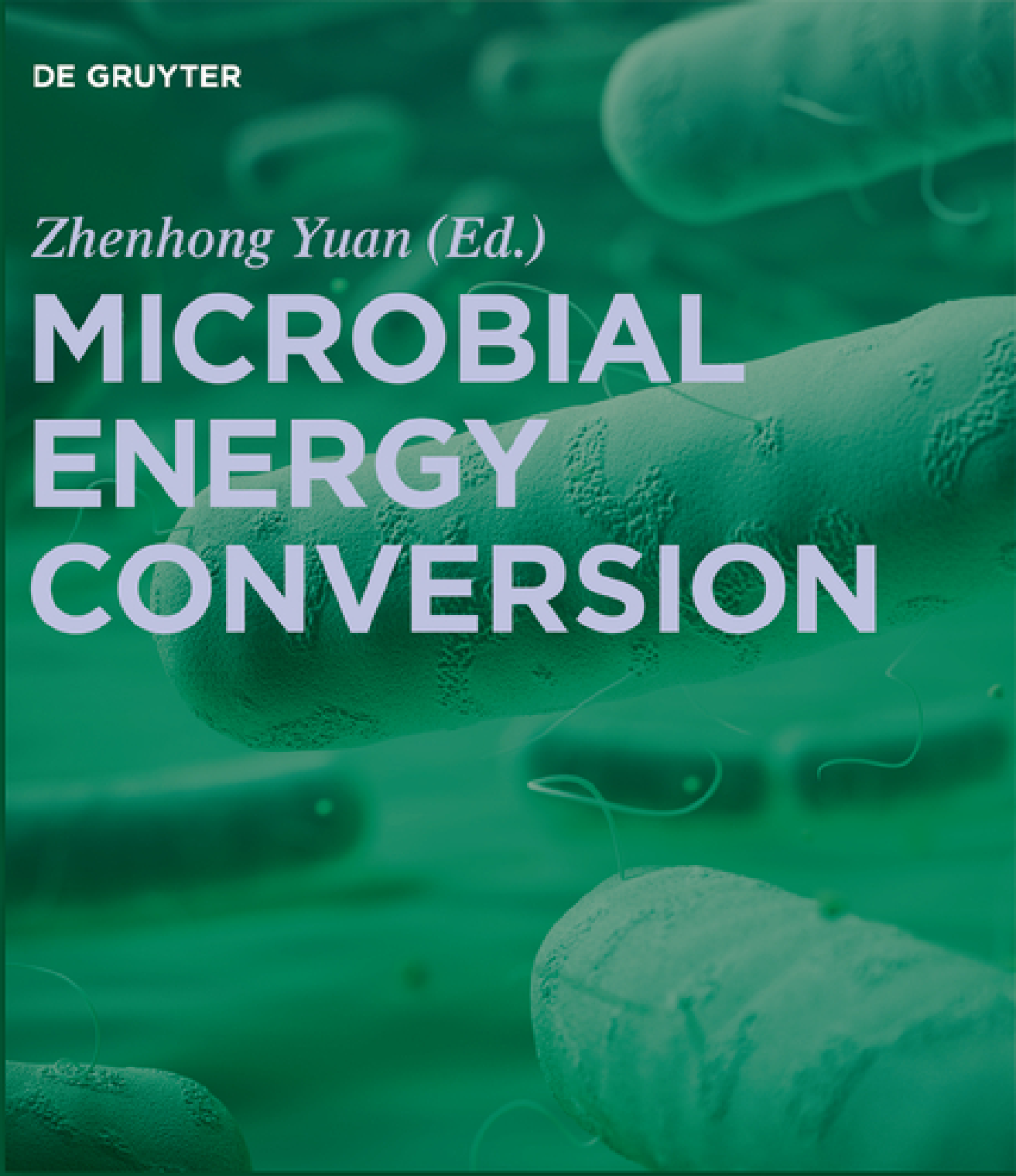

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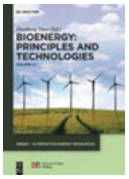

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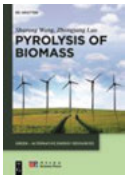

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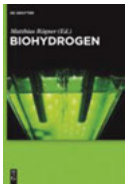

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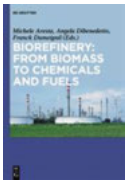

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Edited by
Zhenhong Yuan

DE GRUYTER

Editor
Dr. Zhenhong Yuan
Chinese Aacademy of Sciences
Guangzhou Institute of Energy Conversion
No. 2 Nengyan Road
Wushan, Tianhe District
510640 Guangzhou
PR China

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Contents

List of Contributors

Ying Guo, Jie Xu
Guangzhou Institute of Energy Conversion
Chinese Academy of Sciences
Guangzhou 510640
China

Zhenhong Yuan
Guangzhou Institute of Energy Conversion
Chinese Academy of Sciences
Guangzhou 510640
China
yuanzh@ms.giec.ac.cn

Shunni Zhu
Guangzhou Institute of Energy Conversion
Chinese Academy of Sciences
Guangzhou 510640
China
zhusn@ms.giec.ac.cn

Wen Wang, Jia Feng, Changua Shang, Zhiyuan Wang, Shuna Liu
Guangzhou Institute of Energy Conversion
Chinese Academy of Sciences
Guangzhou 510640
China

Yu Zhang, Qiong Wang, Guixiong Zhou, Cuiyi Liang, Xuesong Tan
Guangzhou Institute of Energy Conversion
Chinese Academy of Sciences
Guangzhou 510640
China

Xinshu Zhuang
Guangzhou Institute of Energy Conversion
Chinese Academy of Sciences
Guangzhou 510640
China
zhuangxs@ms.giec.ac.cn

Jingliang Xu
Guangzhou Institute of Energy Conversion
Chinese Academy of Sciences
Guangzhou 510640
China
xjl@ms.giec.ac.cn

Huijuan Xu, Xiaoyan Chen, Yunyun Liu, Qiang Yu
Guangzhou Institute of Energy Conversion
Chinese Academy of Sciences
Guangzhou 510640
China

Zhongming Wang
Guangzhou Institute of Energy Conversion
Chinese Academy of Sciences
Guangzhou 510640
China
wangzm@ms.giec.ac.cn

Wei Qi, Shunni Zhu, Pingzhong Feng, Lei Qin
Guangzhou Institute of Energy Conversion
Chinese Academy of Sciences
Guangzhou 510640
China

Pengmei Lv
Guangzhou Institute of Energy Conversion
Chinese Academy of Sciences
Guangzhou 510640
China
lvpm@ms.giec.ac.cn

Wen Luo, Changhua Shang, Jin Xu, Changling Mao, Junying Fu, Zhibing Li
Guangzhou Institute of Energy Conversion
Chinese Academy of Sciences
Guangzhou 510640
China

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Yongming Sun
Guangzhou Institute of Energy Conversion
Chinese Academy of Sciences
Guangzhou 510640
China
sunym@ms.giec.ac.cn

Lianhua Li, Yao Wang, Tao Xing, Keqin Hu
Guangzhou Institute of Energy Conversion
Chinese Academy of Sciences
Guangzhou 510640
China

Huijuan Xu
Guangzhou Institute of Energy Conversion
Chinese Academy of Sciences
Guangzhou 510640
China
xuhj@ms.giec.ac.cn

Dong Li
Chengdu Institute of Biology
Chinese Academy of Sciences
Chengdu 610041
China

Dalong Huang, Weizheng Zhou, Lingmei Yang, Zhibing Li, Feng Zhen
Guangzhou Institute of Energy Conversion
Chinese Academy of Sciences
Guangzhou 510640
China

Xiaoying Kong
Guangzhou Institute of Energy Conversion
Chinese Academy of Sciences
Guangzhou 510640
China
kongxy@ms.giec.ac.cn

Ying Li, Gaixiu Yang
Guangzhou Institute of Energy Conversion
Chinese Academy of Sciences
Guangzhou 510640
China

Jingliang Xu
Guangzhou Institute of Energy Conversion
Chinese Academy of Sciences
Guangzhou 510640
China
xjl@ms.giec.ac.cn

Ying Guo, Minchao He
Guangzhou Institute of Energy Conversion
Chinese Academy of Sciences
Guangzhou 510640
China

Ying Guo, Jie Xu, Zhenhong Yuan

1 Introduction

Energy has been regarded as the basis of survival and as a source for the development of human kind. Currently, energy utilization is mainly fossil based; however, with the rapid economic development, fossil fuels, as a nonrenewable source of energy, may not be able to meet the growing demand of our society. Therefore, biomass energy as a source of renewable energy will be helpful in alleviating the current pressure on energy supply and will be important to develop a recycling-based economy.

Microbes can substantially degrade almost all naturally occurring substances and synthetic compounds on Earth. In fact, microbes are a rather important part of the entire life cycle of our planet. Microorganisms and their activated enzymes play a key role for energy conversion. They can utilize agricultural waste, forestry waste, and municipal solid waste (MSW), and transform these materials into clean fuels such as ethanol, butanol, biodiesel, biogas, and hydrogen. This is of great strategic and practical significance for the ecological, the economic, and the social environment.

This book attempts to analyze microorganisms, biochemical conversion processes and mechanisms, strain selection, and applications that are involved in the energy conversion process. The theory of microbial energy conversion and its practical application will be systematically introduced, and the readers will gain basic knowledge of microbial energy production.

Section 1: Energy and Microbes

1.1 Energy Conversion

A wide range of energy is naturally available and several energy forms have already been developed and utilized; examples can be classified into conventional energy (coal, oil, gas, and water) and new energy (biomass energy, nuclear energy, tidal energy, solar energy, geothermal energy, and wind energy). Table 1.1 illustrates their characteristics [1].

Energy can be classified into 2 categories, the primary energy and secondary energy. Primary energy is a form of energy that exists in nature (such as coal, oil, natural gas, biomass, geothermal, nuclear, and wind), while secondary energy (such

Corresponding author: Zhenhong Yuan, E-mail: yuanzh@ms.giec.ac.cn
Ying Guo, Jie Xu, Guangzhou Institute of Energy Conversion, Chinese Academy of Sciences; CAS Key Laboratory of Renewable Energy; Guangdong Provincial Key Laboratory of New and Renewable Energy Research and Development, Guangzhou 510640, China

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Table 1.1: Energy categories.

Classification way	Type
Availability	Primary energy, secondary energy
Source	Energy from outside the Earth, energy from the Earth's interior, energy from other celestial bodies
Regeneration	Renewable energy (cycle), nonrenewable energy (consumption)
Utilization method	Fuel energy, nonfuel energy
Storage and transportation	"Receptacle loads," process energy
Tradition	Conventional energy sources, new energy
Degree of contamination	Clean energy, nonclean energy

as electricity, hydrogen, gasoline, gas, and alcohol) refers to a form of energy that is either directly or indirectly converted from primary energy.

Definitions for renewable and nonrenewable energy sources are provided based on whether energy sources can be recycled. Renewable energy, including biomass, hydro, tidal, solar, and wind energy, generally refers to forms of energy that are not dwindling. However, nonrenewable energy suggests a form that will gradually diminish or be completely depleted by exploitation, and the most typical forms of nonrenewable energy are coal, oil, and natural gas.

On the basis of the degree of contamination energy can be divided into clean energy and nonclean energy; the latter is also known as "green energy." Green energy can be subdivided into two categories: first, it refers to the energy form that can be obtained via utilization of modern technologies, such as biomass, solar, wind, and geothermal; second, it refers to the energy form that can be obtained via utilization of waste such as MSW, agricultural waste, and forestry waste.

A wide variety of energy classifications are currently used, and category approaches for any type of specific energy can be included from different classification perspectives. For example, biomass energy is a renewable energy; however, when it is converted into alcohol, it becomes a secondary energy form.

Primary energy may not meet our demand, and at the same time, primary energy utilization has previously caused severe harm to the ecological environment. In light of this, it is necessary to take specific measures to convert primary energy into secondary energy. Fossil fuels played an indispensable role in the development of human civilization and social progress. However, their nonrenewable nature combined with the sometimes rampant abuse caused significant pressure on the environment. Renewable energy alternatives, such as wind, solar, and biomass, have become an inevitable trend for the future.

Biomass is the only type of renewable energy that can be converted through a variety of ways to meet our needs for all types of energy products, including the three fuel forms (gas, solid, and liquid) in combination with electricity, which can directly replace oil, coal, and other fossil fuels for energy utilization. Furthermore, biomass as carbon resource can provide various types of high value-added chemicals through

biorefining processes; therefore, in this case, biomass can be considered as a substitute for mineral resources. Moreover, the development of biomass energy will be a solution to reduce greenhouse gas emissions and environmental pollution, and thus will be helpful for the ecological restoration and rural economic development.

China has a great abundance of biomass resources. According to recent statistics of the China Agricultural University, China has an annual capacity of 740 million tons of agricultural waste, of which 350 million tons can be utilized as feed, paper, and other industrial raw materials, while the remaining 390 million tons of crop straw are approximately equivalent to 200 million tons of standard coal. For example, industrial organic wastewater and livestock farm waste resources can theoretically produce 80 billion cubic meters of methane, which is the equivalent of 57 million tons of standard coal; forest residues waste can replace 300 million tons of standard coal; municipal waste generation can replace 13 million tons of standard coal; and some crops can also be treated as sources of liquid fuels. In the near future, the potential for the total biomass production in China has been estimated at 500 million tons of standard coal equivalent, which will meet more than 20% of China's energy consumption demand. If rationally processed, this will reduce annual carbon dioxide emissions by nearly 3.5 billion tons, and sulfur dioxide, nitrogen oxides, and dust emissions by nearly 25 million tons [1].

Currently, biomass utilities are applied with a variety of technologies, such as gasification for power, pyrolysis for gaseous and liquid fuel, and synthetic process for chemicals. Biomass gasification is a thermal conversion technology that converts feedstock of low energy density into high energy density fuel, which will then be suitable for storage, transport, and utilization. A gasification approach greatly improves the quality and efficiency of the system.

Fuel ethanol is one of these forms of liquid biomass obtained through chemical and physical hydrolysis of biomass combined with the microbial fermentation of sugars. Ethanol from biomass is one of the closest points of contact with the energy provided by microorganisms. Since the 1970s and in response to the global oil crisis, Brazil and the United States have created specialized agencies to promote the development of fuel ethanol, especially for their most abundant crops (sugarcane for Brazil and corn for the United States). China has also developed its own ethanol industry based on stale rice. In 2010, the global production of fuel ethanol reached 85.8 billion liters [1]. China cannot follow in the footsteps of the United States and Brazil and treat grain as raw material. The technology to develop nongrain fuel based on lignocellulosic feedstock for ethanol and butanol production will be the inevitable choice for the future fuel production of China. At present, China's biomass fuel ethanol technology is thriving, and the golden age for biomass fuel ethanol will commence in the next few years.

Biodiesel is an environmentally friendly liquid fuel and can replace fossil diesel, thus compensating for the shortage of petroleum resources. In Europe and the United States, the main feedstock for biodiesel is either soybean or rapeseed oil. The high cost of resources determine the biodiesel industries in Europe and America, which can only survive with governmental subsidies. Biodiesel production from plant oil cannot

operate in China because of their high price, and the consequential diminishing of the food supply. Therefore, the Chinese biodiesel industry is mainly based on waste oil, and the current distribution of waste oil resources limits the development of the regional biodiesel industry. Biodiesel is also an important part of biomass energy. Jatropha, algae, and other organisms can be converted into biodiesel through either biochemical or chemical processes.

Microbes are of great importance for the biomass energy technology. Biofuels (such as fuel ethanol, butanol fuel, hydrogen, and methane) can be obtained from microbial and the related enzymatic processes. Microorganisms play an important role in the clean utilization of fossil energy such as microbially enhanced oil recovery and coal liquefaction.

1.2 Energy Microorganism

Energy microbes are microorganisms that are closely related to energy utilization from natural resources or waste sources, including bacteria, fungi, and algae [2, 3]. This book mainly describes the types of microorganisms that either can or may be used in the energy conversion processes under the current scientific and technologic conditions. These microbes are mainly involved in biomass conversion, and a small part of them have been applied in oil and coal conversion technologies.

1.2.1 Energy Microorganism Culture

Microorganisms are the most widely distributed and abundant species on Earth, and it is still difficult to estimate the number of biological communities. In addition, microorganisms can live in extreme environments, such as anaerobic, high temperature, and high salt, which may not be suitable for plants and animals. To adapt to such harsh environmental conditions, microorganisms produce numerous substances with specific activities to withstand environmental pressure. Therefore, microorganisms are a rich source of biologically active substances.

1.2.1.1 Microbial Origin

Microorganisms that have been applied for industrial fermentation are divided into three categories based on their sources [4]:

(1) Natural environment
At present, microorganisms that are employed in the industrial process have initially been derived from the natural environment, including from soil, water, animals, plants, and minerals. These are then screened and cultured through a series of steps to meet different needs. It is more likely to find a new species if bacteria samples

would be collected from more extensive sources. Many important species are yet to be developed from extreme environments such as high temperature, high pressure, high salt, high pH, low pH, and the ocean.

(2) Screening

If the species that will produce the desired product has been determined, it is good practice to use many different strains of the species. By culturing microorganisms obtained from various microbiological laboratories and deposited units around the world, target strains can be screened for, analyzed, and ultimately improved. Through a strict selection process, strains suitable for energy production can be verified.

(3) Purchased species (typically for patents bacteria, or applied strains from the industry)

Patents for biotechnology have the following criteria in China: The patent should provide the microbe and its corresponding culture conditions for preservation at preservation agency offices. The deposited and patented strain can be purchased from the agency. In addition, strain can be provided through personal exchanges or as kind gift depending on the circumstances.

1.2.1.2 Breeding of Microorganisms

Although initial production strains have been derived from nature, the efficiency of natural strains is generally low. The rise of the antibiotic industry in the 1940s promoted the rapid development of microbial genetics and laid a solid theoretical foundation for the microorganism fermentation industry. In recent years, because of the rapid development of systems biology, synthetic biology, and protein engineering, the microbial breeding technology continues to advance. It provides engineered strains to meet production requirements. At present, microbial strain selection technologies such as natural selection, mutation breeding, anti-phage strains breeding, cross-breeding, protoplast fusion technology, and genetic engineering techniques are quite extensive.

(1) Natural selection

Microbial strain selection without manual processing and only via natural mutation processes is called natural selection (spontaneous mutation). Such natural mutations (completely free from human involvement) are generally believed to be effected by two factors: mutual mutagenic effects and multiple factors effects.

Natural allosteric effects may end with two different effects: one leads to the target species with decreased product yield or quality decline; the other leads to mutations that are beneficial for production. To ensure stability and to improve production efficiency, regular natural selection should be conducted to eliminate degraded strains and store beneficial strains.

(2) Mutation breeding

Microbes have established a fairly stable metabolic regulation mechanism during their long-term evolutionary process. Their metabolic process is strictly regulated, and a few of the metabolites will accumulate significantly. Natural strains isolated from the environment typically have limited production capacity and generally cannot meet the required efficiency. Mutation breeding is one of the effective methods to improve the strain production capacity of a specific metabolite.

(3) Cross-breeding

Mutagen will decrease production activity in the long term. For example, strains will have prolonged growth cycle, few spores, and slowing metabolism. It is therefore necessary to take advantage of cross-breeding methods to improve the abilities of production strains. The purpose of crossbreeding is to retain good traits of different strains by restructuring the genetic material. Hybridization can expand the range of variation and improve both yield and quality of the product. Because a variety of microorganisms have not yet developed the ability for sexual reproduction, the parent strain should have the appropriate genetic markers.

(4) Protoplast fusion

Protoplast fusion technology is a hybridizing method with full utilization of genetic recombination. Protoplast fusion technology was first developed based on animal and plant cell fusion, and then extended to fungi, bacteria, and actinomycetes. This technology can overcome the boundaries of species and improve recombination frequency.

(5) Genetic engineering technology

Exogenous DNA can insert into the DNA of recipient cells, which will replicate, transcribe, translate, and express in the recipient cells through genetic engineering technology.

The main steps of this genetically constructed process include cloning, DNA recombination, in vitro recombinant DNA insert into a host cell, and positive clone selection. During the construction process, we should focus on the target gene expression yield, the stability of the expression product, the product separation efficiency, and biological activity. A variety of factors should be considered to choose a suitable gene expression system.

1.2.2 Energy Classification of Microorganisms

The microbial classification technique is called microbial taxonomy [5]. Taxonomy covers three steps: classification, nomenclature, and identification. Classification is the process that defines the level of similarity or relevance. Nomenclature is the part that names the microorganism according to regulations and the taxon. Identifica-

tion (or determination) is the step that measures the status of a clear classification or newly discovered microbes.

In this book, microorganisms have been classified into the following eight categories in accordance with their metabolic mode and energy products [6]:

1. Microorganisms for biomass pretreatment and hydrolysis
   Microorganisms with enzymes that can break down starch, degrade lignin, hemicellulose, cellulose, and cellulose.
2. Ethanol fermenting microorganisms
   Hexose fermenting microorganisms, pentose fermenting microorganisms, and synthesis gas fermenting microorganisms.
3. Butanol fermenting microorganisms
4. Oil-conversion and oil-producing microorganisms
   Lipase-producing microorganisms, microalgae, oil-producing yeasts.
5. Methane-producing microorganisms
   Acidogenic fermentation and methanogenic microorganisms.
6. Hydrogen-producing microorganisms
   Photosynthetic hydrogen-producing microorganisms, dark-fermentation hydrogen-producing microorganisms.
7. Electricity-producing microorganisms
8. Fossil energy conversion microorganisms
   Oil and coal conversion microbial organisms.

In the following chapters, these microorganisms will be discussed in detail.

1.2.3 Microbial Energy Conversion Mechanism

The growth and reproduction energy of microorganisms mainly depends on two metabolic pathways, namely catabolism and anabolism. The microbial catabolism absorbs a variety of carbon and nitrogen from the environment to provide energy for its activities. Catabolic pathways include various centers such as TCA, EMP, and HMP, and the outer periphery of the way [5]. Both microbial catabolism and anabolism are interrelated and provide the mutual basis of life activities.

The microbial metabolism is composed of a series of continuous oxidation-reduction reactions, gradually breaking down and releasing energy during reactions, which is also known as bio-oxidation. The energy that is being released in the process of biological oxidation can directly be delivered, stored within energy-conversion compounds (e.g., ATP), or released into the environment in the form of heat. Different microorganisms vary in the substances they are based on, for instance, heterotrophic microorganisms usually rely on organic matter, while autotrophic microorganisms rely on inorganic substances.

1.2.3.1 Bio-oxidation of Heterotrophic Microorganisms

The oxidation process of heterotrophic microorganisms can be divided into fermentation and breathing, based on different electron acceptors. Breathing can be further divided into aerobic and anaerobic respiration.

(1) Fermentation

Fermentation is the oxidative process of converting organic matter within microbial cells into an intermediate substrate with energy release. Under fermentation conditions, the substance and energy release are only partial. Oxidation fermentation is connected to the reduction of organic metabolites, which is produced by initial fermentation catabolites, and the electron acceptor does not need to be provided from the outside environment during the fermentation process.

The fermentation substrate can include sugars, organic acids, and amino acids, of which the most important microbial fermentation source is glucose. Glucose can be degraded in vivo to pyruvate via glycolysis (Glycolysis), which is divided into the EMP, the HMP, and the ED pathways and the phosphoric acid solution transketolase pathway.

(2) Respiration

During the process of respiration, microbes degrade the substrate, thus releasing electrons to $NAD(P)^+$, FAD, or FMN, which then pass through an electronic transfer system to produce energy and water or other reduced products. During the process of aerobic respiration, molecular oxygen forms the final electron acceptor, while in anaerobic fermentation, oxide-type compounds act as the final electron acceptor. The fundamental difference between respiration and fermentation is that an electron is transmitted either directly to the substrate or to the electron transport system, which will then gradually release the energy to the final electron acceptor.

Many organic compounds that cannot be fermented have the potential to be decomposed by respiration, since the reoxidation of NADH and ATP occurred in the biological electron transport system within respiration. Therefore, as long as the oxidation levels of the compound are less than CO2 and with an enzyme capable of electron transfer from the compound to NAD^+, respiration of the compound is possible. Organic matter including certain hydrocarbons, fatty acids, and many alcohols can be decomposed by respiration. However, some human-made compounds possess significant resistance to microbial respiration, which may accumulate in the environment and harm the ecosystem.

1.2.3.2 Microbial Oxidation of Autotrophs

Chemoautotroph microorganisms can obtain energy via oxidation of inorganic substances. They produce ATP through oxidative phosphorylation in the oxidative process of inorganic substances.

(1) The oxidation of ammonia
Nitrifying bacteria use ammonia (NH3) with nitrite ($NO2^-$) as energy form. Nitrifying bacteria can be divided into ammonia- and nitrite-oxidizing bacteria. The process of the oxidation of ammonia to nitrate has two stages: Ammonia is first oxidized to nitrites by ammonia-oxidizing bacteria, and then it is converted to nitrate via nitrification of nitrite-oxidizing bacteria.

(2) The oxidation of sulfur
Thiobacillus is capable to use one or more reduced sulfides (including sulfides, elemental sulfur, thiosulfate, sulfates, and sulfites) to generate energy. H2S is first oxidized to elemental sulfur and then sulfite is oxidized by sulfite-cytochrome C reductase and cytochrome end catalytic system, to produce four ATPs. Oxidation of sulfite includes the direct oxidation of $SO4^{2-}$ bysulfite-cytochrome C reductase and cytochrome end catalytic system to produce one ATP; and the sulfuric acid oxidation pathway by 3'-phospho-adenylylsulfate reductase to generate 2.5 ATP.

(3) Oxide iron
Only a small amount of the generated oxidation energy can be utilized.

(4) Hydrogen oxidizing
Microorganisms for hydrogen oxidizing are Gram-negative and facultative chemoautotrophs. They can be based on CO_2 and other organic substances. Ubiquinone, vitamin K_2, cytochrome, and other respiratory chain components were distributed in the cell membranes of hydrogen-oxidizing bacteria. Hydrogen-oxidizing bacteria can directly deliver hydrogen electrons to electron transfer systems and produce ATP at the same time.

1.2.4 Advantage of Microbial Energy Conversion

Microorganisms are the main participants within the Earth's biosphere. The carbon cycle is the most important and productive cycle. Microorganisms play a major role in energy production process and in the process of the carbon cycle, as microbial decomposition is a final end in the carbon chain of the biosphere [7, 8].

1.2.4.1 High Production Yield

Microbial metabolites are simple compared to chemical methods. For example, in the process of transforming lignocellulosic biomass to ethanol, the change of hemicellulose and cellulose hydrolysis to fermentable monosaccharide is a central step. The

traditional chemical treatment, although with high processing efficiency, will inevitably produce some inhibited substances for the subsequent fermentation process. However, cellulase can directionally hydrolyze cellulose to fermentable sugars, with fewer byproducts.

1.2.4.2 Reducing Environmental Pollution

Microorganisms can be applied for anaerobic digestion to produce a large number of "landfill gases" from landfills for power generation. Biohydrogen can be obtained from urban sewage, garbage, animal manure, and other organic waste by photosynthetic bacteria, and microorganisms can purify water as well.

1.2.4.3 Simple Equipment

The microbial conversion process (such as anaerobic digestion) is very simple and does not require complex or expensive equipment. In many rural areas of China, we can establish household biogas plants from the manure and straw to provide energy for farmers.

1.2.4.4 Reduce Cost

Traditional oil extraction typically requires pressurized, gas injection and complex and expensive flooding methods. However, microbial enhanced oil recovery does not require complex and expensive facilities, and the main operating costs can be significantly reduced.

Section 2: Application Status and Development Prospect of Microbial Energy Conversion

As important members of various microbial populations, energy microorganisms play an irreplaceable function in terms of development and utilization of new energy sources, high-efficient utilization of traditional energy source, and others. In this section, the application summary of the energy microorganism will be discussed.

2.1 Application Status of Microbial Energy Conversion

2.1.1 Application of Microbial Energy Conversion for the Recovery of Traditional Fossil Energy

Coal and oil were the earliest traditional fossil fuels utilized by humans, and microorganisms have also been sufficiently utilized during the oil recovery and coal conversion processes. The study of the biological processing of coal mainly includes two steps: the first is the biological purification of coal, where the sulfur within the coal is removed through the microorganism (bacteria mainly). Studies regarding this have been conducted at first, and consequently many reports exist about those study results; the application prospect for large-scale industry is optimistic. The second is the biological conversion of coal, where coal dissolution and liquidation are realized through the conversion function of fungus, germ, actinomycetes, and other microorganisms, so that coal can be converted to matter that is soluble in water; chemicals with special value are extracted to prepare clean fuel, industrial additive, and plant growth–accelerating agents. The technology originated from the discovery of Fakoussa et al. that untreated brown coal can be dissolved by some fungi. The technique can be conducted under constant temperature and constant pressure. In comparison with the traditional chemical conversion technique, this technique is simpler and saves more energy consumptions; therefore, the exploration of the microbiological degradation coal technique attracts vast attention.

Oil is a nonrenewable energy; after primary and secondary oil recovery, about 60–70% of crude oil in the stratum still cannot be extracted. The world oil recovery industry is widely concerned with improving this crude oil recovery rate. To extract oil that has been left in oil reservoirs, both the oil recovery technique and the enhanced oil recovery technique have been developed. Traditional enhanced oil recovery (EOR) includes the heating power flooding method, the chemical flooding method, and the polymer flooding method. The microbial enhanced oil recovery (MEOR) is also called microbial oil recovery, which is a comprehensive technology for improving the crude oil recovery rate by means of activities and metabolites of the microorganism subsequent to the heating power flooding method, the chemical flooding method, the polymer flooding method, and other traditional methods. Microorganisms for oil recovery are divided into original microorganisms and heterogeneous microorganisms. The original microorganism oil recovery is the MEOR technique for improving oil-flooding efficiency through activating the microorganism in the oil reservoir by purely filling nutrition liquid. The heterogeneous microorganism denotes the MEOR technique for filling separately cultured microorganism bacterial liquid and nutrition liquid to the ground of the oil layer.

2.1.2 Application of Microbial Alcohol Fuel Conversion

Alcohol fuels often refer to fuel ethanol and butanol, and ethanol in particular is the most successful substitute of traditional fossil fuel. Ethanol is blended with gasoline, fluctuating between 5–25% in 15 countries in 2008. Ethanol can be produced via chemical synthesis or biological fermentation; at present, more than 90% of the ethanol output is produced via the biological method. When ethanol is produced by the biological fermentation method, the key factors affecting the production include the performance of the strain, the technical conditions, and devices for fermentation and purification. The most important factor is to utilize an ethanol microorganism with good performance. In a natural cycle, many microorganisms are capable of performing ethanol fermentation, including saccharomycetes, mold, and germs; thus, rich resources are provided for breeding good cultures. The earliest seed lot was basically filtered from nature; however, a naturally filtered wild bacterial strain is generally very unlikely to possess the ideal characteristics for large-scale production in the fermentation industry; therefore, the bacterial strain needs to be further domesticated and improved to equip it with the requirements for major industrial production. Through knowledge accumulation and scientific study for several thousands of years, many feasible breeding technologies have been invented. At present, widely applied breeding technologies include mutagenesis technology, protoplast fusion technology, gene project technology, and gene recombination technology. These methods have provided more possibilities for breeding good cultures and have achieved notable results.

Saccharomyces cerevisiae and *Zymomonas mobilis* are widely applied for ethanol fermentation. These applications gain good quality and great economic benefit in relation to ethanol fermentation: the ethanol output is high, the ethanol resisting ability is strong, and the ethanol yield is also very high. *S. cerevisiae* can ferment a relatively wide substrate, including glucose, mannose, and galactose. This industrial application has a long history. *S. cerevisiae* is one of the best industrial seed lots around the world. The growth rate of *Z. mobilis* is three to four times faster than that of the yeast. Through the fermentation, 97–98% of substrates can be converted into ethanol, and the ethanol yield can reach 0.49–0.5 g ethanol/1 g glucose. Moreover, the residue of the fermentation can be used as a safe animal feed. Thus, microorganism provides insights into the potential of ethanol production from lignocellulosic biomass.

Biomass can be converted into synthesis gas (CO, H_2, and CO_2) through gasification. These synthesis gas is fermented to ethanol by microorganisms. The technique converts various biomasses (including lignin and degradation-resistant parts) into synthesis gas through the gasification with a fluidized bed. Thus, the utilization rate of the biomass is greatly improved and the handling problem of the lignin residue is solved. During recent years, the study about this technique has received wide attention in China and in other countries, and foreign companies engaged in research and development of synthesis gas fermentation mainly include LanzaTech, Auckland,

New Zealand and Coskata, Warrenville, Illinois. These two companies are actively engaged in developing the international market and in approaching the Chinese fuel ethanol domain. Currently, LanzaTech has signed cooperation agreements with China Baosteel, Henan Coal Chemical, and the Biology Bureau of Chinese Academy of Sciences, and has accelerated to occupy the technical market of synthesis gas ethanol fermentation in China. The Guangzhou Energy Research Institute of Chinese Academy of Sciences has conducted substantial early studies on the preparation and purification of synthesis gas, strain breeding of synthesis gas fermentation, low-cost culture medium development for synthesis gas fermentation, and technical optimization of synthesis gas fermentation and design optimization for a high gas-liquid mass transfer rate reactor, obtaining periodic achievements. By taking CO as the carbon source and conducting continuous fermentation, the alcohol concentration in the fermented liquid is noticeably higher than that of similar studies conducted in China, and corresponding preparation, purification, and fermentation devices for synthesis gas are being designed and developed.

At present, the widely applied butanol fermentation-produced solvent *Clostridium* mainly includes four strains: *Clostridium acetobutylicum*, *C. beijerinkii*, *C. saccharobutylicum*, and *C. saccharoperbutylacetonicm*; the reaction conditions and products of these four species are different and are listed in the following: *C. saccharobutylicum*, 30–40°C, pH 5.2–7.0, and suitable for producing solvent; *C. saccharoperbutylacetonicm* 25–35°C, pH 6–6.7, and suitable for producing solvent; the typical solvent rate of *C. acetobutylicum* is butanol:acetone:ethyl alcohol = 6:3:1 (mass percent), the optimal cultivating temperature is 35–37°C, and the optimal fermenting temperature is 37–39°C. The typical solvent ratio of *C. beijerinkii* culture is similar to that of *C. acetobutylicum*; however, some *C. beijerinkii* cultures produce *isopropanol* instead of acetone; *C. saccharobutylicum* and *C. saccharoperbutylacetonicm* are suitable for fermenting green syrup, while *C. acetobutylicum* and *C. beijerinkii* are suitable for fermenting starch type matters. The produced solvent *Clostridium* can perform fermentation from a wide array of sugars, including hexose (glucose, fructose, galactose, and mannose) and arabinose as well as xylose in pentose. However, as for the most butanol fermentation *Clostridium*, they feature slow growth rate, fast degeneration, and sensitivity to bacteriophage.

2.1.3 Application of Microbial Oil Fuel Conversion

Biodiesel is also called fatty acid methyl ester, and it is the liquid fuel obtained by taking oil grease as the raw material and performing a transesterification reaction with short chain alcohol, which is also a high-quality substitute for petroleum diesel. At present, the applied chemical synthesis has a few shortcomings such as complex technique, high energy consumption, deep product color, and environmental pollution – problems that can be avoided through the catalyzed synthesis of biodiesel via the fatty enzymic method. The fatty enzymic approach has good application

prospects. Owing to multiple varieties of microorganisms, fast breeding, and easily occurred heritable variation, the lipase function pH, function temperature scale, and substrate selectivity of the microbial source are more varied than those of animals and plants. Microbial lipase is extracellular, which makes it suitable for major industrial production with high purity; therefore, microbial lipase is an important source for the industrial lipase as well as one of the research hotspot for biodiesel production.

At present, the development of the biodiesel industry was inhibited by the insufficient raw materials. Microbial oil is an ideal oil resource, which is also called unicellular oil. It can be generated in the thallus, with carbohydrate, hydrocarbon, and ordinary oil . Microorganisms, such as, yeasts, molds, germs, and algae, can be the source of the triglyceride and lipid, with similar properties as plant oil. The microorganisms can be the source of unsaturated fatty acid; however, their efficiency is low. The study on microbial oil production is conducted specifically with algae and fungi.

2.1.4 Application of Microbial Gas Fuel Conversion

Gas fuel compounded via the microbiological method mainly includes biogas and hydrogen. Biogas is one of the energy sources that was applied earliest and most widely in China. The Energy Conservation Law was adopted in 1997 and was revised in October 2007, and the Animal Industry Act enforced in 2005 has clearly emphasized the rural biogas construction, listing itas the twenty-first-century agenda in China, and providing a long-term and stable policy support for rural biogas development. In January 2005, the central government proposed to "vigorously popularize rural biogas." On this basis, the Ministry of Agriculture has formulated the Nationwide Rural Biogas Development Plan from 2006 to 2010 and proposed the goal that the rural household biogas scale shall reach 40 million families in 2010, further stating that rural biogas must basically become popular among rural applicable rural areas in 2020. The biogas fermenting process has the theories in two, three, and four stages. Generally speaking, the biogas fermenting process refers to the microbial decomposition of organic matters under specific anaerobic conditions. This step is to convert carbon into CH4 and CO2. During the conversion process, most energy in the decomposed organic carbonization can be converted and stored in CH4, and only a small part of organic carbide is oxidized to CO2; the released energy can be used for meeting the demand of the life activity of the microorganisms. During the process of biogas fermentation, the variety of participating microorganisms is complex, and the series of biological reactions will be discussed in detail in the subsequent chapters.

Hydrogen is an ideal, clean, and environment-friendly reproducible alternative fuel; after burning, it only generates water instead of greenhouse gases. Furthermore, hydrogen has a high calorific value (122 kJ/g), which is three times higher than the oil calorific value. Furthermore, along with the development of the proton film fuel

cell technology, hydrogen can be directly converted into power energy with high efficiency through a fuel cell, and the conversion efficiency reaches up to over 60%. The hydrogen production process can be completed by performing photolysis or fermentation of reproducible, cheap, and inexhaustible water and organic matter through the function of microorganisms under constant pressure and temperature; moreover, various organic waste (including organic wastewater and organic solid waste) have been handled. Therefore, in combination with the continuously increased energy demand of humankind and the continuous concern for environmental protection, the study on the biological hydrogen production has attracted general concerns of the scientific community and even the industry community. According to the variety of microorganisms that participated in producing hydrogen and the difference in metabolic pathways for producing hydrogen, the biological hydrogen production process includes the following five types: (1) algae perform direct or indirect biophotolysis on water; (2) cyanobacteria perform indirect biophotolysis on water; (3) photosynthetic bacteria perform light fermentation on organic compounds; (4) fermenting bacteria perform dark fermentation on organic compounds; (5) Microbial CO-H_2O conversion for nutrition hydrogen production.

2.1.5 Application of the Microbial Fuel Cell

As early as 1910, the English botanist Mark Beutel reported for the first time that a bacterial nutrient solution can generate current; he used platinum as electrode, and put it in a nutrient solution of *Escherichia coli* and ordinary saccharomycetes, and successfully manufactured the first microbial fuel cell (MFC) of the world. Except for generating power energy, the microorganism has several other functions. In wastewater treatment, the electrogenesis microorganism can remove Fe^{3+}, sulfide, organic waste, and others from the agricultural wastewater. The continuous optimization of the MFC design is synchronously conducted with the development of sewage treatment techniques. At an early time, the MFC without medium has been respectively applied to the sewage monitoring sensor and the sewage treatment; a typical study to this respect covers the BOD sensor of MFC without medium.

Electrogenesis microorganisms can play the function of bioremediation. The cost is high for moving bottom mud to the other place and then performing concentrated bioremediation. In situ bioremediation can directly treat bottom mud without dredging; thus, a lot of dredging cost can be saved and the environment disturbance caused by dredging can be reduced; therefore, this is an ideal method for controlling the pollution of bottom mud.

MFC can be used to prepare hydrogen fuel. The Pennsylvania State University recently reported that clean fuel hydrogen can be generated by electrifying table vinegar with germs in wastewater for a short time, and can also be used for driving

automobiles similar to gasoline. The MFC can almost convert any biodegradable organic material into hydrogen with zero carbon emission. Furthermore, the MFC can be performed in the cellulose, glucose, acetate, or other volatilizable acid during the electrolytic process, and water is the only electrolytic product.

By changing the power generation conditions of the microorganism, saline can be desalted. Researchers should first collect samples from ponds or other natural water areas. In several millions of microorganisms of the sample, some germs (its type remains undetermined by scientists) can automatically generate electrons and protons within its cell, and transfer them out of the cell body. Other germs can absorb these electrons and protons, and use them as "fuel," thus creating hydrogen, methane, other chemical matters, as well as other energy matters.

2.2 Development Prospect and Trend of Microbial Energy Conversion

The development and utilization of energy microorganisms have been widely focused since it is featured by clean and high efficiency with good reproducibility. More importantly, energy microorganisms have huge development potential in the whole energy system.

Ethanol is the earliest energy microorganism product, and its history can be traced back to the brewing technique of several thousand years ago. Through refining the obtained ethanol via fermenting, fuel-level ethanol can be prepared; ethanol is mixed with gasoline according to a certain ratio for supply to the engine for burning. Owing to the features of safe burning, zero produced pollution, and renewability, the fuel ethanol industry has been rapidly developing. Brazil uses sugarcane as raw material to prepare fuel ethanol and directly applies it to the automobile engine. America increased the output of ethanol from 23 million tons to 41 million tons from 2006 to 2017. Although the application of fuel ethanol has started late in China, its development is rapid; at present, the production and use of fuel ethanol have been ranked third in the world [9].

Except for Zymophyte of fuel ethanol, the lipase-producing microorganism, oil-producing microorganism, hydrogen-producing strains, and biological fuel cell microorganisms are also hotspots currently studied [10]. Biodiesel is a clean renewable energy source and can be used as fuel to replace oil; its greatest advantage is that biodiesel can be mixed with traditional diesel oil at any ratio and can also be directly applied to locomotives without transforming the engine. The production of biodiesel mainly includes the chemical method and the biological enzyme method; compared to the chemical method, the biological enzyme method is featured by gentle reaction conditions, low energy consumption, simple technique, and

small alcohol usage, and therefore it received tremendous research attention. The development of the oil-producing microorganism provides the raw material with support for the production of biodiesel; microbial oil is developed, the microorganism is short in fermenting cycle and not influenced by field, season, and climate change, and can also be produced by lignocellulose, industrial wastewater, waste gas, and other raw materials with abundant distributions and low price; thus, it can solve the problem of resource shortage, protect the environment, and achieve many other things; therefore, microbial oil has a huge development space. The microbial fuel cell is a special cell; it utilizes natural microorganisms or enzymes as the catalyst and directly converts the chemical energy within the fuel into the electrical energy; the cell is free from pollution, high in efficiency, and gentle in reaction conditions, and can remove harmful matters in the waste water; moreover, the fuel has wide origin and large developmental space. The raw materials for the production of biological hydrogen are urban sewage, household refuse, animal waste, and other organic waste. Through the treatment of photosynthetic bacteria or fermentation bacteria, hydrogen can be obtained; furthermore, the purpose of purifying water quality and protecting the environment can be realized; therefore, in light of environmental protection and new energy development, biological hydrogen production has great development potential, not only in providing clean energy but also in handling organic waste, protecting the environment, and obtaining considerable economic benefit. Hence, this is a sustainable development approach.

The development of energy microorganisms and the biological energy source has important impacts on the adjustment of the current social energy structure. The economic and social development can unavoidably cause energy shortage and environmental degradation. Therefore, vigorous development of pollution-free, clean, and renewable new energy sources, while improving the recovery and utilization rates of the current energy sources, is the trend of the future energy construction as well as an essential road to cope with the increasingly severe energy and environmental problems. Although some technical problems still exist in the conversion and utilization of biological energy source, this is only temporary. Along with the continuous development of the biological technology, the sustainable improvement and domestication of energy source microorganisms, and the project transformation, its utilization rate will greatly improve to completely meet the demand of industrial use. Therefore, it has huge developmental potential and broad application prospect. Furthermore, the microorganism contributes greatly to the use of existing nonregeneration energy since it improves the oil recovery rate and the brown coal utilization rate while reducing the pollution effects of both; therefore, it is worth considering to achieve the energy sustainable development goal. The use of the microorganism-produced energy cannot cause environmental pollution and can also ease the current environmental status of severe pollution. The

development of the energy microorganism technology can also treat pollution and change wastes to valuables. Therefore, the development space and potential for the energy source microorganism are enormous and will bring long-term economic and social benefits.

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Shunni Zhu, Wen Wang, Jia Feng, Changhua Shang, Zhiyuan Wang, Shuna Liu

2 Foundations in Microbiology

Microbes, which are closely related to human life, are widely distributed in air, water, soil, and other natural environments. For example, microbes that exist in the gut can help digestion; consumer goods such as soy, vinegar, and wine cannot be produced without microbes; and food spoilage, disease transmission, drug production, and the development and utilization of energy such as fuel ethanol, butanol, and biogas all have deep connections with microbial activities. In this sense, understanding microbes can help us to take advantage of them.

Researching on the basics of morphology, metabolism, growth and reproduction, heritable variation, ecological distribution, and the evolution of microbes on the molecular, cellular, and colonial levels is useful to generate bioenergy through biochemical conversion. This chapter intends to introduce the basic microbiology required for energy conversion.

Section 1: Prokaryotic Microbes

There are many different prokaryotic microbes that are widely distributed and have relatively simple structures. This section introduces the structural features, groups, and physiological characteristics of bacteria, archaea, and actinomycetes.

1.1 Bacteria

As prokaryotes, bacteria vary widely, have a simple cell structure, and mostly reproduce by binary fission. They are widely distributed and closely related to the daily life of humans. At present, many types of bacteria have been used for energy production.

1.1.1 Bacterial Morphology

The morphology of a single bacterial cell is usually bacilliform, coccoid, or spiral. However, other cell shapes such as triangles, squares, and discs also exist in a handful

Corresponding author: Shunni Zhu, E-mail: zhusn@ms.giec.ac.cn
Wen Wang, Jia Feng, Changhua Shang, Zhiyuan Wang, Shuna Liu, Guangzhou Institute of Energy Conversion, Chinese Academy of Sciences; CAS Key Laboratory of Renewable Energy; Guangdong Provincial Key Laboratory of New and Renewable Energy Research and Development, Guangzhou 510640, China

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of bacteria. The size of bacterial cells is usually measured in microns (μm) and calculated as length × width, which is usually 1–8 × 0.4–2 μm. There are various types of bacilli due to their different shapes, including *Brevibacterium*, *Corynebacterium*, fusiform bacilli, *Mycobacterium*, and *Helicobacter*. The cell arrangements of bacilli can be catenulate, paliform, and so on. The cell size of cocci is often measured by the diameter, on average 0.5–1 μm. According to their different cell arrangements, cocci can be monococci, diplococci, tetracocci, sarcina, *Staphylococcus*, and *Streptococcus*. Spiral bacterial cells are also expressed in length × width, generally 5–50 × 0.5–5 μm. According to their degree of bending, spiral bacteria can be divided into *Vibrio*, *Spirillum*, and spirochetes. The number of spirals present in *Vibrio* species is less than one, while *Spirillum* species have two to six spirals. Spirochetes have more than six spirals.

When a bacterium is inoculated onto solid media, a macroscopic colony with a certain form and structure will be formed after a period of culture. If a colony is formed from a single cell, it is referred to as a pure culture. When a large number of cells are inoculated on the surface of solid medium, lots of "colonies" will grow out and tend to form a bacterial lawn. Bacterial colonies formed on solid media usually have some special characteristics. They can be moist, smooth, transparent, viscous, easily picked, and even in texture. In addition, the color of the front and back or edge of the bacterial colony is the same as that of the central part. Pure bacterial cultures on semisolid media have many unique characteristics. Semisolid media can be used to investigate whether the bacteria are motile or not. Bacteria cultivated in liquid media will be divided into several different groups due to differences in their cell characteristics, proportion, movability, and demands for oxygen. Most bacteria in liquid media appear cloudy, while some form aggregates.

1.1.2 Bacterial Cell Structure

The general structure of bacterial cells from outside to inside is cell wall, cell membrane, and cytoplasm. Gram-negative (G^-) bacteria have an outer membrane outside the cell wall. Additionally, some bacteria have additional structures such as glycocalyx, flagellum, fimbria, and pili, while some form spores.

1.1.2.1 Cell Wall

The cell wall is a thick coat on the outermost layer of the bacterial cell. It consists mainly of peptidoglycan, which is the special component in the cell wall of all eubacteria. The function of the cell wall is to protect the bacterium from adverse factors, such as osmotic pressure and antibiotics. It is required for normal growth and reproduction of bacteria.

Every peptidoglycan monomer of Gram-positive (G^+) bacteria consists of three parts: (1) a disaccharide unit formed by *N*-acetyl glucosamine and *N*-acetylmuramic acid linked with β-1,4-glycosidic bond that can be hydrolyzed by lysozyme; (2) a tetrapeptide side chain consisting of four amino acids alternately connected by l and d types, generally l-Ala–D-Glu–l-Lys–D-Ala; and (3) peptide bridges linking two tetrapeptide side chains.

The peptidoglycan monomers in G^- bacteria are basically the same as for G^+ bacteria. However, the third amino acid of tetrapeptide side chain in G^- bacteria is not l-Lys but meso-diaminopimelic acid (m-DAP), which only exists in the cell wall of prokaryotes. G^- bacteria have no peptide bridges. The linkage of two peptidoglycan monomers in G^- bacteria is through direct connection between the carboxyl group of the fourth amino acid (D-Ala) in the first tetrapeptide side chain and the amino group of the third amino acid (m-DAP). Therefore, G^- bacteria have only sparse peptidoglycan of low mechanical strength.

G^+ bacteria also contain another acidic polysaccharide called teichoic acid in the cell wall. Its main components are glycerol and ribitol. Teichoic acids can be divided into two types: (1) wall teichoic acids covalently bonded to peptidoglycan and (2) lipoteichoic acids that cross the peptidoglycan layer to cross-link with the cell membrane. G^- bacteria have no teichoic acids, but possess an outer membrane whose components are lipopolysaccharide (LPS), phospholipids, and several outer membrane proteins. LPS consists of lipid A, core polysaccharide, and *O*-specific side chain; is thick (8–10 nm); and is located in the outermost layer of the cell wall. Lipid A is the material basis of endotoxin, which is known as pathogenic substance derived from G^- bacteria. The outer membrane proteins anchored to the LPS and phospholipid layer are of more than 20 types, including lipoproteins and porins. There is a narrow periplasm that exists between the outer membrane and cell membrane of G^- bacteria. Many periplasmic proteins exist in the periplasm, including hydrolases, ligases, and transport proteins. Because of the differences in cell wall structure, G^+ bacteria will turn purple and G^- bacteria will become red after Gram staining.

Not all bacteria have a cell wall. Due to long periods of evolution and spontaneous mutation in the lab, a minority of bacteria have no cell wall, including the l-forms of bacteria, protoplasts, spheroplasts, and mycoplasmas. l-form bacteria, found by the Lister Institute of Preventive Medicine (England) in 1935, are spontaneous mutants of *Streptobacillus moniliformis*. The cells are inflated, sensitive to osmotic pressure, and can form colonies that look like "fried eggs" after being cultured on solid media. Generally, l-form bacteria are genetically stable and have cell wall defects formed by spontaneous mutation in the lab or a host. Protoplasts are formed through removal of the cell wall of G^+ bacteria in an isotonic solution with lysozyme or by inhibition of cell wall synthesis with penicillin, and are osmotically sensitive cells only encapsulated by a membrane. Spheroplasts are what remain of a protoplast after the cell wall has been removed by lysozyme and EDTA (ethylenediaminetetraacetic acid). They are often formed by G^- bacteria. Mycoplasma is a prokaryote without a cell wall, which has formed by evolution and has adapted to environmental conditions.

1.1.2.2 Cytoplasmic Membrane

The cytoplasmic membrane, also called the cell membrane, plasma membrane, or inner membrane, is a soft and semipermeable membrane surrounding the cytoplasm and is close to the inside of the cell wall. Its thickness is about 7–8 nm and contains phospholipid (20–30%) and protein (50–70%). Every phospholipid consists of one water-soluble polar head possessing a positive charge (phosphoric end) and one water-insoluble nonpolar tail without charge (hydrocarbon end). The polar head is hydrophilic and faces toward the inside and outside surfaces. The nonpolar tail embeds in the inside of the membrane due to its hydrophobicity. This results in the formation of a lipid bilayer. Proteins are asymmetrically distributed in the lipid bilayer. Some proteins lie on the surface of the lipid bilayer, some are partly or totally embedded inside it, while some span the whole membrane. This structure makes the membrane fluid. This fluid mosaic model of the cell membrane was proposed by American scientists J. S. Singer and G. L. Nicolson in 1972, and is still widely accepted today.

Different bacterial species have different R groups on glycerin C3 of the phospholipid polar head, such as phosphatidate, phosphatidylglycerol, phosphatidylethanolamine, phosphatidylcholine, phosphatidylserine, or phosphatidylinositol. The nonpolar tail is formed by long-chain fatty acid connected to C1 and C2 of glycerin through an ester bond. The chain length and saturation of fatty acids differ depending on the bacterial species and growth temperature. Usually, the higher the growth temperature, the higher the saturation. Moreover, compared to eukaryotes, the cell membranes of prokaryotes (except mycoplasmas) don't contain sterols, such as cholesterol. Hopanoids in the cell membrane of mycoplasmas enhance the rigidity of the cell.

The main function of the cell membrane is to maintain normal osmotic pressure, selectively control the transport of compounds inside and outside the cell, and provide energy for the normal activity of the cell through energy metabolism-related enzymes participating in oxidative phosphorylation or photophosphorylation. The membrane is an important place for various metabolic pathways. For example, the main metabolic activities of microorganisms that produce hydrogen occur on the cell membrane.

1.1.2.3 Cytoplasm and Inclusion Bodies

Cytoplasm is the general term for all translucent, colloidal, granular materials coated by the cytoplasmic membrane, except the nuclear region. It is the main place that cell metabolism occurs. It contains ribosomes, proteins, reserve materials, enzymes, intermediate metabolites, plasmids, nutrients, and monomers for macromolecules. Its water content is 80%. A minority of bacteria have thylakoid, carboxysome, gas vacuoles, or

parasporal crystals, which have specific functions. The cytoplasm of prokaryotes is stagnant, which is quite different from the eukaryote.

Inclusion body refers to a large granular structure in the cytoplasm, including reserve materials, carboxysome, or gas vacuoles (Table 2.1).

1.1.2.4 Nuclear Region

The nuclear region, also known as nuclear body, pronucleus, nucleoid, or genome, is the highly centralized DNA area that is not coated by a nuclear membrane and has no fixed shape. It is the main place for bacteria to store genetic information.

Table 2.1: Components of cytoplasm and their features.

Components		Major constituents	Function	Strain source
Reserve materials	Glycogen	Glucan	Carbon and energy source	*Escherichia coli*, *Bacillus*, etc.
	Poly-β-hydroxybutyrate	Lipid	Carbon and energy source	*Azotobacter*, *Alcaligenes*, etc.
	Sulfur granule	Sulfur	Providing sulfur	*Chromatium*, *Thiothrix*, etc.
	Phycocyano-bilin	Polypeptide of arginine and aspartic acids	Nitrogen and energy resource	*Cyanobacteria*
	Metachromatic granules	Polymetaphosphate	To store phosphorus and energy	*Spirillum volutans*, *Corynebacterium diphtheria*
Ribosome		60% RNA and 40% protein	Synthesizing peptide and protein	Bacteria
Chromatophore		Protein and lipid	Photosynthesis	Photosynthetic bacteria, etc.
Plasmid		DNA	Endow specific functions for host cell	*Colibacillus*, *Pseudomonas aeruginosa*, etc.
Magnetosome		Fe_3O_4	Guiding function	*Aquaspirillum*, *Bilophococcus*, etc.
Carboxysome		RuBP	CO_2 fixation	*Thiobacillus*, etc.
Gas vacuole		Gas	Adjust the specific gravity of cell	*Cyanobacteria*, etc.
Parasporal crystal		Alkaline Dissolved protein	Kill some insects	*Bacillus thuringiensis*, etc.

1.1.2.5 Spores

Spores are dormant structures that are circular or oval, have a thick wall, low water content, and strong stress resistance. A spore is formed in the cell during the late growth period of some bacteria. The spore has no breeding function and every mother cell can only form one spore. The structure of spore from the outside to inside is as follows: exosporium, spore coat, cortex, sporoderm, spore membrane, sporonin, and spore nuclear region. The exosporium is about 25 nm thick and is mainly composed of lipoprotein and a small number of aminosaccharides. The spore coat, about 3 nm thick, is pyknotic, and mainly contains hydrophobic keratins that are high in cysteine and hydrophobic amino acids. It has low permeability for multivalent cations and strong resistance to lysozyme, protease, and surfactant stresses. The cortex contains the special spore peptidoglycan and calcium dipicolinate (DPA-Ca) and is very thick. It has high osmotic pressure, high water content (70%), and strong heat resistance. The spore core is composed of the sporoderm, spore membrane, sporonin, and spore nuclear region. It contains ribosomes and DNA and has a low water content. This special structure has spores that have strong resistance to heat, chemicals, and radiation.

Apart from spores, another bacterial dormant structure is the cyst, which is drought tolerant and nonheat resistant. Some nitrogen-fixing bacteria, such as *Azotobacter vinelandii*, can form cysts under starvation conditions through thickening the ektexine of mother cells and dehydrating the cell. Every vegetative cell can only form one cyst, which cannot reproduce.

1.1.2.6 Glycocalyx

The glycocalyx is a gelatinoid structure of uncertain thickness on the outside of the bacterial cell wall. Its main components are polysaccharides, polypeptide, and proteins. The glycocalyx can be divided into four categories according to the existence of the fixed stratum and the thickness of the layer (Table 2.2).

Table 2.2: Categories of glycocalyx and their related properties.

Categories	Packaging	Existence of fixed stratum	Function
Capsule	Single cell	Yes, thick	(1) Protect microbes from drought damage and poisoning by heavy metal ions, protect bacteria from host phagocytosis, and prevent phage adsorption and dissociation; (2) store nutrients; (3) adhere to surfaces; (4) identify information among bacteria; and (5) accumulate metabolic waste
Microcapsule	Single cell	Yes, thin	
Slime layer	Single cell	No	
Zoogloea	Cell population		

1.1.2.7 Flagella

Flagella are long filiform, wavy protein appendages that grow on some bacterial cells and extend outside the cell wall. They are about 3–20 μm in length, with a diameter of about 0.01–0.02 μm. Flagella can rotate, which propels the cell to orient toward nutrient-rich areas and avoid adverse environments. This is called chemotaxis. There are several ways for flagella to be positioned on the cell, including peritrichous, monotrichous, and lateral flagella. The number of flagella is generally one to ten.

The structure of the flagellum from the insertion site to the exterior of the cell is basal body, hook, and filament. The basal body in G^- bacteria consists of four rings (L-ring, P-ring, S-ring, and SM-ring) located successively from the outermost to the innermost layers of the basal body. The L-ring connects to the outer membrane of the cell wall. The P-ring connects to the inner peptidoglycan layer of the cell wall. The S-ring is located in the periplasmic space. The SM-ring lies in the plasma membrane. The hook links the basal body and the filament. The filament is assembled by flagella protein subunits of 4.5 nm in diameter spiraling along the central pore. Every cycle has 8–10 subunits. The flagellum structure in G^+ bacteria is slightly different from that of G^- bacteria. It is simple, and only has an S-ring connected to the inside of the peptidoglycan layer and an M-ring connected to the plasma membrane. Also, the hook shape of G^+ bacterial flagella is slightly different from that of G^- bacterial flagella.

1.1.2.8 Fimbriae

Fimbriae are hollow, short, and straight protein appendages that grow on the bacterial surface and allow bacteria to attach on the surface of objects. They are simpler than flagella and do not have complicated components, such as the basal body. They grow on the cell membrane, traverse the cell wall, and stretch to the cell surface. Their diameter is about 3–10 nm and they have a hollow shape due to the spiral arrangement of many fimbrial protein subunits around the center.

1.1.2.9 Pili

The pilus, also known as sex-pilus or F-pilus, is a type of special fimbria. It has the same structure and components as fimbriae, but is longer than fimbriae and less numerous (only one to a few per cell). It generally exists in G^- bacteria and plays a role in the transfer of genetic information from the donor to the recipient bacterium. Some pili are also specific adsorption receptors for RNA phages.

1.1.3 Bacterial Reproduction

Bacteria reproduce asexually by fission. However, a minority of bacteria, such as *Nitrobacter*, *Rhodomicrobium*, and *Hyphomicrobium*, reproduce by budding.

1.1.3.1 Fission

Fission refers to a process where a cell splits into daughter cells. After DNA replication, DNA moves to the cell pole as the cell grows. At the same time, the plasma membrane around the cell equator depresses inward in a circular manner, and the cell wall grows inwardly with the plasma membrane. Finally, they assemble together in the center, and then a division is completed.

According to the different methods of division, the fission can be divided into binary fission, trinary fission, and multiple fission. Binary fission is when a cell splits into two daughter cells. If the two daughter cells have the same morphology, size, and structure, binary fission results in equal cells. If not, it is known as unequal binary fission. Most bacteria reproduce by equal binary fission. Trinary fission occurs when a cell splits into three daughter cells, like some cells of *Pelodictyon*. Multiple fission is when cells split into multiple daughter cells of equal length at the same time, such as in *Bdellovibrio*.

1.1.3.2 Budding

Budding is a protuberance that forms on the surface (especially on one end) of the parent cell first, then separates from the parent cell to live independently when it is equal in size to the mother cell. Bacteria that reproduce in this way are called budding bacteria.

1.1.4 Main Groups of Bacteria

According to the first edition of *Bergey's Manual of Systematic Bacteriology* (1984–1989), bacteria are divided into the following groups.

1.1.4.1 G^+ Bacteria

The cellular morphology of G^+ bacteria is spherical or bacillary, and mostly regular. The arrangement of G^+ bacterial cells is single, paired, catenulate, or branched. G^+ bacteria can be further divided into cocci and bacilli that either produce spores or do not.

G^+ cocci that do not produce spores include the genera *Micrococcus*, *Staphylococcus*, *Streptococcus*, and *Leuconostoc*. The G^+ bacilli that do not produce spores include the genera *Lactobacillus*, *Corynebacterium*, *Propionibacterium*, *Mycobacterium*, and *Bifidobacterium*. The G^+ cocci and bacilli that produce spores include the genera include *Bacillus*, *Sporosarcina*, and *Clostridium*. *Clostridium* species are often selected for butanol fermentation.

1.1.4.2 G^- Bacteria

The cellular morphology and arrangement of G^- bacteria are very simple. G^- bacteria reproduce by transverse binary fission. They are free-swimming. Most G^- bacteria grow by heterotrophy, while there are a few autotrophs. They can be divided into spirochetal, spiral, or curved G^- bacteria, G^- bacilli and cocci, mycoplasma, rickettsia, and chlamydia. The main species of spirochetal G^- bacteria are *Cristispira*, *Spirochaeta*, *Treponema*, and *Borrelia*. There are nine genera of spiral or curved G^- bacteria: *Spirillum*, *Aquaspirillum*, *Oceanospirillum*, *Azospirillum*, *Herbaspirillum*, *Campylobacter*, *Helicobacter*, *Bdellovibrio*, and *Spirosoma*. The G^- bacilli and cocci include the genera *Pseudomonas*, *Xanthomonas*, *Azotobacter*, *Rhizobium*, *Methylococcus*, *Methylomonas*, *Acetobacter*, *Escherichia*, etc. Mycoplasmas are between bacteria and rickettsias. Rickettsias are a type of prokaryotic microbe that is parasitic in other living cells. Chlamydia, between rickettsia and viruses, are also a type of prokaryotic microbe that obligatorily parasitizes living cells and can pass through bacterial filters.

1.1.4.3 Photosynthetic Bacteria

According to different photosynthetic pigments and electron donors, photosynthetic bacteria can be divided into oxygenic photosynthetic bacteria and anoxygenic phototrophic bacteria. Oxygenic photosynthetic bacteria include cyanobacteria and prochlorophyta. Anoxygenic phototrophic bacteria include purple bacteria and green bacteria. Photosynthetic bacteria can use biomass or organic wastewater as a substrate to produce hydrogen under light. They can not only produce energy but also treat wastewater.

1.1.4.4 Chemolithotrophic Bacteria

Most of these bacteria obtain energy by oxidizing inorganic substances. CO_2 is their only carbon source. This group includes nitrobacteria, hydrogen bacteria, and colorless sulfur bacteria. In addition, many chemolithotrophic bacteria can use organics, and not CO_2 as a sole carbon source.

1.1.4.5 Appendiculate, Inappendiculate, Budding, and Nonbudding Bacteria

Appendages such as stipe and hyphae are cytoplasmic prominences growing on the cell surface. The diameter of these prominences is smaller than the mother cell. The prominence has a cytoplasm and cell wall. Appendiculate and budding bacteria belong to *Hyphomicrobium*. Appendiculate and nonbudding bacteria belong to *Caulobacter*. Inappendiculate and budding bacteria belong to *Planctomyces*. Inappendiculate and nonbudding bacteria belong to *Gallionella*.

1.1.4.6 Sheathed Bacteria, Gliding Bacteria, and Myxobacteria

Sheathed bacteria grow as a protonema, where many bacteria exist in a tubular sheath and their cells arrange in a line. Their appearance is filiform, like *Sphaerotilus*. Gliding bacteria have multiple physiological and morphological properties, and they are not closely related genetically. Their cells slide instead of moving with visible organs, such as *Beggiatoa*. Myxobacteria are famous for their sporocarps, which can produce mucus, have various shapes, and are colorful and visible.

1.2 Archaea

Archaea are prokaryotes that have various cellular morphologies (globular, heliciform, bacilliform, or discoid) and live in extreme environments. The diameter of a single cell is approximately 0.1–15 μm. The cells reproduce by binary fission, budding, constriction, or fracturing. Cell populations are often colored red, purple, pink, yellow, green, gray, white, and so on. Archaea live mainly in anaerobic, hyperhaline, hyperthermal terrestrial, and aquatic environments, while some live in the alimentary tracts of animals.

1.2.1 Cell Characteristics of the Archaea

The inclusions of archaea are basically the same as for eubacteria. The following mainly introduces their cell wall and membrane structures.

1.2.1.1 Cell Wall

All archaea except *Thermoplasma* have a cell wall, which contains various structures. Based on the components, the cell wall can be divided into pseudopeptidoglycan

cell wall, unique polysaccharide cell wall, polysaccharide sulfate cell wall, glycoprotein cell wall, and protein cell wall. The polysaccharide skeleton of the pseudopeptidoglycan cell wall is formed by alternate linkages between *N*-acetylglucosamine and *N*-acetyltalosaminuronic acid through β-1,3-glucosidic bonds. The peptide tail is formed by l-Glu, l-Ala, and l-Lys. The peptide bridge is formed by l-Glu. The proteins in glycoprotein cell walls are composed of a large number of acidic amino acids, especially aspartic acid.

1.2.1.2 Cell Membrane

Like eubacteria and eukaryotes, the cell membrane of the archaea is also formed by phospholipids, but has its own peculiarities and varieties. First, archaea contain a unique monolayer membrane, or a compound membrane of a monolayer and bilayer in the cell membrane. Second, there are special lipids in the cell membrane, such as cytochromes, carotene, lycopene, retinene, and naphthoquinone. Third, the hydrophilic moieties (glycerol) are connected with the hydrophobic moieties (hydrocarbon chain) through an ether bond instead of ester bond, and the hydrocarbon chain of the hydrophobic moieties is formed by repetitive isoprene units that connect with hydrophilic moieties through ether bonds to form glycerol diether or diglycerol tetraether. Fourth, various groups that are different from those of the eubacteria and eukaryotes may be connected to 3C of glycerol, such as phosphonate group, sulfate group, and saccharide groups.

1.2.1.3 Molecular Biological Characteristics of Archaea

The genome structure of archaea is similar to bacteria, while gene transcription and translation are closer to eukaryotes. The base sequence of rRNA (ribosomal RNA) in archaea is different from rRNAs in bacteria and eukaryotes. The genome of archaea contains circular DNA that is not surrounded by a nuclear membrane. Function-related genes constitute an operon and can be cotranscribed into polycistronic mRNA (messenger RNA). The RNA polymerase for transcription is a multicomponent enzyme consisting of 13 subunits, whose composition and sequence of subunits are similar to those in RNA polymerase II and III of eukaryotes. Some tRNA (transfer RNA) genes have introns. The methionine of the initiator tRNA involved in translation is not formylated. The genes for aminoacyl tRNA synthetase and elongation factor-2 are similar to those in eukaryotes. The translated polypeptides contain inteins that are removed by self-cleavage. Moreover, archaeal cells contain histones. DNA replication requires DNA polymerase, helicase, and gyrase, which is similar to the process in eukaryotes.

1.2.2 Main Groups of Archaea

At present, the archaea include Crenarchaeota, Euryarchaeota, and Korarchaeota. Crenarchaeota contains the genera *Thermoproteaceae*, *Caldisphaera*, *Desulfurococcus*, and *Sulfolobus*. Euryarchaeota contains the genera *Methanogens*, *Halobacteria*, *Archaeoglobus*, *Thermoplasma*, and *Thermococcus*. Korarchaeota cannot currently be cultured in lab. The following will introduce the typical species of Crenarchaeota and Euryarchaeota.

1.2.2.1 *Hyperthermophilic Archaea*

Hyperthermophilic archaea are mainly distributed in broiling soil of geothermal areas or hot water areas that contain sulfur or sulfide. They can survive at over 100 °C although their optimum growth temperature is about 80 °C. Most strains are obligate anaerobes, and use sulfur as an electron acceptor to conduct anaerobic respiration via chemoorganotrophy or chemolithotrophy. The types of respiration are highly diverse. However, no matter which type it is, sulfur always plays a vital role.

1.2.2.2 *Methanogens*

Methanogens are the main bacterial flora responsible for methane production. They have various morphologies, including spherical, sarcina, short bacilliform, long bacilliform, filiform, and disciform. Cells can also be straight bacilliforms or long filiforms. The cell membrane is mainly formed by isoprenoids through an ether bond linkage. They do not produce spores or move, but they have pili. Their Gram stain reaction is often indeterminate. The optimal temperature for mesophilic strains is 37–45 °C, and for thermophilic strains is 55 °C or even higher. Methanogens do not produce catalase or peroxidase, which means that methanogens are obligate anaerobic microbes. They often grow under water, in the rumen of ruminants, and in anaerobic digesters. During autotrophic growth, methanogens use CO_2 as a carbon source, with H_2 as the reducing agent of CO_2 to synthesize organic compounds, and obtain energy through methane fermentation or acetate respiration. Acetic acid can simulate the growth of methanogens. Some species use amino acids, yeast extract, and casein hydrolysate as growth factors. All methanogens can use NH_{4+} as a nitrogen source, and some can fix nitrogen. Moreover, all the methanogens use nickel for coenzyme $F_{43}0$. Iron and cobalt are also very important microelements.

1.2.2.3 *Halobacterium* Species

Halobacterium spp. are G^- archaea. They are bacilliform or bulbiform in shape and have no peptidoglycan in their cell wall. The lipids in the cell membrane are connected

by an ether bond. Bacteriorhodopsin (BR) is used to take advantage of light energy to drive proton pumps and generate a proton gradient for adenosine triphosphate (ATP) synthesis in the cell membrane. They reproduce by binary fission, and have no dormant state or spores. Their DNA is highly repetitive. Large plasmids exist in the cells of *Halobacterium* and *Halococcus*. Most strains of *Halobacterium* are non-motile, while a minority of strains move slowly with their lophotrichate flagella. All strains of *Halobacterium* are chemoorganotrophic and need Mg^{2+} to grow. Most strains of *Halobacterium* can use amino acids or organic acids as carbon and energy sources and require a certain amount of vitamins as growth factors. Most strains of *Halobacterium* are strictly aerophilic, and some are facultatively anaerobic. Some strains perform anaerobic respiration by sugar fermentation, and nitrate or fumaric acid respiration.

1.2.2.4 *Themoplasma*

The most obvious characteristic of *Themoplasma* sp. is that they lack a cell wall. Their cell membranes are mainly composed of LPS, which refers to tetraether lipids containing mannose and glucose units, as well as glycopeptides but no steroid compounds. *Themoplasma* is notable for its lack of cell wall, thermophily, acidophily, aerobiosis, and chemoorganotrophy. It can grow in synthetic media at 55 °C and pH 2.0. Its genome is very small, at about 1,100 kb.

1.3 Actinomycetes

Actinomycetes are prokaryotes that grow with hyphae, reproduce via spores, and are G^+. They are widely distributed in nature and exist in soil, air, and water as spores or hyphae, especially in soil with the low water content, rich in organic matter, and under neutral or alkaline conditions. The earthy smell of soil results primarily from the actinomycetes. Actinomycetes are classified by morphology, not in biology and are still bacteria.

Actinomycetes have extensive applications. Many actinomycetes are used to develop new energy and protect the environment due to their ability to decompose cellulose, paraffins, keratin, agar, and rubber. Antibiotics such as streptomycin and other secondary metabolites like anticancer compounds produced by actinomycetes are important for human health. However, several actinomycetes can cause diseases in humans, animals, or plants, for example, *Streptomyces scabies* is the cause of potato and beet scab.

1.3.1 Morphological Characteristics

Although the morphology of actinomycetes is more complicated than other bacteria, they are still unicellular. Under microscopic observation, actinomycetes exhibit

a ramous filiform texture, named hypha. The diameter of individual hypha is about 1 μm, which is similar to that of bacteria. Most hyphae have no diaphragm. Instead, there is a nucleoplasm inside the cell. The cell wall contains muramic and diaminopimelic acids but no chitin and cellulose. Most actinomycetes can differentiate into substrate hyphae and aerial hyphae when cultured on solid media. The substrate hyphae, also known as vegetative hyphae or primary hyphae, result from the spore that absorbs water, swells, and then sprouts to expand along and inside the matrix when cultured on solid media under appropriate conditions. The diameter of hyphae is between 0.2 and 0.8 μm and they are light colored. Their main function is to take up nutrients and excrete waste metabolites. They can generate water-soluble and fat-soluble pigments that may be colored. Aerial hyphae, also known as secondary hyphae, are an extension of the substrate hyphae outside the media. The hyphae are dark colored and 1.0–1.4 μm in diameter. They can produce pigments that are mostly fat soluble. When aerial hyphae grow to a certain stage, the spore hyphae, also called reproductive hyphae, are formed at the top, which can dissociate from the spore hyphae after spore maturity. However, some actinomycetes have no or undeveloped aerial hyphae, for example, most strains of *Nocardia* and *Micromonospora*.

1.3.2 Actinomycetes Reproduction

In nature, most actinomycetes reproduce with asexual spores that are mainly conidia or minor cystospores. Only a few actinomycetes reproduce through splitting substrate hyphae into spore cells, including *Nocardia*. All actinomycete strains can reproduce from any section of their hyphae.

Conidiospores are formed by diaphragm division. After the maturation of aerial hyphae, spore hyphae differentiate. Either the cell membrane invaginates and shrinks from the outside to the inside to form a diaphragm, and thus divides the spore hyphae into many conidia, or the cell wall and membrane invaginates together and shrinks gradually to finally divide the spore hyphae into a string of conidia. Sporangiospores are formed in the sporangium. The sporangium is formed by expansion of the sporangiophore, or by the winding of the spore hyphae. A sporangium can be formed not only by aerial hyphae but also by the substrate hyphae. When the sporangia grow to a certain stage, a diaphragm is formed and a sporangiospore is produced at the end.

1.3.3 Main Groups

1.3.3.1 *Streptomyces*

Streptomyces, the largest genus in the Actinobacteria, has over a thousand species. They can form well-developed substrate and aerial hyphae. The aerial hyphae grow

to a certain stage to form spore hyphae and generate conidia. The spore hyphae have various shapes, including straight, wavy, unciform, heliciform, and verticillate. The spores also have various shapes, including spherical, oval, bacilliform, cylindrical, fusiform, and half-moon. The shape and arrangement pattern of the spore hyphae and the surface ornamentation of the spores are important characteristics for identifying individual species. *Streptomyces* always exist in the soil. Most strains of *Streptomyces* are saprophytic and aerobic. Their colonies can be tight, rugulose, crusty or smooth, and colorful. *Streptomyces* are widely used to produce antibiotics and other bioactive substances in industry. Moreover, some strains of *Streptomyces* can be used to decompose cellulose into monosaccharides for ethanol production.

1.3.3.2 Cystic Actinomycetes

Cystic actinomycetes multiply using sporangiospores and can be identified by their different fission styles and arrangements of spores. The sporocysts of *Actinoplanes* are globular, rodlike, or irregular. *Actinoplanes* can produce circular or near round planospores with lophotrichaete flagella. Most species of *Actinoplanes* are found in rotten plants and soil. The sporocysts of *Dactylosporangium* appear digitiform or rodlike. Regular spherical spores are generated and arranged in a single row. *Planomonospora* species produce fusiform planospores with peritrichous flagella and are commonly distributed in soil of temperate and tropical regions.

1.3.3.3 Multiple-Cavity Sporocyst Actinomycetes

These actinomycetes directly produce spores through longitudinal and transverse fission. Their hyphae form cell populations or spore clusters. The cell wall contains m-DAP acid. These actinomycetes include genera *Dermatophilum*, *Geodermatophilus*, and *Frankia*. *Frankia* can fix nitrogen together with nonleguminous woody plants.

1.3.3.4 *Micromonospora*

Micromonospora are aerobic and saprophytic and their colonies combine with their growth substrate rising from the substrate surface. Colonies are irregular, plicated, or smooth, and often appear yellowish orange or red, sometimes dark brown, black, or blue. A single spore is formed on the monopodial branching sporophore. Saprophytic *Micromonospora* species can decompose cellulose and chitin and they are often distributed in soils, lakes, compost, and animal manure.

1.3.3.5 *Nocardia* and *Nocardiopsis*

Most strains of *Nocardia*, previously known as *Proactinomyces*, are aerobic saprophytic bacteria, while a minority of strains are anaerobic parasitic bacteria. Most species only have vegetative hyphae without aerial hyphae. Their colonies are varied, and can be smooth or raised, nonluminous or shiny. After being cultured for 15 h to 4 days, the hyphae break into almost the same length of a rod or forked rhabdite or globoid. This genus can produce various antibiotics, and some species are very useful for oil dewaxing, hydrocarbon fermentation, and treating wastewater containing cyanide. *Nocardiopsis* is similar to *Nocardia* based on morphological characteristics.

1.3.3.6 *Actinomadura*

Actinomadura species have no aerial hyphae and cannot form spores. They reproduce through the breakage of vegetative hyphae into "V" or "Y" forms. Most strains are pathogenic.

1.3.3.7 *Thermoactinomyces*

Thermoactinomyces have a distant genetic relationship to other actinomycetes. The substrate hyphae and aerial hyphae can both generate single spores. They are usually found in the hyperthermal compost, animal manure, and natural heat straw stack. Some strains can be used for cellulosic ethanol fermentation and some strains can produce antibiotics.

Section 2: Eukaryotic Microbes

Eukaryotes are organisms made up of cells that possess a membrane-bound nucleus. The cytoplasm of eukaryotic cells contains organelles, such as mitochondria and Golgi apparatus, and has a nucleus enclosed in a nuclear membrane, in which there are nucleoli and chromosomes. Eukaryotic microorganisms include fungi (yeast, mold, and mushroom), slime molds, microalgae, and protozoa.

The cellular structures of eukaryotic microorganisms have both general characteristics of eukaryotic cells and their own peculiarities. Some eukaryotic microorganisms such as fungi and algae have cell walls, while some such as myxomycete and protozoa do not. All eukaryotic microbial cells have cell membranes, a nucleus, cytoplasm, and organelles. Eukaryotic microbial cell membranes are similar to those of prokaryotes in structure and function, but different in their composition. The eukaryotic nucleus

is composed of a nuclear envelope, chromatin, nucleolus, and nuclear matrix. The nucleus is the main site of storage, replication, and transcription of genetic information. The cytoplasm is located between the cytomembrane and the nucleus. It is a transparent, viscous, and constantly flowing solution, filled with many organelles. These organelles are universal eukaryotic organelles such as mitochondria, Golgi apparatus, endoplasmic reticulum, ribosomes, lysosomes, and microsomes, but different microbes also have their own special organelles such as chloroplasts, vacuoles, lomasome, chitosome, and hydrogenosome.

2.1 Fungi

Fungi are microorganisms that have a real nucleus, produce spores, do not contain chlorophyll, and absorb nutrients parasitically or saprophytically. They include yeasts, molds, and mushrooms.

2.1.1 Yeasts

Yeasts are a type of unicellular fungi, which commonly propagate by budding. Yeast can ferment sugars to produce energy. Their cell walls often contain mannan and they usually live in aquatic environments that are acidic and have a high sugar content.

2.1.1.1 Yeast Morphology

Yeast cells can be observed under an optical microscope, are generally spherical, oval, elliptical, columnar, or lemon-like, and have a size of about 1–5 × 5–30 μm, up to 100 μm. The cell walls are 100–200 nm thick, with the main components being glucan, mannan, proteins, and chitin, as well as a small amount of lipids. Glucans are the main components that give yeast cells mechanical strength and are located in the internal layer of the cell wall. They are divided into two categories. One is based on β-(1,3)-connections, like an elongated twisted chain, accounting for 85% of the total glucans; the other is based on β-(1,6)-connection, like a branching net, with a low percentage. Mannan is a branched polymer of mannose with an α-(1,6)-linkage and is located in the outer layer of the cell wall. Proteins are sandwiched between glucan and mannan layers, many of which are enzymes such as glucanase and mannanase, and a few are structural proteins. Chitin is present in low amounts and is distributed around bud scars. However, not all yeasts contain mannan, and the cell walls of different species of yeasts also contain different components. For example, some species of *Schizosaccharomyces*

only contain glucan but no mannan. Yeast cell walls can be digested by snailase made from the gastric juices of *Helix pomatia* to form yeast protoplasts. Yeast cell membranes are composed of a three-layer structure comprising proteins, lipids, and small amounts of sugar. In addition to the nuclear DNA, yeast cells can also contain a 2-μm plasmid.

After yeasts are cultured under suitable conditions on solid media for a period of time, colonies, similar to bacterial colonies, will appear. The colonies are opaque, larger, higher, and thicker than bacterial colonies. The colors of the colonies are most often white or candle colored, but can be partially red or black. Generally, the colony is moist, transparent, smooth on the surface, and uniform. The colors of the front, back, and edge of the colony are consistent with the center. Yeast colonies without pseudohyphae are more elevated, and their edges are extremely round. Yeast colonies with pseudohyphae are relatively flat, and their surface and edges are rough. Since yeast can produce ethanol, their cultures usually have a characteristic smell.

2.1.1.2 Yeast Propagation

Yeasts mainly reproduce asexually by budding, fission, or asexual spores. A few yeasts divide by binary fission, and some yeasts produce spores or chlamydospores to carry out asexual reproduction. Asexual reproduction of most yeasts is by budding (Figure 2.1). First, the cell wall of the mother cell generates a bud body that becomes thin through the action of hydrolytic enzymes. Meanwhile, a large number of new cellular materials, including nuclear materials, accumulate at the site of initiation of the bud body. When the bud body gradually grows, a septum composed of glucan, mannan, and chitin is formed at the junction with the mother cell. After maturation of the bud body, it separates from the mother cell to form a daughter cell. After separation, a bud scar is left on the mother cell, and a birth scar is left on the daughter cell. All cells have only one birth scar, but one to many bud scars. According to the number of bud scars, the age of the cells can be determined.

In addition to asexual reproduction, yeasts can also reproduce sexually through the formation of asci and ascospores. Generally, two adjacent cells that have similar morphology but different gender stretch out a tubular protoplasm protuberance, and make contact with each other. The cell wall at the contact position dissolves into an engagement bridge and then four or eight daughter nuclei are formed through plasmogamy, karyogamy, and meiosis. Subsequently, the daughter nuclei combine with their respective surrounding protoplasts, which are surrounded by the spore walls. Thus, an ascospore is formed, and the original vegetative cell becomes an ascus.

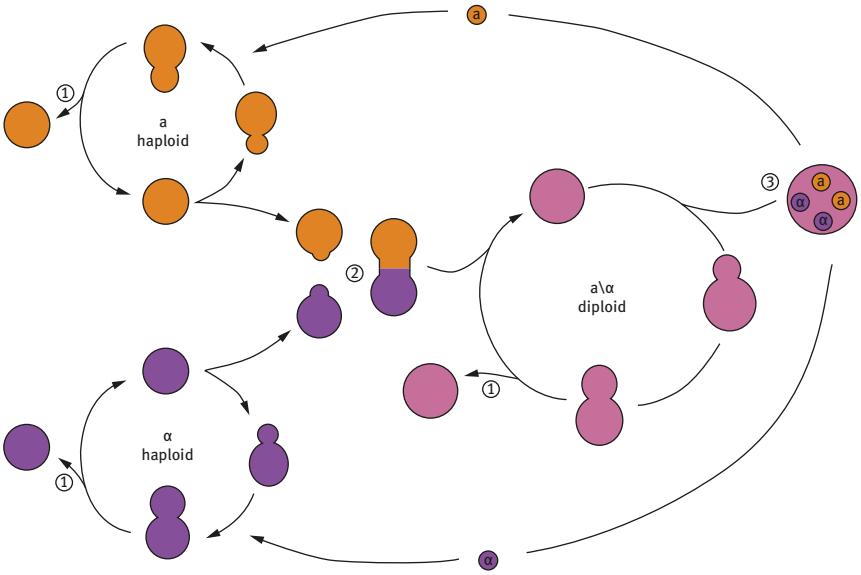


Figure 2.1: The yeast cell's life cycle. (① budding, ② conjugation, ③ spore)

2.1.1.3 Common Groups of Yeast

(1) *Saccharomyces*

The cell shapes of this genus are round, oval, or sausage shaped. Colonies on solid media are white, shiny, and flat, with a neat edge. In liquid culture, a pellicle is not usually formed. Some species can form pseudohyphae, but never form true hyphae. Asexual propagation is by budding, and sexual reproduction generates ascospores. *Saccharomyces cerevisiae* is a typical species of this genus, and its cell shape is mostly round or oval. It can ferment glucose, sucrose, maltose, and galactose; cannot ferment lactose and melibiose; and can use ammonium sulfate as a nitrogen source, but cannot use potassium nitrate. *S. cerevisiae* is widely used in beer, liquor, wine, and alcoholic fermentation and bread making, and is also used to prepare edible, pharmaceutical, and feed single-cell proteins. Moreover, nucleic acids, ergosterol, vitamin C, thromboplastin, and coenzyme A can be extracted from the cells. As an eukaryotic model organism in molecular and cell biology, it was the first eukaryotic organism to have its complete genome determined.

(2) *Candida*

When cells of this genus bud, the daughter cells do not separate from the mother cells, but rather connect with the mother cells to become a chain, similar in to hyphae, known as pseudohyphae. *Candida* cells are round, oval, or rectangle in shape. The colonies' color can vary from creamy white to pale yellow, and some

colonies are mushy, smooth, and glossy, while others are dry, wrinkled, and dim. All the species can produce single or clustered conidia. Individual conidia are round or rectangle in shape. Some conidia can reproduce sexually. Most species can produce long, branched, or curved pseudohyphae, and some species can produce true hyphae, or form chlamydospores. *Candida* is widely used in the production of single cell proteins.

(3) *Pichia*

Pichia cells are oval and their colony morphology is similar to *Candida*. They are facultatively anaerobic. *Pichia* undergoes asexual reproduction by budding or through asexual spores (conidia or chlamydospores). *Pichia* can also reproduce sexually through asci formation. Each ascus has one to four ascospores and is shaped like a hat or are spherical. Some species are opportunistic pathogens, which can infect immunodeficient patients, premature children, low birth weight babies, and long-term hospitalized patients. *Pichia* (e.g., *Pichia pastoris*) are widely used to construct heterologous expression systems, and some species are used for ethanol fermentation, for example, *Pichia stipitis* can ferment glucose and xylose to produce ethanol.

(4) *Schizosaccharomyces*

Schizosaccharomyces cells are oval or cylindrical. They undergo asexual reproduction through division, sometimes forming pseudohyphae. Sexual reproduction is through ascus formation. The ascus has one to four or eight ascospores, and its shape is spherical or oval. *Schizosaccharomyces* can produce ethanol by fermentation and cannot assimilate nitrates. The important species are *Schizosaccharomyces pombe* and *Schizosaccharomyces octosporus*.

(5) *Torulopsis*

Torulopsis cells are spherical, oval, or slightly rectangle. The mode of reproduction is budding. Species of this genus have no pseudohyphae or pigments, can ferment ethanol, can tolerate high osmotic pressures, and can grow in the substrates with high glucose concentrations. Some species can produce glycerol and other polyols, can ferment oil, and produce proteins or other products.

(6) *Geotrichum candidum*

Geotrichum strains are widely distributed in rotten vegetables, silage, pickles, organic fertilizer, animal manure, dairy products, and soil. After being cultured for 24 h in the wort at 28–30 °C, a white, stuffed, or powdered film is produced. The colonies grow fast on the solid media, show planar diffusion, appear flat, white, and have short fiber-like projections, with concentric circle radiation, and some have a central projection. *Geotrichum* produce real hyphae that are branched in some strains. They reproduce by fission resulting in an arthrospore. Arthrospores can be single or connected in a chain.

Spores are long cylindrical and square, and some are oval or round, with blunt ends. Arthrospores are mostly 4.9–7.6 μm × 5.4–16.6 μm. The maximum growth temperature for the strain is 33–37 °C. *Geotrichum* can weakly ferment glucose, mannose, and fructose; can aerobically assimilate glycerol, ethanol, sorbitol, and mannitol; can decompose pectin and lipids; and can assimilate a variety of organic nitrogen compounds and urea. They can also synthesize lipids, use organic wastewater from sugar plants, breweries, and other food factories to produce feed protein, and hydrolyzed protein. Most strains of *Geotrichum* can liquefy gelatin and peptonize milk, while some strains can only peptonize milk and not liquefy gelatin.

2.1.2 Mold

2.1.2.1 Mold Morphology

Molds are commonly known as "filamentous fungi", which is not a taxonomic term. Molds have well-developed mycelia and do not produce large fleshy fruiting bodies. The basic unit of a mold is a hypha, which are long and tubular in shape and 3–10 μm wide. The anterior end of the hypha can continue to grow and branch. When the mold grows on solid media, the part of the hyphae that penetrate into the medium to absorb nutrients is called vegetative hyphae. The vegetative hyphae usually specialize into various structures such as rhizoids, haustorium, appressorium, hyphopodium, sclerotium, rhizomorphs, and stolon. Hyphae that extend into the air are called aerial hyphae. Aerial hyphae mainly differentiate into various forms of fruiting bodies, such as spore heads, sporangia, pycnidia, sporodochia, acervuli, and ascocarps, which are the structure with reproductive functions. A large number of hyphae interweave together into a villous, flocculent, or mesh shape called the mycelium.

According to the presence of the diaphragm in hyphae, hyphae can be divided into nonseptate or septate hyphae. Nonseptate hyphae have no diaphragm, and the whole hypha is a single cell, which contains a plurality of nuclei. The hyphae of lower fungi are generally nonseptate hyphae. Septate hyphae have a diaphragm. A section of hypha separated by a diaphragm is a cell. A single hypha is composed of a number of cells, each of which contains one or more nuclei. There is a plurality of apertures on the diaphragm allowing the exchange of cytoplasm and nutrients between cells. The hyphae of higher fungi are generally septate hyphae.

2.1.2.2 Mold Propagation

Molds can reproduce asexually and sexually. Asexual reproduction produces asexual spores, and sexual reproduction produces sexual spores. The features of various spores are shown in Table 2.3.

Table 2.3: The features of asexual and sexual spores of molds.

	Name	Shape	Formation mode
Asexual spores	Arthrospore	Stumpy, tubular, or with obtuse ends	Hyphae breakage
	Zoospore	Round, pear, or kidney shaped, one or two flagella	Arising from the swollen hyphae within a zoosporangium
	Chlamydospore	Round, fusiform, or rectangular	From the dormant spores produced by the enlargement of several cells in the middle of hyphae, whose protoplasm is concentrated and cell wall is thickened
	Sporangiospore	Round or oval	Produced in the sporangium
	Conidium	Round or oval	Produced in the conidiophores, which are at the top of the aerial hyphae
Sexual spores	Oospore	Round or oval	Developed from a combination of the small antheridium and the large archegonium
	Zygosperm	Round	Homothallism or heterothallism*
	Ascospore	Oval, acicular, or have a diaphragm	Produced in the ascus

Note: * Homothallism refers to hyphae from the same mycelium that contact each other to produce a zygospore, while heterothallism refers to hyphae from different mycelia that contact each other to produce a zygospore.

2.1.2.3 Common Groups of Molds

(1) *Mucor*

Mucor species have well-developed mycelia composed of nondiaphragm and multinuclei hyphae. The hyphae are white, saprophytic, and rarely parasitic. *Mucor* species produce sporangiospores for asexual reproduction, while zygospores are used for sexual reproduction. Amylase produced by *Mucor* species is highly active, so *Mucor* strains are often used to produce amylase or to saccharify starch and are also used for citric acid fermentation. *Mucor* are widely distributed in soil, compost, and on fruits, vegetables, and all types of starchy foods.

(2) *Rhizopus*

Rhizopus species have well-developed mycelia composed of nondiaphragm and multinucleate hyphae. The hyphae are white aericolous and are woven into loose flocculent colonies on solid media. *Rhizopus* species have many similar characteristics to *Mucor* species except that *Rhizopus* species are rhizoid and have stolons. *Rhizopus* reproduce the same way as *Mucor* species. *Rhizopus* species can produce amylase and glucoamylase and are widely used in the wine industry. They are also used for the conversion of steroidal substances and production of organic acids. *Rhizopus* species are widely distributed in soil, air, on surfaces, and various starchy foods.

(3) *Aspergillus*

The hyphae of *Aspergillus* species have diaphragms and are multinucleate. Colonies are fluffy with vegetative hyphae that can form conidiophores. The cells that branch into conidiophores are called podocytes. The top of each conidiophore enlarges to a terminal vesicle, generally spherical. The surface of the terminal vesicle is covered with one or two radial sterigmata. The uppermost sterigmata are bottle-like, on the top of which there are strings of spherical conidia. Conidia are green, yellow, orange, black, or brown. The sexual reproduction of many species is unknown, but some species can conduct sexual reproduction to produce asci and ascospores. *Aspergillus* species are widely used in the fermentation industry and food processing industry to produce food flavorings, amylase, protease, pectinase, and organic acids. Aspergilli are widely distributed in air, soil, cereals, and a variety of organic compounds.

(4) *Penicillium*

The hyphae of *Penicillium* species are similar to those of *Aspergillus* species, but have no podocytes. The colonies are cotton shaped. The top of each conidiophore is not inflated and has no terminal vesicle. After several rounds of branching, the conidiophores produce several symmetric or asymmetric sterigmata. The top of the sterigmata produces strings of cyan conidia with a broom-like spike shape. The mode of sexual reproduction of many species is unknown. Besides *Penicillium chrysogenum* and *Penicillium notatum* that are used for penicillin production, some species of *Penicillium* are used for the production of enzymes, such as phosphodiesterase and cellulase, organic acids, and the bioconversion of sterols. *Penicillium* is widely distributed in air, soil, and on rotten fruits, vegetables, meat, and a variety of damp organic matter.

(5) *Trichoderma*

The hyphae of *Trichoderma* species are transparent, have a diaphragm, and elongate quickly. Colonies are cotton shaped or form dense bundles. Colonies are white initially, and then green, with a surface that often shows concentric rotiform patterns. Conidiophores have opposite or alternate branches that can branch again and again. The top of the branch produces bottle-shaped sterigmatas that are fascicularis, opposite, alternate, or solitary. The terminal end of the sterigmata produces yellow-green conidia that are nearly spherical, oval, cylindrical, or inverted ovals. Several conidia agglomerate to a spherical spore head. In addition, this genus also produces chlamydospores. The sexual stage of this genus was previously known as *Hypocrea*. The antagonism of *Trichoderma* to a variety of plant pathogenic fungi makes it useful for biological control. In addition, *Trichoderma* species have a strong ability to break down cellulose. They can be used to prepare cellulase and hydrolyze cellulosic material to monosaccharides that can be further fermented into ethanol or single cell proteins. Some strains of *Trichoderma* also synthesize riboflavin, produce antibiotics, and convert steroids. *Trichoderma* is widely distributed in rotten wood, seeds, plant residues, organic fertilizers, soil, and air.

(6) *Neurospora*

The hyphae of *Neurospora* are long and multinucleate and have diaphragms. Colonies are loosely reticulodromous. At the asexual stage, *Neurospora* appears like powdery mildew. The sporogenous hyphae branch into double forks. A chain of conidia is generated on the top of the aerial hyphae, which are oval, pink or orange, and easily fall off. At the sexual stage, *Neurospora* performs sexual reproduction by asci and ascospores and is a heterothallic breeding genus. One mycelium forms a protoperithecium to combine with the hyphae of another mycelium. After plasmogamy, they conduct nuclear fission for several rounds, and then perform karyogamy to form multiple diploid nuclei. Each diploid nucleus is packaged into an ascus. The diploid nucleus in the ascus first conducts meiosis to form four haploid nuclei and then each haploid nucleus conducts mitosis again to form a total of eight haploid nuclei. Each nucleus grows to an ascospore. The ascospore is colorless and transparent early, and then turns from olive to light green when it is ripe. There are projections and nerve-like vertical ribs on the outer wall of the ascospores, from which the name *Neurospora* is derived. *Neurospora crassa* has been used as a model organism to study microbial genetics and has recently been used to produce melanin. Furthermore, *N. crassa* can also produce laccase, which is also known as benzenediol oxidoreductase. Laccase is a copper-containing polyphenol oxidase and can oxidatively degrade various phenolic compounds and their derivatives, such as monophenol, catechol, and hydroquinone. Laccase can also degrade some phenol analogs without phenolic hydroxyl and be used for lignin degradation to facilitate ethanol fermentation from lignocellulosic materials.

(7) *Gibberella*

The hyphae of *Gibberella* species have diaphragms and branches. The colonies are flocculent. Conidiophores may be branched or not. Two types of conidia are produced by asexual generation: one is small, single cellular, round, oval, or long columnar; the other is large, multicellular, with a diaphragm, sickle shaped or long cylindrical. Both types of spores join together or accumulate into groups on the top of hyphae. Sexual reproduction produces asci and ascospores. The perithecium is spherical, smooth, and blue. The ascus is shaped like a long rod and contains eight ascospores arranged in two irregular rows. The ascus is straight and narrow. This genus can produce gibberellin, which promotes plant growth.

2.1.3 Mushrooms

Mushrooms are filamentous fungi, and the term usually refers to those fungi capable of forming large fleshy fruiting bodies, including most species of Basidiomycetes and a very few strains of Ascomycetes. Mushrooms are widely distributed and are especially abundant in deciduous forest zones. Humans often eat mushrooms or use them for medicinal

purposes. Edible mushrooms such as *Agaricus bisporus*, *Auricularia auricula*, *Tremella fuciformis*, *Lentinula edodes*, *Pleurotus ostreatus*, *Volvariella volvacea*, *Agrocybe cylindracea*, *Flammulina velutipes*, and *Dictyophora* have high protein contents and a variety of vitamins. Medicinal mushrooms such as *Ganoderma lucidum*, *Coriolus versicolor*, and *Hydnum repandum* are often used to treat cancer and other diseases.

2.1.3.1 Mushroom Morphology

Mushrooms have various shapes, and their basic structures consist of a fruiting body and mycelium with many branching hyphae. They are distributed in soil, rotten wood, and other substrates; decompose substrates; and absorb nutrients. The biggest feature of mushrooms is the formation of large fleshy fruiting bodies of various shapes, sizes, and colors. The fruiting body of a typical mushroom is composed of three parts: the cap on the top (including epidermi, context, and gill), stipe in the middle (often including ring and volva), and mycelium at the base.

2.1.3.2 Mushroom Development

During the development of mushrooms, the basidiospores germinate to form the first-level hyphae composed of a number of mononuclear cells. After conjugation of the first-level hyphae with different genders, the second-level hyphae composed of binuclear cells are formed through plasmogamy. The binuclear cells continuously divide through a unique method of clamp connection, which results in the continuous forward extension of the hyphal tip. Under suitable conditions, a lot of the second-level hyphae differentiate into multiple hyphal strands, namely the third-level hyphae. The hyphal strands will form buttons under optimal conditions and then differentiate and inflate into a large fruiting body. After maturation of the fruiting bodies, the top of the binuclear hyphae enlarges and the cytoplasm becomes dense. Karyogamy occurs to form a diploid nucleus in the inflated cells. After meiosis and mitosis, the diploid nucleus becomes four haploid nuclei. At this time, the top inflated cell develops into a basidium, and subsequently the upper basidium extrudes four sterigmatas. Each haploid nucleus enters into a sterigmata whose top expands to generate basidiospores. Basidiospores are sexual spores that are mostly round, oval, kidney shaped or sausage shaped.

2.2 Slime Molds

Slime molds are similar to both fungi and protozoa. They can produce spores and fruiting bodies and feed by phagocytosis. There are two groups of slime molds: cellular slime molds and acellular slime molds.

2.2.1 Cellular Slime Molds

The vegetative body of a cellular slime mold is composed of a single amoeba-like cell. The amoeba is without a cell wall, shapeless, and can stretch out pseudopodia to prey on bacteria or other particulate foods. During the growth period of cellular slime molds, there are two periods: asexual and sexual reproduction. Diploid cells appear during sexual reproduction. The procedure is roughly as follows: when nutrients are in abundance, cellular slime molds exist in the form of a single cell and survive by eating bacteria; when food runs out, the single cells come together to form a structure similar to a protoplasm mass, in which the amoeba-like vegetative cells lose independence but do not fuse. Following this, some cells conduct gametogony to form diploid gametes and then form new haploid amoeba by meiosis to return to the asexual reproductive cycle. Some cells will form fruiting bodies, producing and releasing haploid spores. The spore shell bursts to release a single cell. Thus the life cycle is completed.

2.2.2 Acellular Slime Molds

The vegetative body of acellular slime molds is not a single cell, but a protoplasmic mass with an unfixed size and shape, also known as a plasmodium. The protoplasmic mass gradually evolves from the initial amorphous sticky mass to be fan shaped, and finally net shaped. It is moist and viscous on its substrate, can be bright yellow, red, pink, or gray in color, and can crawl on the substrate. There are diploid and haploid periods during its growth process. The diploid period starts from a zygote formed by two haploid cells through gametogony. The zygote undergoes mitosis to form a protoplasm mass with many nuclei but only a lump of protoplasm called a plasmodium. After the plasmodium matures, it will form a network and change its surface area in accordance with the required nutrients such as food, water, and oxygen. This period is also known as the vegetative phase and the plasmodium feeds by phagocytosis. Subsequently, the plasmodium becomes a sporangium that will mature to form fruiting bodies. After meiosis, haploid spores are released and are spread through the air. These spores will produce two kinds of gametes: amoeba cells and swarm cells. These two cells can transform into each other, but finally will only carry out gametogony with the same kind of cell to produce a diploid zygote.

2.3 Algae

2.3.1 Algae Morphology

Algae are eukaryotes that differ from plants. They have chlorophyll and can perform photosynthesis. Except *Euglena*, most algae have cell walls composed primarily of

cellulose or other polysaccharides (xylan or mannan). However, the cell wall of diatoms is made of silica, proteins, and polysaccharides. Algal cells contain organelles, vacuoles with membranes, and one or more nuclei in the algal cytoplasm. Algal cells contain not only chlorophyll but also carotene, lutein, and phycobiliprotein chromophores that give algae their characteristic color of green, red, yellow, or brown. Algal energy storage is primarily as starch, as well as polysaccharides and lipids.

Algae have various individual forms and body sizes. According to the body size, algae can be divided into macroalgae and microalgae. Macroalgae are large, measured in inches, multicellular, and mainly include the red green and brown algae. Since macroalgae produce little oil, and their cellulose content is low, they are usually used for the consumption of food. Recently, with the development of new technology, macroalgae have also been used to produce energy in the form of biogas and ethanol. Microalgae are small, measured in microns, and are unicellular. Most algae belong to the microalgae. Due to their high yield of oil, which is a good alternative to petroleum and other fossil fuels, considerable research has been performed on the microalgae. In addition, like macroalgae, microalgae can be also used to produce biogas and fuel ethanol.

2.3.2 Algae Propagation

Algae undergo asexual and sexual reproduction. Asexual reproduction is mainly by the production of spores. Spores can be zoospores or aplanospores, and both are produced by sporangia. Some algae conduct asexual reproduction by fracturing. Each segment can develop into a new algal filament. Sexual reproduction involves gamogenesis. Gametes can be homotypic or heterotypic and can combine by homothallism or heterothallism to form a zygote.

2.3.3 The Main Groups of Algae

2.3.3.1 Chrysophyta

Most algae in the Chrysophyta are single cellular or form cell populations, while a small number exist as multicellular filaments. The motor cell has either one or two flagella. Unicellular Chrysophyta or those that exist as a cell population have one to two chromatophores, which are dominated by carotene and lutein. Green-pigmented algae have one or two of chlorophyll *a*, *c*, or *e*. In addition, Chrysophyta also contain lipids. A representative group of Chrysophyta – the diatoms – can produce lipids through photosynthesis. They reproduce mainly by vegetative reproduction and sporogenesis, and sexual reproduction is extremely rare. Members of the Chrysophyta are mainly

distributed in freshwater, especially in low-temperature freshwater, while some are also distributed in seawater, such as diatoms.

2.3.3.2 Chlorophyta

Most algae in the Chlorophyta are unicellular or form cell populations, while some are also multicellular filaments or plates. The shape and number of chromatophores often changes with the species. Chlorophyta contain chlorophyll *a*, chlorophyll *b*, carotene, and lutein. The motor cells can have two, four, or more isometric and acrogenous flagella. Chlorophyta reproduce by a variety of methods, and some species have alternating generations in their life cycles. Most species of Chlorophyta live in freshwater, and rarely in seawater. They live in planktonic, sessile, or attached forms, while a few species are parasitic or symbiotic. Common genera include *Spirogyra* and *Cladophora*.

2.3.3.3 Pyrrophyta

Most algae in the Pyrrophyta are unicellular, while a small number exist as cell populations or protonema. Although a few species don't have cell walls, most have cell walls composed of cellulose. Their cell walls are called shells. There are upper and lower shells, between which there is a transverse groove that is perpendicular to a longitudinal groove. Two unequal flagella exist at the joint between these two grooves. Pyrrophyta have one or more chromatophores that appear as yellow-green or brown. In addition to chlorophyll *a* and *c*, they also contain large amounts of carotene and lutein. They reproduce predominantly by cell division, or the production of asexual spores in the mother cell leading to sporogony. Sexual reproduction has been found in only a few genera and species. Most species of the Pyrrophyta are planktonic and live in oceans. Sometimes they bloom in the vicinity of the coastline to form red tides. Some species also live in ponds and lakes. The photosynthetic products of the marine species are mainly lipids, while the freshwater species mainly produce starch. The most common genera in the Pyrrophyta are *Ceratium* and *Peridinium*.

2.3.3.4 Euglenophyta

In addition to *Colacium*, other species in the Euglenophyta are unicellular algae that have flagella at the top, are motile, and do not have cell walls. Euglenophyta contain chlorophyll *a*, *b*, and an eye spot, and have some characteristics of both animals and plants. Their storage compounds are mainly starch and a small amount of lipids. Reproduction is mainly by cell division. Under poor environmental conditions, they can form thick-walled cysts. When the environmental conditions improve, the

protoplasts are released from the wall to form a new individual. Many algae of the Euglenophyta are planktonic and live in freshwater rich in animal organic matter. When they bloom, they often turn water green, brown, or red. A few species are colorless and are heterotrophic. A common genus is *Euglena*.

2.3.3.5 Rhodophyta

In addition to individual genera and species, algae in the Rhodophyta are multicellular, usually filamentous, lamellate, or dendritic. The chromatophores are mostly red or purple. In addition to chlorophyll *a*, *d*, carotene, and lutein, they also contain a large amount of phycoerythrin and phycobiliprotein. The assimilation products are the starch-like floridean starch. During their life cycle, Rhodophyta have no flagellate motor cells. Sexual reproduction is by oogamy. The female genital organ is a carpogonium, similar to the oocyst. There is a trichome called trichogyne on the carpogonium. A special spore called a carpospore is produced after fertilization. Except for a few genera and species, the vast majority live in seawater and attach to surfaces. The most common genera are *Porphyra* and *Gelidium*.

2.3.3.6 Phaeophyta

All species in the Phaeophyta are multicellular algae. In addition to chlorophyll *a* and *c*, they contain high levels of carotene and lutein in their chromatophores making them appear brown. The assimilation products are laminarin and mannitol. Vegetative cells have no flagella. The zoospores and male gametes have two paragynous and unequal flagella. They can reproduce by several methods. All species can undergo sexual reproduction. During their life cycle, most species have an obvious alternation between generations. Most species of Phaeophyta live in seawater and are attached to surfaces. The most common species are *Laminaria japonica* and *Undaria pinnalifida*.

2.4 Protozoa

Protozoa are a class of unicellular eukaryotic organisms that are colorless, do not have a cell wall, and can be motile. They are the most primitive and lowest unicellular animals with the simplest structures. The cell structure from the outside to inside includes a cell membrane, cytoplasm, and nucleus. The cytoplasmic membrane is a specialized form of the outer part of the cytoplasm. The cytoplasm has various inclusions of granules and organelles. The nucleus contains a single core, but some protozoa have two or more nuclei.

Generally, the size of protozoa is very small. They can be visible under an optical microscope, while some are visible with the naked eye. They usually eat other microorganisms or organic particles by phagocytosis and obtain nutrients in solution by pinocytosis. In order to meet their nutritional needs, many protozoa can move. Flagellata move using flagella, Ciliatea move using cilia, and Sarcodina move using pseudopodia. Species in the Sporozoa cannot move and live by parasitism. Protozoa are widely distributed in soil, water, freshwater, animal manure, and other organisms.

Section 3: Growth and Metabolism of Microbes

Under appropriate trophic and environmental conditions, microorganisms can grow well and carry out normal energy metabolism. Exploring conditions for the growth of microorganisms can help people to use them better.

3.1 Microbial Nutrition

Nutrition is the process by which an organism takes and uses a variety of substances to meet its own growth and reproduction needs. The various substances are called nutrients.

3.1.1 Nutrients

There are many types of nutrients needed for microbial growth. According to their physiological function, nutrients can be divided into five categories: carbon sources, nitrogen sources, growth factors, inorganic salts, and water.

3.1.1.1 Carbon Sources

Carbon sources, including organic and inorganic carbon, are a type of nutrient that can meet the carbon demand for microbial growth and reproduction. Organic carbon molecules include carbohydrates, proteins, organic acids, alcohols, esters and hydrocarbons. Inorganic carbons mainly refer to CO_2, carbonate, bicarbonate and materials containing inorganic carbon. Microorganisms preferentially utilize carbon sources. Carbohydrates are the priority carbon source. For carbohydrates, monosaccharides are preferred over disaccharides and polysaccharides, and hexoses are preferred over pentoses. Glucose and fructose are preferred to mannose and galactose. As

for polysaccharides, homopolysaccharides are preferred to heteropolysaccharides. In addition, the ability of microbes to use carbon sources is also different. Some species of *Pseudomonas* can use a variety of carbon sources, while some species of *Methylococcus* can only use one or a few carbon sources.

