Spiculiforme	0.25	0.26
A. Hantzschii	0.27	0.25
S. Platensis	0.29	0.4
P. Tricornutum	0.23	0.19
M. Aeroginosa	0.23	0.21
P. Purpureum	0.18	0.2
T. Species	0.13	0.26
G. Verrucosa	0.15	0.13
C. Species	0.2	0.17
A. Cylindrica	0.14	0.14
S. Laxissima	0.22	0.13
G. Planctonica	0.06	0.11
S. Species	0.24	0.09
P. Species	0.17	0.18
A. Africanum	0.17	0.15
E. Magnus	0.09	0.12

Bacterial Biosorbents

Bacteria are the most abundant and versatile microorganisms and constitute a significant fraction of the entire living terrestrial biomass of ~10^{18} g. In early 1980's, some microorganisms were found to accumulate metallic elements with high capacity (Vijayaraghavan & Yun, 2008). Bacteria were used as biosorbents because of their small size, their ubiquity, their ability to grow under controlled conditions, and their resilience to a wide range of environmental situations (Urrutia, 1997). Bacteria species such as *Bacillus, Pseudomonas, Streptomyces, Escherichia, Micrococcus*, etc, have been tested for uptake of metals or organics. Table 4 summarizes some of the important results of metal biosorption using bacterial biomasses, according to some published references (Vijayaraghavan & Yun, 2008).

Table 4. Bacterial biomass used for metal ions removal

Metal Ion	Bacterial Species	Biosorption Capacity (mg/g)	References
Ni(II)	*Bacillus thuringiensis*	45.9	Öztürk (2007)
Ni(II)	*Streptomyces rimosus*	32.6	Selatnia et al., (2004)
Cd(II)	*Staphylococcus xylosus*	250.00	Ziagova et al., (2007)
Cd(II)	*Streptomyces rimosus*	64.9	Selatnia et al., (2004)
Cd(II)	*Enterobacter sp.*	46.2	Lu et al., (2006)

Bacteria may either possess the capacity for biosorption of many elements or, alternatively, depending on the species, may be element specific. It is likely that, in the future, microorganisms will be tailored for a specific element or a group of elements, using recombinant DNA technology which is based on genetic modification using endorestrictive nucleases.

BIOSORBENT IMMOBILIZATION FOR BIOREACTORS AND REGENERATION

The cost of biosorbent preparation must also be taken into account. Although cell entrapment imparts mechanical strength and resistance to chemical and microbial degradation upon the biosorbents, the costs of immobilizing agent cannot be ignored. Free cells are not suitable for use in a column in that due to their low density and size they tend to plug the bed, resulting in large drops in pressure. Support matrices suitable for biomass immobilization include agar, alginate, polyacrylamide, polyvinyl alcohol, polysulfone, silica gel, cellulose and glutaraldehyde (Wang & Chen, 2006). For industrial application of biosorption, it is important to utilize an appropriate immobilization technique to prepare commercial biosorbents which retain the ability of microbial biomass to adsorb metals during the continuous treatment process. The free microbial cells generally are basically small particles, with low density, poor mechanical strength and little rigidity, which may come up with the solid–liquid separation problems, possible biomass swelling, inability to regenerate/reuse and development of high pressure drop in the column mode in real application. Excessive hydrostatic pressures are required to generate suitable flow rates in a fixed or expanded bed reactor. High pressures can cause disintegration of free biomass. These problems can be avoided by the use of immobilized cell systems. The immobilization of the biomass in solid structures would create a biosorbent material with the right size, mechanical strength, rigidity and porosity necessary for use in practical processes. The immobilized materials can be used in a manner similar to ion exchange resins and activated carbons such as adsorption-desorption cycles (recovery of the adsorbed metal, reactivated and re-use of the biomass) as reported by Veglio & Beolchini, (1997). Wang & Chen, (2006) introduces the microbial immobilization techniques in a monograph. Immobilization technique is one of the key elements for the practical application of biosorption, especially by dead biomass. Various kinds of immobilized *S. cerevisiae* have been studied with different support materials, which can be used in practical biosorption (Veglio & Beolchini, 1997). A number of matrices have been employed for immobilization of cells. Important immobilization matrices used in biosorbent immobilization include materials like agar, sodium or calcium alginate, polysulfone, polyacrylamide, polyurethane, silica (Vijayaraghavan & Yun, 2008). The polymeric matrix determines the mechanical strength and chemical resistance of the final biosorbent particle to be utilized for successive sorption–

desorption cycles. Hence it is very important to choose the immobilization matrix. The immobilized *P. maltophilia* cells can be used repeatedly in biosorption–desorption cycles (Tsuruta, 2004). Basidiospores of *P. chryosporium* were immobilized into Ca-alginate beads via entrapment for the removal of Cd(II) ions from aqueous solution in the concentration range of 30-500 mg/L. The alginate-fungus beads could be regenerated using 10 mM HCl, up to 97% recovery.

The entire biosorption process for metal removal include sorption followed by desorption, i.e., to concentrate the solute. Biotechnological exploitation of biosorption technology for removal of heavy metal ions depends on the efficiency of the regeneration of biosorbent after metal desorption. Therefore non-destructive recovery by mild and cheap desorbing agents is desirable for regeneration of biomass and reuse in multiple cycles. Appropriate eluants are necessary to attain the above-mentioned objective, which strongly depends on the type of biosorbent and the mechanism of biosorption. Also, the eluant must meet the following requirements: (i) should be non-damaging to the biomass, (ii) should be less costly, (iii) should be environmental friendly and (iv) should be effective for biosorption (Vijayaraghavan &Yun, 2008). Acidic and alkaline conditions were used for desorption. The eluants such as $CaCl_2$ with HCl, HCl with EDTA and NaOH were reported (Vijayaraghavan & Yun, 2008). Desorption data showed that nearly 99% of the Cr(VI) adsorbed on *Mucor hiemalis* could be desorbed using 0.1 N NaOH. Although continuous process for metal removal by immobilized cells has been realized at lab scale, there is still long way to go for biosorption commercialization. Selection of good and cheap support materials for biosorbent immobilization is important for the improvement of reuse methods and enhancement of properties of immobilized biosorbents such as mechanical intensity and chemical stability. However, immobilization of biosorbents will probably bring about at least two practical problems: mass transfer limitation and additional process cost (Vijayaraghavan &Yun, 2008). The industrial application of biosorption with immobilized dead cells have been performed for some pilot plants of biosorption, but the cost for preparation of the required biosorbents with waste biomass was too expensive by immobilization techniques and by various pre-treatment processes. Process of regeneration and re-use is complex and expensive. The co-existed ions and organic compounds in solution made matters even more difficult and more complex for real effluents (Wang & Chen, 2006). When developing the immobilization and regeneration technology for biosorption application, the above-mentioned problems should be taken into account.

ISOTHERMS AND KINETIC MODELS OF BIOSORPTION

Assessment of a solid–liquid sorption system is usually based on two types of investigations: equilibrium batch sorption tests and continuous-flow sorption studies (Volesky & Holan, 1995). Yu & Neretnieks (1990) reviewed the model isotherms for single component adsorption. Some authors gave a description of the modeling of biosorption in detail (Aksu, 2005; Kratochvil & Volesky, 1998; Pagnanelli et al., 2002; Veglio & Beolchini, 1997). Aksu, (2005) described the kinetic modeling for biosorption in a continuous system. Equilibrium isotherm models are usually classified into the empirical equations and the mechanistic models. The mechanistic models are based on mechanism of metal ion biosorption, which are not only able to represent but also explain and predict the experimental behavior (Pagnanelli et al., 2002). The Langmuir model (L type, based on monolayer adsorption of solute) and the Freundlich model (F type, developed for heterogeneous surfaces) are the most widely accepted and used in literatures. The BET model describes the multi-layer adsorption at the adsorbent surface and assumes

that the Langmuir isotherm applies to each layer. These models can provide information of metal ion uptake capacity and difference in metal uptake between various species (Kapoor & Viraraghavan 1995; Pagnanelli et al., 2002; Volesky & Holan, 1995).

These empirical models do not reflect any mechanisms of sorbate uptake and hardly have a meaningful physical interpretation for biosorption. Volesky & Holan (1995) pointed out that the results from the empirical models cannot be extrapolated, and no predictive conclusions can be drawn for systems operating under different conditions. Both the basic models (the Langmuir model and the Freundlich model) also do not incorporate the effects of any external variable environmental factors, although they are capable of describing many biosorption isotherms in many cases. The mechanistic conclusions from the good fit of the models alone should be avoided. Moreover, biosorption isotherms may exhibit an irregular pattern due to the complex nature of both the biosorbents and its varied multiple active sites, as well as the complex solution chemistry of some metallic compounds (Kapoor & Viraraghavan, 1995; Volesky & Holan, 1995). To describe two- or multi-metal ions biosorption system, various extended Langmuir models (also called competitive Langmuir model) or Freundlich type models have been developed (Aksu, Açıkel, & Kutsal, 1997; Pagnanelli et al., 2002). These empirical models hardly reflect the sorption mechanism. Kinetic models based on the capacity of the adsorbent have also been presented, such as the Lagergren's first-order equation and Ho's second-order expression (Ho, 2006). The first-order equation and the pseudo second-order equation are the most widely used kinetic models to describe the biosorption process. The pseudo second-order equation fitted the data very well in a large quantity of literature for biosorption (Ho & McKay, 1999). Ho, (2006) gave a review on the application of second-order kinetic models to adsorption systems including an earlier adsorption rate equation based on the solid capacity for a system of liquids and solids. Kinetic studies and dynamic continuous-flow investigations, offering information on the rate of the sorption metal uptake, together with the hydrodynamic parameters, are very important for biosorption process design (Volesky & Holan, 1995).

FACTORS AFFECTING BIOSORPTION PROCESS

pH

In metal biosorption, pH is one of the most important environmental factors. The pH value of a solution strongly influences not only the site dissociation of the biomass surface, but also the solution chemistry of the heavy metals, including hydrolysis, complexation by organic and/or inorganic ligands, redox reactions, precipitation, speciation and biosorption availability of the heavy metals (Wang & Chen, 2006). The functional groups on the biomass such as carboxyl and phosphate are almost always acidic and pH dependent. At a high pH, they can be dissociated and become available for metal ion uptake. In general, the biosorption capacity of metal adsorption increases as pH increases. The sorption of Cu(II) by *Dunaliella tertiolecta* was small at a pH level below 4.0, but increased significantly with a rise in pH in the range of 4.0-5.0 and leveled off when pH > 5.0 (Gonzalez-Davila, Santana-Casiano, Perez-Pena, & Millero,1995). As for metal anions, the situation was opposite to that of cation biosorption and a low pH value was favorable for biosorption (Wang & Chen, 2006). In acidic environments, protonation of the functional groups such as carboxyl and amine groups could result in an overall positive charge on the biomass to adsorb the negatively charged heavy metal ions.

Initial Concentrations of Metal Ion and Biomass

Biosorption for the removal of metal ions depends on the initial concentrations of metals and biomass. In general, biosorption capacity increases proportionally with the increase of metal concentrations in the solution, and levels off at a certain metal concentration due to the saturation of the binding sites on the biomass (Yun, Park, Park, & Volesky, 2001). Mehta & Gaur, (2001) found that 70 and 80% of Ni(II) and Cu(II) at a concentration of 2.5 mg/L could be removed by *Chlorella vulgaris*, respectively, while only 37 and 42% of Ni(II) and Cu(II) were removed if metal ion concentration was 10 mg/L, respectively. In general, the removal percentages of metal ion increase with increasing biomass concentrations, while, biosorption capacity of the metal is inversely proportional to the concentration of the biomass when the initial concentration of metal ions is kept constant. Hamdy, (2000) reported that the biosorption capacity of Cr(III), Co(II), Ni(II), Cu(II) and Cd(II) by four different algae decreased with increasing biomass concentrations. Malik et al., (2005) also suggested that the distribution ratio of Cr(III) sorbed on the biomass to the residual Cr(III) concentration decreased with the increase of biomass concentrations. This could possibly be attributed to the limited availability of metal ions, increased electrostatic interactions, interference between the binding sites and reduced mixing at higher biomass concentrations (Mehta & Gaur, 2005). Vasudevan, Padmavathy, & Dhingra, (2003) reported that the biosorption capacity of Cd(II) ion was directly proportional to the initial metal ion concentration and inversely proportional to the biosorbent concentration.

Temperature

Temperature can affect metal biosorption to some extent but different results have been reported. Malik et al., (2005) found that Cr(III) sorption on the sunflower stem increased with the increase of temperature ranging from 10 to 50°C. Park, Yun, & Park, (2004) also found that temperature enhanced the rate of reduction of Cr(VI) to Cr(III) by seaweed *Ecklonia* biomass. The increase of metal biosorption capacity at high temperatures indicated that metal biosorption in these cases was an endothermic process. Goyal, Jain, & Banerjee, (2003) suggested that higher temperature would lead to higher affinity of sites for metal or binding sites on the biomass. In some other studies, the exothermic process seemed to be dominant in metal biosorption. Cossich, Tavares, & Ravagnani, (2002) suggested that the effect of temperature was not as pronounced as that of pH. In application, it is not practicable for the biosorption process to operate at a high temperature, as this will increase the operational cost and deteriorate the structure of the biosorbents.

Contact Time

Contact time is one of the most significant operational parameters in heavy metals removal using bacterial biomass. The need for the investigation of the effect of contact time on the process effectiveness has arisen from the fact that interaction between heavy metal ion and biomass is a dynamic process and amount of metal uptake is a function of time. In the literature most of the research on the effect of contact time was conducted to determine the equilibration period for the interaction.

Flow Rate

In column operations, any volume element of the solution is in contact with a given layer of the bed for only a limited period of time, which is usually insufficient for attainment of equilibrium. Thus the failure of attaining local equilibrium results in a lower uptake of cations from the feed solution (Inglezakis & Grigoropoulou, 2003). Inglezakis, Hadjiandreou, Loizidou, & Grigoropoulou, (2004) studied the ability of natural and modified Northern Greece clinoptilolite for the treatment of solutions containing Pb(II), Cu(II), Cr(III) and Fe(III) in continuous mode at flow rates of 5-15 BV/h (Bed Volume/hour) and concluded that 5 BV/h revealed the best performance among the studied flow velocities.

NICKEL [Ni(II)]

Nickel[Ni(II)] is found in plating, metal pickling and metal cleaning wastewater. The Ni(II) from the plating wastewater can be chelated as nickel sulfamate from sulfamate nickel plating, or nickel lactate from electrolysis nickel plating. Cleaning and stripping solutions of Ni(II) often have nickel cyanide and nickel EDTA complex. Nickel[Ni(II)] that is not complexed or chelated can be precipitated as nickel hydroxide by adjusting the pH to 10.5 or higher. Precipitating the nickel as a carbonate is not effective due to the high solubility of nickel carbonate. The precipitation of nickel phosphate is effective for lightly complexed nickel using a two-step process described in the copper section. To precipitate the it as the metal using ORP, a strong reducing agent, such as ferrous ion or sulfide ion, must be used at a pH greater than 10.5. Electroless Ni(II) solutions should be pretreated by raising the pH to 11.5 and allowing the nickel[Ni(II)] to plate out using the residual reducing agent, sodium hypophosphite, in the solution before continuing the treatment. The plating out or dropping out of electroless Ni(II) solutions releases significant quantities of hydrogen. It can be removed with cation resins and chelated anion resins. Low concentrations can be attained by using a reducing agent and sand filter or other media column to precipitate the nickel metal Ni(II) on the surface of the media. If Ni(II) concentrations below 1 μg/L are required, a three-step process of precipitation, oxidation and then final precipitation or adsorption will be required. Nickel and its compounds are naturally present in the Earth's crust, and release to the atmosphere occur from natural discharges such as windblown dust and volcanic eruptions, as well as from anthropogenic activities. It is estimated that 8.5 million kg of nickel are emitted into the atmosphere from natural sources such as windblown dust, volcanoes, and vegetation each year (Bennett, 1984).

Nickel [Ni(II)] in the Environment

The burning of residual and fuel oil is responsible for 62% of anthropogenic emissions, followed by nickel metal refining, municipal incineration, steel production, other nickel alloy production, and coal combustion (Bennett, 1984). The form of nickel emitted to the atmosphere varies according to the type of source. Nickel species associated with combustion, incineration, and metal smelting and refining are often complex nickel oxides, nickel sulfate, and metallic nickel, and in more specialized industries, the species commonly found are nickel silicate, and nickel chloride. Uncontaminated freshwater and seawater generally contain about 0.30 μg/L of nickel (Barceloux & Barceloux, 1999). Concentrations of nickel in drinking water commonly range from 0.55 to 25 μg/L and average between 2 and 4.3 μg/L. The concentration of nickel in rain has been reported as ≤1.5 μg/L. The speciation and physicochemical

state of nickel is important in considering its behavior in the environment and availability. For example, the nickel incorporated in some mineral lattices may be inert and have no ecological significance. Most analytical methods for nickel do not distinguish the form of nickel; the total amount of nickel[Ni(II)] is reported, but the nature of the nickel compounds and whether they are adsorbed to other material is not known. This information, which is critical in determining nickel's liability and availability, is site specific. Therefore, it is impossible to predict nickel's environmental behavior on a general basis. Most analytical methods for nickel in environmental samples do not distinguish between compounds of nickel or the nature of its binding to soil and particulate matter. It is generally impossible to say with certainty what forms of nickel are released from natural and anthropogenic sources, what forms are deposited or occur in environmental samples, and to what forms of nickel people are exposed. The form of nickel has important consequences as far as its transport, transformations, and bioavailability are concerned. Nickel[Ni(II)] is a natural constituent of soil and is transported into streams and waterways in runoff either from natural weathering or from disturbed soil. Much of this nickel is associated with particulate matter. Nickel also enters bodies of water through atmospheric deposition.

Toxicity of Nickel [Ni(II)]

It is an important environmental inorganic pollutant, with allowed levels under 0.04 mg/L in human consumption water. Higher concentrations affect normal flora in ecosystems and are toxic for human beings. It is considered to be relatively nontoxic to man and a limit for nickel[Ni(II)] is not included in the EPA national interim primary drinking water regulations. The toxicity of nickel to aquatic life indicates tolerances that vary widely and that are influenced by species, pH, synergistic effects, and other factors. Nickel[Ni(II)] is a silver-white metallic element seldom occurring in nature in the elemental form. Nickel salts are soluble and can occur as a leachate from nickel-bearing ores. Nickel salts are used in metal-plating and may be discharged to surface or ground water. The organs which are affected by exposure to nickel metal and soluble compounds are nasal cavities, lung and skin. The toxicity to human beings of nickel or nickel salts through oral intake is low. Nickel salts exert their action mainly by gastrointestinal irritation and not by inherent toxicity. There is sufficient evidence in human beings for the carcinogenicity of nickel sulfate, and of the combinations of nickel sulfides and oxides encountered in the nickel refining industry. There is inadequate evidence in humans for the carcinogenicity of metallic nickel and nickel alloys. There is sufficient evidence in experimental animals for the carcinogenicity of metallic nickel, nickel monoxides, nickel hydroxides and crystalline nickel sulfides. There is inadequate evidence in experimental animals for the carcinogenicity of nickel trioxide, amorphous nickel sulfide and nickel titanate.

Biosorption of Nickel [Ni(II)]

Conventional chemical methods for heavy metal removal from wastewater (precipitation, filtration, ion-exchange, reduction-oxidation) are expensive and ineffective, particularly when metal ion concentration is low (Cañizares Villanueva, 2000). Thus, biotechnological methods such as biosorption are emerging as an interesting alternative. Since cells are metabolically inactive in non-viable biomass systems, metal interactions occur at the superficial level (Yetis & Çeribasi, 2001). Bacteria express a wide range of complex molecules on their cell wall, which confer anionic net charge to the cell surface at acidic pH values. In Gram negative bacteria, the lipopolysaccharide, a highly anionic structure, has been identified as the main binding site for metals.

CADMIUM [Cd(II)]

It is found in aerospace plating shop wastewater. Cadmium is used almost exclusively on aerospace hardware such as landing gear components. It can also be found in very low concentrations in rinse waters from solder cleaning operations where it is a contaminant in the solder. Cd(II) is usually regulated to concentrations of less than 0.1 mg/L. It cannot be precipitated to the regulatory levels by pH adjustment alone. However, the precipitates of cadmium carbonate, cadmium phosphate and cadmium sulfide are all very insoluble. It can also be removed very effectively in chelating ion exchange resins. However, it cannot be removed by reduction, since its oxidation potential is too high. In plating rinse water, the Cd(II) is usually complexed with cyanide and the cyanide must be oxidized before treatment. The equipment used for precipitation includes batch systems for concentrated waste, continuous precipitation for waste of dilute concentrations. When precipitation is used, the two step process must be used. Precipitation of cadmium carbonate, phosphate or sulfide is followed by the precipitation of the carbonate, phosphate or sulfide by the addition of calcium or iron. Cadmium[Cd(II)] occurs naturally in the environment by the gradual process of erosion and abrasion of rocks and soils, and from singular events such as forest fires and volcanic eruptions. It is therefore naturally present everywhere in air, water, soils and foodstuffs. The best known cadmium mineral is greenockite, cadmium sulfide (77.6% Cd(II)). It is one of the heavy metals, which is highly toxic to human, plants and animals. The metal is of special concern because it is non-degradable and therefore persistent. The main anthropogenic pathway through which cadmium enters environment is via wastes from industrial processes such as electroplating, smelting, alloy manufacturing, pigments, plastic, cadmium-nickel batteries, fertilizers, pesticides, mining, pigments and dyes, textile operations and refining (Wu et al, 2010). Various regulatory bodies have set the maximum limits for the discharge of toxic heavy metals in the aquatic systems. However, the metal ions that are being added to the water stream are at a much higher concentration than the prescribed limits by industrial activities, thus leading to the health hazards and environmental degradation. In order to solve heavy metal pollution in the environment, it is important to bring applicable solutions. Thus, the treatment or purification of contaminated water and effluents is one of the major areas of active research. According to WHO's recommendation Cd(II) limit in drinking water is 0.005 mg/L.

Cadmium [Cd(II)] in the Environment

It is a minor constituent of surface and groundwater. It may exist in water as the hydrated ion, as inorganic complexes such as carbonates, hydroxides, chlorides or sulphates, or as organic complexes with humic acids. Cd(II) may enter aquatic systems through weathering and erosion of soils and bedrock, atmospheric deposition direct discharge from industrial operations, leakage from landfalls and contaminated sites, and the dispersive use of sludge and fertilizers in agriculture. Much of the cadmium entering fresh waters from industrial sources may be rapidly adsorbed by particulate matter, and thus sediment may be a significant sink for cadmium[Cd(II)] emitted to the aquatic environment. Some data shows that recent sediments in lakes and streams range from 0.2 to 0.9 ppm (Cook &Morrow, 1995). Partitioning of Cd(II) between the adsorbed-in-sediment state and dissolved-in-water state is therefore an important factor in whether cadmium emitted to waters is or is not available to enter the food chain and affect human health. Rivers containing excess cadmium can contaminate surrounding land, either through irrigation for agricultural purposes, dumping of dredged sediments or flooding. It has also been demonstrated that rivers can transport cadmium for considerable distances, up to 50 km, from the source.

Nonetheless, studies of cadmium[Cd(II)] contamination in major river systems over the past twenty to thirty years have conclusively demonstrated that cadmium[Cd(II)] levels in these rivers have decreased significantly (Cook & Morrow, 1995).

Toxicity of Cadmium[Cd(II)]

It is the most toxic and common among the heavy metal ion pollutants of industrial effluents. It is introduced into the bodies of water from smelting, metal plating, cadmium-nickel batteries, phosphate fertilizer, mining, pigments, stabilizers, alloy industries and sewage sludge. Discharges containing cadmium[Cd(II)], in particular, are strictly controlled due to the highly toxic nature of this element and its tendency to accumulate in the tissues of living organisms (Dianati-Tilaki, Mahvi, Shariat, & Nasseri, 2004). The harmful effects of Cd(II) include a number of acute and chronic disorders, such as *itai-itai* disease, renal damage, emphysema, hypertension, and testicular atrophy (Leyva-Ramos, Rangel-Mendez, Mendoza-Barron, Fuentes-Rubio, & Guerrero-Coronado,1997). Conventional techniques for the removal of heavy metals from wastewater, such as chemical precipitation, ion exchange, activated carbon adsorption and separation processes have limitations and become inefficient and expensive especially when the heavy metal concentration is less than 100 ppm (Yan & Viraraghavan, 2001). When Cd(II) entered into soil, it can be taken up by plants and microbes and then enter into the food chain (Bingham, Page, Mitchell, & Strong,1979).

Biosorption of Cadmium[Cd(II)]

The biological materials that have been investigated for cadmium uptake by bacteria (Chang, Law, & Chang,1997). The maximum cadmium[Cd(II)] biosorption by these materials ranges from 30.35 mg/g fungus to 100 mg/g micro-algae. Waste biomass *Sargassum sp*. (Esteves, Valdman, & Leite, 2000) was reported to remove 100% of Cd(II) from 98 mg/L solution at pH of 4.5. Desorption studies were also reported using $CaCl_2$ solution. They found that ten of the tested materials showed a higher adsorption capacity than that of activated charcoal and ion-exchange resin. Due to high affinity of the adsorbent for the metal ions, the latter is attracted and bound by rather complex process affected by several mechanisms involving chemisorption, complexation, adsorption on surface and pores, ion exchange, chelation, adsorption by physical forces, entrapment in inter and intrafibrillar capillaries and spaces of the structural polysaccharides network as a result of the concentration gradient and diffusion through cell wall and membrane (Basso, Cerrella, & Cukierman, 2002). Different microorganism-derived materials have also been used as biosorbents (Sari & Tuzen, 2008).

BIOREACTORS FOR HEAVY METAL REMOVAL FROM WASTE WATER

Treatments and processes used today in purifying wastewater are expensive and may themselves produce toxic waste (metal hydroxide sludge) as reported by Crusberg, Weathers, & Baker, (2009). Designing a system, including bioreactor configuration and favorable conditions for the elimination of heavy metals from aqueous solutions could provide a possible solution for reducing wastewater contamination. Such a process could be used in wastewater treatment and in fields like bio-mining. There are numerous bioreac-

tors of varying configurations to meet different requirements, but there are two major groups of reactors - batch reactors and continuous reactors. Individual reactors can be classified in four different ways:

- Based on the combination of the mode of substrate addition and the reactor geometry (i.e. continuous tubular packed bed),
- Based on the state of the biomass in the reactor (freely suspended or immobilized),
- The mode of provision of mixing in the reactor (mechanical agitation or gas agitation) and based on the type of biocatalyst or enzyme used in the reactor (aerobic microorganisms or anaerobic microorganisms).

Sequential Batch Bioreactor

The Sequencing Batch Reactor (SBR) is an activated sludge process designed to operate under non-steady state conditions. An SBR operates in a true batch mode with aeration and sludge settlement both occurring in the same tank. The major differences between SBR and conventional continuous-flow, activated sludge system is that the SBR tank carries out the functions of equalization, aeration and sedimentation in a time sequence rather than in the conventional space sequence of continuous-flow systems. In addition, the SBR system can be designed with the ability to treat a wide range of influent volumes whereas the continuous system is based upon a fixed influent flow rate. Thus, there is a degree of flexibility associated with working in a time rather than in a space sequence (Norcross, 1992).

Membrane Bioreactor

Recent technical innovation and significant membrane cost reduction have pushed membrane bioreactors (MBRs) to become an established process option to treat wastewaters. The combination of membrane separation with a suspended growth bioreactor is now widely used for municipal and industrial waste treatment. However, membrane fouling remains a major drawback, limiting the wider application of this process. In recent reviews covering membrane applications to bioreactors, it has been shown that, as with other membrane separation processes, membrane fouling is the most serious problem affecting system performance (Le-Clech, Chen, & Fane, 2006). Fouling leads to a significant increase in hydraulic resistance, manifested as permeate flux decline or transmembrane pressure (TMP) increase when the process is operated under constant-TMP or constant-flux conditions respectively. Frequent membrane cleaning and replacement is therefore required, increasing significantly the operating costs. Membrane fouling results from interaction between the membrane material and the components of the activated sludge liquor, which include biological flocs formed by a large range of living microorganisms along with soluble and colloidal compounds. The suspended biomass has no fixed composition and varies both with feed water composition and MBR operating conditions employed. Thus though many investigations of membrane fouling have been published, the diverse range of operating conditions and feed water matrices employed, and the limited information reported in most of the studies on the suspended biomass composition, has made it difficult to establish any generic behaviour pertaining to membrane fouling in MBRs specifically.

Fluidized Bed Bioreactor

Fluidized bed reactors have several different phases to achieve mixing and mass transfer in the reactor. These reactors are generally used in batch settings. There is normally a liquid phase and a gaseous phase, and a third solid phase within the reactor. The solid phase may be biomass or binding substrate, like glass beads, but may be self-contained if the biomass can survive the physical environment. The general idea is to create mixing by allowing gases to rise through the liquid. The target molecules adhere to the biomass or to the binding material and thus facilitating removal of heavy metals. The performance of the reactor is effected by gas velocity. At high gas velocities, the immobilized biomass gets disrupted due to increased mixing and affects the percent biosorption of selected heavy metal ions.

Packed Bed Bioreactor

Packed-bed column reactors are commonly used in both industry and research for separation of molecules, determination of relative molecular mass, and identification of substances or for purification purposes. These reactors can be used in both continuous and batch settings. Separation takes place in various columns using various characteristics of substrates. The packed-bed columns consist of the container and the bed contained within. The bed can be anything from glass to silicon or plastics beads. Recent application of the reactor is for wastewater treatment by biosorption of heavy metals using several biosorbents. The target molecule or ion is passed through the packed-bed and it gets attached to the assigned ligand or substrate. The final step, in most cases, is to release the target molecule through, for example, a salt gradient.

Increased stability and performance in anaerobic reactors can be achieved if the microbial consortium is retained in the reactor. Two means of achieving this are to use dense bacterial granula as in UASB (Upflow anaerobic sludge blanket) reactors or a microbial biofilm attached to inert carriers as in packed-bed reactors. The packing medium in the packed-bed reactor and the granular sludge in the UASB reactor serve as a filter preventing bacterial washout and also providing a larger surface area for faster biofilm development and improved methanogenesis (Picanco, Vallero, Gianotti, Zaiat, & Blundi, 2001). Specific surface area, porosity, surface roughness, pore size, and orientation of the packing material were found to play an important role in anaerobic reactor performance (Yang et al., 2004). Biofilm or fixed-film reactors depend on the natural tendency of mixed microbial populations to adsorb onto surfaces and to form a biofilm. In nature, microorganisms inhabit the outer and inner surfaces of stone, gravel or sand. This biofilm formation becomes an important factor for water self-cleaning ability. The growth of microorganism in a biofilm is the basis for biological water treatment such as denitrification and for intensification of aerobic and anaerobic wastewater treatment. The use of packed bed reactors to treat different kinds of wastewater has also been reported, for example, dairy and brewery wastewater. The biofilm formation on carrier materials improves the conversion rates by reducing its sensitivity toward concentration variations and inhibiting substance. Picanco et al., (2001) reported that the efficiency of removing organic matter in fixed-bed reactors is directly related to the characteristics of the support material used for immobilization of anaerobes. It is widely accepted that organic support material has a higher affinity than inorganic material. Reticular polyurethane foam has a high specific surface area which can reach 2400 m^2/m^3 and a porosity of 97%. Many kinds of bedding model have been considered for degrading a variety of organic wastes in anaerobic digestion reactors. A simple, low-cost and high efficiency bedding method is necessary for practical use. The development of fixed biomass reactors

has ensured that significant advances in the knowledge and application of anaerobic processes for waste treatment have taken place. Compared to conventional units, fixed film bioreactors perform efficiently at higher organic loading rates (OLR), due to more effective biomass retention in the reaction zone resulting in higher cellular retention times. Immobilized biomass anaerobic reactors also show better responses to organic shock loads and toxic inputs. In many cases, immobilized biomass reactors completely recover their performance after such deleterious occurrences (Caine, Anderson, & Donnelly, 1991).

DESIGN EXPERT SOFTWARE AND RESPONSE SURFACE METHODOLOGY (RSM)

Design Expert is a piece of software designed to help with the design and interpretation of multi factor experiments. The software offers a wide range of designs, including factorials, fractional factorials and composite designs. Design Expert offers computer generated D-optimal designs for cases where standard designs are not applicable, or where we wish to augment an existing design - for example, to fit a more flexible model. Models for categorical parameters often involve a set of terms to represent a single main effect or two-factor interaction, but the interpretation of the effects is similar. In constructing second level factorial designs, Design Expert allows one to add centre points, at which all continuous parameters are set in the middle of their ranges. The inclusion of centre points checks whether the fitted model is adequate, or whether there is a need to include some quadratic terms to take care of non-linearity. Design Expert offers a wide range of different plots to show how the response varies with changes in the parameters. RSM is a technique for designing experiments, building models, evaluate ng the effects of several factors and achieving the optimum conditions for desirable responses with a limited number of planned experiments. RSM helps to demonstrate how a particular response is affected by a given set of input variables over some specified region of interest, and what input values will yield a maximum (or minimum) for a specific response. RSM was initially developed for the purpose of determining optimum operating conditions in the chemical industry but it is now used in a variety of fields and applications, not only in the physical and engineering sciences, but also in biological, clinical, and social sciences (Clesceri, Greenberg, & Eaton, 1998). Response Surface Methodology (RSM) consists of a group of mathematical and statistical methods that can be used to define the relationships between the response and the independent variables. RSM defines the effect of the independent variables, alone or in combination. Rajasimman & Murugaiyan, (2010) carried out batch experiments to find the effect of various parameters such as pH, temperature, sorbent dosage, metal ion concentration and contact time on the sorption of Ni(II) using *hypnea valentiae*. Response surface methodology (RSM) was employed to optimize the process parameters. Based on the central composite design, quadratic model was developed to correlate the process variables to the response. The most influential factor on each experimental design response was identified from the analysis of variance (ANOVA). The optimum conditions for the sorption of Ni(II) were found to be: pH -5.1, temperature -36.8°C, sorbent dosage -5.1 g/L, metal ion concentration - 100 mg/L and contact time - 30 min. At these optimized conditions the maximum removal of Ni(II) was found to be 91.97%.

CONCLUSION

Twenty bacterial species have been isolated from heavy metal ion contaminated wastewater. These isolates have high ability to resist and accumulate one or more of the metal ion mixture Ni(II) and Cd(II). Among all isolated strains, one shown high ability to remove the selected heavy metal ions. The isolated bacterial strain was identified as *Pseudomonas putida* based on physical, morphological and biochemical tests. More than 85% and 80% of isolated pseudomonas species were found to be highly resistant to Ni(II) and Cd(II) heavy metal ions respectively. The present study showed that the maximum biosorption of Ni(II) and Cd(II) was by *Pseudomonas putida* at optimum conditions of contact time of 30 minutes, pH of 4.0, biomass concentration of 2 mg/mL, temperature of 32°C in batch biosorption studies and bed height of 20 cm, flow rate of 300 mL/h, initial metal ion concentration of 100 mg/L in packed bed bioreactor biosorption studies. In batch biosorption studies the effect of pH on the selected heavy metals was studied. Using *Pseudomonas putida*, the biosorption of Ni(II) and Cd(II) was78% and 76% respectively at pH of 4.0. The maximum biosorption for Ni(II) and Cd(II) was 79% and 76% respectively at the biomass concentration of 2.0 mg/mL. The maximum biosorption for Ni(II) and Cd(II) was 81% and 80% respectively at the temperature of 32°C The equilibrium time was 30 minutes for Ni(II) and Cd(II) at which percent biosorption was 79% and 78% respectively. The pseudo first order and second order parameters were calculated for the selected heavy metal ions. R^2 values for pseudo second order kinetics were found to be far higher compared to R^2 values of pseudo first order kinetics. This indicates that pseudo second order kinetics fits the data better compared to pseudo first order kinetics. The recovery of Ni(II) and Cd(II) in single metal ion system was almost 100% when acidic (HCl) solution at different concentrations was used as an eluent showing the involvement of ion-exchange mechanism for the process while, in multi-metal ion system the desorption in acidic medium was reduced to 44.75%. From the batch biosorption experimental work, it is seen that the optimum parameters for batch biosorption studies are pH equal to 4.0, biomass concentration of 2.0 mg/mL, temperature of 32°C and contact time of 30 minutes. Optimization results of Ni(II) and Cd(II) by *Pseudomonas putida* from the Design Expert software were obtained as pH equal to 3.98, biomass concentration of 2.08 mg/mL, temperature of 32.07°C and contact time of 30.24 minutes. The percent biosorption of Ni(II) and Cd(II) is 77.3% and 76.2% respectively. Batch biosorption experiments were carried out at established optimum conditions (using Response Surface Methodology) of contact time of 30.24 minutes, pH of 3.98, biomass concentration of 2.08 mg/mL and temperature of 32.07°C and results of biosorption of Ni(II) and Cd(II) were reproduced and they were in agreement with the predicted results. In packed bed biosorption studies, comparison of the breakthrough curves of Ni(II) and Cd(II) for Agar immobilized and PAA immobilized bacterial strains at optimum conditions of flow rate of 300 mL/h, initial metal ion concentration of 100 mg/L and bed height of 20 cm with weight of biosorbent of 12 g, it was found that the Agar immobilized *Pseudomonas putida* showed maximum percent biosorption and bed saturation occurred at 20 minutes. Optimization (using RSM) results of Ni(II) and Cd(II) by *Pseudomonas putida* from the Design Expert software were obtained as bed height of 19.93 cm, initial metal ion concentration of 103.85 mg/L, and flow rate of 310.57 mL/h. The percent biosorption of Ni(II) and Cd(II) is 87.2% and 88.2% respectively.

The predicted optimized parameters are in agreement with the experimental results. Experiments were carried out at established optimum conditions of bed height of 20.77 cm, flow rate of 309.09 mL/h, and initial metal ion concentration of 109.23 mg/L and results of biosorption of Ni(II) and Cd(II) were reproduced and they were in agreement with the predicted results. The R^2 value of Ni(II) and Cd(II) are 0.9924 and 0.9937 respectively. The adjusted R^2 value of Ni(II) and Cd(II) are 0.9856 and 0.9880 respec-

tively. The predicted R^2 value of Ni(II) and Cd(II) are 0.9437 and 0.9495 respectively. The predicted R^2 is in reasonable agreement with the adjusted R^2. Regression analysis was performed to fit the response functions, i.e. percentage biosorption of Ni(II) and Cd(II). On comparison between batch biosorption and packed bed bioreactor studies, it was noticed that the maximum biosorption yields were obtained in packed bed bioreactor at optimum conditions, the packed bed bioreactor would be a good choice for the removal of Ni(II) and Cd(II) from wastewater using the isolated and identified bacterial species.

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KEY TERMS AND DEFINITIONS

Bioreactor: It is a device or system meant to grow cells or tissues the context of cell culture. These devices are being developed for use in tissue engineering or biochemical engineering.

Biosorption: It is a property of certain types of inactive, dead, microbial biomass to bind and concentrate heavy metals from even very dilute aqueous solutions. Biomass exhibits this property, acting just as a chemical substance, as an ion exchanger of biological origin.

Electro Dialysis: It is a membrane process, during which ions are transported through semi permeable membrane, under the influence of an electric potential. The membranes are cation- or anion-selective, which basically means that either positive ions or negative ions will flow through.

Nanofiltration: It is a relatively recent membrane filtration process used most often with low total dissolved solids water such as surface water and fresh groundwater, with the purpose of softening (polyvalent cation removal) and removal of disinfection by-product precursors such as natural organic matter and synthetic.

Reverse Osmosis: It is a water purification technology that uses a semipermeable membrane to remove larger particles from drinking water. This membrane technology is not considered a proper filtration method.

Ultrafiltration: It is a variety of membrane filtration in which forces like pressure or concentration gradients lead to a separation through a semipermeable membrane.

Chapter 16
Role of Biotechnology in Bioremediation

Tapan Kumar Mohanta
National Institute of Plant Genome Research, India

Yugal Kishore Mohanta
North Orissa University, India

Nibedita Mohanta
North Orissa University, India

ABSTRACT

The global environment is now facing a highly critical situation due to rapid urbanization and industrialization as well as increasing population in the limited natural resources. The population growth reflects the drastic changes of the life style of the people that created anthropogenic stress on the environment. There is requirement of highly developed environmental management systems and search of biotechnological technology to remove the contaminated materials and reestablish the natural resources Bioremediation is now considered as the most useful alternative method for eradicate the contaminated material from the nature for sustainable waste management. Now with recent advancement of the genetic approach multiplies the bioremediation process for protection of the natural environment by recycling the waste materials. This chapter covers detail notes on the use of most advanced technology to boost up the bioremediation process.

INTRODUCTION

Environmental pollutants are now the major global concern due their undesirable recalcitrant and xenobiotic compounds. A variety of polycyclic aromatic hydrocarbons (PAHs), chlorinated and nitro-aromatic compounds and xenobiotics, were depicted to be highly toxic, mutagenic and carcinogenic for all living organisms in the earth. A number of microorganisms are considered to be the best suitable candidates among all living organisms to remediate most of the environmental contaminants into the natural biogeochemical cycle due to their diversity, versatility and adaptability in adverse conditions.