3.1.1.2 Nitrogen Sources

Nitrogen sources, including organic and inorganic nitrogen, are a type of nutrients that can provide a nitrogen source for the growth and reproduction of microorganisms. Organic nitrogen sources include proteins, peptones, polypeptides, amino acids, and nitrogen bases (namely, purine and pyrimidine). Inorganic nitrogen sources mainly include ammonium, nitrate, nitrite, nitrogen, and ammonia. As for carbon source utilization, the use of nitrogen sources for microbes is also selective and differential. For heterotrophic microorganisms, organic nitrogen is preferred to inorganic nitrogen, and simple organic matter is preferred to complex organic matter. Most microorganisms can use a variety of nitrogen sources, while a small number of microorganisms can only use one or a few nitrogen sources. For example, *Clostridium acidiurici* can only use purines and pyrimidines as nitrogen sources. In addition, the vast majority of microorganisms can use ammonium and nitrate. When ammonium salts such as $(NH_4)_2SO_4$ are used as a nitrogen source for culturing microbes, the pH of the culture system will decrease due to the assimilation of NH_4^+. This type of ammonium salt is called a physiologically acid salt. When a nitrate source, such as KNO_3, is used as a nitrogen source to cultivate microorganisms, the pH of the culture system can increase due to the assimilation of NO_3^-. This type of nitrate is known as a physiologically alkaline salt.

3.1.1.3 Growth Factors

Growth factors are organic compounds that are in limited demand and are essential for the growth of microorganisms. They cannot be synthesized by microbes themselves or can only be synthesized in small amounts to meet the needs of microbial growth. Growth factors include vitamins, amino acids, purines, and pyrimidines. The need for growth factors varies from species to species. For example, *Lactobacillus* need all growth factors, while *Corynebacterium glutamicum* requires only one or several growth factors, and *Escherichia coli* does not need any growth factors. In addition, the requirements for growth factors for one microorganism can change under various environmental conditions. Under anaerobic conditions, *Mucor rouxii* requires biotin and thiamine, but under aerobic conditions, it can synthesize these two growth factors. Therefore, it is necessary to provide the proper growth factors for the growth of microorganisms according to the characteristics of each microorganism itself and any changes in culture conditions.

3.1.1.4 Inorganic Salts

Inorganic salts play an important role in maintaining normal physiological functions in microorganisms. Inorganic salts can maintain and adjust the osmotic pressure of the cell, participate in the formation of biological macromolecules and structural components of the cell, act as coenzymes or enzyme activators, as well as provide energy for certain microorganisms. According to the different amounts required for the growth of microorganisms, inorganic elements can be divided into macroelements and microelements. The macroelements are in high demand for microbial growth (within the range of 10^{-3}–10^{-4} mol/L) and include P, S, K, and Mg. The microelements are needed in small amounts for microbial growth (range of 10^{-6}–10^{-8} mol/L) and include Mn, Zn, Cu, and Mo.

3.1.1.5 Water

Water is indispensable for any living organisms and has various physiological functions: to maintain the normal morphology of cells; to ensure normal metabolism; to participate in a series of chemical reactions in the cell; to maintain the stability of biological macromolecules; and to regulate temperature to keep cells in a normal physiological state. Water activity (α_w) is usually used to indicate the water demand for a microorganism and it is defined as the vapor pressure ratio of a solution (P_w) to pure water (P_w^0) at a certain temperature and pressure, namely, $\alpha_w = P_w/P_w^0$. Under normal temperature and pressure, the α_w value of the pure water is 1.00. The higher the amount of solute is, the smaller the α_w value is. Each microorganism has its own suitable α_w value. Both higher and lower α_w values can affect the growth of microorganisms. In general, bacteria require a higher α_w (0.99–0.93) than yeasts (0.91–0.88) or molds (0.94–0.73).

3.1.2 Trophic Type

According to their requirements for inorganic or organic carbon, light or chemical energy, and inorganic or organic hydrogen donors for growth, microorganisms can be generally divided into four trophic types, namely photolithotrophy, photoorganotrophy, chemolithotrophy, and chemoorganotrophy. This division is not absolute, because microbes can be of more than one trophic type. For example, purple nonsulfur bacteria can assimilate CO_2 without organic matter (autotrophy), and can also use it to grow in the presence of organic matter (heterotrophy).

3.2 Microbial Growth and Reproduction

Under suitable conditions, both prokaryotic and eukaryotic organisms can assimilate nutrients to grow continuously. When individual cells are mature, they will be propagated asexually or sexually to increase the number of cells. In an actual production process, we need to make the target strain reproduce continuously to get a desired product. Therefore, the growth of microorganisms needs to be understood. Meanwhile, the propagation of harmful microorganisms during fermentation should be controlled to avoid a loss of yield.

3.2.1 Growth and Reproduction

Although the reproduction of the prokaryotic and eukaryotic microorganisms is different, their growth laws are similar. Microorganisms can grow well with rich nutrients under suitable conditions. As time goes on, their cell number will increase and nutrients will be gradually consumed. As the cell number increases, the culture environment will gradually change, and various factors become disadvantageous for microbial growth. Finally, microbial growth becomes stagnant and gradually moves toward extinction.

3.2.1.1 Growth and Reproduction of Bacteria

Bacteria usually reproduce by asexual propagation. The growth and reproduction of bacteria in a single batch culture is divided into four periods: lag phase, log phase, stationary phase, and decline phase [1].

(1) Lag phase
When bacteria have access to fresh media, a period of time is required to adjust and adapt to the new environment. During this time, there is no increase in cell number, but the cells are not in a static state. The cells are metabolically very active. The amounts of RNA, proteins, and other substances increase, cell volume enlarges, and some specific enzymes and intermediate metabolites are synthesized. These physiological changes lay the foundation for a cell to adapt to the new circumstance. The existence of a lag phase will extend the growth cycle of bacteria, which leads to a cost increase for the actual production. Therefore, it is necessary to shorten or even avoid the lag phase. The length of the lag phase is related to microbial hereditary, strain age, inoculation amount, and other factors. Improving these factors can shorten the lag phase, such as changing the genetic characteristics of strains, inoculating with strains in the log growth phase, and increasing the amount of inoculum.

(2) Log phase

After undergoing the lag phase, bacteria acclimatize to their new environment. Bacterial cells begin to divide and their numbers increase in a geometric series. This period is known as the log phase. In this period, the growth rate of bacteria is the highest; metabolic activity is strong; and the size, morphology, and physiological characteristics of the cells are more uniform. Strains at this phase are often used as seed cultures for inoculation. The number of bacterial cell increases logarithmically and the cellular components increase proportionally, so the characteristics of bacterial growth in this period can be described by the following mathematical model:

$$N = N_0.2^n \tag{2.1}$$

where N refers to the final cell number at log phase, N_0 is the initial cell number, and n is the number of reproductive generations. Equation (2.1) can be expressed as a logarithm in the following equation:

$$n = \frac{\lg N - \lg N_0}{0.301} = 3.322(\lg N - \lg N_0) \tag{2.2}$$

For the individual growth of a bacterium, the time required for each bacterium to reproduce a single generation is called generation time, which is usually expressed as G. The relationship between G and n can be expressed as the following equation:

$$G = \frac{t_2 - t_1}{n} = \frac{t_2 - t_1}{3.322(\lg N - \lg N_0)} \tag{2.3}$$

where t_1 and t_2 are the initial and final culture times at log phase.

(3) Stationary phase

During late log phase, with the rapid propagation of bacteria, the nutrients are mostly consumed, and metabolites and other environmental factors are not conducive to bacterial growth. The growth rate of the bacteria decreases and the mortality rate increases. Once the number of cells reproducing and dying tends to be equivalent, the number of live cells remains stable, and the bacterial growth enters stationary phase. The number of live bacteria in this period is the highest. It is the best time to harvest bacteria and their metabolites. In practice, process design is usually used to keep bacteria grow in the stationary phase, and thus a large number of target products can be obtained.

(4) Death phase

During the late stationary phase, the culture environment becomes more unsuitable for bacterial growth, resulting in a higher number of dead cells than live ones. The number of live cells declines and the bacteria enter into the death phase. During this period, bacterial cells will die due to accumulation of their own metabolites. The autolysis of dead cells will make the culture environment worse for bacterial reproduction. However, some bacterial cells will use their stress response to survive.

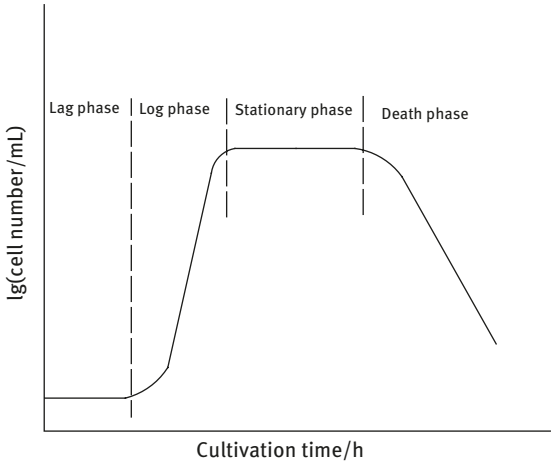


Figure 2.2: Growth curve of the bacterium.

Bacterial growth can be expressed by a growth curve (Figure 2.2). In a closed culture system (no material is added into or discharged from the system), based on the variation of bacterial numbers at different times, the growth curve, whose abscissa is time and ordinate is bacterial quantity, can be plotted to reflect bacterial growth with increasing time.

3.2.1.2 Growth and Reproduction of Fungi

The fungi can grow and reproduce by two methods: asexual and sexual reproduction. Fungi perform asexual reproduction through fission or asexual spore production. Yeast cells can also bud. Sexual reproduction is carried out by producing sexual spores. Yeasts are unicellular fungi and their growth curve is similar to that of bacteria, while the growth curve of the filamentous fungi lack log and stationary phases and are very different from bacteria.

The growth of fungi can be divided into three stages (Figure 2.3): stagnant phase, rapid growth phase, and decline phase [2]. In the period of stagnation, the spores are in a stagnant state or their growth cannot be measured. This phase is also a preparatory phase for growth. After the stagnant phase, the spore germinates, and the dry weight of mycelium increases rapidly, but not in a geometric ratio. The cube root of mycelium dry weight is linear with time. Nutrients are quickly assimilated and the respiratory intensity of fungi reaches its peak. The growth of fungi enters into the rapid period. When fungi grow to a certain stage, the mycelium dry weight begins to decrease, indicating that mycelial growth is in the decline phase. During this period, secondary metabolites will be synthesized and the mycelia will autolyze to different degrees.

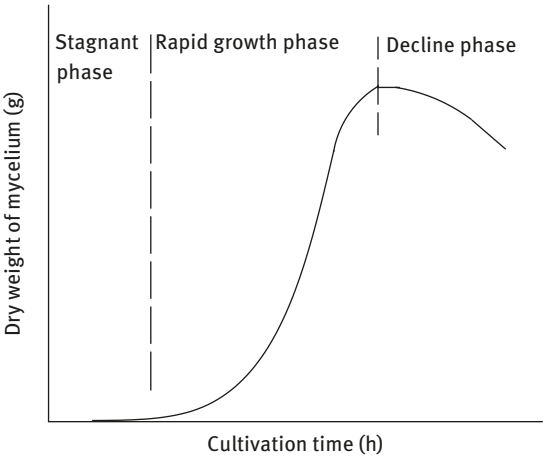


Figure 2.3: Growth curve of the fungus.

3.2.1.3 Environmental Factors Influencing on Microbial Growth

A good environment is a prerequisite for the growth of microorganisms. The main environmental factors affecting the growth of microorganisms include nutrients, water activity, temperature, pH, and oxygen.

(1) Nutrients provide nutrition for microbial growth. The lack of nutrients will impact on the normal physiological and metabolic activities of microorganisms, leading to a reduction or even cessation of the synthesis of some substances, and thus are disadvantageous for the growth of microorganisms.

(2) Microbial growth requires suitable water activity. When the water activity is too high, the water in the environment will penetrate into the cell, cause cell expansion or the cells to burst. When the water activity is too low, the intracellular water will transfer to the environment, leading to plasmolysis or death of the cell due to dehydration.

(3) Temperature can impact on microorganisms in two ways. One way is to influence the fluidity of the cell membrane. The higher the temperature is, the more fluid the cell membrane is. The other way is to affect the activities of biological macromolecules. Intracellular metabolism is mostly accomplished by enzymes that require the appropriate temperature. When the temperature is very low, enzymatic activity is also very low, which makes the metabolism of the microorganism slow or even stop. As the temperature gradually increases, enzymatic activity increases. When the temperature is optimal for the enzyme, enzymatic activity reaches its highest point and the necessary materials can be efficiently synthesized. When the temperature surpasses the optimum point, enzymatic activity decreases and the activity of other biological macromolecules, such as proteins and nucleic acids, is also affected. Metabolism

will be reduced and the cell may die. As a result, there are three temperature points for the microbial growth: the minimum, optimum, and maximum temperatures. The optimum growth temperature for each microorganism is not the same. Based on this characteristic, microorganisms can be divided into three physiological groups: psychrotrophic, mesophilic, and thermophilic. The minimum, optimum, and maximum temperatures for these three types of microorganisms are shown in Table 2.4.

(4) Changing pH can cause the changes in the cell membrane charge and ionization of nutrients, which affect their assimilation and the activity of biological macromolecules. There are minimum, optimum, and maximum pH values for microbial growth. Different microorganisms have different pH requirements. The most suitable pH for bacterial growth is 6.5–7.5, and for yeast and mold is 4.5–5.5. The same microbial species may also have different pH requirements at different growth stages, and during different physiological and biochemical processes. Take *Clostridium acetobutylicum* for example, it mainly grows and reproduces at pH 5.5–7, while it produces acetone and butanol at pH 4.3–5.3. Therefore, it is of great significance to control the pH for industrial production.

(5) According to the different requirements for oxygen, the microorganisms can be categorized into five types: obligate aerobes, facultative aerobes, microaerobes, oxygen tolerant, and obligate anaerobes. Obligate aerobic microorganisms have an integrated respiratory chain, superoxide dismutase (SOD), and catalase and must be in a high oxygen atmosphere (volume fraction ≥20%) to grow. Most bacteria and fungi are obligate aerobic microorganisms. Facultative aerobic microorganisms also have SOD and catalase and can grow with and without oxygen. They produce energy via respiration under aerobic conditions, while they can produce energy through fermentation or anaerobic respiration under anaerobic conditions. This group includes organisms like *S. cerevisiae*. Microaerobic microorganisms can only grow at low oxygen concentrations (volume fraction 2–10%), and include organisms such as *Zymomonas* spp. Oxygen-tolerant microorganisms harbor SOD and peroxidase, but lack catalase. They do not need oxygen to grow, but oxygen does not harm their growth. This group includes organisms such as *Lactobacillus lactis*. Obligate anaerobic microorganisms can only grow under anaerobic conditions due to a lack of SOD. Most of these also lack catalase, and include organisms such as *Clostridium* spp. Differences in the

Table 2.4: Different temperatures for the growth of psychrotrophic, mesophilic, and thermophilic microorganisms.

Microorganisms		Minimum (°C)	Optimum (°C)	Maximum (°C)
Psychrotrophic	Obligate	<0	15	20
	Facultative	0	20	35
Mesophilic		10–20	25–37	45
Thermophilic	Obligate	>40	50–60	55–75, or >75
	Facultative	37		

preference for oxygen between aerobic and anaerobic microbes are dependent on the existence of SOD and catalase. SOD can convert intracellular oxygen free radicals into H_2O_2 that are converted to H_2O by the action of catalase. Thus, harm from oxygen free radicals can be avoided by the cell.

3.2.2 Control of the Growth and Reproduction

During industrial production, in order to effectively obtain the target products, it is necessary to ensure that the whole production process cannot be affected by foreign microbes. Therefore, it is imperative to sterilize the cultivation vessels and related equipments. Sterilization can be performed by physical and chemical ways.

Physical sterilization uses physical factors to inhibit or kill microorganisms. These factors include temperature, water activity, desiccation, filtration, radiation, and ultrasound. Their mechanisms for controlling microbial growth are shown in Table 2.5 [1]. Increasing temperature is the most common method for controlling the growth of harmful microorganisms. There are two indicators for evaluating thermal sterilization.

Table 2.5: The mechanisms of different physical factors that inhibit or kill microbes.

Physical factors	Mechanism of inhibition or sterilization	Examples
Temperature	Low temperature decreases enzyme activity, slows down metabolic activity, and thus inhibits microbial growth; high temperature destroys the structure of membranes and macromolecules, and thus causes death to microbes	Low temperature for preserving food; high-pressure sterilization
Water activity	High water activity causes cells to burst; low water activity results in dehydration and plasmolysis of cells	Pickled fish, meat, and vegetables
Desiccation	Reducing water content down to a point where microbial requirements cannot be met to inhibit microbial growth	Drying preservation of food
Filtration	To filter microorganisms with a filter whose pore diameter is smaller than the cell diameter of a microorganism	0.22–0.45 μm millipore filter, nucleopore filter
Radiation	Ultraviolet radiation induces polymer formation by two adjacent pyrimidines of the DNA chain blocking DNA replication; radiation produces free radicals to break the structure of biological molecules; microwaves kill microorganisms through heating	Clean bench, X-rays, microwave oven
Ultrasound	Through high-frequency vibration, a vacuum cavity is generated in solution, which causes microorganism close to the cavity to break apart due to the internal and external pressure differences	Ultrasonic processor

One is the thermal death time, which is the shortest time for killing a certain concentration of one microorganism at a certain temperature. The other is the thermal death point, which is the minimum temperature for killing a certain quantity of a microorganism in a period of time.

High-temperature sterilization can be performed in dry and moist heat. Dry heat sterilization is carried out in a special dry heat sterilizer, where high-temperature-resistant experimental utensils or other goods can be placed. The sterilization process is conducted at 150–170 °C for 1–2 h to thoroughly kill microorganisms. Burning, the most thorough and efficient form of sterilization, can be only used to sterilize utensils, inoculating needles, inoculating loops, and other wastes, as materials can be destroyed in the process. Under the same conditions, moist heat sterilization is superior to dry heat sterilization. The steam generated by moist heat sterilization process has powerful penetration and can destroy the hydrogen bonds that maintain the stability of biological macromolecules. Moist heat sterilization can be carried out at a high and constant pressure. Moist heat sterilization at atmospheric pressure can meet the lower requirements for sterilization or avoid the destruction of nutrients by high temperature and pressure. This can be performed by boiling for a while, or treating at no more than 100 °C but higher than 60 °C for a certain period of time, such as in pasteurization and tyndallization. Moist heat sterilization at high pressure is usually used for experiments or processes that require rigorous sterilization. Sterilization in the lab is usually carried out in an autoclave at 1 kg/cm^2 pressure, 121 °C for 15–20 min. Sometimes sterilization is performed at 0.7 kg/cm^2 pressure, 115 °C for 35 min to prevent the destruction of glucose and other nutrients. A fermentation plant will usually use continuous high-pressure steam to sterilize the fermentation tank and the culture medium. High-temperature sterilization is effective, but improper operation can affect the experiment or production, such as destroying nutrients and causing reactions between some groups in the medium. It is necessary to perform sterilization according to the actual situation.

Chemical sterilization is similar to physical sterilization. It uses chemical factors to inhibit or kill microorganisms. Chemical factors include chemosterilants and the chemotherapeutics. Their mechanisms of action are shown in Table 2.6 [1].

Table 2.6: Inhibition and killing of microorganisms by chemical factors.

Chemical factors		Inhibiting and killing mechanism	Examples
Chemosterilant		Break up the cell membrane and denature protein	Heavy metals, organic solvents, surfactants
Chemotherapeutic	Antimetabolite	Interrupt normal metabolism due to their similar structure to normal metabolites	Sulfonamides
	Antibiotic	Inhibit the synthesis of cell wall or biological macromolecules, degrade cell wall, or block the transmission of genetic information	Penicillin, lysostaphin, tetracycline, actinomycin D, rifampicin

Chemosterilants are often used for surface disinfection of utensils or materials. Chemotherapeutics play an important role in disease prevention and can selectively inhibit or kill pathogenic microorganisms. In addition, antibiotics are also used for screening target strains in gene engineering methods.

3.3 Microbial Cultivation

It is important to obtain pure cultures of microbes to explore their metabolism and other features. Artificial media are usually used to cultivate microbes. The growth of a microbe requires certain circumstances and nutrients, so the cultivation of microorganisms and preparation of media need to follow established principles.

3.3.1 Medium

Culture medium is a nutrient matrix that is artificial and suitable for microbial growth or the production of metabolites. Its preparation should follow certain principles. First, suitable nutrients that are not only cheap and widely available but also suitable for microbial growth should be selected. Second, the nutrients should be matched properly and evenly, such as the choice of C/N ratio, the matching of inorganic salts and growth factors, and so on. Third, the physicochemical conditions should be appropriate, meaning that the pH, redox potential, and osmotic pressure are fit for the growth of microorganisms. Fourth, the media needs to be sterilized to prevent contamination by undesirable microbes.

According to various purposes and requirements, a variety of culture media have been derived.

(1) According to the known and unknown components in the culture media, culture media can be divided into complex and chemically defined media. Complex media, such as beef extract peptone, contain uncertain chemical ingredients or inconsistent natural organics and can be used to culture microbial strains for the laboratory and fermentation industry. Chemically defined media, such as glucose-ammonium salts medium, is prepared using defined chemical components and can be used for strain identification, microbial physiology, and biochemical research and other more complex quantitative work.

(2) According to the physical state of the culture media, media can be divided into solid, semisolid, or liquid. Solid media are prepared by adding a coagulant such as agar into a liquid media base. Using solid medium to cultivate microorganisms

is known as solid-state culture and is mainly used for microbial purification, characterization, preservation, counting, and solid-state fermentation. Common solid culture methods used in laboratory include test tube slants, Petri dish plates, and Kolle flasks, which are used for culturing aerobic microorganisms, and high-level agar columns, anaerobic Petri dishes, Hungate rolling tubes, anaerobic jars, and anaerobic glove boxes that are used for cultivating anaerobic microorganisms. In practice, the koji method is the most common method for culturing aerobic microorganisms, while anaerobic microorganisms are cultured by stacking. Semisolid media are made through the addition of a small amount of coagulant (0.2–0.7% agar concentrations) to a liquid media base. These types of media are mainly used to observe the characteristics of microbial movement and measure phage titers. Liquid media can be prepared without any coagulant addition. The utilization of liquid medium to cultivate microorganisms is called liquid-state culture or submerged culture and is mainly used in large-scale production and basic research on microorganisms. Common methods of liquid-state culture for aerobic bacteria in the laboratory include test tube culture, shallow liquid culture using bottles, shake flask cultures, and benchtop fermenters. Liquid culture for anaerobic bacteria is the same as for aerobic bacteria, but needs to be performed in the absence of oxygen. In practice, surface and deep liquid ventilation cultivation are often used for culturing aerobic microbes, and deep liquid anaerobic culture is usually used for culturing anaerobic microbes.

(3) Based on the types of culture media, media can be divided into minimal, selective, and differential media. Minimal media contain basic nutrients for the growth and propagation of general microorganisms and can be used for screening for auxotrophic strains. Selective media are designed based on microbial requirements for special nutrients, or resistance to chemical or physical factors, which can be used for screening the strains with particular functions. Differential media are made by adding an indicator that can react with the colorless metabolites of the target microbe in the media to produce a color. These can be used to quickly identify and isolate target strains. However, more types of media exist besides the mentioned above. In actual operation processes, the proper medium should be prepared based on the specific needs.

3.3.2 Batch Culture

Batch culture is carried out in a closed system containing a certain quantity of nutrients and a small inocula of a microbial strain under specific conditions. During the whole cultivation process, no other substances are added or removed from the closed system. Batch culture is convenient, but results in low microbial biomass and product yield.

3.3.3 Fed-Batch Culture

Fed-batch culture is used to prolong the production time of strains or metabolites through replenishing media at some stages of a batch culture and is based on features of the strain growth and the initial culture medium. Fed-batch cultures are also called semicontinuous cultures and represent an intermediate between batch and continuous culture. This kind of culture can eliminate substrate inhibition and dilute out toxic metabolites to achieve high cell density and extend the production time of secondary metabolites.

3.3.4 Continuous Culture

Continuous culture is used to cause the microbial strain to grow continuously at the late log phase through continuous addition of fresh media at a certain speed, meanwhile discharging an equal amount of fermentation broth at the same speed. This culture not only simplifies unit operations such as feeding, sterilization, discharging, and fermenter cleaning, but can also be easily autocontrolled to obtain a stable quality of product. Its disadvantage is that the mass transfer is not homogeneous, which affects the utilization of nutrients and the cultivation of microbial strains, and long culture time can easily lead to microbial degeneration and contamination.

A good control device is essential for continuous cultivation. There are two pieces of equipment used to control continuous cultures: a chemostat and turbidostat. A chemostat is a continuous culture device that tries to keep the flow rate of liquid media constant and the growth of microorganism at a lower rate than its maximum growth rate. It is chiefly used in theoretical research related to growth rates in the laboratory. A turbidostat is a type of continuous culture setup that can obtain high density and constant growth rate of microbial cells through controlling the flow rate of the culture solution. It is mainly applied to produce many cells and/or their primary product. Microbes cultivated by traditional continuous culture are in a free state. Recently, an immobilized cell technology has been developed. This technology fixes microbial cells to the inner or surface of a special carrier by means of embedding, microencapsulation, or adsorption. Compared with free cell culture, the advantage of this technology is that it can provide higher cell density, reduce cell loss, allow the cells to be used repeatedly, and simplify downstream cell separation. However, it has a high fixed cost, is hard to control contamination, can be incrementally resistant to mass transfer, and the hereditary stability of microorganisms can be problematic due to its repeated use.

In traditional culture and fermentation, the above three methods are usually used to cultivate pure microorganisms to meet specific needs. With the development of microbial culture and fermentation engineering, mixed culture fermentation technology has recently appeared. This culture technology mixes microorganisms at certain proportions based on mutualism to achieve specific needs. In a study of ethanol production from lignocellulose, the cellulose-decomposing bacterium *Clostridium*

straminisolvens CSK1 was mixed with noncellulose-decomposing strains to decompose the lignocellulose. *C. straminisolvens* CSK1 is an anaerobic bacterium. The noncellulose-decomposing bacteria can provide good growth conditions for *C. straminisolvens* CSK1 via constructing an anaerobic environment, consuming metabolites and controlling pH.

3.4 Microbial Metabolism

In the process of growth and reproduction, microorganisms need to conduct metabolism. Metabolism is an important feature of life and mainly involves in the decomposition and synthesis of materials, as well as the production and consumption of energy. Microbial metabolism not only has general characteristics like other organisms but also features its own unique points.

3.4.1 Catabolism

Microbial catabolism is mainly related to the degradation of biological macromolecules such as saccharides, lipids, proteins, and nucleic acids that are decomposed into small molecules accompanied by energy generation. Small molecules are either further degraded into smaller substances or as intermediates to form certain substances. Energy production in this process can be stored in the form of ATP that will be used in material synthesis or other metabolic pathways.

3.4.1.1 Decomposition of Saccharides

Saccharides include monosaccharides, oligosaccharides, and polysaccharides. Monosaccharides are not hydrolyzed into smaller molecules and include glucose and xylose. Oligosaccharides can be degraded into 2–20 monosaccharides and include cellobiose and raffinose. Polysaccharides are hydrolyzed to produce more than 20 monosaccharides and include cellulose and hemicellulose. Polysaccharides include homopolysaccharides and heteropolysaccharides. Homopolysaccharides are polymers of one monosaccharide or its derivative such as starch and cellulose. Heteropolysaccharides are polymers of two or more monosaccharides or their derivatives such as hemicellulose and hyaluronic acid.

(1) Decomposition of polysaccharide
There are a wide variety of polysaccharides. The decomposition of each polysaccharide involves different enzymes. The decomposition of starch, cellulose, hemicellulose, and pectin will be introduced in the following section.

(1) Starch

During plant growth, starch is stored in cells in the form of granules. Starch granules are water insoluble and semicrystalline. Natural starch generally contains two types: amylose and amylopectin. Amylose is a linear molecule made up of glucose through α-1, 4-glycosidic bonds. Amylopectin has branches. Every 25–30 glucose units have a branch in which an α-1,6-glycosidic linkage exists. Microorganisms produce amylase to degrade starch. Amylases mainly include α-amylase, α-dextrinase, β-amylase, and isoamylase.

(2) Cellulose

Cellulose is the main component of plant cell walls and is a linear glucan connected by β-1,4-glycosidic bonds. Cellulose is decomposed by cellulases that are produced by molds, basidiomycetes, and some bacteria. Cellulases fall into two categories: cellulosomes and noncomplexed cellulases. Cellulosomes are composed of several subunits in a multienzyme complex with supermolecular structure [3]. They mainly exist in *Acetivibrio*, *Bacteroides*, *Butyrivibrio*, *Clostridium*, *Ruminococcus*, *Neocallimastix*, *Orpinomyces*, and *Piromyces*. They are basically composed of four parts: scaffoldin, cohesin–dockerin interaction, substrate-binding domain, and enzyme subunits. Scaffoldin has noncatalytic activity and contains various cohesins that bind dockerins. Cohesins and dockerins mainly have two types: I and II. Type I cohesins bind to type I dockerins that are contained in cellulosomal enzymes. Type II cohesins bind to type II dockerins that are found on cell-surface-binding proteins. The function of the substrate-binding domain is to bind substrate. There are several enzyme subunits that chiefly possess enzymatic activities, including endo- and exoglucanase, hemicellulase, chitinase, pectinase, and other methods of polysaccharide degradation. Noncomplexed cellulase is a multienzyme mixture including endoglucanase, exoglucanase, and β-glucosidase [4]. Endoglucanase randomly acts on the β-1,4-glycosidic bond of cellulose chains to produce dextrans with a low degree of polymerization. Exoglucanase hydrolyzes glucans in cellobiose units from the nonreduced end to release cellobiose and a small amount of glucose. β-Glucosidase degrades cellobiose into glucose.

(3) Hemicellulose

Hemicellulose, a component of plant cell walls, consists of a variety of heteropolysaccharides, including xylan, glucomannan, galactoglucomannan, xyloglucan, and β-1, 3-glucan [5]. Most of these polysaccharides have side chains, and their molecular size is 50–400 residues. They bind to cellulose via noncovalent bonds in the cell wall. Microorganisms such as molds, basidiomycetes, and some bacteria can generate enzymes to degrade hemicellulose into monosaccharides. When microbes use xylan as a sole carbon source, they produce β-1,4-endoxylanase, β-xylosidase, α-glucuronidase, α-L-arabinofuranose enzymes, and acetylxylan esterase resulting in the degradation of xylan.

(4) Pectin

Pectin is also a component of plant cell walls and mainly exists in the primary cell wall and intercellular middle layer. It is a heteropolysaccharide with a structure that

contains two acidic polysaccharides (polygalacturonic acid and polyrhamnogalacturonan) and three neutral polysaccharides (arabinan, galactan, and arabinogalactan) [6]. Methylesterification of the linear polygalacturonic or polygalacturonic acids at different degrees leads to pectin formation. Different sources produce different molecular weight pectins, which range from 25,000 to 50,000 Da. Pectin also can be hydrolyzed into small molecular substances by microorganism-produced enzymes. Enzymes such as polymethylgalacturonase, endopolygalacturonase, pectin depolymerase, pectinase, and exopolygalacturonase can degrade the polygalacturonic acid chain, while α-L-rhamnosidase can hydrolyze the rhamnogalacturonic acid in the branch chain, and α-L-arabinofuranosidase and endoarabanase can degrade the side chain of L-arabinose and araban.

(2) Decomposition of monosaccharides

Most substances in nature are in the form of polysaccharides or other carbohydrates that must be degraded to monosaccharides or other smaller molecules for assimilation by microorganisms. Decomposition of monosaccharides not only provides energy for microbial growth and metabolism but also provides precursors for the synthesis of other substances. Glucose catabolism has been well studied. In the process of microbial glucose catabolism, the pathways of glucose decomposition include the Embden–Meyerhof–Parnas (EMP) pathway, hexose monophosphate (HMP) pathway, Entner–Doudoroff (ED) pathway, phosphoketolase pathway, and tricarboxylic acid (TCA) cycle [7]. The first four pathways are summarized here and will be described in the following chapters. The TCA cycle is described in detail here.

(1) EMP pathway, also known as glucolysis, is a series of reactions where glucose is converted to pyruvic acid in the absence of oxygen. This process produces two ATP and two NADH + H^+. The EMP pathway is a common method of glucose catabolism and its main physiological function is to provide intermediate products for other metabolic pathways.

(2) HMP pathway, also known as the pentose phosphate pathway, is another mechanism for glucose decomposition. Glucose is first catalyzed into glucose-6-phosphate by glucokinase, following which oxidation and a series of reactions occur, and the glucose is decomposed into pentose and CO_2, producing inorganic phosphoric acid and nicotinamide adenine dinucleotide phosphate (NADPH). The physiological significance of this process lies in providing reducing power (NADPH) and different sugar molecules with different structures.

(3) ED pathway, also known as 2-keto-3-deoxy-6-phosphogluconic acid (KDPG) pathway, is unique to a minority of bacteria that have an incomplete EMP pathway. After four sequential reactions, glucose is degraded to pyruvic acid. The key enzyme in this pathway is KDPG aldolase that transforms KDPG into pyruvic acid and

glyceraldehyde 3-phosphate. Its main physiological significance is to replace the EMP pathway and make up for the lack of intermediate products.

(4) The phosphoketolase pathway has two ways due to different phosphoketolases. One is phosphopentose ketolysis called the pentose phosphoketolase (PK) pathway, which converts pentose into lactic acid and ethanol. The other is phosphohexose ketolysis called the hexose phosphoketolase (HK) pathway, which degrades glucose into lactic acid and acetic acid.

(5) The TCA cycle is also known as the citric acid cycle. Using pyruvate dehydrogenase, the carboxyl group of the pyruvic acid from glycolysis or other metabolic pathways is first removed to produce CO_2 and acetyl coenzyme A, which enters the TCA cycle and is further degraded to yield reducing power and CO_2 through catalysis by various enzymes. The TCA cycle is shown in Figure 2.4. The TCA cycle is carried out in mitochondria of eukaryotes, and on the cell membrane of prokaryotes. The reducing power generated in the cycle participates in the oxidative phosphorylation pathway to

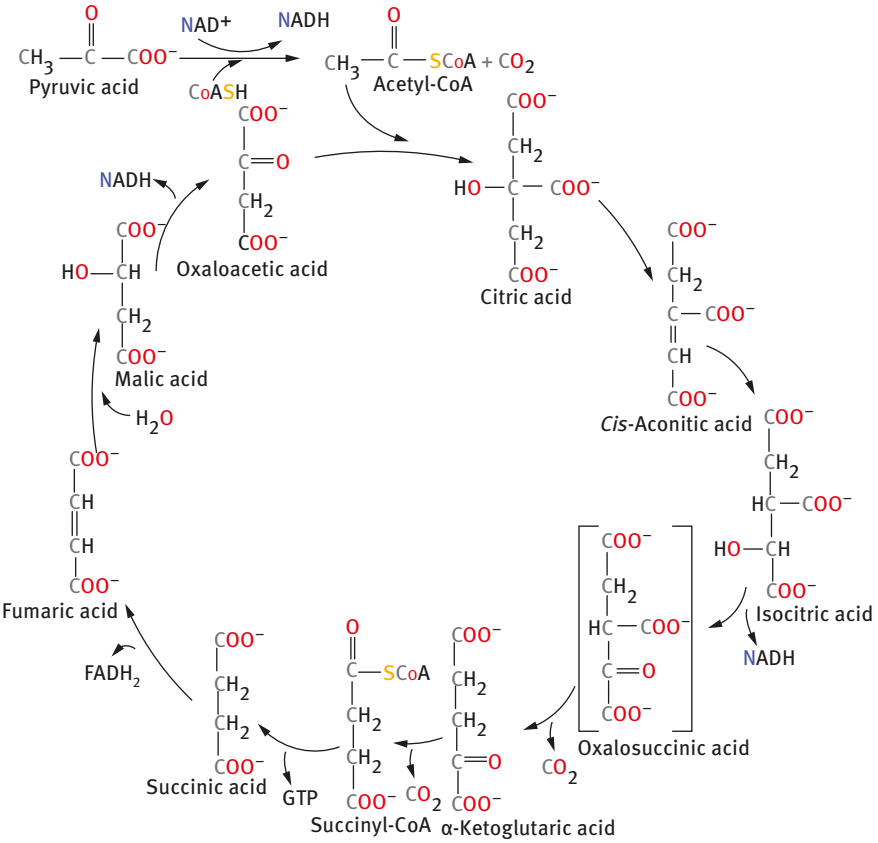


Figure 2.4: TCA cycle.

produce energy. The energy required for the metabolism of an organism predominantly comes from the TCA cycle. TCA cycle is not only the pathway for pyruvate oxidation, but also a common pathway for the oxidation of various molecules like fatty acids, amino acids, and so on. It not only provides the energy needed for life but also supplies precursors for biosynthesis.

3.4.1.2 Lipolysis

Many kinds of microorganisms can use fats as carbon and energy sources such as the bacteria *Pseudomonas fluorescens*, actinomycetes, bacterium prodigiosum, and some species of *Mycobacterium*, and fungi, such as *Geotrichum*, *Penicillium*, *Aspergillus*, and *Fusarium*.

A fat molecule is first split into glycerol and fatty acid by the action of lipases produced in these microorganisms. Some enzymes can convert glycerol to other intermediates that are involved in the EMP or other pathways, such as dihydroxyacetone phosphate. Saturated fatty acids are degraded by β-oxidation (Figure 2.5). The degradation of unsaturated fatty acids requires more enzymes, but is similar to that of saturated fatty acids. The final product of lipolysis is acetyl coenzyme A or propionyl coenzyme A, which are significant intermediates that take part in several metabolic pathways.

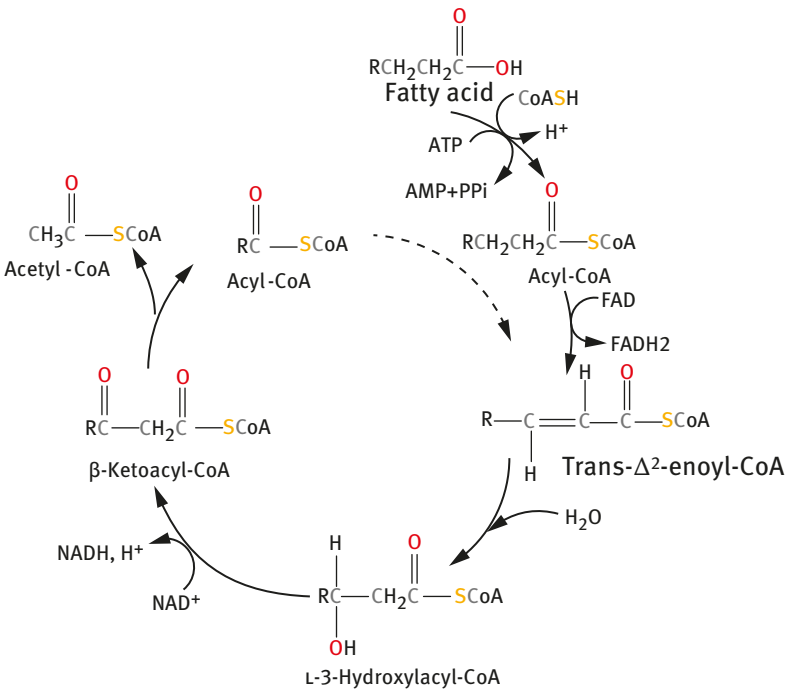


Figure 2.5: β-Oxidation pathway of fatty acid.

3.4.1.3 Decomposition of Organic Nitrogen Compounds

Organic nitrogen compounds include proteins, amino acids, nucleic acids, and bases, and offer a source of nitrogen and growth factors for microbial growth. Proteins and nucleic acids cannot be directly utilized by microorganisms. They need to be degraded into smaller molecules such as amino acids and bases, which can be assimilated by microorganisms. Herein, the catabolism of amino acids and bases will be chiefly elaborated.

(1) Amino acids

There are two basic methods for the microbial decomposition of amino acids: deamination and decarboxylation.

(1) The deamination of amino acids can proceed by three pathways: transamination, oxidative deamination, and transdeamination.

Transamination (Figure 2.6a) is a process where the amino group of an amino acid is transferred to α-keto acid by an aminotransferase to generate the corresponding α-keto acid and a new amino acid.

Oxidative deamination (Figure 2.6b) involves the removal of an amino group from an amino acid by an amino acid dehydrogenase or oxidase to produce the corresponding α-keto acid. In this reaction, the dehydrogenase requires $NAD(P)^+$ as a coenzyme, and the oxidase needs flavin adenine dinucleotide (FAD) as a coenzyme.

Transdeamination (Figure 2.6c) has two further pathways. One is the transfer of the α-amino group of an amino acid to α-ketoglutarate by transamination to form the corresponding α-keto acid and glutamic acid, whose amino group is subsequently removed by glutamate dehydrogenase to generate α-ketoglutarate and release ammonia. The other is a reaction between hypoxanthine nucleotide and aspartic acid that generates an intermediate product – adenylosuccinate – that is cleaved into adenine nucleotide and fumaric acid by a lyase. The adenine nucleotide can be hydrolyzed into adenylosuccinate and ammonia.

Stickland [8] found that deamination exists in a few species of anaerobic *Clostridium*. One amino acid is taken as a hydrogen donor through oxidative deamination, and another amino acid is taken as a hydrogen acceptor to receive the hydrogen from the donor and to remove ammonia via the reductive reaction. As a result, both amino groups are removed from both amino acids. This process is named the Stickland reaction (Figure 2.6d). However, this reaction can be carried out between limited two animo acids. Through the above deamination, products like ketonic acid will enter the TCA cycle through a variety of ways and be further degraded to CO_2.

(2) The decarboxylation of amino acids is conducted with the action of a specific decarboxylase. Generally, one decarboxylase which only works with L-amino acids can act on a corresponding amino acid. Amino acid decarboxylases, except histidine decarboxylase, need pyridoxal phosphate as a coenzyme. The decarboxylation of different amino acids

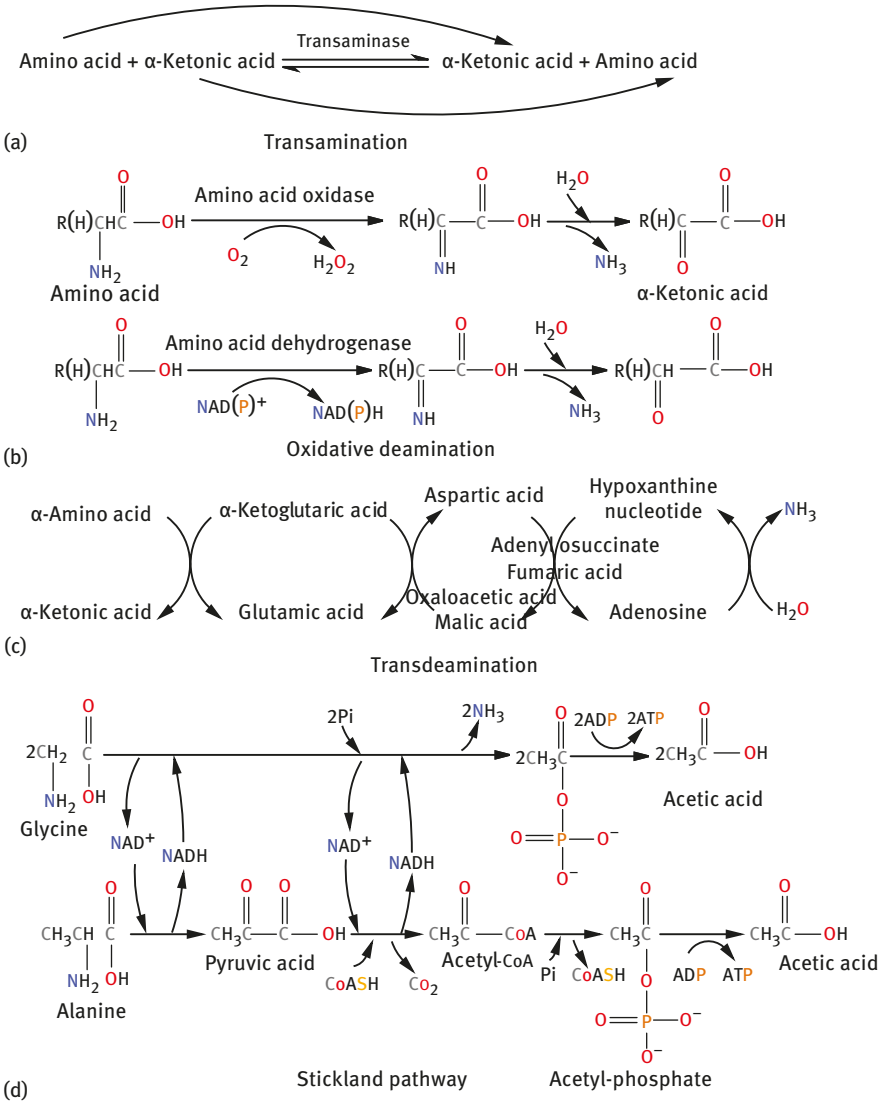


Figure 2.6: Deamination pathways of amino acids.

can produce different amines. After decarboxylation, the amino acid containing one amino group will generate a primary amine, and an amino acid containing two amino groups will form a secondary amine. At the same time, CO_2 is also released.

(2) Bases

Bases include purines and pyrimidines. The decomposition of purines by most microbes is the same. Only a few microorganisms are slightly different. In *Clostridium* species, xanthine is finally converted to glycine and formic acid via the conversion of

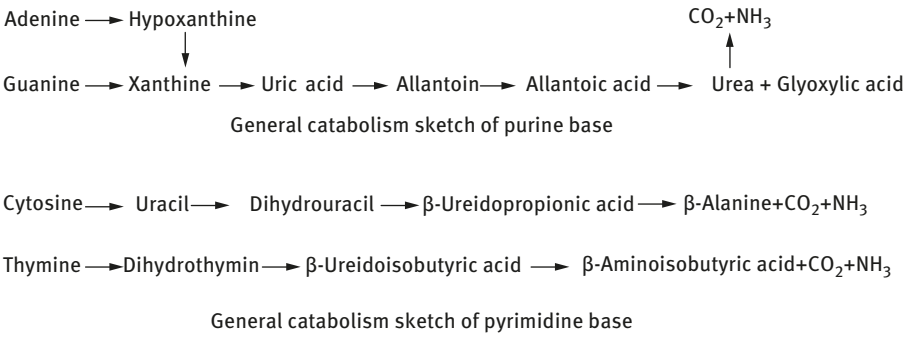


Figure 2.7: General catabolism pathways for bases.

a series of imidazole derivatives. Aerobic and anaerobic microorganisms have different decomposition ways for pyrimidines. Aerobic microorganisms, like *Corynebacterium*, can oxidize pyrimidines to urea and malonic acid because of the presence of an oxidase. Anaerobic microorganisms, such as *Clostridium*, can restore uracil to dihydrouracil by dehydrogenases, and finally degrade uracil into NH_3, CO_2, and β-alanine acid. The general decomposition approaches of purines and pyrimidines are shown in Figure 2.7.

3.4.1.4 Energy-Producing Reactions

The decomposition of biological macromolecules is often accompanied by energy production. This energy is stored in the form of ATP to be used for biosynthesis. Different microorganisms have different ways to produce energy. Energy production has two main pathways: biological oxidation and photosynthesis.

(1) Biological oxidation

Biological oxidation is a process where a substance is gradually degraded through a series of oxidation–reduction reactions in a microbial cell accompanying with energy production. Different trophic types of microorganisms have different types of biological oxidation.

(1) Biological oxidation by chemoheterotrophic microorganisms

Heterotrophic microorganisms must rely on exogenous nutrients to carry out normal physiological functions. According to their different electron acceptors, biological oxidation processes can be divided into fermentation and respiration, including aerobic and anaerobic respiration.

Fermentation is a process where microbes directly deliver the hydrogen resulting from the oxidation of substrates to an endogenous intermediate with energy release, without the use of a hydrogen acceptor. Hydrogen generated from the above-mentioned metabolic pathways, such as EMP and HMP, can be transferred to different

intermediate metabolites to produce different fermentation products, and at the same time it is accompanied by substrate-level phosphorylation to produce ATP.

Respiration is a process where electrons generated by the decomposition of various substances by microbes is transferred by electron carriers, such as $NAD(P)^+$, FAD, and flavin mononucleotide (FMN), to enter the electron transfer chain and is then transmitted to external electron acceptors to produce different products with the release of energy. When the exogenous electron acceptor is oxygen, the process is called aerobic respiration, while the exogenous electron acceptor is another oxidate, the process is known as anaerobic respiration.

During aerobic respiration, the hydrogen removed from a substrate will eventually be accepted by oxygen accompanied by energy production through the complete respiratory electron transport chain. This process is also known as oxidative phosphorylation (Figure 2.8). The respiratory chain consists of a series of carriers that have gradient redox potential and arrange in a chain located in the prokaryotic cell membrane or eukaryotic mitochondrial membrane.

In the process of anaerobic respiration, hydrogen generated from substrates will be finally accepted by oxidative inorganic substances such as NO_3^- and SO_4^{2-} or organic matter, such as fumaric acid and glycine, through a partial respiratory electron transport chain. This process is accompanied by phosphorylation to release energy. The energy resulting from anaerobic respiration is less than that from aerobic respiration due to the transmission of a part of the energy to other intermediate products as the final electron acceptor.

(2) Biological oxidation by chemoautotrophic microorganisms
Chemoautotrophic microbes can obtain energy through the oxidation of inorganic materials. Their main types and energy production mechanisms are shown in Table 2.7.

(2) Photosynthesis

Besides plants, photosynthesis can also be carried out by some microorganisms such as algae, cyanobacteria, purple bacteria, green bacteria, and halophilic bacteria. Energy production by algae and cyanobacteria is through noncyclic photophosphorylation,

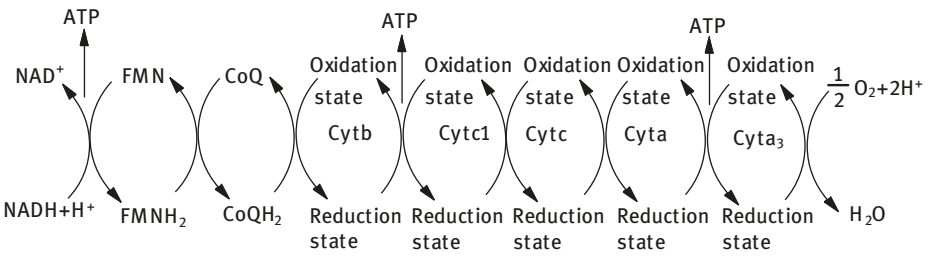


Figure 2.8: Schematic diagram of oxidative phosphorylation.

Table 2.7: Bio-oxidation types of autotrophic microorganisms.

Substrates	Energy-producing mechanism	Examples
Hydrogen	Electrons transferred from hydrogen to the respiratory chain. ATP is produced in the respiratory chain, while hydrogen is oxidized to water. This process requires the hydrogenase and NAD^+.	*Hydrogenomonas*
Ammonia	Ammonia is catalyzed by a hydroxylase and cytochrome P460 in nitrosobacteria to produce hydroxylamine, which will be subsequently catalyzed by a hydroxylamine-cytochrome c reductase and the terminal pigment system to generate nitrite. In this process, hydroxylamine releases two electrons into the electron transport chain, which are transferred to oxygen to produce one ATP. Nitrite is oxidized to nitrate by the action of nitrobacteria. During this process, the generated electrons are also transferred to oxygen to generate one ATP via the electron transport chain.	*Nitrosomonas*, *Nitrobacter*
Sulfur	Utilized by microbes in the form of S^{2-}, S, and thiosulfate. Using thiosulfate as an example, it is first decomposed by enzymes into sulfur and sulfite. Sulfur is oxidized by sulfide oxidase and quinone-cytochrome oxidation system into sulfite. In this process, electrons are generated, which couple with oxidative phosphorylation to generate four ATPs.	*Thiobacillus*, *Thiobacillus thioparus*, *Thiobacillus thiooxidans*
Iron	An electron is released from Fe^{2+} by an oxidase, transferred to a copper protein and cytochrome c, and further transmitted to another cytochrome c and the terminal oxidase of cytochrome a. ATP is produced in the process.	*Ferrobacillus ferrooxidans*, *Thiobacillus ferrooxidans*

while that of purple bacteria and green bacteria is via cyclic photophosphorylation. These two photophosphorylation processes are described in detail in the following chapters. Halophilic bacteria carry out photophosphorylation through BR of the plasma membrane. The reaction center is BR, which uses retinaldehyde as a cofactor. Under illumination, the all-*trans* configuration of BR absorbs a photon to be isomerized into 1,3-*cis*-BR whose recovery is accompanied with proton transport across the membrane, resulting in a proton potential around the membrane. Eventually, ATP is synthesized by the action of ATP synthase.

3.4.2 Anabolism

In the process of growth and reproduction, microorganisms need to synthesize macromolecular substances such as carbohydrates, proteins, and nucleic acids to continue living. The premise for macromolecular synthesis is the supplement of nutrients

from the environment. Macromolecular synthesis is an energy-intensive process and because of the wide variety of microorganisms, anabolic pathways are also numerous and diverse. Some representative metabolic pathways will be introduced briefly here.

3.4.2.1 CO_2 Fixation

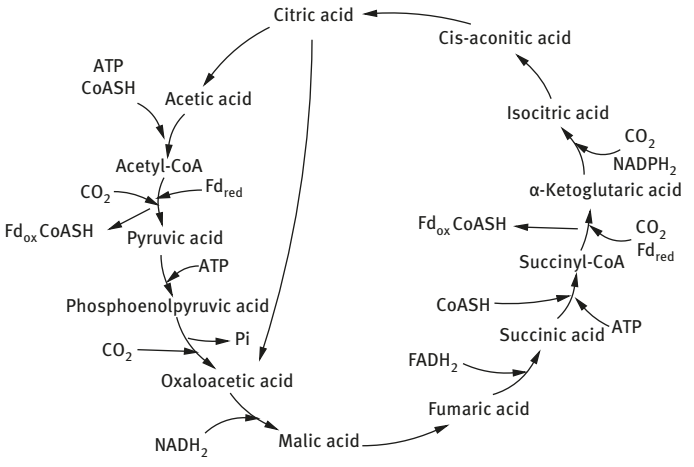
In addition to fixing CO_2 by autotrophic microorganisms, heterotrophic microorganisms can also use CO_2 as a supplementary carbon source.

(1) CO_2 fixation by autotrophic microorganisms

There are three pathways for autotrophic microorganisms to fix CO_2: Calvin cycle, reductive TCA cycle, and reductive monocarboxylic acid cycle. The Calvin cycle will be described in detail in the following chapter. The two latter CO_2 fixing mechanisms are introduced here.

(1) Reductive TCA cycle

This pathway was found in some photosynthetic bacteria such as *Chlorobium thiosulphatophilum*. It is quite similar to the reverse TCA cycle [9]. With the participation of ferredoxin, acetyl-CoA is used to fix one CO_2 molecule to form pyruvic acid, catalyzed by pyruvate synthase. This will subsequently fix one CO_2 molecule through the action of related enzymes to produce oxaloacetic acid, which enter into a similar reverse TCA cycle. The reductive carboxylation of succinyl-CoA to α-ketoglutaric acid by α-ketoglutarate synthase is vital to this process. This reductive carboxylation also requires the involvement of ferredoxin. The whole process is shown in Figure 2.9.



Fd_{red}: Reductive ferredoxin, Fd_{ox}: Oxidative ferredoxin

Figure 2.9: Reductive TCA cycle of *Chlorobium thiosulphatophilum* to fix CO_2.

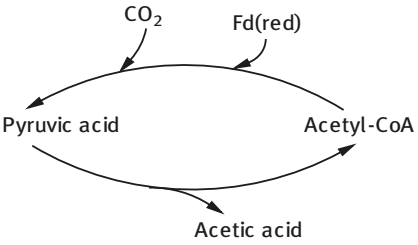


Figure 2.10: Reductive monocarboxylic acid cycle.

(2) Reductive monocarboxylic acid cycle
Some photosynthetic bacteria, like *Clostridium kluyveri*, can directly convert CO_2 into acetic acid. ATP is not necessary for the entire cycle, and only ferredoxin is required for this cycle as shown in Figure 2.10 [10].

(2) CO_2 fixation by heterotrophic microorganisms
As for heterotrophic microorganisms, CO_2 is fixed primarily by organic acids through the action of several related enzymes. The products are mainly intermediates in the TCA cycle. Some major reactions are shown in the following equations. In addition, CO_2 fixation can also occur in the synthesis of fatty acids and nucleotides:

$$\text{Phosphoenolpyruvic acid} + CO_2 \xrightarrow{\text{Phosphoenolpyruvate carboxylase}} \text{Oxaloacetic acid} + H_3PO_4$$

$$\text{Phosphoenolpyruvic acid} + CO_2 + \text{ADP} \xrightarrow{\text{Phosphoenolpyruvate carboxykinase}} \text{Oxaloacetic acid} + \text{ATP}$$

$$\text{Pyruvic acid} + CO_2 + \text{ATP} \underset{}{\overset{\text{Pyruvate carboxulase}}{\rightleftharpoons}} \text{Oxaloacetic acid} + \text{ADP} + H_3PO_4$$

$$\text{Pyruvic acid} + CO_2 + NAD(P)H_2 \overset{\text{Malic enzyme}}{\rightleftharpoons} \text{Malic acid} + NAD(P)$$

$$\alpha\text{-Ketoglutaric acid} + CO_2 + NAD(P)H_2 \overset{\text{Isocitrate dehydrogenase}}{\rightleftharpoons} \text{Isocitric acid} + NAD(P)$$

3.4.2.2 Assimilation of C2 Compounds

C2 compounds are assimilated mainly in two ways: the glyoxylate cycle and glycerate pathway.

(1) Glyoxylate cycle
The glyoxylate cycle can be regarded as a branch of the TCA cycle (Figure 2.11). Exogenous acetyl-CoA enters the TCA cycle to generate isocitric acid, which is split into succinic acid and glyoxylic acid by isocitrate lyase. Catalyzed by malate synthase, glyoxylic acid reacts with another exogenous acetyl-CoA to form malic acid, and thus oxaloacetate is produced. The oxaloacetate will further assimilate foreign acetyl-CoA. Through this

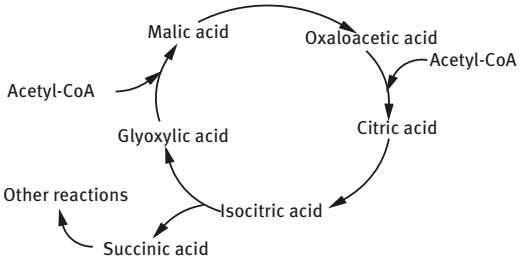


Figure 2.11: Glyoxylate cycle.

cycle, four-carbon dicarboxylic acids can be accumulated and used as the raw materials for other metabolic pathways.

(2) Glycerate pathway

Other C2 compounds such as glycine, glycolic acid, and oxalic acid can be assimilated through the glycerate pathway (Figure 2.12). These are first transformed into glyoxylic acid and then two glyoxylic acids are joined by glyoxylic acid ligase to synthesize a hydroxypropionic acid semialdehyde, which is subsequently transformed into glyceric acid by a reductase. After being phosphorylated, glyceric acid participates in the EMP pathway to produce phosphoenolpyruvate and pyruvic acid, which accepts CO_2 to form four-carbon dicarboxylic acid and enters the TCA cycle.

3.4.2.3 Synthesis of Saccharides

In addition to decomposing saccharides into smaller molecules, microorganisms can also utilize small molecules to synthesize carbohydrates for the establishment of their own cellular structures and growth. The carbohydrates in the microorganism mainly exist in the form of polysaccharides or polymers and are seldom free monosaccharides.

(1) Monosaccharide synthesis

Microorganisms synthesize monosaccharides through glucose-6-phosphate synthesis as part of gluconeogenesis. Glucose-6-phosphate can be converted into other monosaccharides. Gluconeogenesis is a process (Figure 2.13) that synthesizes glucose from

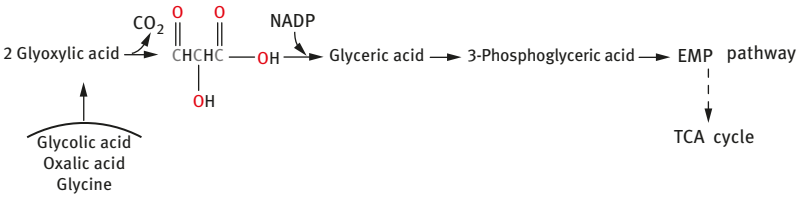


Figure 2.12: Glycerate pathway.

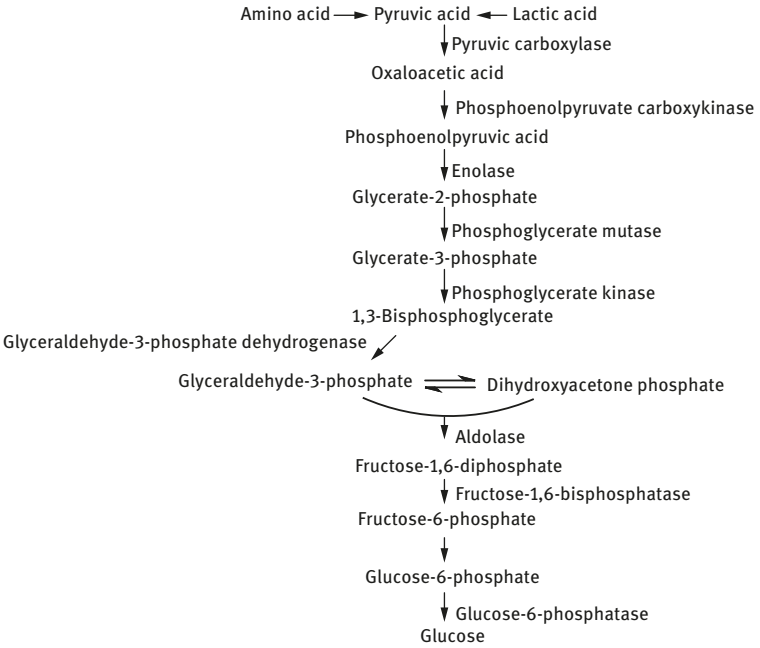


Figure 2.13: Gluconeogenesis.

nonsugar precursors, including pyruvic acid, amino acids, lactic acid, propanoic acid, and glycerol. Diphosphate nucleotides play an important role in the conversion process of glucose-6-phosphate to other monosaccharides. Furthermore, it is also important to provide glycosyl for polysaccharide synthesis.

(2) Polysaccharide synthesis

Both homopolysaccharide and heteropolysaccharide synthesis have common features. The arrangement of subunits in poly chains is determined by the specificity of the transferase. At the initial stage of synthesis, a small section of the polysaccharide chain is required as a receptor. During the synthetic process, catalyzed by corresponding enzymes, the polysaccharide chain is gradually elongated with glycosyl provided by sugar nucleotides as carriers.

3.4.2.4 Lipid Synthesis

According to their chemical composition, lipids can be divided into three categories: simple lipids, compound lipids, and derived lipids. Simple lipids are synthesized from fatty acids and glycerol, including triglycerides and waxes. Compound lipids are composed of fatty acids, alcohol, and other nonlipid components. According to the composition of the nonlipid component, the compound can be classified as a phospholipid

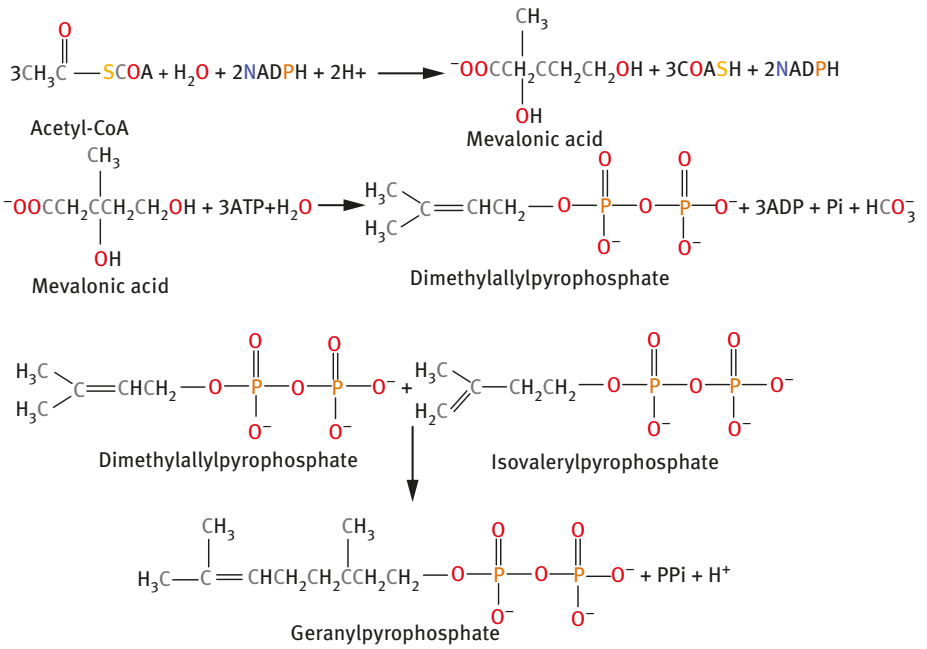


Figure 2.14: Common synthetic reactions of sterol and terpenoid.

or glycolipid. Derived lipids are derived from simple lipids or compound lipids or are closely related to simple lipids and compound lipids, and have general properties of lipids, including hydrocarbons, sterols, terpenes, and vitamins. Before the synthesis of simple and compound lipids, fatty acids are synthesized first, and then fatty acids react with glycerol or other compounds to generate the corresponding lipids through the action of related enzymes. The synthesis of fatty acids is described in detail in the following chapter. Some derived lipids are transformed by structural changes to simple or compound lipids or by the addition of other substances to simple or compound lipids. Some derived lipids, such as steroids and terpenoids, are synthesized from small molecules like acetyl-CoA. The chief intermediate synthetics are mevalonic acid and geranyl pyrophosphate that react with different materials to generate steroidogenic or terpene compounds. Their simplified synthesis pathways are shown in Figure 2.14.

3.4.2.5 Amino Acid Synthesis

Amino acids do not only provide a nitrogen source for microbial growth but also offer raw materials for the synthesis of proteins and enzymes. There are more than 180 types of amino acids found in various organisms, but only 20 basic amino acids are involved in protein synthesis. Based on the different chemical structures of their R groups, these

20 amino acids can be divided into aliphatic, aromatic, and heterocyclic amino acids. There are 15 aliphatic amino acids, while the aromatic amino acids include phenylalanine, tyrosine, and tryptophan; and the heterocyclic amino acids are histidine and proline. Their synthetic pathways are different due to their different structures.

(1) Synthesis of aliphatic amino acids

According to the initial characteristics of their substrates and intermediates, the synthesis of aliphatic amino acids can be divided into four categories: glutamate family synthesis, aspartate family synthesis, pyruvate family synthesis, and serine family synthesis.

The synthesis of glutamate-like molecules requires α-ketoglutaric acid as the substrate for the formation of glutamic acid, which is then converted to glutamine and arginine through catalysis by different enzymes. The synthesis of aspartate family amino acids is through oxaloacetic acid, which accepts an amino group transferred from glutamic acid to generate aspartic acid that is then transformed by different enzymes into asparagine, methionine, and threonine. The synthesis of pyruvate family amino acids is started from pyruvic acid to produce alanine, valine, and leucine via different reactions. Serine family amino acids are synthesized from glycerol acid-3-phosphate to first form serine, which is subsequently converted to glycine and cysteine. In addition, lysine can be synthesized by both synthetic pathways of the glutamic acid and aspartate family. The synthesis of isoleucine requires raw materials from the aspartate family and pyruvic acid family. The general pathways in which the aliphatic amino acids are synthesized are shown in Figure 2.15.

(2) Synthesis of aromatic amino acids

There are three types of aromatic amino acids. The starting materials for their synthesis are phosphoenolpyruvate and erythrose-4-phosphate. Through a series of reactions, these two substances can form chorismic acid, which is subsequently transformed into L-phenylalanine, L-tyrosine, and L-tryptophan via different pathways (Figure 2.15).

(3) Synthesis of heterocyclic amino acids

The synthesis of the two amino acids in the heterocyclic amino acids is completely different. Proline synthesis is similar to the synthesis of glutamic acid family amino acids (Figure 2.15). However, the synthesis of histidine is more complex than that of proline, and its synthesis starts from the reaction of 5′-phosphoribosyl-1′-pyrophosphate with ATP. After several reaction steps, L-histidine is formed.

3.4.2.6 Nucleotide Synthesis

Nucleotides including ribonucleotides (RNAs) and deoxyribonucleotides (DNAs) are composed of a base, pentose, and phosphatic acid. RNAs and DNAs have the same chemical structures, but different pentoses. According to the different bases,

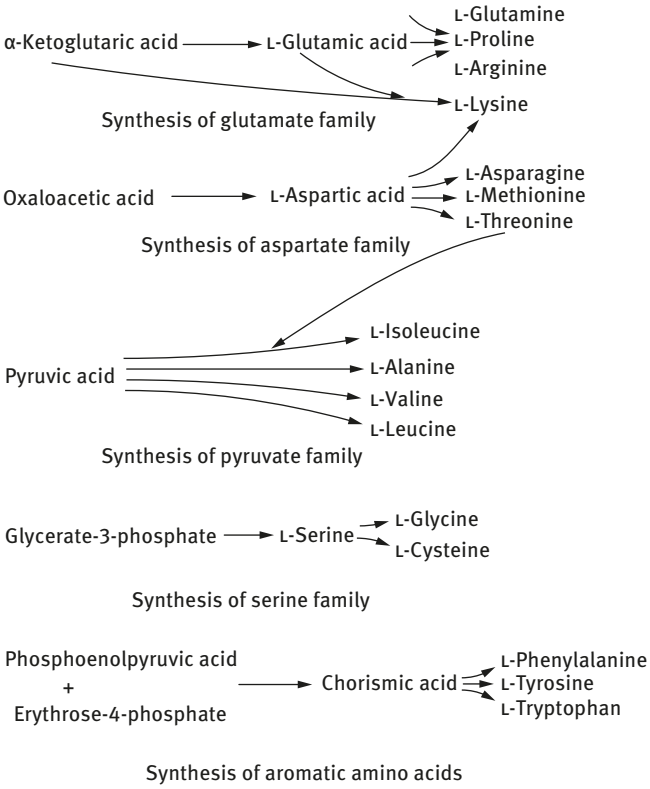
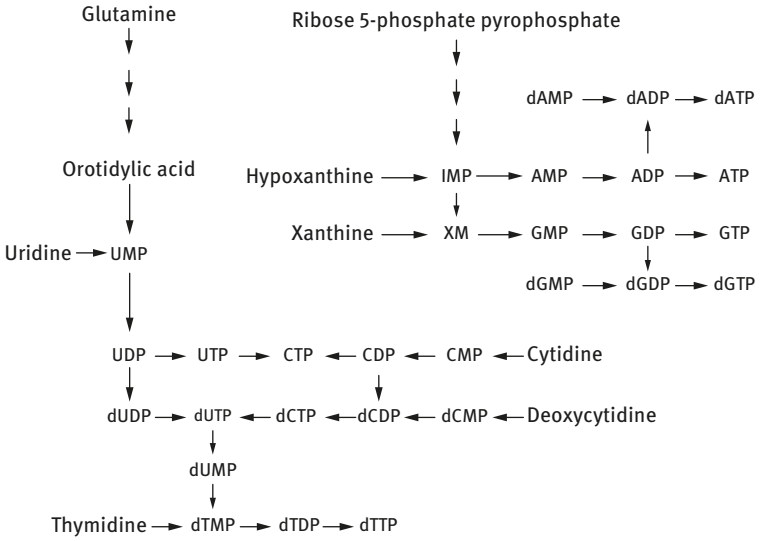


Figure 2.15: Synthetic pathways of different amino acids.

nucleotides/deoxynucleotides can be classified into purine and pyrimidine nucleotides/deoxynucleotides. The purine nucleotides/deoxynucleotides include guanine and adenine nucleotides/deoxynucleotides, and the pyrimidine nucleotides/deoxynucleotides include cytosine, thymine, and uracil nucleotides/deoxynucleotides. RNAs and DNAs can not only be synthesized from simple precursor substances, but can also be imported as preformed bases and nucleosides.

The synthesis of purine RNA starts from 5-phosphoribosyl pyrophosphate. After a series of enzymatic reactions, a hypoxanthine nucleotide is formed and subsequently transformed into adenine and guanine nucleotides (Figure 2.16). Pyrimidine RNA synthesis is different from that of purine nucleotides. The pyrimidine ring is first generated and then binds to ribose phosphate to produce orotidylic acid, which is converted into uracil RNA. Other pyrimidine nucleotides are formed from uracil RNA. Catalyzed by a related reductase, RNAs can be converted into their corresponding DNAs. When the bases and nucleosides are present in the environment, microbes will use their specific nucleoside phosphorylases to catalyze the reaction of a base with 1-ribose phosphate to generate a nucleoside that can be phosphorylated by the appropriate phosphate kinase to form a nucleotide.



AMP: adenosine monophosphate, ADP: adenosine diphosphate, ATP: adenosine triphosphate, dAMP: deoxyadenosine monophosphate, dADP: deoxyadenosine diphosphate, dATP: deoxyadenosine triphosphate, G: guanine, C: cytosine, T: thymine, U: uracil

Figure 2.16: Synthesis of nucleotides.

3.4.3 Secondary Metabolism

Relative to secondary metabolism, primary metabolism is a process through which microorganisms take advantage of a variety of exogenous nutrients to generate substances and energy for maintaining life through catabolism and anabolism. The substances generated by primary metabolism are called primary metabolites. Primary metabolism provides the substances necessary for life in microorganisms, while secondary metabolism is not necessary. Even if secondary metabolism is blocked, the growth and reproduction of microorganisms will not be affected. Secondary metabolism is a process where, after growing to stationary phase, microorganisms use primary metabolites to synthesize chemicals with varied and complex structures. The chemicals produced in secondary metabolism are known as secondary metabolites and have no clear function for microbial life, but can often play a role in other aspects, such as antibiotics, vitamins, and pigments.

Many antibiotics provide examples of the activity of secondary metabolites in other aspects [11]. Antibiotics are substances that can inhibit organisms or even kill them at low concentrations. Since they were discovered, they have been widely used in medicine. A majority of antibiotics are inhibitory to microorganisms, while a minority of antibiotics can kill or lyse microbes. The antibiotics act on microbial physiology to inhibit their growth and reproduction, such as by inhibiting the synthesis of nucleic acids and proteins, changing the cell permeability, interfering

with formation of the cell wall, acting on energy generation systems, or serving as antimetabolites. Antibiotics are not able to influence all microorganisms. Different antibiotics can inhibit different microorganisms, meaning that the inhibition of antibiotics on microorganisms is selective. Penicillin is an example of selective antibiotic as it generally only has an effect on G^+. Overuse of antibiotics can make microbes resistant to the antibiotics. Microbial resistance to antibiotics mainly arises from three methods: producing enzymes to disable antibiotics, changing the sensitive site of an enzyme to an antibiotic, and reducing the cell permeability of antibiotics. Therefore, in the future, it is necessary to take appropriate measures to avoid the generation of antimicrobial resistance when using antibiotics.

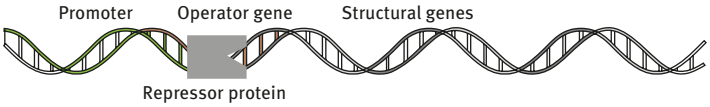
3.4.4 Metabolic Regulation

The metabolic regulation is achieved by the regulation of enzymes in two ways: the regulation of enzymatic quantity and activity.

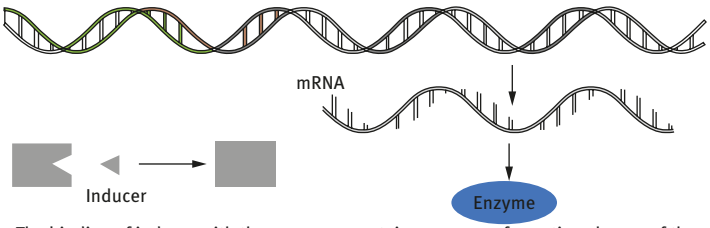
3.4.4.1 Enzymatic Quantity Regulation

When a substance present in the environment is excessive, a microorganism will start or shut down the synthesis of the corresponding enzymes to degrade the substance or stop the synthesis of the substance. With the participation of a repressor protein produced from a regulatory gene, the induction and repression of enzymatic synthesis are carried out through manipulation of operator genes that control the transcription of structural genes. This regulation is known as the operon model [7]. Operons are structural and functional synergistic units that are formed by related functional and structural genes with the same regulatory genes and promoter. When the repressor protein binds to an inducer, it dissociates from the operator gene, and thus enzymatic synthesis is induced. When the inert repressor protein binds to a repressor, it is activated and combines with the operator gene, and thus the synthesis of the enzyme is repressed. A schematic diagram of this regulation is shown in Figure 2.17.

The *lac* operon contains a promoter, operator gene, and three structural genes and is a system for inducing enzymatic synthesis. When lactose is absent in the environment, the repressor protein binds to the operator to keep RNA polymerase away from the promoter region, resulting in an inhibition of transcription of the structural genes. When lactose is present in the environment, lactose is transformed to allolactose due to the catalysis of the low-level expression of β-galactosidase in *E. coli*. The binding of allolactose to the repressor protein causes a conformational change in the repressor protein, which makes the repressor protein unable to recognize the operator gene and combine with it. When this occurs, RNA polymerase binds to the promoter region to transcribe the structural genes. A polycistronic mRNA is produced and guides the translation of β-galactosidase, a permease, and acetyl transferase.

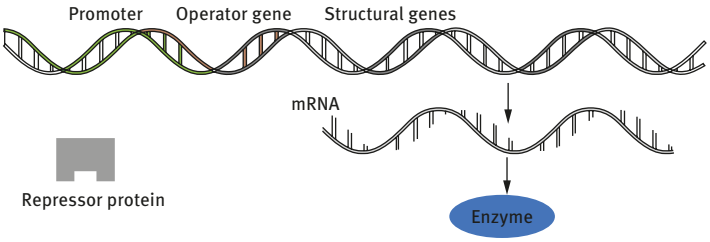


The binding of repressor protein with the operator gene keeps RNA polymerase away from the promoter region to result in unable transcription of the structural genes.



The binding of inducer with the repressor protein causes conformation change of the repressor protein, which makes the repressor protein fall off the operator gene. At this moment, the RNA polymerase binds to the promoter region to transcript structural genes. The polycistron mRNA is produced and guides the translation of the enzymatic protein.

Induction of enzymatic synthesis



The repressor protein cannot bind to the operator gene, which makes RNA polymerase go through the operator gene and transcribe mRNA. Enzymatic protein can be produced.

The binding of metabolite to repressor protein makes its conformational change, which can bind to operator gene and block the transcript of mRNA. Finally, the synthesis of enzyme is inhibited.

Repression of enzymatic synthesis

Figure 2.17: Regulation of enzyme quantity.

The *trp* operon contains five structural genes and is a repressible enzyme system. When the tryptophan is present in excess in the environment, it binds to the free repressor protein and results in a conformational change that makes the repressor protein identify and bind to the operator gene to block transcription of the structural genes by RNA polymerase. When the tryptophan is absent in the environment, the repressor protein cannot bind to operator gene, and RNA polymerase can bind to the promoter region and transcribe the structural genes to generate five enzymes. In addition, attenuation of the *trp* operon can also be seen. Its regulation occurs at a stretch of DNA in the operon's leader region, which is similar to the terminator. This DNA sequence can code for a small length of a leading peptide containing 14 tryptophans.

3.4.4.2 Enzymatic Activity Regulation

Conformational and structural changes can significantly alter the activity of an enzyme. There are many factors affecting the conformation and structure of an enzyme, such as the features and concentrations of substrate and product, temperature, pH, ionic strength, pressure, and osmotic pressure. Under the influence of these factors, enzymatic activity can be activated or inhibited.

(1) Activation

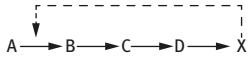
Activation is a phenomenon where some enzymes that originally had no or low activity become active or obtain higher activity through the action of substances known as activators. The activator is mostly an inorganic ion or simple organic compound. Metal ions that can be activators include Na^+, K^+, Mg^{2+}, Fe^{2+}, Zn^{2+}, Mn^{2+}, Ca^{2+}, and Co^{2+}, and the anions are Cl^-, Br^-, I^-, CN^-, and PO_4^{3-}. Simple organic compounds that can act as activators include cysteine and glutathione. In addition, some proteases that can activate zymogens can also be used as activators. The action of an activator on an enzyme is selective, such that one activator can activate one enzyme, but inhibit another enzyme. The activation of an enzyme requires the appropriate concentration of activator. If the activator concentration exceeds the optimum level it can inhibit the enzyme. As an example, $(5–10) \times 10^{-3}$ mol/L of Mg^{2+} can activate $NADP^+$ synthase, while 30×10^{-3} mol/L of Mg^{2+} inhibits the activity of the enzyme.

(2) Inhibition

Inhibition is the opposite of activation and can result in a decrease or loss of enzyme activity. A substance that inhibits enzymatic activity is known as an inhibitor. Inhibition of an enzyme by an inhibitor is selective, in that one inhibitor can only inhibit one enzyme or a class of enzymes. There are two types of inhibition: irreversible and reversible inhibition. Irreversible inhibition is that the covalent binding between the essential group of the enzyme and the inhibitor causes a loss of enzyme activity, and the inhibitor cannot be removed by dialysis, ultrafiltration,

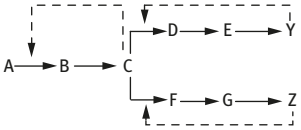
and other physical methods to revitalize the enzyme. These inhibitors include heavy metal salts, organic phosphorus compounds, organomercury compounds, organoarsenic compounds, and cyanide. Reversible inhibition is that the noncovalent binding between the enzyme and the inhibitor causes a decrease or loss of enzyme activity, and the inhibitor can be removed by dialysis, ultrafiltration, and other physical methods to revitalize the enzyme. Reversible inhibition can be divided into three types: competitive, noncompetitive, and uncompetitive inhibition. Competitive inhibition is that an inhibitor competes with a substrate for an enzyme-binding site to affect the binding of the substrate to the enzyme. Noncompetitive inhibition is that an inhibitor and substrate combine with an enzyme at the same time and no competition exists. Uncompetitive inhibition is that an inhibitor can bind to an enzyme only after the enzyme binds to its substrate. Inhibitors of metabolic processes are mainly reversible, and most of these are the results of feedback inhibition.

Feedback inhibition refers to the inhibition by end products generated in metabolism on enzymatic activity. Depending on the branch of the metabolic pathway, feedback inhibition can be divided into nonbranching metabolic pathway inhibition and branching metabolic pathway inhibition [12]. In nonbranching metabolic pathway inhibition, the end product usually inhibits the enzyme that performs the first step of the pathway (Figure 2.18a). There are many types of branching metabolic pathway inhibition: sequential feedback inhibition, isoenzyme feedback inhibition, cooperative feedback inhibition, cumulative feedback inhibition, and synergistic feedback inhibition. Sequential feedback inhibition is that the synchronous excess of several end products inhibits the first reaction steps after the branch point, in order to accumulate a common intermediate product at the branch point that thereby inhibits the first enzyme in the metabolic pathway. The excess of a single end product will not inhibit the first enzyme (Figure 2.18b). An isoenzyme is a type of enzyme that can catalyze the same chemical reaction, but has obvious differences in its molecular structure, physicochemical properties, and other aspects. In the metabolic pathway, the first step has several isoenzymes with different structures. The excess of one end product can only inhibit one enzyme. Once all the end products are in excess, the reaction is completely inhibited (Figure 2.18c). Cooperative feedback inhibition is that the first enzyme in the metabolic pathway is inhibited only when several end products are in excess at the same time (Figure 2.18d). Cumulative feedback inhibition is that every excess end product can inhibit the first enzyme in the metabolic pathway, and when several end products are simultaneously in excess, the inhibitory effect on the first enzyme is greater (Figure 2.18e). Synergistic feedback inhibition is that the excess of a single end product can partially inhibit the first enzyme in a metabolic pathway, and when several end products are in excess, the inhibition of the first enzyme is greater than the sum of the inhibition caused by a single end product (Figure 2.18f).



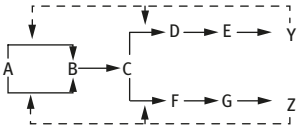
Nonbranching metabolic pathway inhibition

(a)



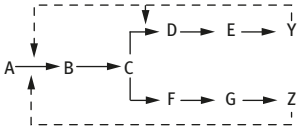
Sequential feedback inhibition

(b)



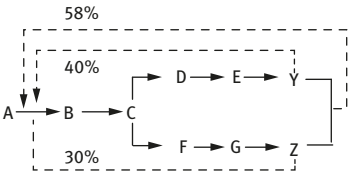
Isoenzyme feedback inhibition

(c)



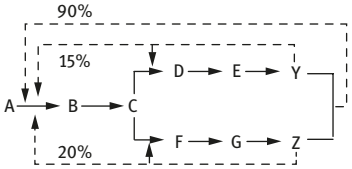
Cooperative feedback inhibition

(d)



Cumulative feedback inhibition

(e)



Synergistic feedback inhibition

(f)

Figure 2.18: Examples of various types of feedback inhibition.

Section 4: Heredity and Mutation of Microorganisms

Microorganisms transfer their genetic information to their descendants through nucleic acids, which allow their descendants to inherit their own characteristics. Nucleic acids will change gradually with changing environments to adapt to the new environment in the long evolutionary process. Nucleic acids retain much original information, and meanwhile have new features. Nucleic acids continually change under environmental stress, which makes it possible for microorganisms to adapt to a new environment. According to this characteristic, microorganisms are genetically modified to meet the demands of production and life.

4.1 Material Basis of Heredity and Transfer of the Genetic Information

Whether the genetic material was protein or nucleic acid caused widespread controversy. British bacteriologist Fredrick Griffith injected mice with S-type (smooth) and

R-type (rough) *Streptococcus pneumoniae* in 1928. The result showed that only S-type cells could kill mice. When injected with inactivated S-type cells, the mice survived. However, after injecting a mixture of inactivated S-type and live R-type, the mice also died. At the same time, live S-type bacteria were isolated from the dead mice. Therefore, it was speculated that some material from the dead S-type cells could transform the live R-type cells into the S-type. In 1944, Avery et al. purified multiple components from dead S-type cells (DNA, RNA, protein, and capsular polysaccharide), and mixed them with live R-type. They found that only a mixture of DNA and live R-type cells resulted in the appearance of live S-type cells. Subsequently, they mixed live R-type cells and DNA treated with DNase, and were unable to recover live S-type cells. These results confirmed that DNA could transform the live R-type to the live S-type, and that DNA was the carrier of genetic information. Subsequently, DNA and proteins were radioactively labeled with ^{32}P and ^{35}S, respectively, in phage T2 by Alfred D. Hershey and Martha Chase in 1952, and *E. coli* was infected with the labeled T2 phages. The result showed that only the labeled DNA entered the bacterial cells. Phage infection demonstrated that DNA was the carrier of genetic information. H. Fraenkel-Conrat split and rebuilt tobacco mosaic virus (TMV) containing RNA in 1956, and proved that RNA was also a form of genetic material. He separated protein and RNA of the wild-type and variant TMV, and mixed the protein of the wild-type TMV with RNA from the variant TMV to reconstruct a new complete virus that was used to infect tobacco plants. The results showed that specific disease spots appeared when the variant TMV was used to infect tobacco, and the capsids of the progeny phages isolated from the disease spots were from the variant TMV. The above experiments proved that nucleic acids were the genetic material.

4.1.1 Material Basis of Heredity

Nucleic acids, including DNA and RNA, form the material basis of heredity. DNA is a double-stranded biomolecule, the basic building block of which is a DNA. DNAs consist of a nitrogenous base (adenine, guanine, cytidine, or thymine), a D-2-deoxyribose, and one phosphate group. RNA is a single-stranded biomolecule, the basic building block of which is an RNA. RNAs consist of a nitrogenous base (adenine, guanine, cytidine, or uracil), a D-ribose, and one phosphate group.

4.1.1.1 DNA

Due to its structural complexity, DNA has primary, secondary, and tertiary structures. The primary structure is a linear or annular polynucleotide chain with the DNAs linked through 3′,5′-phosphodiester bonds. The secondary structure consists of two antiparallel polynucleotide chains that are connected together by the base

pairs through hydrogen bonds. Guanosine (G) pairs with cytosine (C), and adenine (A) pairs with thymine (T). The secondary structure has several conformations such as A-DNA, B-DNA, C-DNA, D-DNA, E-DNA and Z-DNA. Except for Z-DNA, which is a left-handed double helix, others are right-handed double helices. A-DNA and B-DNA are the basic conformations. The tertiary structure is a special conformation formed through twisting and folding of the DNA double helix. Supercoiled DNA is one tertiary structure. Plasmids are typically supercoiled. In addition, DNA can also form a more complex structure along with proteins, such as in eukaryotic chromosomes.

4.1.1.2 RNA

RNA consists of mRNA, tRNA, and rRNA, which are related to the synthesis of proteins. mRNA can convey genetic information to guide the synthesis of proteins, while tRNA is responsible for the transport of amino acids. rRNA combines with mRNA to ensure accurate binding to the ribosome translation initiation site to initiate protein synthesis.

4.2 The Transfer of Genetic Information

Genetic information is generally transferred according to the central dogma, which has been described as "genetic information can be replicated from parental DNA to descendant DNA, then transferred from DNA to RNA through transcription, and finally embodied in protein by translation." However, in some viruses, the genetic information is first transferred from RNA to DNA, and then transferred according to the central dogma. In other viruses, the genetic information is transferred directly from RNA to protein [7]. This section will introduce the general transfer of genetic information.

4.2.1 DNA Replication

DNA replication is a complex process, where DNA itself is used as a template and four DNAs are used as the raw materials to synthesize the descendant DNA strand through complementary base pairing. This process is catalyzed by DNA polymerase. DNA replication has two obvious characteristics. One is that replication is semiconservative, which means that one strand of the descendant DNA is derived from the parental DNA, while the other is newly synthesized. The other is that DNA replication is semidiscontinuous, which means that one strand of the descendant DNA, called the leading strand, is continuously synthesized, while the other, called the lagging strand, is discontinuously synthesized. The lagging strand is formed by first synthesizing discontinuous Okazaki fragments that are joined together by DNA ligase.

In the DNA replication process, some related proteins first identify the origin of replication and trigger the binding of helicase and topoisomerase to this site. Catalyzed by these enzymes, the DNA helix is relaxed, the topological tension is eliminated, the double-stranded structure is opened, and finally a single strand is formed. The single strand is bound with a single-stranded binding protein to prevent it from restoring its double-stranded structure. Meanwhile, a length of complementary RNA primer is synthesized at the origin of replication, catalyzed by a primase. Then the descendant strands are synthesized from the primer using the parental strands as templates and DNAs as substrates. The process is catalyzed by DNA polymerase and the whole principle is based on complementary base pairing. When termination information is encountered, DNA replication is terminated, and the new DNA strand is synthesized.

4.2.2 Transcription

Transcription is a process that uses RNA polymerase to synthesize a new RNA strand using DNA as a template. Transcription has four stages: template recognition, transcription initiation, extension, and termination. At the beginning of transcription, RNA polymerase recognizes and binds to the promoter (a length of DNA sequence recognized, bound, and transcribed initially by RNA polymerase) under the guidance of the σ subunit. The DNA double strands are partially resolved to form a transcription bubble structure, and a DNA–RNA heteroduplex (–2–9 bp) at the active center of the transcription bubble is synthesized. Subsequently, the σ subunit breaks off from RNA polymerase and RNA polymerase leaves the promoter region and moves along with the DNA strand to catalyze RNA synthesis, which is performed according to complementary base pairing with a DNA strand used as a template and RNAs used as the materials. After the transcriptional terminator is encountered, RNA polymerase detaches from the template and releases the RNA.

4.2.3 Translation

Translation is a process in which 20 basic amino acids are used to synthesize proteins using the ribosome and tRNAs, guided by mRNA. Prokaryotic protein synthesis involves the prokaryotic ribosome, which is composed of a 30S small subunit and a 50S large subunit. The 30S small subunit contains 16S rRNA, and the 50S large subunit contains 5S rRNA and 23S rRNA. When protein synthesis starts, initiation factors bind to the 30S small subunit to promote binding of fMet-tRNAf to the small subunit. This complex initiates protein synthesis via matching 16S rRNA and mRNA sequences. Then, the 50S large subunit binds to the 30S small subunit and the initiation factors are released. The ribosome starts to move along

the mRNA strand and the elongation reaction is initiated by elongation factors. When the translational termination signal is encountered by the moving ribosome, protein synthesis is completed, and the protein is released with the help of releasing factors. Protein synthesis in eukaryotes follows a similar process to that in prokaryotes. However, the eukaryotic proteins need to be processed to become mature and functional proteins.

4.3 Culture Preservation

Strains need to be preserved in a reasonable way for scientific research and industrial production. Strains decline in performance readily through long-term storage and repeated use. Declining strains need to be rejuvenated to restore their original features and then preserved again.

4.3.1 Microbial Recession and Rejuvenation

Many factors can cause the recession of strains, such as increasing passage number, and inappropriate cultivation and preservation conditions. [13]. Therefore, improper operation should be avoided. Once strains decline in performance, the strains need to be rejuvenated. Rejuvenation methods mainly include the isolation of the strain in pure culture isolation and host rejuvenation. The strains, retaining the original characteristics, can be selected from the population of recessive strains by isolation of the pure culture. For pathogenic bacteria, they can be inoculated into the corresponding animal and plant hosts. After multiple rounds of selection, the strains, with their restored original virulence, can be isolated from a typical infectious lesion.

4.3.2 Culture Preservation

4.3.2.1 Methods of Culture Preservation

Culture preservation is used to inhibit microbial growth and reproduction, and to reduce variation of the strains under artificial conditions based on their physiological and biochemical characteristics [14]. There are many methods for culture preservation. The main steps for culture preservation are as follows. First, the strain must be isolated in pure culture. If cultures produce conidia or spores, it is better to preserve these. Second, an optimum environment should be built for the dormancy of microorganisms, such as dry, low temperature, hypoxia, darkness, lack of nutrition, and the addition of protective agents or acid neutralizers. Some common methods for culture preservation are shown in Table 2.8.

Table 2.8: Common preservation methods for microorganisms [2].

Preservation method	Preservation condition	Preservation time	Suitable species	Evaluation
Preserved in the refrigerator (slant)	4 °C	3–6 months	All types of microbes	Simple
Preserved in the refrigerator (semisolid)	4 °C, avoiding oxygen	6–12 months	Bacteria, yeasts	Simple
Sealed by *paraffinic oil*	4 °C, blocking oxygen	1–2 years	All kinds of microbes	Simple
Preserved in a glycerol suspension	–70 °C, 15%–50% glycerol	10 years	Bacteria, yeasts	Simple
Preserved by sand	Dry, without nutrients	1–10 years	Microbes producing spores	Simple, effective
Freeze-drying preservation	Dry, anaerobic, low temperature, with protectants	5–15 years	All kinds of microbes	Troublesome, efficient
Liquid nitrogen preservation	–196 °C, with protectants	>15 years	All kinds of microbes	Troublesome, efficient, high cost

4.3.2.2 Agencies for Culture Preservation

In addition to laboratory preservation, microbial strains also need to be sent to professional organizations for preservation and storage, which are conducive to the exchange of resources, and also prevent the loss of resources. Additionally, when identifying and nominating a new species, or applying for patents on a engineered new species, it is necessary to obtain validation from a specialized collection agency (Table 2.9) with international recognition to ensure that the isolated species is novel.

Table 2.9: Professional microbial collection agencies.

Collection of microorganisms	Country	Microorganisms preserved
China Center for Type Culture Collection, http://www.cctcc.org/	China	Bacteria, yeasts, fungi, microalgae, human and animal cell lines, hybrid tumor, viruses from animals and plants, phages
China General Microbiological Culture Collection Center, http://www.cgmcc.net/	China	Microorganisms and cell lines
American Type Culture Collection, http://www.atcc.org/	America	Cells and microorganisms
Agricultural Research Service Culture Collection, http://nrrl.ncaur.usda.gov/	America	Bacteria and fungi

Table 2.9: (continued).

Collection of microorganisms	Country	Microorganisms preserved
German Collection of Microorganisms and Cell Cultures, http://www.dsmz.de/	Germany	Microorganisms, plant viruses, human and animal cell lines, and plant cell cultures
National Collection of Type Cultures, http://www.hpacultures.org.uk/collections/nctc.jsp	Britain	Bacteria and mycoplasmas, cell lines and hybridomas, viruses, and fungi
National Collection of Yeast Cultures, http://www.ncyc.co.uk/	Britain	Yeasts
Culture Collection of Algae and Protozoa, http://www.ccap.ac.uk/	Britain	Cyanobacteria, protists, and macroalgae
National Collection of Industrial, Food and Marine Bacteria, http://www.ncimb.com/	Britain	Industrially and environmentally valuable microorganisms
All-Russian Collection of Microorganisms, http://www.vkm.ru/	Russia	Bacteria, archaea, yeasts, and filamentous fungi
Culture Collection of Yeasts, http://www.chem.sk/activities/yeast/ccy/	Slovakia	Yeasts, mutant cultures, type, and patent strains
Spanish Type Culture Collection, http://www.cect.org/	Spain	Bacteria, archaea, filamentous fungi, and yeast
Belgian Co-ordinated Collections of Microorganisms, http://bccm.belspo.be/index.php	Belgium	Bacteria, cyanobacteria, diatoms, biomedical fungi, and yeasts
National Bank for Industrial Microorganisms and Cell Cultures, http://www.nbimcc.org/en/about.htm	Bulgaria	Bacteria, fungi and yeasts, animal viruses, plant viruses, and animal cell lines
Czech Collection of Microorganisms, http://www.sci.muni.cz/ccm/index.html	Czech	Bacteria and archaea, filamentous fungi and yeasts, and bacteriophages
Collection National Cultures Microorganisms, http://www.pasteur.fr/recherche/unites/Cncm/index-en.html	France	Bacteria, hybridomas, animal or human cell lines, fungi, yeasts, phages, and viruses from animal or human sources
National Collection of Agricultural and Industrial Microorganisms, http://web.uni-corvinus.hu:8089/NCAIM/index.jsp	Hungary	Bacteria, yeast, and filamentous fungi
Centraalbureau voor Schimmelcultures Fungal Biodiversity Centre, http://www.cbs.knaw.nl/	Holland	Fungi, yeasts, bacteria, plasmids, and phages
Korean Culture Center of Microorganisms, http://www.kccm.or.kr/	South Korea	Microorganisms
Korean Collection for Type Cultures, http://www.brc.re.kr/English/ekctc.aspx	South Korea	Bacteria, yeasts, molds, algae, and animal and plant cell lines
Microbial Type Culture Collection and Gene Bank, http://mtcc.imtech.res.in/	India	Bacteria, fungi, yeasts, and plasmids

Section 5: Microbial Breeding

Isolating microorganisms from nature is generally difficult to meet the needs of modern production practices. Microbial strains are usually modified in accordance with certain principles to obtain desirable features to meet industrial needs. The process of breeding species for a special purpose is called microbial breeding. Methods of microbial breeding usually include mutation breeding, genetic engineering breeding, protoplast breeding, and cross breeding.

5.1 Mutation Breeding

Mutation breeding is a process where microbial cells are first treated with a variety of mutagens to increase the frequency of genetic mutation, and then an appropriate screening method is used to obtain desirable strains. The mutagenesis and screening are the main steps. Mutagenesis is random, whereas screening is directional. The choice of screening method is more important than the type of mutagenesis for mutation breeding.

5.1.1 Theoretical Basis of Mutation Breeding

The theoretical basis behind mutation breeding is genetic mutation, which refers to heritable changes in the DNA sequence and includes gene mutations (point mutations) and chromosome aberration. Point mutations are genetic changes that are caused by changes in at least one pair of nucleotide bases. Chromosome aberration is a heritable change caused by a change in the number (increase or decrease) or structure (deletion, insertion, repeat, and translocation) of chromosomes.

5.1.1.1 Characteristics of Gene Mutation

Gene mutation can occur spontaneously or be induced. These mutations have the following characteristics: (1) They are spontaneous, meaning that the mutation of a variety of traits can occur without any artificial conditions; (2) noncorrespondence, meaning that there is no direct correspondence between the mutation traits and the factors causing the mutation; (3) infrequency, meaning that the spontaneous mutation frequency is very low, generally about 10^{-6}–10^{-9}; (4) independence, meaning that mutation of one gene does not affect other gene mutations and has no influence on other gene mutations; (5) inducibility, which means that the mutation frequency can be improved under artificial conditions; (6) reversibility, which means that the mutant strain can change back to the original wild-type strain, a

process known as back mutation or reverse mutation; and (7) stability, meaning that the new characteristic formed by the mutation is stable and heritable.

5.1.1.2 Mechanism of Gene Mutation

Gene mutation results from changes in the nucleotide sequence of DNA. Mutation mechanisms are summarized in Table 2.10. After base substitutions occur, the following cases are possible: (1) synonymous mutations, which occur within a triplet codon, but do not lead to changes in the amino acids of the encoded protein; (2) silent mutations, where a DNA mutation results in an encoded amino acid change, but this has little effect on the structure and function of the protein, and leads to no change in cell phenotype; (3) missense mutations are DNA mutations in a triplet codon that result in substitution of the original amino acid in the encoded protein by another amino acid; and (4) nonsense mutations are when the codon encoding amino acid is mutated to a termination codon, resulting in a truncated protein.

There are two situations that result from frameshift mutations: (1) when the nucleotide number inserted or deleted is not 3 or a multiple of 3, the reading frame of the gene will be altered leading to significant changes to the protein; (2) when the nucleotide number inserted or deleted is 3 or a multiple of 3, only one or several amino acids of a certain section will be altered, and other amino acids will be unchanged. If the amino acid change has little effect on the protein function, the original feature of the protein will be maintained, otherwise it will be changed.

Table 2.10: Mechanism of gene mutation.

Point mutation	Base substitution	Transition	Pyrimidine (or purine) base of the original strand is replaced by another pyrimidine (or purine) base
		Transversion	Purine (or pyrimidine) base of the original strand is altered to a pyrimidine (or purine) base
	Frameshift mutation	One or a few nucleotide pairs of the DNA sequence are inserted or deleted, resulting in a frame shift in the downstream genetic code, leading to incorrect transcription and translation	
Chromosomal aberration	Number variation	Increase or decrease in the number of chromosomes	
	Structure change	Chromosomal deletion, insertion, repetition, inversion, and translocation	

5.1.1.3 Repair of DNA Damage

Base mispairing can occur during DNA replication, leading to mutations. In order to ensure the accuracy of replication, corrective and restorative functions have evolved. DNA polymerase plays a key role in the process of DNA replication and not only has 5′ → 3′ polymerase activity but also has 3′ → 5′ exonuclease activity, meaning that mismatched bases can be repaired during replication. This repair can only work on a small number of mutated bases, and once there are too many mutated bases, they cannot be repaired by DNA polymerase alone. Therefore, in addition to DNA polymerase, other repair systems have evolved that include photoreactivation, excision repair, recombination repair, and SOS repair.

(1) Photoreactivation. Ultraviolet (UV) irradiation makes dimers (TT, TC, and CC) of adjacent pyrimidine bases on the same DNA strand, which affects the normal function of DNA, causing mutations and cell death. When microorganisms are immediately placed under visible light after UV irradiation, the mortality rate can be significantly reduced. This phenomenon is known as photoreactivation. The principle of this phenomenon is related to the *phr* gene in microorganisms, which encodes a photolyase (Phr) that can exclusively identify and bind pyrimidine dimers in the dark. The photolyase is activated by light and depolymerizes the dimers into monomers. At the same time, the enzyme dissociates from DNA and restores the mutated sites.

(2) Excision repair. After the reactions of various enzymes, the damaged portion of the DNA molecule is excised. The excised portion is synthesized using the other complete strand as a template to restore the normal structure of the DNA. This is a common repair mechanism that can repair most DNA damage except for base mismatches and the insertion of single nucleotides. *E. coli* excision repair system requires excisionase, helicase, DNA polymerase I and DNA ligase. The *E. coli* excisionase consists of three subunits: UvrA, UvrB, and UvrC. Initially, UvrA and UvrB form an $UvrA_2B$ complex that will bind to DNA to search for the damaged region. Once the damaged region is found, the UvrA dimer will dissociate immediately from the complex, and UvrB is left to bind strongly to the DNA. UvrC combines with UvrB, and UvrB cuts the fifth phosphodiester bond at the 3′ end of the damaged region, while UvrC cuts the eighth phosphodiester bond at the 5′ end. Then the fragment containing 12–13 nucleotides and UvrBC is released with the help of UvrD helicase, and finally the single-stranded gap left at the excised position is restored by DNA polymerase I and DNA ligase.

(3) Recombination repair is also known as postreplication repair. In the process of DNA replication, if the replication enzymes cannot synthesize the new DNA strand at the damaged site through base pairing, they will skip the damaged site and synthesize new primers at the corresponding position of the leading strand or at the starting position of the next Okazaki fragment to complete replication of the downstream DNA,

resulting in a gap opposite the damaged site of the new DNA strand. Using a recombinase (RecA) and other enzymes, the corresponding fragment of the homologous parent chain of the DNA is used to fill the gap, and the gap left in the parent chain is synthetized by the polymerase using the corresponding matching subchain as the template. Finally, the repair process is completed by the action of DNA ligase. In the above process, the damaged DNA strand is not removed, and its impact on replication still exists. However, its influence on DNA damage will be gradually eliminated with continuous replication and cell division.

(4) SOS repair is an emergency response induced by significant DNA damage. SOS repair can induce not only the production of certain key proteins and enzymes for excision repair and recombination repair, but also the production of DNA polymerase lacking proofreading activity that repairs the damaged site with a high mutation rate. SOS repair is caused by the interaction of RecA and the repressor LexA. LexA represses the expression of multiple repair-related genes. RecA participates in the repair of DNA damage in the following way: when single-stranded DNA and ATP exist together, RecA is activated to promote the proteolytic activity of LexA, which degrades LexA, and finally a variety of repair-related genes are expressed to participate in repair of the DNA damage.

5.1.1.4 Phenotypic Changes Induced by Gene Mutations

The phenotype is the visible individual traits or characteristics that are expressed by genes under certain environmental condition. When genes are mutated, the phenotype of an organism will vary from its original features (Table 2.11). The microorganism before acquiring the mutation is called the wild type, and the microorganism after mutation is the mutant. To obtain the desired strain, appropriate screening methods should be selected based on the phenotypic changes before and after mutation.

Table 2.11: Phenotypic changes caused by mutations and their characteristics.

Phenotypic changes	Characteristic
Auxotrophy	It cannot synthesize the necessary nutrients for growth, but only take them up from the environment to carry out normal growth and reproduction
Resistant mutant	Resistant to certain factors (antibiotics, temperature, etc.)
Conditional lethal mutant	It can normally grow well under certain conditions, but will die under other conditions
Producing mutant	The yield of a certain metabolite of this mutant is significantly different from that of the wild type
Morphological mutant	Individual cells or groups of cells vary in their morphology from the wild type

5.1.1.5 Mutagens and Mechanism of Mutagenesis

Mutagens can improve the mutation frequency and are divided into physical, chemical, and biological mutagens. The types, nature, mechanism, and major genetic effects of common mutagens are listed in Table 2.12.

5.1.2 Mutation Breeding

The general steps involved in mutation breeding include selecting the original strain, preparing the bacterial suspension, choosing and using mutagens, culturing the strain after mutagenesis, screening the mutant strains, and testing the performance of strains to obtain the required strains. The key steps are the selection of the starting strain, choice and use of the mutagens, and the screening of mutants.

5.1.2.1 Selection of Starting Strain

The starting strain is the original strain to be mutated. Choosing an appropriate starting strain will improve the breeding efficiency. The morphological, physiological, and biochemical characteristics of the starting strain should be known. Then the starting strain is selected according to the actual needs. Usually strains that have the ability to synthesize the desired product, have excellent performance, are effective after several rounds of mutagenesis, and have other positive characteristics, such as fast growth rate and low nutritional requirements, should be chosen.

5.1.2.2 Selection and Use of Mutagen

A simple and effective mutagen is usually selected. When physical and chemical mutagens have the same effect, the most convenient mutagen should be selected, and under the same convenient conditions, the most effective mutagen should be chosen. It has been shown that UV radiation is the simplest physical mutagen, while "supermutagens," such as NTG, are powerful mutagens. After selection of the mutagen, its dose should be controlled. Different doses are used for different microbes. In general, the mutation rate increases as the dose of mutagen increases. However, when the dose reaches a certain level, the mutation rate will decrease with increasing doses of the mutagen. In addition, when a single mutagen is ineffective, two or more mutagens can be mixed to improve the mutagenic effect.

Table 2.12: Common mutagens and their mutagenic mechanism [15].

Classes	Name	Properties	Mechanism of action	Genetic effect
Physical mutagen	Ultraviolet radiation	Nonionizing radiation	UV activity is consistent with the absorption spectrum of nucleic acids. DNA molecules strongly absorb UV, which promotes the atomic inner electron energy of the irradiated material	DNA breakage, DNA cross-linking, pyrimidine hydration, formation of pyrimidine dimers can cause base mismatches
	X-ray,γ-ray	Ionizing radiation	The electrons of the atoms or molecules of the irradiated object are ionized to generate free radicals that act on DNA	AT⇌GC conversion, frameshift mutation, chromosomal aberration
	Heavy ion	Ionizing radiation	The physical collision of the accelerated heavy ions and biomolecules results in chemical changes, the molecular structure, or conformation	Changes in biological function of macromolecules
Chemical mutagen	5-Bromouracil (BU), 2-aminopurine (AP)	Base analog	Incorporation of base analog to replace normal bases in DNA	Base-pair conversion
	Ethyl methanesulfonate (EMS), diethyl sulfate (DES), nitrosoguanidine (NTG), ethylene imine (EI), nitroso-methyl urea (NMU), nitrogen mustard (NM), ring acetic acid (EA)	Alkylating agent	Alkyl groups can be transferred to a location of high electron density in DNA, alkylating the bases and phosphate groups, causing base mismatches	Base mismatches and mistakes in DNA repair, AT⇌GC conversion, AT⇌TA transversion, GC⇌CG transversion
	Acridine compounds, ethidium bromide (EB)	Frameshift mutagen	The structure is very similar to purine–pyrimidine base pairs, which makes it embed between two adjacent DNA base pairs	Frameshift mutation
	Nitrous acid	Deaminating agent	Oxidative deamination of bases	Base-pair conversion, DNA cross-linking
	Hydroxylamine	Hydroxylating agent	Hydroxylation of cytosine amino group to form *N*-4- hydroxy-cytosine	GC⇌AT conversion
Biological mutagen	Phage, plasmid, transposon	Inducing the resistant mutation	Base substitution, DNA recombination	DNA deletion, duplication, insertion

5.1.2.3 Screening of Mutant Strains

Appropriate screening methods are designed based on the purposes of breeding and the characteristics of the mutant strain to quickly select the desired strain. Screens can be based on morphological characteristics when the morphology of the mutant strain is significantly different from that of the original strain. When the metabolites of a mutated strain can react with certain substances, this substance can be added to the plating medium to identify the desired mutants. When the mutant strain has other physiological and biochemical characteristics, the screening method should be designed according to the actual situation.