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These microorganisms exhibit a remarkable range of contaminant degradable capacity that can proficiently restore the natural environmental conditions. However, some contaminants have been shown to be uncommonly recalcitrant, i.e. microorganisms neither metabolize nor transform them into certain other nontoxic metabolites. As a result, it may be more productive to discover new catabolic pathways that might lead towards complete mineralization of these toxic pollutants. Due to complexity of microbial physiology which allows response and adaptability to various internal and external stimuli, the perfect knowledge of mechanisms of microbial degradation pathways is quite incomplete. (Fulekar, 2007).

Bioremediation, a biological process mediated by microorganisms, is now considered to be one of the most sustainable approaches to degrade and detoxify environmental contaminants. Though bioremediation approach has been used at varying degrees for more than 60 years, for example petroleum land farming, it historically has been implemented as a very 'black box' engineering solution where amendments are added and the pollutants are degraded (Chakraborty, Wu, & Hazen, 2012). This approach is often successful but practically admiration of less result as desirable, i.e., instead of degradation of the contaminant, even production of more toxic daughter products is found. The key to successful bioremediation is to trap up the naturally occurring catabolic potential of microorganisms to effectively catalyze transformations of environmental pollutants. Small scale experiments using distinct microbial consortia in the laboratory is an immense starting point in providing crucial initial indication of the bioremediation process within definite control condition. However *In situ* bioremediation in real execution is a complex phenomenon involving more than one contaminant and simultaneously mediated by different microorganisms involving different metabolic pathways, across geochemical gradients, geophysical and hydrological complexities (Purohit, 2003).

Recently, modern tools of "omics" such as metagenomic, transcriptomics, proteomics, metabolomics, have been applied to explore biological phenomena of microbial communities. Bioremediation also offers many interesting possibilities from a bioinformatics point of view which is gradually going to be explored. This discipline requires the integration of huge amounts of data from various sources: sequence, structure and function of proteins, chemical structure and interaction of organic compounds; comparative genomics; environmental microbiology etc. Development of biosensor is also another milestone to integrate in remediation process. Biosensors are analytical tools, which use the biological signals in sensing the target molecule. Different types of reporter systems have been described and as well as their application in tracking of levels of pollutants, nitrogen, phosphorus, dissolved oxygen in different habitats and toxic compounds. Systems biology is an integrated research approach to study complex biological systems, by systematic searching of interactions and networks at the molecular, cellular, community, and ecosystem levels. Combination of the results from the various 'omics' tools and approach has provided decisive insights into the survival, metabolism and interaction of microorganisms in their native environments together with groundwater and marine systems (Benndorf, Balcke, Harms, & von Bergen, 2007; Hemme et al., 2010; Edward, 1997; DeLong, 2005), extreme milieus (Baker & Banfield, 2003), deep-sea sediments and vents (Harmsen, Prieur, & Jeanthon, 1997; Hu et al., 2010), and animal microbiomes (Gill et al., 2006). A systems biology approach is being adopted to unravel key processes to understand, optimize, predict and evaluate microbial function and survival strategies in the ecosystem. However, successful application of this approach requires over coming several challenges, including the high cost, materials and reagents, large amount of samples,, the need for skilled personnel to process the samples, massive amount of data generated, and the time consuming nature in integration and synthesis of the data (Chakraborty et al., 2012).

This book chapter describes in detail all the possible application of biotechnological processes for effective bioremediation.

OMICS IN BIOREMEDIATION

Metagenomics

Metagenomics is the analysis of the collective genomes of a given habitat, provides direct access to the entire pool of environmental genomes without the limitations associated with lab-based cultivation of microbial species (Eyers et al., 2004). Metagenomics offers a great possibility to retrieve unknown sequences or functions from the environment. The isolation of genomic DNA from complex environmental matrices, such as sludge, waste water, sediment or soil, is particularly highly challenging in terms of quality, molecular size and adequate representation of all microbial genomes (Cowan et al., 2005). Screening strategies for metagenomic libraries can be designed to explore functional and/or genetic diversity. In the function driven analysis, clones of interest are screened or selected through the detection of heterologous expression of a desired trait. Hosts must be carefully chosen, in order not to spontaneously express the targeted activities. The function driven screening strategy has allowed the discovery and characterization of many new enzymes, antibiotics and other chemicals for biotechnological and therapeutic applications (Daniel, 2005; Langer et al., 2006). Fortunately, library screening can be facilitated by automated colony picking, pipetting robotics, use of microtiter plates, informatics assisted data management and sensitive assays targeting a broad range of biomolecules. Due to several constraints, such as the size of inserts, the effective heterologous expression of the cloned genes (with associated post-transcriptional and (post)-translational events) and the secretion of the foreign protein, the screening of "hits" is very low (Lorenz & Eck, 2005).

However, the screening hit rate can be improved by

- The use of different expression hosts,
- The use of diverse strategies to enrich/select for community genomes with desired traits before metagenomic library construction,
- The use of liquid enrichment cultures for the screening of multiple clones simultaneously, and
- The development of novel high throughput screening strategies.

For instance, individual clones can be identified through promoter activity rather than phenotypic expression. Intracellular fluorescent biosensors (Handelsman, 2005; Williamson et al., 2005) or 'substrate induced gene expression screening' (Uchiyama, Abe, Ikemura, & Watanabe, 2005) are based on this concept and are compatible with fluorescence-activated cell sorting of the clones, the latter allowing a throughput level of 109 events per day (Robertson & Steer, 2004). In the sequence-driven analysis, clones of interest are identified based on the presence of conserved regions in phylogenetic marker genes (e.g., 16S rRNA genes) or in functional genes coding for key processes, such as oxygenases (Erwin et al., 2005). PCR and hybridization are commonly used for screening using suitable primers or target-specific probes, which is designed according to the information available in databases. Therefore, the sequence-driven analysis can only be applied for the identification of members of known gene families or novel variants of known functional classes of proteins (Daniel, 2005). Large DNA insert libraries

(DeLong et al., 2006) offer more possibilities to retrieve entire cluster of functional genes and link potential metabolic functions to specific microorganisms in case phylogenetic and functional genes are present on the same insert. In contrast, the entire metagenome of a given environment can be accessed through large-scale shotgun sequencing of small DNA insert libraries (Deutschbauer, Chivian, & Arkin, 2006). Tringe et al. (2005) have successfully characterized and compared the metabolic capabilities of marine and terrestrial microbial communities using largely unassembled sequence data. In this approach, each environment is characterized by a particular 'fingerprint' of environmental gene tags (short DNA sequences that contain fragments of functional genes). Although the shotgun sequencing approach may be valuable in the quest to compare different environments, it remains unclear whether such sequencing will bring insights on particular genes and associated functions. In fact, both large and small DNA insert libraries offer advantages and disadvantages (Whitaker & Banfield, 2006), and a combination of these approaches (Hallam et al., 2004) is probably the best solution to enhance the accuracy, coverage and reliability of genomics-based efforts to understand complex microbial communities (DeLong et al., 2006).

Advantages

The advent of new sequencing technologies like pyrosequencing, which achieve an approximately 100-fold increase in throughput over current sanger sequencing technology, is revolutionizing large-scale sequencing projects in terms of speed and cost. Their application to environmental samples is rapidly expanding, but due to some drawbacks of the technology (like the short length of assembled contigs, in frame stop codons or frame shifts), it necessitates the development of new algorithms for prediction of protein coding genes in environmental metagenomes (Krause et al., 2006). In addition, once such high-throughput sequencing technologies will become commonplace in research labs, there will be an overwhelming demand for computational power beyond anything that is readily available. It is also likely that additional downstream studies will still be necessary to determine the function of many genes discovered within such sequencing projects.

Disadvantages

It is very costly method of approach for bioremediation process. Apart from that very very skilled personells are required for driving such technology in the large scale utility in bioremediation process. As metagenomes are completely based on uncultivable organism genes, so it sometimes may unstable after one to two generations.

Single Cell Sequencing Approach

Sequencing DNA from single cells has opened new windows onto the microbial world. It is becoming routine to sequence bacterial species directly from environmental samples or clinical specimens without the need to develop cultivation methods. Recent technical improvements often allow nearly complete genome assembly from these otherwise inaccessible species. New bioinformatics methods are also improving genome assembly from single cells. The use of single-cell sequencing in combination with metagenomic analysis is also emerging as a powerful new strategy to analyze bacterial communities (Lasken,2012).

Next Generation Sequencing

Next-Generation Sequencing (NGS) obviously ignited a real revolution in environmental sciences, and it triggered the spread of its novel, cutting-edge disciplines *e.g.* metagenomics and metatranscriptomics. Bioremediation and biodegradation entered into an *omics* era (Ma & Zhai, 2012), and nowadays the state-of-the-art technologies play a common role in several molecular microbiological laboratories. Modern NGS platforms are capable of producing gigabases of monoclonal and digital DNA data in a massively parallel fashion. In line with the decreasing sequencing prices, more and more convenient solutions and tools are offered for the community of the environmentalscience (Bihari, 2013).

Microarray

Microarrays (or microchips) are based on the property of a single stranded DNA or RNA molecule ("target molecule") to hybridize to a complementary molecule ("probe") attached to a solid support (Zhou 2003). Compared to traditional nucleic acid membrane hybridization, microarrays present the advantage of miniaturization (thousands of probes can be spotted on a slide), high sensitivity and rapid ("real time") detection (Eyers et al. 2004). Probe target specificity is ensured by the presence of single mismatch probes on the array, that allow distinguishing sequence specific signals from non specific ones. Fluorescent dyes can be enzymatically or chemically incorporated in the sample to be hybridized, therefore readout of the microarray is based on the detection of a fluorescence signal. In addition, environmental samples can be incubated in the presence of a radioactively labeled substrate prior to hybridization, in order to identify microorganisms involved in the metabolism of a specific substrate (i.e., "isotope arrays")

In the field of environmental genomics, three major classes of microarrays have been developed: (i) phylogenetic oligonucleotide arrays (POAs), which contain oligonucleotide probes targeting taxonomic genes (e.g., 16S rRNA gene), (ii) functional gene arrays (FGAs), where probes target genes encoding key enzymes involved in specific processes, and (iii) community genome arrays (CGAs), which are constructed from whole genomic DNA of multiple cultured strains or species (Zhou 2003;Wu et al. 2004). The design of probes for POA and FGA formats is based on sequences retrieved from databases. For this reason, such microarrays do not give access to unknown phylogenetic affiliations and functional activities. Considering the tremendous reservoir of unknown sequences in natural environments, this is a major drawback of "traditional" microarray analysis (Gentry, Wickham, Schadt, He, & Zhou, 2006). To circumvent this limitation, clone libraries of an environmental bacterium whose genome sequence is still unknown can be spotted on a microarray (called Whole Genome (open reading frame) Array, or (WGA). For instance, a clone library of a specific microorganism can be printed on a microarray and hybridized with mRNA isolated prior to and after exposure of this microorganism to a specific treatment. Differences in hybridization patterns will allow the identification of clones harboring genes expressed (or repressed) in response to this treatment. A further step would be to use probes produced directly from environmental DNA without any cultivations step, i.e. combining metagenomics (direct extraction and cloning of collective genomes from environmental samples) with microarrays. The MGA (metagenomics array) technology is still in the early stages of development, but it holds great promise for high throughput screening of natural environments, as it does not require prior sequence knowledge of the microbial communities being analyzed (Gentry et al. 2006). Another important step towards the use of microarrays for studying the dynamics of microbial communities in situ lies in the recent development of a comprehensive FGA (functional gene array). This microarray is aimed at providing direct linkages

between biogeochemical processes and functional activities of microbial communities in a variety of environments. It contains 24,243 oligonucleotide (50 mers) probes covering N10, 000 genes involved in nitrogen, carbon, sulfur and phosphorus cycling, metal reduction and resistance, and organic contaminant degradation. A new generation of this microarray, called Geochip 3.0, has several additional features, including phylogenetic markers such as gyrB (He et al., 2007).

Environmental application of array technology poses great challenges in terms of specificity, sensitivity and quantification. The specificity issue is especially critical for POAs hybridized with rRNA, because some regions of the rRNA molecule are highly conserved and the stability of the secondary structure of the small subunit rRNA can hamper proper hybridization specificity and sensitivity. The specificity issue can be resolved by using thermal dissociation studies. By linearly increasing the temperature of the microarray and recording signal intensities, dissociation curves are obtained. These dissociation curves allow discriminating between perfect-match and single-mismatch probe target duplexes (Fantroussi et al., 2003). The second challenge lies in the sensitivity of microarrays applied to environmental samples. Such samples may contain various contaminants (e.g., humic acids) that inhibit enzymatic reactions and generate a high signal background on the microarray. Various protocols have been developed to extract RNA and DNA of sufficient purity from complex environments like soils or sediments (Fantroussi et al., 2003). When DNA or mRNA is present in the target molecule, an amplification step is necessary to reach the required level of sensitivity. PCR amplification must be used with caution because of its associated bias and artifacts. Alternatively, whole genome amplification can be carried out with phi29 DNA polymerase and random exonuclease resistant primers without thermal cycling. Such an amplification is surprisingly uniform across the genomic target compared to PCR based whole genome amplification. More recently, Wu, Liu, Schadt, & Zhou, (2006) successfully hybridized microbial community DNA amplified using the phi29 DNA polymerase to a community genome microarray to analyze the structure of environmental microbial communities and demonstrated its application to groundwater samples containing sub-nanogram quantities of microbial DNA. For mRNA based analyses, a T7 polymerase-based linear amplification approach using fusion primers was proposed by Gao et al. (2007). The authors reported that it provides sufficient and representative amounts of mRNAs for functional analysis of microbial communities. With FGAs and CGAs, Wu et al. (2006) measured a linear relationship between signal intensity and DNA concentration over four fold concentration of DNA isolated from pure cultures and mixed populations. On the other hand, quantifying microbial populations with POAs is challenging because of cross-hybridizations that may occur when dealing with complex environmental samples containing perfect and mismatch targets in unknown abundance (Chandler et al. 2006). Again, the use of dissociation curves holds great promise for correct quantification of environmental samples.

Metatranscriptomics

Metatranscriptomics refers to the analysis of the collective transcriptomes of a given habitat. Poretsky et al., (2005) developed an environmental transcriptomic approach based on the direct retrieval and analysis of microbial transcripts from marine and freshwater bacterio-plankton communities. In this approach, environmental mRNA, obtained from total RNA by substractive hybridization of rRNA, was reverse transcribed, amplified with random primers, and cloned. mRNAs analyzed belonged to diverse taxonomic groups, and that many of them could be linked to environmentally important processes such as sulfur oxidation, assimilation of C1 compounds, and acquisition of nitrogen via polyamine degradation. Environmental transcriptomic procedure may be a promising tool for exploring functional gene expres-

sion within natural microbial communities without bias towards the known sequences. This approach, which could be easily applied in a high throughput format, has not been tested yet for the analysis of microbial communities in contaminated sites.

However, their study was restricted to the transcriptome analysis of the dominant species living in this particular environment, *Leptospirillum ferrooxidans*, using a species specific whole-genome DNA array. While microarray based meta-transcriptome analysis undoubtedly provides valuable information about the response of microorganisms to environmental parameters, the information remains restricted to the number and nature of the probes spotted on the array.

Advantages

It is very advanced technique that can be applied to a specific type of bioremediation process by using specialized organisms.

Disadvantages

It is not a common technique that can be used widely in different area for bioremediation process and it is habitat specific.

Metaproteomics

Metaproteomics is the study of the entire protein content of a given habitat (Wilmes & Bond, 2006), has greater potential than genomics for the functional analysis of microbial communities.

Indeed, mRNA expression levels (called the transcriptome) may be unreliable indicators of the abundance of the corresponding proteins, as they do not account for post-transcriptional events (Pradet-Balade, Boulme, Beug, Müllner, & Garcia-Sanz, 2001). In meta-proteomics, complex mixtures of proteins from an environmental sample are typically separated with two-dimensional (2D) gel electrophoresis or high performance liquid chromatography. Following protein separation, fractions of interest (e.g., protein spots on a 2D gel) are analyzed by high-throughput mass spectrometry based analytical platforms. Protein prediction and subsequent identification are greatly facilitated by available relevant metagenomic sequence data (Wilmes & Bond, 2006). For instance, in the proteo-genomic study of an acid mine drainage (AMD) biofilm (Tyson et al., 2004; Ram et al., 2005), a total of 12,148 proteins could be predicted from the extensive data set generated by large scale shotgun sequencing. As expected, for each functional category of proteins, there were discrepancies between the fractions of genes in the genome associated the function and the proteins in the proteome associated with the same function. Nevertheless, the AMD metaproteome contained 2033 proteins from the five most abundant species in the biofilm, including 48% of the predicted proteins from the dominant biofilm organism, *Leptospirillum* group II. Eighty unique proteins were identified by 2-DE/MS from *Pseudomonas putida* KT 2440 cultured in the presence of six different organic compounds (Kim et al., 2006). Based on 2-DE analysis, an enzymatic pathway has been proposed for phthalate metabolism in *Rhodococcus* sp. Strain TFB (Tomas-Gallardo et al., 2006). A modeling of the 3-D protein homology of a psychrophilic bacterium *Colwellia psychrerythraea* 34H reveals changes in proteome composition that may enhance enzymes' effectiveness at low temperature (Methe et al., 2005). Other than 2-DE, MS-based proteomics approaches were used to profile a variety of specific enzymes, such as epoxide hydrolases, peroxisomal antioxidant enzymes, and sarcosine oxi-

dase (SOX) associated with marine pollutant exposure (Mi, Orbea, Syme, Ahmed,& Cajaraville, 2005). 2-DE-based identification of these proteins and enzymes during bioremediation makes them candidates as catalysts for cell-free remediation that can direct the use of biomolecules, extracted or removed from cell and/or cell-free systems for field bioremediation.

Advances in MS have improved the analysis of peptides for protein identification and helped to create the field of environmental proteomics. MS deduces the composition of molecules by determining the particular peptide mass that results from certain combinations of amino acids. To study these combinations of amino acids, proteins are routinely digested with proteases to generate peptides small enough for MS analysis, referred to as PMF. In this process, the controlled fragmentation of a peptide yields a series of overlapping fragment ions that differ by the mass of a particular amino acid, which allows the full or partial amino acid sequence of the peptide to be deduced. This phenomenon is also called MS/MS or tandem MS, as it typically uses one MS analyzer to select ions for fragmentation and a second to measure the fragment ions.

MALDI-TOF-MS is routinely used to identify proteins of interest from 2-D gels, as well as to detect and identify viruses, bacteria, fungal spores, and low-mass compounds in environmental samples (Lay, 2001; Ruelle, Moualij, Zorzi, Ledent, & Pauw, 2004). The complex mass spectra of environmental samples can be used to create characteristic fingerprinting databases to detect many site-specific microorganisms. In terms of bioremediation, MALDI-TOFMS can detect specific bacterial signature proteins and biomarkers (primary and secondary metabolites) from site-specific samples for the taxonomic identification of potential microorganisms.

A form of direct sample analysis on a microchip using MALDI-TOF-MS-SELDI-TOF-MS- is another promising analytical technique for site-specific samples. A variety of differentially expressed signature proteins in blue mussels (*Mytilus edulis*) exposed to PAHs and heavy metals were analyzed using SELDI-TOF-MS (Knigge, Monsinjon, & Andersen, 2004). Although SELDI analysis has been useful for identifying potential biomarkers in clinical research, some have questioned its reproducibility and specificity. Another emerging technique, Fourier transform ICR (FT-ICR) MS, may allow multistage MS experiments (MS*n*) with an enhanced detection limit of, 30 zmol for a, 10 kDa protein (Laskin & Futrell, 2005). Adding ESI and LC to MS has opened new analytical windows for the detection and identification of potential contaminants in the environment (Bossi, Seiden, Andersen, Jacobsen, & Streibig, 1999). A direct analysis of a peroxisomal protein pattern associated with marine pollutant exposure was performed using ESI MS/MS to identify various epoxide hydrolases, peroxisomal antioxidant enzymes, and SOX (Mi et al., 2005). Hence, 2-DE- and MS-based identification of proteins/enzymes from any site specific bacterium would eventually pave a way toward cell free bioremediation.

Advantages

It is a very sophisticated and useful cell free technique that can be easily used for bioremediation process in a large quantity. As it is cell it has no any risk of viability for prolong period during the bioremediation process.

Disadvantages

Sophisticated laboratory facility is required for produce the cell free materials for bioremediation.

Metabolomics

Metabolomics refers to the comprehensive analysis of all low molecular weight (b1000 Da) primary and secondary metabolites present in and around cells growing under defined physiological conditions (Mashego et al., 2007). Metabolomics characterizes and quantifies the end products: the metabolites, produced by an organism under a given set of conditions. Unlike past studies based on predefined metabolites, metabolomics examines all the metabolites present in a biological system; thus, there is no bias associated with the choice of metabolites to be studied.

However, metabolites in a site specific organism are part of an *in vivo* metabolite flux that regulates entire metabolic pathways. Additionally, metabolism-based wide fluxes (fluxomes) allow us to pinpoint scenarios of physiological regulation in an organism. The key issue in metabolomics is how to exploit the hidden information that exists in different metabolite compositions. A microbial cell liberates hundreds of primary and secondary metabolites during its life span in response to environmental or cellular changes. These metabolites were previously identified through a process of metabolite finger printing (Shockcor & Holmes, 2002). Metabolomics takes the next step beyond metabolite fingerprinting; instead of simply making an inventory of the metabolites in a cell, it aims to quantify every single metabolite in its functional role.

The quantification of all metabolites in a cellular system is an ultimate principle for any metabolomics experiment. This can be achieved by technologies combining automation and miniaturization that have been developed to isolate and characterize metabolites, including technologies for sampling, extraction of specific molecular classes, storage temperature, sample preparation, and analysis. So the changes in metabolism of the reporter organisms during the confrontation of any contamination is easily detected and the end products i.e. Metabolites clearly indicated the level of contamination and the possible strategies for bioremediation.

Advantages

It has great advantages of use of each individual metabolites of a cellular system can be used for bioremediation process.

Disadvantages

Screening and quantification of metabolites for bioremediation process is quite lengthy and tedious.

BIOINFORMATICS APPROACH IN BIOREMEDIATION

Bioinformatics is the combination of biology and information technology which focuses on cellular and molecular levels for application in modern biotechnology. Attempts have been made to interpret some areas of genomics and proteomics which have been employed in bioremediation studies. Bioinformatics requires the study of microbial genomics, proteomics, systems biology, computational biology, phylogenetic trees, data mining and application of major bioinformatics tools for determining the structures and biodegradative pathways of xenobiotic compounds.

Bioremediation offers many interesting possibilities from a bioinformatics point of view still slightly explored. This discipline requires the integration of huge amounts of data from various sources: chemical structure and reactivity of organic compounds; sequence, structure and function of proteins (enzymes); comparative genomics; environmental microbiology; and so on (Pazos, Guijas, Valencia, & De Lorenzo, 2005). The accumulation of huge amounts of data on individual genes and proteins allowed the first studies of biology from a 'Systems' perspective (Alves et al., 2002). Biological systems are as being composed of components in complex relationships whose ultimate properties cannot be understood by studying these components separately and later summing their properties, but only by studying the system as whole. In a similar manner, data related to bioremediation (genome sequences, structures of chemical compounds, enzyme sequences and structures, etc.) are being accumulated in public databases (Ellis, Hou, Kang, & Wackett, 2003). This allows the first studies of bioremediation from a Systems Biology perspective (Pieper et al., 2004), which complement the traditional approach focused on individual components (microorganisms, enzymes, etc.). The bioinformatics resources devoted to bioremediation are still scarce. Some interesting projects are being carried out to organize and store this huge amount of information related to this subject, The University of Minnesota Biocatalysis/Biodegradation Database (UMBBD) (Ellis et al., 2003) being the more prominent resource.

Bioinformatics incorporates the development to store and search data and of statistical tools and algorithms to analyze and determine relationships between biological data sets, such as macro-molecular sequences, structures, expression profiles and biochemical pathways. Bioinformatics is the focus on cellular and molecular levels of biology. Biology and computers are becoming close cousins who are mutually respecting, helping and influencing each other and synergistically merging more than ever (Fulekar, 2008). The huge data from biology mainly in the form of DNA, RNA and protein sequences is putting heavy demand on computers and computational scientists. Bioinformatics has taken on a new glittering by entering in the field of Bioremediation. Bioinformatics is the application of computer sciences and related technology to the industries for using the huge available database for computational biology. Computational biologists are those who are specialized in using of computational tools and computer systems to solve the problems of biology in the area of bioinformatics (Westhead, Ucbasaran, & Wright, 2003). Yet, most information available in the literature of microbial biodegradation of xenobiotics and recalcitrant chemicals deals with duos consisting of one pollutant versus one strain and thus, lacks essential aspects of the natural scenarios, like the interchange of genes between bacteria or their metabolic co-operation. The sequencing of 'genomes' of communities and ecosystems, instead of single organisms can answer the question very well.

Computational Biology

A computational biology is a sub discipline within bioinformatics concerned with computation-based research devoted to understanding basic biological processes. It encompasses the fields of:

- Bioinformatics, which applies algorithms and statistical techniques to the interpretation, classification and understanding of biological datasets. Datasets typically consist of large numbers of DNA, RNA, or protein sequences. Sequence alignment is used to assemble the datasets for analysis. Comparisons of homologous sequences, gene finding, and prediction of gene expression are the most common techniques used on assembled datasets; however, analysis of such datasets have many applications throughout all fields of biology (Fulekar & Sharma, 2008).

- Computational biomodeling, a field within biocybernetics concerned with building computational models of biological systems.
- Computational genomics, a field within genomics which studies the genomes of cells and organisms. High-throughput genome sequencing produces lots of data, which requires extensive post-processing (genome assembly) and uses DNA microarray technologies to perform statistical analyses on the genes expressed in individual cell types. This can help to find genes of interests for certain diseases or conditions. This field also studies the mathematical foundations of sequencing.
- Molecular modeling, which consists of modeling the behaviour of molecules of biological importance.
- Systems biology, which uses systems theory to model large-scale biological interaction networks (also known as the interactome).
- Protein structure prediction and structural genomics, which attempt to systematically produce accurate structural models for three dimensional protein structures that have not been determined experimentally.
- Computational biochemistry and biophysics, which make extensive use of structural modeling and simulation methods such as molecular dynamics and Monte Carlo method-inspired Boltzmann sampling methods in an attempt to elucidate the kinetics and thermodynamics of protein functions.(Nair, & Laurencin, 2007)

Bioinformatics Tools

Homology and Similarity Tools

Homologous sequences are sequences that are related by divergence from a common ancestor. Thus the degree of similarity between two sequences can be measured while their homology is a case of being either true of false. This set of tools can be used to identify similarities between novel query sequences of unknown structure and function and database sequences whose structure and function have been elucidated.

Protein Function Analysis

This group of programs allows one to compare a certain protein sequence to the secondary (or derived) protein databases that contain information on motifs, signatures and protein domains. Highly significant hits against these different pattern databases allow one to approximate the biochemical function of the query protein.

Structural Analysis

This set of tools allows one to compare structures with the known structure databases. The function of a protein is more directly a consequence of its structure rather than its sequence with structural homologs tending to share functions. The determination of a protein's 2D/3D structure is crucial in the study of its function.

Sequence Analysis

This set of tools allows one to carry out further, more detailed analysis on the query sequence including evolutionary analysis, identification of mutations, hydropathy regions, CpG islands and compositional biases. The identification of these and other biological properties are all clues that aid the search to elucidate the specific function of the sequence.

MetaRouter

MetaRouter is a system for maintaining heterogeneous information related to Biodegradation in a framework that allows its administration and mining (application of methods for extracting new data). It is an application intended for laboratories working in this area which need to maintain public and private data, linked internally and with external databases, and to extract new information from it (Pazos et al., 2005). The system has an open and modular architecture adaptable to different customers. This multiplatform program, implemented in Postgre SQL (standard language for relational databases) and using SRS as an indexing system (used to connect and query Molecular Biology databases), works using a client/ server architecture that allows the program to run on the user station or on the company server, so it can be accessed from any place in a secure way just by having a web browser.

The University of Minnesota Biocatalysts/Biodegradation Database (http://www.labmed.umn.edu/umbbd) begins its fifth year having met its initial goals. It contains approximately 100 pathways for microbial catabolic metabolism of primarily xenobiotic organic compounds, including information on approximately 650 reactions, 600 compounds and 400 enzymes, and containing approximately 250 microorganism entries. It includes information on most known microbial catabolic reaction types and the organic functional groups they transform. (Ellis, Hershberger, & Wackett, 2000).

Database and Web Interface

The current set of data includes 740 chemical compounds (2167 synonyms), 820 reactions, 502 enzymes and 253 organisms. For the chemical compounds, the following information is included: name, synonyms, SMILES code, molecular weight, chemical formula, image of the chemical structure, canonical three-dimensional structure in PDB format, physicochemical properties (density, evaporation rate, melting point, boiling point and water solubility-the user can define and insert new ones) and links to other databases. For the reactions: substrates and products, catalyzing enzyme and links to other databases (Pazos et al., 2005). For the enzymes: name, Enzyme Commission (EC) code, organisms where the gene is present, database sequence identifiers and links to other databases. The main public sources of information for obtaining the initial set of data were UMBBD (Ellis et al., 2003), ENZYME (Bairoch, 2000) and Swiss-Prot (Boeckmann et al., 2003).

The core database uses the PostgreSQL relational database management system. This database can directly be interrogated at the low level by the user using SQL queries or via data-mining programs. Although it is not possible to make complex queries about the structure of chemical compounds, since the SMILES string contains a linear description of that structure, it can be used to perform some simple limited structural queries. In the representations of the results, all the elements (e.g. enzymes, reactions and chemical compounds) are active links that lead to other sections in the database or to external public resources. For example, in the pages containing information on chemical reactions, the substrates and

nhyenzymes are linked to the corresponding enzyme pages which in turn contain links to the entries for those enzymes in external sequence databases, like Swiss-Prot (Boeckmann et al., 2003) and so on. The client/server architecture allows the system to run in a central server and be used simultaneously by any number of client machines with the requirement of a standard web browser. For local/private installations of the system, a distinction between normal users and administrators (the ones who can modify the database) is incorporated. The administrator can add new information (for example, new reactions or compounds discovered/ isolated in his/her laboratory) filling web forms in the web interface. Working with the system at a lower level (below the web interface) it is possible to incorporate new information on a large scale, for example, writing scripts to insert the full metabolism KEGG (Kanehisa, Goto, Kawashima, Okuno, & Hattori, 2004) into the SQL database.

Data Mining

The current system for extracting new information from the database allows locating biodegradative pathways for an individual compound or a set of compounds. That is, it enables finding pathways between those compounds and the central metabolism. In general, it must correspond to all the pathways in KEGG (Kanehisa et al., 2004) apart from 'biodegradation of xenobiotics'. The system also permits to find pathways between one compound and another (or between two sets of compounds). The pathways are displayed together in a versatile representation where the user can choose what to see (compound names only or images of the chemical structures, synonyms, properties, formula, enzymes, etc.); and where the chemical compounds, reactions and enzymes can be colored according to their properties. All the elements in the representation (compounds, enzymes, reactions) are hyperlinked to the corresponding information pages in MetaRouter database. This system allows the exploration and design of biodegradative strategies for chemical compounds depending on conditions such as environment; bacterial ecosystem and others. Although a similar system for locating pathways is available at UMBBD (Ellis et al., 2003), the one presented here provides graphical and more versatile representations and also additional features like the selection/restriction of pathways.

Yet, most information available in the literature of microbial biodegradation of xenobiotics and recalcitrant chemicals deals with duos consisting of one pollutant versus one strain and thus, lacks essential aspects of the natural scenarios, like the interchange of genes between bacteria (Wilkins, 2002) or their metabolic cooperation (Pelz et al., 1999, Abraham, Nogales, Golyshin, Pieper, &Timmis, 2002). This study of genomes and 'functionomes' from a community point of view (in contrast to organism point of view) is leading, for example, to the sequencing of 'genomes' of communities and ecosystems (Venter et al., 2004), instead of single organisms. These circumstances expose the need to qualify and to represent the information available in biodegradation databases in a fashion in which the entire known biodegradative potential of the microbial world can be crossed with the whole collection of compounds known to be partially or totally degraded through (mostly) bacterial action.

The system presented here can help in assessing the environmental fate of compounds or mixtures and in designing biodegradative strategies for them. Among our future prospects are a plan to include in MetaRouter a system for predicting biodegradative pathways for new compounds not present in the database, the possibility of more advanced queries on chemical structures, links to MedLine and other databases, and an easy interface for studying and representing the global properties of the bioremediation network.

BIOSENSORS IN BIOREMEDIATION

Biosensors are par excellence rapid and cost-effective tools for evaluating pollutant concentration and toxicity based on the response of living organisms to contamination. Biosensors are analytical tools, which use the biological specificity in sensing the target molecule. Within the area, biosensors have become one of the newest dimensions of this revolution. Biosensor technology uses biological outputs to monitor processes. The signals from biosensors are picked up by microelectronic elements and processed through various types of processors. Bio-molecules, utilized as biosensors, are selective in their interaction with other molecules and the reactions always follow the same kinetics. This property of molecular specificity is used as the basis in designing biosensors. Therefore, a biosensor can be case-specific in its approach or with application of genetic engineering; more flexible biosensors can be also envisaged. The second component of this analytical device is a signal generating surface, which feeds the information flow to a signal-processing unit. However, a biosensor is mostly case-specific where the different components are interfaced; and are costume designed to accommodate the shelf life and stability of the biological component.

Biosensors are designed using a specific bio-active component for the desired conversion to yield a signal that can be monitored. Based on the component of the cells or the cell itself, there are various biological components that have been developed by different research groups that may be useful as biosensors. Whole cells may be used when a desired activity is targeted for expression of a recombinant protein and the reaction catalyzed through it. An example of this may be the potentiometric microbial electrode designed for detection of organo-phosphorous pesticides. This system uses the surface-expressed organo-phosphorous hydrolase (Mulchandani, Mulchandani, Kaneva, & Chen, 1998). There could be another option where the inherent property of a cell or tissue is used to generate the signal (Wang, Kane, Liu, Symth, & Rogers, 1996). Of the different types of biosensors, the enzyme based biosensors have been extensively explored. Biosensors using enzymes in their biological function generate the signal either by product formation, the disappearance of substrate, or co-enzyme conversion. Sometimes these reactions are superimposed with another biochemical event such as use of inhibition kinetics or coupling with other reaction(s). Various enzyme based biosensors are reported for the detection of pesticides in different samples. The enzymes most often applied are cholinesterase and choline oxidase for the detection of organo-phosphorous pesticides (Rekha, Thakur, & Karanth, 2000).

Molecular Biosensors

Design and application of molecular biosensors for use in bioremediation has been in great focus now these days. The promoter selected from a genetic operon has been described as an interactive biological component for the target molecule to generate the signal. Different types of reporter systems have been described and as well as their application in tracking of levels of pollutants, nitrogen, phosphorus, dissolved oxygen in different habitats and toxic compounds. The paper emphasizes that in order to extend the applications of this scientific area, specialized research is needed in the aspects pertaining to bringing the biological recognition element into close proximity to the target molecules so that it can be integrated with the signal analysis system.

Methodology of Molecular Biosensor

The methodology of molecular biosensors can be discussed at two levels. First, the designing of the biosensor and secondly, in its application as a monitoring tool where the appropriate signal generation options are utilized. In constructing a molecular biosensor the protocols use different genetic engineering steps. Prior knowledge of the gene or genetic operon is the starting point of conceptualizing any biosensor. A typical physiological response is due to expression of a series of genetic events in the cells. However, in this sequence of reactions, there could be a gene or an operon that plays a crucial role in the manifestation of that physiological response. Therefore, the selected physiological response could be assigned to a key gene or operon. Hence, the promoter of this gene could be considered as a candidate for developing the biosensor, to target and monitor this physiological response under different conditions. Once a biosensor is developed, then capturing the generated signal and its quantification is the second step.

The generalized scheme for a recombinant plasmid as a molecular biosensor has a configuration like any other expression vector. It has a reporter system with multiple cloning sites at the 5' end to sub-clone the promoter. The multiple cloning sites are designed in a way that it does not interrupt the coding sequence for the reporter protein. The signal is generated as a response to target molecule; and its level could be determined as a function of target molecule and promoter interaction. The construction of the recombinant plasmid used in this article shows that the target promoter was amplified using the polymerase chain reaction (Hastings, 1996; Morise, Shimomura, Johnson, & Winant, 1974). The primers used in the amplification provide the restriction digestion sites for directional sub-cloning.

The quantification of the generated signal is the most crucial step. The signal could either be directly a protein or something that acts as a functional protein to bring out the biochemical reaction. In the later type, the exhaustion of substrate or generation of product is correlated with the signal. In the luciferase expression system, the luminescence signal could be quenched on the photographic film or by using a luminometer to quantify the signal. The signal captured on the photographic plate can be analyzed by the densitometric analysis. The light signal generated in the luciferase system also can be received through the fiber optic device, which is connected to a data processing unit to digitize the signal, so that it can be processed quantitatively. In case of GFP, the protein is synthesized and to generate the signal, it has to be excited with a specific wavelength to produce the fluorescence phenomenon. GFP fluorescence is stable, species-independent and can be monitored noninvasively using the technique of fluorescence microscopy and flow cytometry.

Promoter as Biosensor

Promoters are 5' flanking sequence in a gene or operon, that respond to changes in cellular physiology and accordingly the genetic information stored in DNA is transcribed in terms of a messenger RNA. Promoters, either directly take the response of a target molecule or the target molecule interacts with promoter via a receptor system. In either of the situations, the response could be modulated kinetically, which is proportional to expression of m-RNA or any reporter molecule. To design a biological component for a biosensor, a selected promoter sequence could be placed at the 5' region of the reporter system, where the selection of promoter is based on the target molecule to be monitored in the samples. Thereby, at the molecular level, the promoters are the actual sensing components of biosensors. Thus, these kinds of biosensors have three main components. The vehicle or vector molecule (plasmid) is the principal component of this system, which allows the maintenance and survival of biosensor in appropriate host.

The other two components are the promoter, which is the sensing system at genetic level and the reporter system, which could be *lux* operon, GFP or any other signal producing molecule. The following are the promoters derived from various genes and which have been applied in environmental monitoring.

Generalized Stress Promoters

Different kinds of environmental stress trigger groups include heat shock proteins. These are stress-induced proteins synthesized by some bacteria in conditions like nutrient starvation, exposure to toxic organics, heavy metals, and others. The promoters from *Escherichia coli*, such as *uspA*, *grpE* or *dnaK* were sub-cloned in the *lux* expression vector. These promoters have been demonstrated for their non-specific response to various stresses when *E. coli* is used as the host. The resultant expression systems derived from these recombinant plasmids as biosensors, have been observed to have a response time of less than 5 min, when challenged with different toxic molecules. The studies were done in batch and continuous culture of these recombinant bacteria. The experiments were designed to monitor the signal through fiber optic probes. The cells in the reactor were challenged by the stresses with different levels of target molecules and the expression of *lux* operon as the light signal was recorded on-line (Van Dyk et al., 1994; 1995).

Monitoring of Nutrients

The bioremediation approach requires a typical C: N: P ratio for the success of biological degradation. The biosensors can be designed for monitoring the level of nitrogen and phosphorus in the medium with an expression system using promoters, *glnA* and *phoA*, respectively. The nitrogen starvation promoter *glnA* can be turned on with the nitrogen limiting conditions and *phoA* under the phosphorus limiting condition. Similarly, the carbon source specific promoters can be applied. In case of *Pseudomonas* RB1351 the *lux* system driven by *Pnah* promoter has been expressed and it generates the signal in response to naphthalene in the medium. This promoter is derived from the operon which is responsible for biodegradation of naphthalene (King et al., 1990). A monitoring tool for contaminated sites with petroleum products has been designed deriving the promoter from the *tod* operon. The plasmid based molecular biosensor multiplies in the host cells. Depending upon the conditions if survival of host bacterium or stability of the plasmid cannot be maintained, then, instead of using a plasmid as the vector the biosensors are introduced through the transposon system. In this scenario, the biosensor at the molecular level is flanked with the insertion sequences or sub-cloned in the transposon vector. This recombinant DNA is then transferred to a host, which has been shown to survive in the selected environmental conditions. The DNA molecule transferred in this type of experiment becomes lodged on the chromosome of the host DNA. The biosensor developed with this strategy has been demonstrated for monitoring of benzene, toluene, ethyl-benzene, and/or xylene in the contaminated sample (Applegate, Kehrmeyer, & Sayler, 1998). The levels of a pollutant in a medium is a critical parameter observed in the bioremediation program; where a bacterium that uses a pollutant as a carbon source could find the same pollutant as toxic above a particular concentration. Considering this principle, the signal generation systems were designed; where, different bacterial strains were used as hosts with the *lux* reporter system to monitor the toxicity of phenol (Shaw, Beaton, Glover, Killham, & Meharg, 1999), polycyclic aromatic hydrocarbons (Reid, Semple, Macleod, Weitz, & Paton, 1998), and heavy metals (De Weger, Kuiper, van der Bij, & Lugtenberg, 1997).

Monitoring of Metal Ion

In the case of *E. coli*, the universal stress protein A is encoded by *uspA* gene. The promoter *uspA* derived from this gene can be switched on non-specifically by the conditions that limit cell growth. This includes nutrient starvation and exposure to toxic chemicals (Van Dyk et al., 1995). The heat shock promoter *grpE* derived from *E. coli* has been observed to have a similar type of functioning. The generalized biosensors based on *uspA* and *grpE* have been demonstrated in the monitoring of heavy metals such as Cu^{2+} and Cd^{2+}. However, as these biosensors are nonspecific in their expression, usage is possible only under defined conditions. The metal ion specific biosensors are much explored in the area of mercury levels in the environmental sample. The biosensors designed for mercury can target both Hg (II) present in the inorganic or organic form. These independently developed biosensors use the specific promoters derived from *mer* operon, characterized for detoxification of both the forms of Hg (II) (Dodd, Stewart, & Waites,1990). A single-use Hg (II) biosensor comprised of immobilized cells has been designed which uses latex copolymer film and is able to detect with a sensitivity of 1 nM concentration (Lyngberg, Stemke, Schottel, & Flickinger, 1999). For monitoring the bioavailabilty and water extractable mercury, a soil isolate-based biosensor has been designed. This was engineered by transferring a *mer-lux* reporter system to a *Pseudomonas putida* strain (Hansen & Sorensen, 2000).

Monitoring of Physical Parameters

Other than nutrient availability in the growth medium, parameters like pH, dissolved oxygen (DO), and temperature, influence the growth of bacteria. Biosensors that can be influenced by these parameters have also been reported. The effect of pH and toxicity of chlorophenols has been monitored by lux-based biosensor. The sensitivity of this system has been evaluated using different heterologous hosts; and was compared with commercially available toxicity monitoring kits. Results have shown that under low pH conditions, dicholorphenol is more toxic; and for such monitoring the strains of *E. coli* were observed to be more sensitive hosts (Sinclair, Paton, & Meharg, 1999). The promoter based on oxidative stress *KatG* has been demonstrated for its application in monitoring DO level in the systems (Belkin, Samulski, Vollmer, Van Dyk, & LaRossa, 1996). The expression of this promoter decreases with decreased concentrations of DO level in the medium. Redox agents such as hydrogen peroxide, methyl viologen, organic peroxides, and other redox cycling agents can also induce this promoter. Temperature monitoring in bioremediation is not critical; however, in tropical countries with higher temperatures throughout the year, biosensors that function at an optimum temperature of 30^0C would not be efficient. In this scenario, a *lux* reporter system derived from *Photorhabdus* could provide a more temperature stable expression system, since the *lux* operon derived from this bacterium is stable up to 42^0C. Another promoter derived from the *recA* operon, which deals with the DNA damage, has been extensively explored for its application in biosensors. This promoter could be induced by chemicals, which are responsible for genotoxicity and even UV radiation exposure. The property of this promoter has been extended to evaluate the relative quotients for genotoxicant molecule (Rosen, Davidov, LaRossa, & Belkin, 2000).

Online Monitoring

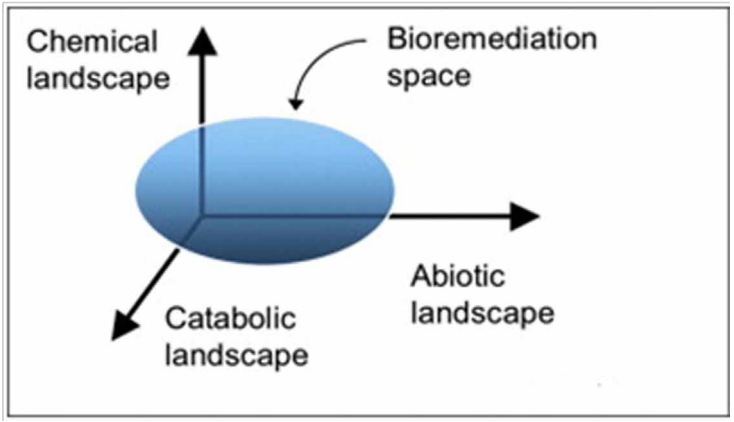
The on-line monitoring using *lux* as a reporter system in biosensors has been demonstrated by various groups. The bacterial cells are either used as immobilized cells or in suspended growth culture. However,

it has been shown in suspended growth cultivation either in the batch or continuous mode, that it is the growth phase, which is crucial for sensitivity of the response (Rupani et al., 1996). With the immobilized cells, chemicals such as benzene in the vapor state have been shown to induce the light response on-line. The immobilization is carried out in agar medium layered in a polypropylene tube, which is fixed at the end of fiber optic probe. The resultant sensor, on exposure to gases carrying hydrocarbons, generates the light signal mediated through the *lux* reporter system, (Gil, Mitchell, Chang, & Gu, 2000). A more specific promoter of a similar nature designed to monitor benzene and its derivatives has been reported, which uses the promoter *xylS* under the control of *xyl*R, a regulatory protein. At the molecular level, the *xyl* R protein senses the hydrocarbon and after activation, reacts with the promoter to turn on the *lux* reporter gene which is used for generation of the light signal (Ikariyama et al., 1997). To assess the toxicity of a wastewater stream, a two-stage mini bioreactor has been developed. In one reactor, the biosensor strain is kept in a continuous cultivation mode with the desired dilution rate, which gives the cells a physiology having maximum sensing capacity. The second reactor has physiologically consistent cells, which are stimulated by a wastewater stream or challenged with a toxic molecule. The signal generated is received through a fiber optic probe and correlated after digital processing for the level of toxicity. Thus, the second reactor behaves as a reaction vessel for light signal quantification (Gu, Gil, & Kim, 1999). The interaction of the target molecule to recombinant bacteria is essentially addressed in the aforementioned monitoring tools. However, all these studies used *E. coli* as a host, which harbors the molecular biosensor. The signal is generated from the stimulation received by the promoter, which is converted into the light response. But, under stress conditions i.e. exposure to a toxicant, how the toxicant will affect the host system, which in turn would modulate the generation of the signal, has not been thoroughly studied. Since *E. coli* is a natural inhabitant of intestine, a bacterium from the soil or bacteria often observed in waste water treatment plants, could be a choice of host for these types of studies.

SYSTEM BIOLOGY APPROACH IN BIOREMEDIATION

Bioremediation involves the exposure of a whole mixture of chemical structures to an intricate multispecies metabolic network present in a polluted scenario. The complexity involved in such events is growingly amenable to the conceptual frame and the tools of systems biology. The availability of genes, genomes, and metagenomes of biodegradative microorganisms make it possible to model and even predict the fate of chemicals through the global metabolic network that results from connecting all known biochemical transactions (Chakraborty et al., 2012). Microbial communities thus embody a landscape of pan enzymes that is shaped by the freely diffusible metabolic pool (*epimetabolome*). Recent computational resources increasingly help the design of superior biocatalysts for biodegradation and biotransformations of desired chemicals, an objective that capitalizes on the new field of synthetic biology. The factors that play in bioremediation scenarios include more elements than just the biological catalysts and the contaminants discussed above. Their dynamic interactions occur in concrete abiotic settings which are defined by a whole of physico-chemical conditions: O_2 tension, electron acceptors, water, temperature, granulation, and others, many of which change over time and the course of the catalysis. Such abiotic conditions determine the species composition of the endogenous microbial communities as much as (or more than) the availability of given chemical species as C and energy source. To get started, one should navigate the various layers of complexity that separate the occurrence of distinct gene clusters encoding catalytic activities in single genomes all the way to extensive implementation of such catalysis on a target site.

Figure 1. The bioremediation space. There are three dimensions to the effectiveness of any bioremediation process, only one of them (the catabolic landscape) being biological. The chemical landscape of the place, including nutrients-to-be, electron donors/acceptors and stressors has a dynamic interplay with the biological vector of the system on the abiotic background imposed by the micro/macro-geography of the location at stake. This includes humidity, conductivity, temperature, pressure texture, matric conditions, redox (O_2) status, etc. Each of these vectors can be formalized for the sake of modeling such a complex process.

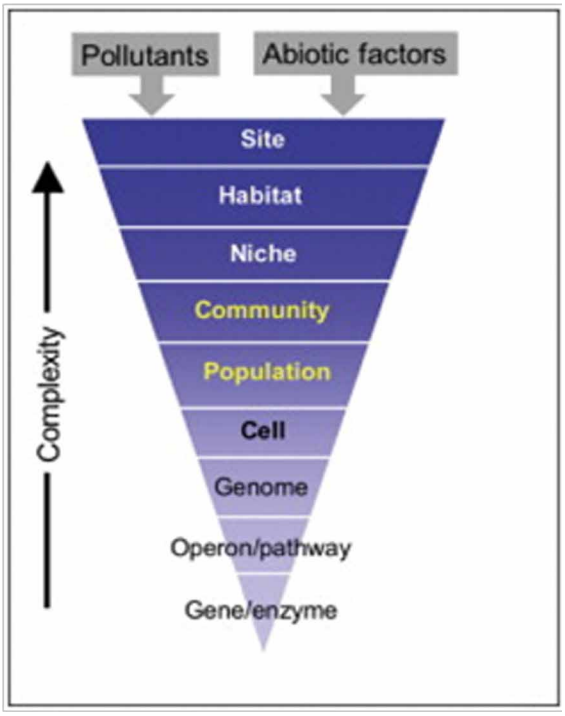


The Catabolic Gene Landscape: Method and Abstractions

Once we have a good catalog of genomes and catalytic strains and information on a large collection of genes encoding biodegradation and detoxification reactions, we can move up in the complexity pyramid of Figure 2 and ask how we can get a picture of the complete catalytic potential of the bacterial communities that thrive in polluted sites. In the best possible scenario, one can have the complete genomes of the main players at works in a given place and try to merge and model all their metabolic transactions. But to go on with our comprehension of bioremediation processes along the complexity pyramid of Figure 2 we need to make a number of abstractions and simplifications. One dramatic generalization (but quite a useful one) is assuming that what matters in bioremediation is the presence and performance of the catabolic activities available in the site, regardless of the particular species that carry them. This generalization is not devoid of a solid rationale. Catabolic genes for recalcitrant and xenobiotic compounds are frequently encoded in mobile elements (broad host range plasmids and transposons) and it is often the case that similar genes/enzymes appear in diverse species. One can thus envisage a situation in which the pool of biodegradative genes (i.e. those which connect unusual chemical structures to central metabolic routes, see below) move freely through the microbial community regardless of the specific ID of the hosts. For the sake of simplification, one can even take that the species composition is dictated by the abiotic conditions of the place, while the profile of biodegradative genes is determined by the C (N, P) sources and electron acceptors available.

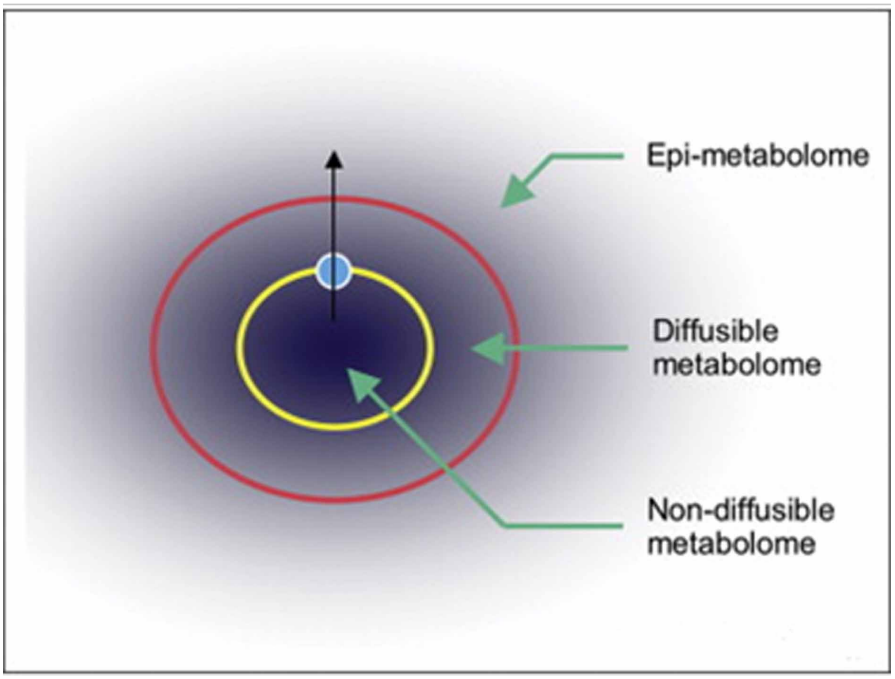
A second abstraction deals with diffusion of chemical species through the metabolic network that may result from having a large number of biodegradative activities acting simultaneously on one or more substrates. The notion of a *metabolism-without-walls* is too far from reality for being useful in our context. In the other extreme, a fully compartmentalized scenario dependent on the morphology

Figure 2. Layers of multiscale complexity associated to bioremediation. The figure sketches the sort of complexity pyramid that one has to go through for taking aboard all factors that intervene in the implementation of any bioremediation strategy. Note that this is a highly dynamic situation, as the course of the biocatalysis changes both the chemical profile of the pollutants and the structure of the microbial community and vice versa. Pollutants and the side products of their metabolism can also have a strong mutagenic effect on the microbial genomes as well as affect the architecture of the abiotic scenario.



and transfer rates of the various cell types and abiotic matrixes adds an extraordinary complexity to any attempt to model metabolism for the sake of bioremediation. One attractive way to overcome this deadlock is to divide the metabolome of any given micro-organism in three categories with distinct diffusion abilities (Figure 3). These include firstly, an intrinsically *nondiffusible pool* of metabolites which never make it outside the cells (for instance, phosphorylated intermediates, nucleotides, etc.), secondly, *a diffusible metabolome* composed of molecules that can occasionally be secreted depending on the catalytic rate of the bacteria which produce them (amino acids, organic acids, etc.), and thirdly, a peripheral metabolome (*epi-metabolome*) formed by the pool of compounds which are transformed so slowly that they can diffuse out the cells between one step of a metabolic pathway and the next one or/and are actively secreted because of its toxicity. This last scenario is in fact very frequent in bacteria endowed with biodegradative capacities. Intermediates of metabolic pathways are most often found in the supernatants of bacteria exposed to the corresponding substrates. These might result from simple diffusion or may imply an active transport by extrusion pumps often found in degradative bacteria (Ramos et al., 2002). In other cases, bacteria that degrade xenobiotics have specific systems; a sort of chemical security valves that expulse the excess of toxic intermediates that may accumulate intracellularly (Endo, Aoki, Yoda, Kimura, & Hama, 2007). In a mixed community, such secreted compounds can diffuse and

Figure 3. Categories of metabolites at stake in a bioremediation site. Bacteria that inhabit any environmental niche posses a non-diffusible metabolome that unless cells are lysed is never secreted. Other metabolites (typically amino acids and organic acids) can diffuse out the cells under certain circumstances, although the ease of their consumption makes their presence very uncommon. On the contrary, compounds that are metabolized slowly have a chance to diffuse out and become available to members of the community other than those that produce them. We call epi-metabolome such a freely diffusible pool of chemicals in a microbial consortium.



be captured and further metabolized by other members of the consortium. It is thus obvious that such a free-diffusible *epi-metabolome* is the only fraction of the chemical pool of the polluted site that can be subject of the combined metabolic network of a bacterial community.

Pan Enzymes

The term *pan-enzyme* explains, "Evoking an enzymatic activity without borders" to designate the result of pooling all activities that bring about an identical reaction on the same substrate and originate the same product(s). There is one more abstraction that needs to be made before attempting to move up the complexity pyramid as shown in figure 2. Microbial communities do contain multiple variants of enzymes that execute the same reaction on the same substrate (Witzig, Junca, Hecht, & Pieper, 2006), albeit with nonidentical efficiencies (Junca, Plumeier, Hecht, & Pieper, 2004). These variants are often encoded in the same genome (duplicated or not: see e.g. the many oxygenases borne by *Rhodococcus* sp. RHA1) (Mcleod et al., 2006) as well as in different species present in the site. Most often, the first enzymes of peripheral biodegradative pathways while having an optimal specificity for a given substrate have considerable activity also on other compounds (Pazos, Valencia, & De Lorenzo, 2003), even if such

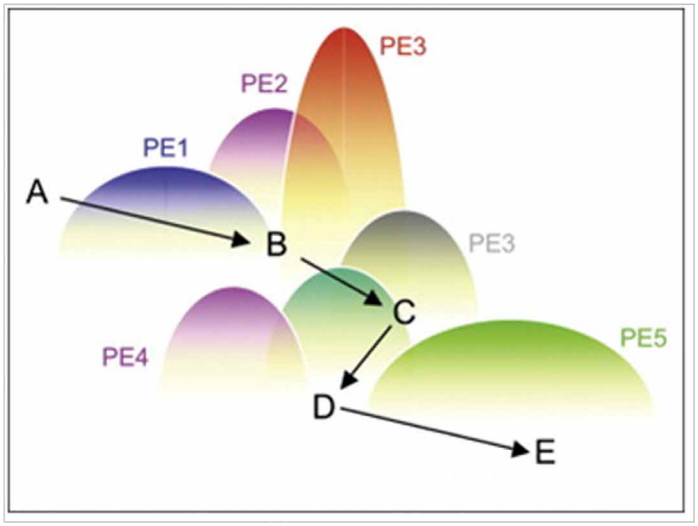
side-reactions lead to dead-end products that cannot be metabolized by the same bacterium (Skiba, Hecht, & Pieper, 2002). This means that the conversion of substrate **A** into product **B** in a microbial community will be the result of the additive action of all activities that can execute such a reaction, regardless of which encodes the enzyme and which benefits metabolically.

Figure 4 explains the adoption of the three key abstractions argued above originates a basic scenario in which pollutants facing a complex microbial community transit through a biodegradative landscape that integrates all possible reactions available in the site until the intermediates find a minimum energy state that ends up into the central metabolism for the production of biomass, or CO_2 and water. Needless to say that this is a highly dynamic situation, as the progress of the bioremediation process does alter the species composition and the overall catabolic gene landscape, while the *pan-enzymes* available at each point do change the chemical composition of the *epi-metabolome*.

Global Biodegradation Network

Public resource for quantitative studies on microbial biotransformations is the University of Minnesota Biocatalysis/Biodegradation Database (UMBBD, http://umbbd.msi.umn.edu). The system permanently maintained and updated represents a colossal effort to collect primary data from literature on such processes (Ellis, Roe, & Wackett, 2006). At the time of writing this article, UMBBD lists 177 pathways, 1220 reactions, 1133 compounds, 786 enzymes, and 462 microorganisms of environmental interests. A

Figure 4. The catabolic gene landscape. The picture results from the adoption of the three abstractions regarding bioremediation discussed in the text: firstly, biodegradative genes, not species is what matters, secondly, only the diffusible epi-metabolome, not the central metabolites is important in bioremediation, thirdly, biotransformations are executed by pan-enzymes, not by singular enzyme species. On the basis of these, one can visualize and model biodegradation processes in a complex microbial community as the flow of epi-metabolites A → B → C → D through an uneven landscape of pan-enzymes (PE1, PE2, etc.). This scenario of complex microbial and enzymatic networks could be approached with the tools of, for example, ecological control analysis for modeling mass flux and process rates (Röling, 2007).

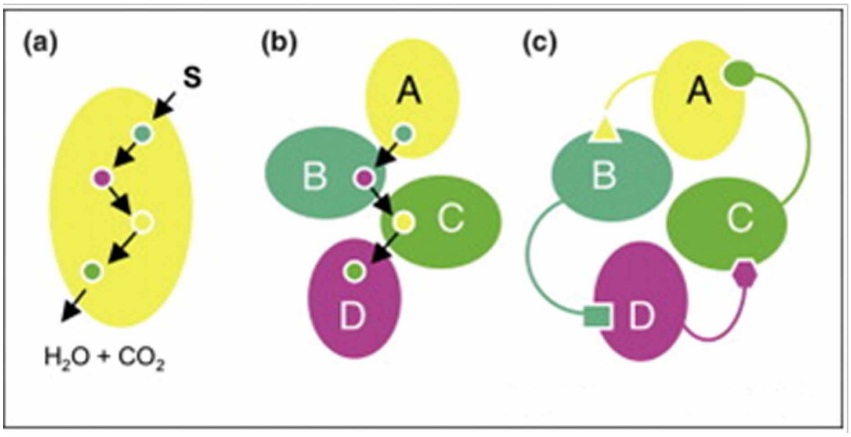


first systematic analysis of the body of data deposited in the UMBBD was attempted in 2003 by connecting all known reaction intermediates of the database in a fashion independent of the microbial host (Pazos et al., 2003). The resulting network had a scale-free organization with connectivities not unlike those found in metabolic networks of single organisms. The study permitted to visualize a considerable *funnel effect* as well in which many peripheral compounds soon become connected to a lesser number of attractor nodes closer to the central metabolism. The network appears to evolve such that new compounds enter the system by becoming connected to metabolic hubs through a minimal number of steps. Such an analysis also exposed generic links between biodegradability, molecular size, and hydrophobicity, aspects that were taken up later for predictive purposes. These early analyses allowed the rigorous formulation of what has been called the *Global Biodegradation Network* regardless of their origin and host species. Such a network reflects the complete biodegradative potential of the global microbiota. This is of course a strong abstraction, as not all reactions might be available at any time simultaneously and some of them can even be incompatible (for instance, aerobic versus anaerobic) for occurring in given sites. However, the concept provides a solid basis to the catabolic gene landscape scenario. One key aspect of both schemes is that any diffusible reaction intermediate can be shared by catabolic pathways present in different micro-organisms until it finds its lower energy location in the landscape. This expands the catabolic potential of a community to reach out many more compounds than the simple addition of the individual capacities of singular strains.

The Environmental Fate of Chemical Pollutants

Once we have the big picture of the reactions available for bioremediation and how they may work together, how can we predict the fate of specific chemicals spilled in a given site and even guide inter-

Figure 5. Implementation of metabolic activities in the environment. Biodegradation of any given substrate S through a multistep biochemical route $S \rightarrow \rightarrow \rightarrow CO_2 + H_2O$ *may occur through the action of a single performer micro-organism, endowed with all enzymes required for complete mineralization of the compound (a). Alternatively, the same process can be executed by a combination of metabolic steps made present by different bacteria A–D (b). Systems and synthetic biology offer the opportunity of setting non-natural biodegradative consortia by playing with their metabolic gravitation (Gilbert, Walker, & Keasling, 2003) and/or by forcing their physical association by means of artificial adhesins (c).*



ventions aimed at accelerating the process? The answer is simpler if we deal with compounds for which some information is known beforehand. The MetaRouter system (Chain et al., 2006) allows visualization through a web interface of all possible pathways that a large number of recalcitrant compounds can take through known steps of all the reactions taken from the UMBBD. The system searches in the database all possible combinations of enzymes (and wherever available, their cognate genes) needed to convert a certain substrate into standard metabolic intermediates or into any other products. The interesting part is that the exercise often results in virtual pathways which are a patchwork of genes/enzymes that come from different bacteria, sometimes having very different lifestyles (for instance, aerobic and anaerobic). Although such combinations may not exist or may have not been discovered yet in nature, these hybrid pathways reflect plausible processes that can occur at different stages and locations by dissimilar microorganisms. In this respect, the MetaRouter system says nothing on the kinetics or thermodynamics of the proposed pathways, although it can certainly guide metabolic engineering attempts. Although MetaRouter handles only compounds for which some biodegradation information is available, it gives a picture of how given chemicals could be degraded if passed through the merged metabolism of a complex community rather than how they could be metabolized by one specialist strain.

CONCLUSION

To address the challenge of cleaning up contaminated sites, a number of advanced technologies have emerged in the field of environmental biotechnology, the "omics" approach, bioinformatics, biosensors and system biology will give a new insight to build up a *In situ* model of successful bioremediation .However, the implementation of such bioremediation techniques in situ is not always successful, because of the difficulty to control and scale up key bio-degradative processes from the laboratory to full-scale. Bioreactors and other confined environments offer conditions far more controllable and manageable than most open large-scale ecosystems, in terms of predictability, dynamics of catabolic microbial populations and process monitoring. Still the new advancement of the process technology will definitely provide a great hope for the successful bioremediation process.

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KEY TERMS AND DEFINITIONS

Bioremediation: Bioremediation is a waste management technique that involves the use of organisms to remove or neutralize pollutants from a contaminated site.

Biosensor: Biosensors are analytical tools, which use the biological signals in sensing the target molecule.

Contaminants: Biological, chemical, physical, or radiological substance (normally absent in the environment) which, in sufficient concentration, can adversely affect living organisms through air, water, soil, and/or food.

Genetic Engineering: Genetic engineering, also called genetic modification, is the direct manipulation of an organism's genome using biotechnology.

Mass Spectrometry: Mass spectrometry (MS) is an analytical chemistry technique that helps identify the amount and type of chemicals present in a sample by measuring the mass-to-charge ratio and abundance of gas-phase ions.

Metabolites: Metabolites are the intermediates and products of metabolism. The term *metabolite* is usually restricted to small molecules.

Metabolomic: Metabolomics is the scientific study of chemical processes involving metabolites.

Metagenomic: Metagenomics is the study of genetic material recovered directly from environmental samples.

MetaRouter: It is a system for maintaining heterogeneous information related to Biodegradation in a framework that allows its administration and mining.

Microbial Degradation: Interest in the microbial biodegradation of pollutants has intensified in recent years as humanity strives to find sustainable ways to clean up contaminated environments.

Microbiomes: A microbiome is "the ecological community of commensal, symbiotic, and pathogenic microorganisms that literally share our body space."

Proteomics: Proteomics is the large-scale study of proteins, particularly their structures and functions.

Pyrosequencing: Pyrosequencing is a method of DNA sequencing (determining the order of nucleotides in DNA) based on the "sequencing by synthesis" principle.

Transcriptomics: The transcriptome is the set of all RNA molecules, including mRNA, rRNA, tRNA, and other non-coding RNA transcribed in one cell or a population of cells.

Xenobiotic Compounds: A xenobiotic is a foreign chemical substance found within an organism that is not normally naturally produced by or expected to be present within that organism.

Chapter 17
Bioremediation: New Prospects for Environmental Cleaning by Enzymes

Vikram Singh
Agricultural Research Organization, Volcani Center, Israel

ABSTRACT

Pollution is the biggest menace to the living being in this planet today. Enzyme bioremediation is a "breakthrough technology" that holds the potential of pollutant eradication through exploiting the enzyme potential by using the various techniques. Enzyme biocatalysis is referred as white biotechnology and work by green chemistry concept. Moreover, developments in the design and application of enzyme cocktails, mutienzyme complexes, promiscuous enzymes and protein families (cupin and VOC superfamily) has recently emerged a new opportunity in bioremediation. The implementation of various enzyme modification approaches intended for potential bioremediation has been done by adopting enzyme immobilization using magnetic nanoparticles, designer enzymes generation through enzyme engineering, nano-technological advancement for single enzyme nanoparticle generations, electro-bioremediation and carbon nanotube construction. Hence, enzyme bioremediation have greater positive effects and propose significant promise to pollutant bioremediation. In conclusion, the enzymatic bioremediation open the new era of pollutant eradication for clean, safe and green environment.

INTRODUCTION

Environmental pollution is one of the serious issues of the present and future climatic scenario. The soaring environmental pollution affects the all forms of living and non-living globally. Environmental contamination has become a serious problem with the advancement of industrial revolution in developed and producing nations. The increasing population growth and urbanization stretch the use of innate resources for the maximum yield. The carrying capacity of the earth is significantly smaller than the demands with increasing populations. To satisfy the existing demands, the overuse of natural resources often results in nature's degradation (Santos, 1990). The new agricultural practices have been carried out to meet the food demand of soaring population. Pesticides play a significant part in modern agriculture

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to increase the productivity. However, pesticides may have a good effect on agricultural productivity, conversely, their indiscriminate use in these farming practices has contributed to serious concern for the health of living being and the environment. (Diez, 2010; Coutinho et al., 2005). Pesticide poisonings are a massive problem worldwide, especially in developing countries. Out of the million tons of pesticides applied annually, most of them get accumulated in the environment with the degradation of only a small fraction (Eerd, Hoagland, Zablotowicz, & Hall, 2003). Pesticides are recalcitrant and remain for a long time in soils and enter directly into the food chain. To overcome these problems researcher has released the new degradable pesticides. The fate of pollutant in the environment is influenced by biological, chemical and physical degradation processes which determine their persistence and mobility (Gavrilescu, 2005). Consideration of the current technology for removal of pollutants clearly shows that physical and chemical methods are often uneconomical. Nevertheless, biological treatment technology may provide a resolution to this problem. The pollutant degradation by biological means such as microorganisms (Microbioremediation) or plant (Phytoremediation) etc. is termed as "Bioremediation". The use of bioremediation technologies provides a dependable and economic alternative to commonly used physical and chemical handling. Biological remediation is regarded as the most dependable, least disruptive and most cost-effective treatment method (Mueller, Cerniglia, & Pritchard, 1996). Principle of bioremediation is to utilize the pollutants, use them for their growth and metabolism or convert them from toxic to nontoxic form. Bioremediation by microorganism offer more advantage over phytoremediation as microbioremediation is a well established technology for the pollutant removal. Simultaneously, microbes act as an 'eco-friendly nano-factories' for environmental cleaning. Microbioremediation offers natural, environmentally friendly and economical solution for environmental cleaning (Vidali, 2001). Bacteria and fungi are well known to degrade hazardous organic contaminants to environmentally less toxic or nontoxic compounds. These organic compounds are readily incorporated into the microbes and get oxidized under aerobic or anaerobic conditions. Heterotrophic bacteria and fungi are the chief agents causing biodegradation. Recently, genetically modified microorganisms have been directed to formulate bioremediation (Paul, Pandey, Pandey, & Jain, 2005). The *Pseudomonas fluorescens* HK44 possesses a naphthalene catabolic plasmid is the first genetically engineered microorganism approved for bioremediation testing (Ford, Sayler, & Burlage, 1999). The major problems associated with microbioremediation is the mass transfer, additionally they also require aeration and nutrient on contaminated sites (require bio-stimulation) and also suffer from thermal condition problems (Fantroussi & Agathos, 2005; Scow & Hicks, 2005). Now a day the bioremediation is shifted from microbes to enzymes because of their eco-friendly attributes. The pollutant degradation by an enzyme is known as "enzymatic bioremediation" and particularly suited for rapid remediation. This method is adventitious over to microbial bioremediation (either natural or genetically engineered microbes), because of the efficiency, time consumption and expense (Ahuja, Ferreira, & Moreira, 2004). The exploited enzyme potential using the various techniques of in-vitro enzyme evolution is being revolutionized in present scenario. The enzymatic bio-efficiency can be appraised by the adoption of two recent methods Biostimulation and Bioaugmentation (Sutherland, Russell, & Selleck, 2002; Scott et al., 2008). Biostimulation provides the optimal environment to enzymes or microbes for their maximum efficiency" in terms of temperature, pH, moisture, aeration, etc. (Baker & Herson, 1994), however "Bioaugmentation employ the superior biodegradable agent (enzymes or microbes) for the environment cleaning" (Scragg, 2005). Enzymes are having narrow (chemo, region and stereo selectivity) or broad specificity and can be applied to a vast range of compounds effectively under extreme conditions. Moreover, the use of enzyme may present advantages over traditional technologies and represent a good alternative for overcoming most disadvantages related to the use of physical,

chemical and biological degradation (micro/phytobioremediation) (Nicell, 2001; Gianfreda & Bollag, 2002; Gianfreda & Rao, 2008). All these characteristics render enzymes as eco-friendly catalysts and enzymatic techniques as environmentally friendly operations.

The intention of this chapter is to assess the response of enzymes in bioremediation by providing the insight of the major enzymes involved. The attempt was aimed to focus the importance and significance of enzymes in terms of their superiority over microbes, their properties and current and future advancement of enzyme bioremediation.

SUPERIORITY OF ENZYMES OVER MICROBES IN BIOREMEDIATION

Before the emergence of white biotechnology, microbes have been utilized extensively for bioremediation. The microbial bioremediation was effective compared to other physical and chemical method of bioremediation because of its eco-friendly attributes. Although, the use of microbes for bioremediation is hampered by many rate-limiting factors such as it is costly and time-consuming. Furthermore, drastic conditions such as chemical shock, toxins, extremes temperature and pH and high pollutant concentrations or their products irreversibly damage or metabolically inactivate microbes. Difficulty in maintaining an active cell culture during transportation to the polluted site also limits the use of whole-cell detoxification technologies. Other factors that restrict the use of microbes include the limited mobility of the cells within the soil, alternate carbon sources and weakness of the inoculated microorganisms in competition with the indigenous population. Therefore, most of these adverse factors can either be eliminated or mitigated if the enzymes are used alternatively to the microbes. Enzyme bioremediation is a "breakthrough technology" that holds the potential to offer copious benefits which are not tendered by other conventional technologies (Gianfreda & Rao, 2004.) Enzyme bioremediation have greater positive effects and propose significant promise to pollutant bioremediation. Hence, the application of enzymes in function-directed remediation may minimize the pollution due to persistence and further spreading of pollutants. The superiority of enzymes over microbes is governed by following features:

- Enzymes attack on the substrate more efficiently.
- Enzymes are more mobile than microorganisms because of their smaller size.
- Enzymes are stereo specific with broad substrate specificity (Gianfreda & Bollag, 2002).
- Enzyme can work on more harsh operational conditions (contaminant concentration, extreme pH and temperature, salinity etc.).
- They are effectively applicable to recalcitrant compounds.
- Enzyme does not require nutrient and biomass acclimation.
- Easy to control and does not form any toxic by-products.
- Lower mass transfer limitation on contaminants compared to microorganisms.
- Most efficient in even modest quantity.

PROPERTIES OF ENZYMES FOR BIOREMEDIATION

The concept of using enzyme systems for enhancing bioremediation is relatively new and adopted globally. The effectiveness of enzymes in bioremediation is absolutely depends upon their efficiency

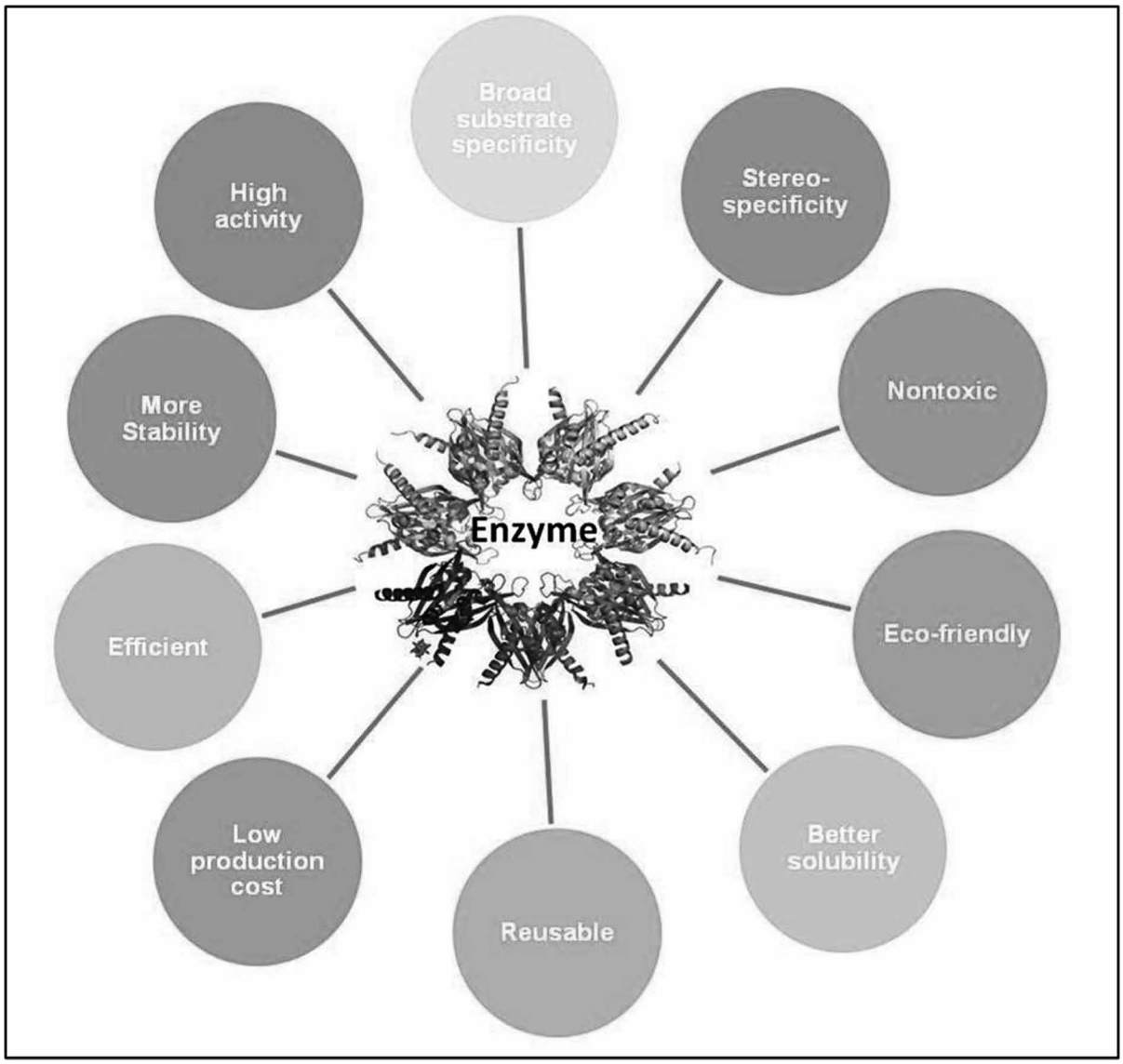
and specificity (Kulshreshtha, 2013). Enzymes are considered as eco-friendly catalysts and enzymatic techniques are seen as environmentally friendly processes (Rao, Scelza, Scotti, & Gianfreda, 2010). The boost in enzymatic bioremediation can be exploited to engineer enzyme properties: active-site topology, enlarge binding pockets and alter the substrate specificity and stability (Chica, Doucet, & Pelletier, 2005). Consequently, the enzyme properties have been altered through modification in protein structure to make it more stable, more resistant to self destruction with additional target directed degradation properties (Arnold, 2001; Tao & Cornish, 2002). Biological remediation can be rationalized by specific finite measurements of following enzyme properties: maximal enzymatic rate (V_{max}), substrate specificity (K_m), turnover number (k_{cat}), enzyme efficiency (k_{cat}/K_m). The pollutant eradication by enzymatic catalysis depends on the following attributes:

- Enzyme should target pollutant to substantially less toxic products.
- They should not require cofactors, which would be prohibitively expensive.
- Enzyme should have high K_m (substrate affinity) and K_{cat} (turn over) values with broad substrate specificity.
- The enzyme must be robust to the wide range of pH, temperature and ionic environments.
- The enzyme should fulfil all the required performance criteria in relation to stability, kinetics and cost of production for a bioremediation (Raillard et al., 2001).