(A) Screening of auxotrophic mutants. Three types of media are involved in this screening process: basal medium, supplemented medium, and complete medium. The basal medium can only meet the growth demands of the wild-type strain, while it cannot be used to culture an auxotrophic strain. Supplemented medium is made by the addition of a certain nutrient to the basic medium to meet the growth demands of the auxotrophic strain. Complete medium can meet the growth demands of both the wild-type and auxotrophic strains. First, an appropriate method should be selected to remove the wild-type strain, and concentrate on the defective strain. Then the strains are plated on the complete medium plate. When colonies appear, they are inoculated into the basal medium by spot inoculation or replica plating method. After culturing, the colonies that appear on the complete medium but not in the basal medium may be the auxotrophic mutants for a certain nutrient. These colonies are picked and cultivated to collect the strains and prepare bacterial suspensions that will be plated onto the basal medium or mixed with the basal medium to prepare plates. Filter paper discs with different combinations of nutrients are placed on different areas of the plate. After being cultured for a period of time, the growth of the strains in different regions is observed to determine the type of nutritional factors required by the strain.

(B) Screening of anti-feedback repression and feedback inhibition mutants. Generally, structural analogs that are structurally similar to metabolites are used for screening. The structural analogs can bind to repressor proteins or allosteric enzymes to repress or inhibit the synthesis of metabolites; however, their concentration will not decrease because they are unable to replace the metabolites and participate in metabolism. The combination of the structural analogs with the repressor protein or allosteric enzyme is irreversible, which irreversibly inhibits the enzymes responsible for the synthesis of the metabolites. Anti-feedback repression and feedback inhibition mutants are not affected by structural analogs and can continue to synthesize and accumulate metabolites in the presence of the structural analogs.

(C) Screening of resistant mutants. The screening of resistant mutants can be designed according to their resistance phenotype. For example, antibiotic-resistant mutants can be identified by adding the required antibiotic to the medium according. Mutants that are resistant to the drug can grow on this medium while wild-type cells cannot.

5.2 In Vivo Gene Recombination Breeding

Gene recombination refers to the process where genomic DNA from different individual cells recombines to form a new stable genome. Gene recombination occurring in vivo is called in vivo gene recombination, otherwise it is in vitro gene recombination. In vivo genetic recombination breeding is a breeding method to obtain strains, using genetic engineering methods and technologies such as transformation, conjugation, transduction, protoplast fusion, quasi-sexual hybridization, and sexual hybridization. In this way, genes recombine within microbial cells to increase the chances of producing a strain with desired traits or leading to the production of multiploid organisms.

5.2.1 Transformation

Transformation is a method of horizontal gene transfer, where homologous or heterologous free DNA molecules are absorbed by a competent recipient cell and expressed in the cell. Competence is a physiological state of the cell meaning that it can absorb exogenous DNA from environment. Transformation can be divided into natural and artificial transformation depending on the method of cellular competence.

5.2.1.1 Natural Transformation

Natural transformation refers to the process where microbial cells grow to a certain stage and absorb exogenous DNA from the environment without human intervention. The transformation mechanisms of G^+ and G^- bacteria are different. When G^+ bacterial cells grow to a certain stage, they begin to secrete competence factors that not only make normal cells become competent cells, but also make surface-exposed DNA-binding proteins and nucleases. Competent cells have the ability to bind DNA. The DNA-binding protein can only bind double-stranded DNA, while the DNA entering into the cell is single stranded. After the DNA binds to the competent cell, exonuclease in the recipient cells will randomly cleave the exogenous DNA and generate new DNA fragments. One strand of the DNA fragment is degraded by exonuclease, and the other strand binds to the competence-specific protein, enters into the recipient cell in the form of single strand, and integrates into the recipient chromosome through homologous recombination. G^- bacterial cells form transformation globules during the formation of competent cells and absorb DNA through the transformation globules that transport DNA to the cytoplasmic space after fusing with the inner and outer membrane of the cell. The DNA is degraded and becomes single strand before entering into the cytoplasm, and the single-stranded DNA will integrate with the recipient DNA in the same manner as for G^+ bacteria. The single chain replaced after homologous recombination is degraded by the nuclease.

5.2.1.2 Artificial Transformation

The artificial transformation is used to artificially induce microorganisms to become competent and take up exogenous DNA by physical or chemical methods. Generally, there are two types of artificial transformation: chemical transformation and electroporation. Chemical transformation is a process that utilizes a high concentration of Ca^{2+} and Mg^{2+} to treat microorganisms, making them competent and able to take up exogenous DNA. Electroporation is a process where the cell membrane of the microorganism is placed in a strong electric field and is polarized to generate a transmembrane potential that exceeds a threshold, causing transient holes to appear on the cell membrane. These holes allow a variety of macromolecules (including DNA) to enter the cell to complete the transformation process.

5.2.2 Conjugation

Conjugation is the transfer of genetic information by direct contact between bacterial cells. The ability to perform conjugation is ascribed to the existence of a conjugative plasmid that carries a set of genes that control bacterial pairing and plasmid conjugation, as well as the genetic information for replication. The F plasmid is a conjugative plasmid that has been extensively studied. Bacterial cells can be classed into F^-, F^+, F′, and Hfr types based on the existence of the F plasmid and its state. An F^- cell has no F plasmid, while an F^+ cell contains the F plasmid in double-stranded and circular form and without any genes or DNA fragments from the host cell. The F plasmid in an F′ cell exists in the same manner as the F plasmid in the F^+ cell, but carries genes or DNA fragments from the host. The F plasmid in Hfr cells (high-frequency recombination cell) integrates into the host chromosome at different sites as linear DNA. Conjugation is usually performed between the cells with and without a plasmid.

5.2.2.1 Conjugation Between F^+ and F^- Cells

An F^+ cell usually has two or three F-pili on the cell surface, while an F^- cell does not. The F^+ cell contacts the F^- cell using the F-pili. The F-pili will shrink to make the two cells connect tightly (this process may be completed by the depolymerization or redissolution of the cell membrane of donor or recipient cells). Then a nickase–helicase encoded by the *traY* gene identifies the OriT site and cuts one strand of the F plasmid. The nickase–helicase immediately combines the 5′ terminal. The single chain of the F plasmid is transferred from the donor cell to the recipient cell via the hole formed at the connection site. The single DNA strand is immediately cyclized after it is completely transferred into the recipient cell. The complementary strand is left in the donor cell. They turn into double-stranded structures by rolling-circle replication. Lastly, the recipient cell without an F plasmid becomes an F^+ cell.

5.2.2.2 Conjugation Between Hfr and F⁻ Cells

Conjugation between the Hfr cells and F⁻ cells is similar to that between F⁺ cell and F⁻ cell. The leading region of 5′ terminus combines with the donor DNA and is transferred to the recipient cell after the OriT sequence is recognized and cut by the enzyme encoded by *traY*. The F plasmid exists in the Hfr cell in a linear form and most parts of F plasmid are located at the terminus of the chromosome except for the leading region. It is difficult to completely transfer the F plasmid into the recipient cell as the transfer process requires a long time and is easily interrupted. The recipient cell often accepts a partial copy of the F plasmid and donor DNA. Finally, the recipient cell is still F⁻ after conjugation.

5.2.2.3 Conjugation Between F′ Cell and F⁻ Cell

An F′ cell carries part of the donor DNA. When it conjugates with an F⁻ cell, parts of the donor DNA chromosome are transferred to the recipient cell along with the F plasmid. The plasmid can be expressed without integrating, and the F⁻ cell turns into an F′ cell. This form of gene transfer is called sexduction.

5.2.3 Transduction

Transduction is a process where small DNA fragments of the donor chromosome are transferred to the recipient cell with the help of phages. A recipient cell that obtains new properties via transduction is called a transductant. Transduction can be classified into generalized transduction and specialized transduction.

(1) Generalized transduction is a phenomenon where a phage packages any DNA from the donor cell by mistake and transmits the genetic material of the donor to the recipient cell. The mechanism of generalized transduction is as follows. After the phage infects the donor cell, the donor chromosome is degraded into many fragments and the phage reproduces in the donor cell. When the phage assembles, it mistakes donor DNA fragments for its own DNA and encapsulates them into its capsomere to form a new phage without its own DNA. When the donor cell lyses, the phages are released to infect the other cells and they will inject the donor DNA into the recipient cells. The donor DNA will recombine with homologous regions of recipient cell chromosome via double-stranded DNA exchange. Sometimes the injected donor DNA does not recombine with the recipient cell chromosome; it may be transcribed, leading to expression of those genes. This phenomenon is known as abortive transduction.

(2) Specialized transduction is a process where a phage carries particular donor genes that are transferred to the recipient and integrate with the recipient chromosome. The mechanism of specialized transduction is as follows. When a temperate phage infects

a donor strain, phage DNA can integrate into the donor chromosome. The phage DNA will be excised when it is affected by environmental factors, such as UV radiation. If the excision process was abnormal, a part of donor DNA connected to one end of the phage DNA can be excised together with the phage DNA. Meanwhile, the prophage loses a corresponding length of its own DNA fragment on the other terminus. The heterozygous DNA containing a part of the donor and phage DNA can replicate and package like other phages to form a transducing particle that will inject the heterozygous DNA into a recipient cell. The heterozygous DNA will integrate into recipient chromosome.

5.2.4 Protoplast Fusion

Protoplast fusion is a process where two different protoplasts from two kinds of strains with different features are fused into a new cell by artificial methods. The new cell is called fusant. Protoplast fusion includes parental selection, protoplast preparation, protoplast fusion, protoplast regeneration, and fusant screening (Figure 2.19).

5.2.4.1 Parental Selection

To gain fusants with good properties, two steady hereditary parental strains with complementary advantages are usually selected. In order to easily identify the fusant, it is

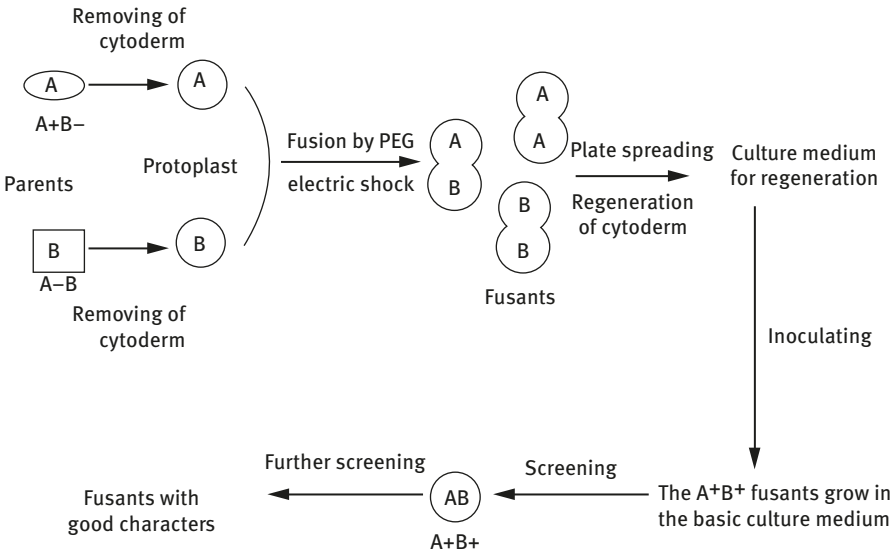


Figure 2.19: Technical routes for protoplast fusion

better to choose the parental strains with discriminating genetic markers. Meanwhile, the parental strains should be detected by the way of back mutation for avoiding false positives.

5.2.4.2 Protoplast Preparation

First, the cell wall should be removed as it hinders fusion. A suitable method should be adapted to remove the cell wall based on the composition and structure of the cell wall. Generally, lysozyme is often used to treat G^+ strains, while lysozyme and EDTA are used to treat G^- strains, cellulase or lywallzyme isolated from fungi are used to treat molds, and snailase is used for yeasts. Protoplasts should be placed in a hypertonic solution to avoid rupture.

5.2.4.3 Protoplast Fusion

A suitable fusogenic agent should be chosen for protoplast fusion. The usual fusogenic agent is polyethylene glycol (PEG), which exists in various types with different molecular weights. With increasing molecular weight and concentration of PEG, it is more viscous and harmful for the cell. It is necessary to choose PEG with a suitable molecular weight and concentration and to control the processing time. Generally, the molecular weight and concentration of PEG used for protoplast fusion of bacteria is 6,000 and 40% (weight per volume), PEG1000 is often used for *Streptomyces* species, and PEG 4000 and 6000 are used for fungi. Processing time is very short, usually one to several minutes or half an hour. In addition, some physical factors such as UV radiation and pulsed electric fields can also improve protoplast fusion.

5.2.4.4 Protoplast Regeneration

The fused protoplasts need to regrow their cell walls and recover their complete morphology and structure. Fused protoplasts are placed in regeneration media, which is prepared by adding trophic factors and osmotic stabilizers into usual media ensuring normal growth of the strains. Trophic factors supply nutrients to accelerate synthesis of the cell wall and guarantee the normal physiological metabolism of the fusant. Osmotic stabilizers maintain the hyperosmotic state of the media to avoid rupture of the protoplasts. Generally, regeneration medium for fungi contains yeast extract, peptone, sugars, or amino acids as the nutrition source, while for bacteria casein hydrolysate, serum albumin, amino acid, and sodium succinate are added as nutrients. Inorganic salts, such as KCl, NaCl, $CaCl_2$, and $MgCl_2$, and organics, such as sucrose, sorbitol, mannitol, inositol, and sodium succinate, are used as osmotic

stabilizers and are added to the regeneration media. The number of fused protoplasts that can regenerate their cell wall in the regeneration media is only a fraction of the total protoplasts. Generally, the regeneration rate of bacteria is 3–10%, fungi is 20–80%, and *Streptomyces* can reach a maximum level of 50%. Besides nutrition and osmotic pressure, factors such as the age of the parental strains, amount of enzyme used for degrading the cell wall, the time for degrading the cell wall, and temperature of regeneration can affect the regeneration of the protoplasts.

5.2.4.5 Fusant Screening

If the parent strains carry recognizable genetic markers, fusants can be identified in a similar way to mutation breeding. If the parental strains have no recognizable genetic markers, fusant can be identified by their morphology or other factors. After the fusants are isolated, their physiological and biochemical features, as well as their productivity, should be tested to determine their suitability for the requirements.

5.2.5 Parasexual Hybridization

Parasexual reproduction is similar to sexual reproduction, and can occur to somatic cells of different strains from the same species where there is low-frequency genetic recombination without meiosis after cell fusion. Parasexual hybridization often takes place in some fungi, especially the imperfect fungi. First, hyphal anastomosis is carried out among the somatic cells of different strains from the same species that have no morphological differences, but genetic differences. Second, the cell nucleus of a hypha enters into another hypha to form a heterocaryon that contains two or more than two genotypes. The heterocaryon can live independently and has better viability. Two cell nuclei from the heterocaryon undergo karyapsis under certain conditions to form a heterozygous diploid. During mitosis of the heterozygous diploid, chromosomes in the nuclei of the rare heterozygous diploids can exchange and form a haploid heterozygote with new characteristics. If the heterozygous diploid is treated with physicochemical factors, such as UV radiation or nitrogen mustard, it can lead to chromosomal aberration or uneven distribution of chromosomes in the daughter cells. Some haploid heterozygotes with different features may be generated.

Parasexual hybridization is a process for obtaining new species by artificially improving hybridization of the parents with different characteristics based on the principle of parasexual reproduction. Its procedure is as follows. First, parents with identifiable genetic markers are chosen, for example, auxotrophs. One parent that cannot synthesize nutrient A but can synthesize nutrient B is marked as $A^{-}B^{+}$.

The other parent that can synthesize nutrient A but not nutrient B is marked as A^+B^-. The conidia of these two parents are mixed and plated onto basal media without A and B. Meanwhile, every parent is also individually plated onto basal media and used as the control. After culturing for a period of 48–72 h, several colonies appear on the mixed culture plates, but not on the control plates. Only heterocaryons can simultaneously synthesize A and B to survive on the basal media. It can be thought that the heterocaryons appear. Subsequently, the colonies are transferred to another new basal medium plate. After a period of cultivation, the conidia are collected and prepared in suspension to plate on basal media plates. After a period of culturing, complete medium is poured over the basal medium and culturing is continued. If there are more colonies appearing on the complete medium than the basal medium, it indicates that the heterocaryon forms are unstable. If there is no difference in the number of colonies appearing on the complete medium compared to the basal medium, it shows that the heterocaryon is stable. Some physicochemical factors, such as UV radiation are used to treat the stable heterocaryon to further increase the probability of new variants with different characteristics in the future generations. At last, the desired strains are identified by detecting a series of characteristics.

5.2.6 Sexual Hybridization

Sexual reproduction is a mode of reproduction where male and female cells fuse into a zygote that will further develop into a mature individual. Sexual hybridization is a method of breeding to obtain good heterozygotes where the probability of chromosomal recombination is enhanced by the artificial union of male and female cells. Take the sexual hybridization of *S. cerevisiae* as an example. *S. cerevisiae* forms both haploid and diploid generations during reproduction. When it enters from a haploid generation into a diploid generation, it conducts asexual reproduction by budding. It carries out sexual propagation under specific conditions. The efficiency of gene recombination is very low during asexual reproduction. However, genetic recombination can occur during sexual propagation to produce new characteristics. Desired strains can be gained by sexual hybridization.

Sexual hybridization of *S. cerevisiae* can be performed as follows. Two parental diploid cells are inoculated onto sporulation media containing sodium acetate to produce asci. Every ascus generates four ascospores via meiosis. The asci are broken by snailase or mechanical processes to release the ascospores. The ascospores from two parents are prepared into a suspension to be plated out. Monoploid cells are formed after a period of culturing. Then two parental haploid strains are mixed together to increase the appearance of various diploids of the sexual hybrids. Finally a method should be chosen to identify the desired strains.

5.3 Genetic Recombination Breeding In Vitro

Genetic recombination breeding in vitro mainly refers to genetic engineering breeding and also includes technologies such as DNA shuffling and random chimeragenesis on transient templates that have been developed based on genetic recombination.

5.3.1 Genetic Engineering

Genetic engineering is when a desired nucleic acid molecule is used in vitro and linked to a special carrier to form a new combination of genetic material that will be introduced, replicated, and expressed in the recipient cell without this genetic material, and finally be inherited steadily by the offspring. Its general steps include acquisition of the target gene, linking of the target gene and the cloning vector, amplification of the recombination vector being introduced into the recipient cell, acquisition of the amplified target gene, linking the target gene and the expression vector, expression of the recombinant vector that is transferred to the recipient cell, and screening and identification of the recombinant (Figure 2.20).

(1) Acquirement of the target gene
According to the breeding requirements, the target gene can be gained by restriction enzyme digestion or polymerase chain reaction (PCR) technology.

(2) Linking of a target gene and cloning plasmid
A suitable cloning vector should be chosen first before ligation. The vector needs to meet the following conditions: first, it should have the ability to autonomously replicate;

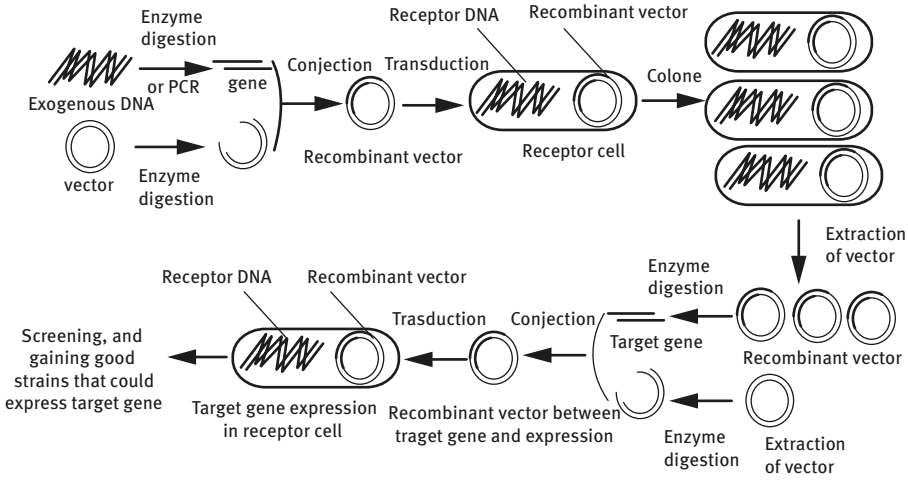


Figure 2.20: Method of genetic engineering.

second, there should be one or several recognition sites for restriction endonucleases; third, it needs to have one or several detectable genetic markers such as drug resistance, chromogenicity, and so on; finally, it should have multiple copies in the cell. A relaxed plasmid is usually used as the cloning vector. The target gene and the cloning vector are digested by the same restriction endonuclease to generate complementary cohesive ends, or the complementary cohesive ends are artificially linked to the ends of the vector and the target gene. The vector and target gene are joined using DNA ligase.

(3) Amplification of the recombinant vector being introduced into the receptor cell
The ligated recombinant vector is introduced into the recipient cell, which will be cultured on selective media. Positive clones are isolated and cultured in selective liquid media to amplify the target gene.

(4) Acquisition of the amplified target gene
The recombinant recipient cells are gathered by centrifugation. The recombinant vector is extracted and digested by the restriction endonuclease to recycle the target gene.

(5) Ligation of the target gene and the expression vector
As in step 2, a suitable vector should be first chosen. The expression vector should meet the following requirements: first, it has a promoter and terminator; second, there should be one or several recognition sites for restriction endonucleases; and third, it needs to have one or several detectable genetic markers. After the choice of vector, the target genes are handled in the same way as for step 2 and ligated to the expression vector.

(6) The recombinant vector is transferred into the recipient cell for expression.

(7) Screening and identification of the recombinants
A suitable way to identify the desired recombinants based on the expression of genetic markers and the characteristics of expression products is designed.

5.3.2 DNA Shuffling

DNA shuffling is also called sexual PCR. Its mechanism is as follows. A set of different original genes with the same functions are digested into random fragments using DNase I. These fragments are directly used for PCR without adding primers. In the PCR process, fragments with complementary homologous regions bind together by base pairing and are amplified with each other as templates and primers. This is when the genetic recombination happens. When the amplified DNA is the length of the parent DNA, the recombinant DNA is amplified by PCR with newly designed primers. The amplified DNA is then ligated to a vector to introduce into recipient cells. The desired strains are identified in the same way as traditional genetic engineering (Figure 2.21).

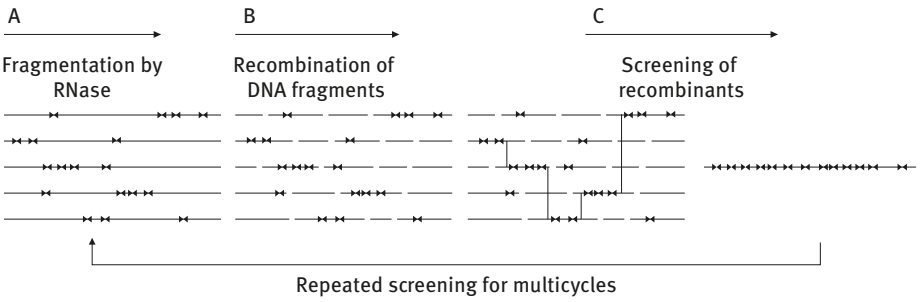


Figure 2.21: DNA shuffling [16].

5.3.3 Random Chimeragenesis on Transient Templates

The mechanism of random chimeragenesis on transient templates is as follows. A gene is chosen from homologous genes that have different origins but same function. It is used as a template for PCR during which 2'-deoxyuridine 5'-triphosphate (dUTP) is added to participate in the synthesis of the new DNA chain. The new synthesized double-stranded DNA is digested into single-stranded DNA that will be used as the transient template. Other homologous genes that are complementary to the above DNA are digested into single-stranded DNAs that will be degraded into random small fragments by a nuclease. These small fragments are annealed to the transient template. Parts of some fractions cannot bind to the template and can be in the single-stranded state whether the sequences overlap or mispairing is used for complementary binding. The single strands are sheared by a nuclease, and gaps will be left. The gaps between the random fragments are polished to form a complete chain. Then, the transient template is digested to release a heterozygous single chain that can be used as the template for PCR amplification. The desired strains are identified in the same way as the traditional genetic engineering (Figure 2.22).

5.3.4 Sequence Homology-Independent Protein Recombination

The mechanism of sequence homology-independent protein recombination is as follows. Two functionally related gene fragments are ligated into a gene dipolymer by a short DNA sequence that contains restriction enzyme sites. The dipolymer is cut randomly by DNase I to produce random fragments that are then treated by DNase S1 or T4 DNA polymerase to generate blunt ends. The random fragments with blunt ends are ligated to form circular molecules. The circular DNA is linearized by restriction endonucleases to obtain the chimeric gene products. Finally, the heterozygous genes are ligated into an expression vector and transferred into suitable recipient cells, or amplified by PCR to be bred in the same way as traditional genetic 0engineering [18].

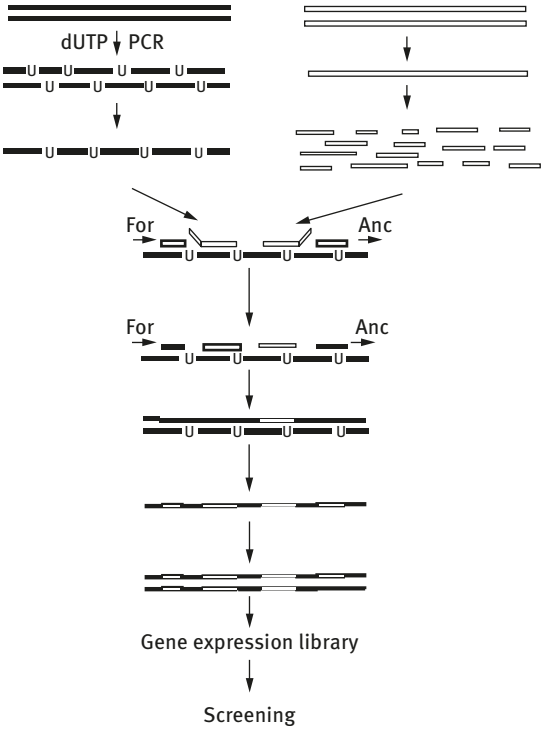


Figure 2.22: Random chimeragenesis on transient templates [17].

5.3.5 Gene Knockout and Knockin

Gene knockout and knockin are used to delete or introduce new genes to a strain. The constructed targeting vector is introduced into a target cell through transformation. The vector DNA is integrated at a certain site of the genome in the target cell by recombination between the vector DNA and homologous DNA sequences of the chromosome in the target cell, or replaces a certain gene section in the genome of the target cell. A marker gene is inserted at the same time that will change the hereditary character of the cell [19]. The cell chromosomes can be modified accurately by gene knockout and knockin methods. The modified genes can be replicated steadily by chromosome replication.

Gene knockout and knockin technology can be used to hinder metabolic shunt products or introduce mutations to change the yield or quality of the target product. In addition, gene knockouts can be used to increase the yield and purity of a fermented product along with a decrease in the amount of by-product and also change the biosynthetic pathway of a microorganism to obtain new products. Therefore, it is an important method of breeding for the fermentation industry.

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Yu Zhang, Qiong Wang, Guixiong Zhou, Cuiyi Liang, Xuesong Tan, Xinshu Zhuang

3 Microbial Biomass Pretreatment and Hydrolysis

Organisms that live in air, water, and land produce biomass through the process of photosynthesis. That is, the organic substance that can grow is known as biomass. In general, biomass includes plants, microorganisms, animals, and their waste products. Crops, crop waste, wood, and wood waste are the typical biomass. From a narrower perspective, biomass refers to straw, trees, lignocellulose that are produced during agricultural and forestry production processes, and other waste and dung from farming industry, except for food and fruit.

At present, the production of ethanol, butanol, and other biomass liquid fuels requires a series of steps including physical, chemical, or biological transformation, which involve pretreatment, enzymatic hydrolysis, and fermentation. Advantages of the use of microorganisms and enzymes over physical and chemical conversion methods include the use of milder conditions, less pollution, and there is less equipment required. This chapter introduces the properties and applications of lignin degradation microorganisms and its degradation enzymes: amylum, cellulose, and hemicellulose decomposition bacteria and related enzymes.

Section 1: Microbial Starch Hydrolysis and Amylases

Starch is the most abundant polysaccharide compound found in plant seeds, roots, and stems. Starch can be used for the production of glucose through amylase hydrolysis. Glucose can be easily digested and absorbed by the human body. The general designation of all enzymes catalyzing the hydrolysis of glycoside bonds in starch (including glycogen and dextrin) amylase is widely distributed in almost all plants, animals, and microorganisms. At present, amylase is mainly produced by microorganism fermentation. Many types of microbes, such as prokaryotes and eukaryotes, contain genes for the expression of amylase and have the ability to produce amylase. Currently, amylase is the most studied enzyme, is the earliest produced, the most widely used, and has the

Corresponding author: Xinshu Zhuang, E-mail: zhuangxs@ms.giec.ac.cn
Yu Zhang, Qiong Wang, Guixiong Zhou, Cuiyi Liang, Xuesong Tan, Xinshu Zhuang, Guangzhou Institute of Energy Conversion, Chinese Academy of Sciences; CAS Key Laboratory of Renewable Energy; Guangdong Provincial Key Laboratory of New and Renewable Energy Research and Development, Guangzhou 510640, China

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highest yield. Due to the large-scale applications in starch industry and food industry, especially since the 1960s, the demand for amylase has greatly increased. Although many new enzymes have been studied and applied, amylase is still the main carbohydrate-hydrolyzing enzyme, accounting for over 25% of the total enzyme production.

1.1 Amylolytic Microorganisms

1.1.1 The Composition and Structure of Starch

Starch is composed of amylose (10–30%) and amylopectin (70–90%). Amylose is composed of the long chains of glucose molecules connected by α-1,4 glycosidic linkages. In addition to α-1,4 glycosidic linkages, amylopectin contains more complicated structures, including α-1,6 glycosidic linkages and a small percentage of α-1,3 glycosidic linkages. The hydrogen bonds make amylose molecules coil into a helix formation with an average of six glucose units forming the helix. Addition of iodine causes iodine molecules to be embedded into the spiral structural space and connected to amylose through van der Waals forces. This compound uniformly absorbs visible light, except for blue light (400–750 nm); therefore, a starch solution has a blue appearance. Amylopectin molecules are also comprised of helixes, and the short average length of each branch is responsible for the small number of helical coils of each helix. When combined with iodine molecules, only short-chain complexes are formed. Thus, when mixed with iodine, a starch solution will show a light red color.

1.1.2 Classification of Amylolytic Microorganisms

Starch can be hydrolyzed by many different types of microbes. Moreover, a large number of highly active amylolytic microorganisms are present in bacteria, fungi, such as *Rhizopus*, *Mucor*, actinomycetes, and yeast. The predominant amylase producing bacteria is *Bacillus*, which has been widely used in industry. Amylase producing strains are also found among many types of fungi, of which *Aspergillus niger* and *A. oryzae* are glucoamylase superior industrial producing strains. Moreover, Actinomycetes in *Micromonospora*, and Actinomycetes such as *Micromonospora*, *Streptomyces*, and *Nocardia* can also hydrolyze starch with weaker activities (Table 3.1).

1.2 Classification of Amylase

According to different reaction modes on starch, amylase is divided into the following four types: Class 1, α-amylase, uses glycogen or starch as a substrate, and when the

Table 3.1: Amylolytic microorganisms.

Enzyme	Classification of strains
α-Amylase	*Bacillus subtilis, Bacillus licheniformis, Aspergillus oryzae, Aspergillus niger, Aspergillus kawachii, Aspergillus nidulans, Rhizopus* sp., *Rhizopus microsporus, Thermococcus* sp., *Thermobifida fusca, Streptomyces* sp., *Saccharomycopsis fibuligera, Schwanniomyces. occidentalis, Lipomyces kononenkoae, Chalara paradoxa, Thermomyces lanuginosus, Clostridium acetobutylicum, Aeromonas media,* etc.
β-Amylase	*Bacillus cereus, Bacillus megaterium, Bacillus coagulans, Bacillus polymyxa, Bacillus licheninformis, Bacillus subtilis, Thermoanaerobacterium, Thermoactinomycete,* etc.
Glucamylase	*Aspergillus awamori, Aspergillus foetidus, Aspergillus niger, Aspergillus oryzae, Aspergillus terreus, Mucor rouxians, Mucor javanicus, Neurospora crassa, Rhizopus delmar, Rhizopus oryzae, Chaetomium thermophile, Thermomyces lanuginosus, Thielavia terrestris,* and *Arthrobotrys amerospora.*
Isoamylase	*Aerobacter aerogenes, Pseudomonas, Bacillus* sp., *Klebsiella oxytoca, Thermomonos poraceae, Thermus, Lipomyces kononenkoae, Clostridium thermosulfurogenes,* etc.

intramolecular α-1,4 glycosidic bonds are cut, glucose is formed. And the limit dextrin and oligosaccharides that contain more than four glucose residues are also formed. Class 2, β-amylase, sequentially hydrolyzes from the nonreducing end of the starch molecule and cuts a maltose unit from each α-1,4 glycosidic bond. Class 3, glucoamylase, is short for glucamylase, and hydrolyzes sequentially from the nonreducing end of the starch substrate and branches glycosides and key glucogenic residues from α-1,4 and α-1,6 glycosidic bonds. Class 4 is a branching enzyme called isoamylase or pullulanase, which can only hydrolyze α-1,6 glycosidic bonds of glycogen or amylopectin branching point, and cuts the entire collateral, thereby forming different lengths of amylose. In addition, recent studies have demonstrated that numerous amylases with novel features have been found. These include, for example, maltotetraose amylase, maltopentaose amylase, maltooligosaccharide amylase, and raw starch hydrolysis enzymes.

1.2.1 α-Amylase

α-Amylase, classified as 1,4-α-D-glucan glucanohydrolase (EC 3.2.1.1), is an endoamylase that hydrolyzes catalytic starch to dextrin, and the molecular weight is about 50 kDa. α-Amylase is widely found in animals, plants, bacteria, and fungi and is a class of hydrolytic enzymes that is closely related to human α-amylase. The majority of α-amylase derived from microbial sources belong to glycosyl hydrolase family 13 and have similar characteristics, including (1) acting on α-glucosidic linkages of the starch molecule, thereby forming monomers or oligomers; (2) having a $(\beta/\alpha)_8$ TIM

barrel, containing catalytic site residues; (3) having four conserved regions, some of which are conserved catalytic sites of amino acids, whereas others maintain some of the conserved TIM barrel topology with important effects.

1.2.1.1 Hydrolysis Mechanism of α-Amylase

α-Amylase acts on starch and glycogen, randomly cuts α-1,4 glycosidic bonds, and generates the α-configuration of maltose, a small amount of glucose, and oligosaccharides and maltodextrin with a different molecular weight. When α-amylase uses amylose as a substrate, the reaction is generally carried out in two steps. First, amylose is quickly degraded to the small molecules dextrin, maltose, and maltotriose. In this step, α-amylase basically reacts in a random manner based on the type of starch. The viscosity of amylose drops rapidly in this step. The second step of the reaction is much slower than the first step and allows oligosaccharides to slowly hydrolyze to glucose and maltose. Thus, α-amylase finally produces glucose, maltose, and a range of limit dextrin (manufactured by four or more oligosaccharides composed of glucose), and oligosaccharides. However, the different sources and characteristics of α-amylase that hydrolyze limit dextrin is significantly different. This also explains that the different characteristics of α-amylase hydrolysis and the branched α-1,4-glycoside bond are different. In general, α-amylase hydrolysis of starch is carried out as follows: the α-amylase catalytic site is the cleavage site and α-amylase has 5–10 cleavage sites, each cleavage site hydrolyzes hydrogen bonds and hydrophobic interactions.

The mechanism of the α-amylase reaction involves multiple steps. McCarter and Withers [1] stated that the α-amylase catalytic reaction includes three steps and that the displacement reaction occurs twice. In the first step, the sugar residue acts as a substrate in the active site binding and the sub-binding sites of the enzyme are the active site of -1. The glycosidic oxygen atom O acts as a proton donor to acidic amino acids as protonated (e.g. Glu261). In the second step, -1 sub-binding sites and other nucleophilic amino acids (such as Asp231) of the sugar residue attack the anomeric C1 carbon atom of the sugar residue of nucleophilic amino acids, thereby forming a covalent intermediate with the substrate. Cleavage of the C1–OR bond occurs at the same time, and the aglycone portion of the substrate is replaced. In the final step, water molecules are activated by deprotonated Glu261, and the covalent water molecules bond hydrolyzes the nucleophilic oxygen atom and the sugar residues between C1 and Asp231 swap. This causes replacement of the enzyme molecule of nucleophilic Asp231 residues and completes the hydrolysis reaction. In the second displacement reaction, if the water molecule is not the offensive group but a free hydroxyl group of sugars (oligosaccharides) ROH, then, after displacement of nucleophilic Asp231 residues, transglycosylation hydrolysis reactions take place rather than transesterification. This is also a reason in which glycoside hydrolases and glycosyltransferases are considered a family of α-amylase.

1.2.1.2 Characteristics of α-Amylase Catalysis

Each α-amylase molecule contains a calcium ion (Ca^{2+}). The enzyme molecule is strongly combined with calcium ions, and α-amylase with calcium ions are removed under low pH conditions and in the presence of a chelating agent. If the enzyme molecule of calcium is completely removed, it will reduce stability and inactivate the enzyme by heat, acid or urea, and other degenerative factors. The calcium-induced changes in the molecular structure of amylase only changes the secondary structure, and is not involved in the construction of the enzyme-substrate complex. Therefore, it does not change its tertiary structure directly, but maintains the optimal conformation of the enzyme so that the enzyme has the highest activity and stability. In addition, α-amylase amino acid content is high in dihydroxy aspartic acid and glutamic acid, whereas the content of sulfur-containing amino acids as methionine and cysteine are particularly low, which may maintain the stability and catalytic activity of α-amylase.

In general, α-amylase is stable at a pH between 5.5 and 8.0, which is the optimal pH. A pH value of 4.0 or less will easily inactivate the enzyme. However, varies sources of enzymes have a different optimal pH. The α-amylase derived from animal has an optimal pH of 7.0 when in the presence of chloride ions, whereas α-amylase derived from plants will be inactivated at a pH of 3.6 or less. Acid resistance of α-amylase has an optimal pH of 4.0, and is stable between a pH of 2.5 and 6.5.

Activity of α-amylase is greatly influenced by temperature, and when the temperature increases, the reaction rate of the enzyme increases. In general, when the temperature is increased by 10 °C, the reaction rate is increased one- to twofold. However, excessively high reaction temperatures may cause denaturation of the enzyme and loss of catalytic activity, thereby decreasing the reaction rate of the enzyme. The thermos-tolerance from different sources of α-amylase is not the same. Usually, thermos-tolerance of α-amylase is animal α-amylase> malt α-amylase> filamentous fungus α-amylase> bacterial α-amylase. Purification of α-amylase above 50 °C results in the deactivation of the enzyme. When starch is hydrolyzed, the thermos-stability of the enzyme will increase when a large number of Ca^{2+} ions, starch, and dextrin are present.

1.2.2 β-Amylase

β-Amylase is also known as the starch 1,4-maltoside enzyme (1,4-α-D-glucan maltohydrolase, EC 3.2.1.2). β-Amylase is an exo-type glucoamylase that is widely distributed in plants and microorganisms, but is not present in mammals. Plants are the main producers of β-amylase, and in general, β-amylase is present alone or coexists with α-amylase. The reactions of β-amylase in microorganisms and plants are substantially the same, but the thermotolerance of β-amylase in microorganisms is superior, and can absorb and hydrolyze raw starch. However, compared with α-amylase and glucoamylase, the hydrolysis efficiency of raw starch is relatively low.

1.2.2.1 Hydrolysis Mechanism of β-Amylase

When β-amylase acts on starch, it can cut a maltose unit from a nonreducing end of the next α-1,4 glycosidic bonds, thereby producing maltose and macromolecules of limit β-dextrin. Due to the enzyme reacting with substrate, a Walden inversion was performed, and the product was changed into an α-β-maltose type, named β-amylase. The hydrolysis of β-amylase to amylopectin does not affect the α-1,6 glycosidic bond, and is unable to go through the amylopectin branching point to act on the α-1,4 glycosidic bond. It stops while encountering a branch point, leaving the 1–3 glucose residues at the branching point, resulting in the hydrolysis end products maltose and limit β-dextrin. Thus, β-amylase acting on amylopectin is an incomplete reaction, it needs to be combined with α-amylase and hydrolyzed together.

1.2.2.2 β-Amylase Hydrolysis Characteristics

The effects of α-amylase and β-amylase are opposite. The Ca^{2+} stability of β-amylase can be reduced at 70 °C, and a pH of 6 to 7 in the presence of Ca^{2+}. Thus, β-starch inactivates the enzyme-purified α-amylase. The optimal pH of different microbial-derived β-amylase is different, therefore the stability may also be different (Table 3.2).

1.2.3 Glucoamylase

Glucoamylase (EC 3.2.1.3), also named α-1,4-glucan-glucohydrolase, is an enzyme containing a single-chain acid that has glycoside hydrolysis activity, and having excision enzyme activity. It will be fully hydrolyzed from starch to glucose; it is generally used for starch saccharification, so it is customary to call glucoamylase. It enables to change the conformation of glucose conformation, and formate β-D-glucose.

Table 3.2: Characteristics of β-amylase from different microorganisms.

Strain	Optimal temperature (°C)	Optimal pH
B. polymyxa ATCC8523	37	6.8
B. polymyxa NCIB8158	30	6.8
B. polymyxa AS1.546	45	6.5
B. megaterium	60	6.9
B. cereus	40–60	6.0–7.0
B. circulans	60	6.5–7.5
B. subtilis	65	6.0
Pseudomonas sp.	50	6.5–7.5
Nocardia sp.	60	7.0
Sulfobacillus thermosulfidooxidans	75	5.5

1.2.3.1 Hydrolysis Mechanism of Glucoamylase

Glucoamylase (GA, EC 3.2.1.3), also known as amyloglucosidase or γ-amylase, is an exo-amylase. This type of amylase has a lower substrate specificity, and is cut from not only the nonreducing end of the α-1,4 glycosidic bond of the starch molecule but also from α-1,6 and α-1,3 glycosidic bonds. However, the hydrolysis rates of the latter two bonds are slower. Glucoamylase can hydrolyze starch molecules and larger molecules and oligosaccharides that have single-chain functions, whereas smaller molecules of hydrolyzed oligosaccharides have multi-chain functions. The larger the substrate molecule, the faster the hydrolysis rate. The enzymatic hydrolysis rate is also affected by the next bond in the molecular arrangement. Panose contains an α-1,6 glycosidic bond that can be hydrolyzed by glucoamylase, while the hydrolysis of isomaltose, which contains an α-1,4 glycosidic bond, is challenging. Moreover, hydrolysis of isomaltosic maltose, which contains two α-1,6 glycosidic bonds, is not possible. It is harder to hydrolyze glycogen containing many branches, therefore the hydrolysis rate of amylopectin is controlled by the number of α-1,6 glycosidic bonds.

1.2.3.2 Characteristics of Glucoamylase Hydrolysis

Glucoamylase is a glycoprotein with a relative molecular mass of about 6.9 kDa. Glucoamylase producing strains tend to produce different kinds of isozymes. In addition, different sources of glucoamylase have different isoelectric points, which are dependent on the amino acid composition. Furthermore, the optimal saccharification temperature and pH are different due to the different sources of glucoamylase. The characteristics of glucoamylase are presented in Table 3.3.

Table 3.3: Characteristics of glucoamylase.

	Characteristic
Relative molecular mass (Da)	50,000–112,000
Carbohydrate content	3.2–20%
Isoelectric point (PI)	3.4–7.0
The optimum pH	4.0–5.0
pH stability	3.0–7.0
The optimum temperature	40–60 °C
Thermostability	Below 60 °C
Ca^{2+} ions	No
Hydrolysis of the substrate	Amylose, amylopectin, dextrin, glycogen, maltose
Catalytic bond	α-1,3, α-1,4, α-1,6 glycosidic bonds
Cut mechanism	Exterior
Origin strains	*Rhizopus*, *Aspergillus*, *Neurospora*

1.2.4 Isoamylase

Isoamylase (IAM, EC 3.2.1.9) is a common debranching enzyme, also known as glycogen 6-glucanohydrolases, and its system name is amylopectin α-1,6 glucan hydrolase. Isoamylase specifically acts on branch points of, for example, amylopectin and glycogen, and was first discovered in 1940 by Maruo et al. in yeast cell extracts. Previous studies indicated that the role of isoamylase involves the conversion of rice starch to a glutinous substance, which a few days later forms a white precipitate. Under iodine, the reaction color turns from red to blue, and the starch polymerization degree increases. Therefore, it has long been mistaken for a synthetic amylase, named starch synthase. However, later studies showed that the change in reaction color was due to the specific properties of branched starch and amylose. It was also verified that the effect of the enzyme was not due to "synthetic" starch, but because of "cut off" of α-1,6 glycosidic amylopectin bonds; therefore, the enzyme was renamed to debranching enzyme or isoamylase. There are strict requirements for isoamylase to act on the α-1,6 glycosidic bond. Isoamylase can only hydrolyze a constitute branch point of α-1,6 glycosidic bonds but cannot hydrolyze linear molecules consisting of α-1,6 glycosidic bonds.

Another example of a debranching enzyme is pullulanase (EC 3.2.1.41, also known as pullulan enzyme), which is an enzyme that catalyzes α-1,6 glycosidic bonds in amylopectin, pullulan, and limit dextrin; therefore, it is also known as pullulan 6-glucan hydrolase. Pullulanase can hydrolyze amylopectin with corresponding β-limit dextrin α-1,6 glycosidic bonds, and can cleave the combination of α-limit dextrin α-1,6 glycosidic bonds of maltose and α-maltotriose residues. However, it is unable to remove glucose units from α-1,6 glycosidic bonds. Moreover, pullulanase cannot act on glycogen, but can degrade amylopectin by using Prussian blue as the primary substrate. The second best substrate is starch, and the catalytic product is mainly IMO. Therefore, pullulanase is suitable for use in both the food and pharmaceutical industry.

Different sources of substrate specificity result in different actions of amylase. This is mainly manifested in decomposing various branched oligosaccharides and pullulan. The activity on amylopectin and glycogen is high, and can be completely debranching; however, the side chains of β- and α-restrictions of limit dextrin, which consist of two or three glucose units. are not hydrolyzed. Moreover, the activity on pullulan (both α-1,4 and α-1,6 glycosidic bonds) is very low.

1.2.5 Other Amylases

Previous studies have shown that, based on the characteristics of the enzyme, different amylases have different modes of action. For example, cyclodextrin glycosyltransferase (CGT enzyme, EC 2.4.1.19) not only has the same hydrolytic activity but also

has four different catalytic transglycosylation reactions (hydrolysis, disproportionation, coupling, and cyclization). Cyclodextrin glycosyltransferase can cut six or seven glucose chains from the end of the starch molecule and form a circular structure, which may be transferred to a straight rear maltose group or another straight-chain receptor. It is mainly used in the production of circular dextrin. Moreover, α-1,6-glucosidase is capable of hydrolyzing α-1,6 glycosidic bonds of starch to form maltose (hydrolyzes two glucose residues from a branched structure). Correspondingly, the starch Branching Enzyme (referred to as BE, EC 2.4.1.18) belong to glycoside hydrolase family (GH13). The BE enzyme can be widely used for modifying starch by catalysis to form α-1,6 glycosidic bonds. On the one hand, the role of cyclodextrin glycosyltransferase is mainly the degradation of linear α-1,4-glucan chains (amylose and amylopectin linear region). On the other hand, it is attached to α-1,6 glycosidic bonds by the degradation of sugar receptor fragments, thereby increasing the molecular density of starch. In addition, G4, G5, and G6 starch forming enzymes hydrolyze maltooligosaccharide and are able to cut off the starch from nonreducing ends of glucose molecules, forming a considerable number of oligosaccharides.

Due to different sources of amylases, the characteristics of amylase may also be different. Some species may have enzymes with multiple roles. Similar to cellulases, these amylases may have synergistic effects in the catalytic hydrolysis of starch. Glucoamylase is required for the production of glucose; however, the formation rate of glucose is rather low. The addition of α-amylase helps to improve the overall yield of glucose. Thus, accelerating the rate of the glucogenic enzyme can be achieved by using a combination of α-amylase, β-amylase, and glucoamylase.

1.3 Screening of Amylolytic Microorganisms

In recent years, the screening of amylase producing microorganisms has primarily focused on extreme enzymes that are produced by microorganisms that can grow under extreme conditions, such as a low or high pH, low or high temperatures, and maintain a high yield and high enzyme activity.

Thermostable α-amylase usually refers to enzymes with optimal reaction temperature of 90–95 °C and a thermostability of above 90 °C. In starch liquefaction terms, thermostable α-amylase, when compared with amylase that is produced by normal bacteria producing amylase, can save energy, reduce costs, and has a high thermostability. Therefore, it is stable in a wide range of storage conditions, is easy to store and transport, and is widely used in wine, food, pharmaceutical, and textile and other industries. Moreover, it is used for environmental treatment. In foreign countries, α-amylase producing strains with a high thermostability have been separated from thermophilic fungi, thermostable actinomycetes, and especially from thermophilic bacterium *B. stearothermophilus* and *B. licheniformis*. Cold active amylase is generally

produced from microorganisms that tolerate a low temperature. In general, they have an optimal temperature of less than 30 °C, and maintain enzymatic activity at 0 °C. In recent years, this has gained interest in research because tolerating lower temperatures has a very important effect on starch-free cooking processes, low-temperature food processing, and chemical pharmaceutical purposes.

Acid α-amylase has a wide industrial application, with a reaction pH ranging between 4.0 and 5.0. Due to maintaining a high activity under acidic conditions, this enzyme is widely used for the fermentation of corn starch to wine, lactic acid fermentation, in sugar and food industries. Moreover, acid α-amylase has great potential in applications in many fields. It is not only used in simplifying the liquefaction and saccharification process, and reducing the production costs of starch processing, but is also used in increasing the concentration of maltose syrup, developing novel digestives, and in industrial wastewater treatment area. In recent years, scientists from all over the world have become interested in screening acid amylase producing microorganisms.

In view of the molecular biology field, a new path has opened for the rapid development of genetic engineering technology for the efficient production of amylase. Genetic engineering of the amylase molecule includes three major aspects: (1) chemical modification of the enzyme, (2) site-directed mutagenesis, and (3) directed evolution. Numerous amylase genes were successfully expressed in *Escherichia coli* (*E. coli*) and *Pichia pastoris*. Tamamura et al. [2] used *Bacillus* sp. AAH-31 (AmyL) producing thermophilic salt amylase as research objects. The amino acid sites of substrate acceptors were optimized and used to operate the enzymes Y426S/K549M, H431A/K549M, and I509A/K549M sites with double-point mutations, which then showed increased activity of 340%, 252%, and 271%, respectively. Mehta et al. [3] mutated the *Geobacillus thermoleovorans* (Gt-MamyIII) of maltogenic amylase of the seven amino acids of four salt bridge positions. Since the connection between the buried salt bridge network domains was exposed, Gt-MamyIII played an important role in the thermostability, which may provide guidance to improve the thermostability of amylase.

Currently, studies in China have also focused on amylase gene cloning from extreme microorganisms. Yang et al. [4] used *Bacillus alcalophilus basophils* and successfully expressed the thermostable amylase gene in *B. subtilis* WB600. Liu et al. [5] applied the thermophilic *B. amyloliquefaciens* α-amylase gene fragments of which the three sites Asp231, Asp233, and Asp438 were mutated to Asn231, Asn233, and Gly438, respectively. After introducing the mutations, the enzyme activity showed varying degrees of increase. Niu et al. [6] demonstrated an overall restructuring of *Pyrococcus furtosus* α-amylase genes (Figure 3.10). After a specific PCR amplification, a plasmid pSK-amyF was combined, and pBL-amyF was expressed in *B. licheniformis*. The results indicated an optimal pH of 5.0 and an optimal temperature of 95–98 °C. This recombinant enzyme production has greatly improved compared to previous studies that focused on other recombinant strains.

1.4 Application of Amylase

As an important industrial enzyme in starch hydrolysis, amylase was initially used as a drug for the treatment of symptoms of digestive disorders. Presently, applications of amylase have been extended to food processing, textile industry, paper making, detergents, pharmaceuticals, analysis of clinical chemistry, food additives, environmental management, exploration for oil, fuel production of ethanol and butanol, and many other industries.

1.4.1 Food Processing

Glucose production by enzymatic hydrolysis is a major innovation in glucose industry since the 1960s. The main reaction mechanism involves α-amylase that selectively liquefies starch in cereals, and includes corn and crude material such as manioc to produce glucose. Starch syrup that is produced from amylase hydrolysis is also known as liquid glucose, and includes small amounts of maltose and maltotriose, and 35% is dextrin with polymerization more than 7, therefore its flavor is more bitter than that of caramel. However, due to the low moisture absorption, liquid glucose is widely used to produce candy. In Japan, liquid glucose is combined with bacteria to produce α-amylase, which can be saccharificated to produce water syrup that has a similar composition as acid syrup. Moreover, because it contains a large amount of isomaltosyl maltose, this maltoletraose has a sweet taste, and can be used to flavor products. After enzymatic conversion, starch is converted to high-fructose corn syrup (HFCS). Due to the high level of sweetness, HFCS is used as soft drink and sweeteners.

In addition, other types of amylase are also widely used in food processing industries, and some ordinary types of amylase are used in food processing as shown in Table 3.4.

Table 3.4: Applications of amylase in food processing.

Enzyme	Applications
α-Amylase	Manufacture of dextrin, maltodextrin, preservatives, antiseptics
α-Glucoamylase	Production of glucose, pretreatment of fermentation material
α-Amylase, β-amylase	Production of caramel, maltose, beer production
Pullulanase	Production of amylose
Glucoamylase pullulanase	Production of glucose
β-Amylase pullulanase	Production of maltose, high maltose syrup
α-Amylase, glucoamylase	Production of fructose, high fructose corn syrup, sweetener
α-Amylase, cyclodextrin glycosyltransferase	Production of cyclodextrin

1.4.2 Textile Industry

In modern fiber manufacturing processes, there will be an increase in the use of large amounts of bacteria in the process weaving of yarn. To prevent yarn from not being cut, a removable protective layer is generally added to the surface of yarn. Many materials can be made from these surface layers, among which starch is an ideal good choice, because of its low costs, easy accessibility, and easy removal. By using α-amylase, starch can be selectively removed without damaging the yarn fibers. In addition, starch can be randomly degraded into dextrin, which is soluble in water and can easily be washed off. With hydrolysis of the starch slurry by the efficient and specific activity of amylase, there are high values of enzyme desizing, such as smaller mechanical action of desizing, the less water using, the more faster desizing, and – low pollution level, it can act at a low temperature condition to achieve the desizing effect, the products are better than the acid- or alkali methods, and more flexible. And that, it does not broke the fibers and has – environment – friendly.

1.4.3 Paper Production

In the paper industry, amylase is mainly used for modifying starch paper coatings. The paste on the paper primarily protects the paper from mechanical damage during pretreatment and improves the quality of paper production. The paste is a sufficient paper coating that not only improves the hardness and strength of the paper but also enhances the removing of paper. When the paper passes through two rollers, starch pulp is added, and this process is controlled at a temperature of 45–60 ˚C, to give the starch a stable viscosity. Through the grinding process, the viscosity of starch also controls different grades of paper. The concentration of natural starch is too high for paper sizing, and can be regulated by α-amylase hydrolysis.

1.4.4 Detergents

Enzymes are an important family member of efficient modern detergents. Amylase can remove dirt by degrading starch. The distinct feature of enzymes in detergents is their cleaning role under mild conditions. From 1975, α-amylase has been applied to detergents, and may be combined with proteases, lipases, and nonionic surface active agents, used in dishwashing detergents or detergent additives. It has been demonstrated in a previous study that by the substitution of methionine of *B. licheniformis* α-amylase protein on site 197, leucine greatly enhanced the oxidative stability in resistance to oxidant enzyme composition, and also enhanced enzyme stability during storage. Due to the increasing needs, foreign countries have successfully used

alkaline α-amylase in the production of industrial detergents. Another study reported that amylase can prevent contamination after low molecular weight starch degradation and prevent the redeposition of rust-like particles.

1.4.5 Alcohol Fuels

The traditional method of starch fermentation to produce ethanol and butanol fuels requires the use of high temperatures and a pressure cooking process. Nevertheless, the energy consumption of this process accounted for 30–40% of the total energy consumption throughout the entire process. Raw starch can be directly hydrolyzed and saccharified by amylase, thereby eliminating the need for a high temperature cooking pasting process, and avoiding high temperature conditions and the loss of fermentable sugars. This lowers energy consumption, simplifies the operation process, and reduces production costs. Although the process of amylase hydrolysis of starch to produce ethanol and butanol is very mature, new strains and novel technologies are constantly being discovered, which will help reduce the production costs of ethanol, and butanol fuel to supply the gap of fossil fuels.

Section 2: Microbial Lignin Degradation and Ligninolytic Enzymes

Lignin is a complex, high molecular weight compound, which is present in most land plants. Together with cellulose and hemicellulose, they constitute the basic skeleton of a plant. In woody plants and herbaceous plants, the lignin proportion is about 20–35% and 15–25%, respectively. Lignin is the second richest organic material on earth. In fact, it is considered that lignin is synthesized in a plant's secondary metabolism process and that it functions to support mechanistic properties, prevent organism degradation, and transport water. Lignin-glucide compounds, which are formed by cross-linking lignin and several glycosyl groups of hemicellulose, cover the net structure on the surface of microfilaments, which are composed of cellulose. Through the linkage between lignin and glucide, lignin connects with hemicellulose that exists in microfilaments to form the compact lignocelluloses structure. Because of this compact structure, raw lignocelluloses need to be pretreated when utilized as a resource to remove lignin, and decrease the crystallinity of raw lignocellulosic materials.

Common lignocelluloses pretreatment methods include physical methods, chemical methods, and biological methods. Compared with physical and chemical methods, biological methods have major potential to further develop, because of its advantages such as mild action conditions, low energy consumption, strong

specificity, nonenvironmental pollution, and low disposal costs. Biological pretreatment methods utilize lignin-degrading microorganisms to degrade lignin from lignocellulose-containing materials. In these methods, not only the lignin itself is removed but the structure of lignocelluloses materials is also destroyed. Therefore, studies that focus on lignin-degrading microorganisms are of significant importance for utilization of raw lignocelluloses as a resource.

2.1 Lignin and Lignin-Degrading Microorganisms

2.1.1 Constitution and Structure of Lignin

Lignin is a natural aromatic high molecular polymer, which has a complex structure. Commonly, it is considered that the steric macromolecule of lignin is composed of its basic unit phenyl propane by connecting C–C linkage and ether linkage. It contains various active functional groups in which ether linkages account for 60–75%, and carbon bonds account for 25–30%. Natural lignin cannot be resolved in water and most other solvents and can hardly be hydrolyzed by acids and enzymes, and is one of the most difficult high polymers to be degraded.

Based on the different basic unit structure, lignin can be classified into three types: (1) hydroxyphenyl lignin, composed of ethyl-*para*-hydroxyphenyl propane units, (2) guaiacyl lignin, composed of guaiacyl propane units, and (3) syringl lignin, composed of syringl propane units. The three basic unit structures are shown in Figure 3.1.

Based on the biosynthetic process, these three units are first transferred from glucose to Shikimic acid through aromatic ring action, then the above lignin basic units are formed by Shikimic acid. Finally, these three units polymerize to form several basic plant compositions with a higher molecular weight. A total of six most common basic structures of lignin exist, which are shown in Figure 3.2.

Through random combination and co polymerization, the basic units form the polymer lignin, whose structure is uneven, nonoptical, crossing, and highly dispersible (Figure 3.3).

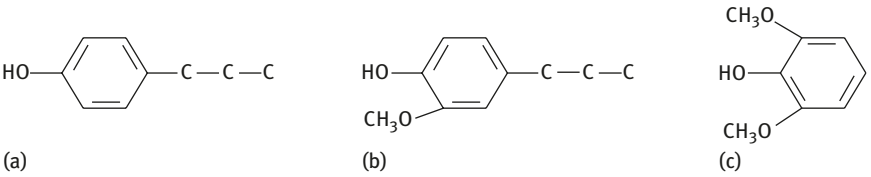


Figure 3.1: Basic unit structure of benzene propane.

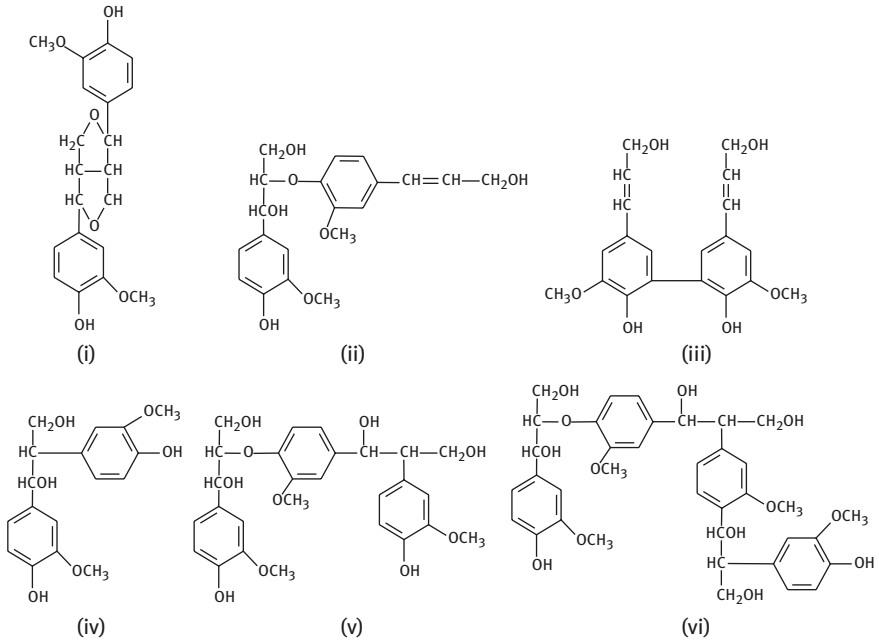


Figure 3.2: Six common basic structures of lignin.

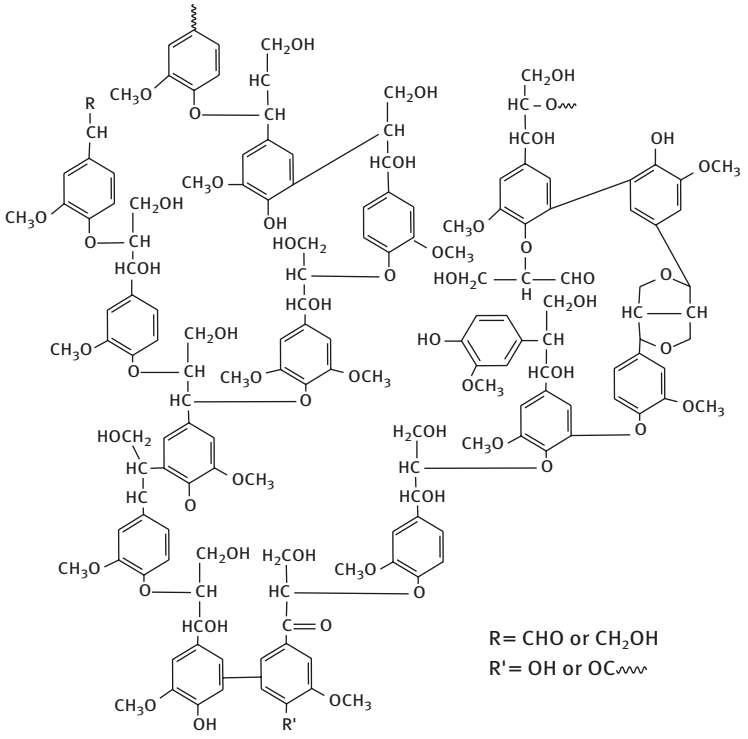


Figure 3.3: Structure of the lignin molecule.

The lignin structure contains many functional groups, including -OCH_3, -OH, and -CO. The existence and distribution of these functional groups within the lignin structure are relevant to the type of lignin. Extraction methods will influence separation of the lignin structure. The existence of many functional groups causes lignin to possess multichemical properties to take various actions.

2.1.2 Classification of Lignin-Degrading Microorganisms

In nature, only a few organisms can degrade lignin and produce lignin-degrading enzymes simultaneously. The complete degradation of lignin depends on cooperation of fungi, bacteria, and the relevant microorganism community. Fungi play the leading role in the degradation process. According to the color presented in the wood decay process, fungi that degrade lignin can be classified into three types: white-rot fungi, *Monilinia* spp., and soft-rot fungi. In addition, there are other fungi, which are capable of degrading lignin, such as the commonly found strains *Trichoderma viride* and *T. koningii*. Previously, it was reported that microorganisms that have better capacity to degrade lignin mostly belong to the white-rot fungi. Because of the extracellular oxidase that white-rot fungi secrete, these fungi are regarded the primary microorganisms to degrade lignin. During a 30d degradation period, white-rot fungi have a better capacity to degrade 30–40% lignin. Moreover, white-rot fungi and *Monilinia* spp. belong to Basidiomycetes, while soft-rot fungi belong to Ascomycetes or fungi *Imperfecti*. White-rot fungus is a type of mycelial fungus, which first degrades lignin that is present in lignocellulose without producing pigment. This results in the formation of white decay in wooden materials. Currently, white-rot fungi that are frequently being studied include *Phanerochete chrysosporium*, *Coriolus versicolor*, *Thametes versicolor*, *Pleurotus ostreatus*, and *Bjerkandera adusta*. Among these fungi, *Phanerochete chrysosporium* is being widely studied as the model fungus for lignin degradation. *Monilinia* spp. can decay celluloses derived from wood, but has little effect on lignin. Soft-rot fungi have a better capacity to degrade polysaccharides than lignin. In general, it can degrade cellulose but completely maintains lignin. Most soft-rot fungi can decay from lumen to compound middle lamella. Soft-rot fungi and *Monilinia* spp. have a higher capacity to degrade cellulose compared to lignin. First, they start to degrade lignin and secrete tawny pigment to make woods tawny, then they begin to partly and slowly degrade lignin.

Apart from fungi, bacteria are also an important participator in the lignin biodegradable process. However, most studies have shown that the lignin degradation capacity of bacteria is weaker than that of fungi. Bacteria mainly play an indirect and supplementary role in the lignin biodegradable process. They only change, to a certain extent, the structure of lignin so that it can be dissolved in water. Bacteria seldom mineralize lignin. However, because of the extensive bacterial sources, rapid growth rate, easy large-scale application, various microorganisms are needed to play

different roles in the lignin degradation process. Therefore, quite a few studies are committed to focus on the role of bacteria in the lignin degradation process. Additionally, bacteria mainly play an indirect role in the degradation process, that is, bacteria cooperate with soft-rot fungi to make lignin easily accessible for other fungi. Moreover, this allows for the removal of substances that are poisonous to the rotten fungi. In fact, when bacteria degrade aromatic compounds ranging from simple phenol compounds to complex high polymer lignin, various metabolic pathways are displayed in the degradation process. The study on the bacterial role in degrading lignin started decades ago. In the 1950s and 1960s, it was shown that wood decay was related to bacteria. Research in the 1980s and 1990s showed that bacteria can degrade and metabolize dioxanonane lignin from cottonwood, vulcanized lignin with low molecular weight, and Kraft lignin fragments. From then on, the bacterial function on lignin degradation gradually became clear. Bacteria can change the structure of lignin to increase water-solubility and produce a modified poly lignin, which can be dissolved in water and precipitated in acid. Numerous bacterial species can degrade lignin. These include actinomyces such as *Streptomyces*, *Arthrobacter, Micromonospora*, and *Nocardia*, which are considered as strong degradation bacteria. Because actinomyces can penetrate undissolved stromas such as lignocellulose, actinomyces participate in the initial degradation and humification of organics in neutral and slightly alkaline soil or compost. Among these are actinomyces. *Streptomyces* mold fungi such as *Streptomyces badius* can degrade as much as 20% of lignin. Some studies indicated that actinomyces played the leading role in lignin degradation in the later period of compost. Non filamentous fungus can also lead to lignin degradation, however only few studies focus on this. The main nonfilamentous fungi that can degrade lignin include *Acinetobacter, Flavobacterium, Micrococcus, Pseudomonas*, and *Xanthomonas*. The lignin degradation rate of nonfilamentous fungi is less than 10%, and nonfilamentous fungi can only degrade low molecular weight lignin and degradation products of lignin. However, it can strengthen the water-solubility of lignin during the degradation process.

2.2 Lignin-Degrading Enzyme and Its Mechanism

The spatial structure of lignin is not regular. Compared with other macromolecules, enzymes that degrade lignin should be more nonspecific. Lignin is a macromolecule polymer that is composed of inner structure units through the connection of C–C linkages and ether linkages. Therefore, the degradation of lignin relies on the oxidizing action but not on the hydrolytic action. Lignin degradation processes are mainly related to three lyoenzymes: lignin peroxidase (LiP), EC1.11.1.14; manganese peroxidase (MnP), EC 1.11.1.13; and laccase (Lac), EC 1.10.3.2. The typical structures of these three enzymes are presented in Figure 3.4 [7–9].

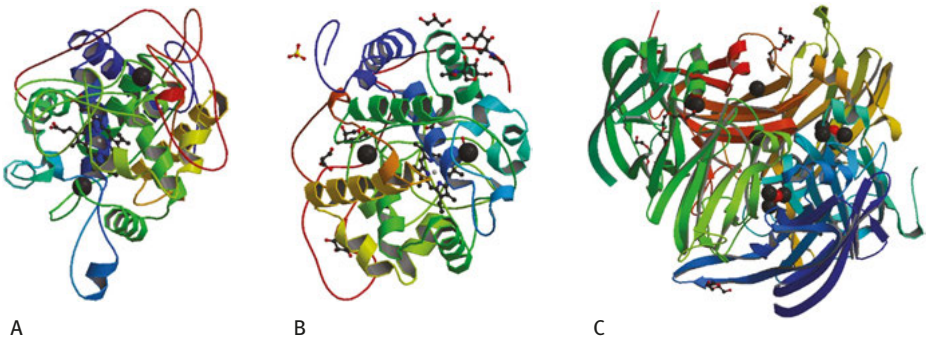


Figure 3.4: Structures of three typical lignin degradation enzymes. A: Lignin peroxidase, B: manganese peroxidase, C: laccase.

2.2.1 Lignin Peroxidase

LiP is also called lignin enzyme. It is a peroxidase that contains a series of Fe^{3+}, IX, and hemachrome prosthetic groups. LiP-producing microorganisms are widely distributed in nature. Many white-rot fungi and *Monilinia* spp. in decayed woods can produce LiP that has a molecular weight of 40 kDa, and isoelectric point of 3.5, and the optimal enzyme temperature is 35–55°C. The optimal pH value of LiP is 2–5. LiP was the first lignin degradation enzyme to be discovered in *Phanerochaete chrysosporium*. It features to oxidize phenols that are rich in electrons or to oxidize nonphenolic aromatic compounds so as to degrade lignin. When LiP attacks lignin through the electron transport system, it can capture an electron from a benzene ring of phenol or nonphenol and oxidize it to be a free radical. Subsequently, many different free radicals are being produced through chain reactions. This allows for main linkages of the lignin molecule to break, followed by several scission reactions. LiP can catalyze the breakage of Cα–Cβ linkage, which is located in the side chains of propyl. This scission reaction is regarded as the most important part in the lignin degradation process of white-rot fungi. The catalytic reactions of LiP mainly contain the following types: (1) benzyl alcohol oxidation, (2) break of C–C linkages, (3) hydroxylation, (4) demethoxylation, (5) demethylation, (6) oxidative dechlorination, and (7) dibasic phenol merization or polymerization. The catalyst degradation process is a series of free radical chain reactions that is started by H_2O_2.

2.2.2 Manganese Peroxidase

MnP and LiP are ectoenzyme peroxidases with glycosyl groups. They are also called hemachrome peroxidases because they contain hemachrome. The molecule structure

of MnP is similar to that of LiP. MnP is a type of glycosidoprotein with a hemachrome prosthetic group and Mn^{2+} constitutes its action center.

Mn^{2+}, as the necessary electron supplier in catalysis and oxygenation processes, can intermediate to produce enzyme, which lacks an electron transfer to the original state to produce Mn^{3+}. In this process, organic acid chelons such as oxalate and glycollate are necessary. Mn^{3+} is regularly produced by these chelons and can promote Mn^{3+} to be released from the active sites of enzymes.

Chelated Mn^{3+} is a transmittable oxidizer that can work in the relatively far distance to the MnP active site. However, it is unable to oxidize nonphenol structures, which takes great proportion in lignin. Moreover, it can only oxidize phenol structures, which account for 10% in lignin and have stronger resistibility through Cα-aryl residue linkage breaks and other degradation reactions. Because the chelated Mn^{3+} molecule is small, it can penetrate wood and start degradation to promote the attacks of LiP with a stronger oxidability.

2.2.3 Laccase

Lac is a protein that can catalyze the lacquer solidification process, and was first identified from the lacquer liquid of *Rhus verniciflua*. Most Lacs are distributed in microorganisms, such as Basidimycetes, *Polyporus*, and *Podospora*. The molecular weight of Lac is 64–390 kDa. Except for the Lac that is produced by *Podospora anserina*, which is a tretramer, all other Lacs are single polypeptides that are composed of about 500 amino acids. The isoelectric point of Lac is partially acidic. Its enzyme activity is the highest when the pH is between 3.5 and 7.0. Lac can simultaneously participate in multiple reactions during the lignin degradation process, and it can use oxygen as the oxidizer. Therefore, H_2O_2 is not required to participate in this process. Lac is a Cu-containing polyphenol oxidase. It can catalyze phenols' redox reactions, and plays an important role in the biological degradation of lignin. Lac is a type of protease that uses O_2 as an electron acceptor. Regarding its mechanism of action, what has currently been mastered is the polyphenol compound catalytic process, such as hydroquinone. This process requires four times a single electron transfer.

First, hydroqueinon substrate transfers an electron to Lac and produces a semiquinone-oxygen free radical intermediate. Then, two semiquinone molecules are transferred to one hydroquinone molecule, and one benzoquinone molecule, and oxygenfree radical intermediates can be transferred to carbon free radical intermediates. These two intermediates can be combined or coupled with each other. In the thallis interior, Lac cooperates with other enzymes, which can oxidize lignin and degrade it.

In the external environment of thallis, when O_2 exists, lignin units will undergo polyreaction with Lac. The deoxygenized Lac is being oxidized, and O_2 is being

deoxygenized to be water. This process occurred through the cooperation of four copper ions of electron transfer and valence change. In this reaction, Lac receives an electron from the oxidized substrate molecules to form a free radical, which is not stable and can undergo further polymerization and depolymerization. The oxidized substrates of Lac have a wide range, and include phenol and its ramifications, arylamine and its ramifications, and aromatic carboxylic acids and its ramifications.

Although LiP, MnP, and Lac are the main enzymes involved in lignin degradation, many other enzymes produced by various microorganisms such as glyoxal oxidase (GLOX) and aryl-alcohol oxidase (AAO) also participate in lignin degradation. At present, the specific function of each enzyme in the lignin degradation process is still unknown. We can assure that these enzymes cooperate with each other, though synergy to degrade lignocellulose and other substances are hard to be degraded (Table 3.5).

2.2.4 Biochemical Mechanism of Lignin Degradation

Oxidation reactions play a dominant role while reduction reactions play a supplementary role in the lignin degradation process. The process of lignin degradation by microorganism mainly occurs in secondary metabolism stage. Based on the catalysis of LiP and MnP and a series of free radical chain reactions started by enzymes, highly active enzyme intermediates are formed firstly so as to oxidize RH-like lignin to be various R•, which include •OH with strong oxidability. Therefore, free active intermediates of lignin with chemical instability are formed, and a series of automatic degradation reactions occur,

Table 3.5: Enzymes related to lignin degradation and their working mechanisms.

Enzyme	Cofactor or stroma mediator	Main reactions or functions
LiP	H_2O_2, veratryl alcohol	Aromatic ring is being oxidized to be carbon-free radical
MnP	H_2O_2, Mn, organic acid is used as chelon, mercaptan, unsaturated lipid	Mn^{2+} is being oxidized to be Mn^{3+}, chelated Mn^{3+} phenol compounds are being oxidized to be phenol-oxygen-free radical, other reactions after adding other compounds
Lac	O_2, medium (such as hydroxybenzotriazole or ABTS)	Phenols are being oxidized to be phenol-oxygen free radicals, other reactions when mediator exists
GLOX	Glyoxal, anisyl glyoxal	Glyoxal is being oxidized to be dihydroxy acetic acid to produce H_2O_2.
AAO	Aromatic polyols (anisic alcohol, veratryl alcohol)	Aromatic polyols are being oxidized to be aldehydes to produce H_2O_2
Other H_2O_2 enzymes	Some other compounds	O_2 is being deoxygenized to H_2O_2

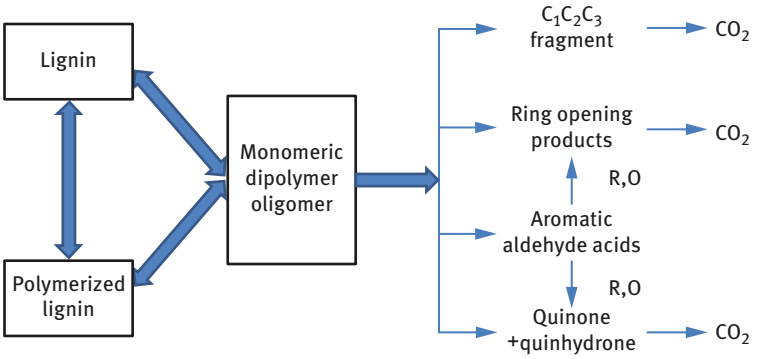


Figure 3.5: Metabolic pathways of lignin polymers.

thereby realizing the biological degradation of lignin. Figure 3.5 shows the lignin polymerizing process. The specific reactions are listed as follows:

(1) C_α–C_β scission

The C_α–C_β scission mechanism of the lignin model compound has presently been confirmed. Through single electron transfer mechanism, LiP catalyzes and oxidizes β-1 nonphenol lignin model compound to be ayle positive ion free radicals. Then, 3,4-dimethoxyphenyl acetic alcohol free radicals and protonated veratraldehydes are formed through C_α–C_β scission. In the presence of oxygen, the former can release superoxide ions and form carbonyl group or alcohol after the addition of oxygen. In the absence of oxygen, 3,4-dimethoxyphenylacetic alcohol free radicals will react with solvent water to form alcohol.

(2) Mechanism of C_α-oxidization

LiP catalyzes the β-O-4 lignin model compound, which involves C_α–C_β scission to form veratraldehydes and 2-metoxyphenol. The latter is easily polymerized, and quite a few will be transferred to be C_α oxidation products. Through positive ions, free radical intermediates losing protons, or by directly losing hydrogen, C_α oxidation products are formed. In the presence of active oxygen, the latter will easily undergo this reaction.

(3) Mechanism of aromatic ring replacement

Ayle positive ion-free radicals formed in solvent water or other moderate reagents will be different when different substrates are used. For instance, when the substitutional group, which is located in the benzene ring, is a methoxyl group, demethoxylation reactions will occur and quinone will be formed because of oxidization.

(4) Oxygen activation

Oxygen activation is very common in the lignin model compound oxidization process. Oxygen is activated through the reaction between an oxygen molecule and a phene-free

radical, which is replaced by the hydroxyl group. Oxygen is being deoxygenized to superoxide ions, which then react with hydrogen protons to produce H_2O_2 and O_2. This process occurs between oxygen molecules and phenyl intermediates, and is a complete chemical reaction. This reaction also occurs in catalytic cycle processes of LiP, which results in the production of O_2 as the final electron acceptor.

(5) Oxidation of veratryl alcohol and its ramifications
Veratryl alcohol is the secondary metabolite of *Phanerochaete chrysosporium* and is broken down by LiP. The main breakdown product is veratryl alcohol, and the secondary products are ring opening products and quinones. Products arrangement is controlled by the pH.

(6) Aromatic ring scission
Previous studies on aromatic ring scission reactions of many model compounds by means of labeling the substrate and reactant have shown that the mechanism of veratryl alcohol ring opening can also be applied to lignin units, dipolymer, and oligomer model compounds. In many cases, a mixture containing various products is being produced.

(7) Oxidation of single anisyl aromatic substances
The 3,4-dimethoxy aromatic ring can be metabolized by *Phanerochaete chrysosporium*, but can also be oxidized by LiP. Although a single anisyl aromatic ring can be metabolized by *Phanerochaete chrysosporium*, it cannot be oxidized by LiP. However, by the addition of a small amount of veratryl alcohol or other dimethoxy aromatic substance, the reaction speed of single anisyl aromatic substances can be significantly increased.

(8) Formation of quinones/quinhydrone
Laccase is a type of phenol oxidase that contains copper. It can catalyze phenol dipolymer models such as b-1 and β-O-4 to produce quinones/quinhydrone that can be replaced by anisyl through C_α–arene scission. HRP also can achieve familiar results. Lignin unit models such as vanillic acid, vanillina, and vanillic alcohol can also be oxidized to quinones/quinhydrone and can be replaced by anisyl.

(9) The mechanism of laccase catalyzes
Laccase uses O_2 as electron acceptor to catalyze a polyphenolic substance so as to produce quinones and phenol oxidase free radicals, which contain copper. This process does not require H_2O_2. For lignin, the reactions include for example demethoxylation, dehydroxylation, C–C linkage scission.

2.3 Application of Lignin-Degrading Microorganisms

Lignin degradation is of great importance in industrial processes such as lignocellulose resource utilization, bio-pulping, bio-bleaching, and effluent disposal. Using

microorganisms to degrade lignin has several advantages, including mild reaction conditions, low energy consumption, and minor influence on the environment.

Lignocellulose is widely available in nature, cellulose and hemicellulose derived from lignocellulose can be hydrolyzed to various reducing sugars. Therefore, lignocellulose has been used to produce various microorganism-fermented products such as alcohol, itaconic acid, and astaxanthin. However, the structure of lignocellulose is compact, and the external surface of cellulose is often covered by lignin and hemicellulose. Therefore, degrading lignocellulose-derived lignin may increase the utilization efficiency of cellulose and hemicellulose, which are present in lignocellulose-derived materials. Moreover, lignin is a common industrial chemical, and the use of microorganisms to degrade lignin into different compounds is a common method to produce relevant industrial chemicals.

Bio-pulping is a necessary part in the paper industry, and lignin-degrading microorganisms are frequently applied to the bio-pulping process, which involves the mostly studied application field. In the bio-pulping process, lignin-degrading microorganisms can be used to pretreat lignocellulose materials, or they can be directly applied to the pulping process. Several lignin-degrading microorganisms and degrading enzymes can selectively degrade lignin of lignocellulose-containing materials. Biological pretreatment prior to pulping can decrease the consumption of chemicals and energy.

Bio-bleaching is mainly achieved by lignin-degrading microorganisms to remove residual lignin. Because the original color of lignin is brown, the lignin degradation process will result decoloring of products to achieve bleaching.

The effluent disposal process mainly aims at effluent with high levels of lignin, which are disposed of by using lignin-degrading microorganisms. In this way, poisonous substances are removed and levels of the effluent's COD (chemical oxygen demand) and chroma decrease. The most common effluent with high levels of lignin is a paper producing industrial effluent featuring high chroma, high toxicity, and a high COD concentration. This effluent contains lignin, which is difficult to be degraded, or other compounds that are connected to lignin. Adopting lignin-degrading microorganisms to dispose paper making effluent can effectively dispose effluent. Moreover, compared with chemical or physical effluent disposal methods, this method uses mild conditions, is low in costs, and has a high removal efficiency.

Section 3: Microbial Cellulose Hydrolysis and Cellulases

Almost all cellulose is present in the cell walls of plants, which accounts for more than 50% of the carbon content in the plant kingdom. Only a small number of animals and bacteria can produce cellulose. Cotton is the most pure form of cellulose among the natural resource of cellulose, and the cellulose content of cotton is close to 100%. In

addition, the cellulose of the stem of woody plants content accounts for 40–50% and the cellulose content of leaves accounts for 15–20%. Moreover, the cellulose content in crop straws and other herbs accounts for 25–40%. So far, the annual output of cellulose has been over 100 billion tons, making cellulose become the most widely distributed compound and the most abundant renewability resource in nature. How to make full use of cellulose has become a hot topic in countries around the world. The process in which cellulose is decomposed into glucose, cellobiose, and other oligosaccharides by microorganism producing enzymes, and further into ethanol, butanol, and other bio-fuels, is regarded as a cellulose utilization strategy with very high potential.

3.1 Cellulose Hydrolyzing Microorganisms

3.1.1 Composition and Structure of Cellulose

Cellulose is composed of D-glucopyranose base (glucosan), and its chemical elements are carbon, hydrogen, and oxygen atoms. The molecular formula of cellulose is $(C_6H_{10}O_5)_n$ (n is the degree of polymerization). For natural cellulose, the value of n can be up to 10,000, while for regenerated cellulose, it is around 200–800. The chemical formula of cellulose can be one of two different types, including the Haworth conformation and the Chair conformation (Figure 3.6).

The Haworth conformation of cellulose is composed of many D-glucose groups that are bound by 1,4-β links, while the position of OH and H groups that connect to the ring of the carbon atoms at both ends are different. As for the chair conformation of cellulose, the geometric arrangement of atoms in molecules changes constantly,

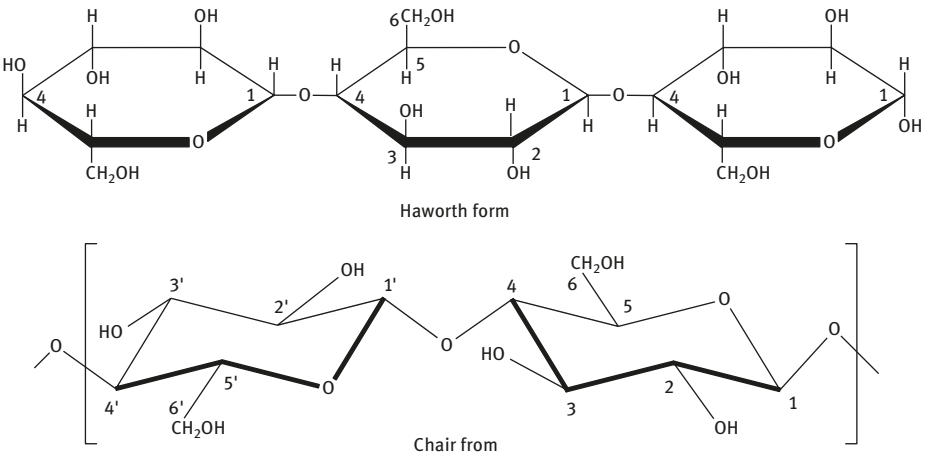


Figure 3.6: Chemical conformation of cellulose.

thereby creating various types of internal rotation isomers, named molecular chain conformations, depending on their internal rotation. The 5th bit C–O key and the C–O key between the 5th and 4th bit can have three different conformations when the 6th bit C–O key rotates about the C–C key between 5th bit and 6th bit (Figure 3.7). It is generally accepted that natural cellulose is present in the gt conformation, whereas regenerated cellulose is present in the tg conformation.

Within the cellulose chain, hydrogen bonds are present (Figure 3.8). The hydrogen bond connects O_6 with O_2, and O_3 with O_5 and creates the entire polymer chain

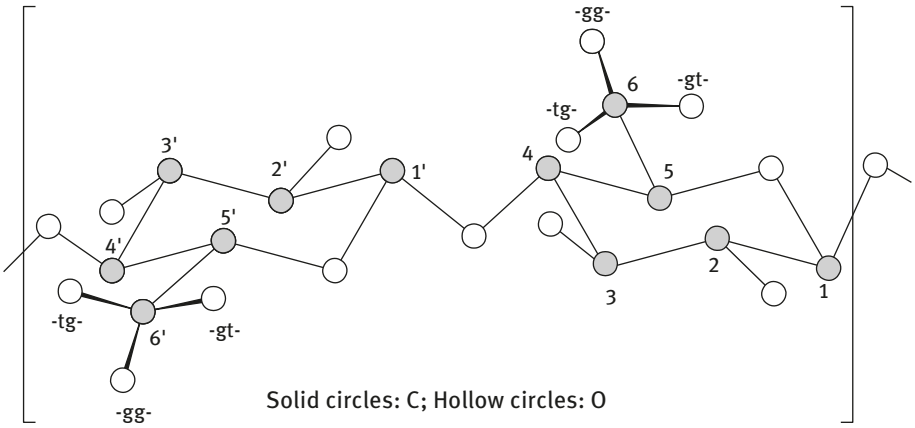


Figure 3.7: The OH group conformation in the 6th bit, g is Gauche and t is Trans.

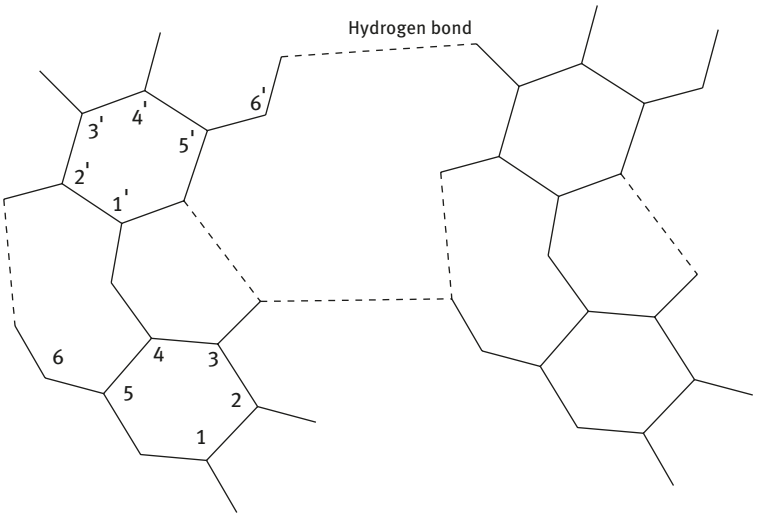


Figure 3.8: Hydrogen bond in the same or different chain of the cellulose molecule.

belt that leads to a higher rigidity. After embedding of the crystal, it can produce interchain hydrogen bonds between one O_6 of a macromolecule chain and the O_3 of an adjacent macromolecule.

3.1.2 Classification of Cellulose-Hydrolyzing Microorganisms

Several cellulose-hydrolyzing microorganisms exist, including aerobic and anaerobic microorganisms, bacteria, actinomycetes, and fungi.

Fungi are representative aerobic microorganisms involved in organics hydrolysis, especially fiber substrate. For example, *Mucor* has a stronger ability of hydrolyzing cellulose and a better effect of using soluble substrates among almost 700 strains of *Zygomycetes*. In contrast, *Ascomycetes*, *Basidiomycetes,* and *Decuteromycetes* consist of over 15,000 strains of decomposing cellulose, such as *Bulgaria*, *Chaetomium*, *Helotium* of *Ascomycetes*; *Coriolus*, *Phanerochaete*, *Poria*, *Schizophyllum*, and *Serpula* of *Basidiomycetes*; *Aspergillus*, *Cladosporium, Fusarium, Geotrichum, Myrothecium, Paecilomyces, Penicillium,* and *Trichoderm*a of *Decuteromycete*s. The classification of fungi is largely based on different stages of the life cycle of mycelial morphology and reproduction modes, rather than on the ability of the substrate. This may explain why fungi of degrading cellulose distribution is widespread.

Gram-positive bacteria of aerobic cellulose-hydrolyzing microorganisms include *Bacillus*, *Acidothermus*, and *Caldibacillus,* whereas gram-negative bacteria include *Pseudomonas*, *Cellvibrio*, *Cellulomonas,* and *Erwinia*. Most mesophilic temperature aerobic microorganisms of these genera have the ability of lively movement and a poor ability of decomposing natural cellulose. The *Cellfacicula* of *bacterium* have a strong ability of decomposing cellulose, thereby making filter paper swell and glutinous. *Cellfacicula* belong to gram-negative bacteria. *Bravibacterium* is short, slightly curved, pointed at both ends, the flagellum produces green, light yellow or light brown slime in the cellulose silicate medium. They exist in the above-mentioned mesophilic temperature aerobic cellulose-decomposing microorganisms, in the cecum of rumen, pasture-fed single stomach animals, as well as in humans. In addition, halophilic cellulose-decomposing bacteria are distributed in coastal and inland saline soil. In contrast, aerobic gliding bacteria have a stronger ability of decomposing cellulose, making the fibrous materials swell and mucous, such as filter paper and cotton cloths, such as *Cytophaga*, *Sporocytophaga*, *Polyangium. C. rubra, C. johnsonii, C. diffluens, C. lytica* of *Cytophaga* are strict aerobic bacteria, whereas *C. fermentans* and *C. salmonicolor* are facultative anaerobic bacteria. *Polyangium* bacteria are rhabdoid, which are usually heaped up to sporocarp by several or hundreds of cysts. They have strong ability to decompose cellulose, resulting in the production of different colors of filter paper.

Anaerobic bacteria of degrading cellulose contain several types of genus of gram-positive and gram-negative bacteria. Gram-positive bacteria include *Clostridium*, *Tuminococcus*, *Anaerocellum*, *Butyrivibrio*, and *Eubacterium*. Several types of *Spirochaeta* also

have the ability to degrade cellulose, such as *S. thermophila*. Gram-negative bacteria include *Caldicellulosiruptor*, *Anaerocellum*, *Fibrobacter, Halocella,* and *Thermotoga*. The above-mentioned anaerobic bacteria include many thermopylae bacteria. These mainly include *Ruminooccus albus*, *R. flavefaciens*, *Butyrivibrio fibrisolvens*, and *Bacteroides succinogenes* in the rumen bacteria, and are strictly anaerobic gram-positive bacteria. *Ruminococcus albus* and *R. flavefaciens* have a strong ability to decompose cellulose, whereas others are poor and show the ability in mixed culture conditions. In the rumen of ruminants, anaerobic fungi are involved in the degradation of cellulose, for example, chytrid fungi of *Chytridiomycetes* can produce highly active cellulase.

3.2 Hydrolysis of Cellulase and Its Mechanism

Cellulase is a type of hydrolase that is involved in the degradation of cellulose into glucose, cellobiose, and glucose. According to its structure, cellulase can be divided into two categories: non complexed cellulase and cellulosome.

3.2.1 Non complexed Cellulase

In 1950, the C_1/C_X hypothesis of the enzymatic hydrolysis mechanism was put forward by Reese et al. [10]. Based on this, Wood and McCraec [11] put forward a synergistic action model of endoglucanase and exoglucanase, which is now widely accepted. In brief, both endoglucanase and exoglucanase act on the surface of cellulose. Next, endoglucanase acts on the β-1,4 glycosidic bond in the cellulose molecule to produce short-chain cellulose and new chain ends. Exoglucanase only acts on glucan chains above 10 polymerization degrees, thereby cutting off cellulose in cellobiose from the chain end to produce cellobiose and glucose. Under the joint action of endoglucanase and exoglucanase, cellulose is degraded into soluble oligomeric glucose (the polymerization degree is less than 7), and then degraded into glucose by the action of β-glycosidase [12–15].

To degrade cellulose, cellulase acts on glycosidic bonds, and two molecular mechanisms exist: (1) retaining mechanism and (2) inverting mechanism [15–17]. For the retaining mechanism (Figure 3.9a) [18], two catalytic carboxyl groups, which are about 5.5 Å from two sides of sugar circle surface, cut off the glycosidic bond and remain in the same conformation on the substrate anomeric carbon (C1). The process was as follows: the first step involved glycosylation where H was provided for one glycosylation from one catalyzed carboxyl by acid catalysis. Another catalyzed carboxyl was formed to glycosylation-enzyme intermediates by nucleophilic attack of anomeric carbons. The second step involved deglycosylation, where the catalyzed carboxylic group that provided H in the first step, captured an H from the nucleophilic group in the reaction system. Thereby, nucleophilic material (a water molecule or a new sugar-based

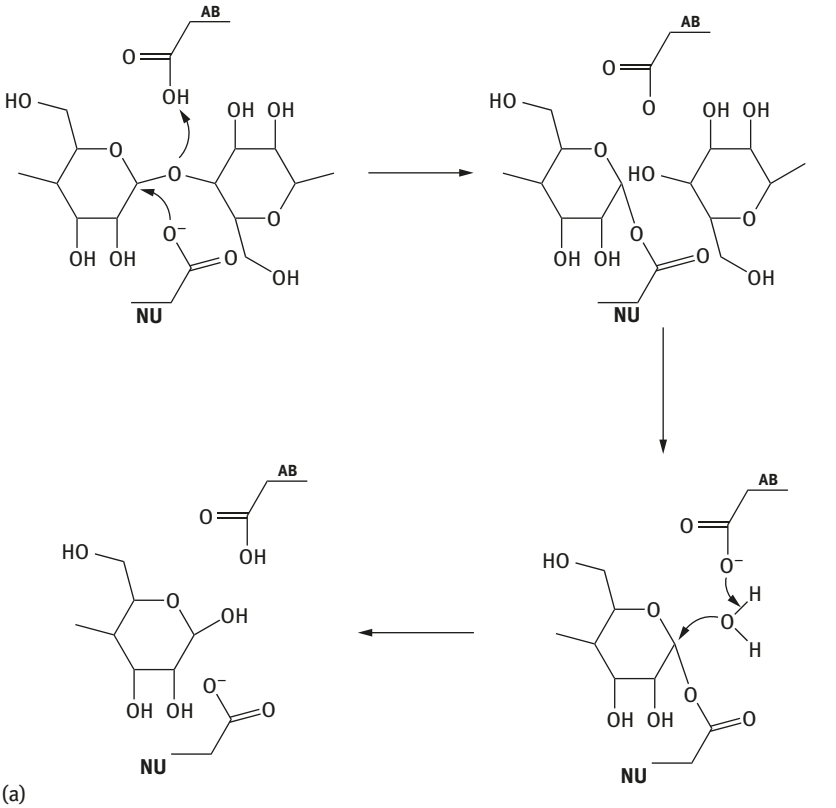


Figure 3.9: Hydrolysis of glycosidic bonds by cellulose, (a) Retaining mechanism, (b) Inverting mechanism. [18]

hydroxyl group) is activated, and reacts with carbohydrate-enzyme intermediates, to replace the glycosylation and break the glycosidic bond. For the inverting mechanism (Figure 3.9b) [18], cellulase binds cellulose and two catalyzed carboxyl groups, located between 6.5 and 9.5 Å from two sides of the sugar circle surface break the glycosidic bond. Therefore, the conformation of the substrate anomeric carbon (C1) becomes the opposite. In other words, the β conformation on C1 becomes an α conformation, and vice versa. Two catalyzed carboxyl groups are involved in the inverting mechanism, where one carboxyl obtained a H from H_2O by acid catalysis, and another carboxyl group provided a H for one glycosylation by acid catalysis. Then, the OH group of H_2O was linked to glycosylation of anomeric carbon from the opposite side of the sugar base circle surface by nucleophilic reactions, causing breakage of the glycosidic bond.

3.2.2 Complex Cellulase System

The representation of microorganisms to produce complex cellulase (cellulosome) is present in an anaerobic environment. A symbiotic relationship exists with other microorganisms, which can either hydrolyze cellulose or not. Cellulosome that is present close to bacteria cells coordinate enzymatic activity of every component to maximize the synergy of the cellulose enzyme system. Moreover, cellulosome also can minimize the diffusion distance of cellulose hydrolysis, resulting in host cells that can effectively extract these oligosaccharides.

Cellulosome is produced in bump of bacterial cell walls, which can decompose cellulose. These bumps are stable enzyme systems that are stably combined with bacterial cells. In previous studies, cellulosome has been studied and is derived from *C. thermocellum*, *C. cellulolyticum*, *C. cellulovoran*s, *C. josui* and other types of *Clostridium* and *Ruminococcus*. Their structure is very similar, although the cellulosome of these microorganisms has different components.

In general, cellulosome consists of two groups of module proteins, as shown in Figure 3.10. One part of cellulosome is necessary to build a supramolecular structure, the other part contains several types of enzyme components. The key noncatalytic subunit of cellulosome is termed scaffoldin, which contains several cohesins. These cohesins fix all types of enzyme proteins on the supramolecular structure of the complex by specifically interacting with dockerin of enzyme proteins. Moreover, scaffoldin and several parts of the enzyme protein also contain a carbohydrate-binding module (CBM) that can be combined with cellulose, to achieve a directive effect on the substrate.

Scaffold protein anchors are attached to the cell wall by II adhesion protein domains, and their catalytic modules contain endonuclease activity (CelA, CelB, CelC, CelD, CelE, CelF, CelG, CelH, CelN and CelP), exonuclease activity (CbhA, CelK, CelO, and CelS), and other hemicellulase and chitinase activities. Understanding the assembly, components, and synergies of catalytic modules of scaffoldin is challenging. It can be assumed that the composition of cellulosome changes and that the catalytic

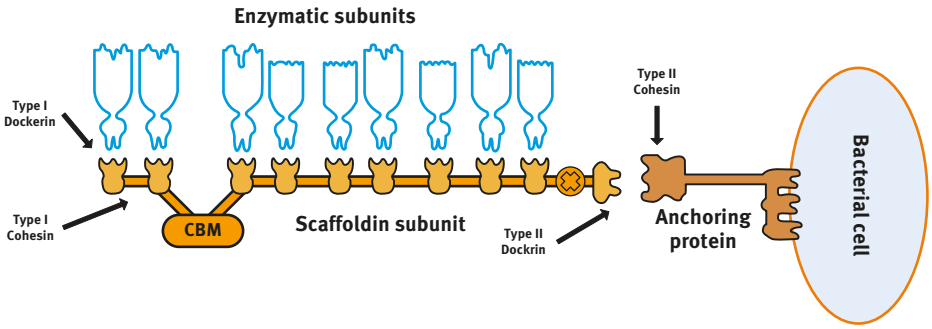


Figure 3.10: Cellulosome and two types of synechia-anchors. CBM: carbohydrate-binding module.

domain does not combine with specific adhesion proteins. Exonuclease CeIS is a progressive cellulase that appears on the cellulosome. CeIS preferably acts on micro-crystalline cellulose or amorphous cellulose that act on CMC. Hydrolysis products are predominantly composed of cellobiose with subordinate cellotriose. Cellobiose has a strong inhibitory effect on CeIS. CeIA is mainly an endonuclease that is related to cellulosome. Moreover, cellulosome is a large complex of 2-6MDa, which, in a polymerization state, can be up to 100MDa. Cellulosome, especially scaffoldin, is highly glycosylated (the carbohydrate content is 6–13%). Sugar-based compounds protect cellulosome from hydrolysis by proteases and may play a role in the synechia-anchor.

Cellulosome efficiently hydrolyzes micro-crystalline cellulose, and its efficiency is attributed to the following three reasons: (I) proper proportion of the catalytic domain can maximize the synergistic effect, (ii) appropriate distance of single enzyme components contributes to the synergistic effect, (iii) existence of different enzyme activities of cellulosome (decomposition of cellulose and hemicellulose) can remove physical steric hindrance of heterogeneous plant cell materials.

3.3 Molecular Breeding of Cellulase

The enzyme production performance of a wild-type cellulose enzyme production strain is generally low and different from the cellulose that is present nature. Therefore, it is required to molecularly breed cellulose strains, to obtain high performance stains. Molecular breeding methods mainly include mutation breeding, protoplast fusion, genetic engineering efficient enzyme production strains, and molecular modification.

3.3.1 Physicochemical-Induced Breeding

Mutation breeding is a simple and effective approach that can substantially change the genetic characteristics of stains, resulting in a high-yield mutant strain. Commonly

used mutagen include ultraviolet (UV), 60 co-gamma rays, ethyl methyl sulfonic acid (EMS), *N*-methyl-*N*'-nitro-*N*-nitrosoguanidine (NTG), nitrite, and diethyl sulfate (DES). UV mutation breeding is the main breeding method and is widely used to improve the performance of strains. Strains that show a high enzyme activity and good resistance will be selected. Composite mutagenesis combined with a variety of mutagenesis techniques has been widely used mutation breeding of cellulase strains. In the mutagenesis screening strategy, the end-product glucose stops further synthesis of the enzyme, which is considered as a major cause of the relatively low cellulase production of natural strains. Therefore, screening repression-resistant mutant strains is an approach to obtain high-yield cellulase stains.

Fang et al. [19] established an excellent mutant strain of high-yield cellulase by UV radiation starting with *Trichoderma*, which has high genetic stability. The values of CMCase activity and FPA were 1332.9 and 301.61 U/g, respectively. The value of CMCase activity is 2.19-fold that of the starting strain *Trichoderma*, and the value of FPA increased twofold compared to the original strain. Ike et al. [20] screened the two mutant strains M2-1 and M3-1 for better enzyme production performance through UV mutagenesis processing of the *Trichoderma reesei* mutant strain, and used ATCC66589 as the parental strain. After 8 days of cultivating these strains with cellulose as the sole carbon source, their FPA improved 1.1- and 1.2-fold compared to that of the parental strains; the values were 257 and 281 U/g respectively. When a mixture of glucose and cellobiose was used as the carbon source of continuous charging cultivating process, the FPA values were 214 and 210 U/g compared to the FPA value of parental strain, which was 140 U/g, indicating their enzyme activity was significantly improved. Chandra et al. [21] selected the resistance mutant strain of stable enzyme production with a complex mutation of EMS and ethidium bromide to *Trichoderma citrinoviride*. The activity of FPase, endoglucanase, β-glucosidase, and cellobiase was 2.14, 2.10, 4.09, and 1.73 times that of parental strain, respectively. Fang et al. [22] separated a mutant strain of high enzyme activity by treatment with UV and NTG, using *Penicillium janthinillum* as the original strain. Compared with the starting strain, the FPase activity increased by 44.7%. In addition, the β-glucosidase activity was also significantly improved compared to parental strain. Du et al. [23] obtained glucose repression-resistant mutant strains E2-26, E5-65,U6-31, and EU7-22 by using mutagen multiprocessing of UV, LiCl, and EMS. The CMC enzyme activity increased 1.7-, 1.4-, 1.3-, and 2.0-fold, respectively, when compared to the parental strain.

At present, *Trichoderma reesei* Rut C-30, which has been studied in depth, and has been widely used in the production of wild-type T.reesei QM6a as the original strain, was obtained by a series of mutation breeding. This mutant stain produces enzyme in which the ability of decomposing cellulose is more efficient compared to that of the wild-type strain. Moreover, expression of the cellulase gene is not repressed by the end-product glucose [24]. Presently, many companies adopt this strain to produce cellulase, because it has great value as a commercial enzyme production. Mutation breeding technology is one of the most effective means of providing high-yield cellulase

strains, has a comparatively high positive mutation rate, and has resulted in a variety of excellent mutant strains and new useful gene types. However, there is some blindness and randomness when using mutation breeding. In the actual application, reasonable mutagenesis methods should be chosen according to the starting strains, experimental conditions, and other concrete conditions.

3.3.2 Protoplast Fusion Breeding

In addition to the traditional methods of microbial mutation breeding, protoplast fusion is widely applied in breeding of high-yield cellulase. This technology allows for construction of new strains between species, genus, and family, and is an important way of strain improvement, gene location, and exogenous DNA transformation. In recent years, some studies reported on protoplast fusion between intraspecific, interspecific, and intergeneric strains in improving high-yield cellulase *Trichoderma* strains. Prabavathy et al. [25] separated protoplasts from *Trichoderma reesei*, performed endogenous strains protoplast fusion after different pretreatment methods, and screened fusants of which 80% showed very high CMC enzyme activity. The enzyme activity of two of the strains, SFTr2 and SFTr3, increased twofold when compared to the parental strains. Dillon et al. [26] obtained fusants through protoplast fusion between *Penicillium echinulatum* and *T. harzianum*. The FPA enzyme and β-glucosidase of cellulase were significantly improved compared to the parental strain. In solid medium, fusant can produce cellulase in a fast and efficient way. Moreover, *Trichoderma reesei* can synthesize a large number exoglucanase and endoglucanase. However, β-glucosidase of *Trichoderma reesei* has a low activity, while β-glucosidase of *A. niger* has a high activity. After protoplast fusion between *Trichoderma reesei* and *A. niger*, fusants can be screened, which have advantages to the two genera, by realizing the complementary advantage between two distant relations.

The protoplast fusion technique is not affected by affinity so that an ideal fusant can be chosen. It represents an effective strain improvement method for bacteria with a high-yield cellulase content; however, the genetic stability needs to be further elucidated.

3.3.3 Genetic Engineering

In recent years, several studies have focused on the cellulase gene of *Trichoderma reesei* derived from the fungus *Trichoderma* genera. The exoglucanase gene of *Trichoderma reesei* cbh1, cbh2, the endoglucanase genes eg1, eg2, e.g3, eg4, eg5, and β-glucosidase gene bg1, bg2, and other cellulase genes have been cloned, and expressed in *E. coli*. Nakazaw et al. [27, 28] analyzed eg I, eg II, and eg III gene sequences of both combination area and catalytic area of *Trichoderma reesei* by fusion expression and methods of fixed point mutation. Expression in *E. coli* laid the foundation for the design and modification of

the eg gene. Although cellulase genes have been cloned into *E. coli*, cellulose expression is limited because of the low expression and secretion level of *E. coli*. At present, several companies use recipient bacteria as the ideal host system of expressing fugal enzymes, which grow and exocytose fast, are easy to cultivate, and do not produce toxins, compared to, for example, *Pichia pastoris*, *Saccharomyces cerevisiae*, *A. niger*, *Fusarium venenatum*. For example, Ouyang et al. [29] cloned *Trichoderma reesei* RutC-30's xyn II into *Pichia pastoris*, obtaining an enzyme activity of up to 1.45 IU/mL after G418 selection, Polymerase chain reaction (PCR) amplification, methanol induction, and cultivation for 60 h. Qiao et al. [30] transferred the *Trichoderma reesei* endoglucanase gene eg2 to *Pichia pastoris*, obtaining *Pichia pastoris*-engineered bacterium Gp2025 with efficient expression of *Trichoderma reesei*'s exdoglucanase II. After fermentation induced by methanol, the enzyme activity can be up to 1573.0 U/mL. Liu et al. [31] cloned the eg III gene from *T. viride* AS3.3711 by using RT-PCR technology. The homology of the eg III gene and the eg III gene of *Trichoderma reesei* is 99.6%. The eg III gene has been successfully expressed in *Saccharomyces cerevisiae*. The enzyme activity of the transformant was 120 U/mL, whereas the stability of gene inheritance was 99.17%. Chen et al. [32] cloned the MANN gene from *Aspergillus sulphureus*. The amino acid sequence was similar to *Trichoderma reesei's* β-glycosidase, which was successfully expressed after transformation into *Pichia pastoris*. The β-glycosidase activity was up to 344.83 U/mg and was not affected by ionic strength and ethylenediaminetetraacetic acid (EDTA). In addition, it is the research hot spot of strain improvement by genetic engineering that the *Aspergillus niger's* β-glucosidase enzyme gene is cloned to the *Trichoderma reesei* expressing efficiently to improve the activity of β-glucosidase or the own homologous cellulase gene of *Trichoderma reesei* is multiply expressed to build *Trichoderma reesei* engineering strain of efficient production of cellulase.

3.3.4 Molecular Modification

Previous studies have shown that molecular modification of cellulase can be successful. For example, Wang et al. [33] used error-prone PCR and made a directed evolution of the eg III from *Trichoderma reesei*, thereby causing fault mutant strains. A mutant N321T strain was obtained, for which the pH had an offset of alkaline pH 0.6 units from the optimum pH. Ozawa et al. [34] transferred Asn179 and Asp194 of the alkaline mesphilic cellulase eg1-64 of basophil bacillus (*Bacillus* sp. Strain KSM-64) into Lys, which increased stability. Moreover, Kim et al. [35] screened a mutant strain of which the CMC enzyme activity was 2.2-fold that of the wild-type strain as determined by DNA reshuffle. Kaper et al. [36] established a glycosidase mutant strain by using DNA reshuffle technology. The hydrolytic activity of the mutants were 3.5-fold and 8.6-fold that of the original strain. Through molecular modification of the enzyme, the properties of cellulase met the desired needs. Although the results are promising, there is still a lot to be elucidated and the molecular modification of cellulase has a great potential.