ENZYMES IN BIOREMEDIATION

Enzymes are a good alternative for bioremediation overcoming disadvantages related to the use of microorganisms (Gianfreda & Rao, 2008). Enzymes are stereo-specific with broad specificity and can be applied to a vast range of compounds. They can be used under extreme conditions with more mobility compared to microorganisms. All these characteristics render enzymes eco-friendly for bioremediation. Enzyme biocatalysis is referred as white biotechnology (Alcalde, Ferrer, Plou, & Ballesteros, 2006) and work by green chemistry concept (Sheldon & van Rantwijk, 2004). "White biotechnology engrosses the use of biotechnology in the bulk production of fine chemicals such as antibiotics, drugs, amino acids, vitamins, enzymes, organic acids and polymers". It is relevant to consider white biotechnology as green chemistry as it focuses on the development of clean bioprocesses that lead to reductions in greenhouse gas emissions, energy and water usage. The most common enzymes used in bioremediation are hydrolases, phosphotriesterases, amidases, proteases, cellulases, amylases, depolymerise, dehalogenases, transferases, oxidoreductases etc. The major oxidoreductase participate in the bioremediation are monooxygenase, di-oxygenases, dehalogenases, reductases, phenoloxidases (laccases, tyrosinases), cytochrome P450 monoxygenases and peroxides. Bacterial hydrolases such as carbamate or parathion hydrolases from *Nocardia, Flavobacterium*, *Pseudomonas*, *Achromobacter* and *Bacillus cereus* have been successfully used in the biotransformation of carbofuran, carbaryl, parathion, diazinon and coumaphos (Sutherland et al., 2004). Similarly, carbohydrases, depolymerases, proteases and phosphates are suitable for the transformation of carbohydrates, plastics and proteins (Nakamura, Tomita, Abe, & Kamio, 2001; Singh, 2002). The combination of xylanase and cellulase (hydrolases) is routinely employed in pulp and paper industry. Nitrilases produced by *Nocardia* sp., *Rhodococcus* sp., *Fusarium solani* and *Aspergillus Niger* act on nonpeptide C\N bonds (Sornyotha, Kyu, & Ratanakhanokchai, 2010). Recombinant *Pseudomonas*

Figure 1. Some additional properties recommended for bioremediation



aeruginosa rhodanese (r-RhdA) is used for r-RhdA cyanide detoxification (Cipollone, Ascenzi, Frangipani, & Visca, 2006). The lignolytic enzyme is capable of degrading lignin compounds, dyes and other environmental pollutants (Pointing, 2001; Sanchez-Porro, 2009). Laccase produced by *Trametes versicolor* and *Funalia trogii* successfully degrade compost, azo dyes, phenols, anilines and aromatic thiols. Recently, laccase is being used extensively for olive oil treatment in Mediterranean countries. Manganese peroxidase (*Lentinula edodes*) eradicates manganese ions. Chromium reductases (*Pseudomonas putida* MK1 and *Escherichia coli*) effectively reduce a toxic, mutagenic and carcinogenic Cr^{6+} to non-toxic Cr^{3+} (Cheung & Gu, 2007). Recently, marine enzymes are gaining more consideration for bioremediation strategy as they have exceptional biocatalytic properties: high salt tolerance, hyper-thermostability, barophilicity and cold adaptability. Many proteases, carbohydrases and peroxidases have been isolated from

marine *Penicillium raistrickii* CBMAI 931, *Aspergillus sydowii* CBMAI 933, *Trichoderma* sp. CBMAI 932 and *Penicillium miczynskii* CBMAI 930 (Martins et al., 2011). Ligninolytic enzyme produced by marine fungi *Mucor racemosus* CBMAI 847, *Cladosporium cladosporioides* CBMAI 857 and *Aspergillus sclerotiorum* CBMAI 849 has the greatest potential of bioremediation (Bonugli-Santos, Durrant, Da Silva, & Sette, 2010). Large number of enzymes act effectively on pollutants and employed for their eradication. Some of the major enzyme groups engaged successfully in bioremediation are as follows:

Mixed Function Oxidases (MFO)

MFO also known as dependent cytochrome P-450 monooxygenases or P450 system. MFO incorporates one atom of oxygen molecule into the substrate and the other atom is reduced to water in presence of Nicotiamide-adenine dinucleotide phosphate (NADPH). MFO contains two enzymes, cytochrome P450 and NADPH-cytochrome P450 reductase. The MFO genes encoding the different isozymes comprise a superfamily of over 200 genes grouped into 36 families based on their sequence similarity (Alzahrani, 2009). The cytochrome P450, family comprises a group of monooxygenase enzymes having a remarkable potential in many industrial processes. Cytochrome P450 enzymes have a broad substrate range and catalyses biochemically potent reactions such as the oxidation or hydroxylation of non-activated carbon atoms (Khaled, Miia, Arja, Jukka, & Olavi, 2012). These properties are ideal for the remediation of environmentally persistent pesticide residues. MFO is actively involved in the metabolism of both endogenous and exogenous substances and can metabolize organophosphates, carbamates, xenobiotics and pyrethroids.

Oxidoreductases

Oxidoreductases are involved in the electron transfer reaction in the presence of electron donors or electron acceptors cofactors. These enzymes have been sub classified into 22 subclasses depending on the reaction catalyzed. Oxidoreductases detoxify toxic xenobiotics, phenolic, and anilinic compounds through polymerization and copolymerization (Park, Park, & Kim, 2006). The enzyme also reduces the radioactive metals from an oxidized soluble form to reduce insoluble form. The most promising application of oxidoreductase is the removal of endosulfan (1, 2, 5, 6, 7, 7-hexachloro-5-norbornene-2,3-dimethanolcyclic sulfite), an organochlorine insecticide, which is highly toxic and endocrine disruptor. Endosulfan is banned in the European Union and several countries because of their toxicity. *Mycobacterium tuberculosis* oxidoreductase ESD efficiently degrades both beta and alpha-endosulfan.

Oxygenases

These enzymes participate in substrate oxidation in the presence of molecular oxygen (O_2) employing FAD, NADH or NADPH. Oxygenase plays a key role in the metabolism of organic compounds by aromatic ring cleavage. Oxygenases are grouped into two categories on the basis of the number of oxygen atoms used for oxygenation.

Monooxygenases

These enzymes incorporate one atom of the oxygen molecule into the substrate. On the basis of presence of cofactors used for their action monooxygenases are classified into two subclasses: (a) Flavin-dependent monooxygenases- Contain Flavin as prosthetic group and require NADP or NADPH as coenzyme. (b) P450 monooxygenases- Heme containing oxygenases. The monooxygenases comprise a versatile super-family of enzymes that catalyzes oxidation of alkanes, steroids, fatty acids, aromatic and aliphatic compounds. Monooxygenase catalyzed the biotransformation, dehalogenation, hydroxylation, desulfurization, ammonification, denitrification, and biodegradation of compounds. Methane monooxygenase is involved in the degradation of substituted alkanes, alkenes, haloalkenes, cycloalkanes, methanes, ethers, aromatic and heterocyclic hydrocarbons (Grosse et al., 1999). The specialized proteins ese and esd, the part of the unique two component Flavin dependent monooxygenase (*Arthrobacter* sp. KW) are efficient for the endosulfan degradation (Weir, Sutherland, Horne, Russell, & Oakeshott, 2006). Similarly, monooxygenase from *Pseudomonas aeruginosa* and *Burkholderia cepaeia* is also effective for the degradation of endosulfan (Kumar, Devi, Krishnamurthi, Kanade, & Chakrabarti, 2007).

Dioxygenases

Dioxygenases are multicomponent enzyme systems that incorporate molecular oxygen into their substrate. Aromatic hydrocarbon dioxygenases belongs to a large family of rieske nonheme iron oxygenases. Different enantiomers of dioxygenases catalyze the oxygenation of a wide range of substrates and oxidize aromatic compounds into aliphatic products. The intradiol dioxygenase utilize Fe (III), while the extradiol dioxygenase utilize Fe (II) and Mn (II) for their action (Que & Ho, 1996). Two dioxygenase superfamilies are engaged most proficiently for pollutant degradation.

Cupin Protein Superfamily

This family comprises three types of aromatic ring cleavage dioxygenases: intradiol catechol dioxygenases, extradiol catechol dioxygenases (EDOs) and non-catechol dioxygenases or EDO-type enzymes (Vaillancourt, Bolin, & Eltis, 2004). Catechol dioxygenases degrade the chlorinated derivatives via meta and ortho pathways (Adams, Singh, Keller, & Jia, 2006). EDOs and EDO-type enzymes are Fe (II) or Mn (II) containing enzymes with a facial capping triad of protein derived ligands. All catechol dioxygenases have a common two-his-one-carboxylate structural motif in the active site, where two histidine and one glutamate residues bind the central metal. These dioxygenase are dexterous in the breakdown of aromatic rings in a variety of pollutants such as catechols, protocatechuates, gentisates, salicylates, aromatic amino acids, 2-aminophenols, 3- hydroxyanthranilates, hydroxyquinones and hydroquinones.

The Vicinal Oxygen Chelate (VOC) Superfamily

This superfarmily is having a βαβββ structural motif for the effective substrate binding. The rearrangement of βαβββ motif leads to the multi-functionality for these enzymes (Gerlt & Babbitt, 2001). The VOC superfamily has bi-dentate vicinal oxygen chelation of substrates in the metalloenzymes FosA, GLO and type I EDOs. The divergent proteins of VOC superfamily such as bleomycin resistance protein (BRP), fosfomycin resistance proteins (FosA), methyl-malonyl-CoA epimerase, glyoxylase 1 (GLO) and type I extradiol dioxygenases are valuable pollutant eradicator (Armstrong, 2000).

Laccases

Laccases (p-diphenol:dioxygen oxidoreductase) constitute a family of multicopper oxidases that catalyze the oxidation of phenolic, chlorinated biphenyls and aromatic substrates with concomitant reduction of molecular oxygen to water. Intra and extracellular laccases are capable of catalyzing the oxidation of ortho and para diphenols, methoxyphenolic, aminophenols, polyphenols, polyamines, lignins, aryl diamines and inorganic ions (Couto & Toca Herrera, 2006). Laccases are prominently involved in the depolymerization of lignin. Artificial substrate such as ABTS (2,2_-azino-bis-3-ethylbenzthiazoline- 6-sulphonic acid), HBT (hydroxyl benzotriazole) or violuric acid, enabling the oxidation of non-phenolic compounds by laccases and expand the range of applications of these enzymes (Smith, Thurston, & Wood 1997).

Peroxidases

Peroxidases catalyze the oxidation of lignin and other phenolic compounds at the expense of hydrogen peroxide (H_2O_2). The peroxidases have classified into three distinct groups (a) Class I is intracellular enzymes, including cytochrome c peroxidase (yeast), ascorbate peroxidase (APX) (plants) and catalase (bacterial) (b) Class II consists of the secretory fungal peroxidases such as lignin peroxidase (LiP) and manganese peroxidase (Mnp) from *Phanerochaete chrysosporium*, *Coprinus cinereus* peroxidase and *Arthromyces ramosus* peroxidase (ARP) (c) Class III contains the secretory plant peroxidases such as horseradish peroxidases (HRP). These enzymes are biosynthetic enzymes involved in plant cell wall formation and lignifications. Class II peroxidase are most competent among the classes for pollutant eradication. Some major class II peroxidases are discussed in detail.

Lignin Peroxidases

Lignin peroxidases are heme proteins which degrade lignin and other phenolic compounds in the presence of H_2O_2 and the mediator (veratryl alcohol). During the reaction, H_2O_2 gets reduced to H_2O with the gain of electrons from LiP (which itself gets oxidized). Lignin peroxidase (LiP) plays a central role in the biodegradation of the plant cell wall constituent lignin and aromatic compounds. The high redox potentials of LiP make them effective in single-electron abstraction during the reaction.

Manganese Peroxidases

MnP is an extracellular heme enzyme that oxidizes Mn^{2+} to the oxidant Mn^{3+} in a multistep reaction. Mn^{2+} stimulates the MnP production and functions as a substrate for MnP. The Mn^{3+} chelate oxalate generated by MnP acts as a mediator for the oxidation of various phenolic compounds. The Mn^{3+} chelate oxalate is small compound which can easily diffuse into areas inaccessible even to the enzyme. Lignin and xenobiotic which are buried deep within the soil and not accessible to the enzymes can efficiently attacked and degraded by these Mn^{3+} chelate oxalate (Have & Teunissen, 2001).

Versatile Peroxidases

VP enzymes directly oxidize Mn^{2+}, methoxybenzenes, phenolic and aromatic substrates. These enzymes have extraordinary broad substrate specificity and tendency to oxidize the substrates in absence of manganese compared to other peroxidases (Wong, 2009). VP is also able to oxidize both phenolic and non-phenolic lignin dimers. Hence, at present the VP is gaining much more attention and being produced at a high level for many industrial processes and bioremediation (Ruiz-Duenas et al., 2007).

Hydrolases

Hydrolyses disrupt major chemical bonds of toxic molecules and reduces their toxicity. Hydrolases catalyze the substrate via condensations and alcoholysis. Effectiveness of hydrolases in the absence of redox cofactors formulate them ideal candidates for current bioremediation strategies. This mechanism is valuable for the biodegradation of oil spills, organophosphate and carbamate insecticides. Organochlorine insecticides, DDT and heptachlor are stable in aerated soil nevertheless, readily degrade in anaerobic environments (Vasileva-Tonkova & Galabova, 2003). The degradation of these compounds by hydrolases is very vital for environmental cleaning. The main advantages of this enzyme are ready availability, lack of cofactor, stereo-selectivity and perform efficiently in the presence of water-miscible solvents. Extracellular hydrolytic enzymes such as pullulanases, lipases, amylases, DNases, xylanases and proteases have quite diverse potential usages in the biomedical sciences, food industries, chemical industries and feed additive (S´anchez-Porro, Martin, Mellado, & Ventosa, 2003). The hemicellulase, cellulase and glycosidase are of much importance due to its application in biomass degradation.

Esterases

Esterases has classified into three groups according to the nature of their interactions with substrate: carboxylic esters (carboxiesterases), amides (amidases) and phosphate esters (phosphatases). Carboxylesterases belong to the group of ali-esterases and B-esterases. Insecticides mainly, organophosphates, carbamates, pyrethroids and xenobiotics have associated a carboxylic ester bond. The Carboxiesterases (type B esterases) hydrolyze xenobiotics, amide, thioester, phosphate esters (parathion, paraoxon) and acid anhydrides (DIPFP). Esterases have a wide range of substrate specificities; they are capable to hydrolyze phosphate esters, triesters, thioesters, amides and peptides (Dary, Georghiou, Parsons, & Pasteur, 1990). The specificities of esterases A and B depends on their active site residues containing a Cys and Ser residue, respectively. Esterases A form a bond between P=S via organophosphates interaction with the functional group-SH, which is easily hydrolyzed by H_2O. Similarly, esterase B form P=O bond through organophosphates interaction with the SER-OH that is not hydrolyzed by H_2O. Esterases protect the target site of acetylcholinesterase by the hydrolysis of insecticides (Reiner, Aldridge, & Hoskin, 1989). Because of this property currently, esterase has been routinely used for the treatment of several neurological disorders.

Lipases

Lipases are ubiquitous enzymes which catalyze the hydrolysis of triacylglycerols to glycerol and free-fatty acids. Lipases degrade lipids derived and drastically reduce hydrocarbon from contaminated soil. Lipase activity is a useful indicator for testing hydrocarbon degradation in soil. These enzymes catalyze: hydrolysis, inter-esterification, esterification, alcoholysis and aminolysis (Prasad & Manjunath, 2011). A lipase from *Candida rugosa* is found proved to be effective for Triolein hydrolysis in the biphasic oil-water system. The lipase breaks the ester bonds of triolein to produce consecutively diolein, monoolein, and glycerol. Lipase is of much interest in the production by region-specific compounds which are employed in the pharmaceutical industry. In addition to diagnostic usage in bioremediation, lipase has many prospective applications in cosmetic, detergent, food, chemical, manufacturing, and paper making industries (Sharma, Sharma, & Shukla, 2011).

Cellulases

Cellulases are a mixture of endoglucanase (EG, endo- 1,4-D-glucanohydrolase), exoglucanase or cellobiohydrolase (CBH, 1,4-β-D-glucan cellobiohydrolase) and β-glucosidase, which attacks regions of low crystallinity in the cellulose fiber. These enzymes degrade the cellulose molecule to cellobiose units and ultimately to glucose units. Cellulases convert waste cellulosic material into foods to meet burgeoning population and have been the subject of intense research (Bennett et al., 2002). Microbes produce cell bound, cell envelope associated and extra cellular cellulases. During the enzymatic hydrolysis, cellulose is degraded through the cellulases to reducing sugars that can be fermented by yeasts or bacteria to ethanol (Sun & Cheng, 2002). The alkaline cellulases produced by *Bacillus strains*, and neutral and acidic cellulases by *Trichod*erma and *Humicola* are efficient for bioremediation. In paper and pulp industry, cellulases have been employed for recycling of paper along with xylanase. The cellulases are supplemented during brewing process to increase the juice liberation from fruit pulp and for the production of ethanol from cellulosic biomass.

Proteases

Proteases hydrolyze the peptide bonds in the protein. Proteases are divided as endopeptidases and exopeptidases depending on the peptide chain catalysis. Based on the position of the active site, endopeptidases further grouped into subclasses such as aspartic endopeptidases, serine endopeptidase, cysteine peptidase and metallopeptidases. The endopeptidase acts on the inner regions of the peptide chain, while exopeptidases act only near the terminal amino or carboxylic position of the chain. The protease that acts on free amino and carboxyl terminals is termed as aminopeptidase and carboxypeptidase, respectively. Proteases have wide range of applications in detergent, leather, food and pharmaceutical industry (Beena & Geevarghese, 2010). The alkaline proteases are used in leather industry for the removal of hairs.

Glutathione S-Transferase (GST)

The GSTs are a group of enzymes that catalyze the conjugation of hydrophobic components with the tripeptide glutathione. The thiol group of glutathione reacts with an electrophilic moiety in the target compound to form a metabolizable conjugate which can be excreted. GST is also involved in detoxification of endogenous and xenobiotic compounds (Khersonsky & Tawfik, 2010).

Phosphotriesterases (PTE)

PTE are the most suitable pesticide degrading enzymes that hydrolyze and detoxify organophosphate pesticides (OPs).The most effective PTEs are: organophosphate hydrolase (OPH), methyl parathion hydrolase (MPH), organophosphorus acid anhydrolase (OPAA), di-isopropylfluorophosphatase (DFP) and paraoxonase 1 (PON1) (Bigley and Raushel, 2013). OPH, isolated from *Pseudomonas diminuta* or *Flavobacterium* ATCC 27551, catalyzes the hydrolysis of a broad range of OP pesticides. Although, carboxylesterases (CbEs) is also involved in OPs hydrolysis, nevertheless hydrolysis of OPs by PTEs is more efficient in the detoxification than the CbEs (Sogorb & Vilanova, 2002). The profenofos degradation by PTEs involves cleavage of the phosphorothioate ester bond to yield 4-Bromo-2-chlorophenol, followed by conjugation with glucose. The PTE isolates from *Aspergillus Niger* showed high biodegradation of malathion pesticide (Ramadevi, Nath, & Prasad, 2012), however *Aspergillus flavus* and *Aspergillus sydowii* PTEs are capable of degrading pirimiphos-methyl, pyrazophos and malathion (Hasan, 1999).

MAJOR POLLUTANT AND THEIR ENZYMATIC BIOREMEDIATION

Pollutants are the substances that adversely affect the environment. A pollutant may cause long or short-term damage to the environment and living beings. Some pollutants are biodegradable; they degrade with time and will not persist in the environment for the long term, although, some are non-biodegradable, either they did not decompose or takes many years to decompose. The degradation products of some pollutants are themselves act as a pollutant, such as the products, DDE and DDD produced from the degradation of DDT. Pollutant such as synthetic organic substances (SOCs), pesticide, DDT, etc. accumulates in the environment because of the slow decomposition rate and known as "persistent organic pollutants (POPs)". Some of the most common examples of POPs are DDT, DDE, PCBs, toxaphene and metal ions. The persistence of these toxic compounds ultimately leads to "Biomagnifications". Biomagnification is the phenomena in which the concentration of a substance exceeds the required diet of an organism (Jorgensen and Faith, 2008). These un-degradable compounds enter into the food chain and mount up their concentration in higher organism, e.g. in the aquatic food chain, the fish bioaccumulates approx. million times the concentration of methyl-mercury (herring ~0.01 ppm and shark > 1 ppm mercury). Similarly, DDT is stored in animals and bio-magnify rapidly as it takes many years to its breakdown. Some of the devastating compounds, for example DDT's, heptachlor, endrin, chlordane, lindane, Aldrin and toxaphene has been banned because of their lethal toxicity. These pollutants need to be remedied effectively for a cleaner environment. Enzymes play a vital role in the removal of a range of pollutant. The bioremediation potential of enzymes for pollutant removal is discussed subsequently.

Methyl Tert-Butyl Ether

MTBE (methyl-tert-butyl ether) is the most widely used fuel ether. The peculiar attribute of these MTBE is that they do not adsorb in soil and therefore move quickly to groundwater. The persistence of MTBE in the environment can be ascribed by its molecular structure and high steric hindrance which makes it recalcitrant to degradation. MTBE degradation is governed by *Pseudomonas putida* CAM, *Pseudomonas aeruginosa*, *Rhodococcus ruber*, *Mycobacterium austroafricanum and Methylibium petroleiphilum* PM1. The MTBE biodegradation efficiency of these microbes is exclusively dependent in the key enzyme

cytochrome P-450 monooxygenases. Some kinds of hydrolases (MdpA) belong to the alkane hydroxylase (AH) family are responsible for the initial step of MTBE biodegradation pathway (Lopes Ferreira, Malandain, & Fayolle-Guichard, 2006).

Heavy Metals

Heavy metals are extremely toxic as bioaccumulates. The biodegradation of these heavy metal ions is anticipated by metalloenzymes. The metalloenzymes metal sites belong to iron sulfur clusters (2Fe-2S), (3Fe-4S), (4Fe-4S) and H_2-activating sites. The (Fe-S) cluster that is proximal to the active site is essential to H_2 activation in metalloenzymes (Lovley, 1991). During the reaction cytochrome c3, Hase and hydrogenase lead to the reduction of the base metal. Metallothioneine, arylsulfatase, β-glucosidase and dehydrogenase plays an important role in heavy metal metabolism. Chromium (VI) reduction using a soluble chromium reductase enzyme is achieved by the electron supply with the assistance of either NADH or cytochrome c3. Under anaerobic conditions Cr is reduced by hydrogenase or cytochrome c3/c7. The bioremediation of vanadium, selenium, uranium, cobalt, manganese, iron and technetium follow similar enzymatic pathways. Explosives, such as trinitrotoluene (TNT) are enzymatically biodegraded by NADPH dependent heme cytochrome cd1 or nitrite reductase. Organomercurial lyase is effective in hydrolyzing the stable mercury-carbon bond by binding Hg (II) in the active site with cysteine sulphydryl residues. Mercuric reductase (the product of the merA gene) reduces the Hg^{2+} to the less toxic and volatile Hg^0 species (Silver & Walderhaug, 1995), similarly, mercuric reductase along with glutathione S-transferase is effectual for the removal of high mercury concentrations. Periplasmic acid phosphatase (named PhoN), accumulate high levels of uranium, nickel and zirconium by the formation of insoluble metal phosphates, however polyphosphate kinase remove the uranium. Wildung et al., (2000) reported the enzymatic reduction of U (VI) is governed by *c*-type cytochrome. Tetra-heme cytochrome *c*3 in combination with hydrogenase is more helpful for uranium reduction (Lovley & Phillips, 1994). Similarly, Lloyd et al., (2003) identified the role of homologous cytochrome (PpcA) and tri-heam periplasmic cytochrome *c*7 of Fe (III) reducing bacterium *Geobacter sulfurreducens* in U (VI) reduction. Radionuclei, Tc is a long-lived radionuclide, having strong ligand-complexing capabilities and is difficult to remove using conventional chemical methods. Three major components of formate hydrogenlyase (FHL) complex and Tc (VII) reductase catalyses the Tc via electron transfer (Alliot, Alliot, Vitorge, & Fattahi, 2009). Th, Np, Pu and Am also reduces by heme dependent reductase enzymes with the similar mechanism (De Luca, De Philip, Dermoun, Rousset, & Vermeglio, 2001; Tamponnet & Declerck, 2008).

Pesticides

The most common pesticide employing in agriculture practices are glycophosphate, parathion, propham, carbaryl, carbofuran, coumphos, diazinon, trazine and 2,4-D. Enzymes mediated degradation of pesticides is an innovative technique for removal of these chemicals from polluted environments as these are more effective than existing chemical methods. Enzymes degrade pesticides, both in the target organism via intrinsic detoxification mechanisms and directly in the environment (Scott et al., 2008). Pesticide degradation is governed by the: hydrolases, Phosphotriesterases (PTEs), Esterase, mixed function oxidases (MFO) and glutathione S-transferases (GST) system. Pesticide degradation is catalyzed by hydrolysis, oxidation, addition of oxygen, oxidation of an amino group, reduction of a Nitro group, the addition of a hydroxyl group to benzene ring, dehalogenation, side chain metabolism and ring cleavage reactions.

Table 1. Enzyme engaged in pesticide degradation (Source: Hernández et al., 2013)

S. No.	Enzymes	Pesticides
1.	Oxidoreductases (Gox)	Glyphosate
2.	DMO	Dicamba
3.	Monooxygenase: ESd, Ese	Endosulphan, Aldrin, Malation, DDDT, Endosulphato etc.
4.	Phosphotriesterases: OPH/OpdA	phosphotriester
5.	P450	Hexachlorobenzene and Pentachlorobenzene
6.	Dioxygenases (TOD)	Trifluralin
7.	Dehalogenases: LinA LinB	Hexachlorocyclohexane (γ isomers) Hexachlorocyclohexane (β and δ isomers)
8.	Cyp76B1, Cyp1A1/1ª2	Atrazine, Norflurazon, Linuron, Chlortoluron and Isoproturon

Atrazine (2-chloro-4-ethylamino-6-isopropylamino- 1,3,5-s-triazine) belongs to a class of s-triazine herbicides, widely used for weed control is having a half-life of greater than 170 days and difficult to biodegradation. The AtzA, B and C enzymes of *Pseudomonas* sp. ADP is most promising for atrazine eradication. These enzymes convert tyrosine to cyanuric acid, which is subsequently mineralized to carbon dioxide and ammonia by the soil micro-flora (Sadowsky, Tong, De Souza, & Wackett, 1998). Monooxygenase from *Pseudomonas* sp. is effective for parathion degradation (Singh & Walker, 2006).

Organophosphate Pesticides (OPs)

Organophosphates (paraoxon, parathion, disulfoton, carbophenothion, chlorpyrifos, ruelene and dimeton) are neurotoxins used as insecticides. OPs are being used worldwide in agriculture practices, municipal hygiene, disease control and pest eradication. The main OPs are thiophosphotriesters, phosphotriesters and phosphorothiolesters. The OPs are highly toxic pesticides containing potent irreversible acetylcholinesterase (AChE) inhibitors activity and have a profound effect on the nervous system (Edwards & Tchounwou, 2005). These compounds suppress acetylcholinesterase by phosphorylation of the serine at the enzyme active site and subsequently prevent them from breaking down acetylcholine at the synaptic junction. Chlorpyrifos, methamidophos, profenofos and acephate are the main active ingredients responsible for food contamination. Profenofos, O-(4-Bromo-2-chlorophenyl) O-ethyl S-propyl phosphorothioate, is a broad spectrum, non-systemic foliar insecticide effectively used against a wide range of chewing and sucking insects and mites. A number of enzymes capable of detoxifying OPs belong to the class of phosphotriesterases (PTE) comprises organophosphate hydrolase (OPH), methyl parathion hydrolase (MPH), organophosphorus acid anhydrolase (OPAA), diisopropylfluorophosphatase (DFP), carboxylesterases (CbEs) and paraoxonase 1 (PON1) (Bigley and Raushel, 2013). The PTEs also known as organophosphorus hydrolase (OPH) are efficient hydrolytic enzyme that can hydrolyze a broad range of organophosphates by the cleavage of P-O, P-F or P-S bonds. Cho, Mulchandani, & Chen, (2002) used the directed evolution approach to enhance PTE catalytic performance, similarly, Chen-Goodspeed, Sogorb, Wu, & Raushel, (2001) performed site-directed mutagenesis of key residues in the active sides mutation on I106G/F132G increased the k_{cat} up to 270 fold.

Petroleum By-Products

Toluene, xylene, BTEX, benzene and ethylbenzene are the petroleum by-products containing short chain aliphatic and simple aromatic hydrocarbons. Gasoline, kerosene, diesel, heavy oils, lubricating oils and Vaseline have varying gradation of increasing carbon chains and complexity. Gasoline contains approximately 1,200 different hydrocarbon, however, diesel and jet fuels contain carbon range of C10 to C28. Heavy petroleum products such as lubricating oil and paraffin wax experience low biodegradation compared to the lighter hydrocarbon, short-chain aliphatics and single-ring aromatic compounds. The branched-chain hydrocarbons are more resistant to biodegradation compared to straight-chain hydrocarbons. Some major microorganisms capable of efficient degradation of petroleum by-products include *Pseudomonas*, *Corynebacteria*, *Mycobacteria* and yeast. The enzymes engaged in the hydrocarbon chain breakdown are AlkB-related alkane hydroxylases, soluble methane monooxygenase (sMMO), particulate methane monooxygenase (pMMO), P450 oxygenase system and dioxygenase (CYP153, class I). Alkane hydroxylases effectively hydrolyzes alkanes from petroleum products. Different types of alkane hydroxylases are known for alkane degradation are soluble methane monooxygenase (sMMO), AlkB-related alkane hydroxylases, particulate methane monooxygenase (pMMO), eukaryotic P450 (CYP52, class II), dioxygenase (CYP153, class I) and Bacterial P450 oxygenase system. Depending upon their mode of action Beilen & Funhoff, (2007) classify alkane hydrolase into three types (i) methane-monooxygenase- degrade methane to butane (C1-C4) (ii) integral membrane nonheme iron or cytochrome P450 enzymes- degrade pentane to hexadecane (C5– C16) and (iii) unknown enzyme- degrade longer alkanes (C17+). Hamme, Singh, & Ward, (2003) discovered the most studied alkane degradation pathways for *Pseudomonas putida* Gpo1, encoded by the OCT plasmid, where the conversion of an alkane into an alcohol is mediated by a membrane monooxygenase and rubredoxin reductase.

Polycyclic Aromatic Hydrocarbons (PAHs)

Polycyclic aromatic hydrocarbons are aromatic compounds made up of two or more fused benzene rings. PAH includes naphthalene, antracene, fluorene, pyrene, benzo(a)pyrene, which accumulated in high concentrations. These compounds are carcinogens and recalcitrant, furthermore stayed for many years in the natural environment. PAHs are conducive to biodegradation by certain enzymes found in bacteria and fungi. PAH degradation under aerobic conditions involves the oxidation of the aromatic ring by specific dioxygenases to complete biotransformation into CO_2 and water (Whiteley and Lee, 2006). The oxidation of PAH is involved by laccases, cytochrome P450 monooxygenases (CYPs) and oxygenases (monooxygenases and dioxygenases). Laccases catalyzes the oxidation of a wide variety of PAHs including phenolic compounds, polyphenols and anilines coupled to the 4-electron reduction of molecular (Alcalde et al., 2005). The CYP oxidized PAHs to catechols, subsequently it is degraded by catechol dioxygenases to harmless products and incorporated into the tricarboxylic acid cycle of microorganisms. The catalytic performance of CYP enzymes have been enhanced by rational design approaches. The modified CYP101 mutants have been generated to hydroxylate naphthalene in the absence of the cofactor NAD (P) H via the 'peroxide shunt' pathway (Joo, Lin, & Arnold, 1999). Directed evolution holds exciting potential for improving the performance of the enzyme. Bulter et al., (2003) developed a mutant of *Saccharomyce scerevisiae* by directed evolution with 8-fold enhanced laccases activity with increased k_{cat} value. Harford-Cross, (2000) developed CYP101 mutant through selective mutations on active site residues F87 and Y96 of the cytochrome P450. Carmichael and Wong, (2001)

introduced two mutations into CYP102, R47L and Y51F, with increased in activity by 40- and 10-fold for phenanthrene and fluoranthene was respectively. Li, Ogawa, Schmid, & Shimizu, (2001) created a triple mutant, A74G/F87V/L188Q, with improved activity on acenaphthalene, acenaphthylene, naphthalene, fluorine and 9-methylanthracene.

Chlorinated Solvents

Pentachlorophenol, trichloroethylene (TCE) and chlorinated solvents are used extensively as cleaning agents. TCE is removed by the action of methanotrophs using methane monooxygenase or toluene monooxygenase (Shields, Reagin, Gerger, Campbell, & Somerville, 1995). Recombinant rhizobacteria (*Pseudomonas* and *Rhizobium*) express a cystein-rich peptide at the cell surface for TCE degradation.

Polychlorinated Biphenyls (PCBs)

Polychlorinated biphenyls include the group of 209 chemical compounds collectively known as **congeners.** These compounds differ in their degree of chlorination and the position of chlorinated sites. PCBs are used as an insecticide, which adversely affects the global environment. They are highly toxic and more resistant to degradation. The 4-chlorobiphenyl and 4,4-dichlorobiphenyl used in industrial applications are recalcitrant and carcinogens. Oxygenases progress the polychlorinated biphenyls metabolism through the addition of two oxygens to the aromatic ring. PCBs can be degraded by microorganisms via a meta-cleavage pathway to yield tricarboxylic acid cycle intermediate and (chloro) benzoate (CBA) (Bruhlmann &Chen, 1999). Biphenyl dioxygenase is a multicomponent enzyme consisting of a terminal dioxygenase, ferredoxin and ferredoxin reductase efficiently degrade PCBs. Enzyme incorporates two hydroxyl groups into the aromatic ring of a PCB congener, which increases the reactivity of the PCBs and make them more susceptible to enzymatic ring fission (Bruhlmann & Chen, 1999). The *Burkholderia cepacia* strain LB400 and *Pseudomonas pseudoalcaligenes* strain KF707 produces stereospecific dioxygenase. Strain LB400 is specific for ortho-substituted PCBs, however KF707 act on para-substituted PCBs (Erickson & Mondello, 1993). DNA shuffling techniques, site directed mtagenesis for *B. cepacia* strain LB400, *C. testosteroni* B-365 and *Rhodococcus globerulus P6,* generates superior region-specific enzymes for PCBs degradation. By using the rational design approach Suenaga, Watanabe, Sato, Ngadiman, & Furukawa, (2002) developed KF707 biphenyl dioxygenase mutant I335F, T376N and F377L which are able to degrade 2,5,2,5-tetrachlorobiphenyl, a PCB congener that remain undegradable by the action of wild-type biphenyl dioxygenase.

CURRENT AND FUTURE ADVANCEMENT IN ENZYME BIOREMEDIATION

Recently, enzymatic bioremediation has become an attractive alternative to the available bio-treatment techniques (physical, chemical, microbial and plant based). From an environmental point of view, the use of enzymes alternative to chemicals or microorganisms has undoubtedly been adventitious. Moreover, development in the design and application of enzyme cocktails for bioremediation has emerged new opportunities (Ji et al., 2014). Enzyme cocktails allow the mixture of enzymes work in combination more effectively. Enzymatic biocatalysts constitute a greener alternative to traditional organic synthesis. This provides appropriate tools for the industrial transformation of natural or synthetic materials under

mild reaction conditions, low energy requirements and minimizing the problems of isomerisation and rearrangement. Mutienzyme and broad substrate specific enzymes are gaining more importance in the recent bioremediation processes. Glutathione S-transferases (GSTs) and cytochrome P450s enzymes are having broad-specificity (Bass & Field, 2011). GSTs a new group of microbial lactonases named as PTE-like lactonases (PLLs) proficiently hydrolyze lactones. Similarly, promiscuous enzymes are furthermore gaining much more significance in current bioremediation processes. Promiscuous enzymes can catalyze more than one reaction (catalytic promiscuity) or show broad substrate specificity (substrate promiscuity). Mutations can increase a promiscuous activity that underlies the divergence of a new enzymatic function (Hult & Berglund, 2007). For the promiscuous enzymes, specificity and promiscuity can reside within the same or different active site. The *Pseudomonas sp*. evolved a kind of promiscuous enzyme atrazine chlorohydrolase (atzA encoded) from melamine deaminase (triA encoded). Melamine deaminase has low activity towards atrazine, however atrazine chlorohydrolase is more effective on atrazine (Scott et al., 2009). Recently, enzyme superfamily proteins focused the significant attentions of researchers or scientist. The members of these protein families are capable of dramatic pollutant deduction. The cupin protein superfamily ring cleavage dioxygenases catalyzes the aromatic ring cleavage in a variety of pollutants such as catechols, protocatechuates, gentisates, salicylates, aromatic amino acids, 2-aminophenols, 3- hydroxyanthranilates, hydroxyquinones and hydroquinones (Vaillancourt et al., 2004). Similarly, VOC superfamily oxygenase is also effectual for bioremediation (Gerlt & Babbitt, 2001). With the advent of molecular engineering the principle of developing a new "designer enzyme" or "novel hybrid enzymes" is being uprising (Okuta, Ohnishi, & Harayama, 1998). The current scenario of enzymatic bioremediation is focused in the development of new innovation on enzyme modification. The implementation of various advancements has been done for the modification of enzyme indented for potential bioremediation. Furthermore, enzyme effectiveness can be improved in vitro using different approaches to generate super bioremediators or superbugs for bioremediation (Furukawa, 2003). Some modern advancement for the enzymatic modifications is as follows:

Immobilization

Immobilization is the method of enzymes fixing by covalent attachment to solid supports, adsorption, entrapment in polymeric gels, cross-linking with biofunctional reagents and encapsulation within a solid support. The immobilization improves the stability and lengthens the half-life of the enzyme. It effectively allows enzymes work in a larger range of environments and therefore allow it to remain active at different temperatures or pH and more resistant to proteolytic degradation. Recently, mutated enzyme has been immobilized with magnetic nanoparticles for the best results (Fang, Si, Tian, Zhang, & Zhou, 2011). A Ser127Ala mutant based on the enzyme glycerophosphodiesterase (GpdQ) from *Enterobacter aerogenes* with improved metal binding abilities has been immobilized using glutaraldehyde on PAMAM dendrimer-modified magnetite nanoparticles (Daumann, Larrabee, Ollis, Schenk, & Gahan, 2014). Immobilized enzymes may prove more economical as they are biochemically stable and can be reused to detoxify pollutant. The foregoing considerations underline the high potential use of immobilized enzymes in detoxification of varied xenobiotics and remediation of petroleum contaminated soils.

Biomolecular Engineering

Biomolecular engineering develops genetically engineer enzymes or microorganisms. Rational design, directed evolution and site-directed mutagenesis are the approaches for enzyme engineering (Steiner & Schwab, 2012). Engineered enzymes with improved activities and stability under selected conditions can be generated by rational site-directed mutagenesis and DNA-shuffling methods. Directed evolution or site-specific mutagenesis improve the existing biodegradation pathways and develop biocatalytic processes with enhanced substrate range and thermo stability (Valetti & Gilardi, 2013). Directed evolution involves the introduction of random mutations in the gene sequence. The desired activity can be changed systematically to all possible other amino acids by site saturation mutagenesis (Cho et al., 2002; Wackett, 1998). The rational design approach constructs a single microorganism containing desirable biodegradation pathways or enzymes from different organisms brought together to perform specific reactions using recombinant DNA technology (Bulter et al., 2003). This approach can be used to engineered whole cell or the enzymes with desired characteristics.

Single Enzyme Nanoparticle (SEN)

The nano-technological approach has been used to improve the enzyme stability in various nanostructures such as nanoparticles, nanofibers, mesoporous materials, single enzyme nanoparticles (SENs) and magnetic nanoparticle. In SENs technique each enzyme molecule is surrounded by a porous composite organic/inorganic network of less than a few nanometers thick mass which stabilizes enzyme activity. The SENs is a new approach distinct from immobilization of enzymes into mesoporous materials or encapsulation (Kim, Grate, & Wang, 2006). On SEN vinyl-group functionality is grafted onto the enzyme surface by covalently modifying the amino groups of lysines with acryloyl chloride. By using small amount of surfactant, these modified enzymes are solubilised into hexane as ion-pair form. SENs can be further immobilized into mesoporous silica with a large surface area, providing a hierarchical approach intended for stable immobilized enzyme systems for various applications such as bioconversion, bioremediation and biosensors. Surface modifications such as silanization, carbodiimide PEG or PVA spacing provide the efficient binding of single/ multienzyme to nanoparticles, further the enzyme stability can be enhanced by the cross linking of glutaraldehyde (Johnson, Park, & Driscoll, 2011). Yang & Zhang, (2013) applied this technique for development of single-enzyme nanoparticles based urea biosensor generation.

Electrobioremediation

Electro-bioremediation as a hybrid technology of bioremediation and electro-kinetics for the treatment of hydrophobic organic compounds (Acuna, Tonin, Pucci, Wick, & Pucci, 2010). It involves the passage of direct current through polluted soil by appropriately distributed electrodes and uses microbe/enzyme. This technique involves the microbiological phenomena for pollutant degradation and electro-kinetic phenomena for the acceleration and orientation of pollutant transport (Li et al., 2010).

Carbon Nanotube

This is the new "one-pot methodology" for a rapid and straightforward fabrication of enzymatically active carbon nanotube (CNT) paper for bioremediation (Mechrez, Krepker, Harel, Lellouche, & Segal, 2014). Carboxylated CNTs are ultrasonically dispersed in an aqueous surfactant solution followed by a microfiltration process to generate a paper-like membrane, which is accumulated from entangled nanotubes. CNT based use of organophosphate hydrolase (OPH) for detoxification of OPs has drawn significant attention. The OPH conjugation to the CNTs is carried out by carbodiimide chemistry during the microfiltration process (Wang et al., 2009). A substantial reduction in methyl paraoxon concentration is accomplished through in situ hydrolysis by the immobilized OPH during the filtration. These thin membranes allow successive filtration cycles to be performed and maintain their enzymatic activity. Due to the unique combination of the mechanically robust CNT scaffold and high OPH loading, this CNT method gained much attention for bioremediation. CNT method presents a new generic approach for the bioactive paper-like scaffold generation, which can be rationally tailored for a various applications.

PROS AND CONS OF ENZYME BIOREMEDIATION

The benefits of enzyme bioremediation are as follows:

- Enzymatic bioremediation is a natural process and perceived as an acceptable waste treatment process.
- The enzymatic bioremediation does not generate toxic side-products.
- Following treatment, the enzymes are digested on-site by the indigenous microorganisms.
- The enzymes significantly enhance their bio-availability efficiently with organic solvents.
- Enzymes are effective even in drastic operational conditions.
- Enzymes are stereo-specific with narrow or broad specificity and can be applied to a vast range of compounds.