3.4 Currant Station of Cellulase Enzymatic Industrialization

To produce cellulase, both liquid and solid cultures can be used. A novel cellulase solid state fermentation reactor, designed to produce a pure culture, along with other supporting technology and equipment was designed by Chen et al. (Institute of Process Engineering, Chinese Academy of Science), who studied the industrialization of solid fermentation technology. Short cycle times of cellulase enzymatic fermentation and high-activity of cellobiase can be achieved when using corn straw as a main raw fermentation material to produce cellulase, This approach obtained a Chinese invention patent in May 2003, and when using the device to produce cellulase, the average cellulase activity reached 120 FPU/g and, after five experiments, peaked at 210 FPU/g.

For over 20 years, Shangdong University, Nanjing Forestry University, Zhejiang University, and other institutions in China have successfully applied enzymatic hydrolysis to produce ethanol. Shandong University breeds their own *Penicillium decumbens* of repression resistant mutants, and uses two-stage temperature cultivation measures, which has shortened the fermentation time, and improved the efficiency of enzyme production. In a 50 L fermentation tank, whole-waste culture medium-produced enzyme experiments showed a substrate rate of 264 IU/g cellulose after inoculation of 48 h. The FPA reached 416 IU/ml, and the yield of the enzyme was 100 IU/(Lh). When using the fed-batch technique, and by adding a small amount of fine hybrid fiber to the batch fermenter after 48 h, the FPA can be further increased to 712 IU/ml. However, this prolongs the fermentation circle, and the enzyme production rate was not further improved. This technology has been used for many years at Xia Sheng Enterprise (Ningxia) and other companies to produce industrial cellulase. Currently, domestic enzymatic cellulase sales have reached 15,000 tons, which includes foreign company sales in China. Due to the high production costs of the enzyme, it can only be used for food, and high value-added textile and food industries.

Novozymes has identified a variety of enzymes that are used to formulate new complex enzymes, which can improve the degradation of enzymes. Combined with NREL pretreatment technology, the enzyme costs was only 1/30 of original, from $5 per gallon in 2001 to $0.10–0.18 per gallon in 2005. In 2009, Novozymes launched the first large-scale supply of composite cellulase CTec Cellic, which was the first to provide enzymes that can be used for optimization and standardization of global cellulosic ethanol production. In 2010, the second generation of CTec2 Cellic was the first commercially available viable source of cellulase in the world, and entered the commercial preparation stage. The production costs of cellulosic ethanol is significantly higher compared with that of corn ethanol production. By the end of 2012, Novozymes put the third-generation cellulase cellic CTec3 into the market, the required amount of cellic CTec3 only counted for 1/5 of the amount of cellic CTec2. Cellic CTec3 contained a high specificity of various types of highly active cellulase, including improved β-glucosidase, and a series of new

hemicellulases, thereby relying on the common role of these enzymes. The conversion efficiency of cellic CTec3 improved at least 50% compared to that of cellic CTec2 and also had a stronger adaptability and tolerance.

In 2007, the world's first commercial cellulase production was launched, and Genencor was the leader in the fuel ethanol enzymatic market. TRIO-Accellerase, a new successful product, was launched in the global market. It had been reported that TRIO-Accellerase can help biofuel producers to greatly improve the efficiency of using renewable nonfood raw materials to produce cellulosic biofuels. Moreover, it reduces ethanol production costs and consumption and has several environmental benefits. TRIO-Accellerase can be used in many aspects, including switch-grass, wheat straw, corn straw, and municipal solid waste.

Section 4: Hydrolysis of Microbial Hemicellulose and Hemicellulases

Hemicellulose mainly exists in the primary and secondary cell walls of plants, and inks cellulose and lignin to tightly link the three important components to form lignocellulose, thereby increasing cell wall strength. Schulze et al. first introduced the name "hemicellulose", and found that these polysaccharides could be extracted from plant cell walls through alkaline liquids. Previous studies confirmed that hemicellulose was not the precursor material for cellulose synthesis, and increasing evidence had indicated that it had nothing to do with the synthesis of cellulose, for example: (1) cellulose is a homopolysaccharide with glycosyl groups of glucose, whereas hemicellulose is a homopolysaccharide with the same glycosyl groups or a heteropolysaccharide containing various sugars; (2) the different polymerization degrees of sugar chains lead to the longer chains of cellulose and the shorter chains of hemicellulose; (3) hemicellulose contains branches with several monosaccharides appearing on the branches, while no braches are present in cellulose.

As an important lignocellulotic feedstock, hemicellulose accounts for 15–35% of plant dry weight and is the second-largest carbohydrate in nature (second after cellulose). It is estimated that the annual amount of hemicellulose by photosynthesis is about 45 billion tons. Due to the currently growing energy shortage, high-grade liquid fuels produced from hemicellulose by biochemical transformation are increasingly gaining worldwide attention.

4.1 The Composition and Structure of Hemicellulose

Different from cellulose, the basic structural unit of hemicellulose is not uniform. Therefore, the components of hemicellulose are complex, including xylan, xyloglucan,

mannan, arabinan, galactan, and arabinogalactan. Hemicellulose is a general term for a variety of complex polysaccharides, and different plants contain different components [37]. In 1962, Aspinall et al. revealed that the main structural unit of hemicellulose is comprised of D-xylose, D-mannose, D-glucose, and D-galactose (Figure 3.11). Typically, hemicellulose is a polysaccharide that is composed by these two to four structural units. Moreover, hemicellulose not only has backbones of varying lengths but may also have structurally different side chains, of which several groups can be modified by nonglycosyl molecules or groups.

Xylan is extremely rich in various forms of hemicellulose and is widespread not only in angiosperms but also in conifer gymnosperms. In monocotyledonous commelinidae (including the most important Gramineae plants such as crops, bamboo, and grass), the main component of primary and secondary cell walls is xylan. The main backbone of xylan is a linear molecular group comprised of β-pyran-xylose connected via β-1,4 glycosidic bonds. Every certain xylose units links a side chain. The glycosyl groups of both main and side chains can be substituted by different substituents, including acetyl groups, acetylglucopyranosides and acetyl arabinopyranosides. These side chains are connected to other components such as lignin, cellulose, and pectin by means of covalent or noncovalent bonds. Not only linear molecular polyxylose but also highly branched heteropolysaccharides may lead to a difference in side chains, resulting in the diversity of hemicellulose structures. In addition, a small degree of homo-polyxylose is present in the shells of grass, tobacco, and several seeds. The chemical composition of typical woods is shown in Table 3.6.

In softwood (coniferous wood), xylan mainly consists of L-arabino-(4-*O*-methyl-D-glucorono)-xylan, with a content of 7–12%. The main chain are D-pyran xyloses

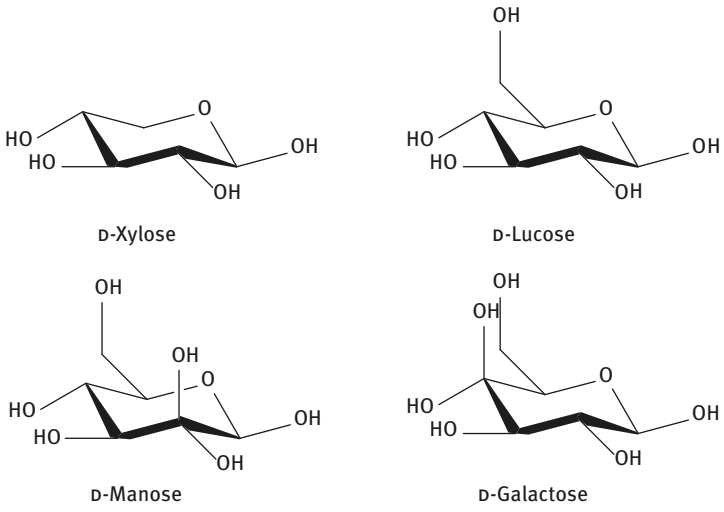


Figure 3.11: The basic structural components of hemicellulose.

Table 3.6: The chemical composition of typical softwoods and hardwoods.

	Cellulose	Lignin	Hemicellulose				
			D-Xylose residues with 4-*O*-methyl-D-glucopyranosyl units	L-Arabino-(4-*O*-methyl-D-glucorono)-xylan	Glucomannan	*O*-Acetyl-galactoglucomannan	Pectin, starch, and ash
Abies balsamea	42	29		9		18	2
Picea glauca Mocuch	41	27		13		18	1
Pinus strobus L.	41	29		9		18	3
Tsuga canadensis L.	41	33		7		16	3
Thuja occidentalis L.	41	31		14		12	2
Acer rubrum L.	45	25	25		4		2
Betula papyrifera March	42	19	35		3		1
Fagus grandi folia Ehrh	45	22	26		3		4
Populus fremnbides Michx	48	21	24		3		4
Ulmus americaua L.	51	24	19		4		2

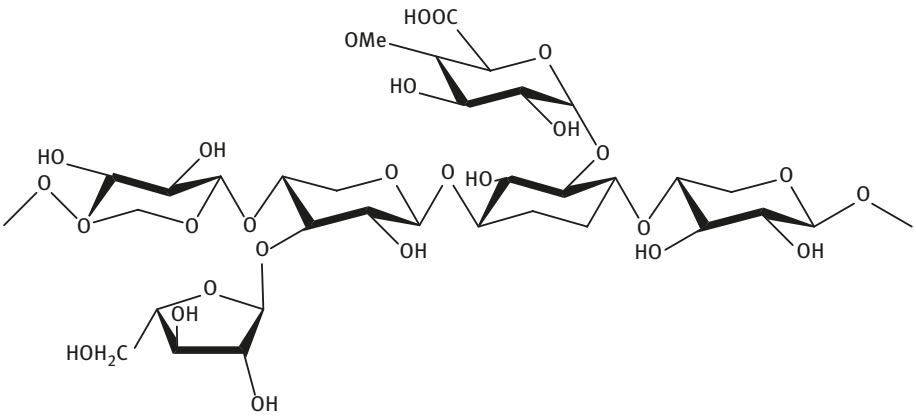


Figure 3.12: L-Arabino-(4-*O*-methyl-D-glucorono)-xylan.

linked by β-1,4 glycosidic bonds. The side chains of 4-*O*-methyl glucuronic acid groups and L-pyran arabinose groups are linked in the C-2 and C-3 position of xylose residues, respectively (Figure 3.12). Only a small number of branches consisted of xylose.

Xylan in hardwood consists of mainly d-xylose residues with 4-*O*-methyl- D-glucopyranosyl units, with a content of 20–25%. The main chain comprises over 70 d-pyran xylosyl units, linked by β-1,4 glycosidic bonds, whereas the side chains of 4-*O*-methyl glucuronic acid groups are linked at every ten xylosyl units in the C-2 position of xylose residues (Figure 3.13). Highly acetylated hardwood has been shown to comprise of 10–13% acetyl groups, which mainly occur in the C-3 position of the xylose residues. The C-2 substitution is rare. The polymerization degree of hardwood xylan is roughly 150–200 and has a longer average length and more branches compared to softwood.

The main component of hemicellulose in Gramineae plants is xylan, which has a similar structure to that of softwood. In wheat straw, L-arabino-(4-*O*-methyl-D-glucorono)-xylan in hemicellulose has a main chain of nearly 73 D-pyran xylosyl units that is linked by β-1,4 glycosidic bonds. The side chains of D-pyran glucuronic acid groups and L-pyran arabinose groups are linked in the C-2 and C-3 position of xylose residues, respectively. The hemicellulose composition in rice straw is similar to that of wheat straw. The side chains of xylan in maize straw are mainly D-pyran xylosyl groups and L-pyran arabinose groups. Furthermore, 4-*O*-methyl glucuronic acid groups are also linked in the C-2 position of xylose residues.

Xyloglucan is another type of hemicellulose, in which the main chain is linked by β-1,4 glycosidic bonds. The side chains consist of the functional groups Xyl, Gal, Fuc, or Ara. The content of xyloglucans in the secondary wall of seed plants is not very high. Except for Gramineae plants, xyloglucan is the most abundant hemicellulose in the primary wall of seed plants, especially in dicotyledons. In the primary wall of monocotyledonous Gramineae plants, xyloglucan has small amounts of glycosyl side chains [37]. In gymnosperms (mainly conifers), xyloglucan is mainly found in the primary wall, but the content is not high (~10%). Xyloglucan is present in Bryophytes, although to a low extent. In Charophytes, common xyloglucan has not been detected [38]. The content of mannosan in hardwood hemicellulose is 3–5%, and the main chain is formed by D-glucopyransoyl groups and D-mannopyranoyl groups linked by β-1,4 glycosidic bonds, with a ratio of 1:1–1:2. There is a small amount of mannosan in hemicellulose of Gramineae plants. Polygalactose is not as widely distributed as the three types mentioned above, in particular, the content of polygalactose in larch hemicellulose is 5–30%.

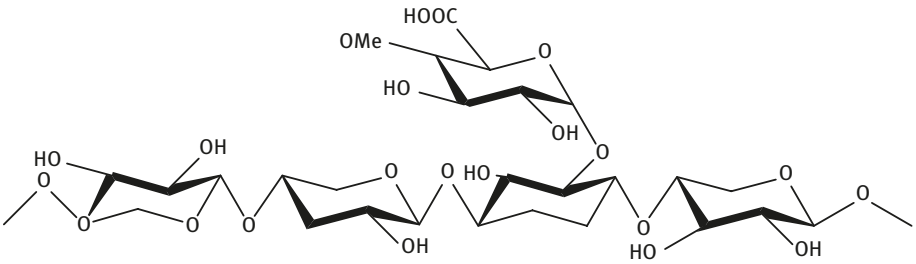


Figure 3.13: D-Xylose residues with 4-*O*-methyl-D-glucopyranosyl units.

4.2 Hemicellulose-Hydrolyzing Microorganisms

In nature, many microorganisms use lignocellulose as a carbon and energy source, and the cycle of carbon elements in the ecological environment is maintained by the degradation and usage of lignocellulose by these microorganisms. Since 1912, Pringsheim et al. were the first to isolate lignocellulose decomposing bacteria from soil. Since then, studies that focus on decomposing microorganisms and their related enzymes have greatly progressed. Currently, the known hemicellulose-decomposing microorganisms include dozens of genera and more than 100 species, mainly including various types of bacteria, actinomycetes, and several fungi. Previous studies have reported that several microorganisms have a beneficial effect on the decomposition of hemicellulose, such as *Aspergillus niger, A. fumigatus, Irpex lacteus Fr., B. subtilis, B. pumilus, B. irrculaus, Trichosporon cutaneum, Cryptococcus albidus,* and *Trichoderma*, and their related hemicellulose composition has been fully investigated.

It is likely that due to the close relationship of cellulose and hemicellulose in plant cells, microorganisms that live in plant residues are capable of producing hydrolases to degrade cellulose and hemicellulose, whereas other bacteria only produce cellulose-hydrolyzing enzymes or hemicellulose-hydrolyzing enzymes. Of these, most bacteria and fungi can secrete extracellular xylanase to treat lignocellulose substances. It is currently believed that rumen microorganisms are the most potential producers of hemicellulase. Most cellulose-decomposing microorganisms degrade hemicellulose and have a high extracellular hemicellulase activity, such as *Trichoderma reesei, T. viride, T. koningii,* and *Penicillium funiculosu*m. However, other hemicellulose-decomposing microorganisms cannot degrade cellulose because they only contain endo-glucosyl enzymes and lack exo-glucosyl enzymes, especially actinomycetes and bacteria. Several commonly-used amylase-producing bacteria can break down hemicellulose. In addition, the yeast strains *Trichosporon* and *Cryptococcus* as well as some protozoa and algae also have this ability.

Hemicellulose-decomposing microorganisms are diverse and widely distributed. Therefore, the degradation behaviors are also quite diverse. Depending on the degradation behavior, microorganisms can be divided into three categories.

One category contains mainly aerobic filamentous fungi, which completely decompose hemicellulose into monosaccharides and disaccharides by the synergy of several exocytosis hemicellulose, such as *Fusarium, Thrichoderma,* and *Aspergillus*.

The second category includes *Bacillus* bacteria that use exocytosis enzymes to decompose hemicellulose into oligosaccharides, and further hydrolyze oligosaccharides from hemicellulose by cell adhesion or intracellular enzymes. Through this approach, this type of microbes has significant advantages in sugar competition, when compared to microbes that do not have the ability of decomposing hemicellulose.

The third category includes multienzyme complex structures, of which the roles of hemicellulose adsorption and hemicellulose degradation could achieve hemicellulose decomposition. These multienzyme complex structures are called fiber bodies

and are mainly composed by a variety of cellulose and hemicellulose assembled by scaffolding proteins, and adhere to the cell wall via cell adhesion proteins. Some bacteria in *Clostridium*, such as *Clotridium thermocellum*, and some anaerobic fungi, such as *Piromyces*, were found in the presence of the fiber bodies [39].

4.3 Hemicellulase

Due to the complex structure and composition of hemicellulose, its complete degradation requires synergy of various enzymes, such as xylanase, xylosidase, arabinosidase, and glucuronidase. Although a variety of hemicelluloses exist, xylan and mannan are the most widely distributed and used in various applications. Therefore, this chapter focuses on xylan- and mannan-degrading enzymes.

4.3.1 The Classification of Hemicellulases

4.3.1.1 Xylan-Decomposing Enzyme Systems

Because xylan is the hemicellulose resource that is most widely distributed in nature, its efficient hydrolysis plays a decisive role on the effective utilization of hemicellulose. Heterozygosity of the xylan structure determines that its complete degradation must be catalyzed by the synergy of a series of degrading enzymes rather than by a single enzyme. According to the different specific degradation sites on the xylan backbone, xylanase can be divided into endo-xylanase, exo-xylanase, and xylanase affecting both the inside and the polysaccharide side chains (Figure 3.14). Based on the catalytic properties of xylanase, it can be divided into the following six categories:

(1) Endo-1,4-β xylanase
Endo-1,4-β xylanase is the most important xylan-degrading enzyme, affecting β-1,4-glycosidic bonds of xylan backbones in an endo-specific manner, thereby decreasing the polymerization degree of the matrix. The hydrolysis products are mainly xylo-oligosaccharides, and generally, no xylose is generated.

(2) β-Xylosidase
β-Xylosidase can have several formats, such as a single dimer, dimers, and four polymers, and are present in bacteria and yeast. β-Xylosidase belongs to the family of exonucleases, and by hydrolyzing xylo-oligosaccharides and xylobiose, it can release xylose from the nonreducing ends.

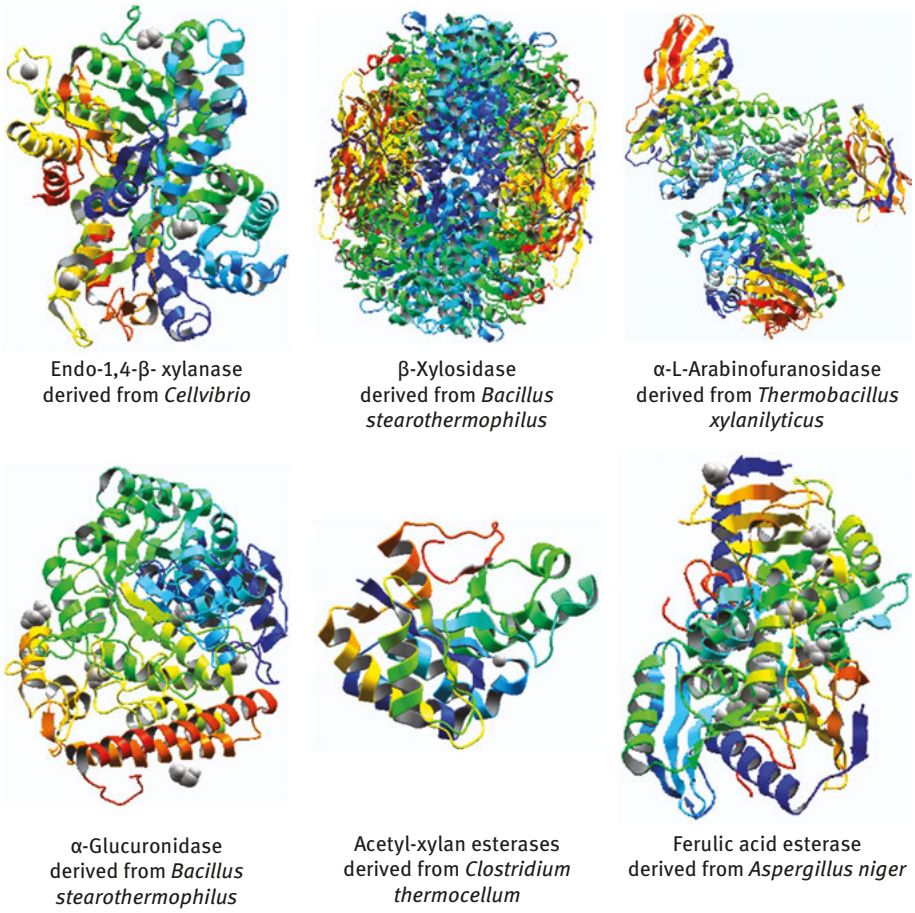


Figure 3.14: Xylan-decomposing enzymes.

(3) α-L-Arabinofuranosidase

Two types of α-L-arabinofuranosidase exist, of which the majority is of the exo-type, acting on *p*-dinitrophenol-arabinofuranosyl and branched arabinan, other endo-types only act on linear arabinan.

(4) α-Glucuronidase

α-Glucuronidase hydrolyzes the α-1,2-glycosidic bonds between glucuronide and xylose residues, and specificity is dependent on the source of the enzyme. For example, enzymes derived from *Aspergillus niger* act on 4-*O*-methyl glucuronic acid xylan polysaccharides, whereas enzymes derived from *A. bisporus* hydrolyze methyl 4-*O*-glucuronic acid oligonucleotide poly-xylose rather than 4-*O*-methyl glucuronic acid xylan polysaccharides.

(5) Acetyl-xylan esterases

Acetyl-xylan esterases act on acetyl substituents of the C-2 and C-3 positions of the xylose residues, thereby eliminating the steric hindrance of acetyl groups on endoxylanase, while enhancing the affinity of endo-xylanases to xylose glycoside.

(6) Phenol acid esterases

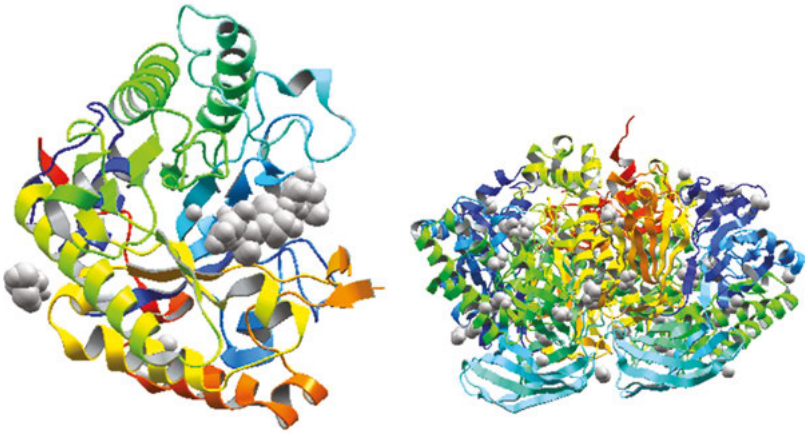
Phenol acid esterases mainly include p-coumaric acid esterases and ferulic acid esterases, the former cuts ester bonds between coumaric acid and arabinose residues, and the latter removes ester bonds between ferulic acid and arabinose residues.

4.3.1.2 Mannan-Decomposing Enzyme Systems

In wood, mannan is glucomannan composed by pyran mannan and glucopyranose and cross-linked by β-1,4 glycosidic bonds. Softwood usually contains glucomannan with acetyl substituents or galactose substituents. Mannan-decomposing enzymes can be classified into the following categories (Figure 3.15).

(1) Endo-1,4-β-mannanases

β-Mannanase is an endo-type enzyme that hydrolyzes the 1,4-β-d-mannopyranose main chain, and acts on the internal glycosidic bonds to decrease the polymerization degree by generating oligosaccharides.



Endo-1,4-β-mannanase
derived from *Pseudomonas cellulosa*

β-Mannosidase
derived from *Bacteroides thetaiotaomicron*

Figure 3.15: The mannan-decomposing enzyme systems.

(2) β-Mannosidases

β-Mannosidase belongs to the family of exo-enzymes and acts on mannooligosaccharides to produce mannose. Furthermore, the complete hydrolysis of mannan requires the synergy of β-glucosidases, α-galactosidases, and acetyl mannan esterases.

4.3.2 Hydrolysis Mechanism of Hemicellulase

Complete hydrolysis of xylan requires synergy of various enzymes. Firstly, endo-1,4-β-xylanases randomly cleave xylan backbones, to produce xylo-oligosaccharides, and decrease the polymerization degree. Then, β-xylosidases decompose xylo-oligosaccharides and xylobiose into xylose. The side-chain substituents hinder the action of xylanase and are hydrolyzed by α-l-Arabinofuranosidases, α-glucuronidases, and acetyl-xylan esterases.

Previous studies have demonstrated that the reaction mechanism of xylanase hydrolysis is an acid-alkali catalytic reaction that is performed by carboxyl groups, similar to those of glucanase and lysozyme. The reaction involves a two-step procedure, of which the carboxyl group of the enzyme active site glutamic acid first provides a proton to the β-1,4-glycosidic bonds, resulting in glycoside bond cleavage. Secondly, the negatively charged aspartic acid, glutamic acid, or histidine stabilize the transition state of carbon. Thirdly, the H_2O molecule provides the hydroxyl to the C-1 position of xylose to reduce sugar residues, and meanwhile provides a proton to the carboxyl of glutamic acid, resulting in enzyme restoration. This reaction arouses reversal of the configuration, and perhaps a single amino acid residue could complete the two-step proton transfer.

Other sources supposed that during the hydrolysis process catalyzed by xylanase, enzyme-glycoside intermediates have been generated. Despite the different xylanase structures, the same double-replacement mechanism was used to hydrolyze glycosidic bonds as follows:

1. Nucleophilic attack to the C-1 positions of initial degraded xylose units by nonionized aspartate or glutamate residues;
2. Generation and removal of polysaccharide fragments from the active site;
3. Generation of covalent bonds between positive carbon ions and ionized glutamic acid or aspartic acid;
4. Additional reactions of hydroxyl groups from water molecules occur to positive carbon ions, and protons to the nucleophilic groups.

In addition, complete hydrolysis of mannan, arabinan, and arabinogalactan are also the results of various hemicellulases synergies, and different enzymes act on different positions (Figure 3.16) [40].

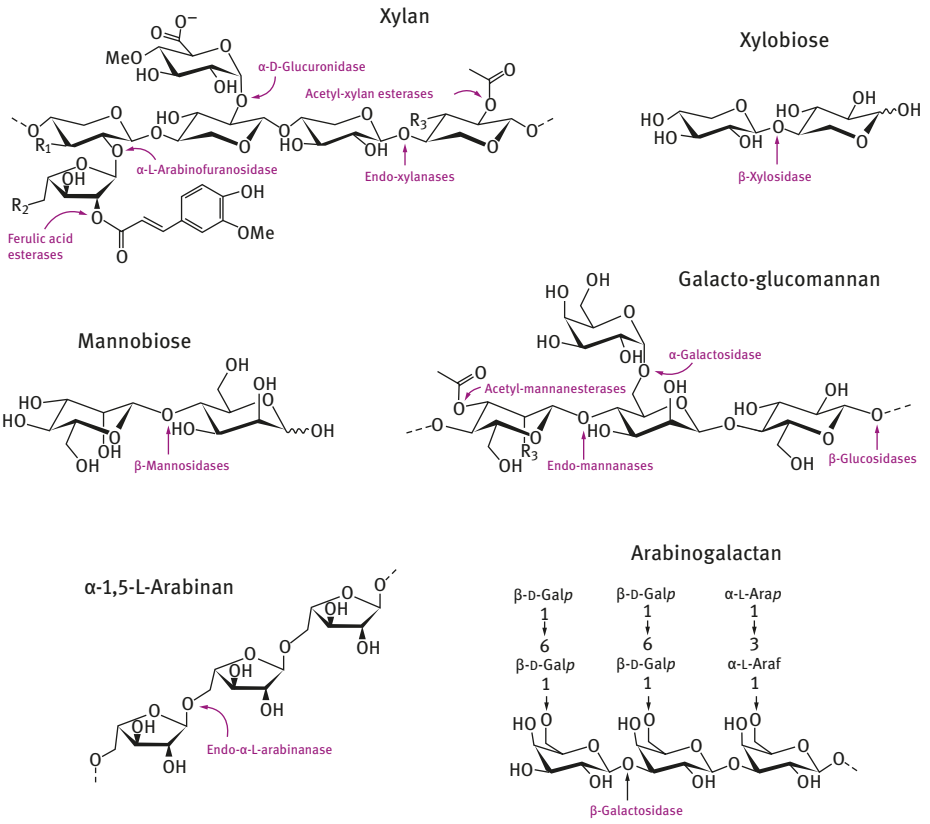


Figure 3.16: The action positions of different hemicellulose.

4.4 Molecular Breeding of Hemicellulases

Compared to the study of cellulases, only few studies were implemented on the biosynthesis and enzyme molecular regulation of hemicellulases. Previous studies have shown that the regulation and production mechanisms of cellulases and hemicellulases are independent. Moreover, increasing evidence has shown a mechanism of joint control on synthesis of the two enzymes. For example, xylanase of fungi is often synthesized with cellulase, and both the cellulase and, in some microorganisms, hemicellulose enzymes cannot be effectively expressed.

In recent years, xylanase-coding genes of nearly 100 different microorganism species have been cloned and are highly expressed in a variety of receptor strains (mainly *Pichia*). In addition, complete gene sequences of many xylanases have been determined to speculate the structure and function of enzymes, which lay a good foundation for the gene expression and molecular orientation transformation of xylanase enzymes. Through cloning and expression of xylanase genes of extremophiles,

xylanases suitable for industrial applications were obtained. In addition, from vector construction and recipient strain selection, several extracellular secreted species were successfully acquired, thereby solving the problem that xylanase from most recombinant strains often retains in periplasm and cytoplasmic regions rather than being secreted extracellularly, thus supplying great convenience for the purification and application of xylanase.

Moreover, large-scale industrial applications of xylanase require that more enzyme products meet the application environment, including better thermal stability, and adaptation to a wider range of pH and temperature. Systems biology techniques are usually adopted to determine the active site of the enzyme, as well as the role of certain residues in catalysis. Site-directed mutagenesis techniques are used for gene-specific operations, to realize rational protein design and improvement to obtain the desired enzyme product.

4.5 Application of Hemicellulase

Hemicellulase can be widely used in many disciplines, such as the feed industry, food industry, pulp and paper industry, and energy industry, thereby fully demonstrating its promising production potential. As a feed additive, hemicellulase improves the feed utilization process by helping animals to decompose nonstarch polysaccharides feed presenting in the plant feed. In the pulp and paper industry, hemicellulase enhances the pre-bleaching process, reduces the use of chlorine and sodium hydroxide, thereby reducing the paper industry-derived pollution to the environment.

During food processing, several inclusions in the plant cells, such as proteins, fat, starch, pigments, and other soluble alkaloids, are difficult to be extracted due to the tightly packed plant cell walls. Pretreatment of these plant materials soften the hemicellulose, which helps swell/collapse the cell walls, thereby facilitating the extraction of the contents, simplifying processing, and improving food quality. In recent years, it has been demonstrated that xylo-oligosaccharides and xylose have many advantages, such as preventing obesity due to the difficulty to digest, low calories, and high proliferative activity to bifidus bacteria. Moreover, dental caries are hard to be developed due to the hardly fermentable and replaceable sucrose as a diabetic diet product. Xylo-oligosaccharides and xylose can be easily obtained by xylanase hydrolysis of cheap and available agricultural waste, such as cotton shells, bagasse, and corn cob.

With the improvement of living standards, energy shortages, environmental pollution, and other issues, the harmonious development of human and ecological environment is seriously restricted. Therefore, the search for novel alternative energy sources is clearly warranted. As one of the most abundant resources on the planet, ligonocellulose could be degraded into pentoses and hexoses through the synergetic action of hemicellulases and other enzymes, and be fermented to ethanol by microbes. This not only provides clean and efficient alternative biomass fuel for

humans but also effectively reduces environmental pollution and promotes healthy and harmonious development of human society.

Although hemicellulose is widely used for production practices, its quality still needs to be increased, including a higher specific activity, higher resilience/stress tolerance and higher stability for large-scale applications. Therefore, system biology technologies, such as mutagenesis, genetic engineering, and protein engineering, are urged to improve the hydrolysis efficiency, stability, and specificity of enzymes, to expand the application of hemicellulase.

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Jingliang Xu, Huijuan Xu, Xiaoyan Chen, Yunyun Liu, Qiang Yu, Yu Zhang

4 Microbial Ethanol Fermentation

Ethanol can be obtained from diverse materials including corn, potatoes, sugarcane, and cellulose by performing hydrolysis, fermentation, and distillation steps. Ethanol is used in many fields, and the most important is the production of denatured fuel alcohol. Denatured ethanol fuel can be obtained by adding some denaturants (unleaded gasoline) to absolute ethanol, which is the dehydration product obtained from distilled ethanol. The addition of denatured fuel alcohol to gasoline in a certain proportion can increase the octane number of gasoline, reduce the CO and hydrocarbon emission of vehicle exhaust, lower the pollution from vehicle emission, and alleviate the energy crisis from the shortage of petroleum resources.

With the increase in global energy crisis and environmental pollution, ethanol fuel has become one of the renewable energies developed worldwide owing to abundant raw material, mature production technology, and wide applications. Although China started bioethanol development in 2000, it is the third in bioethanol production in the world after Brazil and the United States because of its rapid development.

Ethanol can be produced by two methods, namely chemical synthesis and biological fermentation. At present, about 90% of global ethanol is produced by biological fermentation. Biological fermentation is a microbial metabolism process using starch, sugar, or cellulosic biomass as the raw materials. This chapter will describe the theory, process, and applications of microbial fermentation of hexose, pentose, and syngas.

Section 1: Microbial Hexose Fermentation

Yeast, *Rhizopus*, *Aspergillus*, and some bacteria are capable of ethanol fermentation. However, most of these strains cannot hydrolyze polysaccharide. Therefore, for ethanol production, the prehydrolysis method is usually used as the first step to degrade the cellulose or starch to monosaccharides. For starch hydrolysis, fungal amylase is generally used as the catalyst, and the reactions are shown as follows:

$$(C_6H_{10}O_5)_n \xrightarrow{\text{acid/}\alpha\text{-amylase},H_2O} \alpha\text{-1,4-oligomeric glucose} \tag{4.1}$$

$$\alpha\text{-1,4-oligomericglucose} \xrightarrow{\text{acid/}\alpha\text{-amylase},H_2O} nC_6H_{12}O_6 \tag{4.2}$$

Corresponding author: Jingliang Xu, E-mail: xjl@ms.giec.ac.cn
Huijuan Xu, Xiaoyan Chen, Yunyun Liu, Qiang Yu, Yu Zhang, Guangzhou Institute of Energy Conversion, Chinese Academy of Sciences; CAS Key Laboratory of Renewable Energy, Guangdong Provincial Key Laboratory of New and Renewable Energy Research and Development, Guangzhou 510640, China

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Lignocellulosic biomass is composed of cellulose, hemicelluloses, and lignin. Cellulose with highly crystalline microfibrils is parceled by hemicelluloses and lignin. This complex structure hinders the effective contact of substrate with enzyme, making the direct biodegradation of feedstock very difficult. Conversion of lignocellulosic biomass to monosaccharides requires pretreatment, acid or enzymatic hydrolysis. The hydrolysis reactions are shown as follows:

$$\left(C_6H_{10}O_5\right)_n \xrightarrow{\text{acid/cellulase},H_2O} \beta\text{-1,4-oligomeric glucose} \tag{4.3}$$

$$\beta\text{-1,4-oligomeric glucose} \xrightarrow{\text{acid/cellulase},H_2O} nC_6H_{12}O_6 \tag{4.4}$$

A small amount of other hexoses such as galactose and mannose are generated during the hydrolysis. These monosaccharides can also be further converted to ethanol by microbial fermentation.

The existing ethanol production strains have different levels of capacity, including ethanol yield, production rate, and ability to use monosaccharides and disaccharides and withstand extreme environments. Even the capacities of different strains from the same species are different. Therefore, fermentation strains should generally have the following characteristics for industrial production:

(1) High fermentation ability, short circle, and high enzyme activity
(2) Fast proliferation and strong reproduction
(3) High tolerance to ethanol, surviving in the fermenting mash of high ethanol concentration
(4) High tolerance and fast fermentation rate at high temperatures
(5) High contamination resistance
(6) High acid resistance
(7) Stable genetic and slight variation

1.1 Species of Microorganisms for Ethanol Production with Hexose

Many microorganisms including bacteria, yeast, and filamentous fungi ferment sugars to ethanol, while some of them can be applied in large-scale industrial production. Table 4.1 shows some common ethanol fermentation microorganisms and their fermentation substrates [1].

Ethanol tolerance and production yields of the strains determine the quality of products and economic benefits for the next concentrated process. *S. cerevisiae* and *Z. mobilis* have the advantages of high ethanol production and yield with a high tolerance to ethanol. Therefore, they are widely used in the ethanol production industry.

Table 4.1: Species of ethanol production microorganisms and their fermentation substrates.[a]

Microorganisms	Fermentation substrates
Saccharomyces cerevisiae	Glucose, fructose, galactose, maltose, malt trisaccharide, and xylulose
S. rouxii	Glucose, fructose, maltose, and sucrose
S. carlsbergensis	Glucose, fructose, galactose, maltose, malt trisaccharide, and xylulose
S. pombe	Glucose and xylose
Kluyveromyces fragilis	Glucose, galactose, and lactose
K. lactis	Glucose, galactose, and lactose
Pachysolen tannophilus	Glucose and xylose
Candida shehatae	Glucose and xylose
C. tropicalis	Glucose, xylose, and xylulose
Pichia stipitis	Glucose, xylose, mannose, galactose, and cellobiose
Zymomonas mobilis	Glucose, fructose, and sucrose
Thermoanaerobacter brockii	Glucose, sucrose, and cellobiose
T. acetoethylicus	Glucose, sucrose, and cellobiose
Clostridium thermocellum	Glucose, cellobiose, and cellulose
C. thermohydz	Glucose, xylose, sucrose, cellobiose, and starch

[a] Li K, et al. 2008 [1].

S. cerevisiae is the typical strain used for hexose fermentation, and it has been recognized as one of the best ethanol fermentation strain. *S. cerevisiae* can use diverse substrates including glucose, mannose, and galactose. Moreover, it can survive under anaerobic conditions. With a high sugar and ethanol tolerance, it can ferment a high concentration of sugar and achieve up to 15–18% (w/v) ethanol concentration with less metabolic byproducts and significantly save the cost of subsequent ethanol separation such as ethanol distillation [2].

Z. mobilis attracted much attention since its discovery in 1912; it was isolated from a sugar-rich plant fermentation juice [3]. Using glucose or fructose as the fermentation substrate, the ethanol production rate by *Z. mobilis* is three to four times faster than that by yeast. The substrate conversion efficiency can reach approximately 97.398%, where only a small portion of substrate was converted into biomass and a slight amount of cell was accumulated during the fermentation. The substrate utilization efficiency was so high that the final ethanol yield was high (0.49–0.50 g ethanol/g glucose). Many of these strains can grow at 38–40 °C, which is 6–7 °C higher than that for yeast growth; thus, they can be used for high-temperature fermentation. The strains can endure a high osmotic pressure, and most of them can grow in a solution where the glucose concentration is as high as 40% (w/v). For a high ethanol tolerance, the final ethanol mass concentration can reach up to 12% (w/v) at 30 °C. The

high tolerance of hydrolyzates to inhibitors makes these strains regarded as a huge potential microorganism for ethanol fermentation industrialization, and they have been used in starch-based ethanol fermentation.

1.2 Mechanisms of Microbial Ethanol Fermentation from Hexose

Glucose is the main hexose for ethanol fermentation, and its fermentation consists of two stages: The first stage is the formation of pyruvic acid through diverse enzymatic catalysis. In the second stage, the pyruvate is deoxidized to two molecules of ethanol through decarboxylase catalysis, and CO_2 is released under anaerobic conditions. Different microorganisms may have different metabolic processes in the first stage, and there are four main pathways: EMP pathway (Embden–Meyerhof–Parnas pathway), ED pathway (Entner–Doudoroff pathway), HMP pathway (hexose monophosphate pathway), and phosphoketolase pathway [4].

1.2.1 Metabolic Pathway of Microbial Ethanol Fermentation from Hexose

1.2.1.1 EMP Pathway

EMP pathway is also known as glycolysis pathway, and it involves the production of ATP for providing energy to most common organisms under anaerobic conditions. EMP pathway contains a total of 10 reactions (steps) and can be divided into two stages: The first five steps are the preparation phase, where glucose is lysed to C3 sugar through phosphorylation and isomerization. The 1,6-positions of glucose molecules are phosphorylated by hexokinase and phosphofructokinase catalysis, and the generated fructose 1,6-diphosphate produces a final common intermediate glyceraldehyde-3-phosphate through splitting decomposition and isomerization. Each hexose lysis requires two ATP molecules. The last five steps are the energy storage stage for ATP generation. 1,3-Diphosphoglycerate is generated from glyceraldehyde-3-phosphate by dehydrogenase catalysis and then converted to 3-phosphoglycerate through kinase catalysis. 3-Phosphoglycerate is finally converted to pyruvate by a series of catalysis using phosphoglycerate mutase, enolase, and pyruvate kinase; meanwhile, two ATP molecules are released. The entire process utilizes 10 types of glycolysis enzymes. These enzymes are present in the cytosol, and most of them need Mg^{2+} as the cofactor. The overall glycolytic reaction is as follows:

$$C_6H_{12}O_6 + 2NAD^+ + 2ADP + 2Pi \longrightarrow 2CH_3COOH + 2NADH + 2H^+ + 2ATP + 2H_2O \quad (4.5)$$

Pyruvate produced in glycolysis can be further metabolized. Under anaerobic conditions, different microbial organisms degrade pyruvate to generate different metabolites. Many bacteria use lactic acid as the final product under anaerobic or relatively anaerobic conditions. The process in which ethanol is the final product is known as ethanol fermentation, and ethanol production from pyruvate involves decarboxylation and reduction. The reaction is as follows:

$$\text{pyruvate} \xrightarrow{\text{pyruvate decarboxylase}} \text{acetaldehyde} \xrightarrow{\text{ethanol dehydrogenase}} \text{ethanol} \quad (4.6)$$

With a low production efficiency, only 2 mol of ATP for yeast growth can be produced from 1 mol of glucose during the glycolysis. Most of the substrate is converted to ethanol, and the ethanol yield can reach up to 0.49 g ethanol/g glucose (more than 95% of the theoretical yield). Glycolysis provides diverse intermediate metabolites for biosynthetic pathways. They not only provide energy for organisms during hypoxia but also play a bridging role for many important metabolic pathways including citric acid cycle, HMP pathway, and ED pathway. The reverse reaction affords polysaccharides and is closely related to ethanol, lactic, acid, glycerin, acetone, and butanol fermentation.

1.2.1.2 ED Pathway

ED pathway was first discovered in 1952 in *Pseudomonas saccharophila* and later found to be present in many bacteria (widely distributed in Gram-negative bacteria); it is also known as 2-keto-3-deoxy-6-phosphogluconate (KDPG) cleavage pathway. In the ED pathway, 6-phosphogluconate-δ-lactone is produced from glucose-6-phosphate by glucose-6-phosphate dehydrogenase catalysis, meanwhile reducing $NADP^+$ to generate NADPH. The high activity of esterase helps to rapidly hydrolyze the lactone to generate 6-phosphogluconate, finally producing a specific product, 2-keto-3-deoxy-6-phosphogluconate in ED pathway by glucose-6-phosphate dehydrase catalysis. 2-Keto-3-deoxy-6-phosphate gluconic acid is then cleaved to form pyruvate and 3-phosphate-glyceraldehyde under the action of a specific aldolase, and 3-phosphate-glyceraldehyde is finally converted to aldehyde and CO_2 through a unique pyruvate decarboxylase catalysis. The difference between this type of pyruvate decarboxylase and that in yeast is that it does not require thiamine pyrophosphate as a coenzyme to maintain its vitality. Finally, similar to EMP pathway, two types of ethanol dehydrogenase (ADH I and ADH II) reduce acetaldehyde to ethanol, and the same amount of NADH is oxidized to generate NAD^+.

The overall reaction of ED pathway is as follows:

$$C_6H_{12}O_6 + ADP + Pi + 2NADP^+ + NAD^+ \longrightarrow 2CH_3COOH + ATP + NADPH + 2H^+ + NADH \quad (4.7)$$

ED pathway is independent of EMP and HMP pathways and exists alone. It is an alternative ethanol fermentation pathway for a minority of microbial organisms lacking

the complete EMP pathway. In this process, the energy production is low, and 1 mol of glucose generates only 1 mol of ATP. However, glucose requires only four steps to form pyruvate which in the EMP pathway is formed through 10-step reactions. Therefore, ED pathway has a high metabolic rate to ferment ethanol with less cells generated, and its product conversion rate is high. Ethanol production through ED pathway has some other features such as less metabolic byproducts, a high fermentation temperature, and without a regular supply of oxygen. However, the fermentation process is easily contaminated due to a high pH. Bacteria with ED pathway include *Zymomonas* bacteria (*Z. mobilis*), *Pseudomonas saccharophila*, and *P. aeruginosa*.

1.2.1.3 HMP Pathway

HMP pathway is also known as hexose monophosphate branch, pentose phosphate pathway (PPP), glucose phosphate acid pathway, or WD pathway. This pathway is composed of a cyclic reaction system and provides another mechanism for glucose decomposition. Glucose-6-phosphate, the starting material for the reaction system, undergoes oxidative decomposition to generate pentose, CO_2, inorganic phosphoric acid, and NADPH.

The overall reaction for HMP pathway is as follows:

$$6\text{glucose-6-phosphate} + 12\text{NADP}^+ + 7\text{H}_2\text{O} \longrightarrow 5\text{glucose-6-phosphate} + 12\text{NADPH} + 12\text{H}^+ + 6\text{CO}_2 + \text{Pi} \quad (4.8)$$

Every six glucose-6-phosphate molecules become five glucose-6-phosphate molecules in the PPP and produce six CO_2 molecules, Pi, 12 molecules of NADPH, and 12 H^+.

HMP pathway exists in most aerobic and facultative anaerobic bacteria and usually coexists with the EMP pathway. Only a few bacteria possess HMP pathway and no EMP pathway. HMP pathway not only provides the motive force NADPH for microbes but also supplies negative hydrogen ions in reductive biosynthetic process. It is an important source of sugar molecules with different structures within the cell. Moreover, it provides conditions for the interconversion of various monosaccharides and expands the carbon use range for microbial organisms.

1.2.1.4 Phosphoketolase Pathway

Phosphoketolase pathway involves glycolysis through the xylulose-5-phosphate stage and forms di- and tricarbonic acid. It can be divided into HK and PK pathways according to specific enzymes. The overall reaction of HK pathway is as follows:

$$2\text{C}_6\text{H}_{12}\text{O}_6 \longrightarrow 2\text{CH}_3\text{CHOHCOOH} + 3\text{CH}_3\text{COOH} + 5\text{ATP} \quad (4.9)$$

The overall reaction of PK pathway is as follows:

$$C_6H_{12}O_6 + ADP + Pi + NAD^+ \longrightarrow CH_3CH(OH)COOH + CH_3CH_2OH + CO_2 + ATP + NADH + H^+ \quad (4.10)$$

Pentose is cleaved to acetyl phosphate and glyceraldehyde-3-phosphate through phosphoketolase catalysis, which are further reduced to ethanol. Phosphoketolase pathway is more important for ethanol fermentation with pentose.

1.2.1.5 Other Hexose Metabolism Pathways

Other hexoses such as fructose, galactose, and mannose are generated in cellulose hydrolysis. Fructose and mannose can enter the glycolysis pathway, and galactose first needs to pass through Leloir pathway to convert to an intermediate product in glycolysis and then used by microbes.

(1) Fructose can be converted to fructose-6-phosphate by hexokinase catalysis and directly enter the glycolysis; the reaction is as follows:

$$\alpha\text{-D-fructose} + ATP \xrightarrow{\text{hexokinase}} \text{fructose-6-phosphate} + ADP \quad (4.11)$$

Fructose can be continuously catalyzed by six type of enzymes and eventually converted to the glycolysis intermediate product, glyceraldehyde-3-phosphate, and then fermented.

(2) Mannose needs only a two-step reaction to convert to fructose-6-phosphate and then enters the glycolysis pathway. The reaction is as follows:

$$\text{Mannose} \xrightarrow[ATP \rightarrow ADP]{\text{hexokinase}} \text{mannose-6-phosphate} \xrightarrow[\text{phosphom mannose}]{\text{phoshpomannosei somerase}} \text{fructose-6-phosphate} \quad (4.12)$$

(3) Galactose directly serve as a substrate for hexokinase; it needs galactose kinase catalysis and undergoes a complex reaction to convert to the intermediate product of glycolysis and then enters glycolysis.

1.2.2 Key Enzyme for Microbial Ethanol Fermentation from Hexose

Enzyme catalysis is affected by the activity and amount of enzymes. Enzyme activity is regulated by the covalent modification of enzyme and allosteric effectors, and enzyme amount is controlled by transcription according to the metabolic needs.

In a metabolic pathway, enzymes that catalyze the irreversible reactions are the key enzymes that control the metabolic process. Hexokinase, phosphofructokinase, and pyruvate kinase play an important regulatory role in ethanol fermentation.

1.2.2.1 Hexokinase

Hexokinase mainly catalyzes D-hexose (D-glucose, D-fructose, D-mannose, glucosamine, etc.) phosphorylation and generates D-hexose-6-phosphate, while converting an ATP to an ADP. The reaction requires Mg^{2+} [5]. The reaction is shown as follows:

$$\text{glucose} + \text{ATP} \xrightarrow[Mg^{2+}]{\text{hexokinase}} \text{glucose-6-phosphate} + \text{ADP} + H^{+} \tag{4.13}$$

Hexokinase is a regulatory enzyme. Its reaction products glucose-6-phosphate and ADP make the enzyme suffer from allosteric inhibition. The enzyme has four different forms, I, II, III, and IV; these morphological differences are mainly caused by the alternative splicing of the same gene, that is, by the regrouping of the exon in gene. Their distribution in the body is different, and the catalytic properties are also different.

1.2.2.2 Phosphofructokinase

Phosphofructokinase is an allosteric enzyme, which catalyzes fructose-6-phosphate to generate fructose-1,6-diphosphate [6], and the reaction is shown as follows:

$$\text{fructose-6-phosphate} + \text{ATP} \xrightarrow[Mg^{2+}]{\text{phosphofructokinase}} \text{fructose-1,6-biophosphate} + \text{ADP} + H^{+} \tag{4.14}$$

This reaction is irreversible and exergonic. It is the key reaction for glycolysis, and Mg^{2+}, $NH4^{+}$, or K^{+} is required.

Phosphofructokinase is an important regulatory enzyme in the glycolysis pathway; however, its catalytic efficiency is low. The rate of glycolysis depends on the enzyme's vitality. Phosphofructokinase has several binding sites with allosteric activator and inhibitor. Fructose-6-phosphate, fructose-1,6-diphosphate, ADP, and AMP are its activators, and ATP and citric acid are its inhibitors. The regulation of this enzyme is complex.

ATP can be used as a substrate and or an inhibitor. When the amount of ATP is insufficient within the cells, it is mainly used as a substrate to ensure the enzymatic reaction; and when the amount of ATP increases in cells, it acts as an inhibitor to reduce the affinity of enzyme to fructose-6-phosphate. High concentrations of ATP can be combined to a specific adjustment position in phosphofructokinase

and reduce its affinity to fructose-6-phosphate. However, this allosteric effect can be enhanced by AMP; therefore, the ratio of ATP and AMP has a significant regulatory effect on this enzyme.

Glycolysis provides not only energy in hypoxic conditions but also a carbon skeleton for biosynthesis, and the amount of carbon skeleton also affects the rate of glycolysis. The inhibition of citric acid to phosphofructokinase has this meaning. When the citric acid content in intracellular is high, that is, a lavish biosynthetic precursor exists, glucose does not need to degrade to provide a synthetic precursor. Citric acid inhibits the activity of phosphofructokinase by enhancing the inhibitory effect of ATP, thus slowing down the glycolysis. It is a key step for glycolysis regulation to adjust phosphofructokinase because it is the rate-limiting enzyme in glycolysis.

1.2.2.3 Pyruvate Kinase

In the final step of glycolysis, pyruvate kinase catalyzes the conversion of phosphoenolpyruvic acid to ATP and pyruvate [7]. The reaction is shown as follows:

$$\text{phosphoenolpyruvic acid} + \text{ADP} + \text{Pi} \xrightarrow{\text{pyruvate kinase}} \text{pyruvate} + \text{ATP} \tag{4.15}$$

The phosphate group is transferred from phosphoenolpyruvate to ADP, forming pyruvate. It is an irreversible reaction and requires the participation of Mg^{2+} and Mn^{2+}. The relative molecular mass of pyruvate kinase is 250,000 and is made up of a tetramer formed by four subunits with a relative molecular mass of 55,000 and composed of at least three different types of isoenzymes.

Pyruvate kinase is a significant allosteric regulatory enzyme in glycolysis. ATP, long-chain fatty acids, acetyl-CoA, and alanine inhibit pyruvate kinase, while fructose-1,6-biosphophate and phosphoenolpyruvic acid activate this enzyme. When sufficient energy is provided, the allosteric inhibition effect of ATP to pyruvate kinase can slow down glycolysis. The allosteric inhibition effect of alanine to pyruvate kinase can also decrease the rate of glycolysis. Alanine is formed by pyruvate to accept an amino group. The increase in its concentration indicates that excess pyruvate is utilized as a precursor, and thus alanine inhibits the glycolysis and maintains the metabolic homeostasis.

1.3 Breeding of Hexose Fermentation Strains

Although ethanol fermentation strains were initially derived from nature, the production of natural strains is generally low. For example, *S. cerevisiae* has a very complex cell structure and metabolic regulation system. The yeast cell uptake and utilization

efficiency of fermentable sugars for the growth and metabolism of cell to produce ethanol are coordinated and controlled by the cell regulatory network. It is difficult to enhance their metabolism level for large-scale industrial applications.

The rise of antibiotic industry in the 1940s promoted the rapid development of microbial genetics and laid a theoretical foundation for the breeding of excellent industrial microbial fermentation strains. In particular, the development of related technologies such as genetic, cell, and protein engineering gradually improved the breeding technology. At present, numerous fermentation strains with industrial application capacity are available to improve ethanol production and tolerance, temperature resistance, and range of substrate utilization through various breeding methods to reduce the ethanol production cost.

1.3.1 Domestication and Mutagenesis

Domestication utilizes constantly transplanted microbial populations to breed superior strains under specific selected conditions. In industrial breeding, this method can screen practical strains with a stable productivity because of the nature and long-term evolution in the fermentation broth. Bertolini's team screened two yeast strains with resistance to high levels of sugars and high-yield ethanol in a Brazil alcohol plant in 1991[8]. When they were fermented in 30% sucrose syrup, the final ethanol concentration in the mash reached 19.5% and 19.6%. Strains isolated from different materials are generally only suitable to similar types of ingredients in ethanol fermentation. Domestication screening generally relies on the spontaneous mutation of microorganisms. The spontaneous mutation probability is small where the positive mutation probability is smaller, and thus the breeding process is relatively slow.

Mutation breeding utilizes diverse mutagens to treat microorganisms, and positive mutants suitable for production are obtained by appropriate screening methods. It can increase the mutation probability and shorten the breeding period. The initial strain performance is important for the mutagenic effects and efficiency. It is best to select strains with capacities of fast growth and reproduction, low nutritional requirements, strong production ability, and mutagen-sensitive characteristics. Mutagens include physical, chemical, biological, and other substances, and their mutation mechanisms are different. The commonly used mutagens are ultraviolet mutagens, methyl sulfate acetate (EMS), and nitrogen mustard. Different microbial organisms have different sensitivities to mutagen, and the mutation probability also varies after the mutagen is treated. Therefore, it is significant to select appropriate mutagens and their dosage to improve the mutagenesis frequency and at the same time to increase their positive mutation rate. However, a single mutagen treatment often makes the strains sensitive to mutagen drop, and multiple mutagens with compound

mutation can avoid this problem. The combination of mutagenesis and other breeding techniques can provide more superior strains.

Mutation breeding has its weaknesses such as a low beneficial mutagenesis frequency, difficult to effectively control the variation direction and quality, and to induce and identify micromutations. Therefore, it is still required to solve technical problems such as how to improve the mutagenic efficiency, rapid identification, and screening of the mutant.

1.3.2 Genetic Engineering Techniques

Genetic engineering technology is also known as DNA recombination technology. This method involves manual operation to import exogenous DNA in vitro recombination into the recipient cells and makes them replicate, transcript, translate, and express in the recipient cells. Cohen and Boyer invented cloning technology in 1973 and successfully transferred exogenous genes into *E. coli* cells to express them, declaring the birth of genetic engineering. This new technology was not only a milestone in the development of life science research but also brought a revolutionary change to the modern biotechnology industry. In industrial breeding, a lot of strains with better production efficiency have been obtained through genetic engineering, making a great contribution to industrial production and improving the product quality.

Ethanol fermentation strains have been constructed through genetic engineering techniques by many researchers, and certain results have been achieved. Ethanol tolerance of *S. cerevisiae* is an important indicator for selecting ethanol production strains. Zhao et al. [9] built a constructive expression vector SPT3 (SPT3 is an important transcription factor for the ethanol tolerance of yeast) with pYES2.0 as the carrier. They established an SPT3 mutant gene library through error-prone PCR, transformed *S. cerevisiae*, and screened mutant M25 with an increased ethanol tolerance. In a 125 g/L glucose fermentation broth, M25 achieved 11.7% higher ethanol concentration than other strains. Analysis of the amino acid sequence of SPT3 in M25 showed that the isoleucine present in the *N*-terminal conserved region 59 combined with mutant protein and TBP mutated to threonine; this is probably related to the increased ethanol tolerance. In addition, some researchers recombined *Aspergillus niger* glucoamylase GAlcDNA to *E. coli* yeast shuttle plasmid and introduced into *S. cerevisiae* by protoplast transformation to achieve a highly expressed glucoamylase and thus obtained directly starch ethanol conversion strains.

However, genetically engineered bacteria often suffer from plasmid instability in passing from generation to generation. To improve the stability of genetically engineered bacteria, a two-stage culture method is usually used. In the first stage, the main aim is to increase the cell density, and exogenous gene is not expressed. Thus, the specific growth rate between engineered bacteria and plasmid-loss bacteria

is reduced; this can increase the plasmid stability. In the second stage, exogenous genes are induced to express. It can also be achieved by the addition of selective pressure such as antibiotics in the medium to inhibit the growth of plasmid-loss bacteria. To improve plasmid stability, appropriate culture conditions such as temperature, pH, dissolved oxygen, and other culture components should be controlled for a better growth rate of the engineered bacteria, or the culture conditions such as intermittent oxygen and dilution rates should be varied sometimes and the specific growth rate of plasmid-containing strains and plasmid-loss strains should be altered.

1.3.3 Protoplast Fusion

Cell fusion technology was discovered in the late 1950s and subsequently underwent rapid development with gradual increase in applications. Ferenczy from Hungary first reported that polyethylene glycol (PEG) can promote fungi fusion; this extended the protoplast fusion technology to microorganisms and provided a new method for microbial breeding.[10]

Cell fusion, also known as protoplast fusion, is composed of several steps. The cell wall is first removed by enzymatic hydrolysis to release only the plasma membrane wrapped protoplast, and then the parental strain's protoplast is mixed under hypertonic conditions. Meanwhile, a fusogenic agent is added to promote fusion and intercoagulation for genome exchange and recombinant. Finally, the prepotent recombinant is obtained from regenerated cell using reasonable screening methods.

Fusion agent acts as a substrate that induces the protoplast to fuse and can be divided into biological, chemical, and physical pro-thaw agents according to their characteristics. PEG is the most frequently used fusion agent with advantages of stable activity, easier to control and use, and strong ability to promote cell fusion. PEG is a polymer generally fused with a molecular weight of 4,000 to 6,000, and its molecular formula is $H(OCH_2{\cdot}CH_2)_nOH$. These types of polymers are connected by ether bonds with a weak electric charge in the end of the molecule. The fusion mechanism of PEG-induced protoplast fusion is still unknown. Preliminary assumption is as follows: PEG forms many negatively charged hydrogen bonds with water and thus makes the free water in the solution disappear; the resulting high dehydration causes the aggregation of protoplast and makes the neighboring protoplasts closely contact each other. Membrane protein particles in the contact region translocate and aggregate, and then the nearby lipoid molecules are disturbed and rearranged, resulting in the partial fusion of cell membrane in the contact region and forming a small cytoplasmic bridge, followed by gradual expansion for genetic material exchange and restructuring. Fusion process also requires Ca, probably because Ca^{2+} can bind to negatively charged phospholipids and separate the membrane phospholipid molecules from each other and thereby promotes fusion.

Generally, cultured cells in vitro rarely spontaneously fuse (fusion frequency is between 10^{-4}–10^{-5}). Protoplast fusion technology significantly enhances the intercell fusion frequency by artificially adding fusogenic agent to achieve the large-scale restructuring of genetic materials and break the species boundaries. There is no need to know the genetic background of strains to achieve recombination between distant strains and improve the breeding efficiency.

1.3.3.1 Strains with Tolerance to High-concentration Ethanol and Substrates

In traditional ethanol fermentation, the sugar concentration is generally between 160–250 g/L, and yeast can only generate a volume fraction of 6–12% ethanol. Higher concentrations of substrate and ethanol inhibit the yeast growth and fermentation. Strains with resistance to high ethanol concentration and substrate breeding by protoplast fusion technology not only save distillation cost and improve equipment utilization but also increase ethanol production. It is significant for the economic production of ethanol.

1.3.3.2 Thermophilic Yeast

Because of regional difference and seasonal change, when the environment temperature is high, the common yeast fermentation rate slows down, or even the fermentation ability is completely lost, making it difficult for normal ethanol fermentation and decreasing the ethanol yield. Therefore, the production equipment must be cooled to maintain normal ethanol fermentation. However, this will increase the investment in equipment and operation costs. Breeding strains with tolerance to high temperature have a significant economic and practical advantage.

Traditional high-temperature strain breeding mainly focused on high-temperature domestication and natural screening, needing a large amount of work. High-temperature strain breeding with protoplast fusion technology does not require detailed complex resistance to high-temperature mechanism with easier maneuverability.

1.3.3.3 Yeast with Saccharification Function

Saccharification and fermentation are generally carried out stepwise in traditional ethanol fermentation. Starch is cooked and enzymes are added to convert fermentable sugars before yeast fermentation. Yeast with ability to hydrolyze starch and dextrin not only saves equipment investment but also simplifies the production process. At present, protoplast fusion technology has made several achievements by constructing the dual function of saccharification and yeast fermentation. For example, the use of

S. cerevisiae and *C. tropicalis* as parent and single parent inactivated protoplast fusion technology provides fusants with a high glucoamylase activity and high ethanol yield. Moreover, PEG-induced protoplast fusion technology achieves isotype protoplast fusion with yeast haploid and high-temperature and ethanol-resistant yeast haploid to culture fusants with both a high dextrin utilization ability and resistance to high temperature with a high ethanol yield. Protoplast fusion technology can also be used to construct yeasts that can directly ferment raffinose, lactose, and other polysaccharides. These new fusion technologies have provided rich microbial resources for industrial applications.

Section 2: Microbial Pentose Fermentation

China has abundant biomass resources and produces 700 million tons of crop stalks per year. Apart from using them as feedstock, fertilizers, and other industrial materials, at least half of those stalks can be used for bioenergy development. Additionally, there are 134 million-hectare barren hills and wasteland suitable for the development of energy forestry and agriculture. All these resources provide sufficient materials for bioethanol production in China. However, there are still many difficulties in cellulosic ethanol industrial production. First, besides cellulose, 20–30% hemicellulose is present in lignocellulosic biomass. Compared to cellulose, hemicellulose is much easier to hydrolyze, as shown in the following reaction:

$$(C_5H_8O_4)_m \xrightarrow{\text{weak acid, } H_2O} mC_5H_{10}O_5 \tag{4.16}$$

The composition of lignocellulose varies in different raw materials (Table 4.2). Among them, the content of pentose, mainly xylose, is about 6–28% or even higher than the dry weight of plant. To achieve the industrial production of cellulosic ethanol, these pentoses should be utilized. Additionally, during the hydrolysis, a part of cellulose is degraded to fermentation inhibitors including acids, aldehydes, and phenols, further hindering ethanol production.

S. cerevisiae and *pseudomonas* are used to produce ethanol from glucose in traditional ethanol production; however, these microorganisms cannot utilize pentose such as xylose and arabinose. Although natural xylose fermentation organisms can convert xylose to ethanol, they cannot be directly used in industrial ethanol production because of low ethanol yield, weak ethanol tolerance, low fermentation productivity, and sensitivity to inhibitors. Many microbes can utilize arabinose, but many of them cannot transfer arabinose to ethanol directly. Thus, the breeding of efficient pentose ethanol fermentation microorganisms is the key step in the industrial production of lignocellulosic ethanol.

Table 4.2: Sugar composition of various lignocellulosic materials.[a]

Biomass	Component (%)								
	Glucose	Mannose	Galactose	Xylose	Arabinose	Hexose	Pentose	Lignin	Ash
Corn stover	39	0.3	0.8	14.8	3.2	40.1	18	15.1	4.3
Wheat straw	36.6	0.8	2.4	19.2	2.4	39.8	21.6	14.5	9.6
Rice straw	41	1.8	0.4	14.8	4.5	43.2	19.3	9.9	12.4
Rice hulls	36.1	3	0.1	14	2.6	39.2	16.6	19.4	20.1
Bagasse fiber	38.1	–	1.1	23.3	2.5	39.2	25.8	18.4	2.8
Newspaper	64.4	16.6	–	4.6	0.5	81	5.1	21	0.4
Fir	50	12	1.3	3.4	1.1	63.3	4.5	28.3	0.2
Pine	43.3	10.7	2.9	5.3	1.6	56.9	9.8	28.3	7.9
Switchgrass	39.5	–	2.6	20.3	2.1	31	20.4	21.8	4.1
Beech	41.6	2.2	3.6	19.7	0.4	47.4	20.1	21.8	0.3

[a] Aristidou & penttila 2000 [11].

2.1 Pentose-fermenting Microorganisms

The first xylose-fermenting microorganism *Fusarium lini* was discovered in 1928 [12]; then, over 100 organisms that can utilize xylose have been discovered (Table 4.3). The fermentation properties of these bacteria, fungi, and yeasts differ from each other, even different strains of the same species are no exception. Bacteria can utilize many types of sugars, but produce many byproducts and reach a low ethanol yield and titers, about 0.16–0.39 g ethanol per g sugar. Many bacteria can only grow and produce ethanol at neutral pH environment, increasing the risk of other organism contamination. Some bacteria produce toxin during the metabolic process, increasing the difficulty in product purification and production cost.

Fusarium oxysporum and *Neurospora crassa* are the most deeply studied xylose-fermenting fungi. These two strains can secrete cellulases and hemicellulases and utilize pentose and hexose; this significantly simplified the lignocellulosic ethanol process. However, these two strains were used in simultaneous saccharification and fermentation (SSF); their abilities for xylose fermentation have not been widely studied yet. *P. tannophilus*, *C. shehatae*, and *P. stipitis* are yeasts that can utilize xylose. The xylose ethanol yield obtained was 62.7–88.2% of the theoretical value; the highest yield was 0.45 g/g, achieved by *P. stipitis* CBS 5776. Although natural xylose metabolic yeasts can provide high yield of ethanol from xylose, there are some common disadvantages: First, the ethanol productivity from xylose is lower than that from glucose. Second, fermentation was performed under limiting oxygen condition, thus increasing energy consumption and microorganism

Table 4.3: Natural xylose-to-ethanol fermentation microorganisms.[a]

Strains	Xylose (g/L)	Ethanol (g/L)	Yield (g/g)	Productivity (g/L·h)
Bacterium				
Bacillus macerans DMS 1574	20	3.3	0.16	0.03
Bacteroides polypragmatus NRCC 2288	44	6.5	0.15	0.09
Clostridium saccharolyticum ATCC 35040	25	5.2	0.21	0.05
C. thermohydrosulfuricum HG8	35	12.8	0.50	0.37
Thermoanaerobacter ethanolicus ATCC 31938	4	1.7	0.42	0.50
Yeasts				
Candida blankii ATCC 18735	50	5.1	0.10	0.07
C. famata	20	3.9	0.20	0.07
C. fructus JCM-1513	20	4.7	0.24	0.02
C. guilliermondii ATCC 22017	40	4.5	0.11	0.04
C. shehatae CBS 4705	50	24.0	0.48	0.19
C. shehatae CSIR-Y492	90	26.2	0.29	0.66
C. sp. CSIR-62 A/2	50	20.1	0.40	0.42
C. tenius CBS 4435	20	6.4	0.32	0.03
Candida tropicalis ATCC 1369	100	5.4	0.11	–
Hansenula polymorpha KT2	20	3.4	–	–
Kluyveromyces marxianus KY 5199	100	30.0	0.31	–
P. tannophilus NRRL Y-2460	20	6.2	0.31	0.06
P. tannophilus NRRL-Y-2460	50	16	0.32	0.16
P. stipitis CSIR-Y-633	50	21.5	0.43	0.86
P. stipitis CBS 5576	20	22.3	0.45	0.34
P. segobiensis CBS 6857	20	5.0	0.25	0.02
Schizosaccharomyces pombe ATCC 2478	50	5.0	0.10	0.07
Fungi				
Aureobasidium pullulans	20	4.2	0.21	0.09
Aspergilli awamori 3112	50	1.4	0.03	0.01
A. niger 326	50	1.2	0.02	0.01
A. oryzae 694	50	4.7	0.09	0.03
A. sojae 5597	50	5.4	0.12	0.04
A. tamari 430	50	3.5	0.07	0.03
A. foetidus 337	50	3.4	0.07	0.03
Fusarium avenaceum VTT-D-80146	50	12.0	0.24	0.07

Table 4.3: (continued)

Strains	Xylose (g/L)	Ethanol (g/L)	Yield (g/g)	Productivity (g/L·h)
F. clamydosporum VTT-D-77055	50	11.0	0.22	0.07
F. culmorum VTT-D-80148	50	12.0	0.24	0.07
F. graminearum VTT-D-79129	50	11.0	0.22	0.07
F. lycopersici ATCC 16417	50	16.0	0.32	0.17
F. oxysporum VTT-D-80134	50	25.0	0.50	0.17
F. sambucium VTT-D-77056	50	13.0	0.26	0.08
F. solani VTT-D-77057	50	11.0	0.22	0.07
F. tricinetum VTT-D-80139	50	7.0	0.14	0.04
Monilia sp.	50	12.6	0.25	0.08
Mucor 105	50	8.0	0.16	0.08
Mucor indicus	50	11.0	0.22	0.18
M. hiemalis	50	9.0	0.18	0.12
M. corticolous	50	7.5	0.15	0.10
N. crassa NCIM 870	20	6.9	0.35	0.04
Paecilomyces sp. NFI ATCC 20766	100	39.8	0.40	0.24
Rhizopus oryzae B	50	14.0	0.28	0.11
Rhizopus javanicus 2871	50	11.7	0.23	0.16
Rhizomucor pusillus NBRC 4578	20	18	0.18	–

[a] White MG & Willaman JJ. 1928 [13].

contamination and causing a high demand for production control. Third, they showed low tolerance to lignocellulosic hydrolysate inhibitors; a predetoxification is needed, leading to higher production costs. Fourth, a low tolerance to high-concentration ethanol resulted in a low-concentration ethanol output with many byproducts, making the separation process harder; the inhibition from hexose metabolism makes it difficult to use these xylose-utilizing yeasts in large-scale industrial lignocellulosic ethanol production.

Up to now, the known organisms that can produce ethanol from arabinose are bacteria and fungi and a few yeasts (Table 4.4). Most xylose-to-ethanol fermentation yeasts can utilize arabinose, but only a few of them can convert arabinose to ethanol. The ethanol yields are rather low, only one-fifth of xylose ethanol. The low arabinose ethanol conversion makes it impossible for the use of these natural arabinose ethanol organisms in industrial ethanol production.

Table 4.4: Natural arabinose-to-ethanol fermentation microorganisms.[a]

Strains	Arabinose (g/L)	Ethanol (g/L)	Arabitol (g/L)	Sugar utilization (%)
Bacterium				
Sarcina ventriculi	20.4	6.0	–	90
Yeast				
Ambrosiozyma monospora Y-1081	80	0.8 ± 0.2	0 ± 0	14 ± 6
A. monospora Y-5955	80	1.8 ± 0.7	0 ± 0	19 ± 8
A. monospora Y-1484	80	2.3 ± 0.4	4 ± 0	36 ± 4
Candida arabinofermentans YB-2248	80	0.7 ± 0.3	–	–
Candida auringiensis Y-11848	80	1.4 ± 0.6	17 ± 1	32 ± 1
Candida succiphila Y-11997	80	2.1 ± 0.3	8 ± 1	43 ± 2
C. succiphila Y-11998	80	2.3 ± 0.4	8 ± 0	32 ± 6
Candida sp. YB-2248	80	3.4 ± 0.1	4 ± 0	33 ± 5
R. pusillus NBRC 4578	20	7.0	6.0	–
Fungi				
N. crassa (D-arabinose)	20	4.0	–	–
Paecilomyces sp. NF1	–	13.8	–	–

[a] Dien BS, et al. 1996 [14].

2.2 Mechanism of Microbial Ethanol Fermentation from Pentose

2.2.1 Metabolic Pathway of Microbial Ethanol Fermentation from Pentose

2.2.1.1 Xylose Metabolism

Different microorganisms have different xylose metabolic pathways (Figure 4.1) [15]. In bacteria and some lower fungi, xylose converts to xylulose by xylose isomerase (XI), and then xylulokinase (XK) adds a phosphate to generate xylulose 5-phosphate involved in the PPP. The intermediates of PPP, glucose 6-phosphate (G6P) and glyceraldehyde 3-phosphate (G3P), enter the ED pathway and produce ethanol. In some fungi, xylose converts xylulose under the collaboration of xylose reductase (XR) and xylitol dehydrogenase (XDH), which require NADPH and NAD^+ as the coenzyme, respectively. Xylulose is phosphated by XK and converts to xylulose 5-phosphate, and the latter enters PPP. The intermediates, G6P and G3P, enter the EMP pathway and convert to ethanol under anaerobic condition.

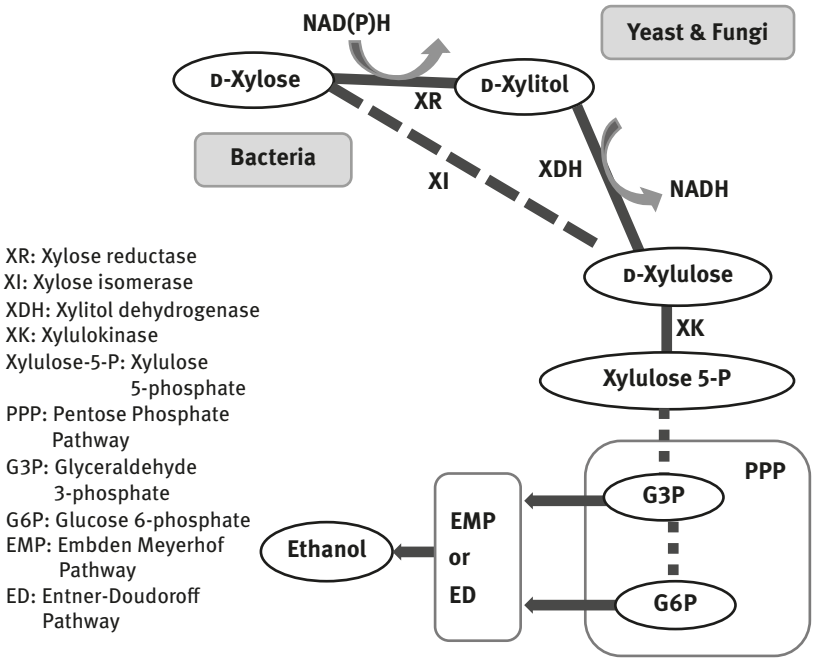

Figure 4.1: Xylose metabolic pathway.
Source: Wang JY, Zhu SG, Xu CF (2002) [15].

The reaction can be described as follows:

$$3C_5H_{10}O_5 + 3ADP + 3Pi \longrightarrow 5C_2H_5OH + 5CO_2 + 3ATP + 3H_2O \qquad (4.17)$$

The theoretical ethanol yield from xylose is the same as that from glucose, 0.51 g ethanol/g consumed sugar.

2.2.1.2 l-Arabinose Metabolism

The L-arabinose metabolic pathways are diverse in prokaryotic and eukaryotic microorganisms are shown in Figure 4.2 [15]. In prokaryotic cells, L-arabinose is catalyzed by a specific isomerase and converts to L-ribulose. L-ribulose is phosphorylated by L-ribulokinase and then epimerized to form xylulose 5-phosphate by L-ribulose 5-phosphate 4-epimerase, which enters the PPP pathway. The L-arabinose metabolism is much complex in eukaryotic cells. Three redox reactions and a phosphorylation are needed to form xylulose 5-phosphate, which enters the PPP pathway.

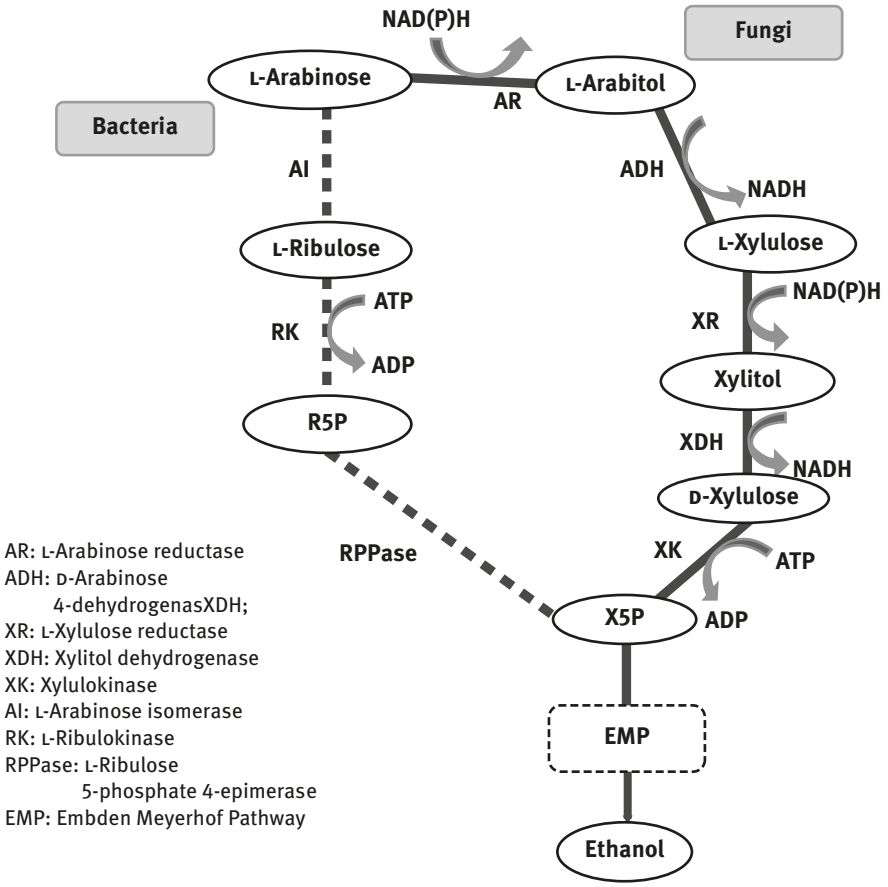

Figure 4.2: L-Arabinose metabolic pathway.

Source: Wang JY, Zhu SG, Xu CF (2002) [15].

2.2.2 Key Enzymes in Pentose Ethanol Fermentation

2.2.2.1 Key Enzymes in Xylose Fermentation

(1) XI

XI is encoded by *XYLA* gene and catalyzes the isomerization of D-xylose to D-xylulose. XI is also known as glucose isomerase, because it can catalyze the epimerization of D-glucose to D-fructose and is widely used in industry to produce high-fructose syrup in large scales.

XI was first discovered in *Pseudomonas hydrophila*; therefore, XI existed in many bacteria and *Actinomycetes* [16]. The specificities of XI from different microbes are diverse; besides D-glucose and D-xylose, some XIs can also utilize many other sugars including D-arabinose, L-rhamnose, D-allose, and 2-dexo-glucose. Additionally, the catalytic efficiencies for D-glucose and D-xylose are different.

All the XIs are nonglycoproteins with an active form of tetramer or dimer, even though their primary structures differ from each other. The subunits of tetramer are connected by noncovalent bonds without a disulfide bridge. The binding force between two dimers is weaker than that between two subunits of a dimer. XI is a metal ion activated enzyme, activated by Co^{2+}, Mn^{2+}, and Mg^{2+} and inhibited by Ca^{2+} and Zn^{2+}. XIs from different resources were crystallized and analyzed to determine their structures. They have similar spatial structures with different amino acids around the active center [17].

According to the sequence homologies analysis, XI can be divided into two types. XIs of type I, mainly of *Escherichia coli, Bacillus subtilis*, and *Thermotoga* species, contain an extended *N*-terminal domain. XIs of type II, mainly of *Streptomyces* species, *Actinoplanes missouriensis*, *Thermus* species, and *Arthrobacter* species, have no *N*-terminal prolonged domain. The homologs of type II XIs commonly excess 50%, and their molecular weights are less than those of type I.

(2) XR and XDH

In bacterium cells, xylose is converted to xylulose by XI. However, in yeast and most fungi cells, xylose is first reduced to xylitol by XR and then converted to xylulose by XDH.

XR is an aldose reductase, because it shows affinity to D-xylose as well as other aldoses including D-glyceraldehyde, L-arabinose, D-ribulose, D-galactose, and D-glucose. At least two types of XR exist. One of them is NADPH-dependent [18], and the other can also utilize NADH as the coenzyme, even though the affinity to NADPH is higher than that to NADH, and the XR activity is about 70% with NADH than NADPH [19].

XDH is a homotetramer with a molecular weight of about 160 kDa. Actually, XDH is a heteronuclear multiple metal protein; one molecule of XDH contains one Zn^{2+} and six Mg^{2+}. XDH is Zn^{2+}-dependent; the lack of Zn^{2+} leads to the loss of enzyme activity. XDH activity is not influenced by Mg^{2+} shortage. Because XDH is NAD^+-dependent, the consumption of NAD^+ results in the imbalance of cofactor and limits ethanol production [20, 21].

(3) XK

XK transfers a phosphate from ATP to D-xylulose and generates xylulose 5-phosphate and ADP, which is the node position of xylose metabolite in PPP pathway and the limit-rating step of xylose metabolism. All the XKs are dimers; the two subunits have molecular weights of 71 and 54 kDa, respectively. Most XKs require Mg^{2+}, Fe^{2+}, Mn^{2+}, or Zn^{2+} as the activator. Those XKs of *Aerobacterogenes*, *P. stipitis*, or *E. coli* can only maintain activity in the presence of Mg^{2+}. The XK of *Lactobacillus pentosus* showed the highest activity under the activation of Fe^{2+} than Mg^{2+}, Mn^{2+}, or Zn^{2+}.

Although *S. cerevisiae* cannot utilize xylose, this yeast grows in the medium with D-xylulose, the isomer of D-xylose, as the sole carbon source and produces ethanol with the same productivity as D-glucose. Overexpression of the autologous XK gene *XKS1* increases the xylose metabolic rate and partly increases the ethanol yield.

2.2.2.2 Key Enzymes of L-Arabinose Metabolism

(1) L-Arabinose isomerase (L-AI)

L-AI can catalyze the conversion of L-arabinose to L-ribulose and the isomerization of D-galactose to D-tagatose. To date, about hundreds of L-AIs have been identified from various microorganisms; their protein sequences share about 20–80% identities. The crystal structure of *E. coli* L-AI has been determined; its monomer contains 498 aa, 16 β-sheets, and 17 α-helixes [22]. It can be divided into three domains: N-terminal (1-176AA), a central domain (177-327AA), and C-terminal (328-498AA). The active form of *E. coli* L-AI is a homotrimer comprising of three subunits A, B, and C with three equivalent clefts appearing on the outer surface. The amino acid residues distributed in the inner surface of each cleft are M185, F279, M351, I373, Y335, and H449 from subunit A and H128, Q125, Y19, L18, and Q16 from subunit B. The residues in other similar enzymes are highly conserved and located in the same loop regions, indicating that this region provides an adjustment space for substrate entry and product release to/out of the active central domain. E306, E333, H350, and H450 have more conservation, indicating that they are residues of the active sites of *E. coli* L-AI.

L-AI exists in mesophilic, thermophilic, and hyperthermophilic organisms, and their enzymatic properties differ from each other. Most L-AIs are thermophilic; their optimal temperature was 30–50 °C. Thermophilic enzymes show the highest activity at 60–80 °C; hyperthermophilic enzymes show the best activity at 85–90 °C. All known L-AIs are optimal at pH 6.0–8.5 and centralized at pH 7.0–8.0. Metal atoms are important for isomerases; they are generally present in the active site and stabilize the entire protein spatial structure. Similar to other aldose–ketose isomerases, metal atoms are also involved in enzyme catalysis. Mn^{2+} and/or Co^{2+} are essential for maintaining the activities and thermal stabilities of many L-AIs. Rhimi et al. [22] found that L-AI from *Bacillus stearothermophilus* US100 are metal-independent; however, the presence of Mn^{2+} and Co^{2+} with a low concentration helps the stabilization of L-AI above 65 °C. It is assumed that the residues of E306, E331, H348, and H447 from *E. coli* L-AI and the residues of E306, E333, H350, and H450 from *E. coli*-like L-AI are the sites binding to metal ions (Mn^{2+}).[23] Additionally, the interactions of E351, D308 with H446, or H348, H447 with E331 promote the central structure.

(2) L-Arabinitol 4-dehydrogenase (LAD1)

LAD1 is the second enzyme in L-arabinose metabolism of fungi cells and catalyzes the conversion of L-arabitol to L-xylulose. LAD1 is indispensable in L-arabinose metabolism and involved in other hexose metabolic pathways. Besides L-arabitol, LAD1 can utilize many other hexose substrates including D-allitol, D-sorbitol, L-iditol, and L-mannitol.

Although LAD1 from different resources show optimal temperatures and different thermal stabilities, they are all Zn-binding enzymes and utilize NAD^+ as the coenzyme. The crystal structure of LAD-NAD^+ shows that LAD is a tetramer. Each monomer

is composed of a catalytic domain and a coenzyme-binding domain, and a big cleft is present between them. Each contains two Zn^{2+}: One is necessary for enzyme structure, and the other helps in catalysis [24].

It was assumed that NAD^+ can bind to the residues of I65, Y307, and R308 of LAD1. The resulting steric space precluded the binding of D-arabitol, only limiting the binding to the L-enantiomers of pentose. The outer space of active central I65, M68, and V284 is extruded inside to cover the binding sites [24].

2.3 Breeding of Ethanol-producing Strains from Pentose

For years, many studies have been conducted for strain selection and breeding to produce ethanol from pentose. These studies were about the four aspects shown below: (1) screening of strains from nature, (2) strain evolution by domestication and induced mutation, (3) strain construction by molecular engineering, and (4) strain fusion of different properties. Because the natural strains cannot always be applied in industrial production, artificial strain engineering provides significant approaches for ethanol production from pentose.

2.3.1 Domestication and Induced Mutation

Domestication is efficient for increasing the ethanol fermentation property of strains using pentose. Song and Zhang [25] obtained strains from natural parent strain by domestication; the xylose utilization rate increased from 62.52% to 92.91%. *P. stipitis* BCRC21777 was acclimated in the neutralized rice straw hydrolysate of sulfate acid; the resulting strain showed a high tolerance to acid and furfural and produced ethanol at a hydrolysate of pH 5.0 and with a yield of 0.45 g ethanol/g sugar, about 87% of the theoretical value [26]. After culturing in acid hydrolysate containing different concentrations of hemicellulose, the growing parent strains were inhibited, and mutations with better ethanol fermentation property were reproduced efficiently. Besides the natural strains, some engineered yeasts were also well acclimated. Domestication requires a long period to achieve good results; however, it is a useful method to screen strains with abilities to produce ethanol from pentose and a high tolerance to the inhibitors of hydrolysate.

Induced mutagenesis is another ideal method to obtain the desired strains using a certain physical or chemical inducer. There are many types of inducers, and their inducing mechanisms are different. Generally, it is necessary to use one inducer for several times repeatedly or several different inducers continuously to obtain a suitable mutation. Bajwa et al. [27] obtained a *P. stipitis* NRRL Y-7124 mutation by continuous UV-light mutagenesis; it showed a higher tolerance to hardwood sulfite pulping

than the parent strain. A suitable mutagenetic method combined with a right inducer titer is the prerequisite for a good mutation. Han et al. [28] tried several combinations of EMS, LiCl, and UV-light on the mutagenesis of *C. shehatae* and obtained the best mutant strain with an increased ethanol yield of 84.9% compared to the original strain. The combination of induced mutagenesis and genetic engineering generated a *S. cerevisiae* mutation, whose growth rate and xylose consumption were 20-fold and 2.5-fold of the parent strain, respectively.

2.3.2 Genetic Engineering

Another useful method to obtain a pentose-to-ethanol-producing organism is genetic engineering. It can be achieved by two ways as follows: (1) by introducing a pentose metabolic pathway to an ethanol-producing strain which can utilize only hexose including *S. cerevisiae* and *Z. mobilis* and (2) by introducing the key enzymes for ethanol production to pentose-utilizing strains such as *E. coli* and *P. stipitis*.

2.3.2.1 *S. cerevisiae*

S. cerevisiae is the traditional yeast for producing ethanol from glucose. *S. cerevisiae* has its own advantages for large-scale industrial ethanol production such as the utilization of high-concentration glucose, less byproducts, high tolerance to ethanol, low cost with less ethanol separation steps, less sensitive to the inhibitors of cellulose hydrolysate, and no need of detox treatment step. Although *S. cerevisiae* cannot utilize pentose, it is still the best candidate strain for pentose-to-ethanol engineering due to its excellent ethanol production performance.

As *S. cerevisiae* can utilize xylulose, an isomer of xylose, introduction of key enzymes for the conversion of xylose to xylulose is an effective way to make yeast produce ethanol from xylose. The enzymes are obtained from two resources as follows: (1) XR (coding gene *XYL1*) and XDH (coding gene *XYL2*) from the natural pentose-utilizing yeast and (2) XI (coding gene *XYLA*) from certain bacterium or filamentous fungi.

Engineered *S. cerevisiae* with the simultaneous overexpression of *XYL1* and *XYL2* can utilize xylose as the carbon source to produce ethanol. However, the XR of *P. stipitis* showed a higher affinity to NADPH than NADH, and XDH is NAD^+-dependent. NAD^+ cannot regenerate under anaerobic condition, resulting in redox imbalance and xylitol accumulation and secretion to extracellular space. Studies have started to solve this problem by improving the genetic property. The site-directed mutagenesis on the XDH of *P. stipites* generated a mutation with better affinity to $NADP^+$ thanks to wildtype strain, but had the same catalytic efficiency. Engineered *S. cerevisiae* with the expression of XDH mutation as well as XR can produce more ethanol from xylose, and less xylitol was detected in the medium [29].

Introduction of bacterial XI gene (*XYLA*) in *S. cerevisiae* is another way to endow the yeast with metabolic ability to utilize xylose. This gene has been successively cloned from diverse bacteria and linked after the yeast constitutive promoters and transformed into *S. cerevisiae*. However, the XI activity could not be detected in *S. cerevisiae* for the first time until 1996, when *XYLA* gene from *Thermus thermophiles* was integrated into the yeast genome [30]. The recombinant XI showed an optimal temperature of 85 °C and maintained only about 4% of the highest activity at 30 °C, the regular ethanol fermentation temperature. Since then, continuous attempts have been made to acquire conducive strains by cloning different *XYLA* genes from bacteria and fungi and expressed in suitable hosts, and good results have been obtained [31, 32]. Because the conversion of xylose to xylulose by XI requires only one reaction without any coenzyme, the development of this pathway is very significant.

XK catalyzes the conversion of xylulose to xylulose 5-phosphate, which is the intermediate of xylose metabolites in PPP pathway; therefore, XK is the key enzyme to improve the xylose metabolism. *S. cerevisiae* has its own XK, but a low expression level. Publications showed that the coexpression of its own *XKS1* gene encoding XK with *XYL1* and *XYL2* from *P. stipitis* provided a higher capacity of xylose utilization and ethanol yield in *S. cerevisiae*. Another example, the *XYL3* gene coding XK from *P. stipitis* under the control of its own promoter also improved the ethanol production of *S. cerevisiae* with less xylitol accumulation, proving the necessity of moderate expression of XK encoding gene. Although the excess expression of *XKS1* in *S. cerevisiae* containing *XYL1* and *XYL2* can increase ethanol production, the utilization speed rapidly decreased. This is probably because the excess XK decreased the concentration of intracellular ATP, which is harmful for cell growth [33].

Although the xylose to xylulose pathway was successfully introduced to *S. cerevisiae*, its ability of utilizing xylose did not improve significantly. NADPH and NAD^+ were necessary in the reactions catalyzed by XR and XDH, and the deficiency in these coenzymes led to a low metabolic speed of xylose. Supplement of NADPH and NAD^+ well improved the ability of xylose ethanol production by *S. cerevisiae*. Studies found that fumarate reductase (FRD) can convert NADH to NAD^+, and the expression of exogenous FRD in recombinant *S. cerevisiae* can convert xylose to ethanol with an increased yield of 60% compared to the parent strain [34].

Acetylating acetaldehyde dehydrogenase (AADH) utilizes NADH generated in xylose reduction reaction and produces NAD^+. In addition, AADH also converts acetyl-CoA to acetaldehyde, and then acetaldehyde is reduced to ethanol. The expression of AADH in yeast containing XR and XDH efficiently solves the REDOX imbalance problem and decreases the inhibitory effects from the acetic acid of lignocellulose pretreatment [35].

Another factor limiting the xylose ethanol production in *S. cerevisiae* is the low efficiency of xylose transport system. Reports on how to enhance the xylose transportation have been published and showed good results. Young et al. [36] found that a peptide sequence of the transmembrane region is related to sugar transportation

selection. Mutation with some amino acids clearly slowed down the glucose transport speed and enhanced the active transport of xylose.

The severe conditions of lignocellulose pretreatment release many toxic chemicals such as furfural, hydroxymethylfurfural, acetic acid, and aromatic compounds, hindering yeast cell growth and ethanol production. Genetic engineering has been applied to improve the tolerance of microbes on these inhibitors. Gorsich et al. [37] found that the deletion of some genes (*ZWF1*, *GND1*, *RPE1*, and *TLK1*) of PPP pathway had negative effects on the yeast tolerance on furfural, which can be used for improving the furfural tolerance by genetic engineering. The reduction ability of furan from lignocellulose hydrolysate is a critical factor for yeast fermentation performance evaluation. Strengthening the regeneration rate of the two cofactors involved in furan reduction is another main aim of metabolic engineering. However, there are few reports on enhancing the tolerance of yeast to weak acids in hydrolysate by genetic engineering.

The construction of arabinose-utilizing strains started in recent years, and a few reports have been published. The introduction of ethanol production pathway to arabinose-utilizing bacteria can achieve the aim of converting arabinose to ethanol, but generally with a low conversion rate. Therefore, more attention has been paid to the introduction of arabinose metabolic pathway to natural ethanol-producing strains. Arabinose metabolic pathway in fungi contains four reactions requiring coenzymes: Two of them need NADPH as the coenzyme, and others need NAD^+ as the coenzyme. Under anaerobic condition, the REDOX imbalance of arabinose metabolism is much more severe than that of xylose pathway, causing the poor ability of utilization of arabinose in the recombinant strains. Thus, more studies were conducted to introduce arabinose metabolic genes from bacteria to *S. cerevisiae*. Becker and Boles [38] cloned the *araA* gene of *B. subtilis* and *araB*, *araD* genes of *E. coli* and expressed in *S. cerevisiae*. The resulting recombinant yeast produced ethanol using arabinose as the sole carbon source at a rate of 0.06–0.08 g/(g·h) under limiting oxygen conditions. Wiedemann and Boles [39] found that in the recombinant yeast mentioned above, L-AI is the limiting factor for arabinose metabolism; the substitution with L-AI from *B. licheniformis* shortened the lag phase of yeast on arabinose utilization. The genetic engineering study of arabinose-utilizing strains are still ongoing; it is believed that more recombinant strains with better performance can be obtained with the development of new technologies.

2.3.2.2 *Z. Mobilis*

Besides traditional yeast *S. cerevisiae*, *Z. mobilis* is another important ethanol-producing microbe. *Z. mobilis* is a rod-shaped bacterium with round ends, has a unique metabolic pathway, and is the only known microorganism utilizing glucose by ED pathway under anaerobic condition till now. Natural *Z. mobilis* can only utilize glucose, sucrose, and fructose and cannot use xylose due to its lack of pentose metabolic pathway. However, *Z. mobilis* has many advantages which make it an

ideal genetic engineering candidate for ethanol fermentation using pentose. First, *Z. mobilis* shows good tolerance to high-concentration sugars, up to 400 g/L glucose; in a fermentation broth with glucose or fructose as the carbon source, the substrate utilization rate can reach as high as 97.3–98%. Second, the cell accumulation speed of *Z. mobilis* is low during the fermentation procedure. Third, fermentation can be preceded under anaerobic conditions with a simple process. Fourth, *Z. mobilis* shows a high ethanol tolerance and fast alcohol productivity.

Early in the 1980s, researchers have made efforts to introduce xylose metabolic pathway to *Z. mobilis*, but the transformation efficiency was very low. Although the XI gene *XYLA* and xylulose kinase gene *XYL3* from *Xanthomonas campestris* or *Klebsiella pneumonia* were successfully introduced to *Z. mobilis*, the resulting recombinant strain could not grow on the medium with xylose as the sole carbon source [40]. The enzymatic activities of 6-phosphaogluconate dehydrogenase and transketolase were very low, and no transaldolase activity was detected, indicating that the recombinant bacteria still lacked a complete PPP pathway. Thus, the introduction and coexpression of XI gene (*XYLA*), xyloluse kinase (*XYL3*), transketolase (*TKL*), and transaldolase (*TAL*) are necessary for the complete xylose metabolism in *Z. mobilis*.

In 1995, Zhang et al. carried out a series of experiments on recombinant *Z. mobilis* strain construction. First, *XYLA* and *XYLB* (*XYL2*) from *E. coli* were placed under the control of a strong promoter of glyceraldehyde-3-phosphate dehydrogenase (*Pgap*) by PCR-mediated overlap extension, and then the xylose assimilation operon was transformed to *Z. mobilis*, generating the recombinant strain. Although all these genes were well expressed, the recombinant strain could not grow on the medium with xylose as the sole carbon source. Moreover, the growth rate of glucose was also hindered by the addition of xylose. These findings inferred that some nonmetabolic intermediates might accumulate in recombinant cells, due to the shortage of transketolase and transaldolase.

Following the PPP pathway of *Z. mobilis*, xylulose 5-phosphate should be converted to intermediate and enter the ED and then converted to ethanol. Based on the previous study, Zhang et al. [41] placed the transketolase and transaldolase genes under the control of *eno* promoter and fused to the *Z. mobilis* plasmid of 2.7 kb length and pACYC184, generating a chimeric shuttle vector (Figure 4.3). The vector brought two promoters along with four genes of xylose metabolic pathway and PPP pathway to *Z. mobilis* and generated strain CP4. When using glucose as the carbon source, *Z. mobilis* CP4 secreted relevant enzymes with specific activities as follows: XI (0.11 U/mg), xylulose kinase (1.5 U/mg), transaldolase (0.88 U/mg), and transketolase (0.16 U/mg). Recombinant *Z. mobilis* CP4 can use xylose as the sole carbon source at a growth rate of 0.057/h with ethanol as the principal product and a yield of 0.44 g/g xylose consumed. In contrast, the control strain with only the shuttle vector and without relevant genes could not grow on xylose or produce ethanol from xylose. CP4 and the control strain utilized glucose as the carbon source with an ethanol yield of 94% and 97%, respectively. The control grew faster and to a higher cell density than CP4, and the growth rate was 0.19 and 0.15/h for the control

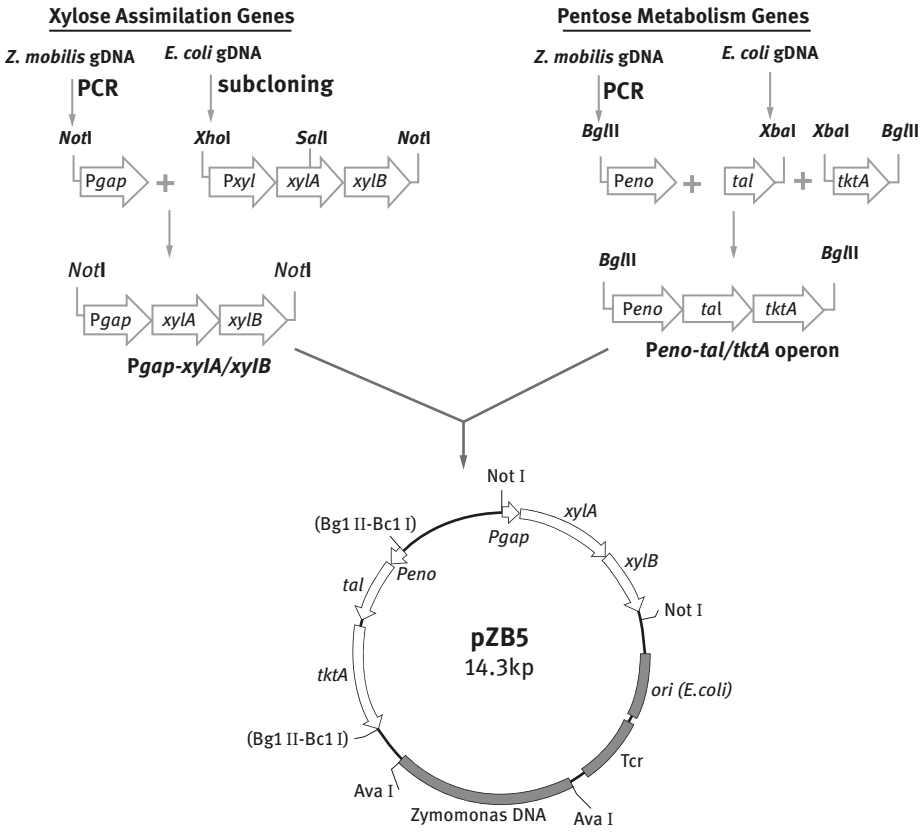

Figure 4.3: Illustration of operon and recombinant plasmid.

Source: Zhang, Eddy, Deanda, Finkestein, Picataggio (1995) [40].

and CP4, respectively. The results probably indicate that the expression of additional four genes led to metabolic repression. In the medium with a xylose and glucose mixture, CP4 produced ethanol with 95% of theoretical yield in 30 h. Although no clear diauxic effect was observed, the utilization rate of glucose was faster than xylose, and the xylose was apparently transported via the inherent glucose transport system and probably suppressed by a high glucose concentration. This study provided a good basis for the design of sugar mixture cofermentation process.

Using similar methods, Deanda et al. [41] cloned five genes including L-AI gene (*araA*), L-ribulose-5-phosphate-4-epimerase (*araD*), L-ribulokinase (*araB*), transketolase (*tktA*), and transaldolase (*talB*) from *E. coli* and introduced to *Z. mobilis* under the control of a constitutive promoter, resulting in a recombinant strain designed as *Z. mobilis* ATCC 39676 (pZB186). The engineered strain produced ethanol in 98% yield of the theoretical value from 25 g/L L-arabinose, even though the arabinose metabolic rate was low. In the mixture medium of glucose and L-arabinose, the ethanol yield was only 84% due to the incomplete L-arabinose utilization. The metabolic rate

of L-arabinose was rather slow; it can start only after the complete consumption of glucose, probably because the L-arabinose transport via glucose transport system was susceptible to the competition inhibitor from high-concentration glucose.

2.3.2.3 *E. coli*

E. coli is a gram-negative bacillus with a size of 0.5 μm × (1–3) μm. *E. coli* is the model strain widely used and deeply studied; it can utilize many substrates including hexose (glucose, mannose, galactose, and fructose), pentose (xylose and arabinose), and furfural (galacturonic acid and glucuronic acid). In *E. coli* cells, hexose is metabolized via EMP pathway, and pentose is metabolized via the coupling of PPP and ED pathway, generating pyruvic acid and then degrading to ethanol, lactic, acetate, and carbonic acids. As ethanol is only a small proportion of the complex metabolites, *E. coli* was generally not selected as the host for ethanol production. However, *E. coli* has the entire set of genes and enzyme of PPP pathway and can ferment under anaerobic conditions with controlled pH. Therefore, it is worthy to construct an engineered *E. coli* strain for ethanol production from pentose or sugar mixture.

The goal of *E. coli* engineering is to make it utilize lignocellulose hydrolysate and carbon source in the cells to produce ethanol. Pyruvate dehydrogenase (PDC) of *Z. mobilis* has a lower *Km* than other isoenzymes acting on pyruvate; its combination with ethanol dehydrogenase (ADHB) can efficiently convert sugar metabolites and pyruvate to ethanol. In 1987, Ingram placed PDC gene and ethanol dehydrogenase gene (*ADHII*) from *Z. mobilis* under the control of a separate promoter and generated a PET operon, which was then transformed into *E. coli* TC4. The resulting recombinant produced ethanol in a yield of 750 mmol/L from 2% glucose. This is the first recombinant *E. coli* that produced ethanol. To enhance the ethanol production efficiency of *E. coli*, a series of exogenous promoters were selected to improve the expression levels of *PDC* and *ADHII*. Under harsh growth conditions such as a high ethanol tolerance, the stability in a nonselective medium and ethanol productivity from xylose were used as the selection criteria, and a strain ATCC 11303 (pLOI297) that can grow in 80 g/L xylose was selected. This strain showed a high growth rate of 0.72 g/(L·h), at 42 °C and pH 6.0 with an ethanol tolerance of up to 53–56 g/L. However, the plasmids containing PET operon showed poor hereditary stability under nonresistance selection conditions. To overcome this shortage, Hespell et al. (1996) selected a conditional legal strain *E. coli* FMJ39 (Δldh-Δpfl) (which lacks lactic dehydrogenase gene *LDH* and pyruvate formate-lyase gene *PFL* and cannot reduce pyruvate to lactic acid in the absence of oxygen and cannot maintain the H^+ recycling of NAD^+/NADH and cause to death.) as the starting strain; plasmid pLOI295 and pLOI297 were separately transformed into the host strain generating recombinant strains, namely FBR1 and FBR2, respectively. These two strains can grow in the absence of oxygen and still maintained the plasmids after 60-time reproductions of nine inoculations. Other

studies on the integration of *PDC* and *ADHII* into *E. coli* chromosome also achieved good results.

From the results mentioned above, it can be concluded that the introduction of ethanol pathway from *Z. mobilis* to *E. coli* is a usable method to acquire ethanol-producing strain. However, the autologous pyruvate formate-lyase of *E. coli* can decompose pyruvate to acetyl coenzyme A and formic acid. Two molecules of acetyl coenzyme A can generate one molecule of ethanol, one acetyl acid, and one ATP molecule by consuming two NADH. Although this is a complex way to produce ethanol, it also provides a theoretical basis for genetic engineering.

These recombinant strains were obtained by the modification of metabolic pathway in natural cells, increasing the substrate resources for ethanol production and improving the ethanol production efficiency. In 2008, Trinh et al. constructed a minimal *E. coli* cell by reducing the functional space of the central metabolic network to those pathways that can only satisfy the demand for cell growth and ethanol production from glucose and pentose. The resulting strains were hardly affected by catabolite repression and produced ethanol with a yield close to the theoretical value by the simultaneous utilization of glucose and pentose.

2.3.2.4 *P. stipitis*

P. stipitis is the most potential natural pentose ethanologenic organism. *P. stipites* utilizes glucose, xylose, mannose, galactose, and cellubiose as well as L-arabinose. Moreover, it harbors many types of cellulases and hemicellulases with the capability of SSF. In addition, *P. stipitis* also metabolizes acetyl acid and disclose the furan ring of furfural and HMF to reduce the toxicity of lignocellulose hydrolysate. On the other hand, *P. stipitis* has many disadvantages as shown below. The pentose fermentation by *P. stipitis* must be carried out under limiting oxygen conditions; ethanol volume productivity is low, increasing the energy consumption and difficulties in controlling organism contamination. *P. stipitis* showed a poor tolerance to the inhibitors of lignocellulose hydrolysate, and pre-detoxic treatment is needed, resulting in a higher production cost. *P. stipitis* is also intolerant to high-concentration ethanol, producing low-concentration ethanol with various byproducts, thus increasing the operation cost of the subsequent separation process. Because the utilization of pentose is inhibited by glucose, pentose can be utilized only after glucose is completely or mostly consumed. Still many difficulties exist for the application of this strain for large-scale ethanol production from plant lignocellulosic hydrolysate.

P. stipitis metabolizes xylose because it contains XR gene *XYL1* and XDH gene *XYL2*. XR uses NADPH or NADH as the coenzyme to catalyze xylose to xylitol. Then, xylitol is catalyzed by XDH and converted to xylulose, and xylulose is phosphorylated by XK and generates xylulose 5-phosphate and then enter the PPP pathway. However, XR prefers NADPH as the coenzyme, and XDH can only use NAD^+ as the coenzyme.

The lack of NADPH leads to the redox imbalance in cells, causing a large accumulation of byproducts xylitol and indirectly decreasing the ethanol yield. To solve this problem, site-directed mutagenesis was performed on *XYL1* and *XYL2* genes aiming to enhance the affinity of XR on NADH or to increase the preference of XDH on $NADP^+$; both can moderate the redox imbalance. The mutated XR or XDH was expressed in *S. cerevisiae*, separately. A comparison between the yeasts containing mutated or original genes was carried out; mutated XR or XDH clearly increased the ethanol production with a concomitant decrease in xylitol production.

There are two sets of electron transport system in *P. stipitis*: One depends on cytochrome C, and the other does not. Shi et al. [42] knocked out cytochrome C encoding gene *CYC1* and generated a mutation whose electron can only be transmitted through cytochrome-independent passage transport system. The respiration and cell growth rate of the mutation are much lower than those of wildtype; however, the ethanol yield increased by 21%, from 0.038 g/g of wildtype to 0.46 g/g of mutated strain. The results indicate that the cytochrome-independent electron transport system is beneficial for oxygen-limiting ethanol production, providing a new way for *P. stipitis* genetic engineering.

The oxygen demands of different strains differ from each other; the fermentation speed and ethanol yield were affected by oxygen supplement. The growth of *P. stipitis* requires oxygen; however, extra oxygen accelerates cellular respiration but produces a low ethanol yield. Therefore, accurate oxygen-limiting supply is very necessary for *P. stipitis* ethanol generation, which would increase the process control and production cost and further limit the application of *P. stipitis* in industrial ethanol production. In contrast, the ethanol fermentation process by *S. cerevisiae* is much simple; this is because *S. cerevisiae* contains a unique dihydroorotate dehydrogenase (DHOdehase). The enzyme makes the yeast germinate with the energy from anaerobic fermentation. This finding was soon applied to obtain a *P. stipitis* recombinant strain with the ability to grow in the absence of oxygen. Shi and Jeffries [43] cloned the *URA1* gene coding DHOdehase from *S. cerevisiae* and transformed into *P. stipitis*. The resulting strain produced ethanol from glucose, but not xylose. This is probably because the metabolic pathways of glucose and xylose utilize different cofactors and electron transport systems; therefore, *P. stipitis* harboring *URA1* gene cannot grow in a xylose medium. Although the construction of *P. stipitis* by genetic engineering is still on the exploratory stage, it also provides a new direction for lignocellulosic ethanol production.

The application of genetic engineering to strain modification to improve the fermentation performances from pentose is theoretically feasible and provided some different types of recombinant strains (Table 4.5). This method laid a certain basis for bioethanol production; however, still much work is required for further application: (1) extension of available substrates, (2) increase in the tolerance to inhibitors including acetic acid, (3) improvement in ethanol tolerance, (4) increase in the inherent stability of recombinant strains, (5) decrease in fermentation byproducts, and (6) refining of the fermentation process. In brief, there is a long way for the currently acquired

Table 4.5: Engineered pentose-to-ethanol-producing strains.

Engineered strains	Sugar concentration (g/L)	Ethanol production (g/L)	Ethanol productivity (g/L·h)	Percent of theoretical yield (%)	References
E. coli KO11	Xyl 90	41.0	0.85	89	[44]
E. coli LY01	Xyl 90	63.2	0.53	88	[44]
E. coli FBR5	Xyl 74	33.8	0.53	92	[45]
E. coli FBR5	Glu:Xyl:Ara 27:36:20	37.8	0.91	–	[45]
K. oxytoca M5A1 (pLOI555)	Xyl 100	46.0	0.96	94	[46]
Z. mobilis AX101	Ara:Glu:Xyl 20:40:40	42.0	0.61	84	[47]
Z. mobilis CP4 pZB5	Xyl 52	25.0	1.67	94	[44]
S. cerevisiae 1400(pLNH32)	Xyl 52	20.5	0.57	78	[44]
S. cerevisiae TMB3400	Xyl 50	13.3	0.12	67	[48]
S. cerevisiae BSW3AP	Ara 20	6.9	–	84.3	[49]

recombinant stains for industrial application. Further studies on recombinant strain construction for pentose metabolic engineering based on the present basis should be continued; this is significant for the bioconversion of plant cellulose to ethanol.

2.3.3 Protoplast Fusion Technique

Protoplast fusion is a genetic modification by which two or more cells are fused together to form a new cell. This fusion is a powerful method for cell modification and can be carried out between intraspecific, interspecific, or intergeneric microbes, or even those with no relationships.

This technique has also been applied for breeding pentose metabolic strains. Value genes of *S. cerevisiae* have been introduced to pentose-utilizing yeast with better xylose fermentation performance and a higher tolerance to ethanol and inhibitors. Li et al. [50] fused *S. cerevisiae* ATCC 4126 with *C. shehatae* CICC 1766, and the yeast fusant produced ethanol with a concentration of 18.75 g/L from 50 g/L xylose, a yield of 0.375 g/g, 73.4% of theoretical value. The ethanol yield increased by 28% compared to *C. shehatae* CICC 1766.

Intraspecific fusion has also been carried out between xylose-utilizing strains. Han et al. (2008) screened a *C. shehatae* fusant with a higher ethanol production of 37.7% than parent *C. shehatae* strain. Limtong et al. [51] found that a fusant strain *P. stipitis*

CBS 5773 can convert xylose to ethanol with a concentration of 1.41% (w/v), lower than the parent strain (1.51%) under the same fermentation conditions. Clearly, the properties of the resulting strains obtained by protoplast fusion technique are different. An effective selection method is very important to obtain the desired strains. There are many disadvantages of protoplast fusion technique including a heavy workload, bad fusion direction, and inherent instability. However, this method has also some advantages such as a low impact from genetic relationship and genetic recombination occurs repeatedly. Therefore, it is still an important method for microorganism breeding.

Besides the methods mentioned above, genome shuffling technique has also received more and more attention. It is based on sequential random mutagenesis and screening. A promising organism is mutagenized to produce a diverse library of random mutants that serve as the initial mutants' library. These individual strains with improved relevant phenotypes were selected for the following protoplast fusion. After multiround recursive fusion, different genes with positive characteristics were introduced into one cell. This technique combines traditional breeding methods with protoplast fusion technique and increases the recombinant rate of positive direction. Bajwa et al. [52] used a *P. stipitis* strain with six-time UV mutagenesis as the starting strain, performed four round of genome shuffling, and acquired two strains, GS401 and GS402, that can tolerate 80% sulfite hardwood pulping. Another two strains GS301 and GS302 that can tolerate 85% sulfite hardwood pulping were also selected. In contrast, parent strain can only survive at 65% sulfite hardwood pulping. In addition, the performances of GS301 and GS302 in 4% (w/v) xylose or glucose containing medium were better than that of the parents. There are very few reports on the application of genome shuffling technique in pentose metabolic strains. However, it is expected that this method would efficiently accelerate the recombinant progress of pentose metabolic strain, because mutagenesis is carried out with the entire genome of starting strains.

Section 3: Microbial Syngas Fermentation

Synthesis gas (syngas) is generated by the gasification of coal, oil, biomass, and organic waste due to the decomposition of carbon-containing substances. The major components of syngas are CO, H_2, CO_2, and CH_4; it also contains small amounts of sulfur and nitrogen compounds. Studies show that syngas is an abundant and inexpensive raw material for biological processing and can be converted to various useful fuels and chemicals via anaerobic fermentation such as methane, acetic acid, butyric acid, ethanol, and butanol. Compared to the chemical conversion of syngas, biotransformation has the following advantages: (1) Reaction is conducted under mild conditions. (2) The specificity of enzymes is higher than that of inorganic catalysts; thus, a high product yield with less byproducts can be achieved. (3) It does not require a fixed ratio of CO and H_2. (4) Most biocatalysts are tolerant to the sulfide in syngas, reducing the cost of

gas purification. Because gasification can eliminate the chemical differences between raw materials, some toxic or refractory organics can also be utilized to produce some useful products through fermentation after converting them to syngas.

Syngas fermentation will play an important role in the conversion of biomass, waste materials, and some materials that cannot be fermented directly. For example, in the conversion of lignocellulosic biomass to ethanol fuel, if biomass (including lignin and recalcitrant part) is first converted to syngas through gasification and then to ethanol through fermentation, technical barriers exist in the acidic or enzymatic hydrolysis of lignocellulose, and the problem that lignin cannot be fully utilized in traditional bioconversion processes can be overcome.

3.1 Microorganisms Capable of Producing Ethanol from Syngas

Microorganisms that can utilize syngas (CO, CO_2, and H_2) as the sole carbon and energy source are anaerobic, and most of them are acetogen. Acetic acid is their major metabolite. Strains capable of fermenting syngas to ethanol are much less. Microorganisms that can produce organic acids and alcohols from syngas are listed in Table 4.6, and those capable of producing ethanol include: *Butyribacterium methylotrophicum*, *C. ljungdahlii*, *C. carboxidivorans*, and *C. autoethanogenum*. These strains utilize CO or H_2/CO_2 to produce ethanol or acetic; the corresponding stoichiometric reactions are as follows:

$$6CO + 3H_2O \longrightarrow CH_3CH_2OH + 4CO_2$$

$$2CO_2 + 6H_2 \longrightarrow CH_3CH_2OH + 3H_2O$$

$$4CO + 2H_2O \longrightarrow CH_3COOH + 2CO_2$$

$$2CO_2 + 4H_2 \longrightarrow CH_3COOH + 2H_2O$$

B. methylotrophicum was isolated from sewage sludge in 1980, but the wild strain cannot metabolize CO. In the early 1990s, a CO-metabolizing mutant was obtained after the domestication. The mutant strain metabolized CO in diverse ways and grew on 100% CO, H_2/CO_2, methanol, formic acid, and glucose. When 100% CO was used as the substrate, the final products are acetic acid, butyric acid, and a small amount of ethanol and butanol. pH significantly affects the formation of products. Acetic acid was the major product at pH 6.8, and the corresponding molar ratio of acetic acid to butyric acid was 32:1. However, at pH 6.0, the ratio was 1:1 [53]. Similar results were obtained in continuous fermentation, and small amounts of ethanol and butanol were detected in the products [54]. When continuous fermentation was performed with cell and gas recycle, butanol concentration reached 2.7 g/L and became the dominant fermentation product [55].

Table 4.6: Microorganisms capable of producing organic acids and alcohols from syngas.[a]

Species	T_{opt} (°C)	$_pH_{opt}$	t_d (h)	Products
C. autoethanogenum	37	5.8–6.0	nr	Acetate, ethanol
C. ljungdahlii	37	6.0	3.8	Acetate, ethanol
C. carboxidivorans	38	6.2	6.25	Acetate, ethanol, butyrate, butanol
B. methylotrophicum	37	6.0	12–20	Acetate, ethanol, butyrate, butanol
Oxobacter pfennigii	36–38	7.3	13.9	Acetate, n-butyrate
Peptostreptococcus productus	37	7.0	1.5	Acetate
Acetobacterium woodii	30	6.8	13	Acetate
Eubacterium limosum	38–39	7.0–7.2	7	Acetate
Methanosarcina acetivorans strain C2A	37	7.0	24	Acetate, formate, CH_4
Moorella thermoacetica	55	6.5–6.8	10	Acetate
Moorella thermoautotrophica	58	6.1	7	Acetate
Desulfotomaculum kuznetsovii	60	7.0	nr	Acetate, H_2S
Desulfotomaculum thermobenzoicum subsp. *thermosyntrophicum*	55	7.0	nr	Acetate, H_2S
Archaeoglobus fulgidus	83	6.4	nr	Acetate, formate, H_2S

Note: nr: not reported.
[a]Henstra et al. 2007 [59].

C. ljungdahlii was isolated from chicken waste in 1987 by Barik and Harrison at University of Arkansas [56]. It is a strict anaerobic gram-positive bacterium. Cells of *C. ljungdahlii* were motile, straight rods, peritrichous, and had a thick (0.1–0.2 μm) coat surrounding each cell, and spores were rarely observed [57]. *C. ljungdahlii* is by far the most widely studied ethanol-producing acetogen. It can grow not only on CO or H_2/CO_2 but also on pyruvate, sugars, and other organic compounds (Table 4.7), where fructose or H_2/CO_2 is the optimal substrate for growth. The major products of syngas metabolization with *C. ljungdahlii* are ethanol and acetic acid, but the results vary. In the previous experiments for strain isolation, 1.14 g/L of ethanol and 4.62 g/L of acetic acid were obtained with CO as the substrate [56]. In batch cultivation with a continuous gas (65% CO, 24% H_2, and 11% CO_2) supply, ethanol concentration reached 7 g/L, and the molar ratio of ethanol to acetic acid was 9:1 [58]. In a continuous stirred tank reactor (CSTR) with cell recycle, the ethanol concentration achieved was as high as 48 g/L, and the acetic acid concentration was 3 g/L [59]. Therefore, *C. ljungdahlii* is a promising strain for ethanol production from syngas. Gaddy [58] and Klasson [60] suggested that acetic acid was growth-associated, whereas ethanol was not. Yeast

Table 4.7: Substrate utilization by *C. ljungdahlii*.[a]

Substrate [b]	Result [c]	Substrate	Result
H_2/CO_2	+	Ribose	+
CO	+	Xylose	+
Sodium formate	+/–	Glucose	+[e]
Methanol	–	Fructose	+
Ethanol	+	Galactose	–
Sodium pyruvate	+	Mannose	–
Sodium lactate	–	Sorbitol	–
Glycerol	–	Sucrose	–
Sodium citrate	–	Lactose	–
Sodium succinate	–	Maltose	–
Sodium fumarate	+	Starch	–
Malic acid	–[d]	Ferulic acid	–
Erythrose	+	Trimethoxybenzoic acid	–
Threose	+	Casamino acids	+/–
Arabinose	+	Alanine	–

[a] Tanner et al. 1993.
[b] Each substrate was tested for the ability to support growth of *C. ljungdahlii*. Gaseous substrates were added to the gas phase of a crimp-sealed tube. Other substrates were added at a concentration of 5 g/L to a medium containing 1 g/L of yeast extract. A 2% inoculum of fructose-grown cells was used; no growth or poor growth results were confirmed by using H_2/CO_2-grown cells as an inoculum.
[c] Growth was measured in each aluminum seal tube. Levels of growth (compared with the control) were scored as follows: +, A_{600} of > 0.1; +/–, A_{600} between 0.1 and 0.01; and -, A_{600} of <0.01.
[d] Malate was metabolized by *C. ljungdahlii* as indicated by a change in the culture pH.
[e] Cultures grown previously on fructose or H_2/CO_2 required adaptation for growth on glucose.

extract in the medium serves as a nitrogen source for cell growth, but a high yeast extract concentration is not conducive to ethanol formation. Similarly, a lower pH favors ethanol production. Under growth conditions (pH 5.0–7.0), acetic acid was the major product of *C. ljungdahlii*, while under nongrowth conditions (pH 4.0–4.5, no yeast extract), ethanol became the dominant product.

C. carboxidivorans is another potential strain for ethanol production; it was isolated from agriculture settling lagoon and named as *Clostridium* strain P7 before. *C. carboxidivorans* can grow on CO or H_2/CO_2; its metabolites include acetic acid, ethanol, butyric acid, and butanol. *C. carboxidivorans* is a gram-positive, motile rod ($0.5 \times 3\ \mu m^2$), occurring singly and in pairs. Cells rarely sporulate, but spores are subterminal to terminal with slight cell swelling. Colonies of *C. carboxidivorans* grown on CO appeared white and opaque, had lobate edges, and were 2–4 mm in diameter after 1–2 weeks of incubation (Liou et al., 2005). Substrates that can be utilized by *C. carboxidivorans* include CO, H_2/CO_2, glucose, galactose, fructose, xylose, ribose, mannose, L-arabinose, rhamnose, sucrose, melezitose, cellobiose, trehalose, cellulose, starch, pectin, citrate, glycerol,

ethanol, propanol, 2-propanol, butanol, inositol, mannitol, glutamate, aspartate, alanine, histidine, asparagine, serine, casamino acids, betaine, choline, and syringate. Methanol, D-arabinose, fucose, lactose, maltose, melibiose, raffinose, amygdalin, sorbitol, gluconate, lactate, malate, succinate, and arginine do not support growth. In Rajagopalan's study [61], artificial syngas (25% CO, 15% CO_2, and 60% N_2) was used; the apparent yields (mole C in products per mole CO consumed) of ethanol, butanol, and acetic acid were 0.15, 0.075, and 0.025, respectively, at steady state in continuous fermentation. Butyric acid was not detected in the products. Datar [62] used real syngas instead of bottled gas in fermentation. *Clostridium* strain P7 stop growing when real syngas was introduced, but growth was observed again when shifted to clean bottled gas. Ethanol was mainly produced once the cells stopped growing, indicating that ethanol is nongrowth associated.

C. autoethanogenum was isolated from rabbit feces; it is strictly anaerobic, gram-positive, rod-like, spore-forming, and motile. The cell size is about 0.5–0.6 × 2.1–9.1 μm, and in old cultures, giant filamentous cells of up to 0.6 × 42.5 μm were observed, formed from several cells housed by capsules (Abrini et al., 1994). In addition, granules formed by cells were observed when *C. autoethanogenum* was cultured in a medium containing yeast extract. *C. autoethanogenum* metabolizes CO or H_2/CO_2, and the major end products are ethanol and acetic acid. It can also utilize xylose, arabinose, fructose, pyruvate, L-glutamate, and rhamnose. Xylose was recommended as the optimal substrate for growth by Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH (DSMZ). Growth occurred at pH 4.5–6.5 and 20–44 °C, with an optimum temperature of 37 °C and an optimum pH of 5.8–6.0. Chloramphenicol, penicillin, ampicillin, and tetracycline inhibit its growth. Compared to the above strains, *C. autoethanogenum* was less studied.

3.2 Mechanism of Microbial Syngas Fermentation

3.2.1 Biochemical Pathway for Microbial Ethanol Production from Syngas

The pathway that autotrophic anaerobes follow to produce ethanol from CO, CO_2, and H_2 is acetyl-CoA biochemical pathway [63, 64], also known as Wood–Ljungdahl pathway to commemorate HG Wood and LG Ljungdahl for their contributions in elucidating the pathway. This pathway has two branches: methyl branch and carbonyl branch, as shown in Figure 4.4. The methyl branch comprises several reductive steps: First, CO_2 is converted to formate by formate dehydrogenase (FDH), and then formate is condensed with tetrahydrofolate to form 10-formyl-tetrahydrofolate, subsequently via the catalysis of 5,10-methenyl-tetrahydrofolate cyclohydrolase, 5,10-methylene-tetrahydrofolate dehydrogenase, and 5,10-methylene-tetrahydrofolate reductase. 10-Formyl-tetrahydrofolate is converted to methyl tetrahydrofolate. The methyl

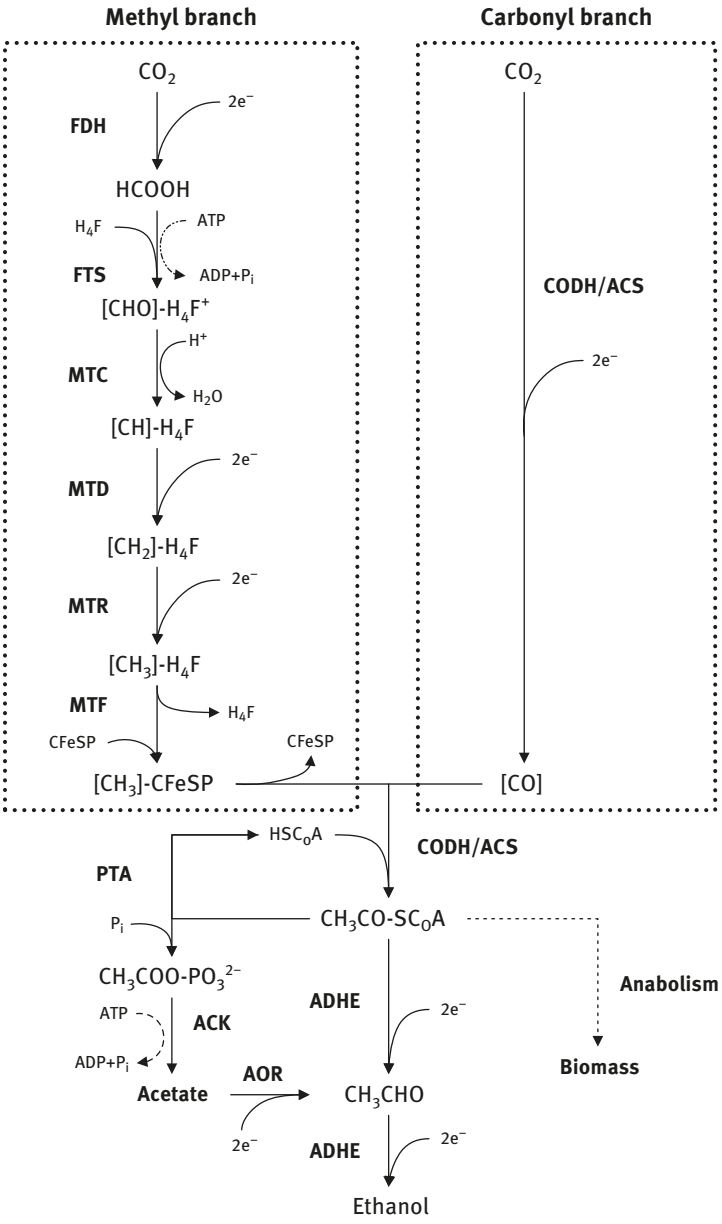

Figure 4.4: Wood–Ljungdahl pathway and reduction of acetyl-CoA to ethanol.

ACK, acetate kinase; CODH/ACS, CO dehydrogenase/acetyl-CoA synthase; CFeSP, corrinoid iron–sulfur protein; FDH, formate dehydrogenase; FTS, formyl-THF synthetase; H_4F(THF), tetrahydrofolate; HSCoA, coenzyme A; MTC, methenyl-THF cyclohydrolase; MTD, methylene-THF dehydrogenase; MTF, methyltransferase; MTR, methylene-THF reductase; Pi, inorganic phosphate; PTA, phosphotransacetylase; ADHE, aldehyde/alcohol dehydrogenase

group of methyl tetrahydrofolate is then transferred to corrinoid iron– sulfur protein (CFeSP) by methyltransferase to form methyl corrinoid iron–sulfur protein, while in the carbonyl branch, one molecule of CO_2 is reduced to CO by CO dehydrogenase (CODH)/ acetyl-CoA synthase (ACS). Finally, by the action of CODH/ACS, methyl from methyl corrinoid iron–sulfur protein is condensed with CO and coenzyme A to generate acetyl-CoA. Acetyl-CoA is an important intermediate for substance and energy metabolism, which can be converted to biomass by anabolism. On the other hand, acetyl-CoA is converted to acetic acid via phosphotransacetylase and acetate kinase reactions. Acetic acid can be further reduced to ethanol, or acetyl-CoA is catalyzed by aldehyde/alcohol dehydrogenase to form acetaldehyde and finally ethanol [54]. Throughout the Wood–Ljungdahl pathway, one molecule of ATP is generated when acetic acid is produced, but it is used to compensate for the ATP required in the formation of 10-formyl-tetrahydrofolate. Thus, there is no net ATP gain.

3.2.2 Key Enzymes for Microbial Syngas Fermentation

The microbial process from syngas (CO, CO_2, and H_2) to ethanol requires many enzymes; among them, FDH and CODH/ACS are closely related to the utilization of CO and CO_2.

3.2.2.1 FDH

FDH catalyzes the reversible redox reaction of formate and CO_2 and is widely found in anaerobic bacteria such as methanogenic archaea and clostridia; it also exists in diverse facultative enterobacteria growing under anaerobic conditions such as *E. coli*, *Salmonella Typhimurium*, *Serratia*, and *Proteus*.

FDH is a highly conserved enzyme. In all the FDHs obtained from different sources, there are 60 completely conserved amino acids, and the homology among some FDHs is up to 75% [65]. FDH from anaerobes is NAD^+-dependent and contains complex redox active sites, which are very sensitive to oxygen. These active sites contain some transition metals such as tungsten, molybdenum, and nonheme iron (mostly iron–sulfur clusters); they also contain cofactors of molybdopterin guanine dinucleotide (MGD).

FDH from *E. coli* is the most widely studied molybdenum FDH. This type of FDH contains selenocysteine, commonly referred to as FDHH. This FDH consists of four domains, namely, domains I, II, III, and IV; its active site is located in the center of the entire molecule – interface of four domains, including a molybdenum atom, two MGD cofactors, a [4Fe–4S] cluster, as well as selenocysteine (SeCys140), His141, Arg333, and other amino acid residues. Molybdenum coordinates to the selenium of SeCys140

and four sulfur atoms of MGD cofactor; domains II and III are connected to a MGD cofactor through a complex hydrogen-bonding network, sulfur bridges, and van der Waals forces [66]. In molybdopterin-containing FDHs, most amino acid residues coordinating to MGD cofactor are highly conserved.

Besides molybdenum-containing FDH, some other FDHs contain tungsten in their active sites. Ljungdahl and co-workers purified and obtained the first tungsten-containing FDH in 1983, which was derived from acetogen *M. thermoacetica*, but this enzyme is extremely anaerobic and very sensitive to pH, resulting in difficulties of separation and purification. Subsequent reports are less likely. To date, the most extensively studied tungsten-containing FDH is derived from mesophilic microorganism *Desulfovibrio gigas*, whose active site is very similar to FDH_H. Except that molybdenum is substituted for tungsten, the key amino acid residues SeCys158, His159, and Arg407 are conserved; it also contains two MGD cofactors and four [4Fe–4S] clusters [67].

3.2.2.2 CO Dehydrogenase/ACS

CODHCODH/ACS is a bifunctional enzyme and has both the activities of CODH and ACS. It not only catalyzes the reversible reaction of CO oxidation to CO_2 but also catalyzes the condensation of methyl, CO, and coenzyme A to generate acetyl coenzyme A. Therefore, it is a key enzyme in the Wood–Ljungdahl pathway.

Diekert and Thauer [68] first discovered CODH in acetogens, but purified enzyme was obtained after a few years because CODH is extremely sensitive to oxygen. Diekert [69] also purified the CODH/ACS of *M. thermoacetica* (formerly *C. thermoaceticum*), indicating that it is a nickel-containing protein and possibly has a $\alpha_2\beta_2$ structure, which was later confirmed. The CODH/ACS of *M. thermoacetica* is a tetramer. The two subunits in the center are β subunits-CODH, and the other two subunits at the periphery are α subunits-ACS [70]. The X-ray crystal structure of CODH shows that it contains five metal clusters (clusters B, C, and D). Each CODH subunit contains a B-cluster and a C-cluster; the D cluster is shared by the two CODH subunits. C-cluster is the catalytic site of CO oxidation, which is a Ni–4Fe–5S or Ni–4Fe–4S cluster. Two channels converge above the Ni center in the C-cluster: One is a hydrophobic channel, proposed to deliver CO to the Ni center, and the other is a solvent channel containing over 40 water molecules to deliver the other substrate-water. Clusters B and D are the redox centers that transfer electrons to and from the C-cluster, which are both [4Fe–4S] clusters. The B-cluster of the adjacent subunit is positioned to mediate the electron transfer between C and D clusters, whereas the D-cluster, which is closest to the molecular surface, is likely to mediate electron transfer between CODH and the terminal electron acceptor (ferredoxin, flavodoxin, etc.). Conversely, a reduced electron donor can transfer electrons to clusters B and D, which first reduce cluster C and then reduce CO_2 to CO via

Cluster C oxidation. ACS subunits consist of three domains: The first domain (amino acid residues 1–312) contains the interface with CODH and a ferredoxin-binding region. The second domain (amino acid residues 313–478) contains a CoA-binding region, and the third domain (amino acid residues 479–729) contains the ligands to A-cluster and CFeSP-binding region. A-cluster is the active site of ACS, which can be described as a binuclear NiNi center bridged to a [4Fe4S] cluster, and the two nickels are referred to as the distal nickel (Nid) and proximal nickel (Nip). Nip may be substituted with Cu or Zn, resulting in the inactivation of enzyme. Furthermore, experiments show the existence of a CO channel between the C-cluster in the CODH subunit and A-cluster in the ACS subunit [71, 72]. CO derived from CO_2 reduction is transferred through this channel to A-cluster, where it is involved in the synthesis of acetyl-CoA.

Section 4: Microbial Ethanol Fermentation Technology and Application

Bioethanol is mainly produced by the hydrolysis and fermentation of sugar crops (sugarbeet, sugarcane, etc.), starch crops (corn, wheat, potatoes, etc.), and lignocellulosic biomass (straw, etc.). Because ethanol fermentation technology from starch feedstock has been mature, this section focuses on ethanol production from only sugar crops and lignocellulosic biomass.

4.1 Ethanol Production from Sugar Crops

Sugar crops are rich in sucrose and hydrolyzed under acidic conditions to glucose and fructose. Then, yeast can use fructose and glucose to produce ethanol under anaerobic conditions. It saves cooking and saccharification compared to starch materials and makes the ethanol production process easier.

4.1.1 Molasses

Molasses is one of the byproducts from cane sugar or beet sugar factory. Because of the high content of sugars, molasses is a good feedstock for the large-scale production of ethanol, requiring only a small amount of yeast. With the improvement in sugar industry in China, yield of molasses has increased rapidly, and many sugar factories have attached ethanol plants for the complete utilization of molasses in ethanol production [77].

4.1.1.1 Pretreatment

Molasses ethanol fermentation contains a high amount of dry materials and sugar, plenty of acid-producing bacteria, and a large amount of ash and colloidal substances, inhibiting the direct fermentation of yeast without pretreatment. The necessary pretreatment procedures include dilution, acidity, sterilization, clarifying, and adding salts.

(1) Dilution

The molasses brix is generally 80–90 Bx, with a sugar content of above 50%. Before the fermentation, dilution must be carried out by adding water into molasses. The concentration of diluted molasses changes with different production processes and operation conditions, and the commonly used technical conditions are as follows: Single concentration process, dilute sugar solution concentration 22–25%; double concentration process, yeast diluted sugar solution concentration 12–14%; basic diluted sugar concentration 33–35%.

(2) Acidification

The purpose of acidification by adding acids is to prevent bacterial breeding, accelerate precipitation of ash and colloidal substances in molasses, and simultaneously adjust the acidity of diluted sugar solution to be suitable for the growth of yeast. Because of the slightly acidic nature of sugarcane molasses, a slight alkalinity of beet molasses, and the optimal pH 4.0–4.5 for yeast fermentation, the technical requirement of adding acid into diluted molasses is necessary. For sugarbeet molasses, the addition of acid removes Ca^{2+} by producing calcium sulfate, thus accelerating the removal of colloid material in molasses by precipitation with ash.

(3) Sterilization

Molasses is always polluted by a large number of microbacteria, mostly including wild yeast and bacteria-producing acid such as *C. albicans* and lactic acid bacteria. To prevent the death of sugar liquid by bacteria, the normal fermentation is ensured. Moreover, the fluid acidity is increased, and sterilization is necessary.

(4) Clarification

Plenty of colloidal substances, ash, and other suspended matter present in molasses are harmful on yeast growth and ethanol fermentation; therefore, they should be removed as far as possible. Typical clarification methods include acidic precipitation with ventilation and hot acidic treatment.

(5) Addition of nutrient salts

A certain amount of nitrogen, phosphorus sources, auxin, and magnesium salt are needed for yeast growth, which is already present in fresh sugarcane juice or sugar beet juice, but most cannot endure the processing of sugar production and molasses treatment. Because of different methods for sugar production, molas ses is different with ingredients present. A diluted sugar solution often lacks yeast nutrients, not only affecting the growth of yeast but also affecting the production of

ethanol. It is necessary to analyze the molasses, check whether nutrition is lacking, know the extent of lacking, and then add appropriate nutrients.

4.1.1.2 Fermentation Microorganism

There is usually a plenty of nonsugar components and bacteria in molasses. Therefore, it is very important to breed yeast species to resist high temperature and acid. In China, yeast Taiwan 396#, As·2·1190, Ganhua No. I, and Chuan 345 and 102 are usually used for ethanol fermentation from sugarcane molasses. Yeast Rass[R] is used for sugarbeet molasses fermentation. National nongrain biomass energy engineering and technology research center breeds high-yielding, high-molasses ethanol special yeast species using comparative genomics methods. The fermented mash alcohol concentration increased from the current 10% to 13.7%: 30% increase for wine, 30% reduction in waste, and 15% reduction in energy consumption. The application of new technologies not only improved equipment utilization but also production increased by 20%. However, energy costs per ton of ethanol reduced by 100 yuan based on the reduced coal and electricity consumption of 15%. Moreover, enterprises reduce waste and emissions and produce more ethanol for the same volume of mash, saving the investment in environmental protection and improving the environment.

4.1.1.3 Ethanol Production from Molasses

Metabolic impurities of ethanol fermentation from molasses generate ester aldehyde, and the fusel oil has a greater influence on the quality of product ethanol. Usually, ventilation is used for yeast culture. Fermented mash with aldehyde generates foam and fouling during the distillation. Therefore, when molasses is used as the raw material to produce high-purity ethanol or ethanol is distilled, a row of aldehyde tower is needed in the middle of the mash tower and rectification tower during the distillation to establish a three-tower continuous distillation process using the vapor or liquid phase over the tower. Currently, the method for producing ethanol from molasses fermentation is almost perfect with a high efficiency. Some domestic and foreign advanced technologies have achieved continuous dilution, continuous fermentation, and continuous distillation to continuously and automatically produce ethanol.

4.1.2 Sweet Sorghum

4.1.2.1 Solid-State Fermentation

Stalk is first ground and scrubbed as shown in Figure 4.5. The length and diameter of the ground stalk are about 10–30 and 2–3 mm, respectively. The ground stalk

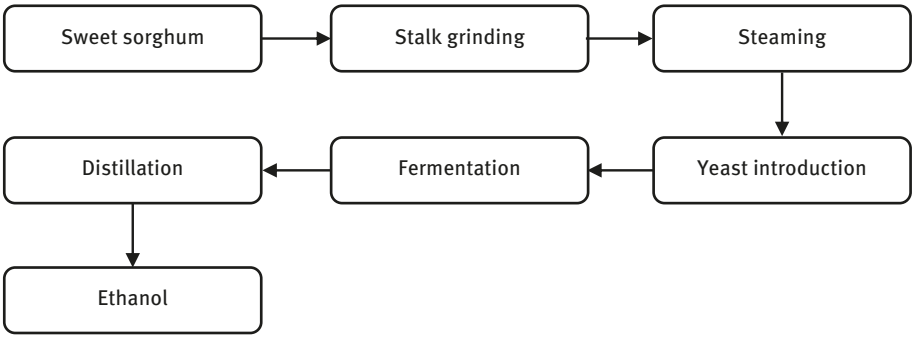

Figure 4.5: Process diagram of solid-state fermentation of sweet sorghum stalk.

must have a uniform size, so that it is favorable for further fermentation. Sterilization, temperature/humidity adjustment, and strain mixing are carried out simultaneously; the mixing degree between stalk and strain is more than 0.7, satisfying the requirement of system capacity. The fermentation cycle is about 1.5–2.5 days (60 h). Difference was observed at different regions and seasons.

4.1.2.2 Liquid Fermentation

The liquid fermentation of sweet sorghum in China was set in the scientific research of "Tenth and Eleventh Five-Year." The yeast immobilization and fluidized bed technology were based on a novel technology in bioengineering field-immobilized active proliferation cell technology, where the traditional free yeast was entrapped in a solid carrier. The selected carrier and yeast were excellent, making the immobilized yeast stable and high-yielding. Therefore, the production was improved, and the difficult continuous fermentation became easy to operate. The technology could be applied to industrial ethanol production from various materials including molasses, cassava, sweet potatoes, corn, sorghum, and canna.

The technology is based on cell immobilization, where the yeast immobilization methods were studied intensively. Repeated carrier tests solved the technical problems such as a low mechanical strength and short life widely present in cell immobilization. The high acid tolerance and active yeast were screened by domestication. A novel ethanol production process suitable for a highly advanced technology was developed according to the physiology and biochemical properties of yeast. After the traditional ethanol fermentation was industrially grafted and modified by the novel biotechnology, an increase in 15–20% revenue was achieved. Besides, the technology effectively decreased energy consumption and saved much water during the ethanol production from sweet sorghum stalk juice.

Live yeast cells were immobilized on a carrier for yeast immobilization and fluidized bed fermentation technology, where there was no yeast loss during the fermentation and yeast concentration was ensured in the primary fermenter. The immobilized yeast has incomparable advantages over the traditional free yeast:

(1) The fermentation was fast, and the conversion rate was high in fluidized bed.
(2) There was a higher ethanol concentration, less residual sugar, and consumption.
(3) The immobilized yeast could be repeatedly used, saving yeast investment.
(4) Ethanol production from sweet sorghum stalk juice has a slight water consumption, and the process was simple.
(5) Rich nutrients and a few impurities were present in sweet sorghum stalk juice. The production cost was low, where the distillation and sewage treatment was simple.

4.2 Ethanol Production from Cellulosic Materials

As shown in Figure 4.6, the main process of cellulosic ethanol production includes pretreatment, enzymatic hydrolysis, and fermentation. To improve microbial polysaccharide utilization, it is critical to break the close cellulose–hemicellulose–lignin structure, reduce the crystallinity and polymerization degree of cellulose, and remove the lignin, and to increase the surface pore size and material surface area.

4.2.1 Pretreatment

Currently, pretreatment techniques of lignocellulosic biomass include thermal pretreatment, chemical treatment, and biological treatment. Thermal pretreatment uses hot water or steam to pretreat lignocellulosic biomass. The reaction temperature is

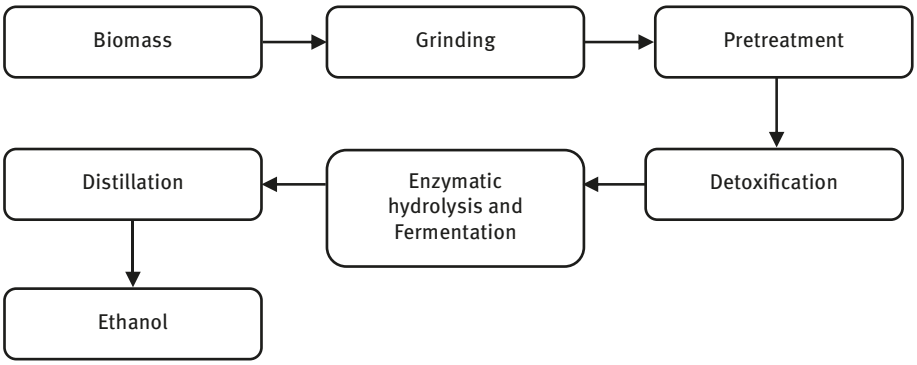

Figure 4.6: Process diagram of cellulosic ethanol production.

generally between 150 °C and 220 °C, and most hemicellulose and some lignin are removed [73–77] Steam explosion and Liquid Hot Water are two methods studied widely. Chemical treatments include acid, alkali, and organic solvents. There are some pretreatments that are widely studied and considered most likely to achieve industrial large-scale application, mainly including dilute acid pretreatment, liquid hot water, and steam explosion.

4.2.2 Enzymatic Hydrolysis

Factors affecting efficient enzymatic hydrolysis can be roughly divided into two categories: One is associated with the substrate such as lignin, hemicellulose, and crystallinity; the other is related to cellulase such as cellulase activity, feedback, and suppression of product.

To improve the enzymatic hydrolysis efficiency of lignocellulose, the effect of negative factors on the enzymatic hydrolysis should be reduced or even eliminated. Pretreatment can eliminate the adverse effects of lignin, hemicellulose, and the lignocellulosic structure itself on cellulose. Adverse effects of substrate concentration on cellulase can be eliminated by reducing viscosity and heat and mass transfer mode of the reaction system. For example, batch feed in enzymatic hydrolysis [79, 80]; the process is carried out as follows: First, the biomass is subjected to enzymatic hydrolysis at a low substrate concentration for some time. Then, the substrate is added, and enzymatic hydrolysis is carried out after a period of time. Then, additional substrate is added according to the actual situation to determine the digestion and feeding time, until the final substrate concentration satisfies requirements and hydrolysis continues for a while. This process ensures that the viscosity of the reaction system is maintained at a low level, which is conducive to efficient digestion and a high concentration of sugar. Adverse effect of product concentration on cellulase can be eliminated by removing the product in time to maintain its concentration at a low level. For example, addition of β-glucosidase degrades cellobiose to glucose, eliminating the inhibition of cellobiose. The inhibition effect of glucose can be eliminated in several ways: (1) Membrane. The biomass enzymatic reaction is carried out in a membrane reactor. The obtained glucose and other small molecules are passed through the membrane and promptly removed by ultrafiltration, while cellulase and other macromolecules in the biomass are trapped in the reactor and undergo enzymatic hydrolysis, thus eliminating the product inhibitory effect on cellulase and improving the biomass efficiency and sugar concentration [81–84]. (2) SSF. In the same reactor, enzymatic hydrolysis and fermentation are performed at the same time, and the monosaccharides from biomass are timely consumed to prevent monosaccharide accumulation, to eliminate product inhibition on cellulose, to promote hydrolysis, and increase ethanol yield [85, 86].

4.2.3 Ethanol Fermentation

Pretreated hydrolyzate of lignocellulosic materials contains large amounts of hemicellulose-derived sugars and fermentation inhibitors such as formic acid, acetic acid, furfural, 5-hydroxymethyl furfural, and aromatic compounds, leading to poor fermentation for traditional microbes. In general, the detoxification treatments of hydrolyzate have the following three aspects: (l) to optimize the pretreatment process for avoiding or reducing the generation of inhibitors, (2) to make detoxification treatment for hydrolysis products before the fermentation, and (3) to build a bacterium with a higher tolerance to achieve in situ detoxification. Lee et al. [87] used activated carbon for the detoxification of liquid hot water hydrolyzate of hardwood pieces and found that 2.5% activated carbon can remove 42% formic acid, 14% acetic acid, 96% 5-hydroxymethyl furfural, and 93% furfural in the hydrolyzate; meanwhile, about 8.9% sugar was lost. Then, they fermented the hydrolyzate after detoxification with a strain thermophilic anaerobic bacterium MO1442 after genetic modification; this bacterium metabolized glucose, xylose, and arabinose, reaching 100% theoretical ethanol yield. Professor Yang Xiushan from Capital Normal University cultivated a new strain of *P. stipitis* Y7 [88]; this strain is capable of in situ detoxification for lignocellulosic hydrolyzate after dilute acid pretreatment, making glucose and xylose in lignocellulosic hydrolyzate converted efficiently to ethanol and reaching 93.6% of the maximum ethanol theoretical value. Detoxification in situ simplifies ethanol production from lignocellulosic materials and reduces the cost of ethanol production; this has important theoretical and practical significance for lignocellulosic ethanol production and commercialization.

4.3 Industrial Production of Ethanol Fuel in China

4.3.1 A Case About Cassava Ethanol Production

In the early April 2008, the National Development and Reform Commission (NDRC) announced that a special planning assessment focused on ethanol biofuel in key provinces has been completed by the China International Engineering Consulting Corporation. The assessment concluded that utilization of tubers as the raw material to produce ethanol fuel is economical. At present, Henan Tian Guan Group's plant that produces 300,000 tons of cassava ethanol fuel has passed certification in July 2012. In November 2012, the cassava ethanol production device of the COFCO (China National Cereals, Oils and Foodstuffs Corporation) Biochemical (AnHui) Co., Ltd., with an annual ethanol production of 150,000 tons, passed the certification as well.

Among all the ethanol fuel production projects from starch feedstocks, Guangxi COFCO Biomass Energy Co., Ltd.'s 200,000 tons/year cassava ethanol

production line is the most representative. The line started in October 2006 in Guangxi with the approval of the NDRC and put into production in December 2007. This production line is China's first set of nongrain fuel ethanol projects and a significant milestone. All the technologies involved in ethanol production from cassava in this plant have self-owned intellectual property rights. A mathematical model of the entire process of ethanol fuel production from cassava was created. Sand cleaning technology to separate sand from a thick mash of cassava, integrated spray liquefaction at high temperatures, low-energy heat exchange, SSF of cassava, thick mash fermentation technology, three-way thermally coupled distillation technology, molecular sieve dehydration coupled with distillation, amplification of a large side-stirred fermenter, high wettability filler, and antiblocking type tray design and manufacturing technology were developed and applied in the plant.

4.3.2 A Case about Cellulosic Ethanol Production

Shandong Longlive Bio-technology Co., Ltd and Shandong University have collaborated and used corn cobs as the raw material, achieving the industrialization of corncob bio-refinery. In "Technology and Application of Cellulosic Ethanol from Corn Cob Waste," they used the waste from corncob xylose processing for cellulase and fuel ethanol production, which not only transfers the cost of raw materials and pretreatment to high value-added products but also makes enzymes on the spot and ferment to ethanol. At the same time, the pretreatment stage converts some corncob hemicellulose to xylooligosaccharides, xylitol, and other high value-added products to solve the low conversion rate of hemicellulose sugars in biomass to ethanol. The remaining lignin is also used in the production of high value-added chemical products, thus improving the overall economic efficiency in the production process and forming a reasonable industrial structure with diversification products. 8 ton dry corn cob produces about 1.5 ton ethanol, 1.5 ton xylose-related products, more than 1 ton lignin, and 1.5 ton CO_2, and the waste can be fermented to produce biogas, thus truly achieving the full use of corn cob. This new technology not only broke through many technical bottlenecks but also took an international lead with building a pilot plant with an annual output of 3,000 ton cellulosic ethanol from corn cob and tons of production of demonstration units, making the ethanol production cost close to the level of ethanol food. Recently, the NDRC has formally approved a project of 50,000 ton cellulosic ethanol to Longlive Company; it has become the first cellulosic ethanol sentinel plant and the world's first industrialization business that achieved the biorefining of cellulosic resources. By collaborating with Sinopec, its products have entered the gasoline sales market.

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Zhongming Wang, Wei Qi, Shunni Zhu, Pingzhong Feng, Lei Qin

5 Microbial Butanol Production

Butanol has been used extensively as an important raw chemical material in the past four decades and is now known as an advanced biofuel owing to its various superior characteristics compared to ethanol and gasoline. It contains 22% oxygen, making it a beneficial fuel extender and a cleaner fuel than ethanol. It is generally produced under strict anaerobic fermentation conditions by Clostridium acetobutylicum, producing butanol, acetone, and ethanol as the main products, with the ratio of approximately 6:3:1. This anaerobic fermentation is known as acetone–butanol–ethanol (ABE) fermentation. The major challenges in biobutanol production are the availability of compatible feedstocks, low butanol titer, and product inhibition. Low price and adequate sustainable feedstocks such as lignocellulosic residues, carbohydrate, and energy crops are needed for butanol production. A large number of hurdles are being resolved using gracious genetic engineering techniques, metabolic engineering strategies, and favorable integrated batch or continuous fermentation process with competent product recovery and purification methods. Because of the rapid development of the petrochemical industry, the ABE fermentation gradually declined at the end of the last century across the world, and China is one of the few countries that continued the fermentative ABE production for several decades. At present, China has made significant progress in strain selection and improvements in the fermentation process and set up several bulk industries for butanol production. This chapter overviews the basic and updated knowledge of the production of butanol and biological long-chain alcohols with economically competitive ABE fermentation process.

Section 1: Butanol Industry Overview

1.1 The Properties and Uses of Butanol

1.1.1 Properties

n-Butanol (butyl alcohol or 1-butanol) is a four-carbon straight chain alcohol with a molecular formula of C_4H_9OH and molecular weight of 74.14 g/mol, respectively. This colorless liquid with alcohol smell is superior to ethanol as a fuel in many regards

Corresponding author: Zhongming Wang, E-mail: wangzm@ms.giec.ac.cn
Wei Qi, Shunni Zhu, Pingzhong Feng, Lei Qin, Key Laboratory of Renewable Energy, Chinese Academy of Sciences; Guangdong Key Laboratory of New and Renewable Energy Research and Development; Guangzhou Institute of Energy Conversion, Chinese Academy of Sciences, Guangzhou 510640, China

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such as higher energy density, lower volatility, less ignition problems, and less corrosion to existing infrastructure [1, 2]. Butanol contains 22% oxygen, making it as a beneficial fuel extender and a cleaner fuel than ethanol. The chemical properties of *n*-butanol are density, 0.808 g/mL at 20 °C; dynamic viscosity, 3.379 mPa s; refractive index, 1.3993; melting point, −89.0 °C; boiling point, 117.7 °C; flash point 35–35.5 °C; autoignition temperature, 365 °C; stoichiometric ratio, 11.21; latent heating (kJ/kg) at 25 °C of 582; viscosity (mm^2/s) at 40 °C, 2.63; and saturation pressure (kPa) at 38 °C of 2.27. The solubility of *n*-butanol in water at 20 °C is 7.7% (wt%), and water solubility in *n*-butanol is 20.1% (wt%). Butanol can be mixed and dissolved with alcohol, ether, and other organic solvents. In general, the toxicity of *n*-butanol is roughly equivalent to that of ethanol, and it is strongly irritating to the skin and eyes causing redness and injury. It is harmful if ingested in the respiratory tract and can cause sleepiness and dizziness. It is classified as a Class 3.3 high flash point flammable liquid according to the domestic chemical safety regulations common classification of dangerous chemicals and signs (GB 13690-1992). The maximum allowable concentration of butanol in the workplace is 300 mg/m^3. Its steam can form explosive mixtures with air, with an explosion limit in the range 0.0145–0.1125 (volume fraction) [3, 4]. Basic physical and chemical properties of methanol, ethanol, butanol, gasoline, and diesel are listed in Table 5.1 [3, 5], and the combustion characteristics of methanol, ethanol, *n*-propanol, and *n*-butanol are listed in Table 5.2 [3, 4].

1.1.2 The Main Use of Butanol

As an important organic raw material and chemical solvent, butanol's uses include the following aspects:

1.1.2.1 Used as Industrial Raw Material in Synthetic Organic Chemistry

In China, butanol is mainly used to produce butyl acetate, butyl acrylate, dibutyl phthalate (DBP), and various pharmaceutical intermediates. Butyl acetate, butyl acrylate,

Table 5.1: Basic physical and chemical properties of methanol, ethanol, butanol, gasoline, and diesel.

Items	Density (20 °C) kg/L	Boiling point (°C)	Heat value of gasification (kJ/kg)	Liquid viscosity (20 °C) (Pa s)	Flashpoint (°C)	Octane number	Cetane number
Methanol	0.7920	64.5	1,088	0.61	12	106–115	3
Ethanol	0.7893	78.4	854	1.20	14	110–112	8
Butanol	0.808	117.7	430	3.64	35	95–100	25
Gasoline	0.72–0.78	40–210	310–340	0.28–0.59	45–38	80–98	5–25
Diesel	0.82–0.86	180–360	250–300	3.00–8.00	65–88	20	45–65

Table 5.2: Comparison of the combustion characteristics of methanol, ethanol, *n*-propanol, and *n*-butanol.

Items	Methanol	Ethanol	*n*-Propanol	*n*-Butanol
Low heat value (LHV) (MJ/kg)	19.916	26.778	32.465	35.103
Volumetric heating value (MJ/L)	15.77	21.26	26.10	28.43
Theoretical hybrid heat value (MJ/kg)	2.6599	2.6700	2.8561	2.8733
Mole heat value (MJ/mol)	638.2	1233.6	1951.0	2601.9
Ignition temperature (°C)	470	434	425	385
Ignition limit when mixed with air (volume fraction) (%)	6.0–36.5	3.5–18.0	2.3–12.5	1.4–11.2
The apparent activation energy when combusted with air (kJ/mol)	172.9	176.7	189.7	202.6
Motor octane number (MON)	91	92	90	94
Research octane number (RON)	106–115	100–112	98–104	95–100
Cetane number	3	8	10	12
Theoretical volume fraction when combusted with air $F/(F + A)$ (%)	12.22	6.51	4.44	3.36
The theoretical ratio of air to fuel (kg/kg)	6.4988	9.0293	10.3788	11.2171
Boiling point	64.5	78.4	97.2	117.7
Hydrophile lipophilic balance (HLB)	8.4	8.0	7.5	7.0
Flash point (°C)				
Open-cup flash point	15.6	17.5	22	37
Closed-cup flash point	12	13	16	35
Theory change coefficient μ	1.0613	1.0653	1.0667	1.0675

Note: The theoretical ratio of air to fuel *(A/F)* is 14.6.

and DBP accounted for 32.7%, 15.3%, and 9% of the total alcohol consumption, respectively. In addition, butanol can be oxidized to produce butyraldehyde or butyric acid.

1.1.2.2 Used as a Solvent

Butanol is an important solvent and can be used for the extraction of grease, pharmaceutical, and flavor. It has also been used as additives of alkyd resin and organic dyes, printing ink solvent, dew axing agent, coatings production, and pigment thinner. In addition, it has been used as flavoring spices of the food industry in China, as the provisions of GB 2760-1996 allows it to use for the preparation of bananas, butter, cheese, whiskey, and other flavors.

1.1.2.3 Used as Biofuels

Butanol is considered as a novel and renewable energy source. Butanol at 85% strength can be used in cars designed for gasoline (petrol) without any modification to the

engine (unlike 85% ethanol), and it provides more energy for a given volume than ethanol and almost as much as gasoline; thus, a vehicle using butanol would return more energy consumption compared to gasoline and ethanol. It can also be used as a blended preservative to diesel fuel to reduce dust discharges. Moreover, it contains 22% oxygen, making it a beneficial fuel extender with cleaner burning characteristic than ethanol. Because of its high octane number, it can also be used as an aviation fuel.

1.2 The Production Process of Butanol

Butanol in industry is mainly produced by three methods, namely, condensation of acetaldehyde and aldol, propylene oxo synthesis, and fermentation. Oxo synthesis and aldol condensation are the chemical synthesis route, both of which use petroleum as the raw materials, and thus need a large investment and have strict technical equipment requirements. The biological fermentation of butanol from renewable resources (biomass) is generally carried out by *Clostridium acetobutylicum* under controlled anaerobic conditions, with butanol, acetone, and ethanol as the main products at a ratio of approximately 6:3:1, and this process is referred to as ABE fermentation.

1.2.1 Aldol Condensation

Aldol condensation of acetaldehyde, followed by hydrogenation, yields crotonaldehyde (butenal), which is converted to butanol by hydrogenation and to butyraldehyde by selective hydrogenation. The aldol condensation of butyraldehyde followed by hydrogenation yields 2-ethylhexyl alcohol (octanol). However, this method has almost been eliminated, because of high production costs:

$$2CH_3CHO \longrightarrow CH_3CHOHCH_2CHO \xrightarrow{-H_2O} CH_3CH{=}CHCHO \tag{5.1}$$

Crotonaldehyde hydrogenates to *n*-butanol in the presence of nickel–chromium catalyst at 180 °C and 0.2 MPa:

$$CH_3CH{=}CHCHO + 2H_2 \longrightarrow CH_3CH_2CH_2CH_2OH \tag{5.2}$$

1.2.2 Carbonyl Synthesis Method

Oxo synthesis method is the world's leading chemical synthetic technique and most common chemical route for butanol production from fossil oil-derived raw materials, involving the reaction of propylene with carbon monoxide and hydrogen in the

presence of an appropriate catalyst [6]. The process of propylene oxo synthesis for butanol production is as follows: propylene reacts with carbon monoxide generating *n*-butyraldehyde and isobutyraldehyde by cobalt and rhodium catalysts, respectively. *n*-Butyraldehyde and isobutyraldehyde are hydrogenated, yielding *n*-butanol and isobutanol, respectively. *n*-Butyraldehyde is converted to octanol by aldol condensation followed by hydrogenation. Depending on the reaction pressure and the catalyst, the process of propylene oxo synthesis of butyl alcohol can be divided into high-pressure cobalt method, cobalt-modified method, high-pressure rhodium method, and modified rhodium method, where the modified rhodium method has the advantages of low temperature, low pressure, high rate, high ratio of desired isomer, less side effects, less rhodium catalyst, long life, recycling of the catalyst, and less equipment investment. In addition, butanol and octanol can switch production, because of the poor selectivity, more by-products (propane), and high boiling point. High-pressure carbonylation synthesis technology has been replaced by low-pressure carbonylation synthesis technology using rhodium catalyst. Low-pressure carbonylation synthesis technology using the oil-soluble triphenylphosphine ligand-modified rhodium as the catalyst is a major breakthrough. The modified rhodium method is divided into gas recycle and liquid recycle methods; the liquid recycle rhodium method is the world's most advanced and widely used method of 2-ethyl hexanol synthesis. It was further improved and has been developed with special characteristics. There are four main technical representative companies, which use the second-generation low-pressure carbonyl synthesis technology. During the synthesis, propylene is used in the presence of rhodium catalyst developed by DAVY and DOW companies together. Low-pressure carbonyl synthesis technology. under rhodium catalyst was developed by Mitsubishi Kasei, and carbonyl synthesis technology and low-pressure carbonyl synthesis technology of BASF were developed by Ruhr Ruhrchemie/Rhone Poulenc [7].

1.2.3 Fermentation Method

Butanol can also be obtained from renewable resources (biomass) by the ABE fermentation. This process is called biobutanol or the ABE production. Butyric and acetic acids are first produced by *C. acetobutylicum* (acidogenesis), and in the subsequent phase (solventogenesis), butanol, acetone, and ethanol are formed [6]. The biobutanol production consists of several stages: prior to the ABE fermentation the biomass consisting of starch-rich, sugar-rich, or lignocellulosic materials is pretreated in the upstream processing and used as the substrate. The pretreatment method differs depending on the type of biomass used. After the fermentation, the desired product is recovered and purified in the downstream processing. Biobutanol production was already developed in the 1910s and has faced numerous production difficulties during the last 20–40 years. In recent years, with increasing depletion of fossil resources, the world crude oil prices continued to increase, thus butanol industry has been

attracting increasing attention from researchers and entrepreneurs. In earlier times, butanol and acetone were produced by the petrochemical process, which is not cost effective, thus bringing new opportunities for the development of the production of acetone and butanol by biological fermentation. The fermentation method has the following advantages compared to the chemical synthesis methods:

(1) Chemical synthesis methods use fossil oil-derived raw material, seeking huge investment and require high technology and costly equipment, whereas microbial fermentation method generally uses starch, wastepaper pulp, molasses, wild plants, and lignocellulosic biomass as the raw material. The process equipment is similar to the alcohol production, thus requires low investment.
(2) The production conditions of the fermentation process are mild and generally can be operated at room temperature and do not need expensive metal catalyst.
(3) Fermentation method has a good selectivity, high safety, less by-products, easy separation, and purification.
(4) It reduces the consumption and dependence on the limited fossil resources.
At present, the new biofuels account for less than 2% of the global transportation fuel market. According to the market forecast, biofuels in the future transportation fuel structure will occupy an important proportion, and the biofuels consumption in the main market is expected to reach 30–20%. Because the biobutanol production is similar to the ethanol production, the existing ethanol production facilities can be turned to the production of biobutanol. Therefore, biobutanol production has a large market potential.

1.3 The Production History of Butanol by the Fermentation Process

As early as 1852, butanol was first found to be a common ingredient of fuel oil by Wurtzt. The formation of butanol in the microbial fermentation was first reported by Louis Pasteur in 1861. He observed butyric acid fermentation by lactic acid and calcium lactate, forming butanol as the by-product. In the following years, Albert Fitz worked actively in the field of fermentation and obtained butanol from glycerol using a mixture of two bacteria. Among others, researchers continuing with this work were Beijerinck, Bredemann Shardinger, and Pringsheim [8, 9]. In 1911, Professor Fernbach isolated a Fitz bacterium, producing acetone besides butanol. However, this bacterium can only use potato starch instead of other grain materials. This process was industrially implemented in Rainham during 1913–1914 and further transferred to another plant of Strange and Graham Ltd at King's Lynn [10]. Later, this process was eliminated, because of the limited availability of the raw material.

In the summer of 1912, Chaim Weizmann left the research group and started his own studies. He succeeded in isolating a bacterium strain (later named *Clostridium*

acetobutylicum), capable of using starch as a substrate for the butanol production process. This process exhibited higher product yields of butanol and acetone, and replaced Fernbach's process [11]. The bacterium strain laid the foundation for the industrialization of the acetone and butanol fermentation and obtained the British patent in 1915. Several countries such as the United States, Russia, Britain, Canada, China, and Japan produced biobutanol in industrial scale during the period 1920–1980 [6]. For this process, locally isolated *Clostridium* strains were used. Since 1911, new discoveries are continuously patented until present [12]. In August 1914, the First World War broke out; the dosage of acetone surged; and in the same year, the first acetone fermentation factory successfully using the Weizmann bacterial strains and craft was founded in Britain [6, 11]. In 1916, this factory migrated to Canada and established as the British Acetone Company in Toronto because of food shortages. After the war, DuPont and Steven Companies discovered the preparation of butyl acetate from butanol. The acetone and butanol fermentation industry was renamed to butanol fermentation industry because butantol and acetone become a valuable solvent and byproduct, respectively.

After the mid-1830s, as the molasses price is lower than corn, the solvent and the fermentation production of butanol by molasses is higher than that of corn fermentation. In addition, because of lower fermentation temperature required by molasses than corn, most of the factories started using molasses fermentation for butanol production. In December 1942, the Second World War broke out and Japan's source of butanol was blocked. Owing to the high octane number, butanol can be used as an aviation fuel, attracting the attention of Japanese researchers to actively investigate on acetone and butanol fermentation technology. Japanese researchers isolated an available molasses fermentation bacterial strain and used it for butanol production from molasses in Taiwan, with an annual capacity of 25,000 tons for general solvent. The United States also used molasses as the raw material for butanol production in 1945.

The synthesis of butanol by the chemical method began to rise in the late 1930s; however, the fermentation method still dominated. By 1949, acetone produced by the United States was 28,892 tons, 39% from the fermentation method and 61% from the chemical synthesis method, mainly because of the low cost of the chemical synthesis method. During 1955, acetone production by Japan was 4,800 tons, 97.5% by the fermentation method, and the butanol production was 10,000 tons, accounting 91.8% of the total production by the fermentation method.

After the peak in the 1850s, because of the frequent phage and sweetening manufacturing progress, the quality of molasses dropped, and as a result, in the western industrialized countries, acetone and butanol fermentation production strength continued to decrease. With the development of petrochemical industry, acetone and butanol production from fossil fuels such as oil was more economical than the fermentation method. In the 1860s, in the western industrialized countries, acetone and butanol fermentation factories were unable to gain profit, thus closed down as a result of costly production facilities, and laboratories, useful microbial strains, and production records were unprotected and destroyed. Until the 1880s, a few acetone and

butanol fermentation factories in South Africa and the former Soviet Union continued their butanol production from the raw material, and the labor cost remains low in those countries. The industrial biobutanol production ceased in the early 1990s. Research and development of the ABE process continued, and during the 1980s and 1990s, studies at the pilot plants were carried out in Soustons (France) and Lower Austria [13]. In recent years, the increasing oil price and the importance of energy and environmental issues opened new development opportunities to the production of microbial butanol fermentation with the shortage of oil resources. At the same time, along with the development of modern biological engineering technology progress, the fermentation is expanding on the selectivity of raw materials, and in addition to starch, sugar is also used gradually as the raw materials for the development and utilization of lignocellulose raw materials.

A number of companies have recently launched projects to produce butanol from biomass, considering novel alternatives to traditional ABE fermentation, which would assist biobutanol to be produced at an industrial scale. In June 2006, DuPont and BP (the largest venture in the area of advanced biofuel) formed a partnership to develop new biobutanol production technology using lignocellulosic feedstocks and announced plans to produce 30,000 tons of butanol per year in a modified ethanol facility of British Sugar in the United Kingdom. On May 24, 2012, Gevo inaugurated production at the world's first commercial-scale 18 MGPY biobutanol plant, developed by the conversion of the former Agri-Energy corn ethanol plant in Luverne, targeting to produce 50,000–100,000 gallons per month of isobutanol by the end of 2014. In January 2012, Green Biologics Limited announced a merger with butylfuel™ Inc., the United States, and operated from its headquarters in the United Kingdom. Green Biologics has developed butanol-producing GM microbial strains and planned to integrate these into a novel fermentation process. This technology advance should result in a step change in the economic viability of the fermentation and enable the large-scale production of Green Biologics' Butafuel™ product. Initially, the facility will continue to produce ethanol, but it aimed to start production of *n*-butanol and acetone in 2016. Previously, Green Biologics was also involved in biobutanol development in India and China. There are a number of biotechnology companies such as ButylFuel, Metabolic Explorer, Cathay Industries, and Tetravitae Biosciences around the world dedicated to providing strains and process solutions for ABE fermentations for industrial customers for developing butanol.

1.3.1 Butanol Fermentation Industry in China

China consumed approximately 34.8% of the world's *n*-butanol in 2012, and approximately 60% of its consumption depends on import [14]. China built the first factory with the fermentation production of acetone and butanol in 1955. During the next 30 years, approximately 30 ABE fermentation factories with an annual production in the range 3000–10,000 tons were built in various provinces, including Beijing, Jiang Su, Yun Nan, Tian Jin, Shan Xi, Zhe Jiang, Shan Dong, He Bei, and Ji Lin. As a result, the annual

production of ABE reached 170,000 tons. Most factories used dry corn and potato as the raw material; however, some factories used molasses and rice as the raw materials [15]. Under the current circumstances of butanol shortage because of heavy industrial use, new opportunities have reemerged for the ABE fermentation industry.

Since 2006, with the rapid rise of the price of *n*-butanol caused by the rapid increase in international oil prices, the ABE fermentation technology in China has been attracting worldwide attention. As the data show, before June 2008, 11 domestic ABE fermentation factories based on corn fermentation were newly established or restored to production, and a few were in the planning stage (Table 5.3). However, with the rapid decline of the butanol price at the end of 2008, resulting from the financial crisis, most of the factories were closed. Some of them closed permanently, whereas others tried to seek alternative feedstocks such as nongrain crops and cellulosic materials. The quality of acetone and butanol in China has reached the international advanced level in the 1980s in terms of raw materials and low energy consumption and started to export overseas countries.

Raw materials are natural resources, and relatively inexpensive access to the fermentation of raw materials is a common issue for all bioproducts. China has begun experimenting with the use of "nongrain crops" to produce bioethanol (development planning of China's bioenergy industry [2007–2016]; Ministry of Agriculture, 2007). With growing concerns over the impacts of biofuel expansion on food security, China has disallowed the use of grain for future expansion of biofuel production since 2007 and recently indicated that it will expand its biofuel target using second-generation feedstock cellulosic materials in the future (medium- and long-term development plan for China's renewable energy; National Development and Reform Commission (NDRC) [14].

In the mid-1990s, because of the increased price of raw materials and the competitiveness of the petrochemical industry, most of the ABE fermentation factories in China were shut down. Early in this century, rapid development of world economy, environmental concern, and other associated reasons provoke the renewable energy demand, increasing the prices of acetone and butanol, and thus the fermentation production of acetone and butanol began to enhance its market competitiveness. In recent years, 11 ABE fermentation companies have been built in China, and the annual output reached approximately 210,000 tons in 2008. At the end of 2008, the butanol price fell from 13,000 yuan to 5,000 yuan per ton, and the acetone price fell from 9,000 yuan to 4,000 yuan per ton.

Although butanol and acetone market prices rebound later, the price of raw material for the production of acetone and butanol (wheat, corn, etc.) was still high, approximately 60% of the total cost of the fermentation, making the industry economically viable. Moreover, policymakers are facing a debate with "food production with increasing population," with negative effects on the enterprises to continue their production of acetone and butanol. However, a few of these enterprises changed the biomass to cassava and lignocellulosic material to produce butanol and ethanol.

Domestic scientific research institutes and some enterprises such as Chinese Academy of Sciences, Shanghai Institute of Plant Physiological Ecology, Shanghai Industrial Microbial Research Institute, Institute of Nuclear and New Energy

Table 5.3: Chinese companies involved in ABE production since 2006 [14, 15].

Companies	Planned (P), newly built (N), or restored from previously shutdown ABE factory (R)	Maximum capacity (tons/year)	Starting date	Location	Feedstocks	Current status
Cathy Industrial Biotech Co. Ltd (Jilin plant)		300,000	2008.3	Jilin		
Songyuan Laihe Chemicals Co. Ltd. (Originally Songyuan Ji'an Biochemical Co., Ltd.)	N	200,000	2007.12	Songyuan, Jilin	Corn; corn starch milk	Corporate restructuring as Lignicell Refining Biotechnologies Ltd. listed in the table
Ji-An Biochemical Co. Ltd	N	150,000	2007.12	Jilin		Running
Guiping Jinyuan Alcohol Co. Ltd	N	100,000	2007.8	Guangxi		
Guangxi Guiping Jinyuan Industrial Co. Ltd.	N	50,000	2007.8	Guiping, Guangxi	Molasses	Shut down
Jiangsu Lianhai Biological Technology Co. Ltd	N	50,000	2008.10	Jiangsu	Corn	Shut down
Lianyungang Lianhua Chemicals Co. Ltd	N	40,000	2008.1	Lianyun-gang, Jiangsu	Corn; mixed corn and cassava	Shut down since trial production in 2010
Kailu Liniu Biochemical Co. Ltd	P	30,000		Inner Mon-golia		Under construction
Anhui Furuxiang Food Co. Ltd	P	30,000		Anhui		Under construction
Lignicell Refining Biotechnologies Ltd (formally Laihe Rockley Bio-chemicals Co. Ltd., a joint venture company by Rockley group and Songyuan Laihe Chemical Co. Ltd.)	R	40,000	2013.12	Songyuan, Jilin	Mixed corn stover and corn cob	Running

Technology, Tsinghua University, Guangzhou Institute of Energy Conversion-Chinese Academy of Sciences, Beijing University of Chemical Industry, Dalian University of Technology, and Zhongke (yingkou) New Energy Technology Development Co. Ltd. have begun fermentation research and development of the production of acetone and butanol. Shanghai Tianzhiguan of the Renewable Energy Co. Ltd., North China Pharmaceutical Companies, including Shanghai Tianzhiguan of the Renewable Energy Co. Ltd and the Chinese Academy of Sciences, Shanghai Institute of Plant Physiology and Ecology's research on fermentation production of acetone and butanol has applied for a national "973 project" and "863 project" and the Chinese Academy of Sciences knowledge innovation project important direction, with a focus on building high yield, high substrate selectivity of acetone–butanol fermentation strains and developing new fermentation production process, including the use of fiber as the raw material for acetone–butanol production, solvent extraction coupling fermentation technology research and development.

Beijing University of Chemical Industry has developed a corn fermentation process for the production of acetone and butanol with the starch conversion and the butanol productivity reaching 70% and 25%, respectively, with only 36 h of the fermentation process.

The process in the North China Pharmaceutical Factory pilot production workshop has "zero" dyeing rate of bacteria for four consecutive years, and thus the industry has the best record and significantly lowered the production cost. Jian New Energy Co. Ltd (China) accepted the Shanghai biobutanol consortium of *C. acetobutylicum* EA 2018 strains in 2010 and the development of the straw hemicellulose butanol fermentation technology with the Chinese Academy of Sciences Institute of Process Engineering achieved an annual output of 600 tons of fuel butanol pilot experimental study.

Guangzhou Institute of Energy Conversion-Chinese Academy of Sciences is focusing on multidimensional research areas to develop a viable and economical fermentation technology for the production of acetone–butanol under the key national "863 project." Several associates financing these projects include the Chinese Academy of Sciences knowledge innovation project and technology innovation project in Guangdong province. This institute uses corn, molasses and cassava, cellulosic biomass plants grown crops, and waste papers as the raw material to produce butanol, and this institute has already exhibited better performance of acetone–butanol clostridium, carried out relevant acetone–butanol fermentation technology research, such as immobilized bacteria and reactor design optimization, the continuous fermentation technology, two consecutive periods of fermentation in the production of new technology, butyl alcohol and butanol fermented liquid separation and enrichment research. In the domestic setting, it has started to provide technical services to related enterprises, and set up more than taking cassava and corn as the raw material to produce acetone–butanol industrial equipment. At present, the production is running normally. In addition, the key study was low acid hydrolysis of lignocellulosic bimass for monosaccharide, disaccharide, and oligosaccharides in differential-type filtration

bed, such as clostridium hydrolyzate by acetone–butanol after the detoxification treatment hydrolyzed fermentation of the sugars into acetone and butanol, and the small parts of process experiment have already completed with good effects. The hemicellulose sugar and cellulose sugar yield reached >90%, and >65%, respectively, mixed with hydrolyzate sugar concentration >5% of corn cob after hydrolysis through differential-type filtration bed low acid hydrolysis. Recently, the small experimental study of the lignocellulose hydrolyzate for 2 kg/h fermented feed product butanol was completed.

Zhongke (yingkou) New Energy Technology Development Co. Ltd. and the Chinese Academy of Sciences-Guangzhou Institute of Energy Conversion in cooperation have devoted to the development of industrialization of lignocellulose raw material to produce butanol, now a lignocellulose hydrolyzate continuous feed 8 kg/h butanol fermentation system test in the experimental study was completed on November 30, 2010. With this target, a 300 ton/year lignocellulose acetone–butanol pilot plant was built, where the mixed hydrolyzate sugar concentration could reach 4–7% and the butanol fermentation production rate was close to 70% after the hydrolyzate detoxification. Domestication 2 *Clostridium* strains were screened, and the solvent conversion rate can reach >35% under different hydrolyzate detoxification conditions. With the total solvent concentration up to 23 g/L, butanol concentration reached 13 g/L.

Section 2: Microbial Butanol Fermentation

2.1 Microbial Species of Butanol Fermentation

Many microbes are proficient in producing acetone and butanol from biomass. The selection of strain used in a butanol production facility depends on a number of factors that must be well studied to the locally available substrate and production condition. At present, almost all industrial strains were screened from natural environments, and momentous modifications have been made through mutagenesis, evaluation engineering, and metabolic engineering to improve butanol yield, titer, and productivity under industrial production conditions [12].

Clostridia, the dominant species for ABE fermentation from the establishment of this industry, can generate butanol through metabolism; most of them are rod-shaped, spore-forming Gram-positive, obligate anaerobes, and toxic to oxygen. According to 16S rDNA sequence homology analysis, solvent-producing *Clostridium* can be divided into four types, that is, *C. acetobutylicum*, *C. beijerinkii*, *C. saccharobutylicum*, and *C. saccharoperbutylacetonicum* [16]. The homology between *C. acetobutylicum* and other three species is 90–95%, while the homology among the other three species can reach 98.2–98.9%. Keis et al. [17] further divided the four species into several subspecies according to DNA blots, as listed in Table 5.4.

Table 5.4: The classification of solvent-producing *Clostridium*.

Subspecies	Bacterial strains	Former name
C. acetobutylicum		
1	ATCC 824T(=DSM 792T =NRRL B527T), ATCC 3625, DSM 1733(=NCIMB 6441), NCIMB 6442, NCIMB 6443, ATCC 43084, ATCC 177922	*C. kaneboi*
2	DSM 1731 (=ATCC 4259 =NCIMB 619 =NRRL B530)	
3	DSM 1737, DSM 1732 (=NCIMB 2951), ATCC 39236	
4	ATCC 8529 (=DSM 1738)	
C. saccharobutylicum		
1	NCP 262^{T}, NCP 249, NCP 265(S), NCP 268, NCP 172(S), NCP 200(S), NCP 202(S), NCP 199, NCP 195, NCP 220, NCP 108, NCP 254(S)/B3, 37/3 IMM2	*C. acetobutylicum*
2	NRRL B643, NCP 258, NCP 272(S), BAS/B3/SW/336(S), 162/BI	*C. acetobutylicum*
C. saccharoper butylacetonicum		
1	N1-4 (=ATCC 13564), ATCC 27021	
2	N1-504 (=ATCC 27022)	
C. beijerinckii		
1	NCIMB 9362^{T}, NCIMB 11373	
2	NCIMB 8052(=DSM 1739 =ATCC 10132 =NRRL B594), NCIMB 8049, NCIMB 6444, NCIMB 6445, NCIMB 8653, NRRL B591,	*C. acetobutylicum*
	NRRL B597214, 4J9	*C. madisonii*
3	3 NCP 193, NCP 172(B), NCP 259, NCP 261, NCP 263, NCP 264, NCP 270, NCP 271, NCP 200(B), NCP 202(B), NCP 280, NCP 272(B),	*C. acetobutylicum*
	NCP 265(B), NCP 260, NCP 254(B), NCP 106, BAS/B/SW/136, BAS/B3/SW/336(B), BAS/B/136	*C. acetobutylicum*
4	ATCC 39058	*C. acetobutylicum*
5	NRRL B593, ATCC 17791	
6	NRRL B592	
	NRRL B596, NRRL B466	*C. acetobutylicum*
7	NCIMB 9503, NCIMB 9504	
8	NCIMB 9579, NCIMB 9580	
9	NCIMB 9581	
10	NCIMB 12404	
11	ATCC 17795	
12	IAM 19015	*C. butanologenum*

Note: ATCC (American Type Culture Collection, Manassas, VA, USA); DSM (Deutsche Sammlung von Mikroorganismen und Zellkulturen); IAM (Institute of Applied Microbiology, Tokyo, Japan); NCIMB (National Collection of Industrial and Marine Bacteria, Aberdeen, UK); NCP (National Chemical Products Ltd, Germiston, South Africa); NRRL (Northern Utilization Research and Development Division, Peoria, IL, USA).

Recent studies reported that *Saccharomyces cerevisiae* also has decisive advantages in industrial processes of butanol isomers owing to its tolerance to alcohols and prospective fermentation conditions, but additional genetic manipulations are required to restore the redox imbalance and drive acetyl-CoA production [18].

2.1.1 Diversity of Fermentation Performances of Butanol-Producing Microorganisms

Solvent-producing clostridia and various nonsolvent-producing species are mainly utilized in the butanol fermentation from long time. *C. acetobutylicum* was isolated in the 1980s from the soil samples collected from the soybean field at Shanghai suburb by prestigious microbiologist Dr. Rui-Shen Jiao at Shanghai Institute of Plant Physiology and Ecology, Chinese Academy of Sciences and has been successfully applied in the ABE fermentation plants in China from long time. This strain can produce solvents butanol–acetone–ethanol with the typical mass ratio of 6:3:1, and its optimum growth and fermentation temperatures are 35–37 and 37–39 °C, respectively [12, 15]. A hyperbutanol-producing strain *C. acetobutylicum* was isolated by Mr. Chiao's research group in 1996 (Chinese patent CN1143677) with a high solvent ratio of butanol–acetone–ethanol of 7:2:1 instead of 6:3:1 [19]. Recently, molecular mechanisms underlying *C. acetobutylicum* EA2018 overproduction have been elucidated by the comparative genomic studies and transcription analysis [20]. Through repetitive evolutionary domestications, a butanol-tolerant strain *C. acetobutylicum* T64 was isolated, which could tolerate 4% (v/v) butanol and produce 15.3 g/L butanol compared to 12.2 g/L produced by the wild-type strain [21]. Another high butanol-tolerant strain *C. acetobutylicum* JB200 was bred in Prof. Shang-Tian Yang's lab at Ohio State University from *C. acetobutylicum* ATCC 55025 by long-term adaptation and could tolerate and produce butanol at a much higher concentration of approximately 20 g/L [22]. *C. saccharobutylicum* is suitable for the production of solvents in the temperature and pH ranges 30–40 °C and 5.2–7.0, respectively, while *C. saccharoperbutylacetonicum* is suitable for the production of the solvents in the ranges 25–35 °C and pH 5.6–6.7. *C. acetobutylicum* produces solvents butanol:acetone:ethanol with the typical 6:3:1 ratio (mass), and its optimum growth and fermentation temperatures are 35–37 and 37–39 °C, respectively. *C. beijerinkii* has the similar solvent profiles of typical solvents as that of *C. acetobutylicum*, but some strains of *C. beijerinkii* produce isopropanol rather than acetone [23].

Solventogenic *Clostridium*, which can use hexoses (glucose, fructose, galactose, and mannose) and pentoses (arabinose and xylose) for fermentation, has a wide range of sugar utilization. From the perspective of industrial application, *C. saccharobutylicum* and *C. saccharoperbutylacetonicum* are suitable for the fermentation of molasses, while *C. acetobutylicum* and *C. beijerinkii* are suitable for the fermentation of starch-based

materials. Shaheen et al. [23] studied the fermentation abilities of the typical strains of these four species in corn, molasses (containing 6% of fermentable sugars), and tryptone–yeast extract agar (TYA, containing 4% glucose) media. The results showed that *C. acetobutylicum* strain exhibited good performance using TYA and corn media, but had a lower yield of solvents using molasses. Among the *C. beijerinkii* strains, the strains obtained from NCP and NRRL B592 showed good performance in molasses medium; other strains exhibited average levels, and some of them had very poor yield. *C. saccharoperbutylacetonicum* strain in the three types of media performed quite well though not the best, and thus was suitable for fermenting mixed media; moreover, this strain produced a higher proportion of butanol.

C. acetobutylicum is sensitive to rifampin, produces riboflavin (vitamin B2) in milk, liquefies gelatin, and is capable of using glycogen and pectin, but cannot use ribose and rhamnose. Most strains of *C. acetobutylicum* cannot utilize glycerin, D-arabitol, L-arabitol, hexitol, inositol, melezitose, melibiose, trehalose, turanose; some strains can utilize inulin and sorbitol.

C. saccharobutylicum is sensitive to rifampin, cannot produce riboflavin in milk, can liquefy gelatin, utilize inositol, melibiose, glycogen, turanose, and trehalose, but cannot use glycerin, hexitol, sorbitol, melezitose, rhamnose and pectin; most strains cannot utilize ribose.

C. saccharoperbutylacetonicum is resistant to rifampin, cannot produce riboflavin in milk, can liquefy gelatin, use glycogen, inulin, D-arabitol, L-arabinose, mannitol, melezitose, melibiose, trehalose, turanose, and pectin, and cannot utilize ribose, glycerin, and pectin. Some strains can utilize hexitol and sorbitol; most strains cannot utilize rhamnose.

C. beijerinkii cannot produce riboflavin in milk, but can use sorbitol, melezitose, trehalose, turanose, and pectin; most strains (except *C. beijerinkii* NCIBM B9579) are resistant to rifampin and cannot liquefy gelatin (except *C. beijerinkii* NCIBM B9785), utilize glycerol and rhamnose. Most strains can utilize D-arabitol, L-arabitol, and glycogen, and some strains can also use ribose.

These four bacterial species can be distinguished according to the above-mentioned properties. For example, only *C. saccharobutylicum* cannot use pectin, while only *C. acetobutylicum* can produce riboflavin in milk. Only *C. beijerinkii* is unable to liquefy gelatin [16]. All the four species could use methyl pyranoside, raffinose, escin, salicin, and apricot glycosides, and none of them can produce indole. All of them do not have catalase and urease.

Milk is not only used for culture preservation (the bacteria were cultured for 5 days at 37 °C, stored at 4 °C), but its curd formation rate is also a standard way to distinguish different strains. *C. saccharobutylicum* cannot solidify milk within 48 h of culture, and the other three species can solidify milk within 24 h. *C. acetobutylicum* solidifies milk most quickly. After 10 days storage at 4 °C, the milk medium inoculated

with *C. acetobutylicum* produces bright yellow substances, but such phenomenon would not happen to other species. The color of such bright yellow substances will continue to deepen in the first few weeks and would become stable afterward. The filtrate of the yellow culture has an absorption peak at 445 nm, which is consistent with the spectrum of riboflavin. When cultured in milk, the significant property for *C. acetobutylicum* is the appearance of such bright yellow substances. Further, in Trypticase-yeast extract glucose (TYG) medium, *C. acetobutylicum* produces only small amounts of butanol (approximately 0.67 g/L), whereas *C. acetobutylicum* produces 3.56 g/L butanol. Thus, if a strain is capable of producing >2.22 g/L butanol in TGY medium, this strain cannot be *C. acetobutylicum*. However, it cannot be determined that the strain which produces <2.22 g/L butanol in TGY medium is *C. acetobutylicum*, because this strain is vulnerable to deterioration [24].

C. saccharoperbutylacetonicum DSM 2152 produced much less acids than *C. acetobutylicum* DSM 792 under all the conditions, and its optimal pH value for the production of solvents was approximately 1 pH unit higher than *C. acetobutylicum* DSM 792 [25], that is, the optimum pH of *C. saccharoperbutylacetonicum* DSM 2152 for producing solvent is 5.5 instead of 4.5. In addition, the chemostat studies have shown that *C. saccharoperbutylacetonicum* DSM 2152 can produce solvents at a dilution rate of 0.3 h^{-1}, while *C. acetobutylicum* DSM 792 can produce solvents at a dilution rate of only 0.2 h^{-1} or less. In other words, *C. saccharoperbutylacetonicum* DSM 2152 is capable of producing more solvent at a higher dilution rate. However, when the dilution rate was <0.1 h^{-1}, *C. saccharoperbutylacetonicum* DSM 2152 produced solvents significantly less than that by *C. acetobutylicum* DSM 792, probably because *C. saccharoperbutylacetonicum* DSM 2152 produced too little amount of acids and cannot provide sufficient acids to form acetone at a very low dilution rate [25].

Related to clostridia, the major benefit of developing butanol-producing strains from nonsolvent-producing species by synthetic biology is the elimination of major by-products acetone and ethanol, and *S. cerevisiae and E. coli* are the two well-established platforms for this purpose [22, 26]. In addition, other organisms such as *B. subtilis*, *C. tyrobutyricum*, *Pseudomonas putida*, and *Synechococcus elongatus* are also under development [12]. Although the advancement in the heterologous expression of the butanol pathway in nonsolvent-producing species is exciting, a deep understanding of the fine control of the heterologous expression of genes and enzymes is still needed to develop for more competent butanol production, making this strategy economically competitive compared to the native pathway in clostridia as well as the chemical synthesis.

2.1.2 Genome Diversity of Butanol Fermentative Microorganisms

All solvent-producing *Clostridium* have *sol* operon. The *sol* operon of *C. acetobutylicum* consists of *aad*, *ctfA*, and *ctfB* genes. *aad* gene encodes aldehyde alcohol

dehydrogenase; *ctfA* and *ctfB* genes encode two subunits of CoA transferase (CoAT); *adc* gene is located in the downstream of the *sol* operon and encodes acetoacetyl decarboxylase. The *sol* operon of the other three species consists of *ald* or *bld*, *ctfA*, *ctfB*, and *adc* genes. *bld* and *ald* are expressed as the butanol dehydrogenase gene and aldehyde dehydrogenase gene, respectively [27, 28].

The solvent-producing gene of *C. acetobutylicum* is located in the plasmid. This plasmid may degrade the fermentation performance during long-term fermentation, when there are only acids without solvents in the fermentation broth. Since the spore-generating gene is also located in this plasmid, maintaining solvents yield using freshly germinated culture for fermentation inoculation is the best way. Solvent-producing gene of *C. beijerinkii* is located in chromosome, and this strain deteriorates more obviously, possibly because of other reasons. Kosaka et al. [27] pointed out that the relationships between the malfunction of the transcript factors of *sol* operon and *C. beijerinkii* N1-4 strain deterioration. Some type of substance extracted from the supernatant of wild-type N1-4 culture can make degenerated N1-4 strain to produce solvents again.

2.2 The Mechanisms of Microbial Acetone–Butanol Fermentation

2.2.1 Metabolic Pathway of Microbial Butanol Fermentation

The process of fermentation essentially depends on the level of metabolic activities of the organism. Insights of the metabolic pathway and the metabolic network analysis are the key steps for metabolic engineering to modify desired organism, leading to better productivity with better fermenting strain.

The metabolic pathway of various clostridia (*C. acetobutylicum*, *C. beijerinckii*, and *C. pasteurianum*) is very similar. The broadly studied *Clostridium* species (*C. acetobutylicum)* produces the following three major classes of end products during fermentation: (a) solvents (acetone, butanol, and ethanol), (b) organic acids (acetic acid, butyric acid, and lactic acid), (c) gases (carbon dioxide and hydrogen). The biosynthesis of acetone, butanol, and ethanol shares the same metabolic pathway as that of glucose to acetyl-CoA, but branches into different pathways thereafter (Figure 5.1 http://link.springer.com/article/10.1007/s10295-009-0609-9/fulltext.html – Fig1).

Bacteria grow exponentially in the first phase of fermentation (acidogenesis phase) along the formation of acids (mostly acetate and butyrate), decreasing the pH to approximately 4.5 [30]. In this phase, acetyl-CoA is converted to butyryl-CoA by catalysis with four enzymes, sulfur kinases, 3-hydroxy butyryl-CoA dehydrogenase, crotonic enzyme, and butyryl-CoA dehydrogenase. Acetyl-CoA, acetoacetyl-CoA, and butyryl-CoA are the key intermediates in the metabolic branch pathway. During

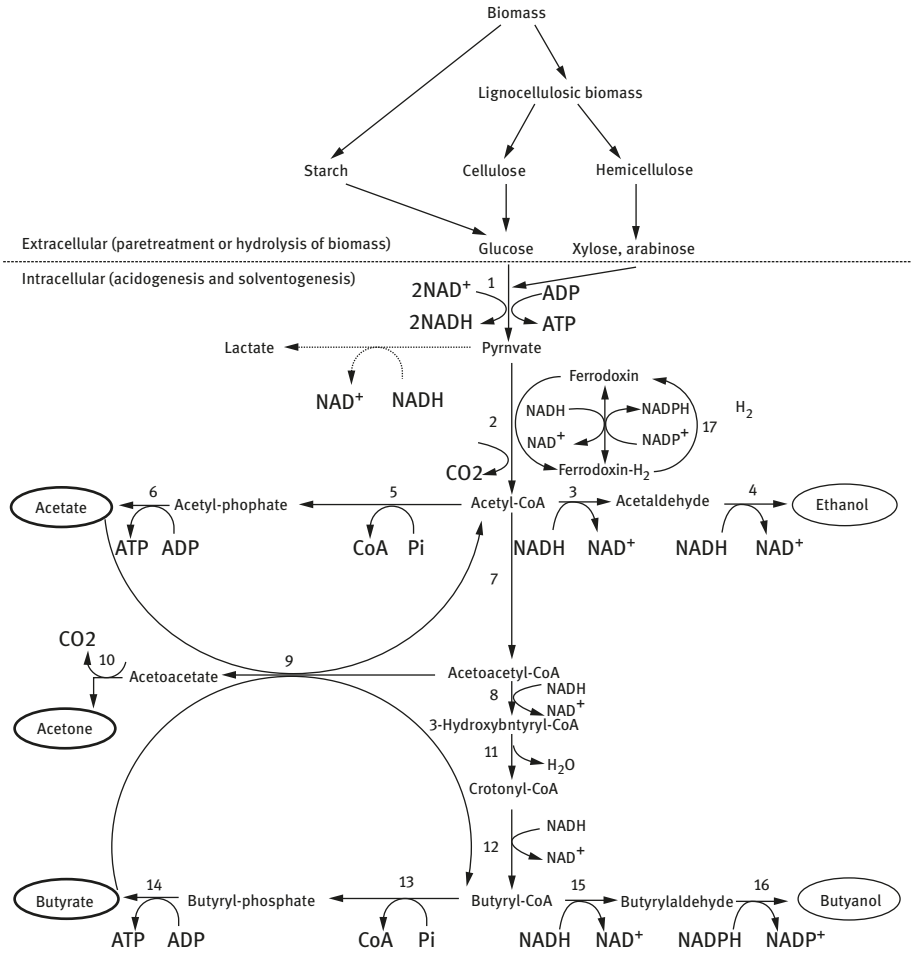

Figure 5.1: Metabolic pathways of *C. acetobutylicum* for acetone–butanol fermentation (adapted from Ref. [29]).

Enzymes are indicated by numbers as follows: (1) enzymes including in the glycolysis process; (2) pyruvate–ferredoxin oxidoreductase; (3) acetaldehyde dehydrogenase; (4) ethanol dehydrogenase; (5) phosphate acetyltransferase (phosphotransacetylase); (6) acetate kinase; (7) thiolase (acetyl-CoA acetyltransferase); (8) 3-hydroxybutyryl-CoA dehydrogenase; (9) acetoacetyl-CoA: acetate/butyrate:CoA-transferase; (10) acetoacetate decarboxylase; (11) crotonase; (12) butyryl-CoA dehydrogenase; (13) phosphate butyltransferase (phosphotransbutyrylase); (14) butyrate kinase; (15) butyraldehyde dehydrogenase; (16) butanol dehydrogenase; (17) hydrogenase

the acidogenic stage, acetic acid and butyric acid are generated through two-step similar reactions from acetyl-CoA and butyryl-CoA, respectively. The formation of acetic acid is catalyzed by phosphate acetyltransferase and acetate kinase, whereas the formation of butyric acid is catalyzed by phosphoric acid butyryl transferase and

butyrate kinase. Acetic acid and butyric acid formation pathways are both important energy production paths. During the stage of the production of solvents, ethanol and butanol are also generated through two-step similar reactions from acetyl-CoA and butyryl-CoA. The formation of ethanol is catalyzed by aldehyde dehydrogenase and alcohol dehydrogenase, while the formation of butanol is catalyzed by butyraldehyde dehydrogenase and butanol dehydrogenases [11, 29]. The amounts of ATP in the production of acetic acid and butyric acid are not the same. Total 4 mol ATP is produced when 1 mol glucose is fully fermented to acetic acid, carbon dioxide, and hydrogen, while only 3 mol ATP can be obtained when 1 mol glucose is fully fermented to butyric acid, carbon dioxide, and hydrogen [31]. Although the formation of acetic acid can produce more energy, this process does not consume NADH produced in glycolysis. To carry out glycolysis continuously, Nicotinamide adenine dinucleotide (NADH) must be oxidized to NAD^+, while the formation of butyric acid can consume most of NADH. Another way for NADH oxidation is that ferredoxin oxidoreductase catalyzes the electrons transferred from NADH to ferredoxin, and then reduced ferredoxin is catalyzed by dehydrogenase to produce hydrogen, indicating that the cells do not have to use additional carbon compound as the final electron acceptor, because the hydrogenase can utilize protons as a final electron acceptor; therefore, it is possible to produce more ATP. The reduced ferredoxin plays an important role in the electronic distribution as it can transfer electrons to produce hydrogen by hydrogenase and can also transfer electrons to the pyrimidine nucleotides by ferredoxin–$NAD(P)^+$ reductase.

At the final stage of acidogenesis phase, acid production slows down because of the low pH, thus organism shifts its metabolic activity from the acidogenesis phase to the solventogenesis phase at the end of the exponential growth phase. In this phase, acetate and butyrate are consumed as the substrates for the biosynthesis of acetone and butanol, respectively, while no growth is observed [30]. The fermentation process changing from acidogenic to solvent-producing metabolism is associated with the changes in the ratio of hydrogen and carbon dioxide. During the acidogenic stage, hydrogenase has high activity, and NADH–ferredoxin oxidoreductase catalyzes most electrons transferred from NADH to ferredoxin to produce more hydrogen, suggesting that the majority of NADH generated in glycolysis is oxidized through the hydrogen-producing pathway. Thus, most electron flux at the acidogenic stage is directed to hydrogen-producing pathway, and the carbon metabolic flux mainly flows to the acidogenic pathway to produce lots of energy. During the solvent production stage, hydrogenase activity decreases, and most of the electrons are transferred from reduced ferredoxin to $NAD(P)^+$ to generate NAD(P)H [32]. The decrease in hydrogen yield indicates that most of the electrons flux and carbon metabolic flux are consumed through the solvent generation pathway, thereby reducing the hydrogenase activity is one way to increase the yield of alcohol.

NADH and nicotinamide adenine dinucleotide phosphate (NADPH) can be excited to produce fluorescence by 340 nm light, while the reduced-state NAD^+ and $NADP^+$ do not emit fluorescence. The intensity of fluorescence is proportional to the concentration

of intracellular NADH and NADPH; therefore, the concentrations of intracellular NADH and NADPH can be obtained by measuring their fluorescence intensity. A large flux of NAD(P)H has to be needed to increase the butanol production, but it is uncertain to be achieved by high NADPH concentration or high circulation rate of NAD(P)H/NAD(P)$^+$. NADH is oxidized to NAD$^+$ by the oxidized-state ferredoxin, while the oxidized-state ferredoxin is reduced; this reaction is reversible. After the cells reach to the solvent production phase, when the forward reaction dominates, the cellular alcohol concentration and NADH concentration change synchronously, whereas when the backward reaction dominates, the NADH concentration must be kept low for the reaction to proceed. However, solvents production requires a lot of NADH; therefore, the circulation rate of NADH has to be accelerated.

Thiolase catalyzes the condensation of the two molecules of acetyl-CoA to acetoacetyl-CoA, and this is a critical reaction at the metabolism branch path. At the acidogenic stage, thiolase and phosphate acetyltransferase compete for available acetyl-CoA, and these two enzymes catalyzing acetyl-CoA drive the metabolic flux to butyric acid and acetic acid pathway, respectively. The amount of ATP produced during the acetic acid formation from per mole of acetyl-CoA is twice as the amounts of ATP during the butyric acid production. Thus, ATP productivity is related to the thiolase activity. At the solvent production stage, thiolase and butyraldehyde dehydrogenase compete for acetyl-CoA, affecting the ratios of acetone, butanol, and ethanol generated. Thiolase has a wide range of optimal pH (5.5–7.9), while intracellular pH in the batch fermentation is generally in the range 5.6–6.2; therefore, in general, the pH does not affect the thiolase activity. Butyryl-CoA accumulation has a feedback regulation on the condensation reaction. CoASH, the reduced state of CoA, strongly inhibits the thiolase activity. The ratio of CoASH and acetyl-CoA has a significant effect on the proportion of acetic acid and butyric acid and also determines the proportion of generated C3 and C4 compounds (acetone, butanol, and butyric acid) and C2 compounds (ethanol and acetic acid). When a genetically engineered bacterium is adopted, the overexpression of thiolase gene can reduce the concentration of acetic acid and ethanol. Hexose produces pyruvate through glycolysis Embden-Meyerhof-Parnas pathway (EMP). About 1 mol hexose produces 2 mol pyruvate, 2 mol ATP, and 2 mol NADH through the EMP (Embden-Meyerhof-Parnas pathway). Pentose can also be utilized by solvent-producing *Clostridium*. Through the pentose phosphate pathway, pentose is sequentially converted to fructose-6-phosphate and glyceraldehyde-3-phosphate catalyzed by transketolase and transaldolase, respectively, passing into the EMP pathway. Pyruvate is cleaved into acetyl-CoA catalyzed by pyruvate–ferredoxin oxidoreductase in the presence of CoA (coenzyme A), together with producing CO_2.

The economy of butanol fermentation industry can be significantly improved by controlling the electron flux and carbon flux to flow to solvent generation pathways to increase solvent (especially butanol) conversion rate. The control of electron flux and metabolic flux can be realized by bacteria engineering, optimization of fermentation process, and so on.

2.2.2 Metabolic Type of Butanol Fermentation

Typical batch fermentation can be divided into two stages: acidogenic stage and solvent production stage. In the initial stage of fermentation, a large amount of organic acids (acetic acid, butyric acid, etc.) as well as CO_2 and H_2 are generated, and the pH decreases rapidly. When the acidity of the fermentation reaction reaches a certain value, the fermentation enters into the solvent production stage. At this stage, the amount of organic acids is decreased, increasing the production of solvents such as acetone, butanol, and ethanol, as well as some CO_2 and H_2. The beginning of the solvent production at the solvent production stage involves the transition from the acidogenic pathway to the solvent production pathway [12]. However, this transition mechanism is still controversial.

Early studies suggest that this transition is inextricably linked with the decreased pH and acid accumulation, but decreasing pH or acid accumulation is not a requirement to this transition. Terracciano and Kashket [33] deemed that the effect of pH on the fermentation is related to the nondissociated butyric acid concentration. When the concentration of nondissociated butyric acid was between 0.528 and 1.584 g/L, the cells began to produce solvents. When the pH was too high or the initial concentration of the substrate was too low, the concentration of nondissociated butyric acid could not reach the threshold, and the fermentation process could not produce solvents. Reducing the pH decreases acid dissociation, thereby is conducive to produce solvents. When the pH is maintained at a higher value, more butyric acid accumulation is needed to make the concentration of nondissociated butyric acid reaching the threshold. In other words, inducing solvent production is required for the concentration of nondissociated butyric acid to reach a threshold. Holt et al. [34] demonstrated that when the pH was maintained at 7.0, adding high concentrations of acetic acid and butyric acid rendered the fermentation broth, which was originally only producing organic acids, to produce solvents. When the pH was maintained at 5.0, adding acetic acid and butyric acid shortened the fermentation time from the beginning of inoculation to the onset of the solvent production.

Harris et al. [35] studied the mutant with an inactive *buk* gene encoding butyrate kinase. They found the mutant began to produce solvents in the early logarithmic phase, when the concentrations of cells and butyric acid are both low. This phenomenon was significantly different from that the typical fermentation solvents produced starting from the late logarithmic phase and was also different from the statement that nondissociated butyric acid-induced cells to produce solvents, because its concentration was less than 0.0352 g/L when the mutant started to generate solvents. Gottwald and Gottschalk [36] noted that most probably the precursors of butyric acid (butyryl-CoA or butyryl-phosphoric acid) instead of butyric acid-induced solvent production. At the late logarithmic phase, increased butyric acid concentration in the wild-type strain increased the butyryl-CoA and butyryl-phosphoric acid concentrations (product feedback regulation), thus inducing cells to produce

solvents. At the initial logarithmic phase, butyryl-phosphoric acid concentration was high, because mutant cells lack butyrate kinase. Zhao et al. [37] studied the variation in the butyryl-phosphate concentration during the fermentation process and found that the first peak of butyryl-phosphate appeared when the cells began to produce solvents. This phenomenon was attributed to the fact that high concentration of butyryl-phosphate enabled phosphorylation of the negative regulatory protein of solvent-producing genes, and the cells expressed solvent-producing genes to generate solvents.

The amounts of ATP generated from the production process of acetic acid and butyric acid are different. Total 1 mol glucose is fully fermented to acetic acid, carbon dioxide, and hydrogen, and can produce 4 mol ATP, while 1 mol glucose is fully fermented to butyric acid, carbon dioxide, and hydrogen, and can only produce 3 mol ATP [31]. Although the formation of acetic acid can produce more energy, this process does not consume the NADH produced in the glycolysis. In order to carry out glycolysis continuously, NADH must be oxidized to NAD^+, and the formation of butyric acid can consume most of NADH. Another pathway of NADH oxidation is that NADH–ferredoxin oxidoreductase catalyzes the electrons transferred from NADH to ferredoxin, and then the reduced ferredoxin reacts with dehydrogenase to produce hydrogen, indicating that the cells do not have to use an additional carbon compound as an ultimate electron acceptor, because hydrogenase is able to use protons as the final electron acceptor, thus producing more ATP. Reduced ferredoxin plays an important role in the electrons allocation, because it can produce hydrogen by hydrogenase transferring electrons and can also transfer electrons to the pyrimidine nucleotides under the catalysis of ferredoxin–$NAD(P)^+$ reductase.

2.2.3 Assimilation of Acids by Butanol Fermentation Microorganisms

Acetoacetylated-CoA:acetate/butyrate:CoAT is a key enzyme in the solvent formation and can catalyze the CoA transfer reaction of acetic acid or butyric acid. CoAT can use acetic acid or butyric acid as a receptor of CoA in the process of conversion of acetoacetyl-CoA to acetoacetate, while acetoacetate is decarboxylated to acetone. Acetic acid and butyric acid are converted to acetyl-CoA and butyryl-Co, respectively, under the catalysis of CoAT. CoAT has a broad specificity for carboxylic acids and can catalyze the transfer of CoA of C1–C7 carboxylic acids. CoAT has a high activity for formic acid, but no activity for benzoic acid and dicarboxylic acid. The cells can convert propionic acid to acetone by adding propionic acid to the normal batch fermentation.

Jones et al. [11] found that the activities of phosphate acetyltransferase, acetate kinase, phosphatase butyryl transferase, and butyrate kinase drop rapidly after the cells began to produce solvents, suggesting that the cellular uptake of acids was not realized by the reverse reaction of acid production. CoAT is a unique enzyme related

to the uptake of acetic acid and butyric acid. The reuse of acetic acid and butyric acid by CoAT is directly coupled to acetone formation. Sillers et al. [38] introduced *aad* gene to strain M5 lack of plasmid pSol1, enabling butanol yield to recover to the level obtained by the wild-type strain. Because strain M5 lacked a gene encoding CoAT, no acetone was produced. The cells cannot assimilate acetic acid and butyric acid, rendering higher concentration of acetic and butyric acids.

Meyer et al. [39] found that feeding carbon monoxide into a continuous fermentation process in which butyric acid was produced at a high concentration at steady state; the cells quickly assimilate butyric acid and produce butanol without acetone production, indicating CoAT is not involved in the butanol production process, and this observation is in contrast that cells can only assimilate butyric acid through CoAT. Under this condition, the cells were more likely to assimilate butyric acid by the reverse pathway of butyric acid formation. Hartmanis et al. [40] observed two peaks of butyrate kinase activity; the first peak appeared at the end of the logarithmic phase, and the second peak appeared at the mid-stationary phase. The intensity of the second peak was greater than that of the first peak. Wiesenborn et al. [41] noted that phosphate butyryl transferase was sensitive to pH for the formation of butyryl-phosphate. Phosphate butyryl transferase had no activity at pH <6.0; however, it was not sensitive to pH for the formation of butyryl-CoA. Therefore, it was not elusive that phosphate butyryl transferase had any effect on butyric acid assimilation at low extracellular pH. Nevertheless, so far there is no clear evidence indicating that cells can assimilate butyric acid through the reverse pathway of butyric acid formation under the catalysis of phosphate butyryl transferase-butyrate kinase.

Assimilation of butyric acid by CoAT is more favorable than that by phosphate butyryl transferase-butyrate kinase from the perspective of energy. It is unnecessary to consume ATP to transfer the energy in the thioester bond to acids in the catalysis reaction involving CoAT. The chemical equilibrium constant of this reaction is 18–46, and the Gibbs free energy is in the range from –7.1 to –9.6 kJ, indicating that this reaction is not favored thermodynamically, requiring the next-step reaction to drive the reaction forward. The reaction exerting this role is the decarboxylation of acetoacetic acid. The equilibrium constant and the Gibbs free energy of this decarboxylation reaction are 4×10^4 and –26.2 kJ, respectively [31]. Hartmanis et al. [40] studied the molar ratio of acetone production and absorbed acids and found that the average ratio was 0.97 with a minimum of 0.8 (this value was explained because of the underestimation of experimental acetone yields). In brief, the assimilation of acids by the cells under the catalysis of CoAT may not be the only reaction pathway, but the most important reaction pathway. The Michaelis constant (K_m) of the reaction involving acetic acid and butyric acid by CoAT is higher than the concentration of intracellular acetic acid and butyric acid, and therefore CoAT is more sensitive to the variation in the concentration of acetic acid and butyric acid. Higher K_m value indicates that the absorption of acids by the cells is a detoxification reaction. The CoAT in vitro experiments showed that the reaction rate of acetic acid was faster

than that of butyric acid, but the inhibition of butyryl-CoA, acetyl-CoA, and CoASH to acetate reaction was stronger than the butyric acid reaction, and the uptake of butyric acid by the cells may be catalyzed by other enzymes; therefore, the concentration of butyric acid declined more rapidly than acetic acid in the normal batch fermentation.

Fond et al. [42] studied the effect of adding organic acids in the fed-batch fermentation with restricted glucose concentration on the fermentation results, indicating that adding acetic acid alone to the fed-batch fermentation could increase the yield of the solvents, but the ratio of butanol and acetone did not change. Tang et al. [43] found that adding 1.5 g/L acetic acid could promote the cell growth and acetone synthesis, with the cell concentration >18.4%, and the final mass fraction of acetone increased by 21.05%, but did not promote the butanol synthesis, whereas adding 1.0 g/L butyric acid promoted the cell growth and butanol synthesis; with the cell concentration >22.29%, the final mass fraction of butanol increased by 24.32% compared to the control, but did not promote the acetone synthesis. Thus, when the glucose concentration is not limited, acetyl-CoA generated by the cellular uptake of acetate is oxidized to acetone instead of being reduced to butyric, indicating that the formation of acetone and butanol is not necessarily coupled.

2.2.4 Alternative Butanol Fermentation Metabolic Pathway

Lactic acid-producing metabolic pathway is an alternative metabolic pathway of inefficient capacity and NADH oxidation. The metabolic pathway may play a role when the hydrogenase activity is suppressed, rendering the cells to produce lactic acid. At neutral pH, when hydrogenase activity is suppressed by carbon monoxide, or the concentrations of ferredoxin and hydrogenase drop because of the lack of cellular iron, fermentation production will change from solvents to lactic acid. It seems that there is a bottleneck at the step involving pyruvate–ferredoxin oxidoreductase. At neutral pH, electrons flow more to the path of lactic acid formation. Only at pH >5, lactic acid could be produced, because lactate dehydrogenase is inactive at lower pH. Under acidic conditions, carbon monoxide could be used to suppress the hydrogenase activity to improve butanol ratio and yield.

Peguin et al. [44] pointed out that the excess formation of NADH by adding methyl viologen may cause cells to produce glycerol. Vasconcelos et al. studied the mechanism of glycerol fermentation by *Clostridium*. They indicated that glycerol could be oxidized to dihydroxyacetone by glycerol dehydrogenase, and then dihydroxyacetone was converted to dihydroxyacetone-phosphate by dihydroxyacetone kinase. Since dihydroxyacetone kinase is not a reversible enzyme, after the addition of methyl viologen, the $NADH/NAD^+$ increased, and glyceraldehyde-3-phosphate dehydrogenase activity would be inhibited under the high $NADH/NAD^+$ condition, accumulating the intermediates dihydroxyacetone-phosphate and fructose-1,6-diphosphate in the cells during the glycolysis process.

2.2.5 Increase in the Solvent Production by Controlling Metabolic Flux

Controlling cell metabolism to increase solvent production is primarily achieved by controlling the fluxes of electrons and carbon to traffic to the route of solvent production, which can increase the solvent yield and reduce the formation of by-products H_2, CO_2, acetic acid, butyric acid, and so on. A variety of chemical and physical measures can be taken to control the microbial metabolism in the fermentation process. According to the fermentation mechanism, reducing the hydrogenase activity is one way to increase the alcohol yield. The ways to reduce the hydrogenase activity include increasing hydrogen partial pressure, the use of carbon monoxide gas stripping, limitation of the iron concentration in the medium, and the addition of electron carriers such as viologen and neutral red.

The concentration of carbon monoxide and hydrogenase activity follows first-order reaction kinetics, indicating high inhibition of hydrogenase by carbon monoxide. When the carbon monoxide concentration in the vapor phase reaches 6%, both the hydrogen formation and cell growth are inhibited. Carbon monoxide changes the direction of electron flow and carbon flux by inhibiting the hydrogenase activity and deviating from the path of the production of hydrogen, carbon dioxide, acetic acid, and butyric acid to the path of the production of butanol and ethanol.

Hydrogen formation can be regulated by increasing the hydrogen partial pressure in the vapor phase during fermentation. Because the reduction potential of H^+/H_2 decreases at high hydrogen concentrations, the electron flow from the reduced ferredoxin to proton is inhibited and redirected to other electron acceptors.

Low concentration of viologen can significantly alter the metabolic process of fermentation. By adding viologen in the fermentation broth, the yields of hydrogen, butyric acid, and acetic acid rapidly decrease, whereas those of butanol and ethanol increase. After the addition of viologen, the color of fermentation broth changes (in the case of methyl viologen and benzyl viologen, the fermentation broth turns blue and purple, respectively), indicating the reduction of viologen by hydrogenase (oxidative viologen is colorless). The reduced ferredoxin competes with viologen for the hydrogenase active site, thereby inhibiting the oxidation of ferredoxin through hydrogenase. The pathway activating the transfer of electrons by the reduced ferredoxin to pyrimidine nucleotides through pyrimidine nucleotides–ferredoxin oxidoreductase is activated; however, this pathway is inhibited by NADH and can be carried out only when the formed NADH is continuously oxidized.

Because of different materials, strains, fermentation temperature, and pH, the chemical reactions are not necessarily the same, and the components and the proportion of reaction product will change significantly. For example, the ratios of fermentation product would be significantly different when using corn and molasses as the raw materials. Furthermore, the reaction product has a variety of low or high boiling point substances such as isopropanol, isoamylol, and esters in the practical industrial

manufacturing. Microbial species are different, resulting in different butanol ratios, indicating that the fermentation of acetone/butanol is an extremely complex biochemical reaction and still now has not been accurately described.

Section 3: The Fermentation Process of Acetone and Butanol

The process of fermentation of acetone and butanol generally includes three stages: fermentation prophase (acid-producing phase), main fermentation phase (acid-declining phase), and fermentation anaphase (balance phase). In this section, the change in the characteristics of the three stages, the main factors affecting the acetone and butanol fermentation process and their ratio, and the microbial breeding for butanol fermentation, and the toxicity of butanol to cells are described.

3.1 The Phase of Fermentation Process

The fermentation process is generally divided into three phases: fermentation prophase, main fermentation phase, and fermentation anaphase. According to the changes in the acidity curve, the fermentation process is also divided into acid-producing phase, acid-declining phase, and balance phase. As for the continuous fermentation (several tanks in a series), the fermentation process can be understood by taking the whole group, even including the parent tank, as a single-stage fermentation tank.

3.1.1 The Prophase of Fermentation (Acid-Producing Phase)

In this phase, the cell number of *C. acetobutylicum* increases sharply, up to 2–5 × 10^{10}/ml. It is also called the acid-producing phase, because the acid value increases rapidly from 0.5 to 5.0–6.0. The major fermentation products are acetic acid, butyric acid, hydrogen, and carbon dioxide. Approximately 30% of the substrate is consumed in this stage. The phase lasts for 13–18 h and begins at an inoculum concentration in the range 2–10%. The fading time of methylene blue gradually decreases. As the acidity increases, the pH decreases.