There is only few limitation of enzyme bioremediation:

- Conversely, the enzymatic bioremediation is still expensive, having short lifetime and low stabilization.

CONCLUSION

Bioremediation is a proficient technique for cleaning up pollution by adopting the same biodegradation processes that occur in nature without damaging the environment and indigenous flora and fauna. Conversely, despite being a panacea to the safe and effective environmental cleaning, bioremediation has limited applications due to the substrate preferences, environmental variability, limited bio-degradative potential and lack of awareness. The development of knowledge about the enzymatic response to pollutant degradation expands the new horizon of cost effective and potentially significant advance environmental cleaning. Enzymes have great environmental advantages and are preferred over chemicals

and microorganisms as they do not generate toxic side products. The enzymes use can be boosted by the production of enzymes at a higher scale with enhanced stability and activity at low cost. The main advantages of using enzymes in these scenarios are their favourable unique properties, basically their biodegradability and high chemo, regio and stereo selectivity. With the recent advances in biomolecular engineering, the prospect of short-circuiting the process of natural evolution to degrade environmental pollutants has been created. Biomolecular engineering is successfully used to improve the capabilities of the enzymes in bioremediation systems. This is opening exciting new panorama for enhancing bioremediation programs in the coming years. Recent introduction and development of novel recombinant DNA technologies such as meta-genomic and directed evolution has a profound positive effect in the expression and production of recombinant proteins with tailored catalytic activities. The engineering of enzyme at a commercially acceptable price and catalytically optimal properties suggest a promising future for environmental biocatalysis. Still, much work yet demands to be done before field applications of engineered enzymes. Future research should emphasize to evaluate the effectiveness of enzymes, the cost of enzyme production, culturing microorganism and develop engineering processes to design parameters and system for optimal enzyme kinetics. In conclusion, enzyme bioremediation has been implemented globally and opened the new era of pollutant eradication for safe, clean and green environment.

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KEY TERMS AND DEFINITIONS

Biomagnifications: The process which accumulates excessive concentration of any substance in an organism at higher level in food chain.

Bioremediation: Technique which degrades the pollutant by biological means such as microorganisms (Micro-bioremediation), plant (Phyto-remediation), enzymes (enzyme-remediation), etc.

Enzyme Cocktails: The mixture or combination of different enzymes, which make it more effective and efficient for any catalytic reaction.

Green Chemistry: Design of chemical products and processes that reduce or eliminate the generation of hazardous substances.

Persistent Organic Pollutants (POPs): Are the organic compounds which are resistant to environmental degradation through chemical, biological and photolytic processes.

Promiscuous Enzymes: Are the enzymes which can catalyze different side reaction in addition to the main catalytic reaction. Although, the promiscuous activity is quite less compared to the main catalysis reaction but this is an additional and significant attribute of enzyme for bioremediation.

White Biotechnology: Uses of enzyme or microorganism in various industries for the production of chemicals, pharmaceuticals, food ingredients, energy, paper and biofuels from renewable resources.

Chapter 18
Fundamentals of Electrostatic Spraying:
Basic Concepts and Engineering Practices

Manoj Kumar Patel
Academy of Scientific and Innovative Research, India & CSIR-Central Scientific Instruments Organisation, India

Chirravoori Ghanshyam
CSIR-Central Scientific Instruments Organisation, India

ABSTRACT

The desired attributes of electrostatic spraying are uniform deposition onto both directly exposed or obscured crop surfaces which minimize the off-target losses of active ingredients to soil, water, atmosphere and provide more effective and economical pest control. This chapter presents an overview of electrostatic spraying technologies in the field of agriculture emphasizing the key role of advanced electrostatic instrumentation and chronicles the scientific innovations in the parlance of providing cost effective and reliable commercial systems along with an insight on the needs of future research perspectives and directives. It is aimed primarily at a familiarization with spraying concepts and engineering practices. This text is to bridge the knowledge and experience gap among researchers and technology developers and the people involved in electrostatic processes applied to agriculture and food processing. It will also introduce the engineering aspects of design and development of an electrostatic spraying nozzle for agricultural applications.

INTRODUCTION

Air-assisted electrostatic sprayers are advanced agri-instruments for efficient use of pesticides to agricultural crops, orchards, plants, trees etc. The electrostatic spraying technique is all about reducing the use of pesticide by increasing the efficiency and bio-efficacy. Bio-efficacy is a measure of the biological efficacy of an active ingredients of agro chemical such as insecticide etc. The methods used to perform the function of bioremediation are known as bioremediators. Electrostatic spraying is to be the one

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among the available spraying techniques in precision agriculture and food processing. For example, the electrostatic spraying technique can be used for protective biomaterial coatings to fruits and vegetables for resistance towards microbial attacks, to enhance the transportation life, to control spoilage microorganisms, antimicrobial sprays for enhanced food safety etc. It is a method which reduces the environmental pollution by reducing contamination to soil as well as air. In totality, it reduces the chemical consumption which is used indiscriminately through conventional methods such as pedestal-mounted sprayers, the high pressure spray guns, the hand pressure swirl nozzles and the consecutive high volume spraying systems etc. The trans-disciplinary aspects of the embryonic field of electrostatic spraying have provided a major motivation to agricultural and food processing researchers for the development of novel techniques for spraying liquid pesticides to crops and orchards, protective coatings to food and food packaging, in addition to other applications of sprays to industrial, manufacturing and transportation, medical facilities and devices etc. This chapter is to be one among the motives behind the renewed curiosity in the usage of the electrostatics in liquid based spraying.

Although, organic measures for crop protection are being preferred, chemical intervention is still the fastest and most economical way for crop protection. However, due to lack of awareness and ignorance, pesticides are being used indiscriminately leading to side effects on human health and ecosystem. Electrostatic method of pesticide application reduces off-target drift, environmental pollution and human health risks and increases the bio-efficacy and mass transfer efficiency onto the biological surfaces of crops and trees with uniform back deposition. Law, (1978); Jia, Xue, Qui, & Wang, (2013) explained the design and development of induction based electrostatic sprayer for agricultural usage and evaluated the performance. So far, the equipment available in the market are uncontrolled in terms of spraying variability. Pesticide application control, targeted pesticide delivery and variable pesticide spraying are the key to improve operation quality, reduce chemical waste, environmental pollution and operational costs. This entices to develop a sensing mechanism which would discriminate between the presence and absence of pesticide application surfaces. He, Yan, & Chu, (2003) developed the automatic target detecting air-assisted electrostatic orchard sprayer. In this spraying system, the sensing mechanism is based on infrared proximity sensors which determine the presence and absence of target to be sprayed. Other than infrared proximity sensors, ultrasonic sensor mechanism is another substitute for target detection and canopy mapping. Sensory attributes stipulate a good approximation of target and canopy mapping for targeted delivery of pesticides to actual target. Automation and mechanization with respect to agricultural pesticide spraying is one of the naive research topics in the present scenario.

The last decade has witnessed the application of existing electrostatic techniques to various fields accompanied with rapid improvements of the spraying technology. Zhang, Srirama, & Mazumder, (2007) have worked on a new approach in signal processing and sampling which shows that electrostatic applications have gone beyond the earth and reached to Lunar and Mars missions. Space research needs electrostatics in dust and particle control. Mazumder et al., (2006); Hamid, & Atan, (2008); Ghayempour & Mortazavi, (2013); Khan, Maan, Schutyser, Schroën, & Boom, (2013) ; Zhang, Kobayashi, Uemura, & Nakajima, (2013) have shown the numerous applications of electrostatic spraying to various fields such as agriculture, medical, transportation, painting and industrial applications, though agriculture remained the main area of research during the last decade. Electrostatic application to agricultural pesticide spraying has revolutionized agriculture farming scenario by making advances and developments via off-target pest control to increase bio-efficacy and deposition efficiency. There is also an increase in deposition efficiency by applying suitable charging techniques, better corrosion resistant material used in electrode design for spray charging and its insulation, liquid atomization methods, post processing of the spray by

air assistance, automation of spray system and cost effective compact design and development for the layman in terms of easy-to-handle and simplicity of use. Developing an automated system for greenhouse spraying is the biggest challenge in the current scenario of research, since working environment inside the greenhouse is unbearable and dreadful due to the presence of increased level of humidity, carbon di-oxide and high temperature. Prolonged exposure of greenhouse workers to these conditions leads to a scratchy and hazardous work environment. Significant novel and innovative thoughts also came into existence; however, technical improvements and applications of the electrostatic spraying technique to various fields remain the main focus.

This chapter reviews the advances made in electrostatic pesticide spraying to agriculture and a brief description has also been made for the charge to mass determination, software like Fluent, CFD (Computational Fluid Dynamics) used in hydrodynamics and aerodynamics processes to simulate the electrostatic models with user defined functions and Deposit Scan to measure the percentage coverage, spray deposition rate and distribution of the charged droplets etc. It seeks to give a fundamental understanding of basics of electrostatic spraying to agriculture; along with the intricacies associated with the design and development of such high performance, efficient, efficacious modern advanced agri-instruments. In this chapter, innovative and technical concepts have been summarized and explained in detail that came into existence in electrostatic spraying along with the prevue of future perspectives and needs in the current scenario. However, before getting involved with the intricacies of electrostatic pesticide spraying, one may discuss the need for and applications of electrostatics in spraying.

Purpose of Pesticide Spraying

The use of pesticides to crops, orchards, plants and trees is among the most important pre-harvest facets of precision agriculture to protect the crops and for boosting the food production. Abhilash & Singh, (2009) discussed the importance of pesticides in the process of development of sustainable agriculture and shown that pesticides have become an important tool as a plant protection agent. However, exposure to pesticides both professionally and ecologically causes a number of human health concerns. The application of pesticides is one of the most frequently used methods to protect crops, orchards and trees against diseases and insects in agriculture. Off-target drift of pesticides is a term used for individuals droplets containing the full of life ingredients that are not lay down onto the target area, when spread-over crop protective pesticides to agricultural targets. The droplets most susceptible to off-target drift are usually smaller in size, less than 150μm in diameter (usually indicates the Volume Median Diameter, VMD) and effortlessly moved off the actual target area by wind, gravitational force or other agro-climatic, harsh and transient environmental conditions. The larger droplets settle down on the ground because of gravitational force. For example the conventional spraying statistics for grape growers shows that almost 60-70% of the pesticides have been lost via off-target drift and non-target deposition. Underside of the leaves and hidden areas remain untouched with the sprayed chemicals.

Efficient use of pesticides can protect and optimize the environment and natural resources, since the use of non-renewable pesticides and fertilizers is perhaps irretrievable, and encompasses an environment cost. Crop protection chemicals deposited in undesirable areas raised serious concerns caused by spray drift, such as surface water contamination, damage to sensitive neighboring crops, health hazards for living individuals as well as masses and possible adulteration to the target and adjoining areas or possible over application within the target area. Off-target drift destruction can be managed by identifying and regulating the factors that affect it such as environmental conditions, equipment and methods used for liquid spraying etc., later being the most predominant factor to avoid such consequences.

Causes of Spray Drift

Managing spray drift improves pesticide bio-efficacy by guaranteeing that the correct amount of dose reaches at the target. Drift takes away the pesticide from the intended target, making it less effective, and deposits it where it is neither needed nor desired. There may be two kinds of drift in agricultural pesticide spraying:

- Finely divided spray drift is off-target movement of the spray particles.
- Vapor drift is the volatilization of the pesticide segments and their movements away from the actual target.

The pesticide then becomes an environmental pollutant in the off-target areas where it can injure susceptible vegetation, contaminate water, or damage wildlife. A number of variables underwrite to spray drift; these are predominantly due to the spray equipment system and meteorological factors:

- Droplet size and spray height.
- Operating speed, direction and wind velocity.
- Air temperature and humidity.
- Crop protection chemicals and carrier volumes.

Various officialdoms conduct rigorous distribution and drift testing. The reliable data is also very important when conducting drift experiments and it comes from independent testing agencies, such as Food and Environment Research Agency (FERA) in the United Kingdom, the Julius Kuhn Institute (JKI) in Germany and the Centre for Pesticide Application and Safety (CPAS) in Australia. Varieties and consumption of pesticides worldwide have been increasing dramatically as increased human population and crop production. In this process, misuse of pesticide becomes more and more serious, has resulted in heavy environmental pollution and health risk of living beings. Study shows that, even a fraction of percentage of efficiently sprayed protective pest by using advanced spraying equipment such as electrostatic nozzle, is sufficient for the desired biological target as compared to conventional methods.

The charged particle evaporation, droplet instability and its implications to agricultural spraying are justifiably explained and presented theoretically as well as experimentally by Law & Bowen, (1975). The surface energy condition can be drastically altered by the presence of unbound surface charge on an air-borne spray droplet. Evaporating droplet carries no charge as a result an increase in surface charge density is witnessed. This increase in charge per unit area cannot increase infinitesimally; approaches a critical level, called Raleigh limit of charge, at which the liquid surface ruptures and gives birth to small daughter droplets.

ELECTROSTATICS AND PESTICIDE SPRAYING: THEORETICAL CONCEPTS

Electrostatic force field application in agricultural spraying is a new technique to apply for the protective liquid pesticides and to reduce the off-target drift. It improves the efficiency of the system and bio-efficacy of the biological crops. For example, air-assisted electrostatic spraying, aerial electrostatic spraying system assembled in Helicopter and Tractors, are the modern systems used in agricultural pesticide

spraying applications. In electrostatic spraying, finely divided droplets are charged by the application of high electrostatic potential to charging electrode. The system should work at lowest possible applied voltage so that it will work on low power consumption and hence the duration of the power supply. For efficient working of the system, ultimately one has to increase the charge to mass ratio at lower applied voltage as there is a trade off between applied potential and power consumption.

Electrostatic space charge and induced image charge forces enhance the uniformity of spray on the target surface, increase the transfer efficiency, bio-efficacy and adhesion. Electrostatic forces minimize the effect of gravitational force which is the main cause of spray drift and ground fall of the pesticide. The electrostatic forces levitate the charged droplets against the gravitational force; however levitation of the droplet depends upon the droplet diameter, surface tension, applied electric field and density of the liquid. The electrostatic spraying process is a very complex phenomenon which can be divided into three basic regions:

Hydro Electrodynamics

It mainly consists of liquid atomization and finely divided particulate matter charging. Liquid atomization can be achieved through centrifugal, pressure, air, hydraulic, pneumatic or electrostatic forces solely or by a combination of two or more of these forces. Chigier, (2007) showed the Challenges for future research in atomization and spray technology. Droplet charging methods include conduction, induction, tribo-electric, and corona charging; the induction charging is predominant in conductive liquid pesticide spraying.

Aerodynamics

It includes charged particles/droplets transport and charge retention. In the droplet transport region, aerodynamic, inertia, electrostatic and gravitational forces work together to determine the trajectories of the charged droplets.

Electrostatics

It mainly involves the actual spray deposition on the target; either it is conducting or non-conducting target. Electrostatic deposition onto a conductive target relies upon a displacement current to transfer electric charge onto or off the target to a degree appropriate for maintaining it at earth potential in the presence of the approaching charged-particulate cloud. In electrostatic spraying, it is assumed that the charged particulate matter is a slow moving phenomenon and therefore magnetic Lorentz force is negligible in comparison to electrostatic force.

In electrostatic pesticide spraying, the following fundamental phenomena are of great importance which have to be taken into consideration for the better understanding of the subject:

- Droplet charging methods.
- In-flight trajectory of charged droplet.
- Optimizing the deposition field and transient effects at deposition targets.
- Charge to mass evaluation and hence efficiency and efficacy of the spraying system.
- Understanding the drop size and its measurement.

Droplet Changing Methods

Research approaches to agricultural spray charging have been based upon several distinct principles. Maski & Durairaj, (2010) showed in their previous work that a major portion of the electrostatic pesticide spraying has been in the development of reliable means for droplet charging. Motion of a charged particle can be easily controlled by the electric force, which depends on the charging level. Therefore, it is desirable to charge the particle to as high as possible and the charged droplet must be acted upon by an electric field. Well known and field-proven methods for imparting the necessary and sufficient charge to pesticide spraying droplets are divided on the basis of conductivity of the liquid to be sprayed:

- Ionized-field droplet charging (for both types of conductive as well as non-conductive liquids).
- Electrostatic-induction droplet charging (only for conductive liquids).
- Direct contact charging (for non-conductive liquids).

Law, (1984) presented a thorough consideration of these charging techniques and pros and cons accompanying with each method, accentuating their applicability as dictated by the physical properties of the pesticide-liquid to be electrified. Each method of charging has advantages and disadvantages in terms of liquid conductivity to be electrified, the level of applied voltage, insulation from the rest of the associated device, power consumptions etc. Induction charging is the most predominant method of charge electrification to fine droplets in pesticide spraying.

Ionized Field Droplet Charging

In corona charging, a sharp electrode is held at a high potential resulting in a local electric field high enough to ionize the surrounding air. The positive charges in the ionized air are less mobile than the negative ones and so remain in the vicinity of the electrode long enough to be picked up by a passing liquid stream. It requires a high applied voltage ranging from few thousands to more than a hundred thousand volts, depending upon the geometry of the charging equipment. Either solid or liquid particles, of diameters larger than approximately 0.5 μm, travelling through this ionized-field region can acquire by ion attachment, a saturation charge dependent upon the particles dielectric constant, its surface area, and electrical characteristics of the corona discharge. Ionized-field charging theory has been well developed mathematically by Law, (1984) and can be used to calculate the net electrical charge imparted to an air-born particle as:

$$q_p = f\left[1+2\frac{K-1}{K+2}\right]4\pi\varepsilon_o E_o r_p^2 \tag{1}$$

where K is the dielectric constant, f is the saturation factor, E_o is the applied electric field and r_p is the radius of the droplet. The fraction of the saturation charge actually attained by the particle depends upon the residence time, and the concentration and mobility of the ion in the charging field. For aqueous-based sprays (K=80) charged to half-saturation (i.e. f=5) in a typical corona-discharge nozzle, droplet charge in coulombs typically attains a value of:

$$q_p \simeq 6\pi\varepsilon_o E_o r_p^2 \tag{2}$$

With an associated charge to mass ratio of

$$\frac{q_p}{m_p} \simeq \left[\frac{9\varepsilon_0 E_0}{2\delta}\right]\frac{1}{r_p}$$

C/kg, where δ represents the surface tension of liquid. While the ionized-field charging method is routinely used in a variety of commercial and industrial processes ranging from xerography to electrostatic precipitation, greater care must be exercised in properly designing the process into agricultural spray-charging devices, in order to maintain long-term charging reliability. Difficulties relate to the fragile nature of the exposed corona electrode, to the elevated ionizing voltage required (typically more than 15kV), and to the onset of reverse ionization from the passive electrode when inadvertently wetted or coated with resistive particles.

Induction Charging

In 1980s, the work led by S. Edward Law at the University of Georgia, developed induction based spray-charging method meeting the engineering design requirements of robustness, simplicity, reliability, energy efficiency, and safety. The electrostatic-induction and the ionized-field spray charging methods are the ones widely used throughout many industrial processes. Agricultural charging based nozzle development has mainly relied upon the induction charging methods. This section presents a detailed consideration of induction spray-charging techniques, emphasizing their applicability as dictated by the physical properties of the pesticide-liquids to be electrified.

Spray liquid is normally electrically neutral. To charge a spray, the normal balance between positively charged protons and negatively charged electrons has to be disturbed so that spray droplets carry either a net positive charge or net negative charge. Droplets with same electrical sign (+ or -) repel while those with opposite charges attract.

Electrostatic induction has proved to be a very satisfactory alternative to the ionized-field method of charging spray droplets for agricultural pesticide applications. Figure 1 indicates the complete picture for understanding the induction charging phenomena. In this method, direct-transfer to the droplet-formation zone of a liquid jet results from electrostatic induction of electrons on to the continuous liquid jet and in order to maintain it at ground potential the presence of closely positioned induction electrode of positive polarity is required. Droplets, formed from the surface of this negatively-charged jet, will depart with net negative charge provided the droplet-formation zone remains subject to the inducing electric field acting between the non-ionizing electrode and the liquid jet.

Gauss's law indicates that maximum droplet charging should occur for the droplet-production zone located at the region which provides maximum field strength at the terminal surface of the liquid jet.

In induction charging of the spray droplets, two time constants are of importance:

1. Time constant of charge-transfer (τ)
2. Droplets formation time constant (t_f)

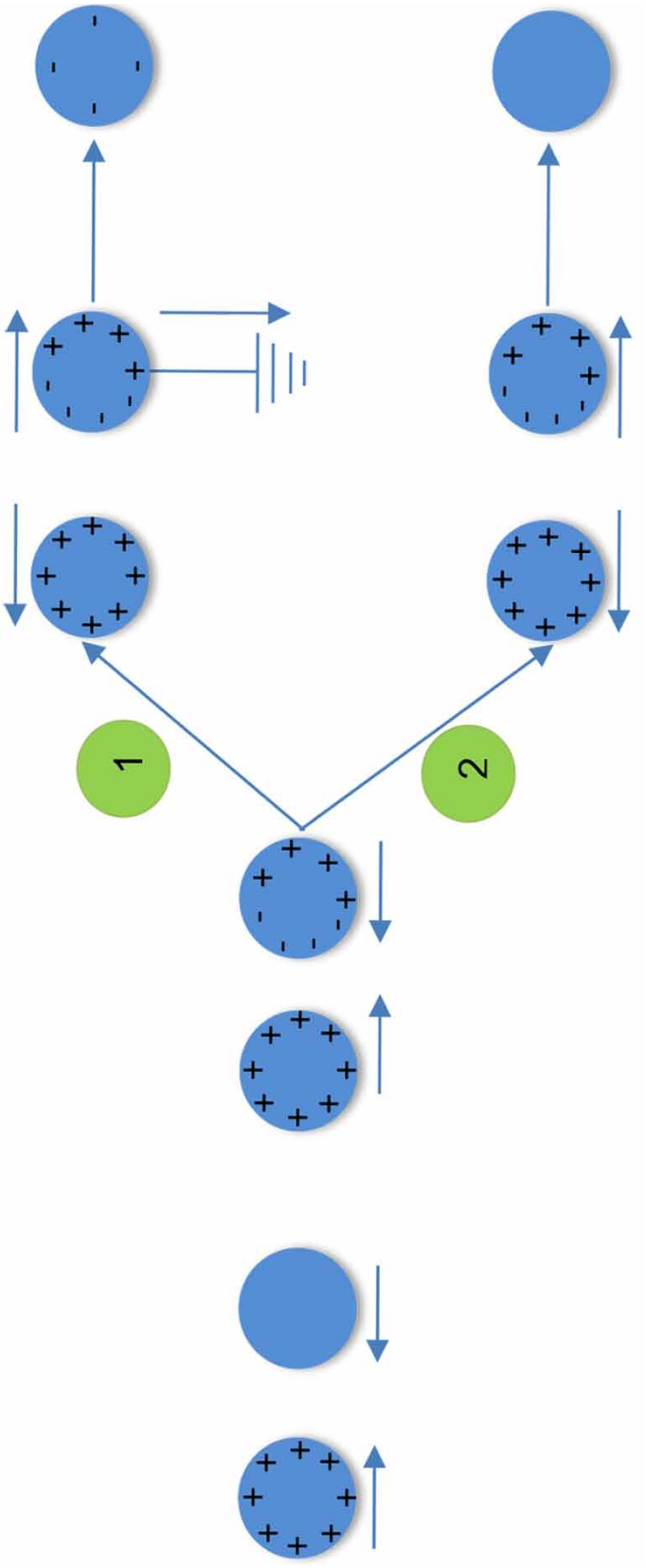


Figure 1. Induction charging of finely divided particulate matter

The level of droplet charge imparted by electrostatic induction depends upon the relative time rate of charge transfer to the droplet-formation zone, as compared with the time required for droplet formation. The charge-transfer capability by induction from a grounded metal nozzle through the issuing liquid jet depends upon the electrical as well mechanical properties of the continuous liquid jet.

The two most important properties of the liquid to be sprayed are:

1. Electrical properties such as conductivity, dielectric constant, permittivity etc.
2. Mechanical properties such as density, viscosity, surface tension etc.

For pesticides, the spray-liquid characteristic may be specified by the charge-transfer time constant, which is a function of the electrical conductivity (σ) and the dielectric constant (K) of the liquid as:

$$\tau = \left(K\varepsilon_0\right) / \sigma \tag{3}$$

If a duration of time t_f, characterizes formation of discrete droplets from the continuous jet, then spray liquids must satisfy the condition $\tau < t_f$, in order to satisfactorily charged by electrostatic induction process. In electrostatic spraying of pesticides, the conductivity of liquid to be sprayed is of inordinate significance and it is to be taken into consideration, when such a system is developed. The system must cover all the ranges of liquid based pesticides used as sprays.

Conduction Charging

In the case of charging by conduction, the DC electric supply is made by contact with the spraying liquid. In case of the conduction charging, the mechanical energy is used for dispersing the liquid into the droplets and the current that leaks to the power supply and assures a part of the electric energy for charging and accelerating the droplets. Both the mechanical energy and the electrical energy drive the charged drops to the target. The electrical energy depends on the nozzle current and the power supply voltage. Each droplet-charging method possesses its unique advantages as well as disadvantages in relation to technical and engineering requirements of vastly different pesticide-application situation encountered.

Droplet Charge Limit

The amount of charge that each individual drop can carry is limited. There is essentially two physical mechanisms limiting the charge that can be retained on the droplet surface, one is Rayleigh limit and other is Paschen limit. *Rayleigh limit:* In an electrostatic spraying process, as the droplets are charged by induction charging, the saturation charge is reached instantaneously. It takes a finite time for the particle to achieve this saturation charge. When the electrostatic force of repulsion is greater or equal to the attractive force due to surface tension, the droplets disrupt to smaller droplets and produce daughter droplets (Allah, 2002). The process results in a fine mist of highly charged droplets.

It has been shown that for dielectric drops in the presence of significant electric fields, particularly within spray plumes, the maximum charge a drop may hold is less than the Rayleigh limit of charge. The Maximum charge acquired during the induction charging process by a conducting sphere is limited by Rayleigh limit as shown in Equation (4) given by (Shrimpton, 2005):

$$q_{max} = \sqrt{64\pi^2\epsilon_0\delta r^3} \tag{4}$$

where, δ is the surface tension of the droplet and *r* is the radius of the droplet. *Paschen limit*: is another mechanism where the avalanche ionization process and localized electrical discharge can occur at the surface of a charged drop when, the drop charge exceeds the limit and that charge to mass ratio can be given by equation (5):

$$\frac{Q_p}{m_p} = \frac{12\varepsilon_o V_p}{\rho D^2} \text{C/kg} \tag{5}$$

where Q_p is the Paschen charge limit, m_p is the liquid mass flow rate, ρ is the density of liquid, D is the diameter of the droplet and V_p is the surface potential.

In-Flight Trajectory of Charged Particulate Matter

The maintenance of charge on electrified clouds of airborne spray enroute from charging nozzles to grounded-plant targets should be maintained. Once the droplets are charged sufficiently, the droplets have to traverse the distance to reach the actual target canopy. Common concern of electrostatic spraying is the exploitation of the most favorable force field for achieving the desired motion and the maintenance of droplet charge during the in-flight trajectory of the electrostatic spray. There is no ideal charge to mass ratio for spraying, however higher the ratio, the better is the efficiency of electrostatic spraying. Various studies reported different minimum level of charge to mass ratio i.e. 0.8, 1.2, 2.0 mC/kg etc. Laryea & No, (2004) have shown that these charge to mass ratio values can be used as references, recent studies have shown that these lower values result in an increased spray deposition on the target.

The change in charge to mass ratio is due to the existence of naturally-occurring free charge and electric fields of the earth's atmosphere. In normal air near the earth's surface, ionization by cosmic radiation and background radioactivity typically provide a charge creation rate c_i=1-7 ion pairs per cubic centimeter per second. The recombination coefficient α_i for these positive and negative ions is approximately $1.6*10^{-6} cm^3 sec^{-1}$. Since the rate of ionic recombination depends upon this coefficient as well as upon concentration (n_i) of both positive and negative species, the air's ion pair concentration is governed by the relation, $\frac{dn_i}{dt} = c_i - \alpha_i n_i^2$. Thus, a charged pesticide spray cloud will encounter some degree of neutralization by the following two actions: 1) traversing a region of ionized air; and 2) causing migration of oppositely charged air ions into the region of charged sprays. Spraying distance and its consequences on the performance of the nozzle has been discussed by Robson et al., (2013), at Federal University of Vicosa, Minas Gerais Universidade Federal de Vicosa, Vicosa- Minas Gerais and it has been concluded that for the liquid deposition, the electrostatic system was affected by the target orientation and spraying distance.

Optimizing the Deposition Field and Transient Effects at Deposition Targets

While inverse-square image-charge forces are significant for achieving deposition at millimeter droplet-to-target spacing, only the space-charge field is relevant for electrically driving charged droplets over greater distances within shielded plant canopies. Significant research effort has therefore been directed toward managing the space-charge electric field for enhanced target deposition. Findings have important practical implications regarding improved designs of portable, human-carried, electrostatic sprayers. A further strategy to enhance pesticide droplet deposition, under constraints limiting the dielectric breakdown of the space-charge field has been theoretically developed. In contrast to deposition benefits derived from surface-charge accumulation on plastic films under and above crops, charge buildup on the actual target plants must be avoided so that they will remain near ground potential. A target's inherent charge-relaxation time constant must accommodate this charge transfer i.e., displacement current to earth as charged conductive droplets impinge Law, (2001).

Charge to Mass Evaluation and Hence Efficiency and Efficacy of the Spraying System

The use of pesticides should be performed efficiently and judiciously to increase the bio-efficacy of the biological plants and trees, since its application without the appropriate technology can cause contamination of the soil and environment, leaving residues in food with an increased risk of farm workers' health. Numerous experimental tests have been conducted over the past quarter century which validate electrostatic crop-spraying technology as currently practiced by various field machines. Spray mass-transfer results by many researchers, both in the laboratory and the field; have generally verified 2-8 fold increase in target deposition, as well as improved spatial distribution on plant surfaces, greater deposition density, attributable to electrostatic forces.

Charge to mass ratio is a key term defining the performance of spray equipment in electrostatic spraying applications. Charge to mass ratio determination may vary as the field of application changes such as agricultural pesticide spraying, medical, powder coating and painting, however, the basics behind the measurement of charge to mass ratio is same. The effectiveness of various electrostatic applications depends directly or indirectly on this parameter. It is a critical measure that needs to be determined in order to accurately predict the behavior of a particle exposed to inertial, electrical and gravitational forces. A brief review has already been given by Toljic, Adamiak, & Castle, (2008) for the determination of particle charge to mass ratio distribution in electrostatic applications. Brown, (1997) divided charge to mass ratio measurement techniques into two categories. First, a static method of charge measurement, measures the amount of charge present in droplets directly. Second, the dynamic method for calculating the charge to mass ratio by observing the particle motion parameters in the presence of external electric field.

Static Method

In static method of charging, Ye, & Domnick, (2003) present a numerical method for the calculation of electric field with space charge in electrostatic powder coating with a corona spray gun and used a suction-type Faraday cup apparatus to measure the charge to mass ratio. An extended commercial Computational Fluid Dynamics (CFD) code was used for numerical simulations. Their calculated charge to

mass profile based on Pauthenier theory showed a good agreement with the experiment for particles with radius less than 60μm. This is a good agreement with surface area theory. For particles with larger diameter, the experimental charge to mass values stayed constant which is in disagreement with theoretical knowledge. They conclude that this was probably due to the experimental uncertainty for the large particles. From the obtained results, it was evident that the sensitivity of this instrument decreased with increasing mean particle diameter.

In agricultural pesticide spraying the common mathematical method for measuring charge is based on charge to mass ratio that is characterized by measured spray current and mass flow rate.

There are two methods of measuring the charge to mass ratio and hence performance of the spraying system:

- Faraday cage method
- Conductive plate method

In both the methods, the charged liquid spray was collected at a specific time and weighed. The spray current was divided by the mass flow rate to determine the charge to mass ratio. Both the performance measuring methods are nicely explained by Mamidi, Ghanshyam, Patel, & Kapur, (2013); Patel, Ghanshyam, & Kapur (2012b) in their previous research work conducted at CSIR-Central Scientific Instruments Organisation, INDIA. In these methods of charge to mass ratio measurement, the spray strikes onto the target, due to potential gradient the current flows and this current can be measured by an electrometer connected to the Faraday cage or conductive plate electrode as shown in Figure 2.

Dynamic Method

The dynamic method for calculating the charge to mass ratio by observing the particle motion parameters in the presence of external electric field involves the Phase Doppler Anemometry (PDA) technique, a nonintrusive optical method for simultaneous measurement of the size as well as the velocity of spherical particles. Kulon, Malyan, & Balachandran, (2003) discussed that it is a noninvasive method of measurement of the charge level on a population of particles by combining the Phase Doppler Anemometry technique and high-resolution computer-controlled traversing system.

The principle of the Phase Doppler Anemometry (PDA) is based on light scattering from two-plane light beams incident on the particle. The intersecting coherent and polarized laser beams form a small measurement region of light and dark fringes within the spray. The phase shift between the signals from different detectors is proportional to the size of the spherical particle for reflection represented by Equation (6),

$$\Phi = \frac{2\pi d_p}{\lambda} \frac{\sin\theta \sin\varphi}{\sqrt{2\left(1 - \cos\theta \cos\varphi \cos\psi\right)}} \tag{6}$$

where, λ is the wavelength of the laser light, d_p is the inter-planar distance, θ is the angle between the incoming laser beams, φ is the scattering angle and ψ is the elevation angle. The velocity measurement is based on the Doppler Effect. As a droplet passes through the measurement region it scatters light at a

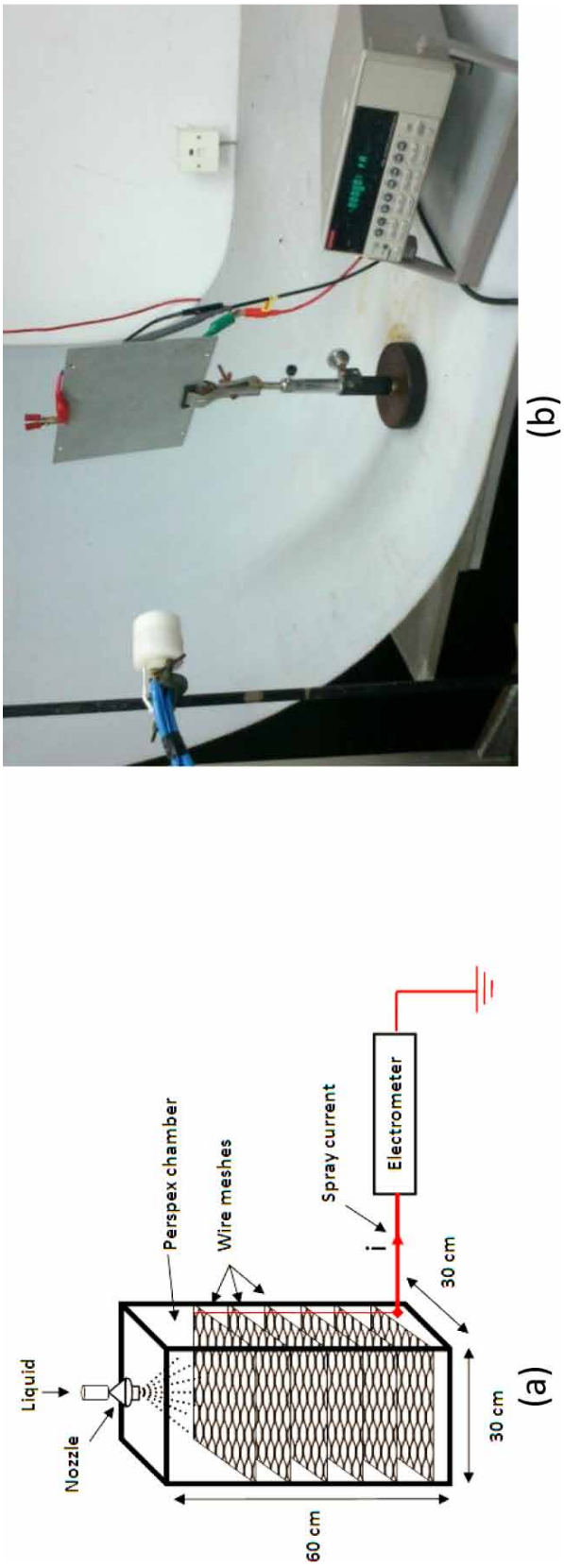


Figure 2. Common methods to measure the charge to mass ratio (a) Faraday cage (b) Conductive plate

frequency based on its velocity normal to the fringes and its spacing. A receiving device measures the frequency of the scattering signal and the spacing of the fringes is determined based on the wavelength of the laser light and the angle between the beams. Knowing the Doppler frequency f_d, frequency of the scattered signal f_s and the spacing of the fringe d_f, the particle velocity v can be calculated as follows in Equation (7):

$$f_d = \frac{f_s + v}{d_f} = \frac{f_s + v2\sin \pi/2}{\lambda} \tag{7}$$

The PDA system was used to track the motion of charged particles in air in the presence of a dc electric field within the space between the parallel-plate electrodes. Charged particles exposed to the external electric field and situated in a viscous medium experience two types of force exerted on them: external electrical force and drag force as a result of a relative motion of a particle in the air. After the relaxation time, a particle attains mechanical equilibrium and reaches a steady state velocity relative to the medium.

Laser Velocity Meter (LDV), an important part of the Electronic Single Particle Aerodynamic Relaxation Time (ESPART) analyzer, is used extensively in non-invasive measurements of fluid flow, turbulence characteristics, and particle dynamics. ESPART analyzer can measure the statistical charge and size distribution of charged particulate simultaneously and it is widely used in industrial applications for the analysis of toner particles, drug powders, oil droplets, dust particles, pesticides charged particulate. In ESPART analyzer, the particle motion in an electric field is analyzed in noncontact manner using LDV or by image analysis. The ESPART analyzer is based on the motion of particles suspended in a gaseous medium when the particles are subjected to an external electric field. The measurements of size and charge of the particles, derived from their relative motion in the gaseous medium, are based on the assumption that the particle motion is within Stokes regime, which is valid only when the Reynolds number of the particles motion in less than 0.1. Some specifications of ESPART analyzer are:

Particle Charge Range

The ESPART analyzer can measure electrostatic charge on each particle in the range from zero to its saturation value, limited by many factors like breakdown strength of the surrounding medium, Rayleigh limit etc., with positive or negative polarity.

Particle Count Range

The maximum count rate will be limited to less than 500 counts per second when signal-to-noise ratio of the Doppler signal is high. At an excitation frequency higher than 2.0 kHz, the maximum count rate depends upon the minimum time required by signal and data processing circuitry for real-time size and charge analysis.

Particle Size Range

ESPART analyzers have been built based on both LDV measurements for small particles (diameter < 50μm) and with image analyzers for larger particles (20μm <d_a<100μm). ESPART analyzer is capable of measuring particle size and charge distributions in different atmospheric conditions.

Understanding the Drop Size and Its Measurement

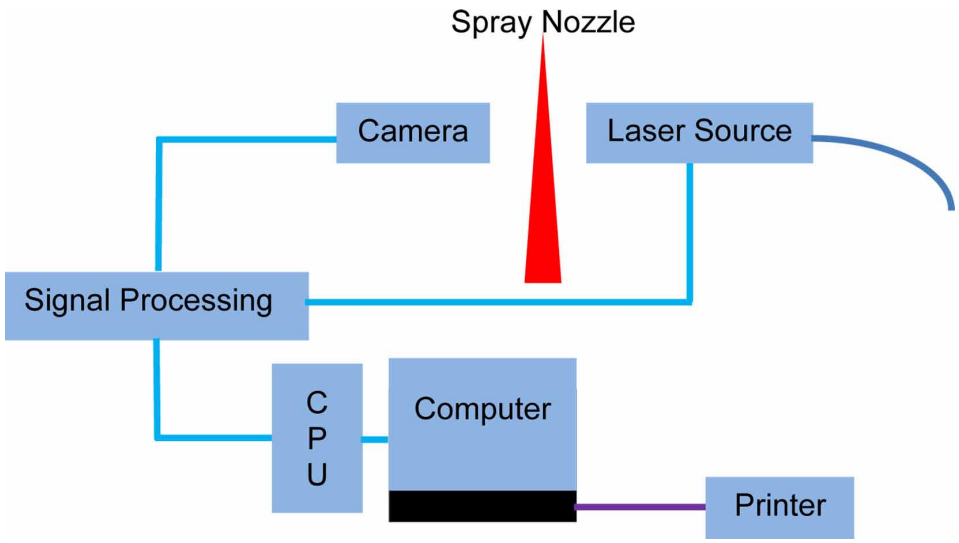
Optical Image Analyzer

Among available methods of droplet size and to visualize the detailed time-resolved structure of electrospray, a simple and sophisticated system i.e. optical image analyzer can be used to study the different mode of spray with the increase in applied voltage and other parameters. A schematic of a typical optical image analyzer is shown in Figure 3. The measurement range of these instruments varies from 1μm with no definite upper bound restriction as the system optics determines the upper range. In such kind of measurements, a high speed camera is implemented to visualize the detailed time-resolved structure of electrospray. The measurement records the shadow images of the droplets by the high speed camera at fraction of micro second intervals. The size of the droplet is determined from the recorded images with the high-speed camera.

Deposit Scan

Zhu, Salyani, & Fox, (2011) developed a portable scanning system to measure the spray coverage and deposition quality feedback information, a system that could quickly evaluate spray deposit distribution and coverage area on deposit collectors such as water sensitive paper or Kromekote card. The system is integrated with a handheld business card scanner, deposit collector, a laptop or computer, and a

Figure 3. Droplet characterization and size measuring system through optical imaging



custom-designed software package entitled "Deposit Scan". Deposit Scan also called ImageJ is a Java-based image-processing program used for the acquisition and analysis of images. It is developed by the National Institutes of Health (NIH), USA and is now available to open access. ImageJ can be used to measure an area and count number of spots in the user-defined areas or throughout the entire image. The shape of selected areas could be rectangular, elliptical, or irregular. It also quickly gives the deposits per centimeter square area of the image.

A Shift from Micro to Nano Scale Measurement

Stark, Zhang, Sharma, & Mazumder, (2008) developed a mathematical model using MATLAB/SIMULINK to measure the charged nanoparticle (particle size distribution ranges from 2 pm to 20 pm and charge to mass ranges from 2 to +24 pC/kg), a modification of the older ESPART analyzer that was restricted to measurement of sub-micron particles i.e. in printing industry applications, space applications, and in applications to distinguish between different types of bacteria. ESPART analyzer at present can measure nanoparticles, which requires measuring signals with a low SNR (Signal-to-Noise Ratio). Resolution of particle size relies heavily on the frequency of the exciting signal, which causes the particle to experience oscillatory motion. With an increase in the exciting frequency, smaller particles experience motion at high frequencies with greater changes. It can be inferred that a higher frequency exciting signal can be used to resolve smaller particle sizes at the limits of the light collection methods and devices such as silicon photodiodes and photomultiplier tubes. Amplitude of oscillation of the particle in the applied AC electric field charges up the particle. The magnitude of oscillation compared with the magnitude of the applied electric field gives the particle net charge.

FLUENT to Model Electrostatic Spraying Processes

The recent development of Computational Fluid Dynamics (CFD) software makes it possible to model the mechanical spraying process with ease. FLUENT is one of such powerful software tools to model the air flow in the continuous phase and the droplet motion in the discrete phase. However, it does not have a direct solution for the electrostatic field formed by the charged droplets. Fortunately, it offers user-defined functions (UDF) and user-defined scalars (UDS) as an extension to suit different needs for various applications.

Numerous studies have been carried out on the charged particle motion under electrostatic space-periodic field in crossed-field systems. In this study, changed particle trajectory was considered in crossed-field systems under periodical electrostatic distribution. Many parameters affect the process such as atomizing air pressure, liquid flow rate, nozzle-to-target distance, droplet size, charge to mass ratio, etc. The mechanical portion can be directly modeled with the computational fluid dynamics software. The droplet trajectories are computed under the discrete phase model. In this mode, the equation of motion is solved by the Lagrange approach where the droplets are tracked by the stochastic tracking (random walk) model in turbulent gas flow. The electrostatic force on the charged droplets due to space charge is incorporated into the FLUENT solver as a component of the droplet body force. The air flow and the droplet discrete phase can be directly modeled in FLUENT.

In practice, the Poisson equation of the electrostatic field is analogous to the general scalar transport equations within FLUENT. In this way, modeling of the electrostatic liquid spraying process can be performed solely by FLUENT without compiling a separate computer program to solve the Poisson field which calls for a vast amount of data exchange. The key of this technique is to find the space charge density for different charging models.

ELECTROSTATICS AND ITS APPLICATIONS TO AGRICULTURAL SPRAYING

The application of pesticides is one of the most frequently used methods to protect crops and trees against diseases and insects in agriculture and agricultural products for the sustainable agricultural development. Laryea & No, (2003) showed that more than 90% of the pesticides are applied as liquid sprays providing more accurate metering and on-site particle-size control but the sprays are highly drift-prone and it is mostly performed by using hydraulic and conventional spray nozzle systems, pedestal-mounted sprayers, high pressure spray guns, hand pressure swirl nozzles and the consecutive high volume sprayers. Sande et al., (2008) showed in their previous work that due to non-uniformity of droplet size and off-target drift, target deposition efficiencies less than 30% is very common in agricultural pesticide spraying. The work was mainly on different spraying techniques and how to optimize spray deposition and minimize spray drift. The smaller droplets are lost in airborne drifts and the larger ones are lost due to gravitational settling by the soil. Off-target drift, aerial spraying losses and uneven deposition are very common in pesticides application that leads to damage of non-target microorganisms, soil quality and environmental pollution. Further, Sayinci & Bastaban, (2011) also worked on spray distribution uniformity of different types of nozzles and discussed the parameters of spray deposition mainly for potato plant. Numerous scientists and researchers have been working on measurement and evaluation of off-target drifts and aerial spraying losses from the target.

Electrostatic force field applications to agricultural pesticide spraying is one of the most efficient and economic methods to protect the crops and trees from insects and diseases (Ru, Zheng, & Zhou, 2007a). Law, (1983) and his students have done a remarkable work in the development of electrostatic pesticide-spraying processes and prototyping devices at the University of Georgia from 1970 to the present-day's commercialization of the technology. Electrostatic technique in spraying has already been commercialized and being used in countries such as USA, China, South Korea etc. In developing countries such India, a small portion of the agriculture farming is being performed in large scale, however, the major chunk of farming is done in small scale. Therefore, the spray equipment available in the market are not viable to developing economies especially in Indian scenario. Mamidi, Ghanshyam, Patel, & Kapur, (2012) have highlighted that the demands of the spraying equipment are very much different in developing economies than developed economies, where the farming is carried out in large scale. On the other hand, earlier electrostatic spray systems are motorized and mounted on tractors, helicopters or any other vehicles because of the complexity and heaviness, bulky which lead to the economic burden to the small scale farmers. Literature shows that a large number of publications and patents are coming from developing economies in today's scenario and these publications are mostly based on simplifying the complex and sophisticated systems to small scale farming such as hand pressure based electrostatic nozzles.

The goal of pesticide spray application is not only the effective deposition onto the target, but also economical, available at the lowest cost. This means that in order to develop a technologically feasible and economically attractive system, more research at both the experimental and theoretical level is needed

(Law, 1995). Even though many parameters are involved in an induction charge based electrostatic spray systems, all the investigations carried out thus far have demonstrated the intricacies of the electrostatic-spray phenomena (Ru, Zheng, & Zhou, 2007b). In this section, the different applications of electrostatics in agriculture spraying and related areas have been discussed in details.

Antimicrobial Sprays for Enhanced Food Safety

Antimicrobial agents are the biomaterials used to sanitize contact surfaces of the food production, processing and supply chain at various stages. The objective of the work 'Food Safety' at University of Georgia by Law and associates was to experimentally establish and control the mass-transfer efficiency and antimicrobial efficacy of the air-assisted induction charged electrostatic spraying process for spread over decontaminator liquids to various surface alignments and compositions commonly come across in food handling and processing operations specifically reconnoitering whether the lower volume of spray carrier liquid put down on the target surface would provide sufficient contact time for microbial cell death, as well as determining adequacy of target charge transfer for various electrically insulating versus conductive food-contact materials (Lyons, Harrison, & Law, 2011). They developed an air-exhausted biosafety chamber, an air-assisted induction charged electrostatic sprayer, commercially provided by Electrostatic Spraying Systems (ESS) Inc., was operated and compared to a conventional hydraulic-atomizing sprayer to determine mass transfer of active ingredient deposited onto the actual targets. Fluoroanalysis of AAIC (air-assisted induction-charged) electrostatic sprayer has given good agreement with theoretical considerations in terms of significant increase in deposition efficiency and surface sanitizing bio-efficacy. This provides backside deposition with greater efficiency and bio-efficacy. The deposition of active ingredients onto backside of the target was 29-times better than any conventional sprayer. However, in these kinds of microbial sanitizing techniques, it is to be noted that the sanitizing media should be biocompatible to food materials.

Postharvest Control of Fruits and Vegetables Spoilage Microorganisms

Maintaining the quality and desired sensory attributes of fruits and vegetables between farm and market is one among the major challenges. It is also called post-harvest treatment of biological commodities. This entices the application of waxes and water-loss barriers onto the surfaces of perishable food product. Law & Scherm, (2005) reported the development of an efficient electrostatic spray application method and processing-line prototype created specifically for postharvest protection of foodstuffs. They developed a prototype electrostatic sprayers unit utilizing an oscillating array of air-assisted induction-charging nozzles for postharvest treatment of fruits and vegetables on processing and packing lines. Example was the demonstration of prototype of Banana packing, a 1 m x 1 m shallow plastic tray containing 16-18 clusters of 5-7 bananas each is pushed along the process roller-line into the treatment chamber ejecting the previously treated tray of fruits. Experimental results of extensive evaluations of electrostatically applied protective sprays onto bananas for international shipment, where both microbiological and mass-transfer data document typically showed the 2-3 fold deposition improvements for food protection.

High Range Electrostatic Sprayers

To develop an electrostatic spraying system for high range spraying, such as defendable forest, shelter trees in urban areas, fast growth forest, field net forest and shelter trees on highways etc., is the current issue to be taken into considerations since performance of such systems deteriorate as the travel distance of charged droplets increases. Also greater the distance between the spray tip and the target area, the greater impact wind velocity can have on drift (Ru, Zheng, & Zhou, 2007c). Charge to mass ratio is one among the parameters which decide the performance and efficiency of electrostatic spraying systems, a process of neutralization due to naturally occurring radioactive phenomena's in ambient atmosphere. It is also very important because in recent years, it has been observed that there are more and more tall trees in worldwide, as forest covering area become wider. Ru, Zheng, & Zhou, (2005) authenticated that to improve the effective spraying range; it should be considered such features of sprayers as spraying height and application efficiency. The work led by Zhou, Professor at the Nanjing Forestry University, China, designed and developed a system to apply liquid sprays to very high trees to control the forestry diseases. The high range spraying systems are commercialized recently and available in the market.

Aerial Spraying Systems

Based on the theoretical analysis and practical experiments and the requirements for the large area forest pest control and farming performed in large scale, Zhou, Ru, Shu, Zheng, & Zhu, (2008) developed an aerial electrostatic spraying system assembled on a helicopter, a hydraulically assisted flight control. The study addressed issues associated with inefficiencies in disease and pest control, including pesticide overuse, spraying difficulties and labor intensity. The spraying system includes electrostatic generator and two sets of nozzles. An electrostatic generator with two high-voltage power levels can output positive and negative high-voltage simultaneously and was used to deliver voltage of 24-36V into a high-voltage generator with output voltage of 10kV in common use and 20kV in maximum and mounted on the aircraft. On the basis of field tests and experimental results, it was observed that combining aerial spray technique with electrostatic spray technique, the invented aerial electrostatic spraying system applied in R44 was provided with scientific design, rational structure, convenient operation, high productivity and high efficiency and had no harmful effects to all the airborne equipment and instruments of R44. It was completely suitable for the safe flight and spraying of R44 helicopter. Electrostatic spraying, which accords with the demands of flight safety and spraying procedures; can evenly distribute droplets while reducing pesticide use that leads to environmental and soil pollution.

Equipment for Improved Deposition in Cotton

Air-assisted electrostatic and hydraulic sprayers have been developed in recent years to improve pesticide penetration and coverage within the plant canopy and to increase the efficiency and bio-efficacy of the biological surfaces of the crops and trees. These sprayers need to be evaluated to determine their effectiveness when compared with conventional sprayers. Sumner, Herzog, Sumner, Bader, & Mullinix, (2000) compared within-canopy penetration and leaf side coverage of spray materials applied using the following spray technologies: (a) air-assisted sprayer (b) over-the-top hydraulic nozzles plus drop nozzles (c) electrostatic air-assisted sprayer (d) over-the-top hydraulic nozzles; (e) over-the-top nozzles plus shielded drop. The results showed that the air-assist nozzles are better than hydraulic and other sprayers in term of coverage and penetration of the canopy of the plant.

Automatic Target Detection and Electrostatic Spraying

The recent work by Xiongkui, Aijun, Yajia, & Jianli, (2011) was based on an infrared sensor detection system to discriminate between the presence and absence of trees for which spray is not needed, when the detecting devices detect the target, an automatic control system activates the spraying system to spray toward the target. Similarly, when the automatic control system receives the signal of a gap between the trees from the "electronic eye", the spraying system shuts off. Infrared sensor detecting techniques have been adopted in automatic target detecting orchard sprayers to discern targets and control the spraying system automatically. The important engineering aspects to keep in mind while developing such systems, is the matching of the electrostatic spray nozzles and sensor actuation. The microcontroller response and response of different sensors should be matched because of the fast actuation of electromagnetic valves. The actuation of controlling devices should be matched for exact target detection along with sensitivity, accuracy etc. An infrared detector is utilized in the automatic target detection system along with electrostatic nozzle and air assistive fans. Multi-sensor fusion techniques can be utilized for this kind of spraying systems.

FUTURE RESEARCH DIRECTIVES IN ELECTROSTATIC PROCESSES APPLIED TO AGRICULTURE AND AGRICULTURAL RELATED AREAS

Real Time Monitoring and Pesticide Delivery

As the population is growing day by day, the demand of nutritious and healthy food is increasing. Therefore, there is a tremendous increase in green house farming to fulfill the demands of billions of people. In greenhouses, it is very difficult to work in the presence of high temperature, increased carbon dioxide and humidity level. Therefore, there is a possibility of automation and mechanization in agricultural pesticide spraying. The automation in pesticide spraying involves majorly three steps: sensor package to sense the target and to map the plant canopy (target detection and canopy mapping), data acquisition to calculate and store the data and the last is navigation to monitor the movement of an automated spraying system. By using sensory attributes, the pesticide dose can be decided as per requirement of the plant canopy and disease.

Pesticide application control and variable pesticide spraying is the key to improve operation quality, reduce chemical waste, environmental pollution and cut down the cost of production. Other factors which have to be considered in pesticide spraying are target detection, quantity and quality of spraying control, directionality of spray, efficient treatment with low pesticide doses, operator safety, ease of use and reliability, flexibility and economic and cost effective spray system.

Characterization of Electrode Material for Spray Charging

Appropriate electrode material selection for spray charging and dimensional design and specifications are very important that need further study. Right electrode material and its insulation may enhance the chargeability of liquid sprays. So far the materials used as an electrode for spray charging were copper, brass and stainless steel. Recently nickel has been reported as a new electrode material for spray charging by Patel, Ghanshyam, Mamidi, Kapur, (2012a). Nickel increases the corrosion efficiency and enhances

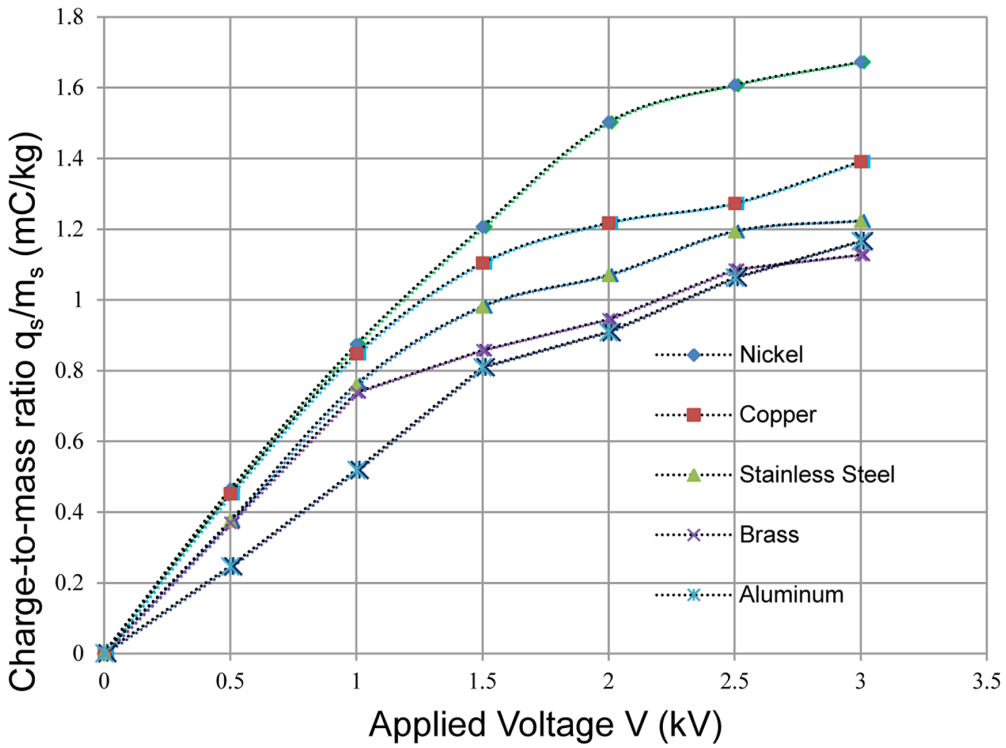
the performance of the system, since Nickel is assumed to be inert in standard temperature and pressure. This may enhance the charge to mass ratio and hence the performance of the electrostatic nozzle system.

Figure 4 shows the performance of the different material electrodes for spray charging in electrostatic spraying processes (Patel, Ghanshyam, Mamidi, Kapur, 2013). It shows that nickel (Ni) electrode has more spray current and hence the charge to mass ratio than rest of the electrodes because of availability of more electron surface density. Here the Fermi energy and work function of the charging electrode material are playing a key role. The performance and characterization of different electrode materials for spray charging is still a naive and unexplored area which requires furthers innovation in material synthesis to work at lower applied voltage for the same performance.

Electrostatic Sprays for Ratnagiri Mangoes to Enhance the Transportation life

Ratnagiri is the largest producer of quality mangoes such as Alphonso, Kesar etc. a Southern part of India. The life of the storage and transportation of these mangoes is about 7-10 days which is very short. The pest level on these varieties is reported by the European Union. This is because, pesticides are being used indiscriminately, affecting the export of perishable commodities, and as a result economic balance

Figure 4. Charge to mass ratio for different material electrodes in spray charging



is affected. The European Union imposed a ban from May 1, 2014, on import of mangoes, after alleging to have found unwanted pests such as "non-European fruit flies" in some consignments. These mangoes are easily attacked by microbial and affected by ambient conditions. There is a possibility of electrostatic sprays or coatings on the surface of these mangoes to protect them from microbial attacks and ambient conditions and also to increase the self-life of transportation.

The electrostatically coated material on the surface of mangoes should be biocompatible so that it will not affect the human being and consumer of the products. The biomaterial should be soluble in water or other solvents so that it can be sprayed as liquid.

Protective Coatings to Apples for Resistance towards Microbial Attacks

An excellent technique for applying protective thin coatings on fruits such as apples, to protect from microbial attacks has been proposed. Further improvements can be done by combining a processing and packaging line with the treatment chamber for automated online processing. An array of nozzles spraying different solutions can be used for applying simultaneous coatings such as waxes and fungicides. These electrostatically coated fruits are more resistive to the ambient conditions and can be stored for a long time.

Ultra High Electric Pulses to Preserve the Liquid Beverages/Foods

Technological advances in food processing industry have evolved various instruments, ranging from simple to complex equipment. These are either processing equipment or preserving equipment. Preservation of food item calls for new techniques, which involve various thermal and non-thermal methods. Pulsed Electric Field (PEF), which is a non-thermal method offers freshness, flavor and nutritional value and is used to improve the shelf-life of liquid food. In this technique, a short pulse is given to foodstuffs for a short duration for effective inactivation of microbes. Method of pulse generation is a still great concern of research, which needs further modifications and improvements. This generator may require power MOSFET, high voltage switching circuit, and microcontroller, to generate high voltage pulses of less than microsecond duration. The instrument should be equipped with data acquisition system and display system for proper and easy operation. Virtual Instrumentation could be used for data acquisition and control purposes.

Air-Assisted Electrostatic Nozzle for Variable Canopy Coverage

In the case of electrostatic spraying, the droplet size is normally in the range of 30-60μm or less and the droplets in this range are most susceptible to spray drift. The electrostatic repulsion among droplets is also the cause of spray drift. Presence of wind is another cause of spray drift and presently available sprayers cannot be used even in normal wind. It can be achieved by shielding the fine electrostatic spray from these harsh wind conditions. It may be simple mechanical means of shielding. The air-assistance forms an envelope around the spray to protect the finely generated charged particles. It also reduces the interaction of charged droplet cloud to naturally occurring charged ionic environment already present and hence applicable for higher range of spraying. The naturally occurring ions are present due the cosmic and radioactive activities. Air-assistance may provide virtual path to the finely divided charged droplets to reach actual target.

Automation in Switching ON/OFF of Power Supply

In the manual operation of the complex and sophisticated electrostatic sprayers, the probability of errors is very high, and calls for automation in the switching (ON/OFF) of the power supply to charge the small droplets in the design of the spraying nozzle systems. There should be an orderly and proper sequence to be followed from the different inputs for the efficient working of the electrostatic spraying nozzle system. A novel idea in the design of an automated circuit is to switch (ON/OFF) the high voltage power supply in electrostatic nozzle system, using the inherent property of water grounding, can be used. This can be achieved with the use of simple electronic and electrical components. Other way to achieve the goal is by using pneumadyne switch to control the high voltage power supply.

CONCLUSION

The aim of this chapter is to provide a basic understanding of an electrostatic spraying processes and the entry of charge to the liquid atomized as well as uniform deposition onto the surfaces of crops and orchards. This chapter has established a theoretical foundation for understanding the basics relying on electrostatic spraying, and relative effectiveness of the electrostatic forces. It emphasized the fundamentals behind the different charging mechanisms along with the charge retention and charge-loss phenomena and underlined the difficulties associated with each method, since it has always been a great challenge to inject the charge into the finely divided liquid droplets. The heart of the charge injection technique is being balance between hydrodynamics and electrodynamics, may generate flow instabilities which leads to turbulence. This requires co-design of the atomizer internal geometry from both hydrodynamics and electrical perspectives, the optimization of which leads to a maximum in the generated spray charge per unit volume contained by the spray.

This chapter links several research areas together to provide an integrated summary of the knowledge relevant to air-assisted electrostatic spraying and electrostatically assisted atomization of electrically conductive liquid specially an attention has been given to pesticide spraying. The emphasis of the review leans towards explanation of physics and description of experimental work, interactions between space charge gradient and electric field produced which in turn can generate instability throughout the bulk of the continuum. It also discussed the interaction between finely divided charged particulate matters and naturally occurring ions present in the atmosphere, leads to neutralization of the charged droplets and hence deteriorates the performance of the spraying system.

This chapter highlighted the various applications of electrostatic spraying to different fields of agriculture, engineering trends and food processing industry along with the future perspectives of the electrostatic spraying. Electrostatic spraying technique can be used for protective biomaterial coatings to fruits, vegetables and perishable food products for resistance towards microbial attacks, to enhance the transportation life, to control spoilage microorganisms, antimicrobial sprays for enhanced food safety etc. However, the biomaterial should be soluble in conducting liquids such as water and other liquid solvents. It is one among the bioremediators since it reduces the pesticide use and ensures the minimum residue level (MRL), an indication of pesticide residue in consumer commodities. Controlled and targeted delivery of pesticides is one of the current fields of interests in pesticide spraying, an automated system which is intelligent enough and trained properly to spray without interventions of human operator. Currently, the fabrication of electrostatically sprayed particles and liquid spray at industrial and user scale

(e.g., ESS Technology, Inc.) is feasible. However, advanced research on electrostatic spraying techniques in the field of food processing and preservation is recommended to enable the applications workable at the industrial scale.

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KEY TERMS AND DEFINITIONS

Bio-Efficacy: In agrochemical, bio-efficacy is a measure of the biological efficacy of an active ingredients of agrochemicals such as insecticide etc. Bio-efficacy of an insecticide is determined by the minimum dose required for maximum control of the disease. For an agrochemical, the bio-efficacy of an insecticide is determined by the minimum dose required for complete kill of the insects or diseases.

Charge to Mass Ratio: Experimentally the performance of the air-assisted electrostatic nozzle can be evaluated in terms of charge to mass ratio, which signifies the chargeability of the spray droplets by the charging electrode. It also signifies the efficiency and performance of the spraying system, higher the charge to mass ratio, better the performance. Charge to mass ratio depends on electrical and mechanical properties of the liquid as well as material of the charging electrode.

Electrode Material: The amount of charge present in the fine droplet depends on many parameters, the one is electrode material. Appropriate and suitable electrode material and its dimensional specifications may enhance the chargeability of finely divided particulate matter. Selection of electrode material for spray charging is as much important as other parameters such as electrical and mechanical properties of liquid to be sprayed. So far, most frequently used materials for electrode in electrostatic nozzle systems are nickel, copper, stainless steel and brass.

ESPART Analyzer: Electronic Single Particle Relaxation Time (ESPART) analyzer is an instrument which is used in many applications to determine both size and charge of micrometer sized particles in electrostatic spraying processes. ESPART analyzer consists of two major units: Laser Doppler Velocimeter (LDV) and particle relaxation apparatus which measures the velocity of a particle and aerodynamic diameter passing through the intersection of two coherent light sources, such as lasers and the signal processing unit, a simulation model for the ESPART analyzer to measure the time lag. The ESPART analyzer can measure the statistical distribution of particle size and charge in electrostatic spraying processes.

Faraday Cage: Faraday cage, a specially designed wire mess like structure which is called a cage, commonly used to collect the charged droplets coming out from the spraying system. The contact of the charge droplets onto the wire meshes of Faraday cage and transfer of the charge to the earth caused an electrical current which is detected by a microampere meter, the charged liquid spray is then collected at a specific time and weight. Then the spray current is divided by the mass flow rate to determine the charge to mass ratio.

Induction Charging: Induction charging is the most commonly used method to charge the finely divided particulate matter in electrostatic spraying processes. In electrostatic induction charging, direct charge-transfer to droplet formation zone of a liquid jet results from electrostatic induction of electrons on to the continuous jet and in order to maintain it at ground potential the presence of a closely positioned electrode of positive polarity is required. Induction electrification process reduces the chances of shock and hazardous to operators of the nozzle system.

In-Flight Trajectory: The path followed by the charged particulate matter in the presence of many forces including electrostatic force is called in-flight trajectory. Once the droplets have been charged inductively, the charged spray-cloud has to travel in a harsh and transient environment, and thus, a charged spray-cloud will encounter some degree of neutralization. The charged droplets are governed by many forces such as gravitational force, force due to surface tension, electrostatic forces, drag force etc.

Rayleigh Limit of Charge: A drop can hold a maximum charge, defined by the Rayleigh limit, or a limit defined by electrical breakdown strength. The maximum limits of charge that can charge mother droplet carry without rupture into the daughter droplets.

Volume Median Diameter: It also refers to average volumetric size or mean size. The Volume Median Diameter (VMD) refers to the midpoint droplet size, where half of the volume of spray is in droplets smaller, and half of the volume is in droplets larger than the mean. For example, A VMD ($DV_{0.5}$) of 50µm, indicates that half of the volume is in droplet sizes smaller than 50µm, and half the volume is in droplet sizes larger than 50µm.

Wraparound Effect: In electrostatic spraying, the pesticide deposits uniformly onto both directly exposed or obscured crop surfaces. The deposition of pesticide onto the backside of the crop surface is called wraparound effect. The wraparound effect occurs due to electrostatic phenomena takes place during the spraying.

Chapter 19
Bioremediation via Nanoparticles:
An Innovative Microbial Approach

Shipra Pandey
NBRI, India

Arpita Bhattacharya
CSIR-NBRI, India

Madhuree Kumari
CSIR-NBRI, India

Shashank Mishra
CSIR-NBRI, India

Satyendra Pratap Singh
CSIR-NBRI, India

Puneet Singh Chauhan
CSIR-NBRI, India

Aradhana Mishra
CSIR-NBRI, India

ABSTRACT

Arena of nanotechnology has revolutionized the field of bioremediation to overcome the problems of environmental pollutions. Approaches applied for the monitoring and treatment of contaminants includes control of pollutants, sensing the pollutants and remediation by nanoparticles. Among the three approaches, the most important is to remediate the pollutants. This chapter highlights the eco-friendly, accurate, cost effective, ex-situ and sustainable approach for the "Green Bioremediation" with the help of nanoparticles. Nanoparticles covers the treatment of surface water, groundwater and industrial wastewater contaminated by toxic metal ions, radionuclides, organic and inorganic solutes and also reduce aromatic recalcitrant compounds from soil and air pollution. There is also a scope of enhancing the remediation potential of nanoparticles by manipulating size and geometry. They have given a new hope towards positive sustainable approach for environment and human welfare.

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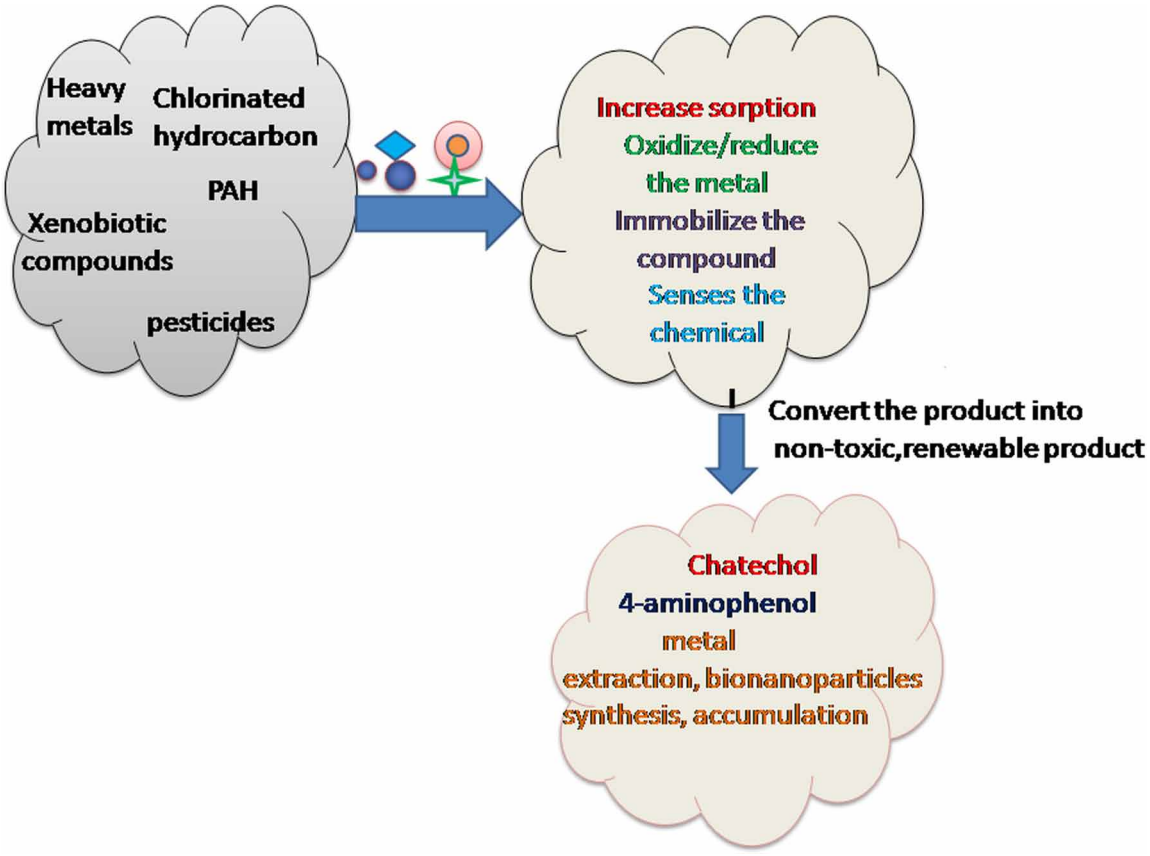
INTRODUCTION

Increment in the population density, necessity of up gradation in agriculture productivity, industrialization and urbanization of human societies are accountable for environmental contamination (Figure 1). The extensive use of anthropogenic materials contaminates the natural ecosystem which degrades the environment in long term by several means such as loss in biodiversity, introducing heavy metals and other organic recalcitrant compounds. On this regard decontamination of these hazardous wastes via eco friendly approaches is an enigma. In the diverse biological species present in nature, many are blessed with the ability to tolerate heavy metals. Progress in science and technology facilitate us to concern the ability of biological diversity for depletion of pollution which is termed as bioremediation. This is promising efficient novel technology for dealing with extensive range of contaminants. This technology mediates phytoremediation (plants), rhizoremediation (plant and microbe interaction) and remediation via biosynthesis of nanoparticles (nanoparticles synthesized by microbes).

Currently maintaining ecosystem and biodiversity has become an increasingly important field of research, as well as a resource management goal. Various industrial chemicals are released daily which are hazardous to the environment and cause harmful effects to the biodiversity by entering the food

Figure 1. Effect of nanoparticles on contaminate sites and their possible mechanism to reduce the contaminants



chain. These chemicals include petroleum hydrocarbon, heavy metals, phenolic compounds, metalloids, radionuclide, effluents and halogenated solvents from industrial sources, explosives, agricultural chemicals. Common contaminants and their health hazards: Polyaromatic Hydrocarbon: Carcinogen, mutagen, single ring compound does not have carcinogen property but after fusion they become carcinogen. Chlorinated hydrocarbon: Easily dissolved in water, mainly affects the respiratory tract, non carcinogenic, causes irritation in eyes.

Phenols: Irritating and corrosive effect on skin and become chronic by affecting central nervous system and kidneys.
Heavy Metal: Cadmium exposure: renal tubular damage, effect on bone and fracture
Arsenic Exposure: Hyperkeratosis, lung cancer
Lead Exposure: Toxic blood, neurotoxic effect of lead at lower levels.
Chromium Exposure: Epigastric pain, nausea, diarrhea, chronic ulcer.

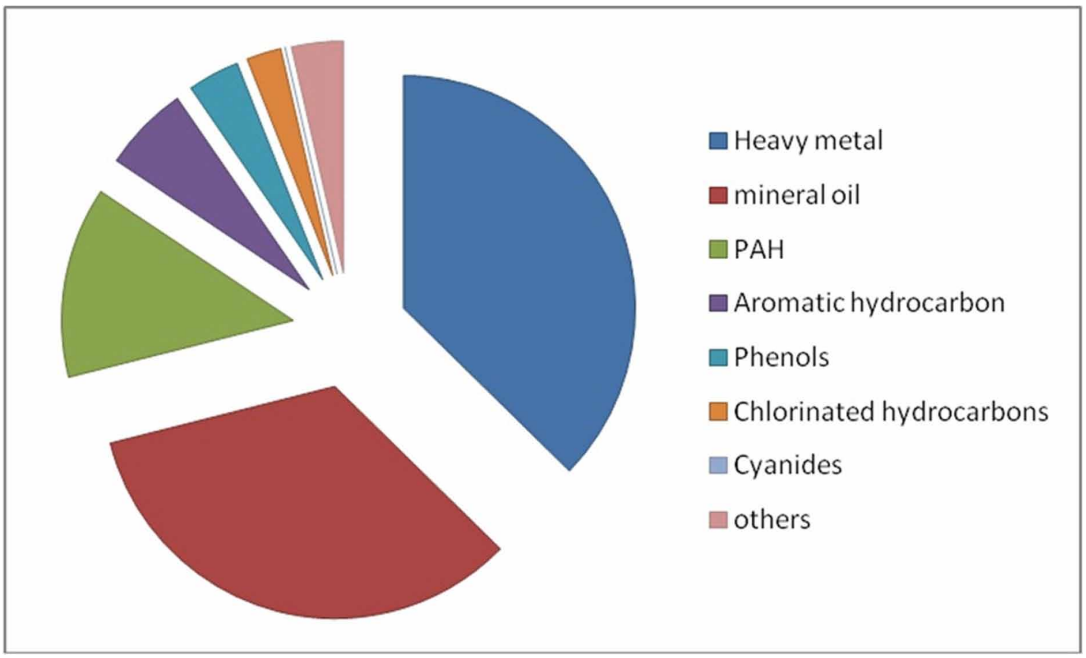
Major part of biosphere which is contaminated with anthropogenic substances is soil and water. Soil, the most important land constituent is contaminated with chlorinated compounds, polycyclic aromatic hydrocarbon, heavy metals and radionuclides (Acevedo et al., 2010). These contaminants sequestered in saturated and unsaturated layer of the soil which underlying between the ground surface and groundwater level. Consequently, sites can have a high concentration of organic contaminants in soil layers in addition to possible groundwater contamination. They can cause detrimental effect on the flora and fauna of affected habitats through uptake and accumulation in food chains, and in some instances, pose serious health problems and or genetic defects in humans.

Water is mainly contaminated by chlorinated hydrocarbon, heavy metals, chlorine compound like Trichloroethene (TCE) and Polychlorinated biphenyls or mixtures of anthropogenic organic chlorinated compounds (He, F) which is highly reactive and water soluble(Arjoon, Olaniran, & Pillay, 2013). These compounds are carcinogenic and naturally non degradable, so it persists in water and soil for long time and become a challenge to remediate these compounds (Figure 2).

To remediate these chemicals several approaches have been applied like incineration, thermal desorption, excavation, but these method release toxic by-products. Alternate of these methods are used of naturally occurring microorganism such as bacteria, fungi, actinomycetes, yeast, plants to degrade pollutants into non-toxic substances. Rhizoremediation, which is the well developed process of bioremediation, entails the elimination of specific contaminants from polluted sites by symbiotic association between host plants and microbes. The drawback of this process is that it is very sensitive to the level of toxicity and environmental conditions of the polluted sites i.e. the conditions must be conducive to microbial activity and need to consider temperature, pH etc. Phytoremediation is excavation of contaminants by the help of plants. This method is specific for soil, where the some of the plants which accumulate the metals.

With the recent development of nanotechnology, the combination of nanoparticles and biological process is successful in enhancing measurement accuracy, improving bioremediation efficiency and broadening biochemical application in environmental research. Nanoparticles related bioremediation has low risk of genetic leakage in the environment and can provide additional functions and characters to the biochemical process.

Figure 2. Emission of different contaminants in different natural resources



BACKGROUND OF NANOTECHNOLOGY

Nanoparticles were firstly used red glasses in late Bronze Age (1200-1000BCE) from Frattesina di Rovigo (Italy) gives coloured due to excitation of phasmon surface modes of copper nanoparticles (Angelini et al., 2004; Artioli, Angelini, & Polla, 2008). In protohistoric age glasses were developed using copper crystals on the top layer by exposing the material to reducing conditions. Along with the use of copper, gold nanoparticles were also used during Roman times (Colomban, March et al., 2003). The well known example is Roman Lycurgus Cup (Freestone et al., 2007). The glass gives ruby colour after transmission of light, although the colour of the cup was greenish-yellow. Change in colour is mainly due to colloidal metal and nanocrystals a silver-gold alloy dispersed throughout the glassy matrix (Barber & Freestone, 1990). Nanoremediation as an emerging field in 2009 commercially applied in 44 cleanup sites around the world.

Nanotechnology is the science which deals with different approaches of nanoparticles. Nanoparticle (10^{-9} m) is defined as a small object that behaves as a whole unit in terms of its transport and properties (Prathna, Mathew, Chandrasekaran, Raichur, & Mukherjee, 2010). Nanoparticles which are found naturally, chemically and biologically synthesized, these are the ultra fine particles which have higher specificity, crystallinity, design, controlled shape and size that differ with different morphology. These specific properties of nanoparticle make it promising tool for the mankind in various fields like pharmaceutical, industrial, medical, genetic engineering, Agriculture, environmental remediation. These nanoparticles are able to remediate easily the different pollutant without any drawbacks and limitations because this technique is very specific for any contaminants without any condition.

BIOSYNTHESIS OF NANOPARTICLES

Nanoparticles are synthesized by "green" approaches by microbes (Mishra et al., 2014). Green technology is the widely accepted method for bioremediation because of their non-toxic effect, clean and eco-friendly approach (Figure 3). Although there are several methods for the synthesis of nanoparticles like sol-gel method, chemical synthesis but biologically synthesis of nanoparticles is most acceptable and eco-friendly method (Mishra et al., 2014). Nanoparticles synthesize by green nanotechnology approach living organisms, plants and microbes. Mostly microbes are used for commercial use and rapidly decontamination process due to their high tolerance and reproduction power. These are usually synthesized from secondary metabolites of extracellular or intracellular metabolism of microorganisms. Synthesis of nanoparticles with different shape and size is an important aspect of nano-biotechnology. These nanoparticles show functional variation along changing in their shape and size (Ahmed & Khan., 2013). Biosynthesis of nanoparticles is also a kind of bottom up approach where the main reaction occurring is reduction/oxidation. The microbial enzymes responsible for reducing properties for reduction of metal compounds into their respective nanoparticles (Prathna et al., 2010).The particles generated biologically have higher catalytic reactivity and greater specific surface area (Riddina, Gerickeb, & Whiteleya, 2010). Biosynthesize nanoparticles do not aggregate due to the presence of capping agent secreted by specific microorganism.