3.1.2 Main Fermentation Phase (Acid-Declining Phase)

In this process, butyric acid and acetic acid are reduced, and the acidity decreases to about half of the maximum acidity. The rate of the acidity decrease in this phase is

almost equal to the rate of acidity increase in the acid-producing phase. More butyric acid is reduced than acetic acid. In addition, in this phase, acetone and butanol accumulate, and the fermentation gas quantity is still increasing, reaching the maximum value. Because hydrogen participates in the production reaction activity of butanol, the proportion of carbon dioxide in the gas (V_{hydrogen}:V_{CO2} = 40:60) increases until the end of the fermentation. During the whole fermentation process, the most vigorous fermentation phenomenon, the strongest bacteria cell vitality, and the shortest time of methylene blue fading all occur in the main fermentation phase. That phase generally lasts for 18–30 h with a sharp decrease in the acidity as the main feature and is also the important phase of the biochemical reaction to produce solvent, because the reduction reactions of butyric acid and acetic acid into butanol and/or acetone are active.

3.1.3 The Anaphase of Fermentation (Balance Phase)

The anaphase of fermentation is also called balance phase or acidity recovery phase. During this phase, the acidity decreases to the minimum level (sometimes also rises slightly) and maintains at a certain pH level (2–3.5), until the end of the fermentation. Balance refers to the cell fission growth and cell death reaching the equilibrium state. Nonvolatile acids and volatile acids mainly accumulate, and the concentration of solvents only increases slightly. Most of the residual acids are acetic acid. The gas continues to produce until the end of fermentation; however, the total amount of gas gradually decreases. *C. acetobutylicum* begins to senesce, thus decreasing the cell vitality. Methylene blue fading time also increases. Because of matrix depletion and solvent toxicity, especially the accumulation of butanol, the cell starts to autolyze or generate spores, ending the fermentation. This last period of this phase is often called as the decline phase. Thus, the process can be divided into four phases. In the decline phase, the solvent is still constantly generated, especially butanol and acetone concentration increases obviously, while that of ethanol increases slightly, and this phase generally lasts for 36–72 h.

3.2 The Change Characteristics of the Fermentation Process

Many factors in fermentation are changing along with the changing fermentation process, such as oxidation of gas production, cell morphology, solvent and acid concentration, fermentation liquid reduction potential, and enzyme activity.

3.2.1 Gas Production Changes in Fermentation Process

Acetone–butanol fermentation process releases two types of gases, that is, hydrogen and carbon dioxide. The greater the amount of gases generated, the more exuberant and violent is the fermentation process. At the beginning and the end of fermentation, very trace amount or no gas is produced. For every 1 ton total solvent production, 1.7 tons of gas will be generated. Among them, the amount of carbon dioxide and hydrogen are 1,653.7 kg (842 m^3) and 46.3 kg (520 m^3), respectively. The weight ratio of carbon dioxide and hydrogen accounts for 97.3% and 2.7%, respectively. The volume ratio of carbon dioxide and hydrogen is 62% and 38%, respectively.

3.2.2 Morphological and Cytological Changes of Bacterial Strains in the Fermentation Process

Westhuizen et al. studied the morphological and cytological changes of *C. acetobutylicum* P262 strain in the fermentation with molasses as the substrate and found that a few cells connected together to elongated rod after being inoculated into the fermentation substrate, and this process only occasionally moved forward. After inoculation for 6 h, the chains connecting the cells were broken, and single cells with high motion activity were released, with the maximum growth rate. Approximately 18 h after inoculation, the majority of cells lost their activity, and thus the growth of the cells stopped. As for *C. acetobutylicum*, sugar, nondissociated butyric acid, and acetic acid are attracting agents, thus cells show a positive growth trend, while acetone, butanol, ethanol, and dissociated acetic acid and butyric acid are the inhibitors, showing a negative growth trend. The directional cell reactions can explain high activity cells having a strong production capacity of solvents. The positive trend refers to the cells to move toward a high concentration, while the negative trend refers to the cells moving toward low concentration. The trend of high activity of the cell reaction is strong, and they are able to move to the high concentration of sugar and leave the place of high concentration of solvent. In general, if used for inoculation of cells with high activity, just after inoculation, most cells become inactive, while approximately 2 h after inoculation, the activity can recover, and then the cells can move linearly with less rapid roll. Along with increasing fermentation process and solvent concentration, the tumbling frequency of cells increases gradually, and approximately after 10 h, only cell rolling can be observed. After 14 h, the proportion of active cells is significantly reduced, and the majority of cells exist in the form of fusiform body after 24 h.

The sporulated degree of strains will also be significantly affected by different medium components. Long et al. [45] designed a sporulated culture medium and observed the formation of spores in *C. acetobutylicum* P262 under an electron microscope. The accumulation of starch grains in the cells began at 1–2 h before

the pH dropped to the lowest. Starch grains can be found in more than 90% of the cells during the 2 h. After 30 h inoculation, more than 90% of the cells expand into Gram-positive fusiform body, and a few cells did not form a fusiform body and were Gram negative, and thus degraded. A layer of capsule or slime was observed outside some fusiform bodies. The cells form fusiform body after the start of endosporulation, and more than 90% of the cells will form endospores in the solventogenic stage. Many starch grains accumulated at the end of the solvent-producing period, when more than 80% substrates, 40–50% of the cell dry weight by the starch grain composition, was consumed. Only the cell growth inhibited, and there were still enough carbon sources, and *C. acetobutylicum* will start to accumulate starch grains.

The capsular polysaccharide from highly acetylated species can be stored as non-reductive compounds and can also be reused by cells. In addition, it is possible to form capsule in the growth and acid production phases and thus can be used for the storage of hydrocarbon capsule.

In general, batch fermentation, ending with the cells in the logarithmic growth phase, has great relationships to solvent, stops cell division, and the cells simultaneously produce solvent. However, the absorbance of the fermentation liquid will still take a few hours for the cells to stop dividing after the increase, and this may be because of the length or volume changes of the cells, cellular memory of the accumulation of material, or a change in the optical properties of cells. The relationship between the cell growth and inducing cells to produce a solvent is not clear in continuous cultivation. The dilution rate has a significant effect on whether cells can produce solvent.

At present, fusiform body is generally recognized as cigar shaped and triggers cell, and there is a close relationship between the solvent and the number of fusiform bodies. As the solvent yield is related to the fusiform body, not forming strains cannot form fusiform body to produce solvent. After the formation of the cells in the fusiform body, spore formation blocked mutants, unable to sporulate, but it can have the normal concentration of solvent. However, Tracy et al. [46] objected, and they thought that the fusiform body cells did not produce precursor solvent, but the main solvent. The chemostat and research culture turbidostats did not show any spore formation and can sift through continuous cultivation, and did not affect the ability of isolates to produce solvent.

3.2.3 The Changes in the Enzyme Activity in the Fermentation Process

In the acetyl phosphate acid production phase, the cells in the transferase, acetate kinase, and phosphoric acid during acyltransferase produced 2–6 times more solvent, rather than the cells with the butyrate kinase activity [11]. From acetyl-CoA to the first three pathways of the generation of dibutyryl-CoA (the enzyme thiolase, 3-hydroxybutyryl dehydrogenase, and crotonic acid enzyme), the cells in the solventogenic

phase have shown higher activity. Aldehyde dehydrogenase and butyl alcohol dehydrogenase only show activity in the solventogenic phase cells. Acetoacetyl-CoA:acetic acid/butyric acid:CoAT showed very high activity in the solventogenic phase cells, but almost no activity in the acidogenic phase cells. The activity of acetoacetate decarboxylase (AADC) in the solventogenic phase cells was approximately 40 times of that in the cells in the acid production phase. The activity of AADC suppressed in the growing cells, and the enzyme exhibited activity only when the cells stopped growing. These results indicate that the activity of most of the enzymes in the acidogenic and solventogenic phases changed significantly.

3.2.4 Changes in the ATP Production in Fermentation Process

The transmembrane pH gradient is necessary for the growth and metabolism of cells and is a result of proton transfer to maintain the ATP enzyme. The consumption of ATP enzyme generates a proton motive. In the end of the acid-producing phase, because of decreasing pH, cells must consume more energy to maintain intracellular pH above the threshold value. The accumulation of high concentration acid in the cells decreases glucose absorption, thus decreasing the yield of ATP. When acid production in the fermentation process transferred to produce solvent, the ATP yield of 3.3 mol decreased to the minimum value of 2.0 mol [31].

3.3 Main Factors Affecting Acetone and Butanol Fermentation Process

3.3.1 pH

pH is a key factor affecting the fermentation process, and it depends on the strains and culture conditions. Solventogenic clostridia can make the intracellular pH higher than that of the extracellular pH; and thus the transmembrane pH gradient is ΔpH. The transmembrane pH gradient by the proton transfer ATP enzyme is required to maintain this enzyme consumption, generating a proton motive. When *C. acetobutylicum* extracellular pH is relatively high, the transmembrane pH gradient is low, and the intracellular pH decreases with decreasing extracellular pH. When extracellular pH decreases significantly, transmembrane pH gradient increases to 0.9–1.5. When the *C. acetobutylicum* ATCC 824 extracellular pH is 6.5, the transmembrane pH gradient is approximately 0.2; when the extracellular pH is 4.5, the transmembrane pH gradient is approximately 1.5. The transmembrane pH gradient for extracellular pH in the range 5.5–6.5 is relatively stable, but the low extracellular pH significantly increased the transmembrane pH gradient; when the extracellular pH was in the range from 6.5 to 5.5, the intracellular pH

decreased from 6.7 to 6. The absolute value of pH decreased with decreasing extracellular transmembrane potential. When the extracellular pH is 6.5, the transmembrane potential is approximately –90 mV; at pH 4.5, the transmembrane potential is almost zero. The intracellular pH is maintained above a certain threshold and is necessary for the synthesis and function of some of the enzymes, because a lot of the enzymes are sensitive to pH. Gottwald and Gottschalk [36] pointed out that maintaining the intracellular pH can induce solvent production at pH >5.5. Nondissociated organic acid (e.g., acetic acid and butyric acid) can affect the function of cell membrane and the proton matrix into the cells. When the concentration of nondissociated organic acids is high enough, the pH gradient would destroy the cell membrane on both sides, thus completely inhibiting the cell metabolism. Nondissociated organic acids can be free to penetrate through the cell membrane and will accumulate in the cell when the transmembrane gradient is larger, thereby reducing the intracellular pH value.

3.3.2 Temperature

The fermentation temperature affects the yield of butanol and acetone, solvent ratio, intensity of production, etc. With increasing temperature, the yield of general butanol and acetone solvents decreases, but the total solvent ratio increased with increasing temperature, and the intensity of production decreases rapidly with decreasing temperature, because the cell growth lag phase varies with lower fermentation temperature.

3.3.3 Fermentation Substrate

3.3.3.1 Matrix Capacity of Buffer

In the fermentation medium with strong buffering capacity, concentration and carbon source could increase the resting cells and the final concentration of alcohol consumption. The buffer material can be maintained within a certain pH range, making butyric acid to exist as ion. The cells are less toxic than the dissociation of butyric acid by cells, and thus before the cells started to produce solvent, higher concentration of butyrate is accumulated.

3.3.3.2 The Nutritional Components of Matrix

Monot et al. [47] used the culture of *C. acetobutylicum* using synthetic medium and studied the effects of different nutritional components in the synthetic medium of *C. acetobutylicum* ATCC 824 on the growth and yield of the solvent. The results show that

the trace elements Fe, Mg, and K are necessary for the cell growth. When the matrix lacked Fe and Mg, the cell growth was limited, producing a small amount of solvent. When Mg was excessive, the cells can grow better, but the solvent production decreased. When Fe was excess, the solvent yield changed slightly. When the K concentration was zero, the fermentation process only produced acid. The solvent production increased with increasing K concentration, and at a certain K concentration, the solvent yield became constant. In the batch fermentation, the initial sugar concentration is too low, and no solvent can be produced in the fermentation. In the continuous fermentation, no solvent will be produced if the flow rate is too low. In order to induce cells to continuously produce solvent, enough sugar must be available. Limited nitrogen source will also have a significant effect on producing solvent. When the concentration of nitrogen in the matrix cell volume decreases to a certain extent, the glucose consumption will be decreased. With further decrease in the nitrogen concentration, cells consume only a small amount of glucose, and thus no solvent is produced. In general, the use of peptone and yeast extract as the carbon source in the medium for fermentation can allow cells to grow well, but the yield of solvent is very low. When the inorganic nitrogen source is used instead of peptone, the solvent yield can be enhanced significantly. When the initial glucose concentration is lower than the threshold value, the solvent yield is proportional to the concentration of glucose; when the concentration of glucose is higher than the threshold, the solvent yield decreases.

3.3.4 Reduction Potential of Fermentation Liquid

Clostridial metabolism needs relatively low reduction potential, because of the conversion of pyruvate to acetyl-CoA ferredoxin, and ferredoxin is accepted at the low potential. Dehydrogenase, ferredoxin, and NADH/NADPH play an important role in determining the electron flow and carbon metabolic flux direction. In the fermentation stage, the main stream of electrons is transferred from NADH to ferredoxin, iron oxide reduction of protein, and the electron transfers proton to produce hydrogen. In the solventogenic phase, the electrons flow mainly from the NADH and NADPH to dibutyryl-CoA. The reduction potential of the fermentation liquid can reduce the flow of electrons to flow more NADH and NADPH, increasing the production of solvent. At lower redox potentials, the yield and substrate solvent consumption will increase, the butyric acid concentration will decrease, while the concentration of acetic acid is not related to the redox potential. In industrial production, commonly used coeruleum methylenum fade situation represents the reduction ability of fermentation. Coeruleum methylenum oxidation–reduction potential is higher, and the reduction of the redox system can accept many reduced substances released from the oxidation of hydrogen, with blue to colorless in the reduced state. When coeruleum methylenum is added to the fermentation liquid, the reduced enzyme system of acetone and butanol

bacteria undergoes a series of reactions. Add 1 mL of coeruleum methylenum solution of 0.04 –0.05% into fermentation liquid (10 mL), and the reaction temperature is maintained at 38–40 °C. The blue color will be faded in the solution. At the same time, measure the time that has been consumed. The greater the reduction capacity and fading ability of the fermentation liquid, the shorter is the time. For example, the mother liquor faded in less than 10 min.

3.3.5 Effects of Agitation

When the stirring rate is less than the threshold, the metabolic rate and solvent cell production rate will increase with increasing stirring rate. When the stirring rate is greater than the threshold, the stirring speed can decrease the production rate with increasing solvent. When the stirring rate increases to a certain degree, the cells will be mechanically damaged.

3.3.6 Degradation of Bacterial Strains

Continuous passage culture will lead to bacterial degradation; however, degradation is not the inevitable result of continuous passage and is more complex; bacteria will degenerate depending on the culture conditions. The pH of the medium, acid-buffering capacity, age and status of strains, interval time of strains passage, and inoculation amount of strains are all related to the degradation of bacterial strains.

Adler et al. pointed out that the degradation and changes of colony form, colony center, and producing strains have a large number of spores of solvent, and after a few days culture, growth in various forms such as extending from the edge of the strain was observed. The most serious strains unable to produce large colonies of solvent are scattered with edge elongation. Both also make spores than the high-yield strains producing less solvent. Therefore, they proposed that the ability of *C. acetobutylicum* producing solvent depends on the morphology of the colony.

3.4 Butanol Toxicity to Cells

In general, for the wild-type strain, when the production rate of solvent in the fermentation process reaches 20 g/L, the cell metabolism will stop. Among the solvents produced in fermentation, butanol is the most toxic for cells. When the concentration of butanol is approximately 13 g/L, the cell will stop producing solvent. Higher acetone and ethanol concentrations could also inhibit the cell growth, and their optimum concentrations inhibiting the cell growth are 70 and 50–60 g/L, respectively.

Butyl alcohol and other long-chain fatty alcohol have a moderate effect on the cell membrane consisting of phospholipids, because of their hydrophobic characteristics. Short-chain alcohols such as ethanol can reduce the cell membrane fluidity, whereas butanol and other long-chain fatty alcohols can increase the cell membrane fluidity. The effect of soluble alcohols on the cell membrane and the cell membrane fluidity increases with increasing carbon chain. The low concentration of alcohol (<5 g/L) had no significant effect on the cell membrane fluidity, but high concentration of butanol (10 g/L) can increase the cell membrane fluidity from 20% to 30%, making the cell membrane unstable and thus affects the function of the cell membrane.

Fatty alcohol can inhibit the ATP enzyme and membrane-associated activity and can destroy the transmembrane pH gradient. In general, at 7 g/L butanol concentration, the transmembrane pH gradient decreases from 1.2 to <1, while at >10 g/L butanol concentration, the transmembrane pH gradient is totally destroyed. When the concentrations of butanol are 10.3 and 20.8 g/L, the activity of the ATP enzyme could be inhibited by 27% and 41%, respectively. In addition, alcohol can decrease the concentration of intracellular ATP and inhibit the cellular uptake of glucose. When the concentration of butanol is 7.4 g/L, the ability of cells to absorb glucose and analogs will decrease by 50%. Therefore, butanol may disrupt the energy transportation system associated with the cell membrane.

The toxicity of butanol to cells relates to the automatic degradation of strains. High concentrations of butanol might cause cells to release autolysin. Westhuizen et al. [48] studied the effects of butanol on the growth and stability of *C. acetobutylicum* P262 and autolysis-defective strains. When the alcohol concentration is in the range of 7–16 g/L, *C. acetobutylicum* P262 will degrade the fusiform body, but not affect the stability of the autolysis-defective strain. In addition, nutrition and physical autolysis-defective strain increase the concentration of butanol.

The increase in the ratio of butanol in the total solvent depends on the tolerance of butanol on the strains. Industrial alcohol tolerance strain is less than 13 g/L, determining the initial sugar concentration in the fermentation substrate as 6–6.5%, posing great obstacles in production and improving the strength of products, thus increasing the extraction cost. Improving tolerance on the solvent of the strain and prolonging the period of cell metabolic activity are crucial for enhancing the solvent yield.

3.5 Factors Affecting the Ratio of Butanol

3.5.1 Material Factors

Different carbon sources can affect strains, sporulation, the ratio of butanol in the total solvent, and the yield of the solvent. In general, if starch-bearing materials (such as corn, sweet potato, and potato) are used for fermentation, the ratio of

butanol, acetone, and ethanol in the total solvent will be 3:6:1. If molasses is used as the raw material, the ratio of acetone, butanol, and ethanol will be approximately (18–34):(76–80):(1–3).

Maddox et al. [49] found that the ratio of butanol:acetone was approximately 10:1, when whey filtrate was used as the raw material for fermentation. Bhal et al. [50] studied the factors increasing the ratio of butanol in the fermentation with whey filtrate and found that the butanol and acetone ratio is only 2:1 in the synthetic matrix containing glucose, lactose, or galactose as the fermentation substrate. Further investigation revealed that the concentration of iron significantly affects the ratio of butanol and acetone. The chemostat culture with restricted iron concentration produced limited amount of acetone. If the dilution rate is larger, butanol and acetone ratio can reach 6:1. Adjusting the pH value of the fermentation substrate will also affect the proportion of butanol and acetone. At pH >5, main fermentation produces lactic acid; at pH 4.4, the proportion of butanol and acetone can reach to 8:1, probably because AADC contains iron ions, and AADC was the last enzyme producing acetone on the pathway. When the concentration of iron ion was restricted, the AADC activity was affected, thus influencing the electron flow. In addition, whey filtrate and lactic acid fermentation can improve the acetone butanol ratio from 2:1 to 8:1. In the *C. acetobutylicum* cells, the electron acceptor in the lactic acid oxidation, which was likely to be able to participate in the formation of butanol and be more efficient than the NADH or NADPH donor, was not clear.

3.5.2 Strain Factors

Different strains have different conversion rates of carbon source, thus affecting the yield of butanol. For example, the transformation rate of general strains of sugar is only 30–35%. *C. beijerinkii* BA 101 produces twice as much butanol as the wild-type strain using the same amount of carbon source. The butanol solvent yield can reach 78%. Improved strains can significantly increase the butanol production, thus reducing the cost.

3.5.3 Other Factors

Chen et al. [51] pointed out that the ratio of carbon to nitrogen (C/N), fermentation temperature, age, additives, and process factors would affect the ratio of butanol. The C/N ratio (carbohydrate and egg mixture from the matter content ratio) had a significant effect on the composition of solvent. With increasing C/N ratio, butanol production increased and alcohol production decreased, whereas opposite results were obtained with decreasing C/N ratio, that is, butanol production decreased and acetone production increased. According to the experiment, adding starch in corn

powder increases the C/N ratio from 4.6 to 8, which in turn increases the butanol yield by 3%. In general, fermentation temperature increased the proportion of acetone, and at low temperature fermentation time prolonged, but the proportion of butanol only increased slightly. For inoculation, the parent strain should satisfy the following requirements: highest acid concentration, large amount of strain, fast fermentation, and low butanol/acetone ratio production. If the inoculated strain is at the middle or end of acid fermentation, the butanol fermentation would become slow or incomplete, while butanol/acetone ratio would increase.

Adding fermented waste liquid can slightly increase the ratio of butanol. Higher butanol ratio and lower ethanol ratio can be achieved in the continuous fermentation than that in the batch fermentation.

3.6 Microbial Strain Selection and Breeding for Butanol Fermentation

3.6.1 Microbial Strain Selection for Butanol Fermentation

Solventogenic clostridia can form spores and obligate anaerobes; however, their separation processes are relatively easy. Because of the heat resistance of spores, the heat treatment method can be used to eliminate the vegetative and weak spores. The soil growing with cereal crops (such as corn and wheat), fruits, and vegetables are all good raw materials for strain separation. The general steps for the separation of bacteria are as follows.

3.6.1.1 Enrichment Culture

An appropriate amount of sample was inoculated in the enrichment medium, heat shocked in the boiling water, then cooled to room temperature, and cultured under anaerobic conditions for a period of time. Normally, gas will release after 24 h of anaerobic fermentation. In general, bacteria-producing butanol can be judged by smelling or observing the mash cover. If there is a smell of butanol in the medium formed on the top cover of mash, further purification of this culture should be carried out.

3.6.1.2 Isolation and Purification

After the last step, the culture medium is inoculated onto the isolation and purification medium plate with anaerobic cultivation. The purity of individual colonies is examined

under microscope to obtain a single colony. If the individual colonies are impure, a single colony with inoculation ring colonies is picked and inoculated to another isolation and purification medium plate until pure individual colony with a single species is obtained.

3.6.1.3 Preliminary Screening

To screen out several strains with the capacity of producing large amount of solvents, flat filter should be administered in the fermentation medium, followed by anaerobic cultivation to the end of the fermentation of the total solvent content in the fermented liquid.

3.6.1.4 Second Screening

First of all, strains are screened to get a high yield of solvents at the beginning of the fermentation. Then, anaerobic culture is performed until the end of fermentation to determine the total production of solvent in the fermented liquid, and finally high-yielding strain is chosen.

3.6.1.5 Strain Preservation

A mixture of calcium carbonate with spores is commonly used in industry for the sterilization of sand soil. The laboratory commonly uses 4 °C sterilized distilled water to save the spore. The enrichment medium can be a natural culture medium (e.g., 5% of the corn mash), or synthetic or semisynthetic medium (e.g., in the basic culture medium adding 5% butanol can be enriched with a range of butyl alcohol-tolerant strains).

Because of separation factors such as different materials and conditions, butyl alcohol fermentation microorganisms isolated from the natural sources will be different. For example, higher fermentation temperature might get high-temperature-resistant strains, and anaerobic culture may lead to oxygen-resistant strains. Pei et al. [52] reported molasses butanol-producing strain isolated from the natural resources (e.g., cow dung, pool, and soil) and characterized. In addition, they enriched the corn culture medium at the beginning with 12 strains and isolated the maximum butanol-producing strains. With 8% molasses fermentation after screening, two strains of *Clostridium* could produce higher amount of butanol, 8.57 g/L and 8.45 g/L, respectively, with butanol ratio as high as 75%. After 16S rDNA gene sequence homology analysis appraisal, the two strains were both found to be bynum's *Clostridium* (*C. beijerinckii*). The activated strain (*C. acetobutylicum* ATCC 824) was inoculated into the corn starch soluble medium (CSSM), and butanol solution was added after culturing for 30 min to reach the final butanol concentration in CSSM of 5 g/L. Culturing was continued until the absorbance

was ≥0.8, and then the culture was transferred to the fresh medium containing a higher concentration of butanol. This process was transferred 12 times to obtain a high concentration of butanol-tolerant strains.

3.6.2 Microbial Mutation Breeding for Butanol Fermentation

The solvent yield isolated directly from the natural environment out of butanol fermenting microorganism is generally not too high and often require further breeding strains to obtain better performance fermentation strain. Mutation breeding, genetic engineering techniques, and other means are used in wild-type butanol fermentation microorganisms to improve their alcohol tolerance, amylase activity, and solvent production and volume ratio.

According to the characteristics of metabolic branch coupled pathway, at the end of the fermentation, metabolic byproducts accumulate yielding higher solvent production. Bromocresol purple plate count agar method with bromocresol purple as an indicator with a size of the transparent circle was used to determine the acid content in the final product. This indicator can serve as a selectable marker to pick lower acid-producing strains, thereby obtaining a high-yield strain. Colonies showing yellow transparent ring around the colonies were normal, and purple around the colony remains lower acid production variants. According to the experimental conditions, the presence of spores in the presence of starch granules will have greater than 90% probability, and thus can be isolated, and purified plates will be exposed to iodine vapor. The colonies containing spores will be dyed blue-black, and that not producing spores will not be dyed blue-black. Propylene alcohol, alcohol dehydrogenase, and alcohol dehydrogenation can thus produce toxic acrolein, while surviving dehydrogenase mutant strain was a mutant or completely missing. There are also very different depending on the use of propylene alcohol mutagenesis strains. Zhang et al. [19] screened several strains of Clostridium with solvent-producing ability from the soil, after several nitrosoguanidine (NTG) and ethyl methane sulfonate (EMS) mutagenesis, and screened several allyl alcohol-resistant strains of high-yielding butanol species, resulting in two strains with good stability, followed by 7% corn mash fermentation, the solvent yields 18 g/L, the proportion reached 70% butanol. Because of alcohol dehydrogenase and butanol dehydrogenase activity of alcohol dehydrogenase, the strain may produce ethanol, but strains with no butyraldehyde dehydrogenase activity do not produce butanol; or strains with no alcohol dehydrogenase activity do not produce alcohol.

Bromate, sodium bromide, and an acid concentration of smaller screening variants can also be used, because bromate and sodium bromide in an acidic environment can release toxic bromine, and thus the survival of variants producing less acid or acid production is very short after quickly converting into a solvent. NTG and EMS enabling

the bacteria acetobutylicum DNA base mutations are the most effective acetobutylicum strain mutagen. Annous and Blaschek [53] used NTG of *C. beijerinkii* NCIMB 805 resulting in higher amylase activity and high solvent strain *C. beijerinkii* BA 101.

3.6.3 Genetic Engineering Modification

Application of genetically engineered microbial fermentation method is an important butanol yielding method to improve especially the ratio of butanol. Usually, the exogenous gene is inserted by taking, overexpression, or removing some of the endogenous genes unrelated to improve the yield of butanol. Genomes of *C. acetobutylicum* ATCC 824 and *C. beijerinckii* NCIMB 8052 had been sequenced in 2001 and 2007, respectively; however, the exact mechanism of transcriptional regulation mechanism of each function of the gene is not very clear.

3.6.3.1 Genetic Background of Microbial Fermentation for Butanol

C. acetobutylicum ATCC 824 strain is one of the most studied strains in the history, with a 210 kb large plasmid (pSol1), containing ctfA, ctfB, adc, and aad genes. ctfA, ctfB, and CoAT genes encode two subunits; adc and aad genes encode acetoacetyl decarboxylase and aldehyde dehydrogenase, respectively. This plasmid loss can lead to strain degradation. The genes aad, ctfA, and ctfB constitute the operon sol. The position of gene adc is very close to the downstream of operon sol, but not a part of the sol operon. Gene adc has its own promoter, and the direction of transcription is opposite to that of the operon sol. Aldehyde/alcohol dehydrogenase has aldehyde dehydrogenase activity and alcohol dehydrogenase activity. The activity of butyraldehyde dehydrogenase is mainly derived from aldehyde/alcohol dehydrogenase. Aldehyde/alcohol dehydrogenase is a major enzyme involved in butanol formation. Genes bdhA and bdhB encode alcohol dehydrogenation isozyme BDH I and BDH II, with alcohol dehydrogenase and butanol dehydrogenase activity, respectively, mainly coenzyme NADH, but BDH I of butyraldehyde activity is 46-fold of the acetaldehyde activity, while BDH II with butyraldehyde activity exhibits two times more activity than the acetaldehyde activity. The role of alcohol dehydrogenase and aldehyde butanol dehydrogenation process isozymes in the formation of ethanol and butanol is still not very clear.

Green et al. [54] thought that these two isozymes may be involved in butanol dehydrogenation to produce ethanol, since gene aad-inactivated mutant produced 35% more ethanol than the wild-type strain. *C. acetobutylicum*-dependent NADH and NADPH are at least two alcohol dehydrogenases. NADH as a coenzyme with alcohol dehydrogenase at optimum pH 5.5 and NADPH as a coenzyme to alcohol dehydrogenase have higher activity under alkaline conditions. Palosaari and Rogers [55]

purified butyraldehyde dehydrogenase and found that the Michaelis constants of acetyl-CoA and butyrylcholinesterase-CoA are 0.055 and 0.045, respectively, whereas the butyrylcholinesterase-CoA relative cycle rate is 5.5 times greater than that of acetyl-CoA; therefore, the proportion of butanol and ethanol production is approximately 6:1. Fisher et al. [56] believed that the production of ethanol is mainly catalyzed by alcohol dehydrogenase with NADPH as the coenzyme. They also believed that the size of the protein is determined by adhE butyraldehyde dehydrogenase purified by Petersen et al., and alcohol dehydrogenase gene encodes a protein of almost the same size. Ding et al. purified aldehyde dehydrogenase for gene encoding, but did not find any evidence that alcohol aldehyde dehydrogenase can be divided into aldehydes and alcohols having dehydrogenase activity 2 protein. Nair and Papoutsakis [57] and Nair et al. [58] reported that sol operon transcriptional repressor is located near the aad gene upstream open reading frame encoding and named it as SolR protein. The C-terminal of the SolR protein has a sequence capable of binding with DNA, indicating that it might regulate gene transcription. The solR gene overexpression can inactivate strain to produce solvents. After the solR gene inactivation, the butanol and acetone production increased.

3.6.3.2 Gene Transformation to Improve the Yield and Proportion of Butanol

In general, industrial fermentation generated butanol, acetone, and ethanol in a ratio of 6:3:1. Since the economic value of butanol and acetone is higher than that of ethanol, it is desirable to reduce the carbon source to form acetic acid and ethanol so that more carbon sources can be used to produce butanol and acetone. Mermelstein et al. [59] reported that adc, ctfA, and ctfB genes consist of re-adc gene operon unified control of ace operon structure, recombinant plasmid pFNK6, and transformed *C. acetobutylicum* ATCC 824 strains, respectively. Compared to the corresponding wild strain, the activity of CoAT and AADC increased from 4 to 33 times and 4 to 38 times, respectively, and the yield of acetone, butanol, and ethanol increased by 95%, 37%, and 90%, respectively; the expression time of the two enzymes is stable and advances to the logarithmic growth in the early phase. Sillers et al. [38] found that the use of strong promoter ptb replaced aad (adhE1) gene promoter itself, resulting in the aad expression in the growth phase, in the place of butyryl-CoA phosphate acyltransferase competition with butyrylcholinesterase carbon flow, reducing the formation of acid and thus increasing the yield of butanol and ethanol.

Nair et al. [57] transferred the plasmid pCAAD containing gene adhE1 into *C. acetobutylicum* M5 to restore its ability to produce butanol, and acetone was not generated, resulting in higher concentration of acetic acid. Lee et al. [60] imported adhE gene in *C. acetobutylicum* M5 and ctfA and ctfB genes, and the proportion of butanol in the total solvent was 84%, but the butanol production did not increase. *C. acetobutylicum* M5 does not seem to be a good production strain,

as its transformation efficiency is not satisfactory. According to a review on the progresses in the genetic engineering modification of *Clostridium* genetics from previous studies, we believe that the research on the solvent tolerance of strains, high biomass fermentation, and nonspore-based issues should be addressed and advanced in the near future.

Section 4: Development in Process Engineering

ABE fermentation was the second largest industrial fermentation process after ethanol (fermentation using yeast) owing to the economic importance of acetone and butanol prior to the introduction of petrochemical solvents. Process engineering for butanol production contains the following main units: handling and pretreatment of feedstock, fermentation, end product recovery, and waste management. The fermentation process can be divided into batch fermentation, continuous fermentation, and fed-batch fermentation according to the feeding mode. Both batch and fed-batch fermentation are appropriate for butanol production at small scale. In general, batch fermentation has been widely practiced in the industry, and fed-batch fermentation has been performed mainly in laboratories. However, continuous fermentation is a good choice for butanol production at large scale owing to its high productivity and less labor-intensive and maintenance investment.

4.1 Batch Fermentation

The general steps of batch fermentation are as follows: the preparation of the fermentation substrate, the up-scale of the culture, and the process of fermentation.

4.1.1 Fermentation Substrate

4.1.1.1 Preparation and Optimization of Fermentation Substrate

According to the different strains and raw materials, the preparation of the fermentation substrate is not the same. Some bacteria can secrete highly active hydrolytic enzymes; raw materials can directly gelatinize fermentation and do not need hydrolysis. For example, when the amylase activity is high enough, the starch can be used as the raw material; corn with a high starch content can be directly fermented without hydrolysis.

Clostridium cannot break down the lignin cellulose, but not cellobiose, glucose, mannose, Arabia sugar, and xylose. Furthermore, the cellulose hydrolysis solution

usually contains substances such as sweet bean acid and ferulic acid, which can strongly inhibit the growth of the strains. Therefore, the lignocellulose raw materials should be hydrolyzed and detoxified before fermentation; however, it also depends on the use of bacteria and raw materials. Qureshi et al. [61] used synchronous hydrolysis fermentation method, simultaneously hydrolyzing and fermenting the raw materials, and achieving the total solvent yield of 11.93 g/L in the absence of nutrients, stirring, and gas.

In addition, because some raw materials are not sufficient to meet the needs of normal fermentation, it is necessary to add a number of essential nutrients. For example, when molasses is used as the raw material, adding additional organic or inorganic nitrogen and phosphate buffer solution is needed. In general, before the use of a new raw material as a substrate, the fermentation substrate including sugar concentration and initial pH value must be optimized in the laboratory. Gu et al. [62] found that *C. acetobutylicum* EA2018 had a superior butanol yield and production strength compared to the traditional strain using corn as the raw material; however, when cassava was used as the raw material, the butanol yield was just 60% compared to that using corn as the raw material. Therefore, optimizing the cassava substrate together with fermentation conditions is necessary. The starch content in cassava is higher than that of corn; however, the fat content and protein content are lower than that of corn. The reason of low butanol yield using cassava may be because of the lack of nitrogen source and fatty acid. Ammonium acetate can significantly increase the solvent production and shorten the fermentation time to approximately 12 h. Ammonium nitrate and acetate ions are necessary to improve the yield of solvent. Adding ammonium acetate in the cassava fermentation can enhance acid accumulation in the acidifying phase and acid assimilation in the solvent production phase. Furthermore, it can also improve the enzyme gene transcription level involved in the solvent production phase. Liu et al. [63] optimized the culture medium of *C. beijerinkii* when fermenting xylose to produce butanol by the response surface methodology. Butanol yield with respect to xylose, yeast extract, vitamin, and trace elements as factors was investigated, and the response surface analysis of these four factors and five levels (–2, –1, 0, +1, +2) were designed according to the central composite design principle. The experimental results suggest that the optimal culture medium for *C. beijerinkii* xylose fermentation was 22.6 g/L xylose, 1.38 g/L yeast extract, 5.55 mL/L vitamin, and 9.67 mL/L trace elements of trace elements; under these optimum conditions, the yield of butanol increased by 18.4%.

4.1.1.2 Butanol from the Cellulose

One of the important factors that restrict the development of butanol fermentation industry is the high cost of raw materials, and thus the production of acetone and butanol using cellulosic materials can be used to address the high cost of raw

materials. Lignocellulosic biomass includes agricultural waste and residue (straw, rice husk, bran, bagasse, etc.), forest (softwood and hardwood) and forestry processing waste, and grasses as the most abundant and least expensive renewable resources on earth. China is the largest agriculture-producing country, yielding approximately 7.4 million tons of crop straw every year; in addition, nearly 100 million tons of other agricultural processing residues including rice husk and bagasse are produced per year. The amount of fuelwood and forestry processing residues is also very large. The traditional cellulose ethanol production process can hydrolyze those resources (acid method or enzyme method) to glucose, xylose, arabinose, and galactose, which in turn can be used in ethanol fermentation relying on the yeast or genetically engineered bacterial strains. Hexose as a carbon source of ethanol fermentation based on *S. cerevisiae* has been widely used in industry; however, the industrial strains, which can be effectively used in pentose to ethanol fermentation or from hexose and pentose by cofermentation, are not well established. Therefore, 30% of the total sugar cannot be converted into ethanol fuel, resulting in low product yields and high cost. In addition, the fiber ethanol prices are relatively low, seriously restricting the industrial process of fiber fuel ethanol. Lignocellulosic biomass is the most prospective raw materials for liquid fuel production. Cellulose and hemicellulose can be converted into monosaccharides or cellobiose by hydrolysis, and further to a new generation of liquid fuel alcohol through the biochemical processes. This can effectively overcome the technical problems (the yeast cannot ferment pentose or coferment hexose and pentose for ethanol production) and economic losses, because of relatively low ethanol prices. China has many fiber ethanol production lines or demonstration plants. The technology and process platform of fiber ethanol pretreatment and glycosylation can be directly used in the production of cellulosic fuel. The fermentation technology and equipment can also be used for the fermentation of butanol, providing a solid technical support for the production of fiber.

Because of the key problems such as difficult degradation of cellulose, the excessive amount of hydrolytic enzymes, and the inhibition of the production of cellulose in the process of producing fuel ethanol, biobutanol and other bio-based materials are needed. In addition, further research on the key issues such as the pretreatment of the fiber material and the hydrolysis of cellulose is needed. At the same time, in order to effectively convert the fiber material into sugar, the metabolic regulation and control, development of low cost, high-efficiency fermentation strains, improving the microbial activity, and enzyme concentration should be investigated.

At present, the fermentation of agricultural wastes such as straw for butanol production has been extensively investigated. Ezeji et al. [64] studied the growth of *C. beijerinkii* BA101 and the inhibitory factors during the butanol fermentation using the hydrolyzate of straw and other agricultural residues. Notably, the growth of *C. beijerinkii* BA101 and solvent yield significantly decreased when 0.3 g/L coumaric acid and ferulic acid were used in the fermentation substrate. Furfural and methyl furfural can stimulate the growth of *C. beijerinkii* BA101 and solvent

yield. Qureshi et al. [61] produced a total of 25 g/L solvent yield. The production strength and productivity were 0.60 g/(L h) and 0.42, respectively, when wheat straw hydrolyzate was fermented by *C. beijerinkii* P260. *C. beijerinkii* P260 strain is proved to have a good ability for the hydrolysis of wheat straw.

Chen et al. [65] studied straw enzymatic hydrolyzate for acetone–butanol fermentation, and the enzyme solution treatment and solvent fermentation indicate that dry straw, corn, and molasses (containing 50% sucrose) can produce 0.103, 0.265, and 0.15 kg solvent per kilogram raw material, respectively. Therefore, 1 kg dry straw can be equal to 0.389 kg corn or 0. 687 kg molasses. Thus, the acetone–butanol fermentation of the rice straw hydrolyzate also has great potential for butanol production industry.

The application of genetically engineered bacteria has increased in the aspects of fiber butanol fermentation. Because the traditional *Clostridium* cannot be effective for the treatment of fiber-rich agricultural waste, the wood fiber raw material must be first pretreated before fermentation. If the strain can be used directly and efficiently, cellulose or xylanase will greatly simplify the fermentation process. There are 11 genes related to the degradation of fiber and 7 genes related to the degradation of xylanase. *C. acetobutylicum* ATCC 824 and *C. beijerinkii* NCIMB 8052 genomes have been sequenced; however, they do not have the ability to degrade microcrystalline cellulose or cellulose. Lopez-Contreras et al. cloned two sections of glycoside hydrolase gene celA (encoding cellobiohydrolase) and celd (encoding an endoglucanase) from fungus *Neocallimastix patriciarum* and transferred them to *C. beijerinkii*. The endoglucanase activity of the transformed strains significantly enhanced, and the ability of the transformation mass to the solvent also improved significantly.

4.1.2 The Expansion of Microbial Species

A large number of mature inoculums can shorten the growth in the lag phase, shorten the fermentation cycle, improve the utilization rate of equipment, and reduce the opportunity of the contaminated bacteria; therefore, the expansion of microbial species is generally necessary in industrial production. Similar culture medium should be used to ensure vigorous growth during the whole process. The good seed should have strong growth activity, short delay period, stable physiological state, strong adaptability, stable production capacity at appropriate seed concentration, and should be free from nonbacterial contamination. The quality of seed should be checked to ensure the proper range of the following parameters such as constitutes of the culture medium, including pH, the content of sugar, amino nitrogen, phosphate, mycelial morphology, concentration of mycelium, and medium appearance. The nature of the species (such as the stability of the bacteria), the number of spores in the bottle, the minimum amount of the seed culture medium, and the volume ratio and the production scale should be determined. All the parameters should be adjusted according the change in the technological conditions.

4.1.3 Fermentation Process

Generally, the batch fermentation process is divided into three stages. The first-stage duration is 13–17 h long. During this stage, acidity reaches the highest level, because a large amount of acetic acid and butyric acid are released, producing a large amount of gas resulting in rapid growth of *Clostridium*. In the second stage, acidity decreases quickly, because acetic acid and butyric acid are converted into acetone, butanol, and other solvents, and the gas production reaches its peak, but unlike the first stage, mainly carbon dioxide is formed. In the third stage, the production of acetone and butanol slows down, and the gas production decreases. The fermentation process generally takes 48–72 h.

The good/bad fermentation process can be determined by observing the changes in the physical properties, including the viscosity of the fermentation substrate, mash cover formation, and the amount of gas production in the laboratory. Mash cover comprises protein, cellulose, and mucus. It suspends on the top of the fermented liquid in the early fermentation and sinks to the bottom at the end of fermentation.

In addition to the substrate, the fermentation temperature is also important for the fermentation results. The optimal selection of these conditions mainly depends on the use of the species. In the laboratory, the optimized combination of the above conditions can be selected by the single factor test to select the optimum value of each factor, and then the orthogonal experiment is carried out to select the best process combination.

The retention of aseptic conditions is most important in the fermentation industry. In order to maintain the sterile condition of industrial equipment (the fermenting tank, pipeline, valve, condenser, heater, and other parts), the steam sterilization must be performed before use.

First, miscellaneous bacteria pollution is the most common lactic acid bacteria pollution. In the presence of some lactic acid bacteria pollution, the mash cover does not form at the top of the fermentation liquid, gas production rarely occurs, and most of the fermentation substrate is unfermented and settled to the bottom of fermenter. Lactic acid bacteria produces toxic lactic acid, which can inhibit the conversion of acid to the solvent by *Clostridium*, thus reducing the pH of the fermentation broth. In industrial fermentation, this phenomenon can sometimes be improved by adjusting pH to the normal level.

Bacillus volutans bacteria are seriously harmful and can generate 1–1.3% lactic acid and a small amount of acetic acid and butyric acid. Under microscopic examination of bacteria after methylene blue staining, two or three points or multipoint color deepening (and sometimes dark red designs) will appear. The cells are rod shaped with size ranging from 0.6–1 μm to 2–4 μm, either alone or chain, but not spores; heterochromosome of mycelium can be visible after staining. The light particle is the characterization of this bacterium. Their growth is much faster than acetone- and butanol-producing bacteria in the range 37–39 °C. The optimum temperature is

36 °C, the highest tolerable temperature is 40–46 °C, and they can be killed at 65 °C in 5 min. The harmful effect of lactic acid bacteria is most vicious, and once infected, the acidity curve abruptly increases. Unlike normal acetone and butanol fermentation, acidity continued to rise after the lactic acid bacterial infection, leading to rancidity and complete stop of the fermentation.

Lactobacillus mannitopoeus cells are rod shaped with a size of 0.35 μm × (4–7) μm. They can exist alone or in sizes more than 25 μm. The optimum temperature range for their growth is 32–37 °C, and the lowest temperature is 10–15 °C. The amount of lactic acid and alcohol production was 1.3% and 2.7%, respectively. If the fermentation is infected by this type of bacterium, the result is also very serious, and the yield of solvents drastically decreases or solvent production completely terminates.

Streptococcus lactis cells are spherical with a diameter in the range of 0.5–1 μm and are arranged in a chain. Their growth stops at temperatures >45 °C, and this complex bacterium often invades the fermentation tank or parent tank from the cooking mash system. Although the harm from this bacterium is less than that caused by *Bacillus volutans*, their presence in excess amount can lead to rancidity.

The second type of pollution is caused by other bacteria and yeast.

Staphylococcus bacteria are aerobic and have a strong proteolytic ability and consume low sugar. The solvent yield will be affected by a small amount of *Staphylococcus*. The acetone and butanol fermentation will be destroyed with serious *Streptococcus* infection. The well-known example of this type of bacteria is *Staphylococcus aureus*.

Coccus includes monococcus, diplococcus, and *Streptococcus*. The shape of *Coccus* is round or oval. The color is very deep after staining; however, a small amount of the bacteria cannot be detected because of the inhibited growth and do not cause serious harm. Nonetheless, they can pose threat to the production when present in excessive amount. These types of bacteria commensurate with acetone and butanol bacteria in the corn mash and turn the fermented mash color to red, as they produce red pigment. The bacteria are able to sporulate.

In addition to lactic acid bacteria, many other bacteria can also infect acetone and butanol fermentation. Those bacteria have varied shapes and generally exist as long rods. These bacterial strains can be distinguished using a microscope. In the production tank with bacteria, the fermentation time, the depth of the dye, and the shape of the cell ends (circle or flat) will change. For example, the color deepens at the two flat ends of large bacillus; color darkens at the two square ends of short *Bacillus*. Yeasts, molds, and filamentous bacteria may also infect the production of acetone and butyl alcohol industry; however, such infections are rare and thus threat to the production is rare too. The size of yeast is more than 10 times of the bacteria; therefore, it is very easy to identify (round or oval). Molds are sometimes found in the sample connection (round or oval). Filamentous bacillus is very thin and long, and thus the microscopic field should be moved to see the whole picture, and the harm is similar to that caused by short bacillus.

The third pollution known as the phage infection is the most serious pollution. The phage of *Clostridium* is widespread in soil, air, raw materials, and so on. If the

ferment tank is infected by phage, the infection will become apparent in 18 h; the fermentation in this period forms the mash cover, and part of this mash cover begins to sink, and almost stops the production of the gas. The pH can be adjusted in the range 6.9–7 using sodium hydroxide to separate the phage used to study. Phage infection is almost fatal to fermentation, and the only solution is to use the strains, which can resist the phage. Most of the fermentation plants will save many species in the face of phage infection, quickly test other strains of this phage resistance, and then inoculate the resistance species into the fermentation tank. The acidity of the sample, the microscopic observation, the turbidity, and other parameters can be used to determine the contamination.

4.2 Continuous Fermentation

Continuous fermentation refers to add fresh medium to the culture system at a certain flow rate (F, L/h) and flow out of the culture liquid at the same rate, to keep constant liquid volume (V) in the fermentation tank. In this type of fermentation, the microbial growth is nearly constant.

In the process of continuous culture, the environmental conditions, such as the concentration of nutrients, the concentration of the product, pH, and the cell concentration of microorganisms, and the growth rate can be maintained, and even can be adjusted according to the needs of microbial cells. Continuous culture eliminates the need for repeated discharge, cleaning, filling, and sterilization step, and avoids the lag phase, so the equipment utilization rate is high and the intensity of the production increases correspondingly.

Herbert et al. [66] studied the basic theory of continuous cultivation, and the specific contents are as follows:

$$\text{Specific growth rate: } \mu=\frac{1}{x}\frac{dx}{dt} \tag{5.1}$$

where x is the cell concentration at time t, g/L; μ is the specific growth rate, h^{-1};

$$\text{Monod equation: } \mu = \mu_m\left(\frac{S}{k_S + S}\right) \tag{5.2}$$

where S is the substrate concentration, g/L; μ_m is the maximum specific growth rate, h^{-1}; k_S is the saturation constant, and its value is equal to the growth rate of the substrate concentration, g/L.

The relationship between the rate of cell growth and substrate utilization is as follows:

$$\frac{dx}{dt} = -Y\frac{ds}{dt} \tag{5.3}$$

where Y is the weight of the cells that consume the substrate of the unit weight.

The cell concentration in the fermentation tank can be expressed as

Cell concentration change = growth − out

$$\frac{dx}{dt} = \mu x - Dx \tag{5.4}$$

where D is the dilution rate, h^{-1}.

The material balance of the substrate in the fermentation tank can be expressed as

Substrate concentration change = Input − output − consumption

$$\frac{ds}{dt} = dS_R - DS - \frac{\mu x}{Y} \tag{5.5}$$

where S_R is the substrate concentration of the substrate into the fermentation tank, g/L. Substituting eq. (5.2) into eq. (5.4) leads to the following expression:

$$\frac{dS}{dt} = x\left[\mu_m\left(\frac{S}{k_S + S}\right) - D\right] \tag{5.6}$$

Substituting eq. (5.2) into eq. (5.5) leads to eq. (5.7):

$$\frac{dS}{dt} = D\left(S_R - S\right) - \frac{\mu_m x}{Y}\left(\frac{S}{k_S + S}\right) \tag{5.7}$$

In the steady state, $dx/dt = 0$, $\mu = D$, $ds/dt = 0$; inserting these conditions into eqs. (5.6) and (5.7) led to the substrate concentration in the steady state:

$$S_{ste} = k_S\left(\frac{D}{\mu_m - D}\right) \tag{5.8}$$

$$x_{ste} = Y\left[S_R - k_S\left(\frac{D}{\mu_m - D}\right)\right] \tag{5.9}$$

Equations (5.8) and (5.9) indicate that the cell concentration and substrate concentration in the steady state are only determined by the value of S_R and D, because for the given strain and the fermentation substrate, μ_m, k_S, and Y are constants. A series of different stable states can be achieved by changing S_R and D.

The critical dilution rate can reach the maximum dilution rate of the steady state:

$$D_c = \mu_m\left(\frac{S_R}{k_S + S_R}\right) \tag{5.10}$$

because $S_R >> k_S$, $D_c = \mu_m$.

If $D > D_c$, $\frac{dx}{dt} < 0$, and the cell concentration in the tank decreases; this is called washout.

Productivity in the steady state can be represented by the following equation:

$$P = Dx_{ste} = DY\left[S_R - k_s\left(\frac{D}{k_s - D}\right)\right] \tag{5.11}$$

The diluted rate and cell concentration at the maximum productivity can be obtained by taking the derivative of eq. (5.9):

$$x_m = Y\left[\left(k_s + S_R\right) - \sqrt{k_s\left(k_s + S_R\right)}\right] \tag{5.12}$$

$$D_m = \mu_m\left(1 - \sqrt{\frac{k_s}{k_s + S_R}}\right) \tag{5.13}$$

Substrate utilization in the fermentation tank is as follows:

$$\frac{S_R - S_{ste}}{S_R} = \frac{S_R - k_s\left(\frac{D}{\mu_m - D}\right)}{S_R} \tag{5.14}$$

At $D = D_m$, the substrate utilization rate is

$$\frac{S_R - S_{ste}}{S_R} = \frac{\left(S_R/S_{ste} + 1\right) - \sqrt{S_R/k_s - 1}}{S_R/k_s -} \tag{5.15}$$

Equation (5.15) indicates that the utilization rate of the substrate is only related to S_R/k_S, and the higher the S_R, the higher the substrate utilization.

The derivation of the equations requires some additional conditions: the liquid in the tank is completely mixed; the relationship between the concentration of the substrate and the growth rate of the cell is in line with the Monod equation; and the cell yield is constant. In fact, it is not possible to meet the above conditions in the continuous fermentation process; therefore, there are some discrepancies between the theoretical and actual production, but the theoretical production amount does provide an important reference.

Because the substrate and product concentrations are difficult to accurately determine, the effect of environmental conditions on the metabolism of strains is difficult to predict in the batch fermentation. In continuous fermentation, the growth parameters can be varied independently for the growth and metabolism of cells.

In 1958, Dyr et al. pointed out to separate the cell growth and solvent production stages to maintain the normal cell growth. In the continuous fermentation, acid and solvent are produced simultaneously, such that the acid production and its reutilization and conversion to the solvent occur at the same time, that is, the cells in the acid production stage and the cells in the solvent production phase exist simultaneously in a single-stage continuous culture system; thus different stages will inhibit the growth of each other. In order to solve this problem, separating the cells in sthe acid production stage and gas production stage is necessary. The conditions in the first stage of the fermentation tank (dilution rate and temperature, etc.) should be suitable for the production of acids, and these bacteria should be mature enough for immediately starting solvent production. Therefore, the first fermentation tank or the first two fermentation tanks of the continuous fermentation process are commonly known as the activated tank, as the majority of the production of the acid occurs in the activated tank, but the cells in this tank should be mature enough to produce a certain amount of solvent in the solvent production stage. For conversion of organic acids to organic solvents, the bacteria should have enough nutrients.

Barbeau et al. [67] studied the conditions to stabilize the ability of strains to produce solvent and inhibit the degradation of strains in continuous fermentation. At sufficient amount of all the nutrient elements and a dilution rate of 0.035 h^{-1}, the production rate of gas, cells dry weight, solvent concentration, and acid concentration increased with time; the cell metabolism stopped at a concentration of 12 g/L and recovered when this value decreased to 5 g/L; however, the stable state could not be achieved. When the dilution rate was 0.16 h^{-1}, the gas production rate and cell dry weight decreased with time, and the steady state could be reached because the concentration of the acid increased with time and the concentration of the solvent was very low. The dilution rate of 0.035 h^{-1} in a continuous culture with limited concentration of ammonium salt provided good stability, although the change in the gas production rate will decrease with time, and the concentrations of solvents and acids would become stable in 28 days. At a dilution rate of 0.18 h^{-1}, the stable fermentation could be obtained in 14 days, but the solvent yield was lower. Thus, following factors could be responsible for the decrease in the solvent production in the continuous fermentation process: higher pH value, the larger dilution rate, and the lack of restrictive nutrient elements. Therefore, the fermentation conditions of the first stage of the multistage continuous fermentation are not the most suitable conditions for the production of acids, because the most suitable conditions for producing acid are most likely to lead to degradation. The acid production rate

increases with increasing dilution rate. At lower dilution rate, the formation rate of the solvent increases to a maximum, and then decreases rapidly. Under continuous culture conditions, the most favorable conditions for the generation of solvent are lower dilution rate, lower pH and phosphate or sulfate concentration, and higher glucose concentration. Cell cycle is an effective way to increase the concentration of bacteria and products in the continuous culture process. The dilution rate is higher than the maximum growth rate, increasing the reactor output. Another characteristic of this reactor is that the dilution rate is almost independent of the growth rate of the cell. Because of the increase in the critical value of the dilution rate due to the cell recycling operation, the concentration of cells in the steady state is not higher than that in the cycle function state [68]. Because of the increase in the biomass, the production strength, solvent concentration, and glucose consumption increase with the extreme value of dilution rate and shift the curve to the right. Thus increasing biomass can increase the production rate at a larger dilution rate. The cell cycle can increase the amount of biomass in the fermentation broth, thus improving the production strength of the solvent.

Automatic chemostat can adjust the input speed of the medium to keep the cell-growth-dependent parameters to maintain stability, and the dilution and the growth rates of the cells are also adjusted accordingly. For the automatic chemostat, the feedback parameters of the selective control including the concentration of the cell (turbidity), pH, and the concentration of the nutrient and the concentration of the product are quite extensive. The control parameter in the turbidostat is the concentration of the bacteria. The signal is transmitted from the sensor to the pump body when the cell concentration is higher than the specified level and the culture medium is automatically added. The concentration of the cells in the reactor is certain, and the dilution rate is adjusted to the steady state.

4.3 Fed-Batch Fermentation

Fed-batch fermentation is a special type of culture mode between the batch fermentation and continuous fermentation. In this operation mode, intermittent or continuous feeding of one or more restricted substrates, cultures are not discharged until the end of the fermentation. This mode can extend the microbial logarithmic growth phase and equilibrium phase duration, and increase the accumulation of biomass accumulation and metabolism of cells.

Compared to the conventional batch fermentation, the fed-batch fermentation can release the inhibition of substrate, product feedback inhibition, and the effect of glucose decomposition. Compared to the continuous fermentation, the fed-batch fermentation does not require strict aseptic conditions and does not have the problem of aging, variation, and pollution.

In simple fed-batch fermentation, the basic medium is added in a certain amount at the beginning of fermentation. The matrix volume is usually less than half of the volume of the fermentation tank. After a certain period of the fed-batch fermentation, the high concentration of substrate is added to the tank at a low flow rate, and the substrate concentration in the fermentation tank is less than that of the toxic concentration. The volume of the culture increases with time, and when the volume of the liquid is close to 75% of the volume of the fermentation tank, the fermentation liquid is discharged. The concentration of the substrate in the fermentation broth can be controlled in the fed-batch fermentation, thus improving the production efficiency. The filling rate of the nutrients is not necessarily constant. According to different purposes, it can be designed in many ways, such as periodic fill material, constant speed feed, linear feed, and index fill material. Constant speed feeding uses a certain speed continuous filling. Linear fill material, index fill material, and logarithmic supplementary material have linear, exponential, and logarithmic relationship to the feeding speed. The use of this type of variable speed feeding purpose is to supplement different needs of the fermentation for achieving the best effect.

The concentration of the substrate is controlled in the vicinity of the target value by adjusting the flow rate and the rate of microbial consumption. This is the key and the core problem to be solved in the fed-batch fermentation. Fermentation can be divided into two stages: growth stage (i.e., the cells growth process) and the production stage (i.e., higher concentration substrate is added to the fermentation tank at a predetermined speed).

Because of the toxic effect of butyl alcohol on the cell, the initial concentration of glucose in the fermentation substrate cannot be more than 50–60 g/L, if not using the in situ extraction technology. When the concentration of butanol reaches 10 g/L, it becomes toxic to the bacteria, and thus can inhibit the growth and fermentation of the strain and reduce the strength and the production efficiency of the fermentation. Adsorption, stripping gas, and pervaporation can be used to separate the fermentation products to reduce the blocking effect of the metabolites in the fermentation process and improve the production efficiency.

Ezeji et al. [69] obtained 23.6 g/L of total solvent yield from 60 g/L glucose by the gas extraction technology coupled to batch fermentation. The total solvent yield increased by 33%, and the fermentation time decreased from 60 to 39 h compared to that without the use of in situ batch fermentation. Thus, the production rate significantly improved. Gas stripping technology can reduce the time of fermentation, because the products inhibiting the cells decrease, leading to better cell growth, and the concentration of cells in the fermentation liquid increases, which in turn increases the production strength. In addition, the gas stripping technique can increase the yield of the solvent, because the gas extraction can induce the cells to completely convert acetic acid and butyric acid into the solvent. In general, when the glucose concentration is >100 g/L, the cell growth can inhibit, and when the

glucose concentration was in the range of 160–165 g/L, the growth of the cells was strongly inhibited. When Ezeji et al. [69] fermented 167 g/L of glucose by the gas extraction technology coupled to the batch fermentation, the lag phase of the cells increased to 67 h, but the intensity of production, which can reach 0.60 g/(L · h), did not decrease.

Roffler et al. [70] studied the in situ extraction coupled to the fed-batch fermentation and found that the intensity of production increased from 0.58 to 1.5 g/(L · h) using the in situ extraction coupling coupled to oleyl alcohol fed-batch fermentation. Using oleyl alcohol as the in situ extraction solvent increased the butanol production strength by 25%; however, the concentration of glucose did not exceed 100 g/L, because the high concentration of glucose inhibited cell metabolism. By the slow addition of high concentration of glucose, the concentration of glucose in the fermentation broth can be maintained under the concentration inhibiting the metabolism. In this way, the fed-batch fermentation can use higher concentrations of glucose, and the concentration of glucose can be increased to 500 g/L, thereby increasing the volume of fermentation wastewater by 3.5–5 times. The butanol yield of the in situ extraction coupled to batch fermentation process is approximately 0.18, and the average value of the butanol production rate of the in situ extraction coupled to the fed-batch fermentation process is 2.2; however, the yield of acetone and ethanol is similar to that in the batch fermentation. This shows that the metabolism of cells in the process of fermentation is directed to produce butanol. The increase in the butanol production rate is accompanied by a decrease in the yield of acetic acid and butyric acid. The yield of acetic acid in the fermentation process was much smaller than that in the batch fermentation.

4.4 Butanol Recovery and Purification

Despite the accomplishment in ABE fermentation, the expensive product removal from the dilute fermentation broth still hinders the industrial production of biobutanol. To alleviate butanol toxicity in the cells and improve the productivity of ABE fermentation systems, various technologies have shortened the fermentation time with continuous removal of butanol removal from the fermentation broth. The technologies so far used for in situ butanol removal are as follows: pervaporation [71], liquid–liquid extraction [72], gas stripping [73], vacuum fermentation [74], perstraction [75], and adsorption [76]. In general, all these recovering technologies are able to recover butanol continuously from the fermentation broth and could shorten the fermentation time. However, the main concern is that none of the technologies could purify butanol efficiently as a final product, even though some of them have high selectivity of butanol to water and low energy consumption. Some scholars claim that much lower butanol titer attained with ABE fermentation could make in situ butanol removal more economically competitive. Recently, Xue et al. [73] reported that two-stage gas

stripping process condensate contained more than 400 g/L butanol. However, until till now no industrial applications have been reported with ABE fermentation coupled to these in situ butanol removal technologies.

In industry, acetone, butanol, ethanol, and other byproducts are fractioned and recovered by distillation. Most novel separation and purification processes so far proposed are for beer and ethanol, and only a few studies have been carried out on butanol separation purification. When butanol is produced at industrial scale such as fuel ethanol, the multistage pressure columns can be combined with the butanol distillation system to recover the latent heat with vapor from those pressurized columns, which then can be used to heat the bottom products from the low-pressure columns to make the system economical. The significance of the improved butanol titer lies in its contribution to develop a high-gravity fermentation process, as achieved for ethanol fermentation by *S. cerevisiae*, through which energy consumption in medium sterilization and stillage treatment can be saved because of a significant reduction of the total mass flow for the whole process.

4.5 Synthesis of Long-Chain Alcohols

Isobutyl alcohol and 2-methyl-1-butanol are the isomers of *N*-butyl alcohol and *n*-amyl alcohol, respectively; they can be synthesized by chemical and biological methods and have a very wide range of usage in the chemical pharmaceuticals, solvents, flavors, organic synthesis, and other industries. Isobutyl alcohol, isoamyl alcohol, and 2-methyl-1-butanol fermentation usually exist in the form of a by-product (fuel oil) in the early fermentation mixture in the brewing industry. The protein in the raw material hydrolyzes into amino acids, which decomposes into fuel oil by the Ehrlich pathway catalyzed by transaminase, decarboxylase, and alcohol dehydrogenase secreted by yeast. The main component is isoamyl alcohol, together with amyl alcohol, isobutyl alcohol, propanol, isopropanol, hexanol, and heptanol; and the isoamyl alcohol content can reach more than 50% [77, 78]. The proportion of various alcohols in the fuel oil will have a significant effect on the wine flavor. More or less proportion of these alcohols will deteriorate the quality of the wine.

Isobutyl alcohol, isoamyl alcohol, and 2-methyl-1-butanol are important chemical raw materials, and they are not only used in organic synthesis but also have better combustion characteristics [79] as the fuel, compared to ethanol and butanol. In the past, there have been no microorganisms capable of synthesizing long-chain length alcohols from glucose. In 2008, the research team of James C. Liao at the University of California, Los Angeles, reported a method for synthesizing highly branched chain alcohols by nonfermentation. They used the common precursor, α-keto acid, of amino acid synthesis as the substrate, producing long-chain alcohol fuel (branched isobutanol, 3-methyl-butanol, and 2-methyl-1-butanol), with higher octane value than linear butanol catalyzed by α-keto acid decarboxylase and alcohol dehydrogenase [79].

The traditional way of fermentation can only produce 1-butyl alcohol. The research team using common precursor α-ketoisocaproate of the isoleucine synthesis as the substrate synthesized alcohols with five to eight carbons by reconstructing the metabolic pathway under metabolic regulation of 2-isopropyl malic acid synthase (LeuA), isopropyl malic acid isomerase complex (LeuC and LeuD), and 3-isopropyl malate dehydrogenase (LeuB) gene [80]. This study was based on the developed amino acid production technology coupled to the production of amino acids with α-keto acid removal via the carboxyl hydrogenation pathway to produce alcohol. The introduction of some of the original metabolic pathways in microorganisms may lead to metabolic disorders, and the accumulation of intermediate metabolites may be cytotoxic. In order to improve the yield of the target product, amino acid synthesis pathway of biological isoleucine, valine, leucine, and alanine can be transformed to fulfill the aim to synthesize alcohols and reduce the transformation of organisms within the original synthesis pathway (Figure 5.2). This innovative thinking has opened up a new exploration direction for the production of long-chain alcohol fuels.

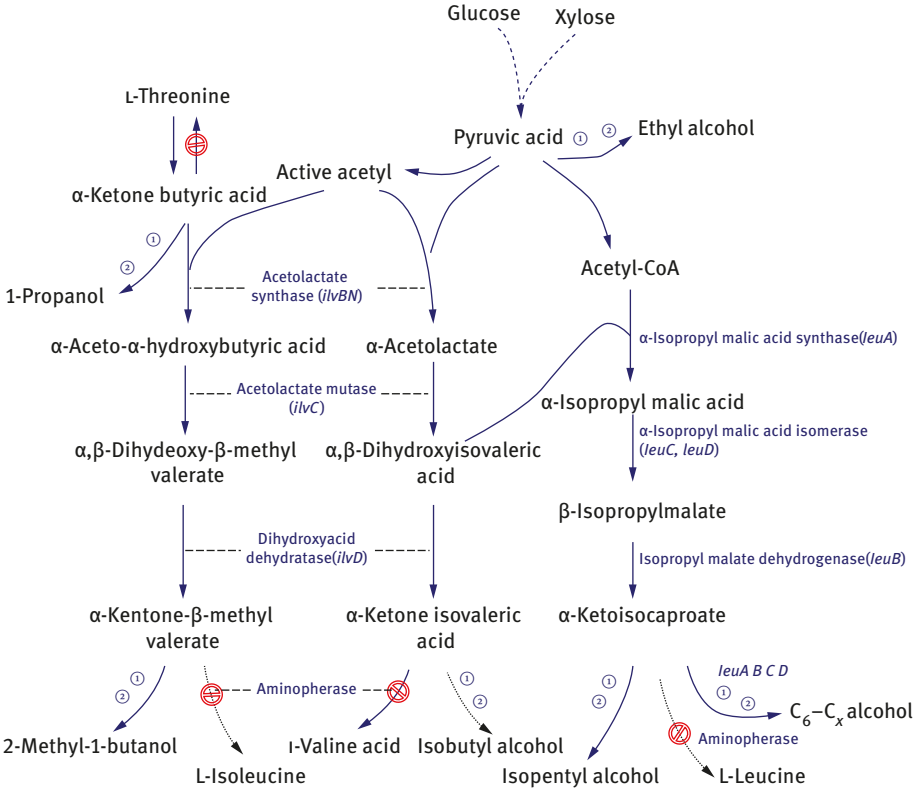

Figure 5.2: Biological long-chain alcohol synthesis pathway ①(α-ketoacid decarboxylase; ② aminopherase).

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Pengmei Lv, Wen Luo, Changhua Shang, Jin Xu, Changlin Miao, Junying Fu, Zhibing Li

6 Microbial Grease Production and Conversion

Grease (oil, lipid, or fat) is a mixture of triglycerides with different alkyl chains. Grease from different materials has different alkyl chains. As an important industrial raw material, the fatty acid structure of grease is very complicated. It has nonpolar carbon chains with appropriate length, and also has many chemically active hydroxyl groups and unsaturated bonds with unequal lengths, so it can be used to prepare different chemical products by pyrolysis, polymerization, isomerization, addition, transesterification, esterification, and hydrolysis.

Biodiesel, also known as fatty acid methyl ester, is one kind of new renewable energy source. It is a superior substitute for diesel fuel, and produced from grease, such as vegetable oils, animal fats, and recycled cooking oils. Biodiesel is typically produced through the reaction of an oil or fat with methanol or ethanol in the presence of a catalyst to yield glycerin and biodiesel. Chemical production of biodiesel involves complex processes, high energy consumption, environmental pollution, and other shortcomings, while lipase-catalyzed synthesis method can effectively avoid the above problems, so it has good prospects for application. However, the high cost and low stability of the lipase enzyme currently restrict the development of enzymatic production of biodiesel. To obtain lipase with high activity and stability is the key to achieve efficient catalytic conversion. Lipase enzyme is widely present in plants, animals, and microorganisms. There are many different types of microorganisms that breed quickly and are prone to variation. The microbial lipase, which is generally an extracellular enzyme, has broader active pH range, temperature range, and better substrate specificity than plant lipase and animal lipase. Therefore, microbial lipase is suitable for industrial production and it is an important source of industrial lipase and a research focus for biodiesel production.

The global biodiesel industry has developed rapidly. Currently, there is a massive demand for raw materials, disrupting the original balance between oil supply and demand. The inadequate supply of raw materials has become a constraint to the further development of biodiesel industry. Therefore, it is important to pay close attention to the development of oil resources and innovative technologies of oil production. Microbial oil, also known as single cell oil, is an ideal new oil resource. Microorganisms produce oil in vivo under certain conditions, using carbohydrates, hydrocarbons, and general oils as the carbon source. Oleaginous microorganisms include yeast, mold, bacteria, algae, and so on. Eukaryotic yeast, fungi, and algae can synthesize

Corresponding author: Pengmei Lv, E-mail: lvpm@ms.giec.ac.cn
Wen Luo, Changhua Shang, Jin Xu, Changlin Miao, Junying Fu, Zhibing Li, Guangzhou Institute of Energy Conversion, Chinese Academy of Sciences; CAS Key Laboratory of Renewable Energy; Guangdong Provincial Key Laboratory of New and Renewable Energy Research and Development, Guangzhou 510640, China

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triglycerides with a composition similar to that of vegetable oil, while prokaryotic bacteria can synthesize special lipids. This chapter mainly focuses on the introduction of microalgae capable of producing oil (hydrocarbon), lipase-producing microorganisms, oleaginous fungi, as well as oil extraction and energy conversion technology.

Section 1: Microalgal Oil and Hydrocarbon Production

Currently, the major sources of global energy are fossil fuels and a few biofuels which are made from animal and plant raw materials. In order to achieve the development goals of human sustainability, it is becoming essential to discover or develop new high energy-yielding as well as environment-friendly resources, without endangering food security.

Algae, as the most primitive form of life, can produce a variety of biomass through cell metabolism, such as proteins, polysaccharides, pigments, and lipids. The carbon-rich oil and hydrocarbons are good raw materials for the lipid biofuel production. Usually, microalgae includes unicellular or aquatic (or terrestrial, aerial, symbiosis) lower plants, which can perform photosynthesis. More than 200,000 kinds of microalgae exist on earth, which can fix 40% of CO_2 from the global net photosynthetic production every year [1]. Thus, microalgae play an important role in energy conversion and carbon cycle. The intracellular stored substances obtained by fixing carbon source through photosynthesis can form various bioenergy sources after biotransformation and processing. For example, some microalgae can accumulate large amounts of fatty acids during their growth and reproduction processes, while some microalgae will produce hydrocarbons in secondary metabolism process. There are many advantages of using microalgae to produce bioenergy. For example, microalgae are photosynthetic organisms with high solar energy conversion efficiency; there are many kinds of microalgae and diverse metabolites can be used to produce a variety of biomass energy; microalgae can breed fast, they have short culture period, and the yield per unit area is several times higher than that of plants; desert and wasteland can be used for production, and not compete with food lands; it also benefits the environment and does not increase the greenhouse gas carbon dioxide, keeping the balance of carbon [2]. Therefore, breeding high-productivity microalgae and exploring the mechanism related to biomass accumulation are of great significance for bioenergy development.

1.1 Microalgal Oil Production

1.1.1 The Classification of Oil-Producing Microalgae

In the early 1950s, many researches were conducted worldwide on unsaturated fatty acids production using microalgae. In order to develop sustainable algae fuels, the

Aquatic Species Program of US Department of Energy has done many works on algae collection, screening, and identification. Ultimately, about 300 oil-producing algae species were screened out, which were mostly green algae and diatoms. So far, the fatty acid contents of several hundred kinds of microalgae have been measured, including Bacillariophyceae, Rhodophyceae, Chrysophyceae, Phaeophyceae, Chlorophyceae, Dinophyceae, Cryptophyceae, and Xanthophyceae [3]. Generally, microalgae cells contain lipids, and the lipid content of different kind of species is different, even in different strains of the same species (Table 6.1) [2]. Recently, researchers are mainly focused on studying a few species of fast-growing algae, because these algae can accumulate large amounts of lipid substances under certain conditions. These microalgae include green algae *Chlamydomonas reinhardtii, Dunaliella salina*, and some Chlorella stains; diatoms *Phaeodactylum tricornutum, Thalassiosira pseudonana*, and so on.

1.1.2 Mechanism of Microalgal Lipid Synthesis

Compared to the higher plants, fatty acid and triacylglycerols (TAG) synthesis mechanism of algae is still relatively less understood. Although a certain degree of progress has been made in the research on lipid accumulation of algae, these metabolic chains remain unclear. Considering the similarity of algae and higher plants in functional homology to gene sequences and biochemical properties of some enzymes, fatty acid and TAG synthesis of algae is considered to be similar to that of higher plants.

Microalgae contain a wide variety of photosynthetic pigments and light-harvesting pigment-protein complexes, so they have a strong ability to photosynthesize. Light is converted into ATP and NADPH through microalgae optical systems PS I and

Table 6.1: Lipid contents of some microalgae species.

Microalgae	Lipid content (%Dry weight)
Botryococcus braunii	25–75
Chlorella sp.	28–32
Crypthecodinium cohnii	20
Cylindrotheca sp.	16–37
Dunaliella primolecta	23
Isochrysis sp.	25–33
Monallanthus salina	>20
Nannochloris sp.	20–35
Nannochloropsis sp.	31–68
Neochloris oleoabundans	35–54
Nitzschia sp.	45–47
Phaeodactylum tricornutum	20–30
Schizochytrium sp.	50–77
Tetraselmis sueica	15–23

PS II, and then ATP and NADPH join in the Calvin Cycle to fix CO_2. One molecule of glyceraldehyde-3-phosphate can be produced by fixing three molecules of CO_2. Glyceraldehyde-3-phosphate provides the raw materials for the synthesis of carbohydrates or other cytoplasmic substances via glycolysis, oxidative decarboxylation, and other types of reaction mechanisms.

Lipid synthesis consists of fatty acid synthesis and triacylglycerol synthesis. The first part is the biosynthesis of fatty acids (Figure 6.1) [4], and a key step in the process

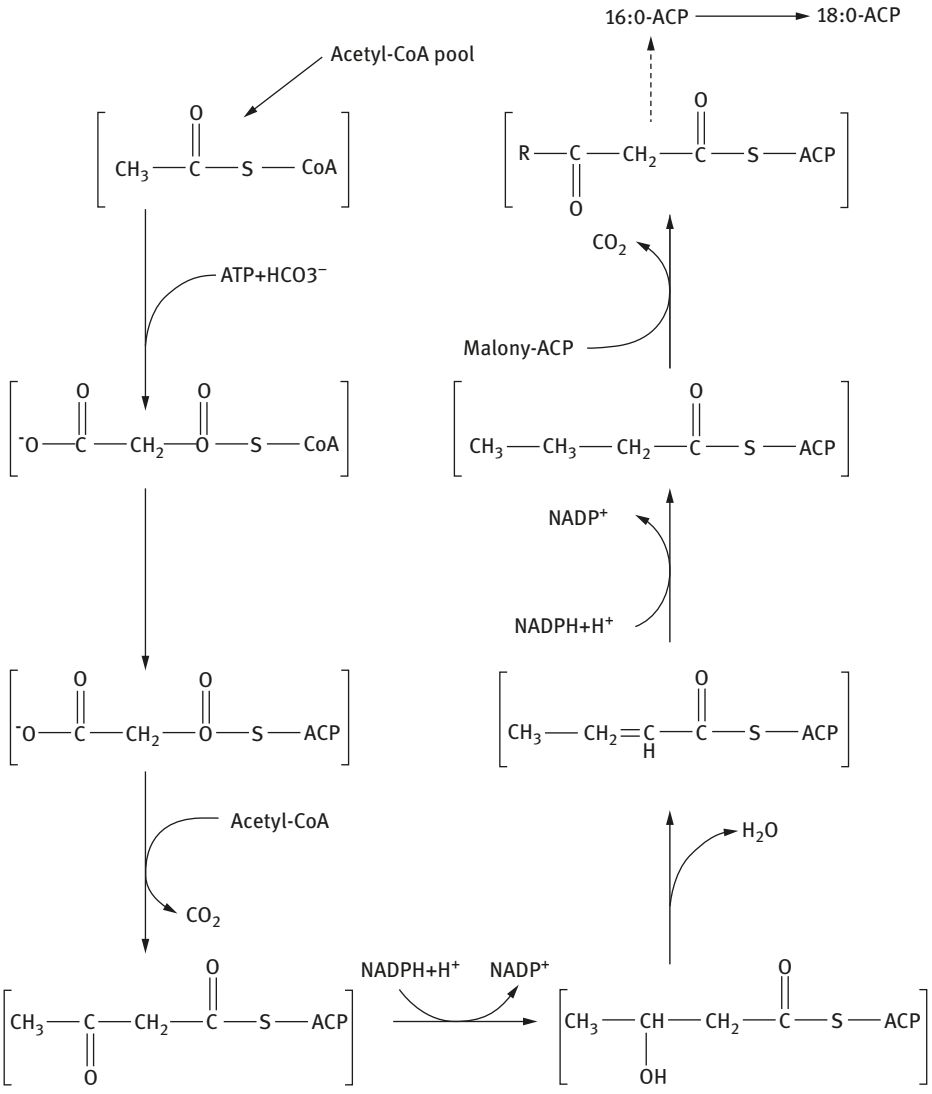

Figure 6.1: The biosynthesis of fatty acid.

is the conversion of acetyl-CoA to malonyl CoA catalyzed by acetyl CoA carboxylase (ACCase); acetyl-CoA as the substrate of Acetyl-CoA carboxylase into the reaction pathway (Reaction 1); acetyl-CoA as a substrate of the starting condensation reaction (reaction 3). Reaction 2: malonyl group is transferred to the acyl carrier protein ACP and forms malonyl-ACP catalyzed by malonyl CoA:ACP, malonyl-ACP is the carbon donor for the extended subsequent reaction; Acetoacetyl ACP restored (reaction 4) by β-ketoacyl ACP reductase, dehydrated (reaction 5) by β-hydroxybutyrate acyl ACP dehydratase, and restored (reaction 6) by enoyl ACP reductase to lengthen two carbon atoms to form saturated 16: 0-ACP and 18: 0-ACP through circular reaction.

The extension of fatty acid was terminated when fatty acids were converted from acyl ACP to 3-phosphate glycerin or 3-phosphate single acyl glycerin by acyltransferase in chloroplasts or free fatty acids were released in acyl ACP hydrolysis through acyl ACP thioesterase. Compared to cyanobacteria, eukaryotic algae usually contain more unsaturated fatty acids. Long-chain polyunsaturated fatty acid is the main component in many algae. Therefore, the initial syntheses of saturated fatty acids need to go through a complex process, to form multi-functional extended unsaturated fatty acids. The second step is the synthesis of triacylglycerols (Figure 6.2) [5]. Fatty acids are converted from acyl group to the first and second location of 3-phosphate glycerin to form central metabolite phosphatidic acid (PA). PA forms diacylglycerol (DAG) through dephosphorylation catalyzed by the specific phosphatase. The third acyl group was transferred to vacancy of DAG to form TAG under catalytic action of diester acyl glycerol acyltransferase.

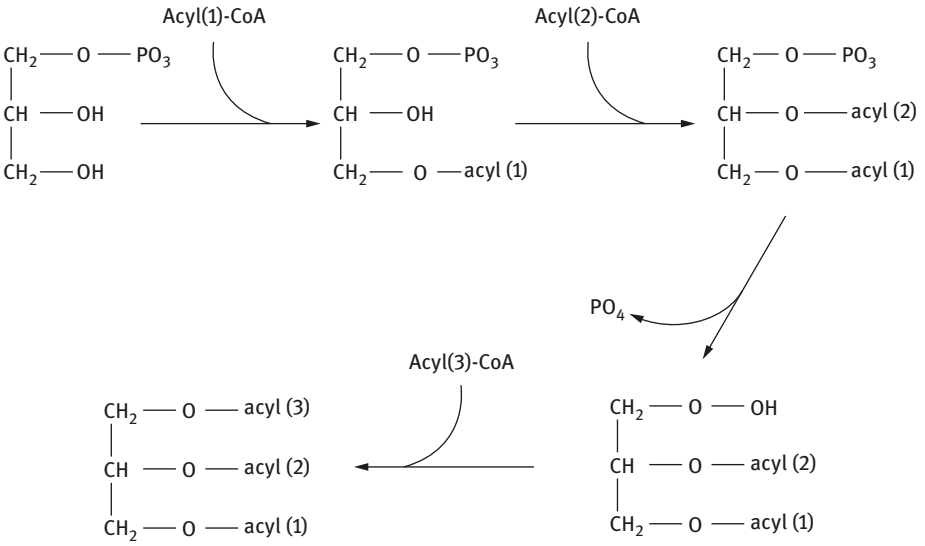

Figure 6.2: The biosynthesis of triglyceride.
(1). Three glycerol phosphate acyltransferase; (2). Lysophosphatidic acid acyltransferase; (3). Phosphatidic acid phosphatase; (4). Diacylglycerol acyltransferase

1.1.3 Oil-producing Microalgae Breeding

Currently, a core issue of microalgae biofuels technology is the very high cost, which prevents its further industrial development. Therefore, cultivating high lipid content microalgae species has very important significance to reduce biodiesel production costs and to develop algae biodiesel.

1.1.3.1 Oil-producing Microalgae Screening

Microalgae are a class of algae with diameter less than 1 mm. The population is extremely large, and the total lipid content in microalgae (% of dry weight ratio) is between 1% and 70%, even up to 85% under certain conditions [6]. Therefore, it is important to select the proper method to measure the lipid content of microalgae. It has been reported that the lipid content of microalgae cells can be determined by the following three methods: weighting method, Nile red staining, and chromatography.

(1) Weighting method
Weighting method is a classic method for the determination of microalgae lipid content. Firstly, lipid is extracted by organic solvent from a large number of microalgae cells, and then directly weighed to calculate lipid content. This method not only needs a large amount of biological samples but also needs a long time for the extraction process. Long times and high energy consumption make this method unsuitable for large-scale use.

(2) Nile red staining
In order to develop quick and simple screening methods, Dr. Keith Cooksey proposed the use of lipophilic oxazine fluorescent dye Nile red staining to screen cells. Nile red is a dye with strong fluorescent properties. It has a strong hydrophobicity and exhibits different fluorescence colors from golden yellow to red depending on the degree of hydrophobicity of the solvent. In water solvent, its fluorescence is completely quenched. In addition, the Nile red dye has photochemical stability. Its unique excitation wavelength range can eliminate some interference on the determination of biomolecules. Research shows that cell fluorescence intensity was significantly associated with intracellular lipid content after Nile red staining. Nile red can directly determinate neutral lipids of cells without extraction. Moreover, the small sample requirement makes this analysis method simple [7].

Since this method is simple, fast, and does not require much equipment, Nile red staining has become the common method for rapid screening of the oil microalgae. However, this method also has some shortcomings. For example, it is only ideal for microalgae with high lipid content. In addition, the method can only obtain the general situation of the total lipid content. It is difficult to get accurate results with this method for a more in-depth analysis of lipid composition.

(3) Chromatography

Since the early twentieth century, chromatography has become one of the most important scientific analysis methods, and it is widely used in many fields, such as petrochemicals, organic synthesis, physiology and biochemistry, medicine and health, and environmental protection. Chromatography combines the processes of separation and determination, thus reducing the difficulty of analyzing mixtures and shortening the analysis period. Compared with staining, this method is not suitable as a screening method for large numbers of samples as it is time-consuming and requires prolonged equipment operation.

1.1.3.2 Oil-Producing Microalgae Improvement

Different algae species have different growth rates, compositions, and contents. For practical applications, we must not only obtain high oil-producing algae species but also improve the quality of the oil. Therefore, it is necessary to make improvements to microalgae to meet the production needs for economic benefits.

Breeding, also known as breed improvement, is one kind of technology to obtain high yield and high quality species by improving genetic characteristics. Oil-producing microalgae improvement is based on the improved genetic characteristics of the microalgae, and requires comprehensive application of ecology, physiology, biochemistry and biological statistics, and other discipline knowledge to achieve high-yielding varieties of microalgae. Nowadays, physical, chemical, or biological methods are generally used to cause biological effects of nuclear chromosome breakage, deletion, nucleotide substitution, gene recombination, and so on and to produce mutant offspring. The main breeding techniques include traditional breeding techniques (selective breeding, cross-breeding) and modern biotechnology breeding technology.

(1) Selective breeding

Selective breeding, selection for short, is one kind of method for selecting good natural variability individuals from the existing germplasm groups to breed new varieties. Its essence is anthropogenic selection of individuals or groups which we need to expand in order to propagate. The breeding methods are easy to operate, low cost, and practical. It is the most basic and effective method of microalgae breeding.

(2) Cross breeding

Cross breeding is a method to breed a new species by combining the advantages of two or more different strains though mating or uniting, selecting, identifying, and breeding. Proper and possible hybridization parents and hybrid combinations should be selected before cross breeding according to the characteristics needed, because correct selection is the key to successful cross-breeding.

(3) Cell engineering applied to breeding

Microalgae cell engineering applied to breeding mainly involves the combination of algae protoplast culture, somatic cell fusion, and hybrid technologies to foster new species. Compared with other breeding methods, this method has the following characteristics: using protoplast fusion to breed heterokaryon hybrid and facilitate the one-time polygenic traits improvement; and overcoming the limitation of distant hybridization, not only between the same species but also between different species. Sexual hybridization only happens among nuclear genetic materials but not among the cytoplasm hybrid. Protoplast fusion can be used to breed cytoplasmic hybrids. Shen et al. used cell chemistry combination method to combine the rapid growth mixotrophic microalgae *Tetraselmis* sp. with autotrophic green microalgae *Pavlova viridis* which was enriched in EPA and DHA [8]. According to the different growth and fatty acid composition characteristics of two kinds of algal parents, a new type of micro algae was selected out. Random amplified polymorphism DNA (RAPD) technology was used to identify fusion and confirm that the fusion product is a new type of micro algae.

(4) Mutation breeding

Compared to cross breeding, mutation breeding requires shorter time and has a better possibility of succeeding. In 1998, Zhiping Wang irradiated different strains and shapes of *spirulina* filaments by γ- rays. The results showed that low doses can stimulate the growth of algae cells. There are significant differences between different strains and forms in the radiation-sensitive filaments. The mutant with larger cell size which could grow well at low temperature was obtained. Sivna et al. [9] mutated *Porphyridium cruentum* strains by MNNG and obtained herbicide DCMU resistant mutant. In 1997, Chuntao Yin et al. [10] treated *platensis* with MNNG, and obtained a mutant whose filaments had obvious morphological changes, and could withstand low temperature. Wang et al. [11], who also used MNNG and EMS for *Spirulina* and *Chaetoceros* chemical mutagenesis and clone screening, found that both morphological and physiological characteristics of microalgae cells changed after mutagenesis. In short, mutation breeding can be applied to screen excellent microalgae strains.

(5) Genetic engineering breeding

For improvement of oil-producing microalgae, the main idea is as follows: in order to accumulate lipid, the metabolism of microalgae must be controlled, which involves weakening or eliminating the self-regulatory mechanisms. Specifically, the goal should be to enhance the main metabolic product flow, cut or hinder branch metabolism, increase the synthesis of precursors, and prevent suppression of the final product. There are three main strategies to improve the lipid content by genetic engineering methods: overexpress key enzymes of lipid synthesis; overexpress related enzymes in lipid synthesis process; and suppress competition-related metabolic pathways. In 2009, Wang et al. [12] successfully achieved a significant increase in TAG content in

nonstarch mutant (sta6) of *Chlamydomonas reinhardtii* compared to the wild type. Dunahay et al. [13] found that *Cyclotella cryptica* overexpressed its acetyl coenzyme A carboxylase gene, which was the key enzyme gene in TAG biosynthesis. Although the activity of enzyme increased by two or three times, the lipid content did not show any significant changes. Therefore, it could meet the requirements of algae improvement to manipulate a single key gene under certain circumstance. But this method also had a secondary bottleneck problem that other steps in the pathway turned into the major rate-limiting steps, restricting the accumulation of metabolites. Therefore, researchers have suggested achieving large-scale lipid synthesis by the manipulation of transcription factors, where specific transcription factors can control and activate the combination of a variety of enzymes in the anabolic process of the target product in order to overcome the defects during transformation of a single key gene. Although algae research in this area is still in its infancy, researchers have found that many transcription factors can regulate the lipid metabolism in animals, plants, and other organisms. Manipulating the expression of transcription factors to regulate lipid metabolism may become a new direction in microalgae genetic improvement.

1.1.3.3 Metabolic Regulation of Oil-producing Microalgae

(1) The impact of nutrients on lipid content of microalgae

The nutrients in growth media, especially some essential nutrients, have a great impact on the lipid content of microalgae. It is generally believed that nitrogen deficiency can cause lipid synthesis to become more active. During the 1980s and 1990s, the US National Renewable Energy Laboratory found that lipid content of microalgae improved when the microalgae were cultured under nitrogen and silicon (for diatom) deficiency. Other researchers found that the lipid content of *Cholorella pyrenoidosa* increased in the case of nitrogen deficiency, and the contents of oil and DHA of *Crypthecodinium cohnii* also became higher. The possible reason is that when nitrogen is sufficient, the cell growth is normal and the proteins, nucleic acids, and lipids synthesis are all in a state of equilibrium; but when nitrogen is deficient, the synthesis of nitrogen compounds will be limited while lipids containing less nitrogen will continue to be synthesized. Therefore, the lipid content increases [14].

Phosphorus is an essential element for the formation of DNA, RNA, ATP, and cell membranes, which are closely related to cell growth and metabolism. There are many researches on the influence of phosphorus limitation on microalgae lipid content. The lipid content of *Phaeodactylum tricornutum, Chaetoceros* sp., and *Pavlova lutheri* increased under phosphorus limitation, while that of *green flagellate, Nannochloris* sp., and *Nannochloris* sp. decreased. In addition, as the polyunsaturated fatty acids (PUFAs) of microalgae are mainly in the form of polar fatty acids, the lipid content is strongly influenced by the phosphorus content in the environment. For example, when the concentration of phosphate is more than 0.5 g/L, *Phaeodactylum tricornutum* cannot grow.

When the phosphate concentration is in the range of 0.05–0.5 g/L, there is no significant influence on microalgae growth, but when the range is from 0.1 to 0.5 g/L, the highest level of eicosapentaenoic acid (EPA) is obtained.

Silicon is an essential nutrient element for diatom growth. In addition to being one of the structural components of cell walls, silicon is also involved in a variety of metabolic and growth processes such as synthesis of photosynthetic pigments, proteins, DNA, and cell division. Silicon deficiency can lead to more carbon being absorbed and used for lipid synthesis, and it also causes previously absorbed carbon to gradually shift from nonlipid compounds into lipids, thereby promoting the accumulation of lipids in microalgae [15].

Vitamins are not necessary for the microalgae growth, but they can be used as a secondary group of enzymes to improve the activity of synthetase, thereby promoting microalgae growth and improving PUFAs content. Trace elements in the nutrient solution can influence the content and composition of microalgae oils. Addition of a certain amount of Mg^{2+} can promote the growth of *Crypthecodinium cohnni* and can also improve DHA accumulation in microalgae. Iron also plays a very important role in the phytoplankton growth process and is the carrier of certain oxidoreductase algae cells and coenzyme components. Iron deficiency can affect various metabolic processes to inhibit the growth of algal cells. Addition of chelating $FeCl_3$ in the last growth phase increases the cell density, but does not increase the accumulation of lipid. Algae were centrifuged and inoculated in media containing various concentrations of Fe^{3+} in the late logarithmic phase, and it was found that the higher the concentration of Fe^{3+} in the medium, the higher the fatty acid content of algae.

(2) The effects of culture conditions on the microalgae lipid content

Microalgae culture conditions include salinity, CO_2 concentration, pH, temperature, light intensity, and so on. These factors can affect the metabolism of microalgae [16–18].

Microalgae can divide into halophilic microalgae and salt-tolerant microalgae based on its salt tolerance. Lipid composition of microalgae cells varies with the change in salinity of the environment. For example, when the concentration of NaCl increased from 0.4 mol/L to 4 mol/L, the contents of saturated fatty acids and monounsaturated fatty acids in *Dunaliella* increased, while polyunsaturated fatty acids content decreased.

Different CO_2 concentrations can also result in changes in the lipid content and composition of microalgae. When the CO_2 content of the air is 1%, the total lipid yield of microalgae increases. Increased CO_2 concentrations lead to reduced content of PUFAs in eukaryotic algal cells. Under the action of high concentrations of CO_2, lipid synthesis increases, but subsequent extension and desaturation are suppressed, resulting in increased proportion of saturated fatty acids.

There are few reports about the pH influence on the content and composition of microalgae oil. Previous studies showed that pH can affect several factors including the availability of CO_2 during photosynthesis, the efficiency of microalgae utilizing organic carbon source during respiration, ion absorption and utilization in microalgae cells, and metabolic product recycling and toxicity. The best pH value for growth of marine microalgae

is close to the normal pH value of water, about 8.0. Some algae can grow under extreme pH conditions. Different microalgae live in different optimum pH values, and deviations from the optimum pH will result in inhibition of the growth of algae and related metabolic activity. For *Chlorella* spp., alkaline pH caused the accumulation of TAG and reduced membrane lipid species; while for *Chlamydomonas* sp., low pH resulted in increase in cell membrane fatty acid saturation and reduced membrane lipid fluidity.

Temperature and light intensity also have some influence on the biochemical components of microalgae. The large-scale cultivation of microalgae outdoors is closely related to the sun's radiation, and the corresponding temperature and light intensity. In some algae, high temperature can increase the protein content while reducing the content of lipids and carbohydrates. The content of PUFAs in many microalgae increases at low temperatures, but at the same time, low temperature results in low microalgae biomass, affecting the total PUFAs.

The relationship between light intensity and microalgae fatty acids shows differences between species. High light intensity can increase the content of most of the saturated fatty acids. In *Nannochloropsis* sp., increasing light can lower unsaturation of fatty acids and cause decrease in content of EPA. In *Cladophora* spp., the total phospholipid content is reduced, but the content of nonpolar fatty acids is increased under high light intensity. Moreover, the content of the polar lipids is increased significantly in low-light conditions. In the study of diatom *Thalassiosira pseudonana*, it was found that light can greatly alter the cellular composition of fatty acids and lipids.

(3) The influence of growth stage and physiological status on lipid content of microalgae

Different growth stages and physiological status will also affect the lipid content and fatty acid composition. Many algae have high content of TAG during their relatively stable growth stages. For example, the content of TAGs in *Gymnodinium* sp. increased from 8% during the exponential growth phase to 30% during the stationary phase [19]. When cells grew to stationary phase, the proportion of saturated fatty acids and monounsaturated fatty acids increased, and polyunsaturated fatty acid ratio decreased. The aging or senescence of microalgae also affects lipid content and fatty acid composition. During cell aging of *Prochlorococcus*, husk *Nitzschia* and *Thalassiosira* spp., their cellular lipid content increased, but in *Phaeodactylum tricornutum*, cell aging or senescence hardly affected the total lipid content [20]. Analysis of the fatty acid composition of aging *P. tricornutum* and *Chaetoceros muelleri* indicated that the contents of saturated and monounsaturated fatty acids increased significantly while the levels of polyunsaturated fatty acids reduced [21].

(4) The effects of cultivating mode on microalgae lipid content

Microalgae cultivating modes can be divided into light autotrophic, mixotrophic, and heterotrophic. Different culture methods have a great impact on the content and composition of microalgae oil [22].

Light autotrophic microalgae reproduce through photosynthesis, and the main limiting factor is the light intensity and distribution of light. Artificial circular runway open ponds were formed earlier for large-scale cultivation, and this kind of equipment is still widely used in Japan, Indonesia, Taiwan, and other regions. Open pond building costs are relatively low, but the microalgae cell density is low, and the open ponds can be easily polluted and evaporated. Closed reactors can obtain higher biomass. In order to get plenty of light, the design of the reactor must have larger surface area, shorter optical path, and less dark area. The commonly used closed reactor types are tubular, plate, and column bioreactors.

Mixotrophic culture methods use light and CO_2 while using organic carbon as carbon source supplement. Researches have shown that so far nearly hundred kinds of microalgae can grow by mixotrophic culture method, including *cyanophyta*, *chlorophyta*, *bacillariophyta*, and so on. When different carbon sources are added in the medium for mixotrophic culture, the production of PUFAs is different. Heterotrophic microalgae can eliminate dependence on light and can be cultured in the absence of illumination in a common stirred tank reactor. The proportion of total lipid in dry mass of all heterotrophic microalgae strains was significantly higher than that of autotrophic microalgae.

1.2 Microalgal Hydrocarbon Production

1.2.1 The Classification of Hydrocarbon-Producing Microalgae

Some strains of microalgae can produce hydrocarbons, but their production capacity is quite different. Also, the content of hydrocarbons in the cells of microalgae is influenced seriously by the different culture conditions. At present, known strains of microalgae with high hydrocarbon production capacity include *Botryococcus braunii*, *Dunaliella Salina*, *Chlorella vulgaris*, *Anacystis montana*, *Dictyopteris acrostichoides* and *Nostoc muscorum*. Among these, the *Botryococcus* algae have the highest hydrocarbon production volume, are most researched, and are most likely to be used for industrial scale production. The ratio of hydrocarbons in dry weight for *Botryococcus* can reach 75% (the following discussion about microalgae producing hydrocarbons uses *Botryococcus* as the example). Maxwell in 1968 found that oils produced by *Botryococcus* were polyunsaturated hydrocarbons and their content can be more than 30% of dry weight of the algae. In the same year, the hydrocarbons detected from *Botryococcus* and *Anacystis* by GelpiE and so on were found to be mainly C17, C27, C29, and C31 which are highly unsaturated aliphatic hydrocarbons with odd carbon numbers that are similar to the carbon number distribution of hydrocarbons in higher plants [23]. These works enable the research of microalgae hydrocarbon production to enter a new stage. At first, the researchers tried to make

algae grow fast and produce more hydrocarbons to provide energy directly, but failed to achieve scale cultivation because of lack of understanding of the growth, physiology, metabolism, causes of blooms, and basic suitable conditions of microalgal hydrocarbon production.

1.2.2 The Mechanism of Microalgal Hydrocarbon Synthesis

Templier J et al. have cultured *Botryococcus* A that produced different fatty acids and inferred that nonisoprene-based long straight chain hydrocarbons were mainly formed by extended decarboxylation with oleic acid as a precursor [24]. The researches of Professor Miao & Wu [25] and Yamaguchi K et al. [26] also support the above conclusions. The synthesis of trans-olefin in strain Gb is based on elaidic acid as a precursor and its synthesis pathway is similar to that of cis-olefin. But the synthesis of "grape hydrocarbon" in strain B is complex and there is no direct evidence to link it to the synthesis pathway of straight-chain hydrocarbon mentioned above. According to the culture experiments with addition of mevalonic acid, it is speculated that "grape hydrocarbon" is synthesized by terpenic pathway. In strain L, the lycopadiene of nC40 is mainly synthesized by connecting isoprenoid hydrocarbons end to end, and a large amount of highly inert and antioxidant long-chain compound named PRB is found in this strain which comes from this hydrocarbon directly. Largeau et al. [27] used Raman spectroscopy and electron microscopy and found that the hydrocarbons mainly exist in the special closed area of cytoplasm and form a continuous and stable "spherical wall" in the extracellular space while the contents of other lipids are very low in these two areas. The species of hydrocarbons inside and outside the cell are the same, but their relative amounts are different: there are more short-chain hydrocarbons in closed area of cytoplasm while the extracellular hydrocarbons account for more than 95% of all hydrocarbons output, and even their spherical volume is larger than the cell itself. According to the researches of Berkaloff, Largeau, and others, it is suggested that the extracellular hydrocarbons may be formed outside the cell wall and the cellular silt-like compound, PRB, is synthesized by the oxidative polymerization of diene [28, 29].

1.2.3 Breeding of Microalgae for Producing Hydrocarbon

Botryococcus braunii is a kind of freshwater unicellular green algae which is found worldwide, and usually grows like grape bunches. Several or even hundreds of cells are linked to each other through irregular and rope-like transparent gelatinous filaments of different lengths (also named refraction filaments) to form different sizes of string-like colonies. These colonies can also form irregular or nearly spherical composite assembled body whose hydrocarbon-bearing amount can account for 85% of dry weight of

the cells, which is much higher than the amount of hydrocarbons produced by other microorganisms. Moreover, the composition and structure of hydrocarbons produced by *Botryococcus* is very similar to that of petroleum, so using *Botryococcus* to produce the substitutes of petroleum should be paid more attention hereafter [28, 30, 31].

Nowadays, researches about hydrocarbons produced by *Botryococcus* mainly focus on the physiological and biochemical aspects, but there are no reports about the culture on a large scale. The main difficulty is that *Botryococcus* grows very slowly and its doubling time is long, so researching the ideal nutrient conditions of *Botryococcus* is essential in order to shorten the generation time, improve growth rate, and achieve high-density cultivation [32].

1.2.3.1 Screening of Microalgae for Producing Hydrocarbon

Many studies have shown that the type of hydrocarbons (carbon number, saturation degree, with or without branching, etc.) in *Botryococcus* may not be the same even from the same algae of different strains. It is related to the type of microalgae, physiological status, and culture conditions. According to the products of *Botryococcus*, particularly the types of hydrocarbons produced, the algae have at least four strains: Strain A (Austin) mainly produces C23-C31 odd-carbon nonbranched linear diolefins and three olefins. Strain B (Berkeley) mainly produces branched C30-C37 class isopentyl diene, which is called botryococcene. Strain L (Lycopadiene) mainly synthesizes isoamyl alkadiene (or lycopadiene) which has a benzene ring or a heterocyclic ring in its branches. Strain Gb has been recently discovered, and its hydrocarbon types produced and synthesis pathways are similar to those of strain A, but there are also cis and trans (to olefin bond) diene in the products. Brown et al. [30] found that there were two different physiological stages in the growth process of *Botryococcus*: green active period and yellow resting period. These stages are different in terms of types and amounts of hydrocarbon production. Strain A in green period produces mainly diene and triene, which account for about 17% of cell dry weight, and produces highly unsaturated branched hydrocarbons in the yellow resting period, which account for 76% of cell dry weight. However, this phenomenon was related to the geographical origin of the algae, and not all strains showed such results.

1.2.3.2 Improvement of Microalgae for Producing Hydrocarbon

Currently, there are few reports about the breeding of *B. braunii*. Motohide et al. used ethyl methyl sulfone (EMS) in order to obtain the mutants that had herbicide resistance toward methylviologen and glufosinate, so as to prevent the contamination of other algae when cultured outdoors on a large scale. By mutagenesis and flat plate selection, *B.braunii* BOT88-2 (mutants of chemical species A) was

obtained that can grow in the medium containing 50 μM of methylviologen and 50 mg/mL of glufosinate, and the growth rate (μ, day^{-1}) was between 0.01 and 0.05. The study showed that it was feasible to culture large scale by improving some traits of *B. braunii*, which provided a useful reference for further innovative research on *B. braunii*.

1.2.3.3 The Nutrition Conditions of Metabolic Regulation of Microalgae Producing Hydrocarbon

Until now, high density culture techniques of *B. braunii* have not been achieved satisfactorily. The research is still in the small-scale laboratory exploration and outdoor cultivation stage, which is mainly due to the slow growth rate of *B. braunii*. Therefore, the most important issues to achieve large-scale cultivation are to determine the optimal growing conditions, increase the cell proliferation rate, and obtain the highest amount of hydrocarbons production. The proper conditions of *B. braunii* growth have been studied by many researchers, and a lot of progress has been made. However, based on the results reported in the last ten years, there are a lot of differences between the works, mainly in the culture media and various basic nutrient elements.

(1) Culture medium

So far, there are many nutritional formulations for cultivating *B. braunii*, mainly containing Prat medium, Chu 13, Chu 10, Jaworski's medium, BBM, BG11, SE, and so on. There are large differences between these media formulations. However, it indicates that *B. braunii* has relatively strong ability to adapt to the environment.

(2) Nitrogen

Nitrogen is the basic element for synthesizing not only proteins, nucleic acids, and chlorophyll of algae but also purine, pyrimidine and amino sugars. Dayananda and others suggest that KNO_3 is the best nitrogen source for the growth and hydrocarbons production of *B. braunii* LB-572 and SAG30.81. Yang et al. [33] studied how the growth process of *B. braunii* utilizes NO_2^-, and indicated that *B. braunii* will continue to utilize NO_2^- after utilizing NO_3^- when NO_3^- and NO_2^- are added to the medium at the same time. Hu et al. [34] found that both *B. braunii* 764 and *B. braunii* 765 can utilize NO_3^-, NO_3^-, NH_4^+ and urea, of which NO_3^- and urea are the most effective nitrogen sources. Ruangsomboon found that at NO_3^- concentrations of 16.5–344 mg/L, there was no difference in biomass amounts of *B. braunii* KMITL-2. But at moderate concentrations of NO_3^- (86 mg/L), the lipid content and yield were the highest. Thus, *B. braunii* can utilize different nitrogen resources in varying degrees, but the nitrogen concentration considerably affects its growth and lipid content. Limiting or lacking nitrogen may affect the growth of algae, while it can increase

the contents of lipid and hydrocarbons. Researches made by Zhila et al. [35] confirmed that limiting or lacking of nitrogen may significantly affect the growth and lipid content of algae *B. braunii* Kütz IPPASH-252, but it increased the content of oleic acid and saturated acids in cells which mainly accumulate glycerol. Due to the different stress times, the maximum lipid components in intracellular space are formed at different times.

(3) Phosphorus

B. braunii usually uses K_2HPO_4 and KH_2PO_4 as phosphorus sources, but some studies consider that increasing the concentration of P in the culture medium will not affect the growth of *B. braunii*, types of hydrocarbons and the relative abundance. The proportion of nitrogen and phosphorus has a very important impact on biomass amounts and hydrocarbon accumulation of *B. braunii*. For fed-batch cultures of the strain *B. braunii*, it is beneficial to accumulate hydrocarbon products with the nitrogen to phosphorus ratio of 1:4, and it is conducive to accumulate biomass amounts and hydrocarbon products when the ratio of nitrogen to phosphorus is 2:1. Ruangsomboon found that the biomass amounts of *B. braunii* KMITL2 reach up to the maximum at the phosphorus concentration of 444 mg/L, and lipid content and yield reach the maximum at the phosphorus concentration of 222 mg/L.

(4) Carbon

In photoautotrophic processes, microalgae can absorb CO_2 in the air for photosynthesis to meet their growth needs. However, the concentration of CO_2 that is dissolved naturally in the general culture is very low, so it is necessary to continue to add carbon source during the growth of microalgae. Wang et al. [36] studied the effects of inorganic carbon source on the growth of *B. braunii* 357 using shake flask. They found that by using $NaHCO_3$ or CO_2 with enriched air as carbon supplement, there was a significant increase in the specific growth rate, significant reduction in the time, and a large increase in the maximum oxygen evolution rate. Ranga Rao et al. found that 2.0% of CO_2 ventilation group significantly increased the biomass amounts of *B. braunii* and β-carotene. Yoo et al. found that *B. braunii* can grow in 10% of CO_2 flue gas (containing about 5.5% CO_2). Ge et al. found that *B. braunii* 765 can grow under 2–20% CO_2 aeration, and biomass and hydrocarbon content increased by increasing the amount of CO_2. Moreover, *B. braunii* can also utilize some organic carbon sources such as glucose, mannose, fructose, galactose, acetate, and so on.

(5) Other nutrients

Xu et al. [37] found that 0.1 mg/L of NaF has a significant effect in promoting the growth of *B. braunii*, while higher concentrations of NaF (0.6–1.0 mg/L) restrain its growth. Jun Wang et al. studied the effect of NaF on the growth of *B. braunii* and found that lower concentrations of NaF can promote the growth of *B. braunii* significantly.

The growth was most enhanced at the NaF concentration of 0.84 mg/L, while higher concentrations inhibited the growth.

(6) Using sewage and waste gases to culture *B. braunii*

As is known, nitrogen and phosphorus in water are necessary nutrients for organisms, but a large content of these elements can cause eutrophication. However, algae can effectively absorb and degrade a variety of organic compounds, such as nitrogen compounds, phosphorus compounds, hydrocarbons, and so on. Recently, many scholars have proposed using waste water and flue gas to culture *B. braunii* on a large scale, which would comprehensively combine the sewage and waste gas treatment and new energy development, and thus achieve energy conservation as well as sustainable social and economic development. Sawayama et al. [38] firstly used the secondary treatment of sewage to develop *B. braunii*. When *B. braunii* was cultured in wastewater in a similar manner as culturingin Chu13 medium, the hydrocarbon content equaled 40–53% of the biomass dry weight (58% in Chu13), and NO^{3-}, NO^{2-} and PO_4^{3-} can be fully utilized. An et al. [39] cultured *B. braunii* UTEX 572 with swine wastewater for 12 days, and found that algal cell dry weight reached up to 8.5 g/L. Yonezawa et al. [40] found that Tofu factory wastewater can promote the growth of *B. braunii*. Yang et al. [33] studied the key components (sulfur oxides and nitrogen oxide) in flue gases, as well as the effects of hydrosulfite and NO_2^- in aqueous solution on *B. braunii*'s growth, tolerance degrees, and utilizing regulation. The results confirmed that the flue gas was a good source of sulfur and nitrogen while also providing a carbon source for growth. Yoo et al. [41] studied the effects of actual flue gas (nonsimulated flue gas) on the growth and metabolism of *B. braunii*, by culturing *B. braunii* UTEX 572 with flue gas containing about 5.5% CO_2. The results showed that growth rate and fat contents of *B. braunii* were 0.077 g/L and 24%, respectively. All of the above indicated that *B. braunii* has a strong ability to deal with sewage and utilize nutrient elements, so the *B. braunii* production and sewage treatment should be combined together.

1.2.3.4 Environmental Conditions

Environmental factors such as light, temperature, salinity, pH, and so on have a great influence on the growth of *B. braunii* and the amount of hydrocarbon generation.

(1) Light

B. braunii is a photoautotroph, so light is an essential regulator of its growth and development. Light intensity can affect the concentration ratio of carotenoids and chlorophyll in *B. braunii* cells, and the cells can be yellow or green accordingly. At the same time, the radiation intensity can also change the doubling time of *B. braunii* and the hydrocarbon content of the cells. By culturing *B. braunii* in 3% CO_2 with different light intensities, Cepak et al. found that the higher the intensity was, the shorter the

doubling time of cells was. When *B. braunii* was cultured with a light intensity of 138 E/m^{2}s, Zhang et al. found that the biomass and cell yield were higher compared to the *B. braunii* cultured in low light intensity. Oyama et al. [42] cultured *B. braunii* UTEX 572 under low light intensity of 23.0–73.6 E/m^{2}s. It was found the contents of carbohydrate, nitrogen, and phosphorus were very low. Light intensity is closely related to the size, morphology and structure of *B. braunii* cells, and has an important effect on the metabolic activity of *B. braunii*. Medium intensity of light (138–414 E/m^{2}s) appears to be more favorable for the growth and production of *B. braunii*. Ruangsomboon found that the biomass of *B. braunii* KMITL 2 was the highest when cultured with 87.5 E/m^{2}s light intensity, while the highest lipid content and yield were obtained when it was cultured with 538 E/m^{2}s. Furthermore, the type of monochromatic light may also affect the growth of *B. braunii*. Baba et al. [43] found that the growth, photosynthesis, CO_2 fixation, and hydrocarbon yield of *B. braunii* Bot-144 (B) under blue light and red light are higher than those under green light. Moreover, red light is the most efficient light source based on light energy supply. Further, Sakamoto et al. [44] studied the effects of monochromatic red light on the growth and yield of *B. braunii* Bot-22 (B). The results showed that the lipid and carbohydrate yields were 40% and 20–30%, respectively, and about half of the lipids were liquid hydrocarbons. Photoperiod could also affect the biomass and lipid content of *B. braunii* KMITL 2.

(2) Temperature

In natural environment, temperature is the main factor that affects the physiological mechanism of single cell algae, which in turn affects its growth and development. For photosynthesis, a certain range of temperature is required by algae. The temperature can also affect the respiration of the algae, so temperature is an important condition for the growth and development of algae. Wang and Xie [45] found that different *B. braunii* strains have different optimal growth temperatures. Kalacheva et al. [46] cultured *B. braunii* at three different temperature conditions (18, 25 and 32 °C), and found that at 32 °C, the synthesis of lipids (except TAG) in the cell was inhibited, and the compositions of fatty acids and hydrocarbon were also affected. The effect of temperature on the growth of algae cells was achieved by changing the reaction rate of the enzyme. The temperature also affected the CO_2 and other nutrient sources in the culture medium.

(3) pH

pH is an important factor that affects many physiological processes of algae such as the growth, metabolism, and so on. Change in pH of the medium will change the dynamic equilibrium of CO_2, HCO^{3-}, and $CO3^{2-}$ in the culture medium, which would affect the availability of CO_2 during the photosynthesis. pH can also directly affect the permeability of cell membranes of microalgae, thus it can affect the utilization of nutrients. Beleher studied the effect of pH, and found that when pH

was 8.0, *B. braunii* had the highest growth rate; when pH was 6.0, it grew best but the growth rate was half of that at pH 8.0 cultivation condition; and when pH was 7.0, the growth rate was between that at pH 6.0 and pH 8.0. Dayananda found that the biomass amount and hydrocarbons amount of *B. braunii* were 0.75 g/L–0.86 g/L and 13–15% (w/w) at the process condition of six pH gradients (6.0–8.5), and there were no significant differences in this result. However, when pH was 7.5, the biomass amount and hydrocarbons amount were the highest.

(4) Salinity

Salinity is also an important factor that affects the growth of algae. Benamotz et al. [47] and Hu [48] found that increasing the salinity can increase the total lipid content of algae. Ruangsomboon found that when the salinity was 5, *B. braunii* KMITL 2 had the largest biomass amount, but when the salinity was 0, it had the highest lipid content and yield. Rao et al. [49] found that addition of 17–85 mmol/L NaCl significantly influenced the growth, palmitic acid content, oleic acid content, and β-carotene content of *B. braunii* LB 572.

(5) Biological factors

In the natural environment, algae and other organisms are in complex systems. In some cases, the bacteria and algae are in cooperation. The bacteria have a role in promoting the growth of microalgae. However, bacteria can also inhibit the growth of algae through competing for nutrition sources, and decomposing harmful substances such as polysaccharides. Jines co-cultured *B. brauni* A strain with different bacteria species and found that the bacteria had a significant influence on the growth and hydrocarbon production of *B. braunii*. The presence of microorganisms can not only affect the total amount of hydrocarbons but also affect the relative abundance of hydrocarbons, and even change the rate of hydrocarbon generation in the cells. Wang and Xie [45] found that the addition of *Bacillus sp*.109 inhibited the growth and production of B. braunii 357. At the same time, the biomass increased from 0.089 to 0.392 g/L, and the total hydrocarbon level increased from 5.6% to 24.2%, when *Corynebacterium* sp. 22-1 was added. Generally, the bacteria which can be co-cultured with *B. braunii* can improve the biomass and the yield of hydrocarbon of *B. braunii* in the natural condition.

Traditional bioenergy mainly utilizes corn, soybeans, and other crops as raw materials, which causes the demand of crops to rise greatly. Moreover, the biggest hidden danger of replanting bioenergy crops on a large scale is “Struggling for land with food”, which makes the price of food rise. Therefore, actively looking for new biomass energy materials to ease the crisis of food and energy has become an issue to concern all countries around the world. Among the many materials that can produce liquid biofuels, microalgae has broad prospects for development and microalgae bioenergy is very likely to become one of the most important renewable energies in the future.

Section 2: Fungal Oil Production

Under certain conditions, some fungi can convert sugar into oil in cells. If the lipid content (based on dry cell weight) of a fungus accounts for more than 20%, then this fungus can be called as oleaginous fungus. Oleaginous fungi include oleaginous yeasts and oleaginous mycetes. Producing oils using fungi has many advantages: rapid growth of cells, raw materials that are abundant and low cost (such as sugar, starch and wastes from food and paper industry), large-scale continuous production to reduce production costs and production of certain functional polyunsaturated fatty acids (such as γ-linoleic acid and arachidonic acid).

Producing oils using fungi can also ease the shortage of vegetable oil and competing for land with food crops. In addition, because the fatty acid composition of oils in most of oleaginous fungi is similar to that in plant oils dominated by C16 and C18 fatty acids (such as palmitic acid, stearic acid, oleic acid and linoleic acid), fungal oils can substitute vegetable oils for biodiesel production to ease the energy shortage.

2.1 Types of Oleaginous Fungi

Since Paul Lindner discovered oleaginous yeast *Metschnikowia pulcherrima* in 1899, many types of oleaginous yeast have been discovered up to now. Many strains, belonging to genus of *Lipomyces*, *Rhodotorula* and *Trichosporon*, are oleaginous yeasts. Common oleaginous yeasts include *Cryptococcus albidus*, *Lipomyces starkeyi*, *Trichosporon pullulans*, *Lipomy slipofer*, *Rhodotorula glutinis* and *Rhodosporidium kratochvilovae*.

Besides oleaginous yeasts, the oleaginous fungi also include oleaginous mycetes. Due to their high lipid content and rich functional polyunsaturated fatty acids, mycetes are studied intensively by researchers. Common oleaginous mycetes belong to the genus of *Rhizopus*, *Aspergillus*, *Penicillium* and *Fusarium*. Some of the well-known oleaginous mycetes include *Mortierella alpine*, *Mortierella elongate*, *Saprolegnia*, *Verticillium*, *Mucor*, *Mortierella ramanniana* and *Cunninghamella echinulate*. The lipid contents of common oleaginous fungi are shown in Table 6.2, and their fatty acid compositions are shown in Table 6.3 [50–52].

2.2 Mechanism of Fungal Oil Synthesis

The process of oil synthesis in fungi is similar to that in animals and plants. Oil biosynthesis starts with acetyl-CoA carboxylase, which catalyzes the biotin-dependent carboxylation of acetyl-CoA to form malonyl-CoA. Fatty acid synthase multienzymatic complex catalyzes the condensation of malonyl-CoA with acetyl-CoA to form ketobutyryl-ACP. The

Table 6.2: Lipid contents of common oleaginous fungi.

Oleaginous fungi	Lipid content (%)
Mucor hiemalis IDD51	41
Mortierella isabellina	86
Rhizopus RC378	47.8
Aspergillus terreus	57
Rhizopus arrhizus	26.5
Rhodotorula	57.73
Lipomyces starkeyi AS 2.1560	57.55

Table 6.3: Fatty acid composition of oils in common oleaginous fungi.

Strains	C14:0	C16:0	C16:1	C18:0	C18:1	C18:2	C18:3	Others
Mucor hiemalis IDD51		25.2		9.6	32.6	11.9	15.4	5.3
Mortierella isabellina	1	29		3	55	3	3	6
Rhizopus RC378	0.6	12.3	2.4	14.6	40.2	15.9	12.6	1.4
Rhodotorula	0.31	10.4	1.68	10.84	52.25	2.94		21.58
Lipomyces starkeyi	0.5	35.2	4.3	4.4	53.0	2.6		0

ketobutyryl-ACP condenses with another malonyl-CoA in the second round of carbon chain extension. This cycle is repeated until palmitic acid or stearic acid is formed. Then, the fatty acids are catalyzed by desaturase to form unsaturated fatty acids.

In the oil synthesis process, acetyl-CoA carboxylase and desaturase are two important enzymes. The first step of fatty acid synthetic pathway is catalyzed by acetyl-CoA carboxylase. Desaturase is the key enzyme during the process of unsaturated fatty acid synthesis. The unsaturated fatty acid is very important for the maintenance of membrane fluidity. Acyl-CoA desaturase is the main desaturase enzyme, which creates a double bond in the acyl-CoA in yeast.

The content of free fatty acids is quite low in the cells. The vast majority of fatty acids are used to produce triacylglycerol (TAG) or phospholipid by the esterification reaction. The TAG synthesis is a multienzyme catalyzed process, which widely exists in animals, plants, and microorganisms. Previous research has indicated that phosphatidate (PA) and diacylglycerol (DAG) are two key intermediate metabolites in TAG synthesis [53]. Similar to other eukaryotes, PA is synthesized through the glycerol-3-phosphate pathway and dihydroxyacetone phosphate (DHAP) pathway in *Saccharomyces cerevisiae*. The enzyme acyl-CoA: glycerol-3-phosphate acyl-transferase (GAT) catalyzes the acylation of glycerol-3-phosphate with an acyl-CoA at *sn-1* position to form lysophosphatidate (LPA) in glycerol-3-phosphate pathway. The corresponding

acyl-transferase can catalyze acylation of DHAP with an acyl-CoA to form acylated DHAP, which is then catalyzed by reductase to form LPA in DHAP pathway. The LPA is then further condensed, catalyzed by acyl-CoA: acylglycerol-3-phosphate acyl-transferase (AGAT), with another acyl-CoA at *sn-2* position to produce phosphatidate (PA). In addition, phospholipase D can also catalyze the hydrolysis of phosphatidylcholine to produce PA.

PA can be dephosphorylated by phosphatidic acid phosphatase (PAP) to form DAG, which is the main reaction to produce DAG. In addition, phospholipase C can also catalyze the hydrolysis of phospholipid to produce DAG. A previous study has found that TAG formation from DAG was mainly performed by *DGA1* and *LRO1* genes in *Saccharomyces cerevisiae* [54]. The *DGA1* gene encoding Dga1p protein belongs to DGAT2 gene family, which is the sole member of this gene family in *Saccharomyces cerevisiae*. The Dga1p protein is standard acyl-CoA:diacylglycerol acyl-transferase (DAGAT), which is similar to the other two acyl-transferases of TAG synthesis pathway, GAT and AGAT. They all use acyl-CoA as an acyl donor. However, the protein encoded by *LRO1* gene catalyzes the acylation of diacylglycerol with an acyl donor phospholipid at *sn-3* position to form TAG.

The *MrDGAT2A* and *MrDGAT2B* genes, two members of DGAT2 family, are expressed in insects. A previous study has found that their products could esterify diacylglycerol [55]. A *DGA1* gene deletion mutant (*dga1Δ*) was generated by homologous recombination in order to determine the physiological function of Dga1p in triglyceride synthesis in *Saccharomyces cerevisiae* [56]. The changes in TAG production in mutant *dga1Δ* strain were assessed by metabolic labeling of cells in logarithmic growth phase with [3H] oleic acid. The result indicated that the *dga1Δ* strain showed a 25% reduction in TAG synthesis compared to the control strain.

The *LRO1* deletion mutant of yeast, which was defective in phospholipid:diacylglycerol acyltransferase (PDAT) activity, showed a 75% reduction in TAG production compared to the control strain [54]. A *lro1Δ dga1Δ* double deletion strain was constructed in order to determine the combined roles of *DGA1* and *LRO1* in TAG biosynthesis [56]. The result showed that the TAG production in double deletion mutant was 2% of that in control, and large amounts of diacylglycerol and phospholipid substrates were accumulated. These results demonstrated that the *DGA1* and *LRO1* genes were responsible for most of the TAG synthesis in yeast.

The study indicated that two proteins, Are1p and Are2p, with acyl-CoA:cholesterol acyltransferase were possibly involved in the esterification reaction of TAG synthesis [57]. There was little TAG production in *lro1Δ dga1Δ* double deletion strain. Oelkers et al. [22] combined the results of Sandager to study the genes which were involved in limited TAG synthesis using three mutants, *dga1Δ lro1Δ are1Δ are2Δ*, *dga1Δ lro1Δ are1Δ*, and *dga1Δ lro1Δ are2Δ*. The result indicated that only Are2p was responsible for the limited TAG synthesis in *lro1Δ dga1Δ* double deletion strain.

A cytosolic 10S multienzyme complex was isolated from oleaginous yeast *Rhodotorula glutinis* [58]. This complex was found to include many kinds of enzymes, such

as lysophosphatidic acid acyltransferase, phosphatidic acid phosphatase, diacylglycerol acyltransferase, acyl carrier protein, and acylacyl carrier protein synthetase. The complex can rapidly synthesize TAG and the related intermediate metabolites using free fatty acids and acyl-CoA. Chemical cross-linking test revealed that this complex was held together by protein–protein interactions. These results showed that the cytosol is one place for TAG biosynthesis in oleaginous yeast.

In summary, there are still many unanswered questions about the TAG biosynthetic pathway of oleaginous fungi, such as the physiological function and the relationship of two pathways of LPA synthesis, and the contribution and relationship of *DGA1* and *LRO1* in TAG synthesis. Further research into these areas will provide important theoretical basis for improving the lipid content of oleaginous fungi by genetic engineering and other methods in production.

2.3 Metabolic Regulation of Fungal Oil Biosynthesis

Accumulation of oil in oleaginous fungi includes two processes: the growth stage and the oil accumulation stage. The fungi rapidly propagate and produce a large number of progeny cells using abundant nutrients in the culture medium during the growth stage. After the consumption of key nutrients (such as nitrogen source and phosphorus source) in the culture medium, the fungi enter into the stage of oil accumulation, and a large amount of carbon source is converted into oil.

At present, the TAG metabolic pathway in oleaginous yeast and mold with glucose as carbon source is well understood. Some major steps, which are associated with the regulation of TAG biosynthesis pathway in oleaginous fungi, are shown in Figure 6.3. When nitrogen source is depleted but excess carbon substrate is present in the culture

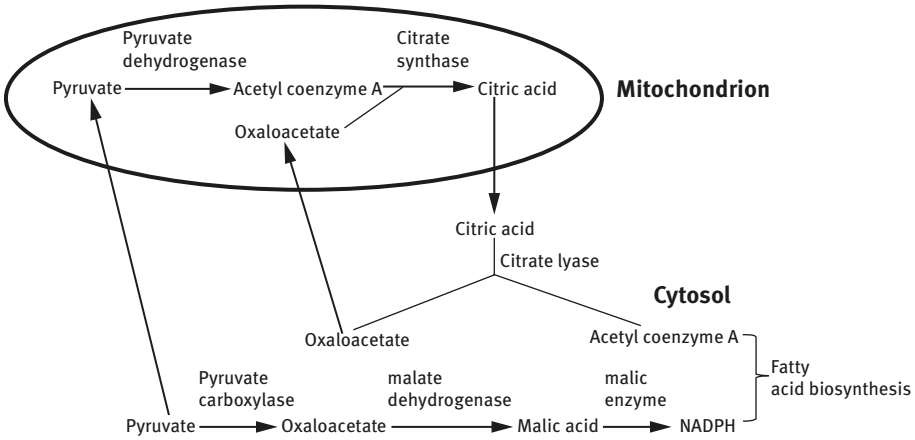

Figure 6.3: Production of acetyl-CoA and NADPH for oil biosynthesis in oleaginous microbes.

medium, the TAG biosynthesis pathway of oleaginous fungi is activated. This activation process involves a series of complex physiological and biochemical processes. When nonoleaginous fungi grow in a medium with a limited amount of nitrogen source, they tend to stop cell proliferation or continue to assimilate the accessible carbohydrate sources. Then, these carbohydrate sources are converted into various polysaccharides (such as glycogen, glucans, mannans, etc.). However, oil accumulation does not occur at this stage. Therefore, it can be concluded that the ability of oleaginous fungi to accumulate large amounts of oil is outside of the directed fatty acid biosynthesis, as this biosynthetic pathway is common to all fungi.

The oleaginicity of fungi may be related to the following two factors. The first factor is the ability to continuously produce acetyl-CoA directly in the cytosol as a necessary precursor for fatty acid synthase (FAS). The second factor is the ability to produce sufficient NADPH as the essential reductant for fatty acid biosynthesis.

The production of acetyl-CoA in oleaginous fungi has been attributed to ATP:-citrate lyase (ACL) which does not appear in the majority of nonoleaginous fungi. The ACL catalyzes the following reaction, citrate+CoA+ATP → acetyl-CoA+oxaloacetate+ADP+Pi. Citric acid should be mainly obtained from the cytosol to ensure effectiveness of this reaction.

Citric acid synthesis is a part of the tricarboxylic acid (TCA) cycle within the mitochondrion. Compared with nonoleaginous fungi, the oleaginous fungi have a unique feature, which is that the activity of isocitrate dehydrogenase of the TCA cycle is dependent on the presence of AMP.

The concentration of AMP is regulated by the activity of AMP deaminase. This enzyme catalyzes the following reaction, AMP→inosine-5'-monophosphate+NH_3. The activity of this enzyme is upregulated when nitrogen limitation occurs in the culture medium of oleaginous fungi. Nitrogen limitation induces a cascade of reactions which result in the formation of acetyl-CoA.

When the nitrogen is exhausted, activity of AMP deaminase increases in oleaginous cells and becomes up to fivefold greater than that before nitrogen limitation. The cellular content of AMP in the mitochondrion decreases because of the increased activity of AMP deaminase, which prevents isocitrate dehydrogenase from working. As a result, isocitrate cannot be metabolized. Then, accumulated isocitrate is readily equilibrated with citric acid via aconitase, which results in the accumulation of citrate in the mitochondrion. Citrate enters the cytosol through an efficient citrate efflux system which exists in the mitochondrial membrane and is cleaved by ACL to produce acetyl-CoA and oxaloacetate. The acetyl-CoA can be used for fatty acid biosynthesis. The oxaloacetate is converted to malate by malate dehydrogenase, and then enters the mitochondrion through the citrate efflux system.

Fatty acids are highly reduced materials. Fatty acid synthesis requires abundant NADPH. The reactions in fatty acid synthesis, which are catalyzed by beta-ketoacyl-ACP reductase and enoyl-ACP reductase, need NADPH as the reductant. The synthesis of 1 mol of a C16 palmitic acid requires 14 mol of NADPH. The majority of NADPH for fatty

acid biosynthesis originates from the reaction catalyzed by malic enzyme. The malic enzyme exists in the majority of oleaginous fungi. The malic enzyme, ACL and fatty acid synthase, form a complete lipogenic metabolon which uses acetyl-CoA for fatty acid biosynthesis [59]. Then fatty acids are esterified with glycerol into triacylglycerol.

Malic enzyme activity is not ubiquitous among oleaginous fungi and may perhaps be absent in some oleaginous yeasts, such as *Lipomyces* sp. and some *Candida* sp. [unpublished work]. In such cases, it is possible that an alternative NADPH-generating enzyme exists, such as NADPH-dependent isocitrate dehydrogenase in cytosol.

In summary, the production of acetyl-CoA and NADPH are two important factors for the regulation of lipid production in fatty acid biosynthesis. Any method which can increase the yield of these two substances (e.g. overexpression of malic enzyme gene) could increase the lipid content of oleaginous fungi.

The flux of carbon from the mitochondrion forms acetyl-CoA in the cytosol through citrate efflux system and then into fatty acid biosynthesis. Finally, long-chain PUFAs (LCPUFA) are synthesized in the membrane of the endoplasmic reticulum. The metabolic system uses pyruvate (from glycolysis) as the supplier of intramitochondrial acetyl-CoA. Then acetyl-CoA is used for citric acid production.

2.4 Factors Affecting Fungal Oil Synthesis

2.4.1 Strains

The lipid content and composition of fatty acids are different in different fungi. The lipid contents of several fungi under same culture conditions are as follows: *Rhizopus arrhizus* (26.50%), *Rhodotorula* (57.73%), and *Saccharomyces cerevisiae* (32.06%) [51].

2.4.2 Culture Conditions

The lipid content of oleaginous fungi is determined not only by the strains but also by the culture conditions including carbon source, nitrogen source, carbon nitrogen ratio, pH, temperature, and aeration rate.

2.4.2.1 Carbon Source

The fungus strain can convert a large amount of carbon source to oil when the carbon source is rich and other key nutrients are restricted. The suitable carbon sources for culture of oleaginous fungi include glucose, sucrose, waste molasses, lactose,

wast water from starch factory, and so on. Among them, the carbon source which is most favorable for cell growth and oil accumulation is glucose.

2.4.2.2 Nitrogen Source and Carbon Nitrogen Ratio

Nitrogen is an important nutrient for cell growth. The fungi can accumulate a large amount of oil when the nitrogen source is limited. Common nitrogen sources include corn steep liquor, amino acids, nitrate, and urea. The effects of different nitrogen sources on lipid accumulation in *Mortierella isabellina* have been studied [60]. The results showed that ammonium nitrate and urea were beneficial to cell growth, but were adverse to oil accumulation. At the same time, peptone and beef extract were beneficial to oil accumulation, but were adverse to cell growth. The conditions of oil accumulation in *Mortierella* strain M14 have been studied, and it was found that low C/N ratio promoted the production of mycelium, and high C/N ratio promoted the conversion of carbon source to oil [61]. Therefore, accumulation of oil in oleaginous fungi can be divided into the growth stage and the oil accumulation stage.

2.4.3 Temperature

Temperature can significantly affect the accumulation of oil. The regulation of temperature can influence the activity of enzymes related to oil biosynthesis, and further affect the yield and composition of oil. The study indicated that moderate temperature (25–30 °C) was beneficial for the production of mycelium, while low temperature (15–20 °C) was beneficial for oil accumulation in cells.

2.4.4 pH

The pH value also affects the accumulation of oil. The most appropriate pH for oil production is different for various fungi. It has been found that the production of mycelium and oil yield were both high when *Mortierella* strain M14 was cultured at pH 4.5–5.5 [61].

2.4.5 Culture time

The culture duration can also affect the oil yield of fungi. The most appropriate culture time for oil production is different for different types of fungi. For instance, the optimum culture times for *Aspergillus niger*, *Aspergillus oryzae*, and *Rhizopus* were 3 d, 7 d, and 7 d, respectively.

In addition, other factors (such as inorganic salts, trace elements and ventilation) can also affect the oil synthesis in fungi.

2.5 Breeding of Oleaginous Fungi

The genetic characteristics of the strains have a decisive effect on the accumulation of oil. Therefore, it is a key step to breed strains with high lipid content. Breeding methods of strains include not only conventional cross breeding and selective breeding but also advanced modern breeding techniques such as cell fusion breeding, mutation breeding, and genetic engineering breeding. Modern breeding technology can significantly shorten the breeding period and raise the breeding efficiency.

2.5.1 Mutation Breeding

After mutation, the mutant cells only account for a small proportion of the living cells. The mutant cells with increased lipid content also account for a small proportion of the mutant cells. Therefore, the workload of strain selection is very large after mutation treatment of oleaginous fungi. In order to improve the working efficiency, and screen the mutant cells that meet the expected target in a short time, we need to adopt the scientific screening program and the screening method with high efficiency. Common screening indicators include growth performance, appearance, biomass, and lipid content. The biomass and lipid content of mutant strain were 26.7 g/L and 15.6 g/L, respectively, when *Lipomyces starkeyi* As 2.1560 was treated by composite mutation (ultraviolet plus lithium chloride) [52].

In order to obtain good results in the mutation breeding process, the following principles should be followed: (1) Try to choose a simple and effective mutagen. Ultraviolet light and NTG with strong mutagenic effects are commonly used mutagens. (2) The performance of original strain should be good. (3) Treat suspension of single cells or single spores. (4) Use appropriate dose. Try to choose a dose which can promote mutation rate and beneficial variation through pre-tests. (5) Fully utilize synergistic effect of composite mutation. The effect of composite mutation is frequently better than that of single mutation.

2.5.2 Cell Fusion Breeding

In traditional breeding techniques, the process of sexual reproduction must be carried out by cross breeding. Modern biotechnology has now replaced the conventional process. Cell fusion breeding is a method which combines the protoplast of two cells with different genetic characters, and then combines the nuclear and genetic materials to produce a fusant with the characters of parents.

Cell fusion breeding has the following characteristics: (1) Oil yield and other quantitative characteristics are often controlled by multiple genes. The transfer of multiple genes is difficult through genetic engineering. Cell fusion technology provides a way

for improvement of traits controlled by multiple genes. (2) Cell fusion breeding can be carried out between the same species as well as between different species, which provides a possible way for distant hybridization. However, distant hybridization is generally difficult to achieve in sexual hybridization. (3) Sexual hybridization only produces nuclear hybrids, but cell fusion breeding can obtain cytoplasmic hybrids.

Cell fusion breeding includes the steps of protoplast preparation, induction of cell fusion, hybrid cell selection, and identification of hybrid cell. Cell fusion can be divided into chemical fusion, electrofusion, physical fusion, and laser fusion. In practical work, chemical fusion and electrical fusion are usually used.

The cells are induced by chemical reagents in chemical fusion. The commonly used chemical reagents include salts (such as sodium nitrate and calcium nitrate) and polymers (such as polyethylene glycol and polyvinyl alcohol). Polyethylene glycol (PEG) is a commonly used chemical reagent. The PEG method has good repeatability and can obtain higher rates of cell fusion. However, PEG has certain toxicity to cells. In addition, it is not easy to optimize the molecular weight, processing time, and concentration of solution.

Electrofusion is a method for cell fusion using short-duration high-voltage electric pulses. Compared with chemical fusion, electrofusion has the advantages of no toxicity, high fusion efficiency, simple operation, and so on. However, the fusant does not easily survive, and the instrument is expensive in electrofusion. Therefore, electrofusion technique is not as popular as chemical fusion.

The population in cell fusion experiment includes fusant, nonfusant, chimera, and multiple fusant. A variety of screening methods are used to isolate hybrid cells in order to obtain the fusants. The main screening methods include the selection method of mutant cell complementation, the selection method of physical specificity, the selection method of growth difference, and the selection method of asymmetric fusion.

Various methods need to be utilized to identify the true fusant after the fused cells are cultured. Common identification methods include phenotyping, cytology method, isozyme method, and molecular biology method. Although cell fusion breeding has been successful in fungi, there are only a few reports on the breeding of fungi with high oil yield using cell fusion breeding technology.

2.5.3 Genetic Engineering Breeding

Researchers have made considerable progress in genetic engineering breeding of oleaginous fungi. A gene encoding acetyl-CoA carboxylase (ACC) was cloned from an oleaginous fungus, *Mucor rouxii* [62]. Then, the recombinant plasmid containing *M. rouxii ACC* was introduced to nonoleaginous yeast *Hansenula polymorpha*. The results indicated that the total fatty acid content in *Hansenula polymorpha* overexpressing *M. rouxii ACC* increased by 40% compared with control. The gene encoding

acyl CoA:diacylglycerol acyltransferase (DGAT) from *Arabidopsis thaliana* was overexpressed in yeast and tobacco respectively [63]. It was found that the triacylglycerol content in transgenic yeast and tobacco was up to 3–9 times and 7 times of control, respectively. The genes encoding malic enzyme from *Mucor circinelloides* and *Mortierella alpine* which are named as *malEMt* and *malEMc*, respectively, were overexpressed in *M. circinelloides*. In the recombinant strains with *malEMt* and *malEMc*, the activity of malic enzyme increased by 2 and 3 times and the oil yield increased by 1.5 and 1.4 times, respectively [64].

At present, genetic engineering breeding of oleaginous fungi is still in progress. It has great developmental potential for improving the yield and content of fungi.

Section 3: Microbial Lipase Production

Lipase is a widely existing enzyme which plays an important role in lipid metabolism. In the oil–water interface, lipase-catalyzed hydrolysis of the ester bond of triglyceride takes place and releases fatty acid glycerides or glycerol with fewer ester bonds. Lipase-catalyzed reactions are characterized by mild reaction conditions and excellent stereoselectivity without any environmental pollution. So, lipase is widely used in many industrial fields such as food, leather, pharmaceuticals, and detergents.

Biodiesel, known as fatty acid methyl ester, is a clean renewable energy source and considered as a high quality substitute for traditional petroleum diesel. It is made from oil reacted with short-chain alcohols. Chemical production of biodiesel involves a complex process, high energy consumption, environmental pollution, and other shortcomings, while lipase-catalyzed synthesis method can effectively avoid the above problems. However, currently the high cost and low stability of lipase restrict the development of the enzymatic production of biodiesel. To obtain lipase with high activity and stability is the key to achieve efficient catalytic conversion.

Lipase is widely present in plants, animals, and microorganisms. There are many different types of microorganisms, which breed quickly and are prone to variation. The microbial lipase is generally an extracellular enzyme and has a broader range of active pH and temperature as well as better substrate specificity than plant lipase and animal lipase. Therefore, microbial lipase is suitable for industrial production, and it is an important source of industrial lipase and a research focus for biodiesel production.

3.1 Classification of Lipase-Producing Microorganisms

Lipase is widely present in plants, animals, and microorganisms. Plant lipase mostly exists in seeds of oil crops, such as castor beans and rapeseed. Lipase can cooperate with other enzymes and play a role in the catalytic decomposition of oil to generate

Table 6.4: Different origins of microorganisms producing lipase.

Microorganisms	Origins
Rhizopus	Isolated from Wine Starter in winery
Mucor	Isolated from plants and animals, food, animal feces, soil
Yeast	Isolated from carbohydrate-rich sources, such as grapes, apples and other fruits or plant exudates
Pseudomonadaceae	Isolated from the spoilage of meat and meat products, fish and shellfish, eggs, milk and vegetables

carbohydrates, which provide the necessary nutrients and energy for seed germination. Animal lipase is mostly present in pancreas and adipose tissue of higher animals. A small amount of lipase is also present in the intestinal juice, to supplement the inadequate digestion of fat catalyzed by pancreatic lipase. A small amount of glyceryl tributyrate lipase is also present in the gastric juice of predators. Various types of lipase control the digestion, absorption, fat reconstruction process, and lipoprotein metabolism of animals. More abundant lipase species can be produced by bacteria, fungi, and yeast.

Lipase (triacylglycerol ester hydrolase, EC 3.1.1.3) is a kind of ubiquitous enzyme with various biological activities, including enantioselective hydrolysis and esterification, chiral resolution, synthesis of enantio-enriched monomers and macromolecules for polymerization, and other enzymatic reactions. It is usually used for hydrolysis of triglyceride formed from glycerol and insoluble long chain fatty acids with more than 12 carbon atoms. Lipase is widely distributed in nature, and 65 species of microorganisms produce lipase. Strains-producing lipase can be screened from soil rich in oil or fat such as soybean or rapeseed planting soil, and soil near cafeterias, kitchens, and oil refinery factories. Different origins of microorganisms producing lipase are shown in Table 6.4.

3.2 Mechanism of Oil or Fat Conversion by Lipase

3.2.1 Enzymatic Characteristics of Lipase

Lipase has its unique catalytic properties and it catalyzes reactions occurring at the oil – water interface. The conventional enzyme kinetics cannot be applied to lipases, so the study of interfacial enzymes called "interface enzymology" has a very important significance for enzyme-catalyzed reactions on membrane. As seen from the structures of several known lipase, the molecular weight of lipase is between 20000 and 60000 Da. It acts in hydrophilic–hydrophobic interface layer of the reaction system, which is a feature different from the esterase enzyme.

The length of most of lipase gene fragment is about 1–3 kb, and only individual lipase gene is up to 6 kb. Lipase gene has a high G + C content, and most exist in the third

codon. The amino acid number of the majority of signal peptide sequence is between 23 and 26, or between 40 and 44. The number of amino acids encoded by the lipase is between 269 and 320, or 460 or so. Lipase is a glycoprotein, and its carbohydrate moiety occupies about 2% to 15% of the molecular weight. Based on mannose, lipase molecule is composed of a hydrophilic part and a hydrophobic part. The active site of the molecule is near the hydrophobic side. Lipase catalytic site containing nucleophilic catalytic triad (Ser-His-Asp) or (Ser-His-Gly) is buried in the molecule. Serine residues of the active site are covered by α helix, protecting triad of the catalytic site. The α- helix will open when lipase is in contact with the interface. Lipase exposes hydrophobic residues by creating an electrophilic area around serine, increases the affinity for lipid substrate and catalyzes the formation of transition intermediate product. The presence of the interface also allows the lipase to form an incomplete hydration layer, which is conducive to an aliphatic side chain of hydrophobic substrate being folded into the enzyme molecule surface.

There is a comprehensive understanding of the gene, structure, function, and other aspects of lipase by molecular biology research. So far, many bacterial lipase gene sequences such as *Pseudomonas, Staphylococcus*, *Streptomyces*, *Bacillus subtilis,* as well as dozens of fungal lipase gene sequences have been cloned, and the primary amino acid structure of these enzymes have been determined. By studying fragments and expression systems for lipase gene, higher expression of microbial lipases can be obtained. Therefore, conventional breeding is not the only method to improve the lipase.

Lipase has a multiple catalytic ability. It may catalyze the hydrolysis, alcoholysis, esterification, transesterification of triacylglycerides and other water-insoluble esters, and reverse synthesis reaction of ester. In addition, it also exhibits catalytic activity of other enzymes, such as phospholipase, lysophospholipase, cholesterol esterase, acyl peptide hydrolase, and so on. Lipase displays different types of catalytic activity depending on the characteristics of the reaction system. For example, the oil–water interface promotes ester hydrolysis, while enzymatic synthesis and transesterification can be catalyzed in the organic phase.

Research on properties of lipase includes several aspects, such as optimum temperature, pH value, temperature and pH value stability, and substrate specificity. So far, a large number of microbial lipases have been isolated and purified, and their properties have been studied. There are differences between their molecular weight, optimum pH, optimum temperature, pH and thermal stability, isoelectric point, and other biochemical properties. Overall, the microbial lipase has broader active pH and temperature range, better substrate specificity and better stability and activity than plant lipase and animal lipase [65].

3.2.2 Lipase-Catalyzed Mechanism

Nonaqueous enzymology research has made great progress in the last 20 years, and applied lipase reaction systems are also greatly improved. The reaction systems

mainly involve enzyme-catalyzed reaction of the organic phase, nonsolvent system under microwater conditions, as well as the microemulsion system and supercritical fluids system.

There are many advantages of lipase catalysis in organic solvent system which cannot be obtained in many other routine conditions: (1) it improves the application range of traditional hydrolases, so the transesterification and esterification reactions may be catalyzed in ammonia solution; (2) water-insoluble materials can be catalytically converted, greatly expanding the scope of enzyme substrate; (3) Since lipase is insoluble in organic solvents, it is convenient for recycling and reusing the enzyme at the end of the reaction; (4) the use of organic solvent may reduce the energy consumption during the reaction process and separation process; (5) the thermal stability of the enzyme is greatly improved; (6) it may reduce or prevent side reactions caused by water; and (7) it may avoid microbial contamination during long-term reactions.

There are many types of organic solvents, and the appropriate solvent should be selected according to the required physical and chemical properties. In recent years, studies on enzymatic resolution and synthesis of chiral drugs in organic solvents have focused on catalytic activity and selectivity of lipase. Enzyme-catalyzed reaction in the organic phase is mainly affected by the water content and solvent polarity, as well as the temperature and pH. Water is still the basic condition required for enzyme catalysis in organic solvents. A layer of water exists on the outside of enzyme protein molecules, which is called essential water. In absolutely anhydrous systems, the enzyme does not exhibit catalytic activity. Generally, the folding of protein molecules reduces the number of exposed hydrophobic groups in a solvent (water) to a minimum, so that the surfaces of the protein molecules have more hydrophilic groups and charged groups. Then the water molecules adsorb on the surface of proteins through these hydrophilic groups forming hydrogen bonds. Water helps to maintain stability of the conformation of natural enzyme (activity), shields interactions between polar groups on the protein surface, and facilitates a certain flexibility of enzyme molecules in the water. To increase the flexibility of enzyme molecules means reducing the resistance of the substrate close to enzyme molecules, the product leaving enzyme molecules, and increasing the catalytic activity of the enzyme. However, most of the important enzyme molecules maintain a certain rigid structure. Due to the rigidity of the structure, the enzyme can maintain conformation memory before it is added into organic phase. Such conformation allows the enzyme to maintain high catalytic specificity and activity. The water content can also have a direct relationship with polarity of the required solvents. Nonpolar hydrophobic solvent cannot easily capture the necessary water around the enzyme molecule, and the enzyme containing necessary water is relatively stable in such media. Polar solvents easily compete with necessary water of enzyme, but as the water content increases, the enzyme maintains a certain activity in the polar solvent.

The water content of the reaction system will have an impact on the water content of the enzyme. In many cases, in order to promote the reaction of ester synthesis, it is necessary to remove the water formed in the reaction, which can be achieved by evaporation, cold trap, molecular sieves, or nitrogen gas. At present, the water content is controlled by direct addition of water to the reaction system or enzyme, or by a hydrated salt balancing with immobilized enzyme in a closed system. Sometimes, the hydrated salt method is also used to control water activity. Several studies showed that adding not only water but also other additives, such as glycerol and polyols in the reaction system, can increase the speed of the transesterification reaction.

Research on the activity and stability of lipase are very important to the industrial applications. In the study of production of functional oils or fats by transesterification, Shimada et al. found that the decrease in activity was not due to the loss of binding water of immobilized lipase. In the course of reaction, excess water of the enzyme is removed by "dry" substrate, and gradually decreased to the critical water content, and then the water content of the enzyme is not affected by the substrate. Moreover, the water content of the system is also difficult to alter as the water content of the enzyme changes. Details on the critical water content of each lipase have not been reported in the literature. Polarity of solvents can also have a significant impact on the catalytic activity of enzyme. It is generally believed that there is higher enzyme activity in nonpolar solvents than in polar solvents. The hydrophobic parameter LogP can quantitatively describe the polarity of solvent. Generally, the larger the LogP of hydrophobic solvent, the greater the enzyme activity.

Selectivity of enzymes includes substrate specificity, enantioselectivity, prochiral selectivity, position selectivity, and so on. Enantioselectivity for identifying an enantiomer plays a decisive role in the synthesis of chiral drugs. Selectivity of enzymes between two substrates is generally defined as the ratio of the specificity constant, so enantioselectivity can be expressed as (Kcat/Km) R / (Kcat/Km) S, where Kcat and Km denote the catalytic number and Michaelis constant, respectively. Obviously, the better the enantioselectivity, the higher the optical purity of the product, and the better the rival drug splits. Enantioselectivity of enzyme is significantly affected by the nature of the solvent, so enantioselectivity of enzymes can be changed by changing the type of solvent. Currently, the exact nature of solvent effect on enzyme selectivity is not fully understood. Some effects can be explained by solvent properties (e.g., hydrophobicity parameter LogP) and interactions between substrate, enzyme, and solvent.

In the enzyme-catalyzed reaction, the use of organic solvents will cause environmental pollution, more complex after-treatment of the products, and also affect the safety of the product. Using other reaction media to replace the organic solvent is a green synthetic goal.

A microemulsion is generally composed of a surfactant, cosurfactant, oil, water, and so on. It is a thermodynamically stable, optically transparent, macros-

copically uniform and microscopically uneven system, which can provide significant oil–water catalytic interface for lipase. Lipase can dissolve in nanoscale "pool" of water-in-oil (W / O) microsolution in the form of molecules, which are very similar to the cellular microenvironment in vivo. However, preparation of the system is complicated and it is difficult to maintain enzyme activity and reuse the enzyme.

The system without any solvent and surfactant can avoid the problems of solvent system and microemulsion system. The after-treatment of product is simple and environmental pollution is small. It meets safety requirements of the product and production and has little effect on activity and selectivity of enzyme. In addition, due to the absence of solvent, the substrate concentration is high and the reaction proceeds rapidly. However, there is a disadvantage that higher temperatures are often required because of the high viscosity of reaction mixture in this system. Nevertheless, solvent-free system has wide range of applications in the preparation of esters with oil or fat as raw material by transesterification.

Enzyme-catalyzed reaction under supercritical conditions is one of the new areas of enzyme catalysis. In a supercritical state, the nature of substrates and mass transfer are very different. Whether enzymatic properties such as activity, stability, specificity, and so on. change or not under these conditions is an issue of great interest. Researches on lipase-catalyzed transesterification under supercritical carbon dioxide have made some progress, and more thorough researches are required for further advances.

3.3 Screening of Lipase-Producing Strains

3.3.1 Isolating, Screening, and Purification

Isolating, screening, and purification steps of strains producing lipase include media preparation, enrichment of culture, primary screening, and secondary screening. A few relevant examples are discussed as follows:

3.3.1.1 Media Preparation

Enrichment medium (g/L): yeast extract 5.0, Na_2HPO_4 1.0, KH_2PO_4 1.5, $MgSO_4 \cdot 7H_2O$ 0.5, $(NH_4)_2SO_4$ 5.0, NaCl 0.5, and olive oil 10 mL / L, sterilized at 115 °C for 30 min.

Plate medium (g/L) : $(NH_4)_2SO_4$ 1.0, K_2HPO_4 1.0, KCl 0.5, $MgSO_4 \cdot 7H_2O$ 0.5, and agar 20.0, sterilized at 121 °C for 20 min.

Primary screening medium: Olive oil and polyvinyl alcohol (2.0%, V/V) were mixed in a volume ratio of 1:3, stirred and emulsified for 5 min. A milky polyvi-

nyl alcohol-olive oil emulsion was formed. After sterilization, 12 mL emulsion was added into 100 mL sterile enrichment medium, which formed the primary screening medium.

Secondary screening medium (g/L): peptone 20.0, yeast extract 5.0, sucrose 5.0, soy flour 20.0, $(NH_4)_2SO_4$ 1.0, $MgSO_4 \cdot 7H_2O$ 1.0, K_2HPO_4 1.0, and olive oil 10 mL / L.

Seed Medium (g/L): YPD medium, glucose 20.0, peptone 20.0, and yeast extract 10.0.

Basal medium for producing enzyme (g/L): peptone 20.0, sucrose 5.0, soy flour 20.0, $(NH_4)_2SO_4$ 1.0, $MgSO_4 \bullet 7H_2O$ 1.0, K_2HPO_4 1.0, and olive oil 5 mL/L [67].

3.3.1.2 Enrichment Culture

About 5 g of each soil sample was weighed and 20 mL of sterile water was added, then the soil suspension was prepared by oscillation. Then, 5 mL of each soil suspension was added to the flask containing 30 mL enrichment medium. The flasks were placed in 28 °C and 32 °C shakers to culture for 3 d.

3.3.1.3 Primary Screening

Plate screening medium was sterilized at 121 °C for 20 min, removed from the autoclave and cooled to 60 °C, 10mL Rhodamine B (0.1g/L) filter sterilized was added to each 100 mL medium, mixed, and poured plates. 1 mL bacteria solution was taken from the enrichment medium from each flask, and diluted serially gradiently with sterile water, then coated plates with three gradients 10–5, 10–6, 10–7. After biochemical incubator at 28 °C, 32 °C for 3d, plates were observed under 350 nm UV light. Fluorescent circle would appear around strains producing lipase. The greater the fluorescent circle was, the higher lipase activity was. Strains screening were based on fluorescent ring size on culture plates.

3.3.1.4 Secondary Screening

After primary screening culture, large colony color-changing circles were selected for streak culture, according to the size of color-changing ring around the colonies on the medium. After separation, single colonies were saved by bevel save. The pure strain was transferred to 30 mL seed medium and shaken at 30 °C for 24 h to make seed liquid. Then, the microbe solution was mixed with a volume ratio of 5% to 100 mL of secondary screening medium, then shaken for fermentation at 30 °C for 3 d. The supernatant was removed after centrifugation to measure enzyme activity.

3.3.2 Breeding Methods of Lipase

3.3.2.1 Conventional Mutation Breeding

With the increasingly high demands of modern applications, mutation breeding has become the typical process for microbial lipase. It has the advantages of being simple, fast, and effective. Mutation breeding, which used to be the main method of industrial microorganisms breeding, is still an important and effective method. Especially, the excellent strains produced in the fermentation industry are mostly obtained by the method of mutation breeding.

3.3.2.2 Screening Novel Lipase from *Extremophiles* and *Archaea*

Extremophiles (including archaea) often secrete extracellular enzymes or intracellular enzymes which are highly stable and have effective catalytic activities. In order to accommodate the harsh environments enzymes are subjected to, it has become a routine way to find new industrial enzymes with novel catalytic properties from *thermophile, psychrophile, acidophile, alkalophile, halophile, piezophilic piezophile, metalophile, radiophile, microaerophile, organic solvent tolerant and other extremophiles,* and *archaea*. Using this method, a variety of microbial lipases from *extremophiles* and *archaea* has been successfully obtained with a number of excellent properties. The genes of these lipases are cloned and effectively expressed in heterologous hosts. Characterization of secreted lipase of several extremophiles and archaea are listed in Table 6.5. Domestic research paid extensive attention to the heat-resistant and cold-resistant lipases, but studies on the other extreme lipases are relatively few. A library of strains producing lipases under various extreme environmental conditions has been established, and the enzymatic properties of *Rolen Cryptococcus* yeast lipase (optimum temperature was 25–30 °C) were studied [68].

Table 6.5: Extremophiles (or archaea) that produce lipase.

Strains	Source	Special property
S. solfataricus	Archaea (not clear)	Half-life at 90 °C is 83 min
C. paurometabolum	lake water of low temperature	The optimum temperature is 25 °C
Pseudomonas sp.	Ground water (700–800 m)	The optimum temperature is 35 °C
S. saprophyticus	Seawater	It has good stability in benzene, toluene, hexane and other organic solvents
B. sphaericus	Soil	It has good stability in xylene, hexane and other organic solvents
B. stearothermophilus	Hot spring	Half-life at 100 °C is 15–25 min

The study of tolerance (or adaptation) mechanisms of heat-resistant and cold-resistant lipases is thorough, while studies on the mechanism of other extreme resistance (or adaptation) microbial lipases are few. It is expected that new types of lipase can be obtained by analyzing its structure, and clarifying its tolerance (or adaptive) mechanism with the help of modern protein engineering techniques and rational design.

3.3.2.3 Screening Novel Microbial Lipase by Metagenome Technology

About 99% of the natural microorganisms fail to achieve pure culture using traditional media, so development of the genetic resources of uncultured microorganisms is one of the hotpots in current microbiology and enzymology study. The technology of screening uncultured microbial genetic resources mainly involves the following aspects: function-based screening, sequence-based screening, and substrate-induced gene expression screening. Using metagenomic techniques, a variety of new gene sequence enzymes such as lipase, protease, amylase, dioxygenase, chitinase, cellulase, ethanol oxidoreductase, and β-lactamase have been successfully cloned. It has been reported by Ranjan that 12 lipolytic enzyme genes were cloned from pool water samples by metagenomic techniques, and homology of the amino acid sequence in one of the gene was only 25% with known proteins. Hårdeman and Jinwei Zhang have utilized metagenomic techniques to clone genes encoding novel cold lipases from Marine sediments. In another study, Tirawongsaroj cloned high temperature lipolytic enzyme-encoding gene directly from the samples of spa mud by metagenome technology.

At present, high-throughput screening technology is the main factor restricting the development of metagenomic technology. Cost of sequence-based screening is expensive, while function-based screening must be based on a mature expression system. Since the gene sequences in the metagenome are highly diversified and individually complicated, there is no expression system that can meet the functional expression of all genes. In order to overcome the limitations of the above screening technology, Bell designed specific primers based on conserved amino acid lipase molecules, and successfully obtained novel gene resources of lipase through direct PCR amplification using metagenomic DNA as a template.

3.3.2.4 Screening Novel Microbial Lipase by Genomic Database

With the completion of more and more microbial genome sequencing, it has attracted the attention and active exploration of researchers on enzymology. They analyze, annotate, and dig out valuable information from the hidden genomic sequences in order to find genetic resources with novel enzymatic properties. A large number of new genes have been annotated as genes encoding lipase (http://www.led.uni-stuttgart.

de/) by pair-wise comparison with the known gene sequences of lipase, but their functions are to be further verified. The annotated gene sequences of lipase are evaluated based on the characteristics of conserved sequences in the primary structure of amino acid sequence, such as the Ser constituting the activity centers located in a conserved pentapeptide -G-X-S-X-G-. The distance between the amino acid conserved region near the first oxyanion hole and the serine in activity centers is about 70 to 100 amino acids. With the gene sequences compiled with conserved structural features of lipase, novel lipase gene resources can be directly, rapidly, and efficiently obtained by primers design, PCR amplification, heterologous expression, and functional verification, thus avoiding omission of the gene library screening method due to sub-threshold expression level. With this method, Kim successfully screened five new lipase genes from nine candidate genes of four strains. In addition, screening efficiency of strains yielding lipase can be dramatically improved by fully digging genomic information and establishing a corresponding orientated high-throughput screening technology. In the screening of lipase microbes through conventional selective medium, a certain kind of microorganism is often unnoticed because it is not dominant bacteria in the environment.

3.3.2.5 Protein Engineering and Optimization of Microbial Lipase

Advances in protein engineering technology are endless. In support of bioinformatics, biophysics and quantum chemistry and other subjects, various rational design and directed evolution technologies are widely used in the optimization and evolution of the microbial lipase. Thus, several novel microbial lipase resources have been obtained, including lipases with increased esterlysis activity, amidolytic activity, phospholipid hydrolysis activity, alcoholysis activity, aldehyde polymerization activity, and enantioseparation activity; lipases with improved temperature stability, organic solvent tolerance, and oxidant tolerance; and lipases that can selectively change the chain length of the substrate, the optimum temperature, and optimum pH, thus expanding the acceptable range of substrates. The various improvements of lipase characteristics by protein engineering techniques are listed in Table 6.6 [57].

There are two key technologies for reconstruction and optimization of lipase using protein engineering: (1) establishing the corresponding expression system for specific lipase gene. This approach requires different conditions for different lipase molecules to form active conformation and undergo activation, so expression system of lipase gene mutant library will be chosen based on gene-specific needs. (2) Establishing the corresponding high-throughput screening technology platform, according to the catalytic activity of different lipases. Tielmann established high-throughput screening technology for enantioseparation activity of lipase using FTIR analysis, which can screen 20,000 transformants every day.

Table 6.6: Reconstruction of lipase molecules using protein engineering technologies.

Improvement of lipase characteristics	Category of lipase	Applied technologies	Mutation site
Improvement of enantioseparation activity	*B. subtilis* lipase	Recombinant technology based on multiplex PCR	N18Q/Y49V
	B. cepacia lipase	Single-molecule PCR	L17F/ F119I/L167G/ L266V; L17F/L167G/ L266I
Change of preference for chain length	*C. rugosa* lipase4	Site-directed mutagenesis	A296I; V344Q; V344H
Expanding the acceptable range of substrate spectrum	*C. antarctica* lipase B	Ring replacement	A283/A283-KRPRINSP
	P. aeruginosa lipase	Combinatorial Active-Site Saturation	M16A/L17F; M16G, M16G/L17F, etc.
Increase in lipolysis activity	*P. expansum* lipase	Site-directed mutagenesis	R182K
Improvement of amidolytic activity	*P. aeruginosa* lipase	Error-prone PCR / site-directed mutagenesis	F207S/A213D
Improvement of phospholipid hydrolytic activity	*B. thermocatenulatus* lipase	Site-directed mutagenesis	L353S
Improvement of a ctivity of aldolase reaction	*C. antarctica* lipase B	Site-directed mutagenesis	S105A
Improvement of alcoholysis activity	*R. oryzae* lipase	Error-prone PCR	K138R
	R. arrhizus lipase	Site-directed mutagenesis and DNA shuffling	E190V
Improvement of temperature stability	*P. expansum* lipase	Site-directed mutagenesis	K55R
Improvement of resistance to organic solvents	*Pseudomonas* sp. lipase	Error-prone PCR	F146L; I289T; V304A
	C. antarctica lipase B	Site-directed mutagenesis	M72L
Change of optimum pH	*R. arrhizus* lipase	Site-directed mutagenesis and DNA shuffling	E190V

3.4 Fermentation of Lipase-producing Strains

Different strains have different fermentation conditions for lipase production. Factors affecting lipase fermentation are nitrogen, carbon, substrate induction agent, surfactants, pH, culture temperature, rotation speed, incubation time, inoculum amount, and so on. In order to improve the level of expression of exogenous protein, the best fermentation conditions are researched. There are two recommended fermentation methods:

3.4.1 Two-Step Fermentation Method

Fermentation is divided into two stages, namely cell growth and exogenous protein expression. In biomass growth stage, only a small amount of inhibitor (usually glycerol) is able to control the rapid growth of the cells, and the expression of alternative oxidase (AOX) is suppressed. In the expression phase, exogenous protein induced expression can be obtained after exhausting the remaining glycerol and adding methanol, when the yeast growth is relatively slow. Usually the secretion of exogenous protein is at the peak level when induced by methanol for 150–200 h. This method can produce high yields of recombinant proteins, but it is more time-consuming.

3.4.2 Mixed Fermentation Method

AOX is increased between the growth and expression stage in this method. Glycerol is added according to the required growth-limiting, not only in the transitional stage but also in the methanol induction stage. This methanol–glycerine combined approach allows some expression strains to grow more actively and secrete protein more rapidly, and the expression stage is relatively shortened. In addition to the above factors, expression of recombinant protein is also effected by the selection of the host bacterium, culture conditions, the protein itself, and so on. Even if fermented strains use the same host strains and the same recombinants, expression levels are often not the same, so screening of high expression strain is very important. In addition, it is necessary to optimize the culture conditions to improve protein expression.

Section 4: Microbial Oil Production and Its Application

4.1 Microalgal Oil Production and Utilization

Mass production and utilization process for microalgae is a complex systematic engineering work encompassing a number of technical steps, mainly including large-scale cultivation to attain considerable microalgae, microalgae harvesting, drying and lipid extraction, and turning the harvested microalgae into biodiesel and other products for comprehensive application. [69–71]

4.1.1 Large-Scale Cultivation of Oleaginous Microalgae

Large-scale microalgae cultivation is a precondition for driving microalgae to commercial applications. Therefore, exploring and developing novel high-performance

microalgal culture systems and achieving high-density cultivation of microalgae have become important aspects of microalgal biotechnology [72, 73].

At present, microalgae culture systems are primarily photoautotrophic. Two main types of reactors are used in photoautotrophic cultivation, namely, open photobioreactor (PBR) and closed PBR [74, 75].

4.1.1.1 Open PBR

The so-called open PBRs refer to open pond culture systems, mainly consisting of four types: shallow ponds, water circulating ponds, raceway ponds, and natural ponds. Among these, the open raceway ponds are the most typical and have been used by most of the companies commercializing large-scale microalgae cultivation. Nowadays, the open raceway PBRs simulate microalgae living environment in natural lakes and utilize man-made open water surface culturing facilities with sunlight as the energy source. The culturing facilities are usually made of cement or polyethylene, are 0.1–0.3 m deep, and have an area as large as thousands of square meters, where culture medium is mixed and circulated by means of impeller rotation (Figure 6.4). To prevent contamination and reduce water evaporation during production, the ponds are often covered from above by some materials such as light-transmitting films. In the design and operation of raceway-type microalgae culture ponds, agitation plays a key role in enabling microalgae to come into contact with sufficient sunlight and CO_2. Water flow is required to have enough velocity to prevent microalgae from settling down to the pond bottom. Normally, a velocity of 10–20 cm/s is able to prevent cell settlement effectively, and the higher the velocity, the better the effect. Nonetheless, when the velocity is greater than 30 cm/s, the whole system consumes excessive energy, thereby hampering its economics [76].

(a)

(b)

Figure 6.4: Images of open PBRs

Open raceway ponds are advantageous in terms of low construction cost and ease of maintenance and cleaning, but open PBRs still have the following drawbacks: (1) being susceptible to external environment, making it difficult to maintain suitable temperature and illumination; (2) being susceptible to contamination from dust, insects, and heterogeneous strains, making it difficult to maintain high quality cultivation of single alga; (3) low utilization of light energy and CO_2. All these factors will result in lower cell culture density, making harvesting cost higher. Microalgae strains adaptable to large pond cultivation have to be capable of growing quickly in extreme environment. For the microalgae strains requiring mild culture conditions and having poor population competence, cultivation can be made only in closed PBRs.

4.1.1.2 Closed PBR

In contrast to the open cultivation system, a closed PBR is not directly exposed to air. Instead, it is covered by one layer of transparent material or composed of some transparent tubes, in order to enhance the photosynthesis efficiency and increase the cell density of microalgae by decreasing pathlength of light and increasing illumination area. Closed PBRs have the following advantages: controllable algae culture conditions and growth parameters, enabling a stable culture environment; ease of contamination control, making sterile culture achievable; long production period all year round with high yield; and facilitating high culture density, which lowers harvesting cost to some extent. Nowadays, closed PBRs are mainly of the tubular type or flat plate type. Most of the novel closed PBRs developed in recent years are based on tubular or plate PBRs, such as columnar airlift type, mixing tank type, and floating plastic film bag type [77].

The tubular PBR (Figure 6.5a) is one kind of extensively applied closed PBR, consisting of enclosed tubular light collection unit, circulating power pump system, culture medium mixing and gas exhausting system, and temperature-controlling system. Driven by the circulating pump, the microalgae culture medium flows circularly within the enclosed loop. The circulating pump can be a mechanical pump, airlift pump, or air-operated diaphragm pump. In order to reduce the damage to microalgal cells due to mechanical shear of the circulating pump, the airlift pump and air-operated diaphragm pump are better choices. There are various forms of the enclosed tubular light collection unit, which are either mounted on the base by means of horizontal coiling or installed in upright fences, mainly aiming at increasing illuminated area of the algal culture. The tubing can be made of glass or transparent plastic pipes. To prevent the attachment and residue of algal cells inside the reactor tubing, flow rate of algal culture in the tubing may be suitably increased. Moreover, the reactor internal wall should be as smooth as possible, to avoid deposits in gaps and cleaning problems.

In the enclosed tubing, microalgal photosynthesis will produce oxygen gas. The longer the tubing, the higher the concentration of dissolved oxygen; it is recommended

(a) (b)

Figure 6.5: Images of closed PBR: (a) Tubular PBR; (b) Flat plate PBR.

that the length of continuously enclosed tubing for PBR is no more than 80 m. Excessively high concentration of dissolved oxygen has poisoning effect on microalgal cells and generates stress to microalgal growth. Therefore, when flowing circularly in a PBR, microalgal culture enters the mixing and gas exhausting unit periodically for "gas exchange" (emission of dissolved oxygen and addition of carbon dioxide) of culture medium.

Flat plate PBRs (Figure 6.5b) were studied slightly later than tubular PBRs, and they have the advantages of larger specific area of illumination, shorter light path, and smaller footprint area. A plate-type PBR is comprised of rectangular transparent vessels with thickness of 1–30 cm, which are normally made of transparent lamellar material facilitating light capture, and are placed obliquely or vertically to increase the illuminated area. Compared to tubular PBRs, plate-type PBRs have larger illuminated area, thus enhancing photosynthesis efficiency. Also, air ventilation is used to facilitate culture mixing and turbulent flow, thus there is less damage to microalgal cells; dissolved oxygen is lower, and capital and maintenance costs are less.

Compared to open cultivation system, closed PBRs increase microalgae density and yield, and simplify cell harvesting, but the high construction and operating costs become the bottleneck for large-scale application of such reactors. Currently, these reactors are used for quick low-volume cultivation of axenic microalgae, for example, providing inoculum algae for large-scale large pond cultivation of oleaginous microalgae, or production of high-value microalgae.

4.1.2 Collection of Oleaginous Microalgae

Biomass harvesting is a process of separating biomass from free water in culture medium and augmenting concentration of suspended solid particles. As microalgal cells are very small in size (normally around 3–20 μm in diameter) and biomass

concentration in culture medium is relatively low, selection of a suitable cell harvesting method is of great importance to improving harvesting efficiency and lowering harvesting cost [78].

Commonly used algae harvesting methods at present mainly include centrifugation method, filtration method, flocculation-sedimentation method, and flotation method.

4.1.2.1 Centrifugation Method

Centrifugation method is a forced mechanical separation method commonly used in bioseparation, and almost all microalgae can be isolated by means of centrifugation. Normally, microalgal biomass is able to yield a harvesting rate above 95% when centrifugal force reaches $13{,}000 \times g$, and when centrifugal force is $6{,}000 \times g$ and $1{,}300 \times g$ respectively, the harvesting rate falls to 60% and 40% respectively. Currently, high-speed flottweg separator with automatic residue discharging function is widely applied in microalgae isolation; it is capable of continuously treating microalgal culture medium in production, which is essential for large-scale water treatment. This method is simple to operate and easy for motorized control, but involves high investment in equipment, high energy consumption and large mechanical force. For some filamentous or cell wall-less microalgae, mechanical vibration during centrifugation readily causes cells to break or disrupt. As a result, the physiological activity of microalgae is affected, and also the released cell content readily contaminates culture medium to lower its value in circulation use [79].

4.1.2.2 Filtration Method

For microalgae having a certain length of filaments, cell harvesting can be accomplished by direct filtration with screen cloth, filter fabric, vibratory screen, or microstrainer. For example, *Spirulina* harvesting usually uses inclined sieve filtration method to gather algae-containing culture medium via water pump. The sieve surface for filtration is normally a 260–300 mesh screen cloth. The *Dunaliella salina* culture medium can be harvested with ceramic hollow fiber membrane, which allows saline water reuse, resulting in low saline consumption, high yield, simple process and low energy consumption [80].

Because a large number of microalgae have tiny cells and there is only a small difference between cells and culture medium in density, selection of an appropriate filter membrane is very important. Large membrane pores would fail to filter while very small pores would be readily blocked. Moreover, as algal matter is lightweight and thus readily floats up, it is difficult to form filter cakes and high filter pressure is needed as well. In case of harvesting microalgae by filtration, attention must be

paid to filter membrane selection, control of operating conditions, lowering filter layer thickness, and decreasing filtration resistance, so that micropores of filter medium will not be blocked.

4.1.2.3 Flocculation-Sedimentation Method

As microalgal cell surface carries a certain amount of negative charge, addition of appropriate amount of flocculants can destabilize suspended cells and help them aggregate into large particles so as to settle down naturally, thereby achieving the objective of concentrating and isolating microalgal cells. Nowadays, flocculants widely applied in industries can be roughly classified into three major types, inorganic flocculants, organic flocculants, and bioflocculants [81].

Flocculation is a very complex physiochemical process, and in summary, there are four types of interactions between flocculant hydrolyzates and micelles: compressed electric double layer, adsorption-charge neutralization, adsorption-bridging, and sweeping-enmeshment. In fact, the addition of flocculants to aqueous solution to make micelles destabilized involves three interactions (micelle versus flocculant, micelle versus aqueous solution, and flocculant versus aqueous solution). A single mechanism is not able to account for all the phenomena. Flocculation process is actually a result of integrated action of several mechanisms, or dominated by a certain mechanism under particular water quality conditions.

4.1.2.4 Flotation Separation Method

As a high-performance, fast, solid-liquid separation technique, flotation method finds applications in wastewater treatment, cell separation from biofermentation broth, and microalgae isolation and harvesting. This method utilizes highly dispersed microbubbles as the vehicle to adhere suspended matter in the system such that their density becomes smaller than that of water and the suspended particles float up to the water surface, thereby fulfilling solid-liquid separation process. The operating process of flotation method is partly similar to that of flocculation-sedimentation method. Normally, flocculants are added to the suspension prior to separation, so that algal cells flocculate and form a large number of highly dispersed tiny bubbles in water. Then, by means of bubble versus floc collision and adhesion, the bubbles undergo nucleation and grow larger in flocs, and by means of flocs enmeshing, wrapping and bridging bubbles, the bubbles and flocs form the bubble-floc coaggregated complexes and ultimately float up to liquid surface. Then, they are scraped by residue scraper into storage tank to fulfill microalgae harvesting objective. This method has the advantages of relatively simple equipment and process flow, mild operating conditions, minimum damage to cells, rapid treatment, and relatively high separation effect [82].

4.1.3 Oleaginous Microalgae Drying

Microalgae can be more stable to continue downstream work after drying, which would also benefit the conservation of microalgae biomass. Several simple microalgae drying methods are listed as follows [83–88]:

4.1.3.1 Rotary Drying

By using an inclined rotating cylinder, the algae mud is rotated from one end to the other by gravity. However, this method is energy intensive: for heating up microalgae with 30% solid content to 120 °C in 10 s, the energy consumption will be 50 Kwh.

4.1.3.2 Spray Drying

In this drying method, the moisture content of material is evaporated rapidly by spraying the liquid into droplets dispersed in a hot stream by sprayer. It can be completed in a few seconds, and the dried product is removed from the bottom of the instrument. However, the thermal efficiency is not high and heat consumption is large.

4.1.3.3 Solar Thermal Drying

The moisture in solid materials can be evaporated and diffused into the air by using solar energy. Solar energy is absorbed by the dry algae mud directly or indirectly. Energy is transmitted to the material inside, so as to achieve the purpose of drying. This method requires less energy consumption and temperature. Between 40 and 70 °C, the content of water can be dried to 4–8% in 5–6 h.

4.1.3.4 Vacuum Freeze Drying

In this method, algae mud is frozen and the water present in the sample is turned into ice crystals. Then, under high vacuum, the water is sublimated so as to achieve the purpose of drying. In dry conditions, Arabic gum and gelatin can protect cells from damage, and new cells can be affected by a stronger anti-temperature difference.

4.1.4 Oleaginous Microalgae Oil Extraction

There are three commonly used methods for extracting algae oil: mechanical crushing method, organic solvent (*n*-hexane) extraction, and supercritical CO_2 extraction.

There are some other auxiliary extraction methods, such as microwave-assisted extraction, enzyme extraction method, and so on.

4.1.4.1 Mechanical Crushing Method

Mechanical crushing method involves the use of mechanical force to squeeze the oil out. It is the primary method of oil extraction from plants and is applied to the microalgae oil extraction also, but algal cells must be dried first. The methods of crushing and squeezing are simple and varied, the process is safe and the maintenance of production equipment is convenient.

4.1.4.2 Organic Solvent Method

Organic solvent extraction of oil is the most commonly used method. The principle of solid-liquid extraction is to use suitable organic solvents which are capable of dissolving grease to extract lipids after exposure to microalgae, by soaking or spraying, and so on. The mixed oil is obtained first, and then the more pure oil is separated from mixed oil. The solvent vapors are collected by condensation. Soxhlet extraction is one of the most commonly used methods. Microalgae oil can be extracted with benzene and ether, but it is widely extracted with hexane solvent now, because the latter is relatively inexpensive. However, the solvent which is used is not only explosive but also toxic. Also, the production process is not safe and may cause solvent residue in oil.

4.1.4.3 Supercritical CO_2 Extraction Method

Supercritical fluid has excellent solubility which can change with temperature and pressure, so that substances with different polar components, low boiling point, and molecular weight can be extracted selectively and sequentially. The process of supercritical fluid extraction is a combined process of extraction and separation under low pressure and high temperature. CO_2 is generally used as an extracting agent which has the following advantages: low operating temperature; not harmful for the effective extracted components; most of the components can be extracted under the optimum process conditions which improves the recovery rate of the product; the process of extraction is simple, efficient, and nonpolluting; supercritical fluid can be recycled after refining; and CO_2 is abundant, inexpensive, nontoxic, nonflammable and nonexplosive.

4.1.4.4 Ultrasonic Assisted Extraction Method

The frequency of ultrasonic sound waves is above 20 KHz. Ultrasonic extraction is a physical crushing process under unique mechanical vibrations and cavitation. When

the ultrasonic vibration is disseminated, it can produce a powerful energy, causing the medium into the vibrating state with large particle velocity and acceleration. As a result, the media's structure is changed, prompting the active ingredient into the solvent. At the same time, cavitation effect can happen in the liquid. Cavitation bubble is formed in the case of considerable damage stress. Cavitation occurs when the intensity of the ultrasonic radiation surface reaches 0.3W/cm^2 in the water. In addition, many secondary effects of ultrasonic waves such as heating, ultrasonic emulsification, diffusion, smashing, chemistry effects, biological effects, and flocculation effects can accelerate the effective diffusion of the components of extract into the solvent. This allows the active ingredients to be mixed fully with solvent, which is conducive to extraction.

4.1.4.5 Enzyme Extraction Method

Enzymes extraction method is a new method in recent years which is based on mechanical and enzymatic degradation of plant cell walls to release oil, also called aqueous enzymatic extraction. Vegetable oil combines with other macromolecules (proteins and carbohydrates) in the cell and forms lipopolysaccharides, lipoprotein complexes, and so on. Aqueous enzymatic method, based on mechanical crushing, involves decomposing the oil using some complex degrading enzyme (such as cellulase, hemicellulase, pectinase, amylase, glucanase, and protease) from lipopolysaccharide, lipoprotein, and so on. Enzymatic hydrolysis oil extraction technology has the following advantages: raw materials without drying, edible vegetable oil obtained by enzymatic hydrolysis and centrifugation, separation of oil and protein at the same time to shorten the process route; mild operating conditions and higher quality of extracted oil and protein, especially for high moisture fuel. Since fat is released under mild conditions, it can improve the quality of oil and by-products and increase the extraction rate. The enzymolysis proceeds in the aqueous phase and later phospholipids enter into the water phase, so the oil can be obtained without degumming. Moreover, enzymolysis process is simple and requires less energy, and also it can make full use of the protein after extraction.

4.1.5 Comprehensive Utilization of Microalgae

With the rapid progress of society, biofuels will play a more and more important role in energy field as the fossil energy sources are being depleted. Biodiesel is a potential renewable liquid fuel and it has been concluded that the use of microalgae for the low-cost production of biodiesel is technically feasible, but its industrialization is limited due to manufacturing costs. Producing algal biodiesel requires large-scale cultivation and harvesting systems, with the challenge of reducing the cost per unit area. Such processes are most economical when combined with sequestration of CO_2

from flue gas emission, with wastewater remediation processes, and/or with the extraction of high value compounds. Also, building a large reactor and using CO_2 from power plant for culture enrichment and replacing oil to reduce GHG emission can help improve the economic benefits of oil from microalgae [83, 89, 90].

4.1.5.1 Microalgae for Medical Industry

Natural β-carotene is widely believed to have a positive effect on tumors, radiation hardened leukocytes, atrophic gastritis, oral ulcer, skin diseases, and so on. The products from microalgae are natural carotene syrups, granules, buccal tablets, water dispersive powders and others. In recent years, the addition of unsaturated fatty acids (DHA, RHA) as a dietary supplement for babies is popular among consumers, and also welcomed in health care products. Moreover, the experts are paying more attention to microalgae colloid (ECP) which has potent antitumor activity.

4.1.5.2 Food Industry

Crude protein content in algae is more than 60%, which is higher than that in any other crops. Algae protein production is booming. *Chlorella, scenedesmus, closterium*, *spirulina maxima* have been used as protein sources. Moreover, *chlorella, spirulina maxima,* and *Dunaliella* are also available in the form of powder, pills, and extracts in the health care products market or used as food additives.

4.1.5.3 Detection of Environment

The growth of microalgae can directly reflect the quality of water and determine the toxic gases in the air. It can match the conventional gas sample analysis and detection. Fixing chlorella on a hydrophobic membrane and connecting membrane electrode to the bioreactor can reflect the content of methanol steam and tetrachloroethylene in the air.

4.1.5.4 Environmental Purification

Chung and coworkers combined the production of single cell protein (SCP) with wastewater treatment by anaerobic fermentation of swine manure to produce methane. The result shows that the production of *spirulina* is 5 g/m^2/day. Using membrane hanging reactor can separate algae and water. Currently, wastewater treatment by removal of NH_4^+, NO_3^-, and PO_4^{3-} ions mostly depends on the activity of microorganisms that can use these water contaminants as nutrients and lower their levels to

meet the national discharge standard. It is possible to combine microalgae growth with a pollution control strategy of other industry. Microalgae can remove nitrogen, phosphorus, refractory organics, Co, Mn, Hg, and other heavy metal ions. Moreover, microalgae can absorb certain concentrations of NO_x, SO_x, and H_2S. Norway and Japan have researched microalgae cultivation for environmental protection for a long time.

4.1.5.5 CO_2 Emission Reduction

Carbon makes up almost 50% of the dry weight of microalgae, and the microalgae growth needs a lot of carbon source. Therefore, to use flue gas emission from an industrial process unit (e.g. from fuel-fired power plants) as a source of CO_2 for the microalgae growth is envisioned to have a great potential to diminish CO_2 pollution and to provide a very promising alternative to current GHG emissions mitigation strategies.

4.2 Biodiesel Preparation Catalyzed by Lipase

Yeast Lipase, Rhizopus lipase, Mucor lipase, and *porcine pancreatic lipase* are capable of catalyzing transesterification reaction of triglycerides with short-chain alcohols to produce biodiesel in lipophilic organic solvents or supercritical medium. Chemical production of biodiesel involves a complex process, high energy consumption, environmental pollution, and other shortcomings. Compared with chemical methods, lipase-catalyzed reactions have many advantages: lower demand for raw materials (refined oil, raw vegetable oil, free fatty acids, and catering waste oil can be directly esterified by lipase), no need for original seed oil pretreatment, mild reaction conditions (the reaction temperature is usually between 20 °C and 60 °C), a small amount of alcohol, a higher fatty acid ester yield, no waste, and the product is easy to recycle. In recent years, biodiesel production has attracted more and more attention [91–96]. However, there are still some problems to be solved: methanol and ethanol can lead to decrease or loss in enzyme's activity; the glycerol and water by-product is difficult to recover; this by-product not only inhibits the formation of products but also has toxicity for the enzyme; and the presence of short-chain fatty alcohol and glycerol affects the activity and stability of the enzyme. These issues are the main bottlenecks in the industrial production of biodiesel, which needs further research to be addressed.

4.2.1 Lipase Catalysis Mechanism of Biodiesel

Lipase is a glycoprotein that consists of mannose primarily, and the sugar base part accounts for approximately 2–5% of the molecular weight. Different types of lipase enzymes are comprised of different kinds of amino acids. Due to the evolutionary

process and biological homology, the catalytic center area for all the lipase enzymes is the same or has similar characteristics. The primary structure of lipase contains a five peptide conservative sequence of -Gly-Xl-Ser-X2-Gly-. Catalytic active center is a catalytic triplet including serine (Ser)–histidine (His)–aspartate (Asp). These three amino acids in highly conservative geometric orientation are located in the central hydrophobic beta fold side of the "ring", and surrounded by a "lid" which consists of two removable α-helixes with hydrophilic external surface. The "lid" covers the active catalytic sites in the "ring", so as to protect the triplet center. When in contact with the oil–water interface, the association of the hydrophilic α-helix with oil–water interface changes the conformation of enzyme, which leads to the movement of α-helix covering the active center to expose the active site. Then, the substrate can easily enter the hydrophobic channels and combine with the active site. Three amino acids in the catalytic triplet begin to interact with each other to generate active site of reactive oxygen species. Afterwards, the reactive oxygen atoms start to attack the substrate through nucleophilic addition reaction to form enzyme-substrate compounds. Then the proton in the n-terminal amino of the lipase transfers to the substrate oxygen atom followed by bond-breakage. At this point, triglycerides are transferred into diacylglycerol, and a free glycerin-OH group is immediately formed accompanied by the combination of fatty acid and the enzyme molecule. The methanol CH_3O^- group is added into the C=O group from enzyme-fatty acid compounds with the formation of intermediate phthalein-based enzyme-alcohol compound. Through the proton transfer, the intermediate pulls away from the enzyme molecules to form biodiesel [97–99].

4.2.2 Substrate Specificity of Lipase

The spatial structure of different types of lipase is highly similar, and all of them belong to the family of a/β type. However, because of the different sources, lipase with different structure has different substrate specificity. The substrate specificity of lipase includes: fatty acid specificity, location specificity, and stereo specificity [100, 101].

4.2.2.1 Fatty Acids Specificity

Fatty acid specificity refers to the different activities of lipases toward the grease substrates with different chain lengths, degree of saturation, and positions of double bond. The specificity of hydrolyzing triglyceride fatty acid for lipases from different sources is greatly different. For example, *Penicillium cyclopium* lipase has high catalytic activity for the fatty acid whose carbon main chain is below C8, *Aspergillus niger* lipase is most suitable for the fatty acid with medium length chain C8-C12, *Geotrichum candidum* lipase shows strong specificity for oleic acid, *Staphylococcus hyicus*

lipase prefers phospholipids as optimum substrate and it also hydrolyzes other fatty acids with different chain lengths, *Staphylococcus aureus* lipase has specific effect on triglyceride formed by short-chain fatty acids but is unable to hydrolyze triglyceride formed by long chain fatty acids and phospholipids, and the lipases from *Penicillium camembertii* U2150 and *Aspergillus oryzae* act only on monoacylglycerol or diacylglycerol and have no effect on triglycerides on their own, but they can assist or accelerate the hydrolysis of triacylglycerol in company with lipases which are able to hydrolyze triacylglycerol. *Geotrichum candidum* lipase has different specific hydrolytic activities for the unsaturated fatty acids with different positions of the double bond in the substrate.

4.2.2.2 Position Specificity

Triglyceride contains three ester bonds, so it can be hydrolyzed by lipase in three positions. The position specificity of lipase is the difference in the recognition and hydrolysis of triglyceride in 1 (or 3)-position and the 2-position. According to different locations of the hydrolysis, lipases are divided into: α-type lipase which only hydrolyzes substrates in 1- and 3-position, β-type lipase which only hydrolyzes substrates in 2-position, and αβ-type lipase which hydrolyzes substrates in all positions. Most lipases belong to α-type lipase. For example, *Aspergillus niger* lipase, *Rhizopus* sp., *Pseudomonas aeruginosa* lipase, and lipoprotein lipase (pure enzyme) from milk and lipoprotein lipase from humans can only selectively hydrolyze ester bonds in the 1, 3-position, and have no effect on the 2-position ester bond. Some lipases can transfer the acyl group in 2-position with slower nonenzymatic catalytic rate to 1 (or 3) -position, so as to further hydrolyze the fatty acid in 1, 3-position; at this time the α-type hydrolysis becomes β-type lipase hydrolysis. The lipases belonging to this type are *Pseudomonas fluorescens* lipase, *Candida rugosa* lipase, *Rhizopus arrhizus* lipases, and *Cryptococcus neoformans* lipase. In addition, *Geotrichums P.* lipase and lipoprotein lipase from the adipose tissue of rat can only hydrolyze 2-position ester bonds, and do not have 1, 3-position specificity which belongs to β-type lipases.

4.2.2.3 Stereo specificity

Stereospecificity refers to the recognition and selective hydrolysis of 1, 3-position of the ester bond in the stereo mapping structure of triglyceride. For example, *Pseudomonas fluorescens* lipase has high stereo selectivity in catalyzing the chiral resolution of racemic compounds, and it can selectively hydrolyze phthalein glyceryl in 1 and 3 positions, but its speed of hydrolyzing phthalein glyceryls in 2, 3 positions is much faster than that of its enantiomer.

4.2.3 Factors Affecting Lipase-Catalyzing Transesterification Reaction

The process of preparing biodiesel by enzymatic method is influenced by many factors, and the mechanism and degree of influence are different [102–105].

4.2.3.1 Methanol to Oil Molar Ratio

In the transesterification reaction, the effect of TG on the reaction is promoted. However, the low carbon alcohols such as methanol, due to strong polarity and strong hydrophilicity, deprive the lipase of essential water and cause very strong damages to the enzyme protein, which then affects the stability and activity of the enzyme. At the same time, because of the immiscibility of alcohols and triglycerides, the combination between the substrate is not smooth, which also hinders the reaction. Currently, the approach for eliminating or reducing the effect of low carbon alcohol on the enzymatic inhibition is to add co-solvent, prompting the short-chain alcohols to dissolve in organic solvent, changing the substrate concentration in water layer which is necessary for lipase. Then, it is not easy for the hydrophobic organic solvent to capture the required water for lipase, which then decreases the substrate ground level or increases the lipase–substrate complex level. Finally, the enzyme activity is affected and the enzyme catalyzed reaction speed is changed. Another method is add the alcohol step by step to mitigate the effect of excessive short-chain alcohol on enzyme, or using other phthalidyl acceptors in place of short-chain alcohols, which completely avoids alcohol damage on enzyme protein.

4.2.3.2 Enzyme Dosage

In the enzymatic reaction, the reaction rate is proportional to the enzyme concentration if the substrate concentration is high enough to make the enzyme saturated. But the actual situation is quite different. When the enzyme concentration is low, the reaction speed is less than ideal. When the enzyme concentration is high, the excess enzyme will agglomerate and increase the resistance of mass transfer, which will affect the process of reaction.

4.2.3.3 Reaction Temperature

Temperature has a great influence on the enzymatic reaction. Each enzyme catalyzed reaction has an optimum reaction temperature. Before reaching this optimum temperature, the reaction rate increases with temperature, and after reaching it,

further increase in temperature will cause the enzyme to deactivate gradually and decrease the reaction rate. Most of the enzymes will denature over 60 °C, and a relatively small number of enzymes can endure high temperature. The high temperature inactivation of the enzyme is because with increase in temperature, the molecular heat of the enzyme protein also increases. The increase in heat energy causes breakage of the noncovalent bonds which maintain the three-dimensional structure of enzyme as well as its activity, thus resulting in the loss of conformation activity of the enzyme protein.

4.2.3.4 Water Content

The hydration of the enzyme protein is necessary for the maintenance of the active conformation of the enzyme, and the activity of the enzyme is closely related to the water content in the reaction system. Under absolute anhydrous conditions, the interaction of the charged group and polar group in enzyme protein forms a closed and inactive structure. When a certain amount of water is added to the system, the noncovalent bonds are formed, and the enzyme molecules begin to become soft. The activity center polarity is enhanced, which improves the enzyme activity. However, excess water in a nonwater system can not only make the enzyme aggregate and increase the mass transfer resistance but also reduce the catalytic efficiency of the enzyme and accelerate enzyme inactivation. Moreover, the presence of water will also result in the occurrence of methyl ester hydrolysis.

4.2.4 Lipase Immobilization

Lipase has high catalytic activity and selectivity, but free lipase is not stable at extreme conditions, such as heat, strong acid, strong base, organic solvent, and so on. In the catalytic reaction, lipase can easily agglomerate and lose activity or be degraded by microorganisms. Free lipase has poor tolerance to the environment and it is difficult to achieve operation and automation for lipase-catalyzed reactions. Also, it is difficult to separate lipase from substrate, resulting in impure products and poor recovery and reusability of the lipase.

In order to overcome the above shortcomings, researchers have explored combining lipase and an insoluble carrier together, making a kind of enzyme derivative which is insoluble in water, but also maintains most of the enzymatic activity during the catalytic reaction. Therefore, the concept of 'immobilized lipase' has grown. Compared with the free enzyme, the immobilized enzyme has good stability and is suitable for industrial production. It is easy to be separated from the substrate and the product. It can be easily purified and used repeatedly to reduce the production cost. The reaction condition is easy to control, which is suitable for continuous industrial production.

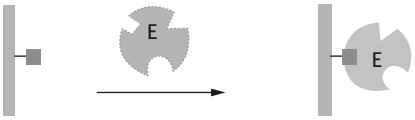

Figure 6.6: Adsorption method

There are four types of methods for the immobilization of lipase, including adsorption method, covalent binding method, cross linking method, and embedding method [106–109].

4.2.4.1 Adsorption Method

Adsorption method involves using ionic bonds or physical adsorption to fix the enzyme on carriers such as cellulose, agarose, porous glass, ion exchange resin, and so on. (Figure 6.6)

The adsorption method can be divided into two kinds, the physical adsorption method and the ion adsorption method. Physical adsorption method uses hydrogen bond, hydrophobic bond, electron affinity, and so on. to fix enzyme on the carrier. The carriers include porous glass, activated carbon, acid clay, bleaching clay, kaolin, alumina, silica, bentonite, hydroxyapatite, calcium phosphate, ceramics, metal oxide, starch, albumin, macroporous resin, butyl or hexyl dextran gel, cellulose and its derivatives, chitin and its derivatives, and so on. Ion adsorption method uses the interaction between the side chains of the enzyme and the ion exchange groups to immobilize enzyme in the appropriate pH value and ionic strength conditions. There are literature reports that lipase was immobilized onto 2-ethyl cellulose and anion exchange carriers such as Duolite (ion exchange resin). The carboxylate and macroporous ion exchange resins, such as IRA DEAE-SephadexAS0 and Amberlite IRA 94, are also effective carriers for lipase immobilization.

The operation for adsorption method of immobilizing enzyme is simple and can be divided into four categories:

(1) Static procedure

In the static procedure, there is no stirring or shaking involved. The carrier is directly added into the enzyme solution and the immobilized enzyme is prepared through natural adsorption, desorption, and re-adsorption process. But this method is inefficient, time-consuming, and is not uniform.

(2) Electro-deposition procedure

In this method, two electrodes are placed in the enzyme solution, and one electrode is placed near the carrier. Upon passage of electricity through the electrodes, the enzyme is deposited onto the surface of the carrier and immobilized. In the process of deposition, some ions related to enzyme activity and stability can be added to compensate for any loss or damage.

(3) Dynamic procedure

Dynamic procedure is a commonly applied method to immobilize enzymes in the laboratory. Different from the static procedure, dynamic procedure is conducted by mixing the carrier and enzyme solution under continuous stirring or shaking. In this case, the immobilization will be far more than that of the static method. The speed of stirring or shaking must be adjusted to protect the structure of enzyme and carrier from being damaged as well as sufficiently mix them.

(4) Reactor loading process

Reactor loading process is commonly applied in industrial scale. It can combine the further application of immobilized enzyme and its immobilization process. This method can be adjusted to be applied in CSTR or PBR method.

The advantages of adsorption method include its simple procedure, mild reaction conditions, high reusability of carrier, and high enzyme activity recovery. However, the adsorption method also has several disadvantages. For example, the enzyme concentration can only be determined by experience, and the pH, ion strength, reaction temperature, and time vary with different enzymes and carrier combinations. The loose connection between enzyme and carrier will lead to reduced enzyme activity or product quality. All of these issues restrict the application of adsorption method. However, the adsorption method is commonly used in combination with other procedures in scientific research to widen its application performance.

4.2.4.2 Embedding Method

The embedding method involves embedding the enzyme molecule into the structure of polymer material to allow the substrates to penetrate into and contact with the enzyme as well as to prevent the release of enzyme protein. This method can easily cover the enzyme with some kind of material and the enzyme is less damaged. However, this method cannot be applied for enzymes with large-size substrate.

(1) Gel embedding procedure

The gel embedding procedure involves embedding the enzyme into the space inside the cross-linked insoluble gel (Figure 6.7): the enzyme is firstly mixed with monomer, then the polymerization is initiated by adding initiator to the mixer. After that, the gel immobilized enzyme can be successfully synthesized. PAGE is the first method used for enzyme embedding. Recently, natural materials such as alginate and KCarrageenan were used in enzyme embedding research. Alginic acid sodium, which is extracted from alga, can be made into a gel by multivalent ions such as Ca^{2+} and Al^{3+} through an easy and economic method. For instance, the immobilized *Rhizopus delemar* lipase which was embedded into alginic acid sodium gel displayed higher thermal stability. This immobilized enzyme was tested in the synthesis of isoamyl valerate in nonwater solution. After repeated tests for six times, it still retained 95%

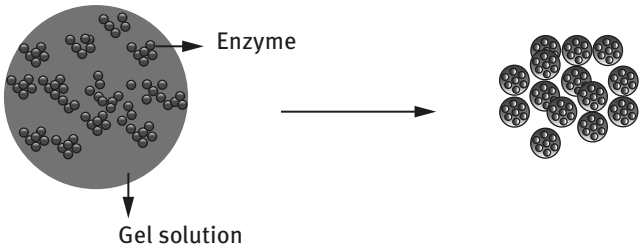

Figure 6.7: Gel embedding method

of its original activity. KCarrageenan is a polysaccharide extracted from carrageen. By mixing the aqueous enzyme with KCarrageenan in physiological saline solution at 40–60°C, the immobilized enzyme was formed by a cooling-down-gelation and a hardening process in 0.3M KCl aqueous solution. The product can be further hardened using curing reagents such as tannins and glutaraldehyde, to produce immobilized enzyme with better stability. With simple process and mild operating temperature, this method can be widely applied for immobilization of other kinds of enzymes.

(2) Microcapsule embedding method

Here, the enzyme is physically embedded into semipermeable polymer membrane to form microcapsules (with diameter of 1–100 μm), which allow the substrate molecules and product to pass through by free diffusion. Materials such as ethyl cellulose, polyethylene, and so on can be used to produce microcapsules. For enzyme stability consideration, hemoglobin is added into the capsules to guarantee a relatively stable environment. In addition, bi-functional reagents such as the commonly used glutaraldehyde and tannic acid can promote the cross-linking of enzyme to increase its stability.

(3) Covalent method

The covalent method involves connecting the enzyme molecule to the solid carrier with covalent bonds formed by the functional groups in both enzyme and carrier. The covalent force is relatively stronger than other methods, so the immobilized enzyme shows higher stability in catalyzing reactions (Figure 6.8). Gao et al. used sodium periodate to oxidize the canvas fiber to form aldehyde group in order to create covalent bond with lipase. The immobilized enzyme synthesized through this method showed higher tolerance to extreme temperature and pH and a longer half-life period of 140 h. Cai et al. chose GMA as the monomer and DVB as the cross-linker. By applying solid-liquid pore-forming process, the synthesized immobilized enzyme showed higher thermal stability and better recovery of activity than free enzyme. Currently, lipase has been successfully attached covalently to cyanogen bromide-activated agarose beads, cellulose, glucan gel carrier, and so on.

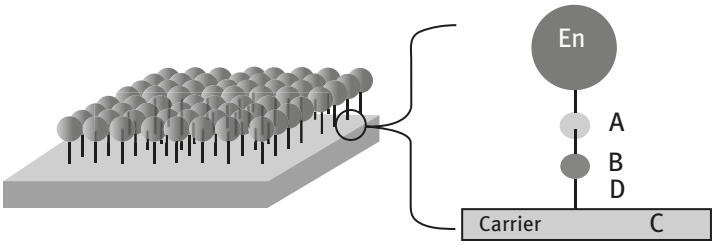

Figure 6.8: Covalent embedding method

The covalent method is the most widely adopted method in enzyme immobilization research because the enzyme molecule has many functional groups that can be covalently connected to the solid carrier: (1) amino group, from ε–amino in lysine or terminal α-NH_2 of peptide; (2) carboxyl group, from β-COOH aspartic acid, or α–COOH and terminal –COOH in glutamic acid; (3) phenolic group, from phenol ring in tyrosine; (4) sulfhydryl group: from cysteine; (5) hydroxyl group, from serine, threonine and tyrosine; (6) imidazole group, from histidine; (7) indole group, from tryptophan. The amino group, carboxyl group, and aromatic ring in tyrosine and histidine are the most commonly functional groups for enzyme covalent immobilization.

Common covalent immobilization methods include the following:

(i) Diazo method

The $-NH_2$ group in the insoluble solid carrier can be transformed into diazo compound, which is able to connect the enzyme molecule by covalent bond in the presence of dilute hydrochloric acid and nitrite. These carriers include polysaccharides, aromatic amino derivatives, amino acid copolymer, polyacrylamide, and so on.

(ii) Alkylation and arylation method

The carrier with functional halogen groups can react with the amino or thiol group (alkylation or arylation reaction) in enzyme to make it immobilized. This method commonly uses carriers such as derivatives of halide acetyl, triazine, or halide isobutylene base. For example, chloride acetyl cellulose, bromine acetyl cellulose, and iodine acetyl cellulose are all halide acetyl derivatives.

(iii) Glutaraldehyde treatment method

Glutaraldehyde reacts with polymers containing amino groups to generate templates with aldehyde functionality. The generated polymer reacts irreversibly with the enzyme protein to form immobilized enzyme, for example the amino ethyl cellulose pretreated by glutaraldehyde can be used to immobilize trypsin. DEAE cellulose, agarose amino derivatives and part of the stripping acetyl chitin, and so on can also be used as carriers in this method. In addition, the glutaraldehyde treatment method can also be applied after the enzyme protein is physically absorbed on the porous material, or connected to polyethyleneimine-pretreated silicon oxide through ion adsorption.

(iv) Peptide helation method

Spheron is a kind of macroporous hydrophilic gel formed by copolymerization of hydroxyethyl isobutyric acid ester and ethylene diisobutylene acetate. With multiple hydroxyl groups, this gel carrier can be applied in peptide chelation method for enzyme immobilization. The peptide salts used in this method are all nontoxic, noncarcinogenic, low-price and recyclable.

(v) Peptide bond method

This method involves the covalent connection between protein and the carrier through peptide bond which can be formed by the functional groups in carrier and enzyme protein, such as the ε–NH_2 in lysine acid residue or the terminal α-NH_2. For example, in the enzyme immobilization with azide derivatives, the cellulose is firstly transformed into methyl ester, then into hydrazide with hydrazine. The hydrazide then reacts with nitrite to generate azide derivatives, which can immobilize the enzyme at low temperature. Poly (methyl glutamate) can be transformed into acyl azide derivative, which can be used as a carrier for the immobilized urease. The immobilized enzyme can be made into films, balls, strips, and so on. By this method, more than 90% of the enzyme activity can be retained and good thermal stability is also obtained. In addition, the cyanogen bromide activated polysaccharides, water-insoluble polysaccharides, cellulose, cross-linking dextran and agaroscan can also be used as immobilized carriers.

In covalent method, the carrier activation and immobilization conditions must be strictly controlled to retain high enzyme activity and stability. Both the functional groups of the protein and physicochemical properties of the carriers can influence the functionality of the immobilized enzyme. Therefore, the cross-linking method is often combined with the covalent method to improve this method.

(4) Cross-linking method

The cross-linking method involves the covalent cross-linking between a multifunctional reagent and the enzyme protein to form a 3D cross-linking framework. The cross-linking can be either intermolecular or intramolecular for enzyme protein. The properties of immobilized enzyme depend on the synthesis conditions and reagents. The methods for producing immobilized enzyme using multifunctional reagent can be classified into the following categories: (1) functionalization with only enzyme; (2) cross-linking after the enzyme is absorbed on the carrier; and (3) enzyme protein connection after the carrier reacts with the multifunctional reagent. Glutaraldehyde is the most commonly used cross-linker among others, such as isocyanate derivatives, double azo benzidine, N, N'-ethylene maleic imide, and so on. The main advantage of this method is the firm connection between enzyme and carrier which makes the immobilized enzyme more stable. However, the complex procedure and the enzyme deactivation during chemical decoration also limit its wider application. Moreover, the cross-linkers are also expensive. It is often combined with other methods to guarantee a better result.

Furthermore, the noncarrier immobilized enzyme can also be synthesized using the cross-linking method. There are two kinds of noncarrier immobilized enzyme synthesis methods: direct cross-linking and indirect cross-linking. The direct cross-linking method involves synthesizing the immobilized enzyme through intermolecular or intramolecular cross-linking of the enzyme itself, but it results in small enzyme particles, low activity recovery and poor mechanical properties. Instead of the direct method, the indirect method is being widely researched. In this method, the cross-linking is performed after the copolymerization of enzyme protein. The most advanced technique in indirect method is called cross-linked enzyme crystals (CLECs). Navia et al. (from Altus Co. Ltd) firstly synthesized the enzyme crystal and then performed the glutaraldehyde treatment to produce a cross-linked enzyme crystal with high activity and stability. For complicated synthesis methods and strict enzyme crystalline conditions, the enzyme prepared by CLECs technique is quite limited.

Shelton's team (from University of Delft, Netherlands) used salts, organic solutions, or nonionic polymers to precipitate enzyme protein to form enzyme copolymer before treating it with glutaraldehyde to generate Cross-linked Enzyme Aggregates, CLEAs. Based on this method, the CLEAs of penicillin acylation enzyme and lipase from different microbial sources have been successfully synthesized for application in the production of ampicillin and high purity chemicals, respectively. Theoretically, any enzyme protein which can be precipitated can be made into CLEAs based on this method, and the CLEAs also show high stability and activity. Moreover, some kinds of lipase CLEAs even show ten times higher activity. Therefore, the CLEAs is seen as a promising enzyme immobilization method.

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Yongming Sun, Lianhua Li, Yao Wang, Tao Xing, Keqin Hu

7 Microbial for Methane Production

With the rapidly growing economic development and the increasing population, the human demands for clean energy grow substantially. Moreover, environmental issues are being accompanied by the overexploitation of fossil fuels because of massive greenhouse gas emissions. Therefore, the development of clean, efficient, and alternative renewable energy has become a global effort.

Biomass energy is the fourth largest source of energy after oil, coal, and natural gas. As an important part of biomass energy, biogas is one of the earliest approaches that is widely used.

Section 1: Principle of Anaerobic Digestion

Overall, the anaerobic digestion process is the conversion of organic matter to CH_4 and CO_2 by anaerobic microorganisms. In this process, most energy derived from organic matter is transferred into CH_4, whereas only a small portion of energy is transferred into CO_2.

1.1 Two-Stage Theory of Anaerobic Digestion

In 1906, scholars isolated pure bacteria that converted cellulose into methane. They believed that the complex organic compounds could be directly decomposed to form methane and carbon dioxide. Previous studies have shown that different microorganisms involved in the complex anaerobic digestion process. In ecological conditions with a higher oxidation–reduction potential (ORP), some protozoans (ciliates and flagellates) and facultative anaerobic bacteria were able to function during this process; however, most bacteria did not directly participate in the formation of methane [1, 2].

Based on the biochemical reaction, the methane production process was divided into two groups of microorganisms, namely fermentation bacteria and methanogenic bacteria, which are commonly referred to as the acidogenic phase and methanogenic phase. First, complex organic compounds are hydrolyzed and converted into fatty acids, ethanol, carbon dioxide, hydrogen, and hydrogen sulfide by hydrolyzing

Corresponding author: Yongming Sun, E-mail: sunym@ms.giec.ac.cn
Lianhua Li, Yao Wang, Tao Xing, Keqin Hu, Guangzhou Institute of Energy Conversion, Chinese Academy of Sciences; CAS Key Laboratory of Renewable Energy; Guangdong Provincial Key Laboratory of New and Renewable Energy Research and Development, Guangzhou 510640, China

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bacteria and acid-producing bacteria. Next, methanogenic bacteria use the products formed in the first stage to form CO_2 and CH_4.

1.2 Three-Stage Theory of Anaerobic Digestion

The two-stage theory of anaerobic digestion considered that methanogenic bacteria can convert various organic acids and alcohols into methane and carbon dioxide in second stage. However, several studies demonstrated that methanogens can only use small-molecular substance such as acetic acid, and it is challenging to explain how methanogens use more complex alcohols or organic acids.

In 1976, Bryant et al. demonstrated that ethanol fermentation by *Methanobacterium omelianskii* was the result of the syntrophic effect of two types of bacteria [3]. One type is known as hydrogen-producing acetogenic bacteria that oxidize ethanol to acetic acid and hydrogen. The other type is known as methanogenic bacteria that use hydrogen to produce methane. In 1979, Bryant proposed that the anaerobic digestion process could be divided into three stages, named the three-stage theory, which highlighted the key role of the generation and use of hydrogen during the process [4].

First phase: Complex organics are hydrolyzed by microorganism. Carbohydrates, such as cellulose and starch, are hydrolyzed into monomers, which then are broken down into pyruvate. Proteins are first decomposed into amino acids, which form organic acids and ammonia. Lipids are hydrolyzed into glycerol and fatty acids. Organic acids are further degraded to propionic acid, acetic acid, and butyric acid, as well as ethanol, hydrogen, and carbon dioxide.

Second phase: The substrates generated in the first stage were utilized by different obligatorily anaerobic bacteria to generate acetic acid, hydrogen, and carbon dioxide.

Third phase: Methanogenic bacteria convert products generated in the second phase to methane. The substrates that methanogenic bacteria utilize include carbon dioxide, hydrogen, acetate, and other one-carbon compounds (e.g., CO, methanol, formic acid, and methylamine).

1.3 Four-Stage Theory of Anaerobic Digestion

The four-stage theory involves that in the first phase complex organic compounds are hydrolyzed into organic acids and ammonia. In the second phase, organic acids are decomposed into acetic acid by hydrogen-producing acetogenic bacteria. In the third phase, homoacetogenic bacteria form acetic acid from hydrogen and carbon dioxide. In the fourth phase, methanogens convert products that were generated in the second and third phases to methane. The microorganisms mentioned above

can be divided into two groups: methanogens and non-methanogens, which mainly include hydrolyzing bacteria, acetogenic bacteria, hydrogen-producing acetogenic bacteria, and homoacetogenic bacteria. It is emphasized that under different conditions, not all anaerobic digestion processes contain all four stages, and they are not strictly sequentially alternated [5].

Section 2: Microbial of Hydrolyzing/Acidogenic and Acetogenic

Based on the physiological roles, they can be divided into hydrolyzing/acidogenic bacteria and acetogenic bacteria. With the development and improvement of separation methods, novel species are continually being discovered.

2.1 Hydrolyzing/Acidogenic Bacterial

2.1.1 Physiological and Biochemical Characteristics of Hydrolyzing Bacteria

Hydrolyzing/acidogenic bacteria are part of a complex bacterial community, which can convert complex organic compounds to acetic acid, propionic acid, butyric acid, hydrogen, and carbon dioxide in anaerobic conditions.

2.1.1.1 The Taxa, Quantity, and Nutrition of Hydrolyzing/Acidogenic Bacteria

Presently, hundreds of hydrolyzing/acetogenic bacteria have been discovered, including *Clostridium*, *Bacteroides*, *Butyrivibrio*, *Eubacterium*, *Bifidobacterium*, and *Spirochetes* [6]. In the thermophilic anaerobic digestion process, Gram-negative facultative anaerobes exist, such as *Streptococcus* and *Enterobacteria*. Several studies have shown that in the sewage sludge mesophilic anaerobic digestion process, the number of hydrolyzing/acidogenic bacteria reached 10^8–10^9/mL. In this process, protein-decomposing bacteria reached about 10^7/mL, whereas cellulose-decomposing bacteria reached 10^5–10^6/mL.

Hydrolyzing/acidogenic bacteria use carbohydrates and several metabolism intermediates as a nutrition source. Some can utilize different types of carbohydrates, whereas others only use one type of carbohydrate. For example, *Butyrivibrio fibrisolvens* and *Bacteroides ruminicola* can ferment glycosides, polysaccharides,

and many other sugars; however *Bacteroides amylophilus* only use starch and its hydrolyzates.

2.1.1.2 Functionality and Living Environment of Hydrolyzing/Acidogenic Bacteria

In the process of anaerobic digestion, hydrolyzing/acidogenic bacteria play an important role, and mainly in the following two aspects [7, 8]:

(1) Hydrolyzing/acidogenic bacteria hydrolyze large-molecule insoluble organic matters to the water-soluble low-molecule organic compounds. Hydrolase is an extracellular enzyme; therefore, the hydrolysis process is performed on the cells surface of bacteria or in the surrounding medium. Most hydrolyzing/acidogenic bacteria can utilize the hydrolyzate; however, only a part of hydrolyzing/acidogenic bacteria can secrete hydrolase.
(2) Hydrolyzing/acidogenic bacteria absorb the hydrolyzate and convert it into small metabolites by a complex system. These metabolites include organic acids, alcohols, and ketones, and were dissolved in water as a substrate.

2.1.2 Species of Hydrolyzing/Acidogenic Bacteria

Hydrolyzing/acidogenic bacteria mainly include obligate and facultative heterotrophic anaerobes. Their community changed with environmental conditions and fermentation substrate. Short generation time of hydrolyzing/acidogenic bacteria makes it impossible to breed a generation in a few minutes [9]. The main species of fermentation bacteria are shown in Table 7.1.

Table 7.1: Species of hydrolyzing/acidogenic bacteria.

Species	Typical bacteria
Cellulose-decomposing microorganisms	*Acidothermus cellulolyticus, C. stercorarium, Bacteroides succinogenes, Butyrivibrio fibrisolvens, Ruminococcus flavefaciens, C. lochheadii, C. longisporum, C. cellobioparus, C. cellulovorans, C. populeii, C. papyrosolvens, B. cellulosolvens, C. stercorarium*
Xylan (hemicellulose)-decomposing bacteria	*Bacteroides ruminicola, B. fibrisolvens, Butyrivibrio fiblvenriso, B. xylanolyticus*
Pectinolytic microorganisms	*Lachnospira multiparus, B. fibrisolvens, B. succinogenes, C. pentinovorum, C. felsineum, B. petinophilus, B. galacturonicus*
Amylolytic bacteria	*B. amylophilus, Streptococcus bovis, Selenomonas ruminantium, Succinomonas amylolytica, B. ruminicola*
Proteolytic bacteria	*C. putrificum, C. thermoputrificum, C. paraputrificum*
Lipolysis bacteria	*Anaerovibrio lipolytica*

2.2 Acetogenic Microbial

The process of H_2 and acetic acid production mainly converts organic acid (except acetic acid) and alcohol into acetic acid, hydrogen, and carbon dioxide.

2.2.1 Function of H_2-Producing Acetogens

H_2-producing acetogens are strict anaerobic bacteria, and most of those are syntrophy bacteria. Their growth and metabolism totally relies on methanogenic bacteria and other microorganisms to transfer and remove metabolites of H_2 [10].

H_2-producing acetogens are located between hydrolyzing/acidogenic bacteria and methanogenic microorganism. They play a linking role in functional niches. Moreover, they convert volatile fatty acids (VFAs) and ethanol into acetic acid and CO_2/H_2, which is then used as the substrates of methanogen. The metabolic characteristics of H_2-producing acetogens are that protons are used as the sole electron acceptor. Moreover, most of the oxidation reaction is an endergonic process under a standard thermodynamic state. Its growth and metabolism are dependent on the elimination of hydrogen or formic acid. Thus, to maintain their growth they must be cocultured with H_2-consuming bacteria. Strengthening the function of H_2-producing acetogens can promote the process of acetic acid and methane production in a reaction system, and will lead to increase in the effectiveness of the entire anaerobic digestion system [10, 11].

2.2.2 Butyrate Degradation by H_2-Producing Acetogens

In the anaerobic digestion process, VFAs that are easier to accumulation are mainly butyric acid and propionic acid. In this process, acetic acid can directly be utilized by methanogens; however, butyric acid and propionic acid must be decomposed to acetic acid and H_2 by H_2-producing acetogens.

Presently, butyrate-oxidation bacteria have been reported, including obligate syntrophomonas bacteria. Based on the analysis of the 16S rRNA sequence, a new family has been established – Syntrophomonadaceae.

Syntrophomonas wolfei are Gram-negative sporeless bacteria, with a cell size of 0.5–1.0 × 2.0–7.0 μm. There are two to eight flagellum on the cell, and this strain is sensitive to penicillin.

2.2.3 Propionate Degradation by H_2-Producing Acetogens

Previous studies have reported several H_2-producing acetogens for propionate degradation. In 1993, Stams et al. reported a coculture of mesophilic propionate-oxidizing

bacteria (MPOB) and *Methanospirillum*, and obtained a pure culture. The 16S rRNA sequence analysis showed that there was some genetic relationship between *Syntrophobacter wolinii*, MPOB, and sulfate-reducing bacteria. Both *Syntrophobacter wolinii* and MPOB can couple propionate oxidation and sulfate reduction. In another study, this bacterium was also obtained and named *Syntrophobotulus glycolicus*. MPOB oxidized glycolic acid to CO_2 and H_2 when cocultured with *Methanospirillum*.

Propionate is hard to oxidize under anaerobic conditions, and there is some relation between sulfate reduction and propionate oxidation. One type of bacteria, belonging to *Desulfobulbus*, can degrade propionate in the presence of sulfate. Boone and Bryant obtained two types of colonies using Hungate anaerobic technology with propionate as substrate, *Desulfovibrio G11* as symbiotic bacteria, and enrichment culture as inoculum. Additional studies have shown that this strain is a dual bacterial strain of H_2-producing acetogen and *Desulfovibrio G11*. It was demonstrated that more H_2-producing acetogens were obtained when propionic acid was used as a substrate. Moreover, it requires *Desulfovibrio* to participate interspecies hydrogen transfer for growth. This bacterium is known as *Syntrophobacter wolinii*, which is Gram-negative, sporeless, solitary, pairing, short chain or long chain, and is sometimes irregular and filamentous. It grows and coexists with sulfate-reducing bacteria, and only oxidizes propionic acid. The doubling time of *Syntrophobacter wolinii*, *Methanospirillum*, and *Desulfovibrio* culture (161 h) without sulfate is twice that of dual bacterial culture with sulfate (87 h).

For propionic acid oxidation, two types of symbiotic metabolic pathways exist. One is the methyl-malonyl coenzyme A pathway, the other pathway is disproportionation. The methyl-malonyl coenzyme A pathway has been found in syntrophism propionic acid oxidants, such as *Syntrophobacter*, *Desulfotomaculum thermobenzoicum* subspecies, *Pelotomaculum thermopropionicum*, and *Pelotomaculum schinkii*. The disproportionation of metabolic pathway was only observed in *Smithella propionica*. *Smithella propionica* produces acetic acid and butyric acid by disproportionation of propionic acid. Two molecules of propionic acid were polycondensed into six carbon intermediates, which were rearranged to form 3-keto acid before it was broken down into acetic acid and butyric acid. The enzymes involved in the reaction are currently unknown.

2.3 Homoacetogenic Microbial

Homoacetogenic bacteria are multinutrition bacteria, which convert organic compound or H_2/CO_2 to produce acetic acid that is the substrates for methanogens to produce methane. Homoacetogenic bacteria consume H_2 to reduce the H_2 partial pressure, which is favorable for H_2-producing bacteria. Keeping the hydrogen partial pressure in the anaerobic digestion system low is an advantage for biogas production.

Studies have shown that, in the sewage sludge, the number of these bacteria could reach up to 10^5–10^6/mL. The main homoacetogenic bacteria include *Acetobacterium woodii*, *Acetobacterium wieringae*, *Clostridium aceticum*, *Clostridium thermoautotrophicum*, and *Butyribacterium methylotrophicum*.

2.4 The Dominant Type for Different Acidogenic Fermentation

The acidogenic fermentation type seeks to determine the microbial physiological metabolic pathways based on VFA distribution in acidic end products. Substances that dominate in acidic end products are defined as corresponding metabolic types. Examples of acidogenic-phase fermentation types include butyric acid-type fermentation, propionic acid-type fermentation, and ethanol-type fermentation. The main end products of butyric acid-type fermentation are butyric acid, acetic acid, H_2, CO_2, and a small amount of propionic acid. The main end products of propionic acid-type fermentation are propionic acid, acetic acid, and a small amount of butyric acid, with a low yield of biogas. Analysis of physiological ecology confirmed that the stability of ethanol-type fermentation is superior to butyric acid-type fermentation and propionic acid-type fermentation. Moreover, the main end products of ethanol-type fermentation are acetic acid, ethanol, CO_2, and H_2. Based on the microbial conversion rate of substrates in the methane-producing phase, the conversion of acetic acid is the "rate-limiting step" for methane production. The fermentation type can to some extent be selected by controlling the rate-limiting factor. Previous studies have suggested that the main limiting factors that affect the consistency of end fermentation products in the acidogenic phase include temperature, pH value, ORP, and inorganic nutrients. Among those, the first four are controllability "causing factors" [12, 13].