Biosynthesis of nanoparticles can be intracellular or extracellular (Table1). Extracellular biosynthesis has gained a lot of attention because of low cost requirement and no downstream processing requirements (Mishra et al., 2014). The secondary metabolites and extracellular components present in cell free extract carry out the redox reaction for biosynthesis of particles after addition of precursor molecule (Figure 4). By varying biological and physical parameters, the configuration of particles can also be varied. These particles can further be characterized by UV-visible spectroscopy, Zeta sizer, Transmis-

Figure 3. Advantages of using Green technology

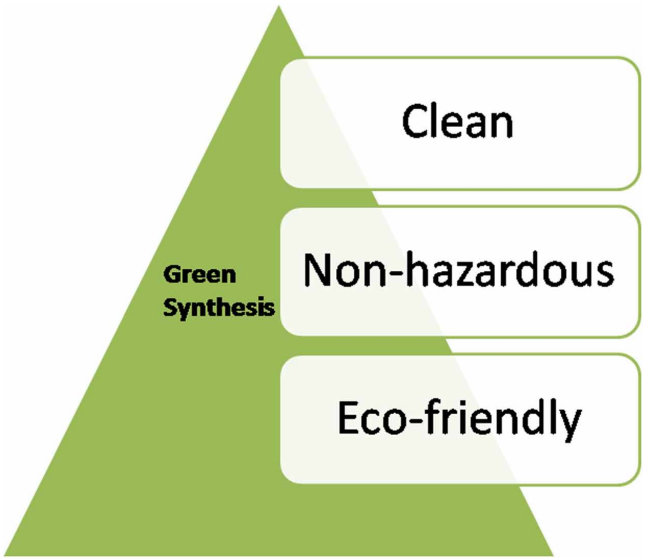


Table 1. Synthesis of nanoparticle by different microorganisms

		Biosynthesized Nanoparticle	Reference
Bacteria	*Bacillus* sp.	Silver	Pugazhenthiran et al.(2009)
	Geobacillus sp.	Gold	Correa-Llantén, Muñoz-Ibacache, Castro, Muñoz, & Blamey, (2013)
	Pseudomonas aeruginosa	Gold	Husseiny, El-Aziz, Badr, & Mahmoud, (2007)
	B. licheniformis	Silver	Kalishwaralal, Deepak, Ramkumarpandian, Nellaiah, & Sangiliyandi, 2008)
	Salmonella typhirium	Silver	Ghorbani, (2013)
Fungus	*Fusarium oxysporum*	Si and Ti	Bansal et al. (2005)
	Fusarium oxysporum	Silver	Ahmad et al. (2003a)
	Fusarium oxysporum	Zirconia	Bansal, Rautaray, Ahmad, & Sastry, (2004)
	Fusarium oxysporum	Gold	Mukherjee et al. (2002)
	Aspergillus fumigates	Silver	Bhainsa and D'souza (2006)
Actinomycetes	*Streptomyces viridogens*	Gold	Balagurunathan, Radhakrishnan, Rajendran, & Velmurugan, (2011)
	Rhodococcus sp.	Gold	Ahmad et al. (2003b)

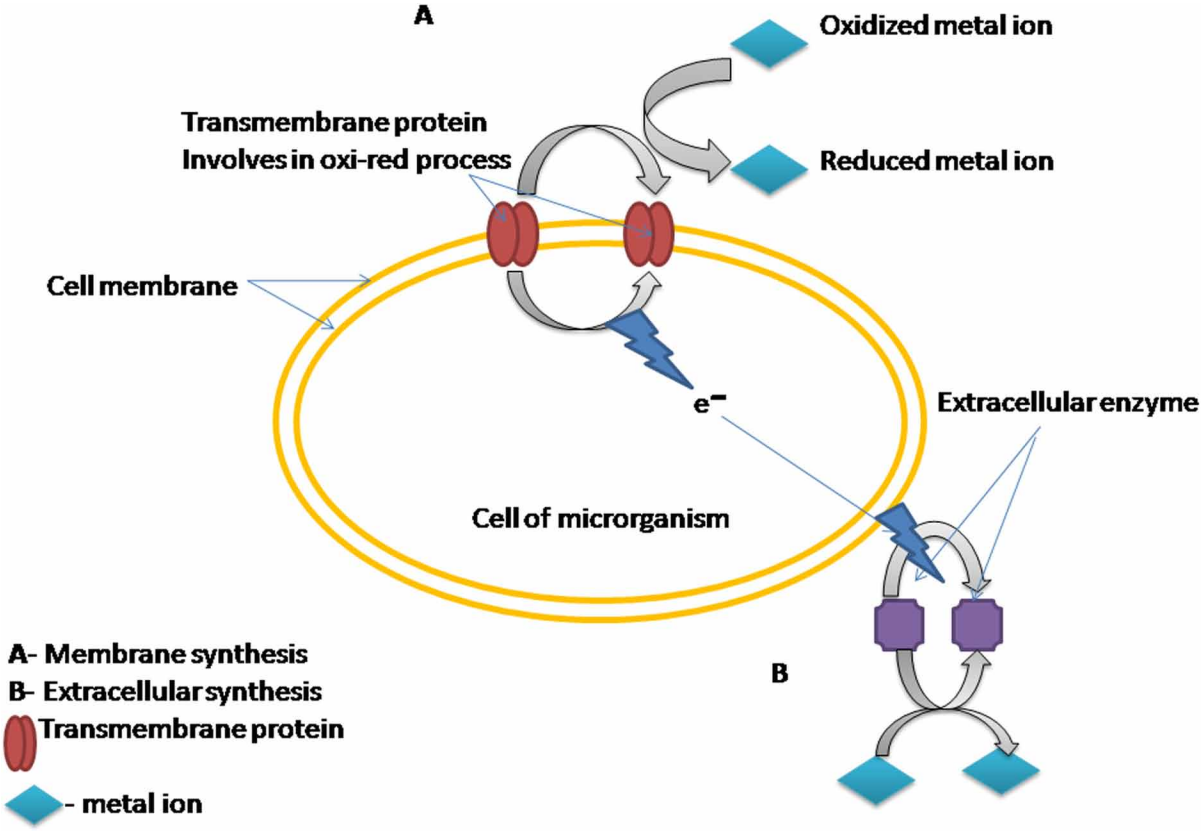
sion electron microscopy, Scanning electron microscope, Fourier transform infrared spectroscopy and X-Ray Diffraction (Singh, Rawat, Khan, Naqvi, & Singh, 2014; Shrivastava, Raghav & Singh, 2012).

The potential of nanoparticles in environmental can be categorized as remediation, sensing and detection, sequestration of elements, pollution control. Field of remediation by nanoparticles is basically soil, groundwater and wastewater.

ADVANTAGES OF NANOPARTICLES

- Nanoparticles have the ability to absorb maximum amount of pollutants due to large surface area and high surface energy.
- It catalyzes the reactions in faster rate in comparison to bulk material, thus reducing energy consumption during degradation or helps in preventing release of contaminants.
- The nanotized form of particles makes it accessible the contaminants hence promote *in-situ* remediation rather than *ex-situ* remediation.
- The ability of the nanoparticles to be coated with various ligands and control of surface area to volume ratio by changing the shape of the nanoparticles enables the design of sensors with high selectivity, sensitivity and specificity (Mehndiratta, Jain, Srivastava, & Gupta, 2013).

Figure 4. Different approaches of microbial biosynthesis of nanoparticles (Source: Mishra et al., 2014)



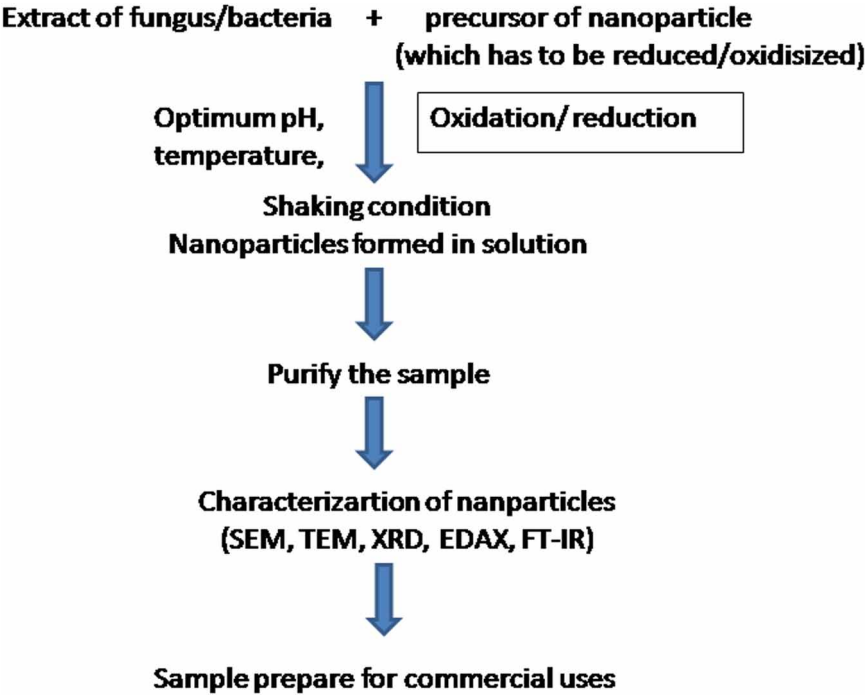
STRATEGIES OF NANOPARTICLES TO CONTROL THE POLLUTION

Control of pollutants from the source of origin is promising step of controlling pollution. The pollution strategies can be regulating by different ways:

- Reduction at the point of source, the controlling process is *in-situ* whereas degradation process is basically *ex-situ*.
- Use less complex products and disposable items
- Prevent release of contaminants at manufacturing sites and control mechanism for the mixing into natural resources
- Prevention of harmful intermediate and by-product formation
- Reduce energy consumption

Biosynthesized gold nanoparticles are efficiently used in bioremediation and biofuel generation. It has been used to generate hydrogen by oxidizing the carbon monoxide which can be used as fuel cell. It is also used to degrade several organic pollutants including para-nitrophenol. A recent study degradation of para- nitrophenol to amino phenol in 30 min using biosynthesized gold nanoparticles by *Trichoderma viride* as heterogeneous catalyst (Ai & Jiang, 2013; Mishra et al., 2014). Along with biodegradation ability *Trichoderma viride* also shows an efficient biocontrol activity against human pathogenic bacteria (Figure 5).

Figure 5. Generalized flow chart for nanobiosynthesis



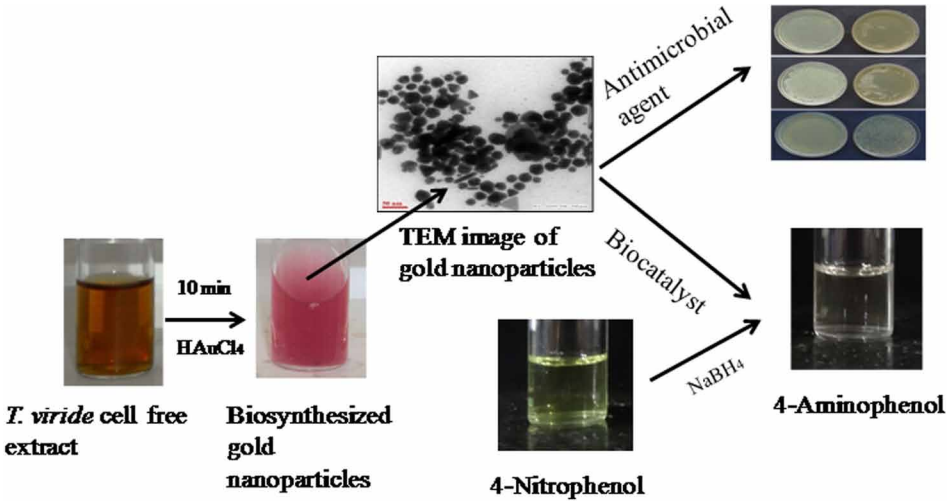
Nanotized zeolites in catalysis and separation process by oxidation of hydrocarbons in presence of visible light and getting high yield of product. Titanium dioxide nanoparticles have photocatalytic property that shows catalytic reaction in presence of light (Shen, Mu, Huang, DU, Zou, & Yang, 2006). It also acts as an oxidizing agent in presence of UV light, so it is also used in pollution control.

The second approach of pollution control is use of eco-friendly non toxic material by the use of nanoparticles. It helps in sustainable development of environment. Cathode ray tubes are replaced by the carbon nanotubes in computers, preventing the use of heavy metals like lead. Another example is use of titanium dioxide and silicon in photovoltaic cell formation (Pizzini, Acciarri, & Binetti, 2005). The use of mesoporous silica nanoparticles (definite size of pores present in silica nanoparticles) cell increased adsorption efficiency results in higher internal quantum efficiency (Figure 6).

NANOPARTICLE AS A SENSOR

Sensing the pollutants in environments is an essential step for controlling the pollution. There are various methods for sensing the pollutants, but these techniques are unable to determine the exact composition and nature of the pollutant under field conditions and time consuming process. Nowadays nanotechnology plays a significant role in sensing the pollutants by improving sensors more specific and sensible for environmental monitoring either by targeting the binding between the contaminant and the recognition element or optimizing the transduction and electronic interface to the sensing layer. Sensor can be used for the sensing of contaminants related to organic contaminants, inorganic contaminants or biological organisms.

Figure 6. Biological synthesis of nanoparticles from T. viride and potential degradation of 4- nitrophenol and convert into 4-aminophenol



Quantum dots (QD) are used as a fluorescent labelling system for detection of microorganisms. Zhu, Ang, & Liu, (2004) reported that conjugation of antibody to QD can be used for the detection of pathogenic micro-organisms viz. *cryptosporidium* and *Giardi*, also it is used in detection of *Salmonella, E.coli,* Staphylococcus endotoxin. Quantum dots exhibit superior photostability and multiplexing analysis as compared to organic dyes. Apart from this it also helps in detection of disease by giving fluorescent colour (Liu, 2006). Detection of heavy metals like Pb, Hg, Cd, gold nanoparticle has been used and for pesticides detection silica nanoparticles are used. Sugunan, Thanachayanont, Dutta, & Hilborn, (2005) functionalized gold nanoparticles with 11-mercaptoundecanoic acid and chitosan respectively for the detection of heavy metal ions like lead, cadmium and mercury. Mercaptoundeanoic acid and chitosan are metal chelating agents. Binding of heavy metal ions to these metal chelators results in aggregation of the nanoparticles yielding a shift in wavelength absorption and resulting change in colour from red to blue. Though it is not specific for a particular ion but the detection of heavy metal ions in general was very sensitive. Liu & Lu, (2004) developed a sensor for Pb detection for those gold nanoparticles were coated with lead dependent DNA enzyme. In the absence of lead, enzyme induced aggregation of nanoparticles whereas enzyme cleaves in presence of Pb resulting the aggregation was prevented resulted in a shift in wavelength absorption. The combination of the exceptional optical properties of gold nanoparticles(Au NPs) with the inherent advantages of microfluidic devices can be used to detection of Ziram, zinc bis (dimethyldithiocarbamate), is a broad spectrum fungicide member of the dithiocarbamate (DTC) family of pesticides (Lafleur, Senkbeil, Jensen, & Kutter, 2012).

SnO_2 and In_2O_3 these two metal oxides interesting for sensing application. SnO_2 shows good sensitivity to reducing gas (for example, CO) and In_2O_3 to oxidizing ones (for example, NO_2) depending, among other parameters, on the method of preparation and crystallite size. To investigate the sensing characteristics of the as-synthesized nanocrystals, they were deposited without addition of any binder or activation layers on a micro patterned alumina substrate and tested for their ability to detect trace levels of NO_2, CO, and CH_4 in air. Due to their specificity and targeted specificity of enzymes have vast capabilities in the areas of chemical conversions, bio sensing, and bioremediation (Durán, & Esposito,

2000). Enzymes can also be stabilized by producing single-enzyme nanoparticles consisting of single-enzyme molecules surrounded by a porous organic–inorganic network of less than a few nanometres thick (Kim, Grate, & Wang, 2006). Immobilized enzymes in biopolymers and carbon nanotubes are another strategy for environmental nanobiosensors. Although their life time is less, but several genetic modification have been used for improvement. Tyrosine is used for the electrochemical detection of phenols and pesticides, when it conjugates with the gold nanoparticles. Tyrosinase (TYR, EC 1.14.18.1) is a copper containing oxidoreductase that catalyzes two different reactions: (i) the *o*-hydroxylation of monophenols (cresolase activity) and (ii) the oxidoreduction of *o*-diphenols to *o*-quinones (catecholase activity) (Seo, Sharma, & Sharma, 2003). Tyrosinase thus offers great potential for the development of biosensors for the detection of mono- and diphenolic compounds (Alkasir, Ganesana, Won, Stanciu, & Andreeceu, 2010).

Another example of single enzyme linked nanoparticle catechol sensitive magnetic core-shell (Fe_3O_4-SiO_2) nanoparticles with laccase enzyme. Catechol concentrations in compost samples were determined by using the laccase sensor (Tang et al., 2008). Catechol is a hazardous phenolic compound which affects the nerve centre system of human beings, inhibits DNA replication, and leads to chromosomal aberration (Topping, Bernard, & O'Donoghue, 2007). The synthesis of Fe_3O_4 magnetic nanoparticles and the immobilization of laccase on the surface of nanoparticles were achieved according to the procedure introduced by Zhang et al. (2007).

REMEDIATION

The most important part to eliminate the pollutants is remediation. The strategies used for elimination of pollutants from water, soil, and sediments by microorganisms, are called bioremediation (Talley, 2005; Wasi, Jeelani, & Ahmad, 2008). The basic concept involved in bioremediation is transformation of harmful pollutants into the harmless form of compound or gases. Microorganisms can transform inorganic pollutants, not necessarily completely, but to compounds with decreased solubility, mobility, and toxicity (Kamaludeen, Megharaj, Naidu, Singleton, Juhasz, & Hawke, 2003; Wasi et al., 2008. Main reservoir of the contaminants are mining areas, refineries and industrial areas releases several radionuclides with heavy metal such as Cd, Ni, and Pb and polycyclic aromatic hydrocarbon (PAH).

Microbes are the geoactive agent who plays a very important role in biotransformation and bioleaching in environment and play down the anthropogenic contaminants. Although this process is quite slow which give the chance to accumulate the contamination by magnification. In the present scenario the scope of nanotechnology is increased in day to day life for remediation. They remediate the pollutants from whole biosphere (air, soil, water) in a cost effective manner. There are various mechanisms for the remediation of various contaminants like heavy metals, Polycyclic Aromatic Hydrocarbon. It is thus not surprising that today some nanotechnological applications for environmental use have been commercialized.

Remediation of Water Pollutants

The importance of water is during the phases of creation, evolution and continuity of life on earth, water remained as its most vital component. Leonardo Da Vinci had described water as "the vehicle of nature" ("vetturale di natura"). Water is basic demand of life that is linked to economical development. Due to

urbanization and rapidly increasing industrialization introducing the unwanted hazardous chemicals in water, drinking water is in critical phase. The major contaminants in water are heavy metal, pesticides, chlorinated compound etc.

Nanotechnology has overcome the conventional method of purification. Due to small size adsorption aptitude increases the surface energy available with each adsorbent particle also increases significantly. Several advantages over there:

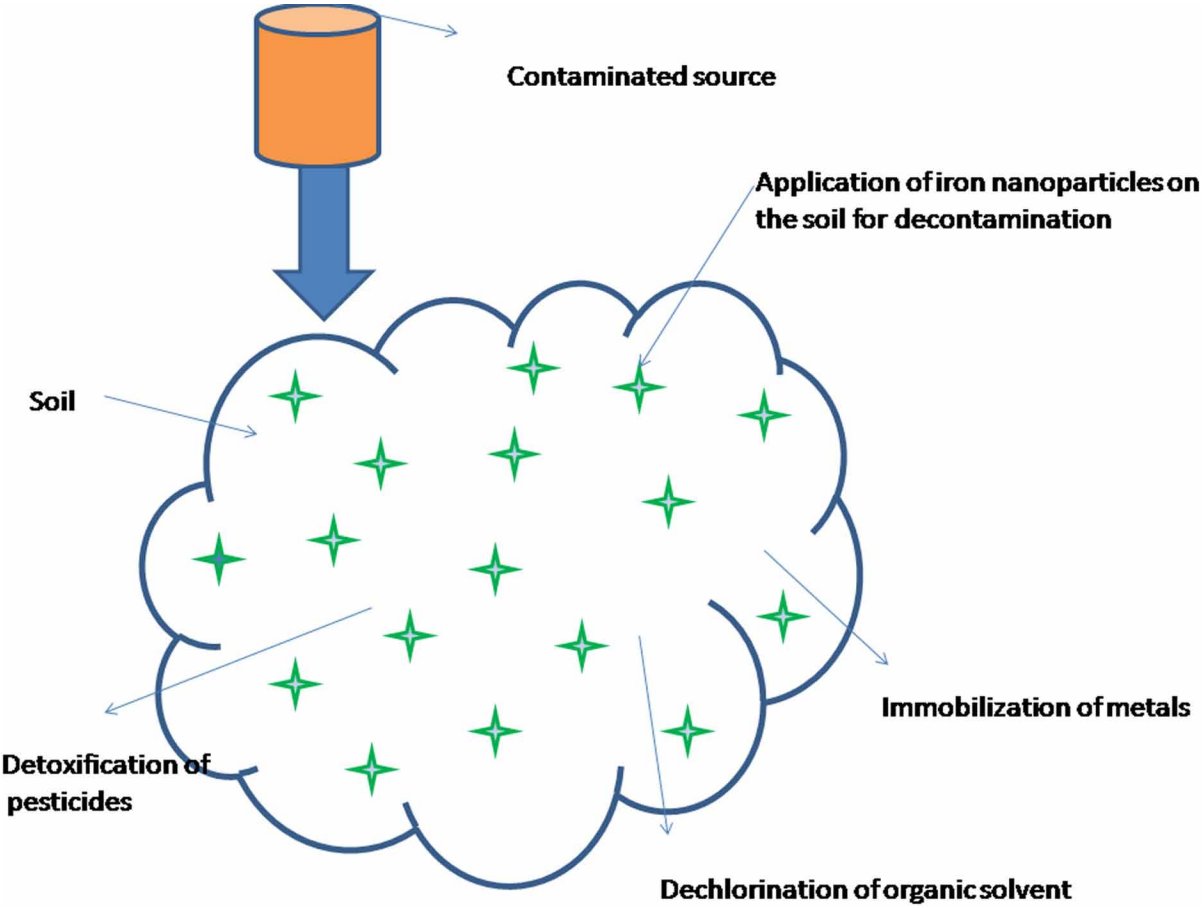
- Large amount of contaminants removed in small quantity of nanoparticles.
- The surface area of nanoparticle can be functionalized by different group to increase the affinity and activity of the particles.
- Post treatment less waste generate in comparison to bulk form.

Zero-valent iron metal (ZVI) is one of the most plentiful metals on the globe (Deng, Luo, Wu, Xiao, & Wum, 2000). Zero-valent iron nanoparticles extensively used for the dechlorination, Fe ion act as reducing agent (Chuang, Larson, & Wessman, 1995), Chlorinated hydrocarbons that have equivalent oxidizing potentials to oxygen can contend with dissolved oxygen as an electron acceptor. Aerobic groundwater enters the iron fillings wall, and causes the oxidation of metallic iron (Fe^0) to ferrous iron (Fe^{2+}) with the discharge of two electrons. Increasing the surface area of iron greatly increased the rate at which carbon solvents respond as electron acceptors, resulting in dechlorination and release of a chloride ion (Junyapoon, 2005).The TCE reduction rate of granular iron particles has been found very slow, with half-lives in the order of days or longer and also form the toxic by product. Further improvement have been made by Pd, Pt, Ag, or Ni which can also accelerate the dechlorination process and thereby prevent formation of toxic byproducts (Wang & Zhang, 1997; Zhang, Wang, & Lien, 1998; Xu, & Zhang, 2000; Schrick,Blough, Jones, & Mallouk, 2002 coating iron particles with a second catalytic metal such). This coating also increases the dechlorination process (Zhang et al., 1998). Zero valent iron (Fe^0) nanoparticles show the redox activity to detoxify the organic and inorganic contaminants. Fe^0 and bimetallic Fe^0 particle and Fe^0/Pd^0, Fe^0/Pt^0, Fe^0/Ag^0, Fe^0/Ni^0 and Fe^0/Co^0 have widely been used in environmental remediation (Figure 7).

Heavy metal pollution is becoming one of the most serious environment problems globally (Fujita et al., 2014; Al-Musharsfi, Mahmoud, & Al-Bahry, 2013; Naser, 2013). Heavy metal pollution fromss both natural and anthropogenic sources results in the accumulation of metals in ecological niches (Kuo & Genthner, 1996). In order to detoxify heavy metals, various techniques like photocatalytical oxidation, chemical coagulants, electrochemical, bioremediation, ion-exchange resins, reverse osmosis, and adsorption have been employed (Fu & Wang, 2011; Hashim, Mukhopadhyay, Sahu, & Sengupta, 2011). Nano based adsorbent are the new approach for the removal of heavy metal. Iron nanoparticles removes As(III), Cu (II) Pb(II), Hg(II), Cd(II) Iron oxides nanoparticles such as magnetite (Fe_3O_4), maghemite (Fe_2O_3),and hematite (Fe_2O_3) based nanoabsorbents for removal of heavy metals from water/ wastewater(Dave & Chopda, 2014).

Carbon nanotubes oxidized and hydroxylated CNT are also good absorbers for metals. Multiwalled CNT has good absorbent capacity for volatile organic compounds (Li et al., 2003). They also shows the higher adsorption for several dyes like ethidium bromide, eosin bluish (Fugetsu et al., 2004).This has been found for various metals such as Cu (Liang, Ding, & Song, 2005a), Ni (Chen & Wang 2006; Lu & Liu, 2006), Cd (Li et al., 2003), Pb (Li & Zhang, 2006), Ag (Ding, Liang, Song, & Xiang, 2006), Am(III) (Wang et al., 2005) and rare earth metals (Liang, Liu, & Guo, 2005b). In most cases adsorption is highly

Figure 7. Potential remedial scheme using ZVI nanoparticles



pH-dependent with increasing sorption with increasing pH as expected for adsorption of metals onto hydroxyl groups. Adsorption of organometallic compounds on pristine multi-walled CNT was found to be stronger than for CB (Munoz, Gallego, & Valcarcel, 2005). Chemically modified nanoparticles have been proposed for environmental cleanup and may therefore be released into the environment (Obare & Meyer, 2004). TiO_2 functionalized with ethylenediamine was tested for the ability to remove anionic metals from groundwater (Mattigod et al., 2005). Nanoparticulate ZnO which was coated with the surfactant sodium dodecyl sulfate was stable in soil suspension for 14 days without changes in particle size distribution (Gimbert, Hamon, Casey, & Worsfold, 2007). Further the "Greener" approach were applied according to Raveendran, Fu, & Wallen, (2003) showed that starch can serve as a good template or dispersant for preparing nanoscale Ag particles in aqueous media. Qi & Su, (2004) have evaluated the sorption of Pb (II) onto chitosan nanoparticles (40-100 nm) prepared by ionic gelation of chitosan and tripolyphosphate. The phosphate-functionalized chitosan nanoparticles have a maximum Pb (II) sorption capacity of 398 mg/g. To inhibit the agglomeration of nanoparticles humic acid, fulvic acid and carbon nanotubes increase the permeability (Hyung, Fronter, Hughes, & Kim, 2007).

Titanium dioxide nanoparticles are great potential catalysts and redox active media due their optical and catalytic properties (Obare & Meyer, 2004). This potential is used in water purification by having

both the properties either oxidation or reduction. TiO_2 catalyzes the organic compound in water in the presence of ultraviolet light. This nanoparticles also used in air remediation and in water it photocatalyzes the organic contaminants (aromatic compounds, chlorinated compounds), reduce toxic metal ions [e.g., Cr(VI), Ag(I) and Pt(II)] in aqueous solutions under UV light (Savage & Diallo, 2005). Now slight-activated titanium dioxide concept attracted, for ability to catalyze the contaminants. Asahi, Marikawa, Ohwaki, Aoki, & Taga, 2001 synthesized N-doped TiO_2 nanoparticles that were capable of photo degrading methylene blue under visible light. Some nanoparticles are used in desalination of water by nanofilteration. Van der Bruggen & Vandecasteele (2003) have reviewed the use of nanofiltration to remove cations, natural organic matter, biological contaminants, organic pollutants, nitrates, arsenic and microbes from groundwater and surface water, U (VI) from sea water by nanofilteration and reverse osmosis. For removing pathogenic microbes carbon nanotubes filters are successfully reported. Srivastava et al. (2004) showed that the filters were effective at removing bacteria (*Escherichia coli* and *Staphylococus aureus*) and *Poliovirus sabin 1* is a type of poliovirus from contaminated water. DeFriend, Wiesner, & Barron, (2003) reported the successful fabrication of alumina ultrafine membranes using alumina (A-alumoxanes) nanoparticles (7-25 nm). When alumina nanoparticles are doped with the Fe and Mn, it increases the permeability and selectivity. Finally the Fe in bimetallic condition the successful preparation of reactive membranes by incorporation of bimetallic Fe^0/Pt^0 nanoparticles into acetate films by Meyer, Wood, Bachas, & Bhattacharyya, (2004). The embedded metal domains were effective for chlorinated hydrocarbon. For removal of pathogenic microbes MgO nanoparticles is an effective biocide against Gram positive and Gram-negative bacteria (*Escherichia coli* and *Bacillus megaterium*) and bacterial spores (*Bacillus subtillus*). Biosynthesized silver nanoparticles in the presence of silver nitrate, also behave as a biocide. Son, Youk, Lee, & Park, 2004 reported that Ag nanoparticles embedded in cellulose acetate fibres is effective against *Staphylococcus aureus*, *Escherichia coli*, *Klebsiella pneumonia* and *Pseudomonas aeruginosa*.

Dendrimers polymers nanoparticles are soft and use for the purification of drinking water from organic and inorganic solutes and free from microbes, toxic metal ions. Dendrimers have centre core, highly inner branched cell and outer branched cell (Frechet & Tomalia, 2001). These ranges from 1-20nm are highly water soluble ligand for toxic metal ions, radionuclides and inorganic anions (Ottaviani et al., 2000; Birnbaum, Rau, & Sauer, 2003). Dendritic polymers have also been successfully used as delivery vehicles or scaffolds for antimicrobial agents such as Ag (I) and quaternary ammonium chlorides (Balogh, Swanson, Tomalia, Hagnauer, & McManus, 2001; Chen & Cooper, 2002). They are found in different forms dendrimers, dendrigraft polymers, random hyperbranched polymers, dendrons (Figure 8 & 9). These Dendritic polymers have also been successfully used as delivery vehicles or scaffolds for antimicrobial agents such as Ag (I) and quaternary ammonium chlorides (Balogh et al., 2001; Chen & Cooper, 2002).

Remediation of Soil Contaminants

Soil is precious natural resource on which contributes the major part in economy. Indian economy 70% depends on the agriculture. Due to the anthropogenic activity land subjected to contaminants from industries, metal from mines, household discharges. Soil remediation has done by excavation followed by incineration landfilling but this method takes several months and also produces harmful gases and by products. Green technology approach of the bioremediation does not harm the environment and also reducing the amount of energy used in chemical processes (Kidwai & mohan, 2005). Contaminants

Figure 8. Different form of nanoparticles

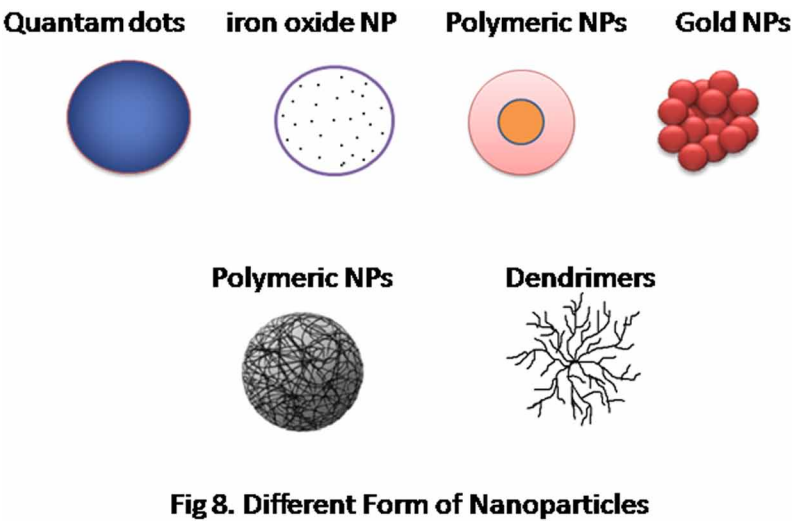
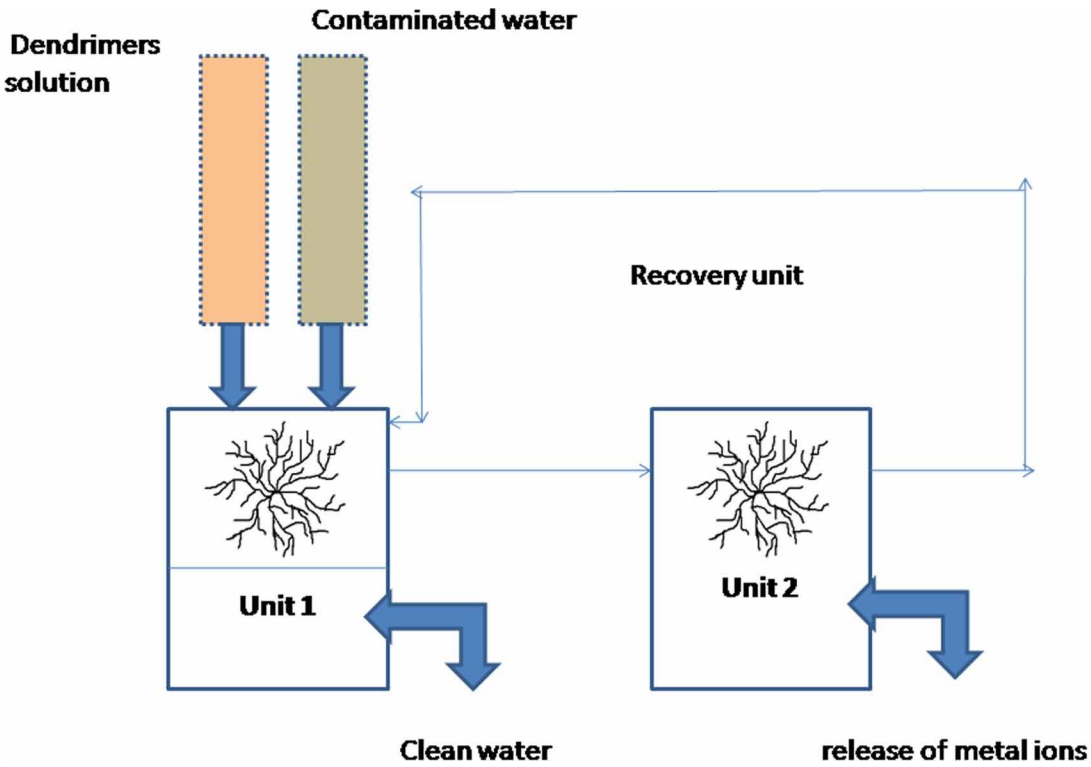


Figure 9. Removal of heavy metals from contaminated solution and its recovery by dendrimers filteration unit



continuously releases from the industries, houses mix with soil present in surroundings. Plants are also able to remediate the soil contaminants by extraction of heavy metals. Plants accumulate the heavy metals by transport channel of plant cell and get accumulate into the vacuole, but due to less biomass and bioavailability to the arial parts of the plants for contaminants, commercially not applied as much as like microbial degradation. Native microflora in soil also contributes in remediation of organic and inorganic contaminants in it. After a long period of time several microflora which are present in contaminated sites are having a good tolerance capacity in comparison to non-contaminated soil, these microbes chelates the heavy metal present in higher amount or sometimes in may converts and accumulate the metal into nanoparticle of corresponding salt or ions present in soil. These biosynthesized nanoparticles can be extracted from the microbe and used in industrial purpose or can enhance the soil and plant growth activity (Mishra et al., 2014).

Zerovalent iron can is extensively used for soil remediation. Use of heavily chlorinated pesticides (Sayles, You, Wang, & Kupferle, 1997) in soil and field soil ph becomes change day by day. These iron used in field to reduce the chlorinated compounds. Malathion is a wide spectrum non systemic organophosphate insecticides (Singhal et al., 2012) used in agriculture. Half lives of malathion is 1-25 days depends on the soil condition, soil binding, the process enhanced in presence of sunlight and moisture/alkaline condition.

Inorganic Contaminants Remediation

Inorganic pollution caused by coal mines, industries, coal combustion, municipal incineration, electroplating, energy and fuel production, power transmission have led to the accumulation of heavy metals which persists and magnifies into the environment. Heavy metals removed by nanoadsorbent iron nanoparticles by magnetic force. In soil mercury (Hg^{2+}) is major component and $HgCl_2$ is found abundantly which a major cause of soil contamination. Another source of contamination of mercury and its derivatives seed treatment, dental fillings. Several bacteria have been reported to show the ability to tolerate high concentration of metal and reduce into volatile elementary form.

Chromium compounds are used in various industries (e.g., textile dying, tanneries, metallurgy, metal electroplating, electronic, and wood preserving); hence, large quantities of Cr have been discharged into the environment due to improper disposal and leakage (Kimbrough, Cohen, Winer, Creelman, & Mabuni, 1999). Cr (VI) is the toxic form and mobile in the environment (Cheryl &Susan, 2000). Zerovalent Fe nanoparticle has a strong reducing capability, Fe^0 nano has a core–shell structure with a Fe^0 core surrounded by an oxide/hydroxide shell, which grows thicker with the progress of iron oxidation (Li & Zhang, 2006; 2007). During oxidation thickness of the shell increases (Martin et al., 2008). Mines are core of several heavy metals are present in mines. The microbial diversity present there, are also tolerant for metal like Cu, Al, Zn, Co, Hg. Microbes are sometime adsorb and get it converts into the nanoparticles which can easily harvest and used in industrial purposes (Salvadori, Ando, Nascimento, & Correa, 2014). Salvadori, Lepre, Ando, Nascimemento, & Correa, (2013) isolated *Hypocrea lixii* from the wastewater of copper mines, the dead biomass of the *H. lixii* acted as a good biosorbant of copper ions and got it converted into the copper nanoparticles. This dead biomass is a good adsorbant, reducing agent and provides the stability during uptake and synthesis of nanoparticles. These microbes used in bioreduction and bioextraction of contaminants in wastewater and groundwater.

Contamination of Organic Contaminants

Polycyclic aromatic hydrocarbons (PAHs) are highly toxic organic contaminants widely distributed in terrestrial and aquatic ecosystems, as products of the incomplete combustion of fossil fuels (Gibson, & Subramanian, 1984; Johnsen, Wick, & Harms, 2005). Manganese peroxidase (MnP) produced by *Anthracophyllum discolor*, a Chilean white rot fungus, was immobilized on nanoclay obtained from volcanic soil and its ability to degrade polycyclic aromatic hydrocarbons (PAHs) compared with the free enzyme was evaluated(Acevedo et al., 2010).Among the enzymes secreted by white rot fungi, lignin peroxidase (LiP), laccase(Lac) and manganese peroxidase (MnP) were found to have a pivotal role in the degradation of PAHs (Collins, Kotterman, Field, & Donson, 1996; Steffen, Hatakka, & Hofrichter, 2002). White rot fungus immobilized MnP by itself secreted on a nanoclay to degrade PAHs in liquid solution.

Nanoparticles can also be used as biocatalysts for reductive dechlorination. De Windt, Aelterman, & Verstraerte, 2005 reported palladium Pd(0) nanoparticles can be deposited on the cell wall and inside the cytoplasm of *Shewanella oneidensis* and charged with H* radicals by adding different substrates such as hydrogen, acetate and formate as electron donors in a bioreductive assay containing Pd(II). When these charged Pd (0)-deposited *S. oneidensis* cells are brought in contact with chlorinated compounds; the H^+ radical on the Pd (0) can catalytically react with PCP resulting in the removal of the chlorine molecule from the chlorinated compounds.

Nanoparticles can be further used to immobilize microbial cells that can degrade or biorecover specific chemicals. Unlike conventional cell immobilization on micron sized media or a fixed surface, magnetic nanoparticles (that is Fe_3O_4) were functionalized with ammonium oleate, coated on the surface of *Pseudomonas delafieldii*. By applying an external magnetic field to these microbial cells, these magnetic nanoparticle-coated cells were concentrated at a specific location on the reactor wall, separated from the bulk solution, and recycled for the treatment of the same substrate. These microbial cells were added into a bioreactor at a high biomass concentration, and were demonstrated to desulfurize organic sulphur from fossil fuel (that is dibenzothiophene) as effectively as non-nanoparticle-coated cells (Shan, Xing, Zhang, & Liu, 2005). Dyes are heterocyclic organic compounds uses as a dye in industries and research organizations, which ultimately mixed with air and waste, after mixing with water bodies it forms a layer on water, which cause hindrance in total incidence of sunlight on photosynthetic plants and bodies, so there is depletion of photosynthesis and less production of oxygen, which become a serious issue for water animal and plants. Methylene blue is aromatic heterocyclic compound its can be degraded by the photocatalyst titanium dioxide in presence of solar/UV light (Jang, Kim, & Kim, 2001), at high light energy it can also decompose the pathogenic bacteria like *Pseudomonas aeruginosa*, *Escherichia coli* and ammonia gas.

CONCLUSION AND FUTURE PROSPECTS

In an ever increasing field of development, environment pollution has become a major concern. To combat with pollution, nanotechnology has emerged as a powerful tool to make the environment clean. It can act as sensor to detect pollutants, controls the release of pollutant and has the potential to remediate *in situ* and *ex situ.* Zero valent iron, gold, silver, titania, quantum dots and carbon nanotubes have proved their potential as efficient sensors and remediators. Gold and silver nanoparticles act very well as heterogenous catalyst for degrading environmental pollutants such as para nitro phenol. With the use

of microbial systems to synthesize nanoparticles, they are giving an added advantage of clean and green nanotechnology in environmental clean-up. Though the field is rapidly increasing, the exact mechanism of biosynthesis and bioremediation through nanoparticles remains unknown which needs to be explored. Nanoparticles will be use as a carrier for sustainable delivery of pesticides and chemical fertilizers for efficient less and equal distribution in soil. Soil microflora can be used to synthesize nanoparticles and further can be used as bioremediator. To elucidate the role of microbes in biosorption of heavy metals from contaminated area and extract for industrial purpose. Microbes can be used as a factory for various purposes to explore the nanoremediation along with fertility of the soil and maintaining the balance in ecosystem. Nanoparticles can also limits the use of pesticides by biosynthesizing the nanoparticles by native microbes which is emerging as a new technology for mankind to protect their crop. The nascent field of green nanotechnology needs to be bloomed up to make the earth more green and clean with the rapid advancement of eco-friendly microbial synthesis procedures.

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KEY TERMS AND DEFINITION

Biomass: Residue of living material present in soil and environment.

Green Technology: It's a continuously involving a technique for the non toxic, sustainable production of products for promoting the viability and innovative effect in the environment.

Nanoremediation: Use of nanoparticles for cleaning or remediate the contaminants and waste product.

Native Microflora: Microbes which are present in their originated place and where they balance the physiology of the environment.

Photocatalysis: Oxidation/reduction or breakdown the compounds in the presence of light. Example: titanium dioxide nanoparticle.

Rhizoremediation: This remediation is performed by means of interaction of soil and microbes and plants. The most active region for the remediation of soil contaminants is near the roots of the plants.

Secondary Metabolites: These are the organic compounds that are not essential for metabolic processes of microbes/plants, although it enhance the growth and development.

Zerovalent Iron: Zerovalent iron is granular form of iron, has reducing and absorption property used in remediation of chlorinated compounds.

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About the Contributors

Shivom Singh joined the Department of Environmental Science, ITM University, Gwalior, India in 2012, where he currently is the Assistant Professor of Environmental Sciences. He also worked as Guest Faculty in the Department of Environmental Sciences, at G. B. Pant University of Agriculture and Technology, Pantnagar. Following a Postdoctoral Fellowship at the Department of Biological Science, G. B. Pant University of Agriculture and Technology, Pantnagar, where, with Dr. Anil Kumar Sharma he filed Indian patent, Govt. of India. Dr. Shivom Singh received two Ph.D. degrees first in Botany from MJP Rohilkhand University, Bareilly and the second in Environmental Sciences from Gurukul Kangri University, Haridwar. His postgraduate degree is from the Gurukul Kangri Univerisity, Haridwar, India in 2003. He is a Fellow of Action for Sustainable, Efficacious Development and Awareness (FASEA) and holds the membership of many national and international professional bodies. Dr. Singh researches on Environmental monitoring of different aspects of pollutions and their biological managements and rhizosphere biology. His current work is aimed on biofertilizer and biopesticides. He has written more than thirty five research and review articles in reputed national and international journals.

Kajal Srivastava is a Post Doctoral Fellow at the G. B. Pant University of Agriculture and Technology, Pantnagar, India. From 2007-2009 she was a guest faculty member at the Department of Botany, at Bareilly College, Bareilly. She received her doctoral degree in Botany, from MJP Rohilkhand University, Bareilly, India and had studied post graduation in Industrial Biotechnology from Ch. Charan Singh University, Meerut, India. Dr. Srivastava is the author of over 35 peer-reviewed publications in national and international journals and also holds membership of many professional bodies. Her research specifically focuses on bryomonitoring, plant biochemistry and environmental biotechnology.

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Nagia Ali is working as professor at Textile research division, Dyeing and Printing Department, at the National Research Center, Cairo, Egypt. She has 42 research articles published in different national and international journals. Currently she is running a project entitled “Green strategy to production smart fibres to be used in pigment handlooms by economic methods.”

Feroz Ahmad Ahanger soon after completing his B.Sc from Degree College Pulwama (2005-2007). Mr Feroz joined the Jiwaji University, Gwalior for post graduate studies in Environmental Sciences. After finishing his postgraduation (2008-2009) the author joiind the M.Phil programme in the same university (2010-2011) and completed it and has published few of research papers in national and international repute. During the same year he qualified UGC-JRF and national eligibility test in Environment Sciences

conducted by UGC. Currently, he is pursuing a Ph.D programme in the Department of Environment Sciences, University of Kashmir and is working on the Air pollution of Mulberry on the *Bombyx Mori* with focus on the impact of the pollution on silk producing ability of *Bombyx mori* and the microbial disease susceptibility to silkworm.

Sajad Ahmad Bhat after completing B.Sc from Degree College Pulwama (2006-2008) joined the Department of Zoology for Post Graduation. After completing M.SC in Zoology (2009-2011), the author qualified to joint UGC - CSIR. JRF and the National Eligibility Test in Life Sciences conducted by CSIR India in 2012. Mr. Sajad joined the Microbiology and Pathology laboratory at Centre of Research for Development, University of Kashmir Srinagar for his Ph.D programme and is working on Actinomycetes from the local geographical region with the target of the research as, novel bioactive compounds. The study involves the isolation of actinomycetes bacteria from soil samples from Kashmir with molecular identification of the isolated samples . The focus of the study is to look for some new novel actinomycete species and culture of the isolated bacteria under different growth conditions to observe the activity of the extracts and search for new novel antimicrobial, antioxidant and anti diabetic compounds. The author has also attended many conferences and workshops and has published many national and international research papers.

Deepesh Bhatt completed his doctorate degree in 2013 in the field of Plant Biotechnology and is currently working as an Assistant Professor in Biotechnology Department at Shree Ramkrishna Institute of Computer Education and Applied Sciences, Surat, Gujarat. Prior to this he was working as an Associate Research Scientist at research and development unit of a leading hybrid seed company Maharashtra Hybrid Seeds Company (MAHYCO). He also worked as a Research Associate at the National Research Center on Plant Biotechnology, PUSA campus, New Delhi. Dr. Bhatt also has strong research experience in plant genomics and molecular biology, microarray analysis, subtractive hybridization, rice transformation, handling callus, suspension and hairy root culture with good list of publications.

Megha D. Bhatt has completed a doctorate degree in Microbiology from G.B Pant University of Agriculture & Technology, Pantnagar in 2013 and is currently working as a Deputy Manager (Agribusiness) at Gujarat state fertilizers and chemical limited, Vadodara, Gujarat. She has good research publication in peer reviewed journals. Dr. Bhatt also assessed soil health, culturable and non- culturable bacterial diversity using DGGE in organic and conventional farming under Indo Swiss Collaboration project.

Arpita Bhattacharya, CSIR JRF, working at CSIR NBRI as a PhD scholar. He has done my graduation from Calcutta University and post graduation from B.H.U, varanasi. At present she is working on microbes and their ability to induce defense modulating strength in plants against various biotic stresses existing in the environment. Her research area of interest involves studying various plant microbe interactions and understanding the various metabolic pathways involved in it.

Puneet Singh Chauhan obtained his PhD from Jiwaji University, Gwalior in Biochemistry. After graduation, he did his postdoctoral research (2009– 2011) at College of Agricultural Chemistry, Chungbuk National University, Cheongju South Korea after that he has worked at CSIR National Botanical Research Institute, Lucknow, India as Senior Scientist Fellow during 2011– 2013. At present he is working as Senior Scientist at CSIR National Botanical Research Institute, Lucknow, India in the area of plant microbe interactions, stress biology, microbial ecology and soil metagenomics.

Arezoo Dadrasnia is a Post Doctoral Research Fellow in Molecular Bacteriology and Toxicology laboratory, at Institute of Biological Sciences, Faculty of science, University of Malaya (UM). Dr. Arezoo Dadrasnia received her B.Sc and M.Sc degrees in Soil sciences (chemistry and fertilizer) from Guilan University, Iran in 2004 and 2007, respectively and Ph.D degree in Environmental biotechnology/ Toxicology from University of Malaya, Malaysia in 2013. Her areas of research interests are soil science, remediation of soil and water resources, solid waste management, microbial degradation, leachate treatment, enhanced oil recovery, environmental pollution treatment, isotope monitoring remediation, and hazardous waste management researches.

Anil Dhar has vast experience in the field of Mulberry research in North West India. As principal scientist, Dr. Dhar has developed a number of technologies which have resulted in significant impact on sericulture industry in north. The major technologies include Package of Practices for mulberry Bush and Tree cultivation, Prunning-Shoot harvesting for mulberry trees, and Mulberry Sapling raising technology for sub tropics. Central Silk Board and Directorate of Sericulture, J&K Govt. have jointly honoured Dr Dhar with the Award in recognition of Development of suitable Technologies for mulberry farming for the North Indian States (2009). He is the only scientist bestowed with this award so far under CSR&TI, Pampore. He is currently working at Regional Sericultural Research Station, Jammu, India.

Monika Dhote has done her M.Sc and Ph.D in Biotechnology. Her Ph.D, (2013) entitled "Studies on polycyclic aromatic hydrocarbon compounds in oil sludge" and has published three research papers and one review paper in International Journals. She has seven years of research experience in the field of Environmental Microbiology and Biotechnology. She has been awarded with Senior Research Fellowship from CSIR for her Ph.D work. She has received the best performance award for an oral paper presentation at the National symposium organised by the National Academy of Sciences, at Kerala University. Currently she is working as a Research Associate at CSIR-NCL, Pune and focusing on studies on molecular tools of bioremediation of petroleum hydrocarbon compounds, especially PAHs.

Chijioke Emenike is a Post Doctoral Research Fellow at the Centre for Research in Waste Management, University of Malaya (UM). Dr Emenike obtained his B.Sc degree (Microbiology/Biochemistry) from University of Nigeria, M.Tech (Environmental Management) and PhD (Environmental Toxicology & Bioremediation) degrees from UM. He has served UM in various research projects for a period of four years. He has experience in environmental toxicology, remediation technologies, waste management and risk assessment. He is versatile with systems which are pivotal to biostimulation, bioaugmentation and bioconversions as required in remedying polluted sites and waste generation systems. His roles in teaching, course development and mentoring have been overwhelmingly positive. Dr Emenike has written and published a number of scientific articles in international journals and papers in proceedings, and contributed chapters to books edited by others.

C. Ghanshyam received his M.Tech degree in Instrumentation & Control Engineering from Jawaharlal Nehru Technology University, Kakinada, Andhra Pradesh, and did his PhD in the area of gas sensors from GND University, Amritsar. He was a DAAD Fellow from 1988-1990 and worked at the Institute of Technical Physics, University of Kassel, Germany. He was an UNDP Fellow from October 1991 to December 1991 and worked at Tampere University, Finland. He is an Honorary Adjunct Professor at BESU, Shibpur, West Bengal. He is also an honorary Professor at the Academy of Scientific

and Innovative Research (AcSIR), CSIR-CSIO, Chandigarh. For the past 35 years, he has been actively involved in R&D in the field of electron and ion beam instrumentation. Currently he is working as Chief Scientist, and Head of the Agrionics Division at CSIR-CSIO, Chandigarh. His research includes thin film instrumentation, gas sensors, electrostatically vapour deposition, molecular beam Epitaxy for III–V compound semiconductors, pulsed electric field technique for preservation of liquid foods etc. He has wide experience in the design and development of sensor structure and applications. He has published more than 180 papers in journals and proceedings. He was a Project Investigator for several national projects. He is a Fellow & Life member of Institute of Electronics & Telecommunication Engineers, New Delhi, Fellow of Institute of Engineers, Kolkata (W.B).

Krishna Giri is Scientist B at the Rain Forest Research Institute in Jorhat, Assam (Indian Council of Forestry Research and Education, Dehradun, Ministry of Environment, Forests & Climate Change, GoI, India) and has acquired M. Sc. and Ph.D. degrees in Environmental Sciences from G.B. Pant University of Agriculture and Technology, Pantnagar in the year 2010 and 2013 respectively. He has published about 10 research papers in peer reviewed scientific journals of National and International repute and also contributed papers to National and International seminars, symposiums and workshops. He is actively engaged in teaching and research activities in the field of carbon sequestration and shifting cultivation in Northeast India.

Mansee Govil has completed her M.Sc. in Agriculture from Govind Ballabh Pant University of Agriculture and Technology, Pantnagar, Uttarakahnd. Mrs. Govil is experienced in the application of biofertilizers, floriculture and landscaping and also having exposure of molecular breeding on Groundnut at the International Crops Research Institute of Semi-Arid Tropics at Hyderabad.

Md. Anwar Hossain is the leader of the Microbial Genetics and Bioinformatics Laboratory. He obtained his PhD in 1991 from the Department of Pharmaceutical Analytical Chemistry, University of Tokyo, Japan and completed his Post-doctoral research from SUNBOR, Japan and the University of Medicine and Dentistry- Robert Wood Johnson Medical School, New Jersey, USA. Dr. Hossain became Professor in March 1997 and was the former chairman of Department of Microbiology, University of Dhaka. He has been serving in different administrative positions in different public and private universities and post-graduate colleges. He is the founder co-chair & currently the Vice-president of Asian Food Safety and Security Association (AFSSA). He is the national expert member of the National Task Force on Biotechnology for Bangladesh. Dr. Hossain is on the board of governors and serves as a council member in the Bangladesh Council for Scientific and Industrial Research (BCSIR). He has received the Best Researcher Award in 2011 from University Grant Commissions, Bangladesh. Dr. Hossain has been working as a dedicated researcher and is currently leading several projects such as – (i) FMDV in Bangladesh: Genome analysis and Vaccine development'; (ii) Environmental Antibiotics and Resistant Gene Pool Pollutions: Its Impact on dissemination of Drug Resistance, and (iii) Food Hygiene and Foodborne Pathogens in Bangladesh. He is supervising as the co-principal investigator of the project "Arsenic Microbiome and its' application in Bioremediation of As-Pollution".

Salmah Binti Ismail was born in Kuala Lumpur, Malaysia in November 1964. She graduated from University of Malaya, Malaysia with a B.Sc. (Hons) in Microbiology (1985-1989), followed by MSc. In Bacteriology (1994-1997) and Ph.D. in Molecular Bacteriology (1998-2000) from the same university.

She is currently a Lecturer in the Biohealth Department, Institute of Biological Sciences, Faculty of Science, and University of Malaya, Malaysia. Her research interests include the application of biofertilizer, plant growth promoting bacteria, biosufactant, biocontrol of agricultural diseases and pests, remediation technologies, vaccine production for mycobacterium tuberculosis and plasturella multocida, wound healing, and molecular bacteriology.

Mamta K. is a PhD student in the Department of Environmental Science (IGAEERE) Jiwaji University, Gwalior, Madhya Pradesh India. She obtained her Master's degree from Jiwaji University, Gwalior, India. She is an Inspire Fellow of the Department of Science and Technology. Her areas of interest include environmental pollution and solid waste management. She has published research papers in various journals and is the author of three books on environmental science.

K. Narasimhulu has been working at NIT Warangal since 17-01-2008 as an Asst. Professor in the Biotechnology Department. He has worked in the field of Environmental Biotechnology and published 20 research papers. He has conducted two national level workshops in the Department of Biotechnology sponsored by TEQIP-II. He has undergone R&D training at Rice University for one month, sponsored by TEQIP-II. He has guided twenty undergraduate projects in the area of Environmental Biotechnology and has been sanctioned a research project (under Fasttrack) on Bioethanol production for three years (2014-2017). He has designed and developed a course on Downstream Processing in Biotechnology as a part of developing suitable pedagogical methods for various classes, intellectual calibers and research in e-learning under National Mission project on education through ICT and developing another course on Novel Separations.

Anil Kumar obtained his masters and doctoral degrees in biochemistry from Lucknow University in 1973 and 1983, respectively. He worked as Lecturer in Biochemistry at the Lucknow University for about 08 years. In 1985, he joined Prof. Jack Preiss lab at the Michigan State University, East Lansing, USA. There, he worked on DNA sequencing and site directed mutagenesis. He is working as Professor & Head of the School of Biotechnology, Devi Ahilya University, Indore, India since June, 1991. He has also worked in many Administrative positions for the University. He has also worked as Acting Vice-Chancellor of the University many times. He is a founding member of the School of Biotechnology at the Devi Ahilya University. He has published nearly 120 full length research papers in various International and National journals of high repute and authored nearly a dozen books including Advanced topics in Molecular Biology (Horizon Scientific Press, UK), Genetic Engineering (Nova Science Publishers, USA), Biochemical Tests (Viva Books Pvt Ltd, Delhi), Enzymes & Enzyme Technology (MV Learning, London & New Delhi), and guided more than 25 Ph.D. students. He also authored number of chapters for many books including Encyclopedia of Molecular Cell Biology & Molecular Medicine. He was invited to host & chair a symposium at the 4th World Congress of Cellular & Molecular Biology held at Poitiers, France in 2005. He hosted and organized the 5th World Congress of Cellular & Molecular Biology (November 02-06, 2009) & was the Congress President. He is a fellow of many Societies including World Society of Cellular & Molecular Biology, France where 21 Nobel Laureates are also the fellows. In 2012, his chapter on Regulation of Gene Expression has been published in Epigenetic Regulation and Epigenomics (Wiley VCH). In 2014, his updated chapter on Regulation of Gene Expression has been published in RNA Regulation and again in Synthetic Biology-Advances in Molecular Biology and Medicine.

Ashish Kumar did his M.Sc. (Ag.) and Ph.D. in Plant Pathology from G.B.Pant University of Agriculture & Technology, Pantnagar. He did his Post Doctorate from International Crops Research Institute for the semi-arid tropics, Hyderabad. His specific areas of research are Molecular breeding for biotic and abiotic stress in plants, functional genomics of crop plants, pathogen population characterization and population genetics, biological control of biotic and abiotic stresses and plant disease management. Prior to his appointment as Scientist, Plant Pathology, Dr. Kumar has been awarded with the Department of Biotechnology funded Research associateship at ICRISAT, Hyderabad. On account of his professional achievements, Dr. Kumar has received several prestigious awards and honours such as Prof. M. J. Narasinhan academic merit award of Indian Phytopathological Society (IPS) at Zonal level, DBT- Research associateship award by Department of Biotechnology and Young Scientist award by professional societies. He has served as Principal Investigator of a national project funded by MPCST. He has so far guided 3 M.Sc. students in Plant Pathology. Dr. Kumar has published about more than 30 research papers/reviews in national/international journals of repute. He has prepared several book chapters published by international and national publishers. He has done pioneering research on Map kinase b from *Trichoderma virens*.

Madhuree Kumari, CSIR-SRF working at CSRI-NBRI, lucknow. She has completed masters in biotechnology from Banasthali Vidyapeeth. At present her research work is Biosynthesis of silver nanoparticles and its role in inhibiting the growth of pathogenic fungus. Along with silver nanoparticles, she also worked on gold nanoparticles biosynthesis and it's application in catalysis degradation of water pollutants.

Aradhana Mishra obtained her PhD from R. D. University, Jabalpur in 2007. Afterwards she joined CSIR-NBRI as Women Scientist Fellow under Women Scientist Scholarship Scheme of Department of Science and Technology, Government of India. She is at present Senior Scientist in the Division of Plant Microbe Interactions, CSIR-NBRI. Her research interests include work on understanding of soil microbial diversity and microbial efficiency of diesel contaminated soil to predict the fate of possible diesel biodegradation under field conditions; development of novel plant growth promoting *Trichoderma* protoplast fusants, and elucidating effect of green house gases on plants, utilising transcriptome, proteomic, and metabolomics. Recently she is working on biosynthesis of nanoparticles and its application in bioremediation of pollutants along with its beneficial role in agriculture.

Gaurav Mishra has completed his M. Sc. and Ph. D degree in Soil Science from G. B. Pant University of Agriculture and Technology, Pantnagar Presently he is working as a scientist B at the Rain Forest Research Institute, Jorhat, Assam. He has published more than 10 research papers in National and International reputed journals.

Shashank Mishra is working at CSIR-NBRI, as a PhD scholar. He has completed his graduation from V.B.S Purvanchal University, Jaunpur and masters from A.P.S University, Rewa. His research area of interest in agricultural microbiology, agriculture biotechnology, proteomics study during biotic and abiotic stress condition, formulation of biofertilizers for improving the crop productivity and fertility of soil.

Yugal Kishore Mohanta, Passed M.Sc. Biotechnology in 2010 (First class first). Currently he is a PhD student in Dept. of Botany, North Orissa University, Orissa, India. He possesses some excellent scientific credit in terms of scientific publication in highly reputed journals like BMC Plant Biology etc.

Tapan Kumar Mohanta, Assistant Professor, Dept. of Biotechnology, Yeungnam University, Republic of Korea. He completed two years (2012-2014) of postdoctoral research associate research, National Institute of Plant Genome Research (JNU Campus), New Delhi. He was awarded a PhD in Environmental and Biosensors Science, Dept. of Plant Biology, University of Turin. He passed MS in Bioinformatics in 2007, University of Turin, Italy. He passed M.Sc. in Biotechnology in 2006 (First class first), North Orissa University, Orissa India. Dr. Mohanta have several excellent scientific credit in terms of scientific publication in highly reputed scientific journals like BMC Genomics, PLoS One, BMC Plant Biology etc.

Nibedita Mohanta, Passed M.Sc. Biotechnology in 2007. She has two years (2008-2010) of research experience as a Research Fellow, Dept. di Biologia Vegetale, University of Turin, Italy. She posses some excellent scientific credit in terms of scientific publication in highly reputed journals like BMC Genomics, BMC Plant Biology etc.

Chandrika M. Nanayakkara is a Senior Lecturer attached to the Department of Plant Sciences, University of Colombo, Sri Lanka. She earned her basic degree in Botany from University of Colombo, M. Sc. in Applied Microbiology from University of Kelaniya, Sri Lanka and Ph. D. in Environmental Microbiology from University of Aberdeen, UK. She is the founder of the two new academic Programmes: M. Sc. in Agricultural Microbiology and Diploma in Microbiological Techniques, conducted by Department of Plant Sciences, University of Colombo. Chandrika holds various positions at the Sri Lanka Council for Agriculture Research Policy and Sri Lanka Accreditation Board for Conformity Assessment. She authored more than 40 publications including books, chapters in books, journal articles and short communications. She also contributed to the publications on national interest by coauthoring National Agriculture Research Policy of Sri Lanka 2012-2016. She has research interests in bioremediation, biofertilizers and antimicrobial compounds of natural origin.

Ruqeya Nazir, completed her Ph.D. in Microbiology (2004-2009) from the Department of Microbiology, Faculty of Medical Sciences, University of Delhi, Delhi, and has pursued her postgraduation in Medical Elementology & Toxicology (2001-2003) from the Department of Medical Elementology & Toxicology, Faculty of Science, Jamia Hamdard, New Delhi, and Bachelor's in Biological Science (1998-2000) from University of Kashmir. She has worked as a research scientist at Division of Veterinary Microbiology and immunology, SKAUST – Kashmir. As a research student from the very beginning of her carrier, she has worked extensively in various aspects of Toxicology, virology and microbiology. Worked as a Senior Research Fellow, (2006 – 2009) and junior Research Fellow (2003 – 2006) in the Department of Respiratory Virology, V P Chest Institute, University of Delhi. She had also pursued her postgraduation dissertation from Department of Hematology, All India Institute of Medical Sciences, Ansari Nagar, New Delhi. She has qualified Graduate Aptitude Test in Engineering (GATE), Conducted by IIT, Chennai, India in 2003. And has received best poster Award in ICAAICON, 41st annual conference of the Indian College of Allergy, Asthma and Immunology held on 9-12, Dec, 2007 at the Golden Jubilee Paintal Memorial auditorium, V. P. Chest Institute, Delhi. She has been also awarded with International Merit Travel Award to attend

IVth Orthomyxovirus Research Conference 2007, at Marine Biological Laboratories in Woods Hole, MA, September 2007, at University of Wisconsin-Madison, USA. After being appointed as Assistant Professor at the Centre of Research for Development University of Kashmir, she has published many research papers in journals of national and international repute and is presently guiding many M.Phil and Ph.D research scholars. She teaches toxicology and Microbiology to the Post Graduate students of Department of Environmental Science, University of Kashmir. She has organized conferences at the national level and has presented her research work in a number of national and international conferences.

Rashmi Paliwal has acquired M. Sc. degree (Environmental Science) from Gurukul Kangri Vishwavidyalaya, Haridwar, Ph.D. degree (Environmental Science) from G.B. Pant University of Agriculture and Technology, Pantnagar and availed UGC-NET and ARS-NET. She is an active member of "Association of Microbiology of India" and "The Indian Science Congress Association". She possesses 6 years of research experience in the fields of bioremediation and biodegradation of recalcitrant compounds. She has published research papers in leading international research journals and has also contributed papers in National and International seminar, symposium and workshop.

Shailesh Pandey is working as a Scientist at Rain Forest Research Institute, Jorhat, Assam. He did his Masters and Ph. D in Plant pathology from G.B.P.U.A & T. Pantnagar. He is actively engaged in teaching, research and extension activities in the field of plant pathology. Dr. Pandey has over 15 scientific publications to his credit in national and international journals of repute. His area of interest is population biology of plant pathogens, biological control and plant disease) management.

Shipra Pandey, PhD scholar, works at CSIR-NBRI, Lucknow. Pandey has graduated from Allahabad University and received a masters in Microbiology from Barkatullah University. Her areas of research work is plant microbe interactions, biosynthesis of nanoparticles, how microbes biosynthesize metal nanoparticles and the mechanism by which these nanoparticles inhibit the growth of phytopathogen and their application in agriculture.

Manoj Kumar Patel received his B.Tech degree in Electronics & Communication Engineering from National Institute of Technology (NIT), Jamshedpur, India and completed his M.Tech in Advanced Instrumentation Engineering from Academy of Scientific and Innovative Research (AcSIR), New Delhi, India. At present, he is working as a scientist in the Agrionics Division, CSIR-Central Scientific Instruments Organisation, Chandigarh. He is also an honorary Assistant Professor in Academy of Scientific and Innovative Research (AcSIR), CSIR-CSIO, Chandigarh. He is a recipient of the Postgraduate Research Program in Engineering (PGRPE) Fellowship- Council of Scientific and Industrial Research, Government of India. His research interests include advanced agri-instrumentation, automation & mechanization in agriculture, electrostatic processes applied to agriculture, electrostatic deposition of antimicrobial liquids for surface decontamination, airborne charged particulate technology for air-pollution abatement and instrumentation & control for perishable food products. He is an active reviewer of many national and international SCI journals.

J. P.N. Rai, Senior Professor & Head Department of Environmental Science, G.B. Pant University of Agriculture and Technology, Pantnagar, Uttarkhand, India, has acquired M.Sc. and Ph.D. degrees (Environmental Science) from North Eastern Hill University, Shillong, India, has 25 years UG and PG

teaching experience in the field of environmental biotechnology and waste utilization and 28 years research experience in the field of plant environment interaction leading to bioremediation and biodegradation of recalcitrant compounds. He has successfully completed a dozen research projects funded by national and international agencies. He has supervised 24 Ph. D and 36 M. Sc. students and has published over 85 research papers in scientific reputed journals, besides contributing several popular articles. He has availed research associate-ship of Council of Scientific and Industrial Research, Government of India, bagged national award of commission on scientific dictionary from Ministry of Human Resource Development, India and has life membership of several academic national and international societies. Dr. Rai has also been recognized and approved as functional area expert on biodiversity and solid waste management by Ministry Of Environment and Forests, GOI, India.

R.J. Rao is a Rector at Jiwaji University, Gwalior and Professor in School of Studies in Zoology Jiwaji University, Gwalior, Madhya Pradesh, India. He has 27 years of research experience. He has completed his MSc and PhD in Zoology from Bhopal University, India. He is a member of various societies and communities and has completed 11 research projects. He has published research papers in various national and international journals.

Santonu Kumar Sanyal has a special interest in Research such as Microbial Bio-remediation of arsenic contaminated water and develop an eco-friendly sustainable technology for arsenic free water. Sanyal is also working on a project "prevalence of White Spot Syndrome virus in the coastal belt shrimp firms of Bangladesh'.

Y. Pydi Setty has completed B. Tech in Chemical Engineering at Andhra University, Visakhapatnam, India in 1976 and M. Tech and Ph. D in Chemical Engineering at Indian Institute of Technology, Madras, India in 1978 and 1984 respectively. Afterwards Prof. Y. Pydi Setty has been very active in Academics and Research work. He has more than 30 years of experience in Teaching and Research. He has guided several students for M. Tech and Ph. D. He has been working in the areas of Fluidization Engineering, Wastewater Treatment, Fluidized Bed Drying, Circulating Fluidized Beds, and Modeling, Simulation and Optimization of Chemical Processes. He has published more than 35 papers in National and International Journals and presented more than 20 papers at national and international conferences. He has received three awards for best paper publication.

Fayaz Ahmad Shah completed B.Sc from Amar Singh College Srinagar during 2006- 2008, the author joined the Department of Zoology at the University of Kashmir for his post Graduate studies (2009-2011). After completing M.Sc in first division the author qualified joint UGC-CSIR-JRF conducted by CSIR India in 2012. The author is currently pursuing his Ph.D at Microbiology and pathology Laboratory Centre of Research for Development, University of Kashmir and is working on the bioefficacy of indigenous medicinal plants against aflatoxin producing Aspergills fungi and aflatoxins. The author is working on the major Aspergillus species damaging stored food items and is also working on the analysis of the aflatoxin producing ability of different Aspergillus species. Further the focus is the quantification of aflatoxins among diverse stored food items. The author has also attended many conferences and workshops and has published many national and international research papers.

Krishna Kumar Sharma did his M.Sc. (Ag.) and Ph.D. in Plant Pathology from G.B.Pant University of Agriculture & Technology, Pantnagar. He did his Post Doctorate from Vivekanand Parvartiya Krishi Anusandhan Shala, Almora (Uttarakhand). His specific areas of research are biological control of biotic and abiotic stresses and plant disease management. Prior to his appointment as an Assistant Professor of Agriculture, Dr. Sharma was serving as a Research Associate at Vivekanand Parvartiya Krishi Anusandhan Shala, Almora. On account of his professional achievements, Dr. Sharma has published more than 15 research papers/reviews in national/international journals of repute. He has prepared several book chapters published by international and national publishers.

Tarlan Sheikhavandi is a third year PhD student in soil science and plant nutrition department in Çukurova university-Turkey. Her research interest is soil contamination with heavy metals and bioremediation, more specifically, phytoremediation of heavy metal contaminated land. After her B.Sc. on soil science at State University of Tabriz-Iran, she studied soil classification and evaluation at the State University of Tabriz, where her main area of study was soil micro morphology. Later, her dissertation became based on usage of organic and synthetic conditioners effects on soil micro-morphological properties. A related paper, Effect of biological sludge and Polyacrylamide on improvement of top soil aggregation, was published. She continued her studies in Ankara, Turkey on soil genesis at Ankara University for a second master's degree, while participating in a project on soil survey and mapping (GIS-based). Her second master's dissertation was a presented project on world's soil and vegetation classification methods.

Mohini Singh is an Assistant Professor in Department of Environmental Sciences, Central University of Haryana, Mahendergarh, India with specialization in Wastewater Treatment and Environmental Management. She has more than two years of teaching experience to Undergraduate and Post Graduate students. She was honored by Uttarakhand Counsil of Science and Technology, India (UCOST) as 'Young Scientist' in 4th Uttarakhand State Science and Technology Congress in year 2009. She was the recipient of INSPIRE Fellowship by Department of Science and Technology (DST), Government of India, New Delhi, India for pursuing doctoral research. She has published her research works at national and international journals of repute. She is the member of International Society of Environmental Botanist, Indian Water Resources Society and Asia-Pacific Chemical, Biological & Environmental Engineering Society.

Satyendra Pratap Singh working as a Ph.D. scholar at CSIR-National Botanical Research Institute, Lucknow. He did his masters from Allahabad Agricultural University, Allahabad in Microbiology. Now a days he is acticily participated in the field of agricultural microbiology. His research area of interest is in the microbial diversity identification and control of disease organisms, often working area is in allied sectors such as food technology and environmental management.

Saurabh Kumar Singh is holding the position of Technical Assistant in AICRIP on Rice at JNKVV, College of Agriculture, Rewa. Dr. Singh is PhD in Life Science. Dr. Singh is having is expertise in Rice agronomy. Dr. Singh is having more than 15 international and national papers to his credit.

Vikram Singh did his doctorate degree at the Govind Ballab Pant University of Agriculture and Technology, Pantnagar, Uttarakhand, India in Biochemistry discipline. Thereafter, he joined the Central Research Institute for Dryland Agriculture (ICAR), Hyderabad, Andhra Pradesh, India as a Research Associate under the project 'National Initiative on Climate Resilient Agriculture (NICRA)' sanctioned

by ICAR, New Delhi, India. In this job, he explored the functional and structural aspect related to mitochondrial electron transport chain complexes protein at heat stress condition in Maize (Zea mays). Presently, he is working as a visiting scientist at the Agricultural Research Organization, Israel. As a researcher in ARO he is investigating the molecular mechanism and cross link of ethylene biosynthesis and ROS generation pathways. He explored the role of major genes responsible for ROS generation, ethylene biosynthesis and signaling, including involved transcription factors. He has published many research papers in reputed journals, co-authored published books, and been an editorial board member of many reputed journals with the membership of various scientific societies.

Munawar Sultana is a young Microbiologist in Bangladesh who is currently serving as an Assistant Professor in the Department of Microbiology, University of Dhaka, Bangladesh. Dr. Sultana has completed her Secondary and Higher secondary School certificate Examination from the Dhaka Board in 1995 and 1997, respectively. She obtained B.Sc. (with honours) in academic session 2001 and M.S. in 2002 from the Department of Microbiology, University of Dhaka, Bangladesh. She got DAAD (German Academy Exchange Service) fellowship for conducting a Ph.D. in Germany and in 2010; she completed her doctoral study with a very good grade (Magna-cum-Laude in German) from the Institute of Bioscience, Faculty of Chemistry and Physics of TU Bergakademie Freiberg, Germany. In 2014, she was selected as a Fulbright visiting fellow to conduct research at University of California, Santa Cruz, USA. Dr. Sultana acquired considerable experience in writing scientific protocols, concept papers and research articles and also contributed substantially to the progress of research in her institution. She is associated with the research group at the Microbial genomics and bioinformatics (www.microbialgen.du.ac.bd) laboratory of the Department. She is currently involved in various national and international projects and is actively involved in grant writing, training and guiding of M.S. and Ph.D. student theses. At present she has more than 17 research articles published in peer reviewed impacted international journals. Dr. Sultana has consistently shown high scientific excellence and research capability, which, is due to her intellectual capacity and arduous perseverance. Her overall contribution in teaching and research in the Department of Microbiology, University of Dhaka is highly praiseworthy and will be beneficial to improve and enhance the capacity of the University of Dhaka, Bangladesh.

Deep Chandra Suyal is actively working in microbial diversity and ecology oriented research work since 2010. He completed his B.Sc. degree in 2008 from S.S.J. Campus, Almora (Kumaun University, Nainital). Next, he successfully completed his M.Sc. and Ph.D. degrees in Microbiology from Govind Ballabh Pant University of Agriculture and Technology, Pantnagar, in 2010 and 2014, respectively. At present, he is engaged in Indo-European International collaborative project "SAHYOG".

R. K. Tiwari is the Senior Scientist in AICRIP on Rice at JNKVV, College of Agriculture, Rewa. Dr. Tiwari's specialty is in Rice Agronomy. Prior to his assignment as Senior Scientist, Dr. Tiwari held positions as SMS and Programme Coordinator at Krishi Vigyan Kendra, Rewa. Dr. Tiwari has more than 50 National and international publications to his credit.

A.N. Tripathi has been serving as a Scientist (Plant Pathology) for the Indian Council of Agricultural Research at the Central Research Institute for Jute & Allied Fibres, Barrackpore, Kolkata (W.B.) since 2011. With a doctorate degree in Plant Pathology, minoring in Molecular biology and Biotechnology with specialization in Molecular Fungal pathology and biological control from G. B. Pant University

of Agriculture & Technology, Pantnagar (U.K.), India in collaboration with ICAR, the National Bureau of Plant Genetic Resources, New Delhi, India. Dr. Tripathi did his M. Sc. Ag. (Plant Pathology) with specialization in Plant Bacteriology at CSK, Himachal Pradesh Krishi Vishva Vidyalaya, Palampur (H.P.) and a B. Sc. (Ag.) from Jawahar Lal Nehru Krishi Vishwa Vidyalay, Jabalpur (M.P.). He has received the ICAR- Junior Research Fellowship (Plant Sciences) in Plant Pathology- 2002; Joint CSIR-UGC, JRF & NET (Life Sciences) 2004 and ICAR-NET, 2006, 2008, 2009 & 2010. He has authored more than 10 research papers, contributed several chapters to books, popular articles, farmers technical leaflets and edited book chapters and research articles, for national and international publishers. He has been bestowed with the Certificate of Merit -94th FOCARS- 2012 from NAARM, Hyderabad and best poster award in 2013 from the Indian Phytopathological Society (Eastern Zone). Dr. Tripathi worked as an Asst. Professor of Plant pathology and Microbiology, in the Division of Crop Protection, of the College of Horticulture and Forestry, at the Central Agricultural University, Pasighat, Arunachal Pradesh in 2010-11. He started his professional career as a Research Associate at the Central Rainfed Upland Rice Research Station (CRURRS), CRRI, Hazaribag, Jharkhand from 2008- 2009 & further joined the Project Implementation Unit, ICAR- Krishi Anusandhan Bhavan–II, Pusa New Delhi from 2009 - 2010. He is the life member of The Indian Natural Fibre Society.

S. K. Tripathi has held the position of Professor and Head of the Department of Plant Pathology, JNKVV, College of Agriculture, Rewa. Dr. Tripathi is actively associated in teaching, research, and extension for the last 30 years. He has vast experience in the field of plant pathology and has guided more than 40 students in Ph.D. and post graduate studies. He has had more than 100 publications in National and international refereed journals. He has been awarded several national awards like the KPV Menon Award, the Crop Research Award, the Best Research Paper Award and the Bioved Fellowship Award.

Praveen Kumar Verma received his Ph. D from the University of Lucknow on the topic 'Studies in the Hepaticae of Nilgiri Hills with special reference to Epiphytic taxa' and also worked as a JRF and a SRF in the AICOPTAX project funded by MoEF, Govt. of India. Presently, he is working at the Forest Research Institute, Dehradun. He has published more than 40 research papers in national and internationally reputed journals. His area of interest is the taxonomy of liverworts, conservation and user aspect, specifically the characterization of the moss genera *Sphagnum*. Currently, he is engaged in various research projects such as the 'Utilization Potential of Sphagnum' and the 'Ecology of an Invasive Species Mimosa.'

Khursheed Ahmad Wani is an Assistant Professor in the Department of Environmental Science, ITM University, Gwalior, Madhya Pradesh (India). He completed his M.Sc. and Ph.D. in Environmental Science at Jiwaji University, Gwalior, India. He has more than five years of teaching and research experience. His areas of interest include vermicomposting, environmental impact assessments and occupational health. He has published research papers in various national and international journals and is the author of three books on environmental science. He is the member of various national and international organizations and is in the editorial board of various journals. He has organized various programmes on environmental awareness and has completed a research project sponsored by Madhya Pradesh council of Science and Technology, Bhopal (India).

Ayoma Witharana is a science graduate with a B. Sc. degree in Forestry and Environmental Sciences. She completed the Master of Science programme in Environmental Science with the specialization in Environmental Science and Technology at UNESCO-IHE, the Netherlands. The research component was based on 'Metal leaching during anaerobic acidification of sewage sludge and potato peal waste.' During the above programme, she had the opportunity to follow taught courses, assignments and research work which mostly focused on technologies that address current environmental problems. She also earned a research-based Master's Degree in the field of Environmental Management from the University of Moratuwa, Sri Lanka. The research was focused on 'Investigation of Zinc Removal Capacities of Different Sorbent Materials to be used in Constructed Wetlands.' At present, she is working towards a Ph.D. degree at University of Moratuwa, Sri Lanka.

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