2.4.1 Ethanol Type

Classic ethanol fermentation by yeast takes place under anaerobic conditions. CO_2 is the gaseous product generated; however, no H_2 is produced. Ethanol type is characterized by a particularly low ORP and pH value. Under these circumstances, the number of acidic end products becomes the limiting factor. To ensure a normal pH of the cell and maintenance of a normal metabolism, anabolic obligate anaerobic acetogen bacteria use a feedback regulation mechanism to produce ethanol. For the generation of 1 mol of ethanol in glucose–ethanol fermentation processes, 2 mol of NADH should be oxidized, while 1 mol hydrogen is generated by NAD^+/NADH coupling. Therefore, ethanol-type fermentation is very stable.

2.4.2 Propionic Acid Type

Generation and accumulation of propionic acid leads to lower pH values and a "propionic acid suppression" phenomenon. This results in inactivation of methanogens, which have an important effect on anaerobic treatment systems. Previous studies have indicated that at the beginning of anaerobic digestion, propionic acid type often occurs. The reason for this may be the higher ORP, and the presence of facultative anaerobic bacteria, which provide an ideal environment for obligate anaerobic bacteria to survive.

2.4.3 Butyric Acid Type

When the ORP is low and pH value is 5.06–6.0, butyric acid-type fermentation will occur. According to Odum's view, in the regulation of pH and the ORP, microorganism community is restricted by ecological factors in the environment, which show a certain succession process. Microorganism selected by environmental conditions will become the dominant strains. This community "evolution" process includes a functional change from a quantitative to qualitative process and includes the following three steps: (1) Complex microbial relationship between various types of fermentation microorganisms is adjusted by physiological metabolism regulations. Dominant populations will quickly adapt to environmental conditions and will grow and reproduce rapidly. (2) Inner balance and feedback regulation of a microbial community with a similar fermentation type. Metabolic products of different fermentation types control and regulate the community. (3) Niches of dominant populations in the community are similar to ecological conditions in the reactor. The dominant population at the beginning of sludge domestication is not easy to replace.

Section 3: Classifications of Methanogens

It is a challenge to classify methanogens by phenotype and nutrient characteristics. Batch et al. proposed a classification method based on the differences in 16S rRNA, which divides methanogens into three orders, four families, seven genera, and thirteen species. Recently, Boone and Whitman proposed another standard to describe the new taxa of methanogenic bacteria, which mainly includes culture purity, general morphology, susceptibility to lysis, gram staining, motility, colony morphology, substrate range, product formation, measurement of growth rate, growth conditions, DNA G + C content, electron microscopy, antigenic fingerprinting, lipid analysis, protein analysis, nucleic acid hybridization, and sequencing. Currently, polyphasic taxonomy is the most efficient method to classify methanogenic bacteria, and objectively and comprehensively reflects the position of methanogenic bacteria. An increasing

number of methanogens can be found this way, which can then be enriched for methanogens [14, 15].

In the ninth edition of the Berger classification of microorganism, recent work is summarized and presents a new classification system that is mainly based on phylogenetic development. Moreover, methanogens are classified into five orders, including Methanobacteriales, Methanococcales, Methanomicrobiales, Methanosarcinales, and Methanopyrales [3, 13].

3.1 Methanobacteriales

The main characteristics that distinguish Methanobacteriales from other methanogens are the substrates they use as well as their shape, lipid composition, and rRNA composition. Commonly, Methanobacteriales are hydrogenotrophic methanogens, which generate methane by reducing CO_2 with H_2. However, some strains can also use formic acid, CO, methanol, or secondary alcohols as an electron donor. The main polymer of the cell wall of Methanobacteriales is pseudomurein, which separates them from Methanomicrobiales. Moreover, based on their 16S rRNA, Methanobacteriales can be divided into Methanobacteriaceae and Methanothermaceae [15].

3.1.1 Methanobacteriaceae

The microorganism in the family of Methanobacteriaceae have a peptidoglycan which is chemically different from that of pseudomurein. Methanobacteriaceae are typically Gram-positive bacteria and can be classified into four different genera, such as *Methanobacterium*, *Methanobrevibacter*, *Methanosphaera*, and *Methanothermobacter*. These are all strictly anaerobic bacteria and use H_2/CO_2 as a substrate to support their growth and metabolism, although some of them can also use formic acid or CO. Methanogens in the family of Methanobacteriaceae are widely distributed in the world, but are less distributed in temperatures of over 70 °C.

3.1.1.1 *Methanobacterium*

The genus of *Methanobacterium* contains 12 species with different lengths and filaments. For example, *Methanobacterium thermoaggregans* form large multicellular aggregates. All species in this genus can use $H_2 + CO_2$ as substrates to generate methane. Some of the methanogens can use formic acid and diatomic alcohol as a substrate, such as *M. bryantii*, *M. thermoformicicum*, *M. formicicum and M. palustre*.

In general, acetic acid, cysteine, and yeast extract can stimulate growth of these microbes. For example, vitamin B is useful for the growth of *M. bryantii*, whereas basophilic methanogenic bacteria, such as *M. alcaliphilum* and *M. thermoalcaliphilum* require yeast extract. Detailed characteristics of *Methanobacterium* are presented in Table 7.2.

Currently, methanogens in *Methanobacterium* can use ammonium, sulfide, and elemental sulfur as nitrogen and sulfur sources, respectively. *M. ivanovii* can use glutamate as the only nitrogen source, whereas *M. thermoautotrophicum* can use glutamate and urea. *M. ivanovii*, *M. bryantii*, and *M. thermoautotrophicum* use cysteine, *M. ivanovii* can also use methionine, and *M. thermoautotrophicum* can use both sulfide and thiosulfate.

3.1.1.2 *Methanobrevibacter*

The genus of *Methanobrevibacter* contains 14 methanogen species (Table 7.3). No flagellum and spores were observed in this genus. They belong to Gram-positive bacteria with a pseudomureinas cell wall composition and are unable to move. Energy metabolism comes from the process of reducing CO_2 to CH_4. Formate, H_2, and CO can be used as an electron donor. The optimal temperature is 37–40 °C and the optimal pH value is around 7.0. Ammonia or N_2 can be used as a nitrogen source, and sulfate or elemental sulfur can be used as a source of sulfur. The required complex nutrients of *M. ruminantium* can be provided by the addition of acetic acid, coenzyme M, or amino acids. Acetic acid, casitone, yeast extract, and vitamin B were essential nutrients for *M. smithii* isolated from human stomach [16].

3.1.1.3 *Methanosphaera*

This genus only contains two species, namely *Methanosphaera cuniculi* and *Methanosphaera stadtmanae*. They are Gram-positive bacteria with a different spheroidal shape. They use methanol and H_2 as a substrate. The rRNA composition is similar to that of *Methanobacterium*. *Methanosphaera* have only been found in mammals, can tolerate cholate, and belong to a chemoorganotrophic type of bacteria that require CO_2, acetic acid, vitamins, and amino acids for growth. In addition, *M. stadtmanae* needs NH_3 as a nitrogen source.

3.1.1.4 *Methanothermobacter*

Included in this genus are thermophilic methanogens, such as *M. thermoautotrophicum*, *M. wolfei*, and *M. thermoformicicum*. Most of them share characteristic properties of Gram-positive bacteria, including bent rods, no spores, and no movement. The cell

Table 7.2: Characteristics of *Methanobacterium*.

Characteristics	1	2	3	4	5	6	7	8	9	10	11	12
Size (μm) width	0.4–0.8	0.5–0.6	0.5–1.0	0.4–0.5	0.8	0.5–0.8	0.3–0.4	0.5	0.1–0.15	0.2–0.6	0.4–0.5	0.7
Size (μm) length	2–15	2–25	10–15	2–10	3–22	1–15	3–10	2.5–5	0.6–1.2	1.9–3.8	3–5	5–18
Substrates $H_2 + CO_2$	+	+	+	+	+	+	+	+	+	+	+	+
Acetic acid	+	–	–	–	–	–	+	+	–	–	+	–
Gram staining	+	–	+	+	+	+	nd	+	+	nd	–	+
Movability	–	–	–	–	–	–	–	–	–	nd	–	–
pH value range	6.0–8.5	7.0–9.9	nd	5.9–8.2	4.6–7.0	6.5–8.5	6.0–8.5	nd	6.5–9.2	6.0–8.5	6.5–8.0	5–.9
Optimal pH	7.0	8.1–8.9	6.9–7.2	7.2	5.6–6.2	7.0–7.5	7.0	7.0	7.8–8.8	nd	7.2	7.5–8
Temperature range (℃)	nd	nd	nd	nd	15–50	15–55	20–42	20–45	3.6–45	15–45	25–50	5–48
Optimal temperature (℃)	37–45	37	37–39	37–42	35	45	40	33–37	20–40	40	37	45
NaCl (M)	nd	0	nd	nd	nd	nd	0–0.4	0.2	0.2	nd	0–0.5	0.05–0.9
Mol (%) G + C	40.7–42	57	32.7	39.5	34	37	31	34	54.5	33.8	38.9	34.9

Notes: 1, the numbers in the first line represent 1. *M. formicicum*^{T}, 2. *M. alcaliphilum*, 3. *M. bryantii*, 4. *M. congolense*, 5. *M. espanolae*, 6. *M. ivanovii*, 7. *M. oryzae*, 8. *M. palustre*,9. *M. subterraneum*, 10. *M. uliginosum*, 11. *M. beijingense*, 12. *M. aarhusense*.
2, "+" represents usable, positive, movable for substrates, gram staining, and movability. "–" represents the opposite of "+", "nd" represents not determined.

Table 7.3: Characteristics of *Methanobrevibacter*.

Characteristics	1	2	3	4	5	6	7	8	9	10	11	12	13	14
Size (μm) width	0.3–0.5	0.4	0.34	0.23–0.28	0.7	0.5	0.6	0.6	0.5–0.7	0.5	0.7	0.3–1.0	0.5–1.2	0.5–1.2
Size (μm) length		1.2	1.6	4	0.9	0.6–1.2	1.0	1.0–1.4	1.0–1.5	1–3	0.8–1.8			
Substrates H_2/CO_2	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Acetic acid	–	+	–	–	–	–	+	–	–	+	–	+	+	+
Gram staining	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Movability	–	–	–	–	nd	nd	nd	nd	–	–	–	–	–	–
pH value range	5.0–7.5	6.5–8.5	6.5–8.5	6.5–7.5	nd	nd	nd	nd	6.2–8.0	nd	Nd	5.5–7.0	6.0–10.0	5.5–10.0
Optimal pH	6.07.0	7.7	7.1–7.2	7.0–7.2	7	7.0	7.0	7.0	6.9–7.4	7.0	7.5–8.0	6.0–7.0	7.5	7.0–8.0
Temperature range (°C)	25–37	10–37	10–30	10–33	nd	nd	nd	nd	25–39	nd	10–45	33–42	28–42	33–43
Optimal temperature (°C)	35	37	30	30	37	37	37	37	36–38	37–39	30–37	37–39	36–40	36–42
NaCl (M)	nd	nd	nd	nd	nd	nd	nd	nd	0.01–0.1	nd	nd	nd	0.45	0.45
Mol (%) G + C	nd	nd	nd	nd	29	38	31	33	28	28–31	27.5	31	27–29	31–32

Notes: 1, the numbers in the first line represent 1. *M. acididurans*, 2. *M. cuticularis*, 3. M. *curvatus*, 4. *M. filiformis,* 5. *M. gottschalkii*, 6. *M. thaueri*, 7. *M. woesei*, 8. *M. wolinii*, 9. *M. oralis*, 10. *M. smithii,* 11. *M. arboriphilus*, 12. *M. ruminantium*, 13. *M. ollegae*, 14. *M. millera*.
2, "+" represents usable, positive, movable for substrates, gram staining, and movability. "–" represents the opposite of "+", "nd" represents not determined.

Table 7.4: Characteristics of *Methanothermobacter*.

Characteristics		1	2	3	4	5	6
Size (μm)	width	0.4–0.6	0.4	0.35–0.5	0.4	0.4	0.36
	length	3–7	3–30	2.5	7–20	3–6	1.4–6.5
Substrates $H_2 + CO_2$		+	+	+	+	+	+
Acetic acid		–	–	–	+	+	+
Gram staining		+	+	+	+	+	+
Movability		–	–	–	–	–	–
pH value range		6.0–8.8	5.0–8.2	6.0–8.2	6.0–8.7	nd	nd
Optimal pH		7.2–7.6	7.0–7.4	7.0–7.5	7.0–8.0	nd	nd
Temperature range (°C)		40–75	38–70	37–74	40–65	nd	nd
Optimal temperature (°C)		65–70	65	55–65	55	nd	nd
NaCl (M)		0.6	0.5	–	nd	nd	nd
Mol (%) G + C		52	52	61	55	62.2	44.7

Notes: 1, the numbers in the first line represent 1. *M. thermoautorophicus*, 2. *M. marburgensis*, 3. *M. wolfei*, 4. *M. thermoflexum*, 5. *M. defluvii*, 6. *M. thermophile*.
2, "+" represents usable, positive, movable for substrates, gram staining, and movability. "–" represents the opposite of "+", "nd" represents not determined.

wall contains pseudomurein and cilium. The optimal temperature for growth is in the range of 55–65 ℃. They can produce methane by converting H_2/CO_2. Some strains can also use formic acid as an electron donor. Ammonia is the only nitrogen source. And sulfur can be reduced to sulfide. G + C content is in the range of 32–61% [17]. Detailed characteristics of *Methanothermobacter* are presented in Table 7.4.

3.1.2 Methanothermaceae

This family only contains the genus *Methanothermus*, which was isolated from a very special environment such as volcanic vent. It contains two species: *Methanothermus sociabilis* and *Methanothermus fervidus*. This genus is characterized by a double cell wall and has a high heat tolerance. The optimal temperature is in the range of 80–90°C, and Methanothermaceae cannot live in an environment with a temperature of <60 °C or >97 °C. The G + C content is in the range of 33–34%, and as a hydrogenotrophic methanogen, only $H_2 + CO_2$ can be used [18].

3.2 Methanococcales

The group of Methanococcales contains two families and four genera. All species in the Methanococcales are spherical in shape, and the cell wall contains

proteinoid. They can move by their fascicular polar flagellums and will rapidly dissolve in the presence of a cleaning agent. Except for *Methanocaldococcus jannaschii*, they lack C40 glycerides. The presence of Se can stimulate the growth of this strain.

3.2.1 Methanococcaceae

Detailed characteristics are presented in Table 7.5. The group of Methanococcaceae contains four thermophilic methanogens, and the G + C content is in the range of 30–41%. In a previous study, it was suggested that *M. thermolithotrophicus* belonged to *Methanothermococcus* [19].

3.2.1.1 *Methanococcus*

The optimal temperature and pH of this genus is in the range of 35–40 °C and 6.0–8.0, respectively. In the stable growth stage, the shapes of the cells are irregular and aspherical with a diameter of 1–2 μm, and are always found in pairs. The diameter of some of the bigger cells can reach up to 10 μm in size and are found in static enriched medium. The cells will rapidly dissolve in distilled water but tolerate NaCl solution at a concentration of 20 g/L.

Methanococcus are a group of strict and obligate anaerobic methanogen bacteria. H_2 and formic acid serve as the electron donor. Acetic acid and methylamine cannot be used as substrates. Moreover, the strains also cannot use alcohol, such as ethanol, isopropanol, isobutanol, and cyclopentanol as an electron donor. Except for *M. voltae*, all *Methanococcus* species can grow in a solid substrate with sulfide as the only reducing agent and CO_2 as the carbon source. The growth of *M. maripaludis* can be stimulated by acetic acid and amino acid, and the amino acids are mainly used to efficiently generate proteins. On the contrary, acetic acid and amino acid have no effect on the growth of *M. vannielii* and *M. aeolius*. Moreover, *M. voltae* needs acetic acid, isoleucine, and leucine for proliferation.

Amine, N_2, and alanine can be used as a nitrogen source for *Methanococcus*. *M. vannielii* can use caffeine to support growth. Sulfide is useful for all species of this genus, which cannot be replaced by for example cysteine, dithiothreitol, and sulfate. Several strains including *M. maripaludis* use thiosulfate as a source of sulfur.

A high magnesium concentration can stimulate the growth of *Methanococcus*. In addition, Ca, Fe, Ni, and Co share this function for *M. voltae*, whereas W and Ni share this function for *M. vannielii*. Moreover, *Methanococcus* species tolerate low levels of antibiotics. However, adriamycin, chloramphenicol and monensin will inhibit growth even when present at very low levels.

Table 7.5: Characteristics of *Methanothermococcus*.

Characteristics	*Methanococcus*				*Methanothermococcus*	
	M. aeolicus	*M. vannielii*	*M. voltae*	*M. maripaludis*	*M. thermolithotrophicus*	*M. okinawensis*
Cell diameter (μm)	1.5–2.0	1.3	1.3–1.7	1.2–1.6	1.5	1.0–1.5
Movability	+	+	+	Slightly	+	+
Substrates	H_2, formic acid	H_2, formic acid	H_2, formic acid	H_2, formic acid	H2, formic acid	H_2, formic acid
Sulfur source	S S^{2-}	S S^{2-}	S S^{2-}	S S^{2-} S2O3^{2-}	S S^{2-} $S_2O_3^{2-}$ SO_3^{2-} SO_4^{2-}	S^{2-}
Nitrogen source	N_2, NH_3	NH_3, caffeine	NH_3	N_2, NH_3, alanine	N_2, NH_3, NO_3^-	NH_3
Temperature range (°C)	20–55	20–45	20–45	18–47	17–70	40–75
Optimal temperature (°C)	46	36–40	35–45	35–39	60–65	60–65
pH range	5.5–7.5	6.5–8	6.5–8	6.4–7.8	4.9–9.8	4.5–8.5
Optimal pH	nd	nd	nd	6.8–7.2	5.1–7.5	6–7
NaCl concentration (M)	0.05–1.0	0.6–2	0.6–6	0.6–2	0.1–1.6	nd
Optimal NaCl (M)	nd	0.3–5	1–2	0.3–5	0.25–0.4	nd
G + C (mol%)	32	31	30	33	32%	33.5
Typical strain	Nankai-3	SB	PS	JJ	SN1	IH1

3.2.1.2 *Methanothermococcus*

This genus includes two methanogens and are characterized by the following characteristics: Gram-negative, irregular and spherical shape, and movement by multiple flagella. They rapidly dissolve in distilled water or dilute sodium dodecyl sulfate (SDS) solutions. Their optimal temperature is 60–70 °C, and NaCl is needed for their growth. H_2 and formic acid can be used as an electron donor, whereas acetic acid, methanol, and aminomethane cannot be used for methane production.

3.2.2 Methanocaldococcaceae

This family contains two genera and the G + C content is in the range of 31–33%. Detailed characteristics are presented in Table 7.6.

3.2.2.1 *Methanocaldococcus*

The genus *Methanocaldococcus* contains five species. The cells present with an irregular spherical shape and they move by multiple flagella. They rapidly dissolve in distilled water or dilute SDS solutions, require NaCl for growth, and Se and W can stimulate growth. Acetic acid, formic acid, methanol, and aminomethane cannot be used for methane production. Penicillin G, ampicillin, kanamycin, and streptomycin do not have an effect on growth; however, growth is inhibited by chloramphenicol and rifampicin.

3.2.2.2 *Methanotorris*

This genus only includes *Methanotorris formicicus* and *Methanotorris igneus*, which do not require Se, W, and vitamins for growth. Chloramphenicol and rifampicin will inhibit *the* growth of *Methanotorris formicicus*, which can tolerate streptomycin and kanamycin at a concentration of 200 μg/mL.

3.3 Methanomicrobiales

Based on 16S rRNA analysis, Methanomicrobiales can be divided into the following three families: Methanomicrobiaceae, Methanocorpusculaceae, and Methanospirllaceae.

Table 7.6: Characteristics of Methanocaldococcaceae.

Characteristics	*Methanocaldococcus*					*Methanotorris*	
	M. indicus	***M. fervens***	***M. vulcanius***	***M. infernus***	***M. jannaschii***	***M. igneus***	***M. formicicus***
Cell diameter (µm)	1–3	1–2	1–3	1–3	1.5	1–2	0.8–1.5
Movability	+	+	+	+	+	Slightly	–
Substrates	H_2/CO_2	H_2/CO_2	H_2/CO_2	H_2/CO_2	H_2/CO_2	H_2/CO_2	H_2/CO_2 formic acid
Sulfur source	S S^{2-}	S0 S^{2-}	S0 S^{2-}	S0 S^{2-}	S0 S^{2-}	S0 S^{2-}	S^{2-}
Nitrogen source	NO_3^- NH_3	NO_3^- NH_3	NO_3^- NH_3	NO_3^- NH_3	NH_3	NH_3	NO_3– NH_3 N_2
Temperature range (°C)	50–86	48–92	49–89	55–91	50–91	45–91	55–83
Optimal temperature (°C)	85	85	80	85	85	88	75
pH range	5.5–6.7	5.5–7.6	5.2–7.0	5.25–7.0	5.2–7.0	5.0–7.5	6.0–8.5
Optimal pH	6.5	6.5	6.5	6.5	6.0	5.7	6.7
NaCl concentration (M)	15–50	0.5–5.0(%)	6.25–56.25	12.5–50	1.0–5.0 (%)	0.45–7.2 (%)	4–60
Optimum NaCl (M)	30	3.0 (%)	25	25	3.0 (%)	1.8 (%)	24
G + C (mol%)	30.7	33	31	33	31	31	33.3
Typical strain	SL43	AG86	M7	ME	JAL-1	KoL 5	Mc-S-70

3.3.1 Methanomicrobiaceae

This family of Methanomicrobiaceae contains six genera. The similarity of their 16S rRNA is in the range of 87–95%, and the similarity of species in the same genus is >95.4%. The cellular shape of this family is spherical (*Methanogenium* and *Methanoculleus*), disc shaped (*Methanoplanus*), club shaped (*Methanomicrobium* and *Methanolacinia*), or helicine (*Methanospirillum*). Methanomicrobiaceae have an S-protein layer, thus they are Gram-negative and can be easily broken up [19, 20].

3.3.1.1 *Methanomicrobium*

In this genus, *M. mobile* is the only species, which is Gram-negative, short rod shaped, and slightly curved, and moves using single polar flagella. H_2/CO_2 or formic acid can be used to generate methane. Acetic acid, amino acid, vitamin, rumen liquid, or a liquid mixture of VFA is required when culturing this species. The temperature range is 30–45 °C, the optimal temperature is approximately 40 °C. The optimum pH value is around 6.1–6.9, and the G + C content is 49%.

3.3.1.2 *Methanolacinia*

After reclassification, the main species in this genus is *Methanolacinia paynteri*. This species is easily dissolved in SDS, and the diameter of this Gram-negative strain is 1.5–2.0 μm. It has flagella but can only move at a very slow rate or not at all. This species can use H_2/CO_2, isopropanol/CO_2, 2-butanol/CO_2, and cyclopentanol/CO_2 to produce methane. Formic acid, acetic acid, aminomethane, ethanol, propyl alcohol, *n*-butyl alcohol, and cyclohexanol cannot be used for methane production. The optimal temperature and pH values are 40°C and 7.0, respectively. Moreover, the optimal NaCl concentration is 0.15 mol/L, and the minimum generation time is 4.8 h. The G + C content is 44%.

3.3.1.3 *Methanogenium*

Methanogenium contains five species. The characteristic of this genus includes an irregular spheroidal shape, Gram-negative, and nonmoving but having flagella. The cell wall consists of protein subunits, so it is easy dissolved in SDS. Growth factors are required and formic acid can be used for growth. The G + C content is 47–53%.

3.3.1.4 *Methanoculleus*

The species in this genus are irregular in shape, gram-negative, and do not move. This genus can use formic acid and the optimal pH value is near neutral. The optimal temperature is in the range of 50–60 °C for *M. receptaculi* and *M. thermophilicum*; however, for *M. marisnigri* the range is lower and between 20–25 °C. The G + C content is in the range of 59–62%. Typical strains include *M. olentangyi* and *M. bourgense*. Detailed characteristics are presented in Table 7.7. *M. frittonii* DSM2832 and *M. thermophilus* DSM2373 have a sequence similarity of 99.9% according to analysis of their 16S rRNA; however, their DNA is only 86% similar. Based on their phenotype and genotype, *M. frittonii* and *M. thermophilus* are suggested to be classified together as *M. thermophilus*. Recently, *M. bourgensis*, *M. olentangyi*, and *M. oldenburgensis* have been proven to be synonyms.

3.3.1.5 *Methanoplanus*

This genus includes four species. All have flagella. According to the shape as determined microscopically, *M. linicola* is a rectangular plate, whereas *M. endosymbiosus* is disc shaped. H_2/CO_2 or formate is the substrate that allows for generation of methane. The optimal culture conditions involve neutral, mesophilic, 0.20–0.25 mol/L NaCl, and yeast extract, peptone, and vitamins stimulate growth. The G + C content is 39–50%. Characteristics of this genus are shown in Table 7.8.

3.3.1.6 *Methanofollis*

M. tationis and *M. lininatans* are classified into the group of *Methanofollis*. Their G + C content is in the range of 54–60%. Some newcomers to this group are shown in Table 7.8.

3.3.2 Methanocorpusculaceae

Methanocorpusculaceae contains one genus (*Methanocorpusculum*) and five species. Its shape is irregular and spherical with single flagella. The cellular diameter is about 1 μm. H_2/CO_2 and formic acid can be used as substrate; however, some strains can also use isopropanol + CO_2. Acetic acid, yeast extract, and rumen liquid are necessary nutrients or can stimulate growth. Methanocorpusculaceae are sensitive to SDS. Detailed characteristics are shown in Table 7.9.

Table 7.7: Characteristics of *Methanoculleus*.

Characteristics	*M. receptaculi*	*M. thermophilicum*	*M. submarinus*	*M. chikugoensis*	*M. palmolei*	*M. marisnigri*	*M. bourgenisi*
Size (μm)	0.8–1.7	0.7–1.8	0.8–2.0	1.0–2.0	1.25–2.0	1.5	1–2
Gram	nd	–		–	–	–	–
Movable	–	– or slightly	–	–	–		–
pH value	6.5–8.5		5.0–8.7	6.7–8.0	6.5–8.0	6.7–7.6	5.5–8.0
Optimal pH	7.5–7.8	6.7–7.2	6.0–7.5	6.7–7.2	6.9–7.5	6.2–6.6	6.7
Suitable temperature (°C)	30–65		11–55	15–40	22–50	15–45	30–50
Optimum temperature (°C)	50–55	55–60	43	25–30	40	20–25	35–40
Suitable NaCl (M)	0–1.3		0.1–0.7	0–0.3	nd	0–0.7	0–0.68
Optimal NaCl (M)	0.2	0–0.3	0.1–0.4	0.1	nd	0.1	0.17
Mol (%) G + C	55.2	55–59	nd	62.2	59	61.2	59

Note: nd means not analysis

Table 7.8: Characteristics of *Methanoplanus* and *Methanofollis*.

Characteristics	*Methanoplanus*			*Methanofollis*				
Size (μm)	*M. petrolearius*	*M. endosymbiosus*	*M. limicola*	*M. liminatans*	*M. formosanus*	*M. aquaemaris*	*M. tationis*	*M. ethanolicus*
Gram	1–3	1.6–3.4		1.25–2.0	1.2–2.0	1.2–2.0	3	2–3
Movable	nd	–	–	nd	–	–	–	–
Optimal pH	5.3–8.4	nd	6.5–7.5	nd	5.6–7.3	6.3–8.0	6.3–8.8	6.5–7.5
Suitable temperature (°C)	7.0	6.8–7.3	7.0	7.0	6.6	6.5	7.0	7.0
Optimum temperature (°C)	25–45	16–36	17–41	25–45	25–42	20–43	20–45	15–40
Suitable NaCl (M)	37	32	40	40	40	37	37–40	37
Optimal NaCl (M)	0–5%	nd	0.4–5.4	nd	0–4%	0–6%	0–7%	nd
Mol (%) G + C	1–3%	0.25	1	nd	3%	0.5	0.8–1.2%	nd

Notes: "–"means no movable; "nd" means no analysis.

Table 7.9: Characteristics of Methanocorpusculaceae.

Characteristics	*Methanocorpusculum parvum*	*Methanocorpusculum aggregans*	*Methanocorpusculum bavaricum*	*Methanocorpusculum labreanum*	*Methanocorpusculum sinense*
Size (μm)	≤1	0.5–2	≤1	0.4–2.0	≤1
Gram	–	–	–	–	–
Movable	Slight	/	Slight	/	Slightly
pH value	nd	6.2–7.5	nd	6.5–7.5	nd
Optimal pH	6.8–7.5	6.6	7.0	7.0	7.0
Suitable temperature (°C)	15–45	27–38	15–45	nd	15–45
Optimal temperature (°C)	37	35	37	37	30
Suitable NaCl (M)	0–47	0–2%	nd	nd	nd
Optimal NaCl (M)	nd	0%	nd	15	nd
Mol (%) G + C	48.5	52	47.7	50	52

Notes: "–"gram-negative; "/"means no movable; "nd" means no analysis.

3.3.3 Methanospirillaceae

The family Methanospirillaceae contains genus *Methanospirillum*, which is mesophilic bacteria. *M. hungatei* is the only species and is helix-like in shape, which has never been observed in any other methanogen.
M. hungatei is Gram-negative and moves slowly by using polar flagella. Formic acid and H_2/CO_2 can be used as substrates to generate methane; however, acetic acid, methanol, and ethanol cannot be used as a substrate. Yeast extract and protein can stimulate their growth. The optimum temperature and pH is 30–37 °C and 6.6–7.4, respectively. G + C content is 45–49%.

3.4 Methanosarcinales

Based on their phenotype and 16S rRNA composition, Methanosarcinales are classified into two genera. Species in this order have acidic polysaccharides, but do not contain peptidoglycans and false murein, and can use a variety of substrates. Methanosarcinales are mainly comprised of *Methanosarciaceae* and *Methanolobu*s [21, 22].

3.4.1 Methanosarcinaceae

3.4.4.1 Methanosarcina

This genus contains nine species. None of the *Methanosarcina* can move. Acetic acid, formic acid, aminomethane, and CO can be metabolized, and some species can also use H_2/CO_2. Table 7.10 presents the detailed characteristics.

3.4.4.2 *Methanolobus*

Methanolobus contains seven species, most of them are Gram-negative. Except for *M. tindarus* and *M. profundi* they are unable to move. The optimum temperature is in the range of 30–40°C, however *M. tindarus* is a psychrophilic methanogen, and 0.5 mol/L NaCl is suitable for growth (Table 7.11). Methanol and aminomethane can be used as substrate, whereas H_2/CO_2, formic acid, acetic acid, and ethanol cannot be used as substrate.

Table 7.10: Characteristics of *Methanosarcina*.

Characteristics	1	2	3	4	5	6	7	8	9
Size (μm)	1.5–2.0	1.7–2.1	1.5–3.0	1.5–2.5	1.0–3.0	1.0–2.0	1.5–3.0	1.5–3.5	0.8–2.1
Gram	+	–	–	+	+	+	nd	+	+
Movable	nd	–	–	nd	–	–	nd	–	–
pH value	6.5–7.5	5.4–8.5	5.0–7.8	5.5–8.0	5.8–8.0	6.0–8.0	4–8.5	4.5–8.5	6.2–8.3
Optimal pH	7.0	6.5–7.0	6.5–6.8	6.0	6.8–7.2	7.5	6.5–7.5	7.0	6.5–7.5
Suitable temperature (°C)	25–50	15–48	15–42	35–55	25–45	20–45	4–27	1–35	18–39
Optimum temperature (°C)	45	35–40	40	50	40–42	40	25	25	30–35
Suitable NaCl (M)	0.1–0.7	0.1–1.0	0.2–0.6	0–1.2	0.1–0.7	0.1–0.5	nd	nd	0–1.4
Optimum NaCl (M)	<0.2	0.2	0.4–0.6	0.6	0.2–0.4	0.1	0.3–0.4	nd	0.2–0.6
Mol (%) G + C	39–44	41	41–43	42	42	36	nd	43.4	nd

Notes: 1. the numbers in the first line represent 1. *M. barkeri*, 2. *M. acetivorans*, 3. *M. siciliae*, 4. *M. thermophile*, 5. *M. mazeii*, 6. *M. vacuolata*, 7. *M. baltica*, 8. *M. lacustris*, 9. *M. semesi*.
"–"gram-negative or no movable; "+"means gram-positive; "nd" means no analysis.

Table 7.11: Characteristics of *Methanolobus*.

Characteristics	*M. tindarus*	*M. bombayensis*	*M. oregonensis*	*M. taylorii*	*M. vulcani*	*M. zinderi*	*M. profundi*
Size (µm)	0.8–1.25	1.0–1.5	1.0–1.5	0.5–1.0	1.0–1.25	0.5–1.0	0.9–1.2
Gram	–	–	–	–	–	nd	nd
Movable	+	–	–	–	–	–	+
pH value	5.5–8.0	6.2–8.2	8.2–9.2	5.5–9.2	5.8–7.8	nd	6.1–7.8
Optimal pH	6.5	7.2	8.6	8	7.0	nd	6.5
Suitable temperature (°C)	10–45	20–42	25–42	5–42	13–45	nd	9–37
Optimal temperature (°C)	25	37	35	37	40	nd	30
Suitable NaCl (M)	0.06–1.27	0.2–2.2	0.1–1.6	0.1–1.5	0.1–1.4	nd	0.1–1.0
Optimum NaCl (M)	0.49	0.5	0.48	0.5	0.5	nd	0.35
Mol (%) G + C	40	39	40.9	40.8	39	42	42.4

3.4.4.3 *Methanococcoides*

This genus contains two species. The cell shape is irregular, and the cell wall has a protein layer of 10 nm. Aminomethane and methanol can be used as substrate; however, acetic acid and formic acid cannot be used. *Methanococcoides* are easily dissolved in SDS solvents, and a suitable NaCl concentration is 0.2–0.6 mol/L. Detailed characteristics are shown in Table 7.12.

3.4.4.4 *Methanohalophilus*

Methanohalophilus contain three species. The cells are irregular in shape and are unable to move. Aminomethane and methanol can be used to generate CH_4; however, methanol becomes toxic when the concentration used exceeds 40 mmol/L. Detailed characteristics are shown in Table 7.12.

3.4.4.5 *Methanosalsus*

Methanosalsum zhilinae is the only species in *Methanosalsus*. The cells are irregular in shape with a diameter of 0.75–1.5 μm. Aminomethane can be used as a substrate, whereas acetic acid, formic acid, and H_2/CO_2 cannot be used. Yeast extract, rumen liquid, and tryptone are not required for culturing this species; however, these compounds can stimulate growth. Antibiotics such as penicillium G inhibit cell growth, whereas chloramphenicol and achromycin completely inhibit growth. Details are presented in Table 7.12.

3.4.4.6 *Methanohalobium*

Methanohalobium mainly contains the extreme halophiles *M. evestigatum*, which cannot use formic acid and H_2/CO_2, but uses methanol at a lower concentration of 5 mmol/L as substrate. Vitamin B and yeast extract are necessary for growth. Table 7.12 presents the detailed characteristics.

3.4.4.7 *Methanosaetaceae*

Species in this family are obligate anaerobes, Gram-negative, do not move, and are unable to form spores. Methanosaetaceae can form immune group aggregation. The outer layer of cell wall consists of proteins. Acetic acid is the only carbon source and the doubling time is 4–7 days at 37 °C. Formic acid can be decomposed into H_2/CO_2, and the G + C content is 50–61%.

Table 7.12: Characteristics of *Methanococcoides*, *Methanohalophilus*, *Mehtanosalsus*, and *Methanohalobium*.

characteristics	*Methanococcoides*		*Methanohalophilus*			*Methanosalsus zhilinae*	*Methanohalobium evestigatum*
	methylutens	*burtonii*	*mahii*	*halophilus*	*portucalensis*		
Size (µm)	1.0	0.8–1.8	0.5–2.5	0.5–2.0	0.6–2.0	0.75–1.5	nd
Gram	–	V	–	–	–	–	nd
Movable	–	nd	nd	–	–	–	nd
pH value	6.0–8.0	6.8–8.2	6.8–8.2	6.3–7.4	nd	8.0–10	6.0–8.3
Optimal pH	7.0–7.5	7.7	7.5	6.5–7.4	nd	9.2	7.0–7.5
Suitable temperature (°C)	15–35	1.7–29.5	10–45	18–42	nd	20–50	25–60
Optimal temperature (°C)	30–35	23.4	35	26–36	nd	45	50
Suitable NaCl (M)	0.1–1.0	0.2–0.5	0.4–3.5	0.7–2.6	nd	0.2–2.1	1.7–5.1
Optimal NaCl (M)	0.24–0.64	0.2	2.0	1.2	nd	0.7	4.3
Mol (%) G + C	42	39.6	48.5	39	41	38	37

3.5 *Methanopyrales*

Methanopyrales only contains *Methanopyrus kandleri*, which can grow at a temperature of 110 °C and have little contingency with other methanogens. *Methanopyrales* are hydrogenotrophic, high-temperature resistance, and Gram-positive. Gene sequencing analyses show that they are closely related to *Methanococcales*. *Methanopyrus kandleri* is the only methanogen that can generate methane in an environment with a temperature higher than 110 °C. It uses CO_2/H_2 as nutrients and belongs to the inorganic autotrophic methanogens. Amine and sulfide can be used as a nitrogen and sulfur source, respectively. The cell wall contains two layers; the inner layer consists of pseudomurein that mainly contains ornithine and lysine. The outer sphere is sensitive to cleaner agent indicating that the main components include proteins. *Methanopyrales* move by flagella. They grow at temperatures between 84 and 110 °C, and the optimal temperature is 98 °C. The optimal pH value is 6.5, and the optimum NaCl concentration is 2–4 g/L. H_2S may be produced when sulfur is present. The content of G + C is 60%. At present, the position of *M. kandleri* in phylogenesis is not clear. Phylogenetic analysis based on 16S rRNA, extension factors, and transcription factor showed it is very different from other methanogens. However, it showed similar properties with other methanogens in analyses with methyl coenzyme M reductase operon, translation factor, and complete genome sequence. Therefore, *M. kandleri* may belong to a branch of methanogens.

Section 4: Pathways of Microbial Methane Formation

Along with the development of modern biotechnology, studies on the pathways of methane formation have gradually developed. It is now known that H_2 and CO_2, methyl acetate, methanol, acetic acid, and methyl amine can be utilized by methanogens to produce methane as follows:

$$4H_2+HCO_3^-+H^+ \longrightarrow CH_4+3H_2O \quad (7.1)$$

$$4HCOO^-+4H^+ \longrightarrow CH_4+3CO_2+2H_2O \quad (7.2)$$

$$4CH_3OH \longrightarrow 3CH_4+CO_2+2H_2O \quad (7.3)$$

$$CH_3COO^-+H^+ \longrightarrow CH_4+CO_2 \quad (7.4)$$

$$4CH_3NH_3^-+3H_2O \longrightarrow 3CH_4+H_2CO_3+4NH_4^- \quad (7.5)$$

Based on different substrate types, methanogens can be divided into two categories: (1) the first category includes Methanobacteriales, Methanococcales,

Methanomicrobiales, and Methanopyrales, which convert H_2/CO_2 and methyl into methane; and (2) the second category including Methanosarcinales, which utilize complex substrates, such as methanol, methyl amine, and acetic acid. Some can also use H_2/CO_2 [14].

4.1 Methane Formation from H_2 and CO_2

H_2 and CO_2 are the most common substrates for methanogens, conversion of H_2/CO_2 to CH_4 provides energy to methanogens, and cell substances are synthetized in the process. In fact, when using H_2 and CO_2 as substrate, the growth efficiency of methanogens is relatively low, because most of the CO_2 is converted into methane [23–27].

Except several species such as *Methanosphara stadtmanae*, which can reduce methanol with H_2, and *Methanolobus tindarius*, which can only utilize methanol and methylamine, most of methanogens can use both H_2 and CO_2 as substrates:

$$4H_2 + CO_2 \longrightarrow CH_4 + 2H_2O \quad \Delta G^{0'} = -131\ \text{kJ/mol} \tag{7.6}$$

In the methanogenic ecosystem, hydrogen partial pressure is usually between 1 and 10 Pa. Given this low hydrogen concentration, the free energy change during the methanogenic process is between 20 and 40 kJ/mol. In cells, at least 50 kJ/mol of free energy is required for ATP synthesis from ADP and inorganic phosphate. Therefore, under physiological growth conditions, less than 1 mol of ATP is synthesized when 1 mol of methane is produced. It can be evidence for the coupling of the energy-producing process of methane formation and energy-consuming process of ADP phosphorylation via the mechanism of chemical osmosis.

Phase 1: Reduction of CO_2 into Formyl MF

$$CO_2 + H_2 + MF \longrightarrow HCO\text{-}MF + H_2O \quad \Delta G^{0'} = +16\ \text{kJ/mol} \tag{7.7}$$

In the first step of this pathway, formyl that is formed by H_2-dependent CO_2 reduction binds methanofuran (MF) to form formylmethanofuran (Figure 7.1). Formylmethanofuran is the first stable intermediate formed in this pathway, and based on the standard free energy, this process is considered as an energy-consuming process. The

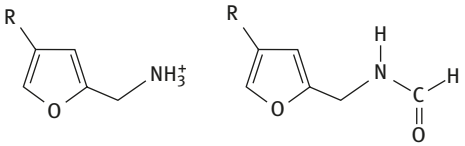


Figure 7.1: Chemical structures of methylfuran and formylmethylfuran.

hydrogen partial pressure under typical methanogenic environments is <1 atm (standard state), which is around 10^{-4}–10^{-5} atm; therefore, the actual free energy change of this reaction is about 40 kJ/mol.

The formation of formylmethanofuran is catalyzed by formylmethanofuran dehydrogenase, which uses sub-molybdenum purine dinucleotide as a cofactor. The enzyme from *Methanobacterium thermoautotrophicum* is a $\alpha_1\beta_1$ dimer with subunits of 60 and 45 kDa. Moreover, 1 mol of the dimer contains 1 mol of molybdenum, 1 mol of molybdopterin dinucleotide, 4 mol of nonheme iron, and acid-labile sulfur. Two formylmethanofuran dehydrogenases are isolated from *Methanobacterium wolfei*, one is a $\alpha_1\beta_1\gamma_1$ structured molybdenum enzyme, containing three subunits of 63, 51, and 31 kDa, respectively. The enzyme comprises 0.3 mol of molybdenum, 0.3 mol of molybdopterin dinucleotide, 4–6 mol of nonheme iron, and acid-labile sulfur. The other enzyme is a $\alpha_1\beta_1\gamma_1$-structured tungsten protein, containing three subunits of 64, 51, and 35 kDa, which consists of 0.4 mol of tungsten, 0.4 mol of molybdopterin guanine dinucleotide, 4–6 mol of nonheme iron, and acid-labile sulfur. Comparing to these enzymes, formylmethanofuran dehydrogenase that is isolated from *Methanosarcina barkeri* is more complex, which is composed of six non-identical subunits of 65, 50, 37, 34, 29, and 17 kDa. Each mole of the enzyme comprises 1 mol molybdenum, 1 mol molybdopterin guanine dinucleotide, 28 mol nonheme iron, and 28 mol of acid-labile sulfur. Gene analysis indicated that obligate hydrogenotrophic methanogens formylmethanofuran is produced by a similar mechanism.

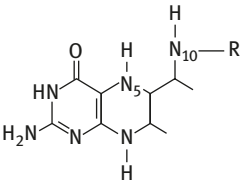
Phase 2: The transfer of the formyl moiety from formyl-MF to H_4MPT and the formation of methenyl-H_4MPT

$$\text{HCO-MF} + \text{H}_4\text{MPT} \longrightarrow \text{HCO-H}_4\text{MPT} + \text{MF} \quad \Delta G^{0'} = -5 \text{ kJ/mol} \tag{7.8}$$

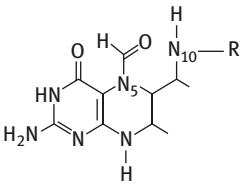
$$\text{HCO-H}_4\text{MPT} + \text{H}^+ + \longrightarrow \text{CH} \equiv \text{H}_4\text{MPT}^+ + \text{H}_2\text{O} \quad \Delta G^{0'} = -5 \text{ kJ/mol} \tag{7.9}$$

In the first step, the formyl moiety of formyl-MF is transferred to tetrahydromethanopterin (H_4MPT, Figure 7.2). This reaction is catalyzed by formyltransferase (Ftr), which has been isolated from methanogens and *Archaeoglobus fulgidus*. This enzyme is a polypeptide monomer or tetramer of 32–41 kDa, devoid of chromophoric prosthetic group, and stable in air. The corresponding gene from *M. thermoautotrophicum* has been cloned, sequenced, and has been expressed in *Escherichia coli*. In solution, Ftr is present as monomer, dimer, and tetramer. The monomer is neither active nor thermally stable, whereas the tetramer are both active and thermally stable.

The next step is the reversible hydrolysis of N^5-formyl-H_4MPT to N^5,N^{10}-methenyl -H_4MPT, which is catalyzed by N^5,N^{10}-methenyl-H_4MPT cyclohydrolase. Under alkaline conditions, N^5,N^{10}-methenyl-H_4MPT can automatically be hydrolyzed into N^{10}-formyl-H_4MPT (Figure 7.2). This reaction can be accelerated in the presence of anions. N^5,N^{10}-Methenyl-H_4MPT cyclohydrolase has been purified from *M. thermoautotrophicus*, *Ms. barkeri*, *M. kandleri*, *A. jidgidus*, and *Methylobacterium extorquens* AM1. It



Tetrahydromethanopterin, H_4MPT



N^5-formyl-H_4MPT

Figure 7.2: Chemical structures of tetrahydromethanopterin and N^5-formyl-H_4MPT.

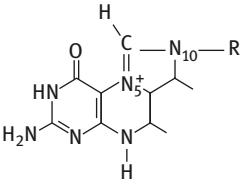
is insensitive to dissolved oxygen, is composed of one or two identical subunits of 40 kDa, and no chromophoric prosthetic groups have been identified. The N-terminal amino acid sequences of the enzyme show a high similarity, and the catalytic efficiency and thermal stability are greatly influenced by salts. The corresponding gene from *M. kandleri* has been expressed in *E. coli*. The crystal structure of the enzyme is a novel α/β fold, consisting of two domains and a pocket is present in between sequences of the Mch domains. Sequences of the Mch domains are well preserved in other organisms.

Phase 3: The reduction of methenyl-H_4MPT to methyl-H_4MPT

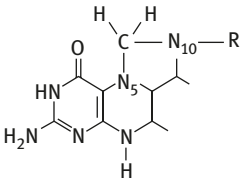
$$CH \equiv H_4MPT^+ + F_{420}H_2 \longrightarrow CH_2{=}H_4MPT + F_{420} + H^+ \quad \Delta G^{0'} = +6\ \text{kJ/mol} \tag{7.10}$$

$$CH_2{=}H_4MPT + F_{420}H_2 \longrightarrow CH_3\text{-}H_4MPT + F_{420} \quad \Delta G^{0'} = -6\ \text{kJ/mol} \tag{7.11}$$

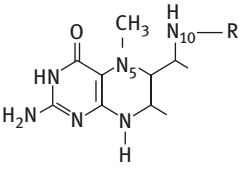
Methenyl-H_4MPT is reduced to methylene-H_4MPT and then to methyl-H_4MPT (Figure 7.3). In both reactions, reduced coenzyme F_{420} serves as a reductant. The F_{420}-dependent reduction of methenyl-H_4MPT is reversible and is catalyzed by methenyl-H_4MPT dehydrogenase. This enzyme has been purified from *M. thermoautotrophicum* and *Ms. barkeri* and has been characterized as a polypeptide with a molecular mass of 32 kDa that is stable in air and is devoid of prosthetic groups. Moreover, another methenyl-H_4MPT dehydrogenase has been isolated from *M. thermoautotrophicum*, which links methenyl-H_4MPT reduction directly with the oxidation of H_2, and composed of one polypeptide with an apparent molecular weight of 43 kDa:



Methenyl-H_4MPT



Methylene-H_4MPT



Methyl-H_4MPT

Figure 7.3: Chemical structures of methenyl-H_4MPT, methylene-H_4MPT, and methyl-H_4MPT.

$$CH \equiv H_4MPT^+ + H_2 \longrightarrow CH_2{=}H_4MPT + H^+ \Delta G^{0'} = -6\ kJ/mol \quad (7.12)$$

This reversible F_{420} H_2-dependent process of methylene-H_4MPT reduction to methyl -H_4MPT is catalyzed by process methylene-H_4MPT reductase (Mer). Mer is a soluble enzyme with an apparent molecular weight of 35–45 kDa, has no chromophoric prosthetic groups, and is stable in air. This enzyme has been purified from *M. thermoautotrophicus*, *M. marburgensi*, *Ms. barkeri*, *M. kandleri*, and *A. filgidus*. The primary structure of the enzyme is highly similar to F_{420}-dependent alcohol dehydrogenase isolated from *Methanoculleus thermophilicu*s.

Phase 4: Transfer of the methyl group from methyl-H4MPT to coenzyme M

$$CH_3\text{-}H_4MPT + HS\text{-}CoM \longrightarrow CH_3\text{-}S\text{-}CoM + H_4MPT\ \Delta G^{0'} = -30\ kJ/mol \quad (7.13)$$

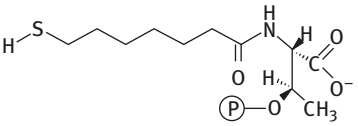
The methyl group of N^5- methyl-H_4MPT is transferred to coenzyme M to form methyl coenzyme M. The methyltransferase from membranes of *Methanosarcina* can be stimulated by Na^+, and act as a sodium ion pump in H_2/CO_2-based methanogenesis. This indicates that the free energy produced from methyl group transfer (–30 kJ/mol) can be stored in the form of a transmembrane electrochemical sodium ion gradient ($\Delta\mu Na^+$). Moreover, this gradient can be the driving force of ATP synthesis via $\Delta\mu Na^+$-driven ATP synthase.

In the case of a methyl transfer, methylated-corrinoid compound accumulation is found in the absence of coenzyme M, and when adding coenzyme M, the compound is then demethylated. It has been demonstrated that the corrinoid compound is 5-hydroxybenzimidazolylcobamide. Based on previous findings, it can be postulated that the methyl transfer from methyl-H_4MPT to coenzyme M proceeds in two steps: first, the methyl is transferred from methyl-H_4MPT to a corrinoid protein, and then it is transferred from methylated-corrinoid to coenzyme M. However, it is currently not clear which step is the driving force of Na^+ translocation. The transfer of methyl from methyl-H_4MPT to coenzyme M is a critical process, and the only site of energy transduction in the carbon dioxide reduction pathway:

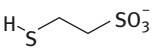
$$CH_3\text{-}H_4MPT + [Co(I)] \longrightarrow CH_3[Co(III)] + H_4MPT \quad (7.14)$$

$$CH_3\text{-}[Co(III)] + H\text{-}S\text{-}CoM \longrightarrow CH_3\text{-}S\text{-}CoM + [Co(II)] \quad (7.15)$$

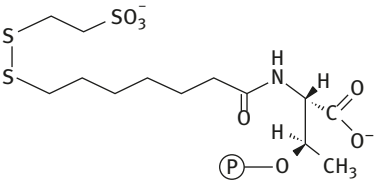
The enzyme that catalyzes the overall reaction has been isolated from *Methanobacterium thermoautotrophicum*, and consists of subunits with apparent molecular weights of 12.5, 13.5, 21, 23, 24, 28, and 34 kDa. The polypeptide of 23 kDa could be a corrinoid-combined polypeptide. Each mol of the complex contains 1.6 mol of 5-hydroxybenzimidazolylcobamide, 8 mol of nonheme iron, and 8 mol acid-labile sulfur.



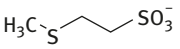
HS-HTP



Coenzyme M



CoM-S-S-HTP



Methyl-coenzyme M

Figure 7.4: Chemical structure of *N*-7-mercaptoheptanoylthreonine phosphate (HS-HTP), coenzyme M, CoM-S-S-HTP, and methyl-coenzyme M.

Phase 5: The reduction of methyl-coenzyme M to methane

$$CH_3\text{-S-CoM} + \text{HS-HTP} \longrightarrow CH_4 + \text{CoM-S-S-HTP}\ \Delta G^{0'} = -45\ \text{kJ/mol} \tag{7.16}$$

The reduction of methyl-coenzyme M is catalyzed by methyl-coenzyme M reductase (methyl-coenzyme M, Figure 7.4). Two unique coenzymes are involved in this reaction, including *N*-7-mercaptoheptanoylthreonine phosphate (HS-HTP, Figure 7.4), which serves as electron donor in the reduction of methyl-CoM to form methane and a mixed disulfide (CoM-S-S-HTP, produced from HSCoM and HS-HTP, Figure 7.4**)**. The other is F_{430}, which serves as characteristic prosthetic group. Methyl-coenzyme M reductase (Mcr) has been purified from *M. thermoautotrophicus*, *M. marburgensii*, *M. kandleri*, *Ms. barkeri*, *Methanosarcina thermophila*, *Methanothrix soehngenii*, and *Methanococcus voltae*. Various studies have been performed regarding the biosynthetic pathway of Mcr from *M. marburgensis*. The molecular mass of this enzyme is roughly 300 kDa, containing three $\alpha_2\beta_2\gamma_2$ subunits with molecular masses of 65, 46, and 35 kDa.

Phase 6: The reduction of F_{420} by H_2

$$H_2 + F_{420} \longrightarrow F_{420}H_2\ \Delta G^{0'} = -11\ \text{kJ/mol} \tag{7.17}$$

It is well known that H_2 is the ultimate electron donor in all reactions of the carbon dioxide reduction pathway. However, the physiological electron carrier for the reaction of formylmethanofuran dehydrogenase has yet to be identified. Several studies have indicated that the reduced factor F_{420} ($F_{420}H_2$) serves as an electron donor for the enzyme, which catalyzes the reaction of methenyl-H_4MPT to methyl-H_4MPT. $F_{420}H_2$ can be regenerated by an F_{420}-dependent hydrogenase, which has been purified from *M.*

vannielii, *M. thermoautotrophicum*, *M. voltae*, *M. formicicum*, and *Ms. barkeri*. F_{420}-dependent hydrogenases consist of flavin, Ni, and Fe-S clusters. Furthermore, the primary structure of F_{420}-dependent hydrogenases purified from methanogens has a high similarity with hydrogenases from other prokaryotes, especially the amino acid residues, which provide ligands to the active site of nickel, are highly conserved.

The structures of F_{420}-dependent hydrogenases from different methanogens are similar but not identical. For example, hydrogenase from *M. thermoautotrophicum* is a $\alpha_1\beta_1\gamma_1$ trimer with three subunits of 47, 31, and 26 kDa, each mole of which consists of 1 mol of flavin adenine dinucleotide (FAD), 13–14 mol of nonheme iron, and acid-labile sulfur. Based on previous findings, assumptions can be made that the subunit of 47 kDa contains nickel, and the subunit of 31 kDa consists of flavin and the site of F_{420} reduction. Several hydrogenases from other methanogens, such as *M. vannielii* and *M. voltae*, consist of selenium in the form of selenocysteine. In addition, these types of methanogens contain a set of genes that codes for selenium-free F_{420}-dependent hydrogenase.

Phase 7: The reduction of disulfide CoM-S-S-HTP to HS-CoM and HS-HTP

$$\text{CoM-S-S-HTP} + H_2 \longrightarrow \text{HS-CoM} + \text{HS-HTP}\ \Delta G^{0'} = -42\ \text{kJ/mol} \tag{7.18}$$

CoM-S-S-HTP that is formed in the methylreductase reaction is reductively cleaved to generate HS-CoM and HS-HTR. This reaction is catalyzed by the H_2-dependent heterodisulfide reductase system. In *Methanosarcina* species, this membrane-bound electron transport system was linked to energy conservation. Studies demonstrated the H_2-dependent reduction of CoM-S-S-HTP, accompanied with the generation of a transmembrane proton potential and of ATP synthesis from ADP and Pi. It is worth noting that in the *M. voltae* strain, the H_2-dependent reduction of heterodisulfide and the generation of a transmembrane sodium ion gradient is the driving force of ATP synthesis.

It is considered that the H_2-dependent heterodisulfide reductase system consists of multiple reactions: first, H_2 is activated and the electrons are conveyed to the electron transport chain. Next, the electrons are transported to CoM-S-S-HTP, and finally catalyzed by heterodisulfide reductase, which reduces CoM-S-S-HTP. The function of heterodisulfide reductase has been identified in vitro. Heterodisulfide reductase has been purified from *M. thermoautotrophicum*, which is a $\alpha_4\beta_4\gamma_4$ trimer with three subunits of 80, 36, and 21 kDa. Each mole of the enzyme consists of 4 mol of FAD and 72 mol of each nonheme iron and acid-labile sulfur. Heterodisulfide reductase from *Methanosarcina* is strictly membrane bound, and the enzymes from *M. thermoautotrophicum* are recovered from the soluble fraction of disrupted cells.

Recently, a complex has been purified from *Ms. barkeri*, which was able to catalyze the complete electron transfer from H_2 to CoM-S-S-HTP. The complex is composed of nine polypeptides with apparent molecular masses of 46, 39, 28, 25, 23, 21, 20, 16, and 15 kDa, respectively. Moreover, it contains nickel, FAD, nonheme iron, and acid-labile sulfur. Previous findings confirm that cytochromes are involved in the

electron transport chain, the role of which is to convey the electrons to the oxidant CoM-S-S- THE. So far, the cytochromes have only been identified in the family of Methanosarcinaceae. Most of the methanogenesis are cytochromes-free, and have been substituted by other electron carriers.

The reactions discussed so far, mainly involve enzymes in the CO_2 reduction pathway. In summary, CO_2 is connected to specific carriers and consecutively reduced into methane, and H_2 serves as a reductant in this process. Two types of hydrogenases with different electron acceptor characteristics have been found in the process of reducing equivalent transfer from H_2 to the various intermediates. The driving force for the formation of H_2-dependent formyl-MF from CO_2 and MF is still unclear, whereas the transmembrane electrochemical potential of protons from H_2-dependent heterodisulfide reduction can be used for ATP synthesis. Furthermore, free energy that is generated by methyl transfer from methyl-H_4MPT to coenzyme M can be related with the generation of a sodium ion potential.

4.2 Methanogenesis from Formate

In addition to H_2 and CO_2, several methanogens can produce methane from formate. The first step in the formate pathway is the oxidation to CO_2 which is then similar as the CO_2 reduction pathway mentioned above. The key enzyme in the formate pathway is formate dehydrogenase, which has been purified from *M. formicicum* and *M. vannielii*. The enzyme purified from *M. formicicum* consists of two non-identical $\alpha_1\beta_1$ subunits with apparent molecular masses of 85 and 53 kDa, molybdenum, iron, acid-labile sulfur, and 1 mol of FAD per mole of enzyme. Molybdenum is the component of molybdopterin cofactor, the spectral properties of which indicate that it is structurally similar to the molybdopterin cofactor purified from xanthine oxidase. The genes coding for formate dehydrogenase have been cloned and sequenced. The DNA sequence suggests that formate dehydrogenase from *M. formicicum* is a selenocysteine-free enzyme. In contrast, one of the two formate dehydrogenase from *M. vannielii* contains selenocysteine [28–30].

4.3 Methanogenesis from Methanol and Methylamines

Members of the *Methanosarcinaceae* are the only methanogens that can use methanol or methylamines as their sole energy source. Members of the genus *Methanosphaera* in *Methanobacteriaceae* can only utilize methyl compounds in the presence of H_2. Most *Methanosarcina* species can utilize both methyl compounds and H_2 + CO_2, whereas others, including *Methanolobus, Methanococcoides,* and *Methanohalophilus*

Table 7.13: Reactions and enzymes in methanogenesis from methanol and methylamines in *Methanosarcina*.

Reactions	Free energy (kJ/mol)	Enzyme (Gene)
Methane formation		
CH_3-OH+H-S+CoM ⟶ CH_3-S-CoM+H_2O	−27.5	Methanol:coenzyme M methyltransferase (*mtaA* + *mtaBC*)
CH_3-S-CoM+H-S-CoB ⟶ CoM-S-S-CoB+CH_4	−45	Methyl coenzyme M reductase (*mcrBDCGA*)
CoM-S-S-CoB+2[H] ⟶ H-S-CoM+H-S-CoB	−40	Heterodisulfide reductase (*hdrDE*)
CO_2 Formation		
CH_3-OH+H-S-CoM ⟶ CH_3-S-CoM+H_2O	−27.5	Methanol:coenzyme methyltransferase (*mtaA* + *mtaBC*)
CH_3-S-CoM+H_4SPT ⟶ H-S-CoM+CH_3- H_4SPT	+30	Methyl- H_4SPT:coenzyme methyltransferase (*rntrEDCBAFGH*)
CH_3-OH+H_4SPT ⟶ CH_3-H_4SPT+ H_2O	+2.5	
CH_3-H_4SPT+F_{420} ⟶ CH_2=H_4SPT+ $F_{420}H_2$	+6.2	F_{420}-dependent methylene-H_4SPT reductase (*mer*)
	−5.5	F_{420}-dependent methylene- H_4SPT dehydrogenase (*mtd*)
CH ≡ H_4SPT+H_2O ⟶ HCO-H_4SPT+H^+	+4.6	Methenyl--H_4SPT cyclohydrolase (*mch*)
HCO-H_4SPT-MFR ⟶ HCO-MFR+ H_4SPT	+4.4	Formyl methanofuran:H_4SPT formyltransferase (*ftr*)
HCO-MFR ⟶ CO_2+MFR+2[H]	−16	Formyl methanofuran dehydrogenase (*fmdEFA CDB*)

only utilize the methyl compounds. *Methanolobus siciliae* and several *Methanohalophilus* species can also utilize dimethylsulfide.

Table 7.13 presents the enzymes involved and the changes in free energy.

4.3.1 Transfer of the Methyl Group

The utilization of methanol starts from the transfer of the methyl group to coenzyme M. With the catalyzation of two distinct enzymes, methyl is sequentially transferred to coenzyme M in two steps. First, it is catalyzed by methanol:5-hydroxybenzimidazolyl cobamide methyltransferase (MT1), the methyl group is transferred to the corrinoid prosthetic group of MT1, then to coenzyme M with the catalyzation of cobalamin:HS-CoM methyltransferase (MT2). MT1 is purified from *Ms. barkeri* and is sensitive to oxygen. It has an apparent molecular mass of 122 kDa. The enzyme consists of two $\alpha_2\beta$ subunits of 34 and 53 kDa, respectively, each mole of which contains 3.4 mol of 5-hydroxybenzimi-

dazolylcobamide. In general, the genes coding MT1 contain one operon. MT2 has been purified from *Ms. barkeri*, which is composed of one subunit of 40 kDa. In addition, the genes coding MT2 are derived from single gene transcription. By culturing *Ms. barkeri* in the presence of trimethylamine, a trimethylamine-specific methyltransferase is detected.

4.3.2 Oxidation of the Methyl Group

In the process methanol conversion, the reduction of methyl-CoM to methane is similar to the reduction of CO_2 to methane. First, the methyl group is transferred from methyl-CoM to H_4MPT. Under standard conditions, the reaction is endergonic, and it has been demonstrated that the reaction is driven by a transmembrane electrochemical gradient of sodium ions. Methyl-H_4MPT is oxidized to CO_2 via intermediates, including methylene-H_4MPT, methenyl-H_4MPT, formyl-H_4MPT, and formyl-MF. When catalyzed by methylene-H_4MPT reductase and methylene-H_4MPT dehydrogenase, methyl-H_4MPT and methylene-H_4MPT are oxidized to reduced F_{420}. However, the physiological electron acceptor of formyl-MF dehydrogenase has not yet been identified. For the H_2-dependent reduction of CO_2, input energy is required; therefore, it can be assumed that the reversible reaction produces energy. Consistent with this assumption, studies using *Ms. barkeri* have revealed that a transmembrane sodium potential is formed in response to the oxidation of formyl-MF.

4.3.3 The Reduction of the Methyl Group

The reducing equivalents that are produced from the oxidation of methyl-H_4MPT are subsequently transferred to heterodisulfide groups. The electron channel from formyl-MF is not yet clear; however, it can be assumed that the electron transfer is linked to energy conversion.

The $F_{420}H_2$ produced from the oxidation of methyl-H_4MPT and methylene-H_4MPT is reoxidized by the membrane-bound electron transport system. Studies have indicated that the $F_{420}H_2$-dependent reduction of CoM-S-S-HTP generates a transmembrane electrochemical proton potential, which serves as the driving force of the synthesis of ATP from ADP and Pi. The $F_{420}H_2$-dependent heterodisulfide reductase system can be processed via two partial reactions: the first reaction involves the oxidation of $F_{420}H_2$ by $F_{420}H_2$ dehydrogenase, and subsequently, electrons are transferred to heterodisulfide reductase, which plays a key role in the H_2-dependent heterodisulfide reductase system. $F_{420}H_2$ dehydrogenase has been evaluated in vitro studies by using an artificial electron acceptor system. Moreover, the enzyme that catalyzes the reaction has been purified from *Methanolobus tindarius*. The apparent molecular mass of the enzyme is 120 kDa, which consists of five polypeptides with molecular masses of 45, 40, 22, 18, and 17 kDa, respectively. Each mole of the enzyme contains 16 mol Fe and 16 mol acid-labile sulfur.

It can be summarized that CoM can be synthetized via methyl transfer by methanogens. The oxidation of one methyl-CoM generates three pairs of reducing equivalents, which can be used to reduce three methyl-CoM to methane. This process includes the formation of CoM-S-S-HTP, which serves as the actual electron acceptor. The reduction of this reaction has been linked to energy transduction.

4.4 Methanogenesis from Acetate

Acetate is a key intermediate in the anaerobic digestion. In 1977, a study was performed, which indicated the anaerobic digestion metabolism of acetate to CH_4 and CO_2 produced 70% CH_4 of the total biomass. Subsequent studies have proposed similar findings. During anaerobic digestion, a variety of organic compounds, such as carbohydrates, amino acids, and long-chain fatty acids produce acetic acid, with glucose ($C_6H_{12}O_6$) as follows:

$$C_6H_{12}O_6 + 2H_2O \longrightarrow 2CH_3COOH + 2CO_2 + 4H_2 \tag{7.19}$$

$$2CO_2 + 4H_2 \longrightarrow CH_4 + 2H_2O \tag{7.20}$$

$$2CH_3COOH \longrightarrow CH_4 + 2CO_2 \tag{7.21}$$

The overall reaction:

$$C_6H_{12}O_6 \longrightarrow 3CH_4 + 3CO_2 \tag{7.22}$$

Some CH_4 that was generated from CO_2 and H_2 was produced from $C_6H_{12}O_6$ oxidation, whereas two-thirds of CH_4 was generated from acetic acid. Several strains belonging to *Clostridium* can use H_2 and CO_2 to synthesize acetic acid as their substrate of methanogens.

In nature, acetate is the main precursor of methane; however, only a minority of methanogenic species can utilize acetate as a substrate. Those methanogens belong to the *Methanosarcina* and *Methanothrix*, both of which are members of the Methanosarcinaceae. The main difference between the two genera lies in the ability of *Methanosarcina* to utilize H_2/CO_2, methanol, and methylamines in addition to acetate. Moreover, *Methanothrix* are unable to utilize any substrates other than acetate. Furthermore, the affinities of these two methanogens for acetate are different. This difference highly affects the predominance of *Methanosarcina* and *Methanothrix* in a given environment. Due to its higher affinity for acetate, *Methanothrix* is the dominant acetotrophic species when acetate concentrations are below 1 mM. However, *Methanosarcina* favor higher acetate concentrations due to their faster growth rate. The reaction of acetic acid involved in the anaerobic digestion process is shown in Table 7.14.

Table 7.14: The reaction of acetic acid in methanogenesis.

Reaction	Free energy	Bacterium
Acetate + CoA ⟶ acetyl-CoA + H_2O	+35.7	Sarcina methane uses acetate kinase (akt) and phosphoric acid acetyl transferase (pta), Methanogens for thiokinase (acs)
Acetly-CoA + H_4SPT ⟶ CH_3- H_4SPT + CO_2 + CoA + 2[H]	+41.3	CO dehydrogenase/acetyl coenzyme A synthetase (cdh ABCXDE)
CH_3- H_4SPT + HS-CoM ⟶ CH_3- S - CoM + H_4SPT	−30	Methyl-H_4SPT: coenzyme M methyltransferase (energy storage) (mtrEDCBAFGH)
CH_3- S-CoM + H-S-CoB ⟶ CoM-S-S-CoB + CH_4	−45	Methyl coenzyme M reductase (mcrBDCGA)
CoM-S-S-CoB + 2[H] ⟶ H-S-CoM + H-S-CoB	−40	Miscellaneous disulfide reductase (hdrDE)

4.4.1 Activation of Acetate and Synthesis of CH_3-H_4MPT

When acetate is used as a substrate, methanogens have a lower growth rate. The difference between acetate and acetic acid are two carbon atoms at a different position, which causes the transfer rate between methane and carbon dioxide to be different. Carbon labeling experiments have shown that approximately 65% of the ^{14}C-labeled methyl was transferred to methane, whereas the portion of ^{14}C-labeled carboxyl that was transferred to carbon dioxide was 16%. Therefore, in some studies, it was suggested that methane in which carbon was obtained from various matrices decreases in the following order: CH_3OH > CH_4 > C-2 acetic acid > C-1 acetic acid. However, when the environment contains an auxiliary matrix such as methanol, the metabolism of acetic acid dramatically changes, resulting in altered methyl carbon flows.

For utilizing acetic acid, methanogens start by activating acetyl-CoA. *Methanosarcina* and *Methanothrix* use different approaches for acetate activation. *Methanosarcina* use acetate kinase and phosphotransacetylase, whereas *Methanothrix* use acetyl-CoA synthetase. Both acetate kinase and phosphotransacetylase have been isolated and purified from *M. thermophilato*. Moreover, acetyl-CoA synthetase has been isolated and purified from *M. soehngenii*, and all three enzymes are soluble and sensitive to oxygen.

The acetate kinase isolated from *Ms. thermophila* is composed of two identical subunits of 53 kDa. The crystal structure of the acetate kinase indicated that *Ms. thermophila* is a member of acetate/hexokinase/Hsc70/actin (ASKHA) in phosphotransacetylase, and kinetic and biochemical studies have shown that the transfer of phosphate from ATP to acetic acid involves a direct in line mechanism.

Phosphotransacetylase consists of one polypeptide of 42 kDa, and its activity can be significantly stimulated by potassium and ammonium. The crystal structure of phosphotransacetylase indicates CoA-SH bonds with active site residues. In addition, acetyl-CoA-synthetase purified from *Mtx. Soehngenii* consists of two identical 73 kDa subunits, and has a K_m of 48 μm for coenzyme A.

4.4.2 The Cleavage of Acetyl-CoA

The breakdown of acetyl-CoA is catalyzed by CO dehydrogenase (cdh) in a very complex reaction. First, the cdh complex catalyzes the cleavage of acetyl-CoA, thereby giving rise to a methyl group, a carbonyl group, and CoA, all of which are transiently bound to the enzyme. In a second step, the cdh complex catalyzes the oxidation of the carbonyl group, resulting in the formation of CO_2. CoA is released from the enzyme, whereas the methyl group is transferred to corrinoid-Fe-S protein. Subsequently, methyl is transferred to H_4SPT and eventually forms methane.

Methanogens from *Methanosarcina* and *Methanothrix* characterize cdh. The cdh derived from *M. thermophila* and *Ms. barkeri* contains five subunits (α, β, γ, δ, and ε). When detergent is used, Ni/Fe-S components (α and ε subunits), Co/Fe-S components (γ and δ subunits), and β subunits can be determined. The Ni/Fe-S crystal structure from *Ms. barkeri* shows that the α subunit contains a false cumene (pseudocubane) Ni-3Fe-4S cluster, which primarily contains acid-labile sulfur, connected to an exogenous Fe. Thus, Ni and exogenous Fe play a role in activating C–O bonds. Structural analysis of the ε subunit displays a possible FAD binding site, which is consistent with the FAD component reduction effect of Ni/Fe-S isolated from *Ms. barkeri*. In addition, the FAD binding site is located in the subunit of the 4Fe-4S cluster. Therefore, it is considered that the ε subunit transports electrons from the 4Fe-4S cluster to initiate FAD function. The β subunit from Cdh of *M. thermophila* contains an "A" cluster, which may likely be the site to break down or synthesize acetyl coenzyme. The cdh components Co/Fe-S from *Methanosarcina* contain factor III and participate in the transportation of methyl groups of acetyl coenzyme A to H_4SPT. The Co/Fe-S component also contains a [4Fe-4S] cluster that can be used as a direct electron donor. The encoded δ and β subunits of the cdhD and cdnE genes have been cloned and sequenced. The cdhE gene contains a 4-cysteine motif, and a possible 4Fe-4S cluster.

C. thermoaceticum is a homoacetogenic bacteria, in which the cdh complex catalyzes the fracture reaction of acetic acid and forms acetyl coenzyme A. Accordingly, this enzyme is acetyl coenzyme A synthetase. Briefly, the fracture of acetic acid may be considered a reversible process of this mechanism. Interestingly, enzymes isolated from *M. thermophila* catalyze not only the breakdown of acetyl coenzyme A, but also synthesize CoA, CO, and methyl iodide in a similar fashion as the enzyme isolated from *C. thermoaceticum*. Depending on the reaction mechanism proposed by previ-

ous studies, acetyl coenzyme A is degraded by the effect of Ni/Fe-S, and methyl and carboxyl groups are bound to the active site of the metal center, while CoA is bound to other sites of Ni/Fe-S and is then being released. Carboxyl side groups bound to metal sites are oxidized to CO_2 and subsequently released. Methyl is transferred to the Co(I)/Fe-S component and methylated Co (III) corrinoid protein is generated. Then, methylated corrinoid proteins transfer methyl to H_4MPT to generate methyl-H_4MPT.

4.4.3 The Electron Transfer and Energy Conversion of Acetate to Methane

When methanogens utilize acetic acid and H_2/CO_2 as a substrate from methyl-H_4MPT to methane, the carbon flow is similar, whereas the flow of electrons is altered. Moreover, when utilizing H_2/CO_2 as a substrate, H_2 activated by the membrane binds hydrogenase, and electrons are transferred through alienation disulfide reductase. In contrast, when acetic acid was used as a substrate, the electron carrier has not yet been identified. In vitro experiments have shown that electrons generated by the oxidation process in the carboxyl group of acetyl coenzyme A are transferred to ferredoxin. However, how exactly electrons are transferred from ferredoxin to alienation disulfide remains to be elucidated. In *M. thermophila*, the ferredoxin causes cdh transfer electrons to the membrane-related hydrogenase. Presumably, the ferredoxin is oxidized in the membrane. This process involves energy conversion and electron transport chain, primarily using alienation disulfide as a terminal electron acceptor. However, this system has not yet been identified. It may be assumed that cytochromes participate in the electron transfer processes of methanogenesis, because *Methanosarcina* and *Methanothrix* both contain membrane-bound electron carriers. Furthermore, acetoclastic methanogens contain an unclear energy-absorbing substance that is involved in the acetic acid cleavage reaction described below:

$$\text{Acetyl-S-CoA} + \text{HS-CoM} \longrightarrow \text{CO} + \text{CH}_3\text{-S-CoM} + \text{CoA}\ \Delta G_0' = +40.3\ \text{kJ/mol} \qquad (7.23)$$

In summary, the conversion of the matrix of methanogens, including the formation of methyl-CoM and the reduction of alienation disulfide, has been investigated in several studies. Different substrates differ in their reactive electron acceptors for H_2, $F_{420}H_2$, and the acetyl coenzyme A carboxyl group.

4.5 Methanogens Genome Research

In 1996, a study at the University of Illinois (United States) first completed the genome sequencing of methanogenic archaea *Methanocaldococcus jannaschii*. Since 2006,

additional studies have been performed, resulting in complete gene sequencing of methanogenic. So far, genome sequencing has been completed for 14 genera, 9 types, and 22 strains of methanogenic archaea. The length of the methanogens' genome is 1.57–5.75M bp. In general, the genome of *Methanococcus* and *Methanobacterium* is less than 2M bp. In contrast, *Methanosarcina* usually has a larger genome, and at present, *Methanosarcina acetivorans* have been reported to have the largest genome. Most of their genome contains predictable open reading frames, which encode the function of those proteins [31–34].

By analyzing transcriptomics and proteomics of *M. acetivorans* in different environments, cells contain more protein when acetic acid is used as a matrix. Similarly, mRNA expression levels for F_{420} genes also increased when acetic acid was used. The expression level and protein abundance derived from microarray analysis showed a good correlation at each of the conditions tested. Therefore, access to new information regarding the electron transfer component is mainly related to bacteria that use acetic acid as a substrate, CODH-ACS's replication capabilities, conventional stress responses, and a large number of regulatory proteins. Other proteomic studies involve the CO_2 reduction methanogenic pathway of *M. acetivorans* with CO as a substrate. This approach uses a novel methyl transferase, a novel energy conversion mechanism, $F_{420}H_2$, and CoM-SS-CoB oxidoreductase. Thus, these studies indicate that the number of single-function CODH increases when growth is dependent on CO.

Proteomic analysis of extreme methanogens has been found to be relatively exile, which is different from that of mesophilic bacteria. Saunders et al. focused on genomics and proteomics of the psychrophilic methanogenic archaea *Methanococcoides burtonii* and they found that major differences were amino acids, tRNA composition, and protein structure by genomic analysis. Indeed, psychrophilic archaea contained more uncharged polar amino acids, especially glutamic acid and threonine, and less hydrophobic amino acids such as leucine. Having the information of the genomes of nine methanogenic archaea, a protein structure model was established. The model showed that cold-adapted archaea has a higher water-soluble region, and contained glutamic acid and threonine, hydrophobic amino acids, and less charged residues.

Section 5: Microecology of Methane Production and Its Application

5.1 Microecology of Methane Production

On the one hand, non-methanogens and methanogens are interdependent in creating desired environmental conditions for each other. On the other hand, they are mutual restraint and maintain a state of relative equilibrium.

5.1.1 Non-methanogens Provide Substrates for Methanogens

Non-methanogens convert all types of organic matter into H_2, CO_2, NH_3, VFAs, and methanol. Propionic and butyric acid can be decomposed and converted into H_2, CO_2, acetic acid by hydrogen-producing acetogens. This provides carbon precursors the electron donor and nitrogen for methanogens that synthesize cytoplasm and methane. However, methanogens and non-methanogens compete for H_2 and other matrixes.

5.1.2 Non-methanogens Provide Anaerobic Environment Suitable for Methanogens

Methanogens are strictly anaerobic microorganisms that will die fast under aerobic conditions. When oxygen is reduced to water, toxic intermediates can be formed, including hydrogen peroxide, superoxide, and hydroxyl radicals. Aerobic microbes contain enzymes that can decompose these products, such as catalase and superoxide dismutase enzymes, whereas in strict anaerobic microorganisms these enzymes are lacking. Therefore, under aerobic conditions, the activity of methanogens is inhibited. In microorganism community, oxygen in the environment can be consumed by non-methanogens, thereby reducing the redox potential of the environment to reach an optimum Eh (–350 mV) value. The optimal Eh value is dependent on the ordered replacement of anaerobic microorganisms, for example, cellulose-decomposing bacteria, sulfate-reducing bacteria, nitrate-reducing bacteria, and bacterial production of ammonia producing bacteria and acetic acid.

5.1.3 Non-methanogens Alleviate Toxic Effects for Methanogens

Industrial waste water or waste as feedstock, which might contain phenols, cyanides, benzoic acid, long chain fatty acids, and several heavy metal ions and these substances may inhibit methanogens. Many non-methanogens microorganisms have the ability to metabolize and remove poison for the above-mentioned substances. The H_2S metabolites of some non-methanogens can react with heavy metal ions to form insoluble metal sulfides, thereby reducing the toxic effects of these ions on methanogens.

5.1.4 Methanogens Relieve the Feedback Inhibition of Biochemical Reactions for Non-methanogens

Under anaerobic conditions, and in the absence of an exogenous electron acceptor, non-methanogens convert various organic substances into, for example, H_2, CO_2, organic acids, and alcohol. These fermentation products can suppress the activity of methanogens, and acid accumulation can inhibit acid-producing bacteria to continue producing acid. When the concentration of acetic acid in an anaerobic digester exceeds 1,000 mg/L, acidification will occur and anaerobic digestion cannot be performed effectively, resulting in failure of methane production. In a normal-operated system, methanogens can continuously use metabolites from non-methanogens to produce methane, and relieve the inhibition to non-methanogens. In previous studies, the effects of 88 types of substrates used by *Methanosarcina* sp. (DSM2906) and *Desulfotomaculum nigrificans* (DSM 574) as methanogens and sulfate-reducing bacteria were investigated. The results showed that nine types of substrates inhibited *D. nigrificans*; however, no effects were found on the activity of methanogenic bacteria.

5.1.5 Regulation of Methanogens in an Anaerobic Digestion Process

The proton regulators, electronic regulators, and nutrition regulator involved in anaerobic digestion were given in Table 7.15. The proton regulator function of the methanogen metabolism can remove toxic protons, resulting in an anaerobic digestion environment that does not include acidification and making the microorganisms in anaerobic digestion to live in a suitable pH range, which is the main ecological role of methanogens. The electronic regulator function of the hydrogen metabolism by methanogens creates the most favorable condition for hydrogen-producing acetogenic bacteria that metabolize multicarbon compounds, such as alcohols and fatty acids, from the perspective of thermodynamics. Moreover, it increases substrate utilization efficiency by hydrolyzing bacteria.

Table 7.15: The regulation of methanogens

Regulation	Metabolic reactions	Role
Proton regulation	$CH_3COO\text{-} \longrightarrow CH_4+CO_2$	Remove toxic protons Maintain a proper pH range
Electronic regulation	$4H_2+CO_2 \longrightarrow CH_4+H_2O$	Create optimal conditions for hydrogen-producing acetogens and improve the efficiency of hydrolyzing bacteria for utilizing matrix
Nutrition regulation	Secrete growth factors	Stimulate the growth of heterotrophic bacteria

5.2 Application of Anaerobic Digestion Technology

During anaerobic digestion process, organic material is decomposed to methane, carbon dioxide, and water by the anaerobic microbial metabolism. A biogas project is considered as environmental engineering and effective treatment of organic waste that is not only a clean and convenient energy project but also a type of ecological engineering for biomass multilevel utilization.

5.2.1 The Present Situation of Biogas Projects at Home and Abroad

Development of biogas technology in Europe began in the 1970s, when people started to pay attention to renewable and green energy. However, pre-built anaerobic digestion facilities did have some issues, including pipe blockage, poor equipment quality, backward utilization of biogas, and inefficiency, resulting in poor economic efficiency of biogas projects. Over the last decade, the number of biogas projects in Europe has reduced; however, the technology and equipment quality are significantly improved, resulting in relatively lower project costs. Rapidly developing countries are mainly Denmark, Germany, the United Kingdom, and Sweden.

Since the energy crisis in the 1970s, studies in Germany have continuously focused on biogas. At the end of 2013, Germany had 7,720 biogas projects, of which the power generation by biogas plants took up 4.5% of the total capacity. These biogas projects, however, are still far from meeting all requirements, and if all waste and energy crops were treated, Germany would need 30,000–40,000 biogas projects. Currently, the German medium-sized biogas projects are applied to solid waste treatment including sanitary landfill, livestock or poultry farms, and private farms.

Interests in biogas energy research in the United States started with an energy shortage pressure in the twentieth century. In the twenty-first century, methane, as a clean energy source, started to gain attention of Congress. The US government and Congress had developed strategies and projects related to biogas and other types of biomass energy development. The congressional 2112 amendment requires the Ministry of Agriculture to establish university-based studies, extension, and education programs to promote biomass energy technology, as well as products and economic development in rural America. From the perspective of research demonstration, Cornell University began to implement a biogas fuel cell project and converted a dairy farm's manure into electricity by using biogas and fuel cells to provide green fuel for trucks. Britain has 45 large biogas projects and can treat farm waste to produce renewable energy, and by using its nutrients soil improvement can be achieved. Within 2 years after the year 2000, Sweden had built five large-scale biogas projects and treated 2.6 to 80,000 tons of agricultural waste per year. During

this process, the fermented liquid was used as organic fertilizer. Denmark had built 19 centralized biogas projects and 18 farm biogas plants and treated up to 160,000 tons of agricultural waste per year. Moreover, Switzerland and Japan built 18 biogas garbage treatment plants, containing 12 waste-treatment plants with an annual capacity of 10,000 tons or more. The French company Steinmueller Valorga Sarl used a self-developed Valorga process to treat organic waste, and maintained a solid content of 25–35%.

In China, biogas projects involving large and medium livestock and poultry farms started at the end of 1970s. The entire development process was closely related to the scale and intensification of aquaculture, but was also related to the level of concern for the environment. In the late 1970s to mid-1980s, the development of biogas projects for livestock and poultry farms mainly focused on the production of biogas energy to alleviate the shortage of energy supply in rural areas. At that time, the number of large and medium farms was smaller; therefore, several early projects also used straw as raw material instead of stool. This was fermented at room temperature at a gas volume rate of 0.2 m^3/m^3·day. The fermented liquid could directly be used as organic fertilizer. From the early 1990s, China started to pay attention to the environmental benefits of large biogas projects by conducting utilization to increase economic benefits of the project. By studying and developing a variety of novel and efficient fermentation processes, the anaerobic digestion processing capacity has increased 2- to 10-fold and the gas production rate increased 1- to 3-fold. Moreover, the chemical oxygen demand (COD) removal rate increased by 10–20%.

5.2.2 The Biogas Technology Gap at Home and Abroad

5.2.2.1 The Scale of Biogas Projects

At the end of 2010, China's total rural household biogas projects population reached 38.51 million, including 337 large-scale biogas projects for treating industrial waste, 4,631 large biogas projects for treating agricultural waste, 22,795 medium-sized biogas projects, and 45,259 small-scale biogas projects. The power generation capacity was 73,587 kW, and the annual generated capacity was 277.06 million kW hours. The biogas production is nearly 14 billion m^3 per year, which saves 25 million tons of standard coal and reduces carbon dioxide emission by about 50 million tons.

Since 2000, the German "Renewable Energy Law" was implemented, and through demonstration projects, the number of biogas projects increased rapidly. By the end of 2010, 6,000 biogas projects were built at a scale over 500 m^3 all over Germany. The total installed capacity was 2,700 MW, including 40 biogas plants with an installed capacity of 2 MW, and the minimum capacity was 50 kW. Germany has 470 gas companies engaged in biogas project design, construction, and equipment supply.

Moreover, Germany plans to build 12,000 biogas energy projects in 2020. With a total installed capacity of power generation of 9,500 MW, the biogas electricity generation accounts for 7.5% of the country's total energy-generating capacity.

5.2.2.2 Biogas Production Technology

In China, the substrates concentration of biogas fermentation is low, especially at room temperature. Only a few projects require high-temperature fermentation. The biogas production rate and the proportion of codigestion is low; however, with the development of large-scale biogas projects, China now has several companies that focus on biogas project design, construction, and equipment supply, but the general level of technology is not very high.

Germany has advanced equipment and technology, such as feed equipment, mixing equipment, desulfurization equipment, gas storage facilities, and cogeneration equipment. Moreover, the volume load and volume gas production rate are higher compared to those in China. The biogas projects are mainly performed at a high or medium temperature. With the implementation of combined heat and power (CHP), biogas production is still efficient in the winter. The biogas production rate at medium temperature system is 1.2–1.8 m^3 (m^3/day), whereas the biogas production rate at high-temperature system is 3.0 m^3 (m^3/day). The economic benefits of biogas are significant.

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Huijuan Xu, Dong Li, Dalong Huang, Weizheng Zhou,
Lingmei Yang, Zhibing Li, Feng Zhen

8 Microbial Hydrogen Production

Utilization and over-exploitation of fossil fuels have caused many environmental issues, such as the acid rain, ecological destruction or degradation, and global warming. From the point of view of environmental protection, the development of nonpolluting, renewable energy is an inevitable choice. Hydrogen combustion generates only water without producing any greenhouse gases, due to which it is environmentally friendly. Due to these reasons, hydrogen is likely to be an ideal alternative energy source.

Currently, hydrogen is mainly produced from fossil fuels (natural gas reforming or pyrolysis, partial oxidation of heavy oil, and coal gasification), water (electrolysis or photolysis), and biomass (thermochemical or biochemical conversion). However, hydrogen production from fossil fuels requires large amounts of oil, gas, and coal, which are nonrenewable, whereas the reactions need critical conditions, such as high temperature and high pressure. Furthermore, the electrolysis of water into hydrogen is highly energy sensitive, as 1 m^3 of hydrogen is produced at the cost of 4–5 kWh of electricity consumption. Therefore, fossil fuels and water are unlikely to be used for hydrogen production on large scale for their high consumption of resources or energy. On the other hand, microbial hydrogen production can operate at normal temperature and pressure. In addition, water or various renewable organic materials are utilized for photolysis or fermentation, while a variety of organic wastes, including organic wastewater and organic solid wastes, can also be treated as an added advantage to the process. Therefore, the process of microbial hydrogen production will have a broad prospect of applications. With the increasing demand for energy and the growing concern for environmental protection, research on biohydrogen production has attracted widespread attention.

According to the types of hydrogen-producing microorganisms and their metabolic pathways, microbial hydrogen production is mainly divided into five types.

(1) Direct or indirect biological photolysis of water in algae.
(2) Indirect biological photolysis of water in cyanobacteria.
(3) Light fermentation of organic compounds in photosynthetic bacteria.
(4) Dark fermentation of organic compounds in fermentative bacteria.
(5) Water-gas shift reaction in carboxydotrophic bacteria.

Corresponding author: Huijuan Xu, E-mail: xuhj@ms.giec.ac.cn
Dong Li, Dalong Huang, Weizheng Zhou, Lingmei Yang, Zhibing Li, Feng Zhen, Guangzhou Institute of Energy Conversion, Chinese Academy of Sciences; CAS Key Laboratory of Renewable Energy; Guangdong Provincial Key Laboratory of New and Renewable Energy Research and Development, Guangzhou 510640, China
Dong Li, Chengdu Institute of Biology, Chinese Academy of Sciences, Chengdu 610041, China

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This chapter will discuss biological hydrogen production with regards to key enzymes, hydrogen metabolism, microorganisms and strain screening, and its potential applications.

Section 1: Hydrogenase and Nitrogenase

1.1 Hydrogenase

In 1931, Stephenson and Stickland discovered hydrogenase in *Escherichia coli* [1]. In 1974, Chen et al. [2] isolated and purified soluble hydrogenase from *Clostridium barati*. Since then, a variety of hydrogenases have been isolated and purified from fermentative bacteria, photosynthetic bacteria, cyanobacteria, nitrogen-fixing bacteria, eukaryotic algae, and fungi. As a matter of fact, a vast majority of microorganisms contain hydrogenase, which is directly involved in reversible oxidation-reduction for catalytic hydrogen production ($H_2 \leftrightarrow 2H^+ + 2e^-$) and plays an important role in the regulation of biological oxidation-reduction reactions. When excessive electrons or reducing power is produced during biological oxidation processes, the microorganisms use proton reduction of hydrogenase to release excessive electrons or reducing power, so as to ensure the oxidation-reduction balance during cellular metabolism. When the cell lacks electrons or reducing power, and H_2 is present, microorganisms employ the hydrogen oxidation of hydrogenase to produce electrons or reducing power. In addition, the catalytic hydrogen oxidation of hydrogenase can also be used as the sole source of anabolic energy for cells. For example, the acetyl coenzyme A route is performed to fix the homoacetogenic bacteria, hydrogen nutrition methanogens, and sulfate-reducing bacteria of CO_2. These bacteria only use H_2 and CO_2 as growth substrates for cellular synthesis. The production of hydrogen is carried out through reduction of acetic acid, methane, and sulfate under dark and anaerobic conditions.

$$2H^+ + 2e^- \xleftrightarrow{\text{hydrogenase}} H_2 \tag{8.1}$$

$$NAD(P)H + H^+ \xleftrightarrow{\text{hydrogenase}} NAD(P)^+ + H_2 \tag{8.2}$$

Hydrogenase can be categorized into two groups depending on its position within the cell: soluble and membrane-bound. Membrane-bound hydrogenase is associated with the membrane components and is generally involved in cellular metabolism. On the other hand, soluble hydrogenase includes hydrogenases in cytoplasm and periplasm and is involved in the regulation of intracellular metabolic balance, release of intracellular excessive electrons, and absorption of exogenous electron donors, such as hydrogen.

Hydrogenase can also be categorized according to the metals in its active center, such as FeFe-hydrogenase [3–5], NiFe-hydrogenase [6–8], and Fe-hydrogenase [9–12].

The active centers of these enzymes are different from each other in their structures and catalytic properties. Fe-hydrogenase is found only in hydrogenotrophic methanogens. Substantial evidence has revealed that NiFe-hydrogenase and FeFe-hydrogenase belong to two evolutionary classes. Existing genome sequences have revealed that the genes encoding mature protein of NiFe-hydrogenase have no matching sequences in organisms containing only FeFe-hydrogenase.

1.1.1 FeFe-hydrogenase

FeFe-hydrogenase was first isolated from *Clostridium pasteuianum* [13] and can be induced by external conditions. For example, *Desulfovibrio desulfuricans* produces FeFe-hydrogenase under anaerobic conditions in the presence of H_2 and sulfates and in the absence of Ni, whereas it mainly produces NiFe-hydrogenase in the presence of Ni [14]. FeFe-hydrogenases can be categorized according to their location in cells. They are specifically distributed in cytoplasmic and periplasmic enzymes. The cytoplasmic enzymes catalyze the reduction of protons, while the periplasmic enzymes catalyze the oxidation of hydrogen. In general, the catalytic reduction of protons by FeFe-hydrogenase is superior to the catalytic oxidation of hydrogen by FeFe-hydrogenase.

FeFe-hydrogenase usually consists of a single peptide chain, a prime example being the FeFe-hydrogenase from *C. pasteurianum* I (abbreviated as CpI), which has the molecular weight of 60kD [15]. FeFe-hydrogenase can be composed of two peptide chains, such as in the FeFe-hydrogenase of *D. desulfuricans* (Dd). In this case, the two polypeptide chains have molecular weights of 46kD and 10kD. According to the crystal structure, the enzyme can be roughly divided into two subunits of different sizes regardless of the fact if it is composed of one or two peptide chains. The larger subunit is the conserved region of enzyme located at the C-terminal and contains an active center composed of six Fe atoms. The center is correlated with the activation of H_2, which is known as the H-cluster. The smaller subunit plays a role in electron transfer and is mainly for Fe-S cluster, which is known as the F-cluster. Based on the source of the hydrogenase, [4Fe-4S] clusters and [2Fe-2S] clusters are obtained. For example, *D. desulfuricans* contains only two [4Fe-4S] clusters, while *C. pasteurianum* contains three [4Fe-4S] clusters and one [2Fe-2S] cluster.

The active center of FeFe-hydrogenase is bimetallic and is composed of two Fe atoms (the two Fe atoms are denoted as Fe_I and Fe_{II}) [16]. Fe_{II} is connected to the proximal [4Fe-4S] cluster through sulfocysteine by a mercaptan metal, while the entire active center is connected to the surrounding amino acid by the iron of [4Fe-4S] cluster as well as the enzyme protein, which is very different from NiFe-hydrogenase. Each Fe atom has two diatomic ligands (CN^- and CO). Each Fe atom is connected to N atom of another ligand (dithiocarbamate methyl amine; DTMA) through two S

atoms. There is a bridging ligand between the two Fe atoms. Therefore, Fe_{II} has six ligands and forms a highly distorted octahedral structure, while Fe_{I} has only five ligands with a vacant site, which is associated with the binding of H_2 and can be occupied by CO to inhibit the activity of FeFe-hydrogenase. During catalysis, Fe_{I} is involved in the oxidation-reduction of iron, while Fe_{II}, which is associated with the Fe-S cluster, maintains its low-spin and divalent form.

1.1.2 NiFe-hydrogenase

NiFe-hydrogenase of *Desulfovibrio gigas* is a heterodimer globular protein [7], which has a radius of about 3 nm and is composed of two subunits having different sizes (molecular weights are 63kD and 26kD). The junction area of the two subunits is 3,500 $Å^2$. The larger subunit contains an Ni-Fe binuclear active center that is buried deep inside the protein, while the smaller subunit consists of three Fe-S clusters. Both the subunits are closely connected to each other. The active center is close to the two inner Fe-S clusters and is located on the functional plane between the two subunits. Hydrogenase contains a total of 12 Fe atoms, 1 Ni atom, and 12 acid-labile sulfurs. Among these, 1 Fe atom and 1 Ni atom form the Ni-Fe active center, and the other 11 Fe atoms constitute 3 Fe-S clusters (1 [3Fe-4S] cluster and 2 [4Fe-4S] clusters). These 3 Fe-S clusters are arranged in a nearly straight line, whereas the distance between them is about 1.2 nm. The cluster nearest to the active center is called $[4Fe\text{-}4S]_p$, while the fastest one is called $[4Fe\text{-}4S]_d$. Furthermore, the middle cluster is called $[3Fe\text{-}4S]_m$. The distance between $[4Fe\text{-}4S]_p$ and the active center is about 1.3 nm, while $[4Fe\text{-}4S]_d$ is close to the hydrogenase surface. There is a Mg atom near the histidine (His) of C-terminal of the larger subunit (in case of *Desulformicrobium baculatum*, the atom is of Fe).

The active center of NiFe-hydrogenase is bimetallic and is composed of Ni and Fe. It is attached to the protein through four Cys residues *via* a sulfur bond [17]. In the oxidized state, the Ni atom has three close and two distant ligands, thus forming a highly distorted orthogonal pyramid structure, where the position of the sixth ligand on the axis is empty, while the Fe atom has eight ligands and forms a distorted octahedral structure. Four Cys residues on the larger subunit are connected to Ni by S atoms. In the oxidized state, two Cys residues are also connected to Fe atoms. The nonprotein ligands that are connected to Fe atoms are CO and two CN^- in *D. gigas*, whereas SO, CO, and CN^- are present in NiFe-hydrogenase of *D. vulgaris*. Additionally, the photosynthetic bacteria *Chromatium vinosum* may have three CN^- and one CO. The bridging ligand (X) is also present between the two metal atoms, whereas S is the bridging ligand in *D. vulgaris*, while it is O in *D. gigas*. The enzyme activity may be associated with the loss of bridging ligands and the contraction of the distance between Ni and Fe. In all reduced states, the bridging ligands between the two metals may not exist.

1.1.3 Fe-hydrogenase

In 1990, Thauer et al. reported the existence of Fe-hydrogenase for the first time. However, its structure and function were not studied until recently. At present, the most frequently studied Fe-hydrogenase is from *Methanothermobacter marburgensis* [18]. However, a homology analysis of its amino acid sequence indicated that all sources of Fe-hydrogenase were similar. Fe-hydrogenase exists only in hydrogenotrophic methanogens. In the reduction of CO_2 to methane, it can reversibly catalyze the reaction between methyl tetra hydrogen methane pterin (CH-THMP) and H_2 to form methylene tetra hydrogen methane pterin (CH_2-THMP), which is why it is known as H_2-forming CH_2-THMP dehydrogenase (Hmd hydrogenase) [12]. In addition, the active center of Fe-hydrogenase does not contain a binuclear metallic center or Fe-S clusters, due to which it is also known as non-Fe-S cluster hydrogenase and therefore is a metal-free hydrogenase. NiFe-hydrogenase and FeFe-hydrogenase activate H_2 by their binuclear metallic centers, and use Fe-S clusters to shunt electrons ($H_2 \leftrightarrow 2H^+ + 2e^-$), while Fe-hydrogenase employs mononuclear Fe center to catalyze H_2 and produce hydride ($H_2 \leftrightarrow H^+ + H^-$). The hydrogen is subsequently transferred to the methotrexate of CH_2-THMP. Notably, Fe-hydrogenase cannot reduce dyes, such as methyl viologen.

Fe-hydrogenase is a homodimer, in which each monomer has the molecular weight of 38kD. The C-terminals of the two subunits form a central helical structure, while the two peripheral N-terminals are similar to the structural domain of Rossmann, including a classical Rossmann curler (βαβ) and an α helix. Table 8.1 compares the physical and chemical properties of three types of hydrogenase.

Table 8.1: Physical and chemical properties of four types of hydrogenase.

Types of hydrogenase	FeFe-hydrogenase	NiFe-hydrogenase	Fe-hydrogenase
Sources	*Desulfovibrio desulfuricans*	*Desulfovibrio gigas*	*Methanothermobacter marburgensis*
Locations	Extracellular periplasmic	Extracellular periplasmic	Extracellular periplasmic
Molecular weight (kD)	53	85	76
Number of subunits	2	2	2
Number of Fe atoms	14	12	1
Number of Ni atoms	0	1	0
Other metal atoms	0	1 Mg	0
Nonprotein ligands	2 CN, 2 CO, 1 DTMA	2 CN, 1 CO	Except for 1 CO and 2-pyridinol, the others are unknown
[4Fe-4S]	3	2	–
[3Fe-4S]	0	1	–
Hydrogen production rate[a]	6000~9000	700	–
Hydrogen consumption rate	28000	700	–

[a]Number of H_2 molecules catalyzed per second by each molecule of hydrogenase at 30 °C [17].

1.1.4 Mass Transfer in Hydrogenase

The above discussion mainly introduced the active centers of three types of hydrogenase. In addition to the active center, the catalytic production of hydrogen also requires electron transfer, proton transfer, and hydrogen transfer channels. Protons and electrons are transferred to active center in the interior through proton transfer and electron transfer channels, respectively, while H_2 is delivered to the surface of enzyme through hydrogen transfer channel. The mass transfer channels for FeFe-hydrogenase and NiFe-hydrogenase are described below; however, studies are still being conducted on the mass transfer channel of Fe-hydrogenase.

1.1.4.1 Electron Transfer

As discussed above, the electron transfer is carried out mainly by Fe-S clusters [19].

1.1.4.2 Proton Transfer

Proton transfer is realized by the rotation or vibration of carrier and receptor groups. These groups include amino acids, sulfur ligands, hydroxyl radical, and carboxyl radical. They all have suitable pK_a values for the transfer of protons. The space between the protons in protein is about 0.1 nm. In addition, internal water molecules can provide or receive two hydrogen bonds, due to which they also serve as a good transfer group. Since the group movement is very fast, the proton transfer does not limit the catalytic reaction rate.

1.1.4.3 Hydrogen Transfer

According to the analysis of topological structure carried out using the X-ray diffraction of xenon diffusion and molecular dynamic calculations of hydrogenase, the entry of H_2 is mediated by the hydrophobic internal cavity that is composed of narrow channels [19]. One end of the network-like tunnel is connected to the vacancy point of the active center, while the other leads to the external medium. Most of the residues in the channel are hydrophobic and extend to the molecular surface to form several hydrophobic sites, which formulate the gas inlet region. Oxygen molecule may also utilize the same channel to enter the active center. Almost all hydrogenases can be inhibited by CO, however the inhibition is reversible, while CO can be removed by the continuous addition of H_2 in the system.

1.1.5 NiFe-uptake Hydrogenase

Based upon the catalytic function, hydrogenase can be categorized into uptake hydrogenase and reversible hydrogenase. Uptake hydrogenase, such as in cyanobacteria, only

catalyzes the oxidation of H_2, whereas the reversible hydrogenase can reversibly catalyze the reduction of H^+ and the oxidation of H_2. In particular, the reversible hydrogenase catalyzes the reactions in different directions based on the intracellular physiological conditions. This type of hydrogenase is generally present in green algae, cyanobacteria, photosynthetic bacteria, and fermentative bacteria. In addition, some microorganisms may contain several different types of hydrogenase. For example, cyanobacteria possesses uptake and reversible hydrogenases, both of which are NiFe-hydrogenase (FeFe-hydrogenase has not been found in cyanobacteria to-date). Only the reversible hydrogenase is found in green algae and is mainly FeFe-reversible hydrogenase. In some ancient bacteria, Fe-hydrogenase is reversible hydrogenase. The following discussion will introduce NiFe-uptake hydrogenase and NiFe-reversible hydrogenase in cyanobacteria, as well as the FeFe-reversible hydrogenation in green algae. It is worth mentioning that related hydrogenases in other microbial sources are generally similar.

There are at least two different subunits in uptake hydrogenase, which have the molecular weights of around 60kD and 35kD. The smaller subunit HupS has eight cysteines, which are involved in the formation of Fe-S clusters, while the smaller subunit lacks the N-terminal twin-arginine signal peptide and the hydrophobic motif in the C-terminal. The larger subunit HupL contains Ni-binding sites, which are unique to N-terminal and C-terminal of the uptake hydrogenase.

For nitrogen fixation, uptake hydrogenase usually functions with nitrogenase, whose role is to catalyze and recycle the "wasted" reduction force and Adenosine triphosphate (ATP) in the form of H_2 (Figure 8.1). Happe et.al showed that, after the deletion of *hupL* gene that encodes the larger subunit of uptake hydrogenase in *Anabeana variabilis*, the yield of H_2 increased by 3 times as compared to the wild type [20]. In some of the filamentous cyanobacteria, the uptake hydrogenase is expressed only in heterocyst, and there is no or very little activity in the nutrient cells. In addition, the uptake hydrogenase is also present in the unicellular, nonnitrogen-fixing cyanobacteria *Synechocystis* PCC6301 that comes from gene mutation of reversible hydrogenase.

1.1.6 NiFe-reversible Hydrogenase

Reversible hydrogenase is widely present in nitrogen-fixing and nonnitrogen-fixing cyanobacteria, heterocysts, and nutrition cells. It combines with other

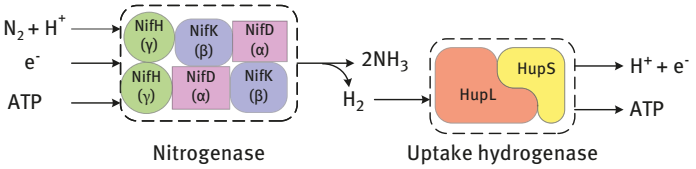

Figure 8.1: Function of the uptake hydrogenase.

proteins to form a complex. During cell division, it is easily dissolved or bound to the membrane. In unicellular *Synechococcus* PCC6301, reversible hydrogenase loosely binds with the cell membranes, while in *A. variabilis* and *Synechocystis* PCC6803, it binds with the thylakoid membranes [21]. Reversible hydrogenase is sensitive to O_2 and has poor thermal stability. It is a type of NAD^+ reductase, as it has a homologous NAD^+ reductase in *Alcaligenes eutrophus*. Because this enzyme is not involved in nitrogen fixation, the combined-state nitrogen in the culturing medium has almost no effect on this enzyme, while the combined-state nitrogen has strong inhibitory effects on nitrogenase. The activity of this enzyme relies on its electron donor, NAD(P)H, and electron acceptor, $NAD(P)^+$. The intracellular activity of reversible hydrogenase in cyanobacteria with heterocysts significantly improved under anaerobic and micro-aerobic conditions. However, the partial pressure of oxygen seemed to have no effect on the activity of this enzyme in non-nitrogen-fixing unicellular cyanobacteria. The physiological function of reversible hydrogenase is not very clear. It may be used in anaerobic environments either to release excessive reducing force or to release excessive electrons produced in photoreactions. In addition, reversible hydrogenase may be responsible for the distribution of electrons to respiratory chain.

NiFe-reversible uptake hydrogenase is a heterologous tetramer (Figure 8.2), which contains an NiFe-hydrogenase part and a diaphorase part. The larger (54.8 kD) and smaller (22.5 kD) subunits of NiFe-hydrogenase are encoded by *hoxH* and *hoxY*, respectively, while the smaller (26.9 kD) and larger (57.9 kD) subunits of diaphorase are encoded by *hoxU* and *hoxF*, respectively. In some cyanobacteria, the diaphorase may also be encoded with the involvement of *hoxE*. Notably, there are open reading frames (ORF) or spacers of different sizes and in different amounts between the subunits [22].

1.1.7 FeFe-reversible Hydrogenase

Green algae hydrogenases discovered to date are mainly FeFe-reversible hydrogenases. Firstly, the hydrogenase in *Chlamydomonas reinhardtii* was isolated and identified. It was composed of a 47.5kD monomer, which comprised of FeFe-reversible

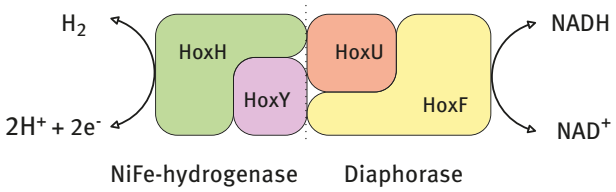

Figure 8.2: NiFe-reversible uptake hydrogenase.

hydrogenase [9]. Later, FeFe-reversible hydrogenases were also found in *Chlorella fusca, Chlamydomonas moewusii, Chlorococcum littorale*, and *Platymonas subcordiformis*.

Among the three categories of reversible hydrogenase, FeFe-hydrogenase attracts extensive attention due to high conversion of H_2 from the catalysis of protons. FeFe-hydrogenase has been isolated and obtained from *C. reinhardtii, C. fiscal, C. litiorale*, and *S. obliquus*. When compared to FeFe-hydrogenase in bacteria (such as *Clostridium pasteurianum*), the sequence homology is high in the C-terminal of FeFe-hydrogenase (including the catalytic center). In addition, the sequence in N-terminal has a large fragment deletion, while all the Fe-S cluster structures are lost. The electrons are transferred directly to the reaction center from the thioredoxin (Trx). The catalytic efficiency of FeFe-hydrogenase in green algae is much higher than that in the bacteria, which might be due to the loss in structure that benefits the electron transfer.

One type of green algae contains a unique variety of FeFe-hydrogenase. Wunschiers et al. found a completely different FeFe-hydrogenase gene in *Scenedesmus obliquus*, whose expression was subjected to constitutive expression rather than the anaerobic regulation. Forestier et al. and Zhang et.al also amplified *HydA2*, which was different from *HyaA1* in *Chlamydomonas reinhardtii*. Its encoding protein was slightly larger (49kD) than that of HydA1 (47.5kD). The similarity in the sequence of amino acid of both was 68%, while it was found highly similar to the hydrogenase in anaerobic bacteria, as all of the conservative motifs in the catalytic center were retained.

The FeFe-reversible hydrogenase gene is transcribed and rapidly induced for expression only when the cells grow under anaerobic conditions. Western blot and Northern blot analysis indicated that the FeFe-reversible hydrogenase began expression 10 min after the culture medium became anaerobic. Furthermore, the lack of oxygen could reduce the inhibition of expression of FeFe-reversible hydrogenase, while the start of gene expression was not directly related to factors, such as how the anaerobic environment was established, whether the illumination was available or not, and photosynthesis.

1.2 Nitrogenase

1.2.1 Classification of Nitrogenase

Nitrogenase participates in the photosynthetic hydrogen production in nitrogen-fixing bacteria, photosynthetic bacteria, and cyanobacteria. So far, four types of nitrogenase systems have been identified. Among these, three are very similar, and mainly differ in the metal atoms present in their atom cluster of the active center.

(1) Nitrogenases containing molybdenum and iron are abbreviated as MoFe nitrogenase. They are encoded by the gene *nif1* in heterocysts, whereas those in vegetative cells are encoded by the gene *nif2*.
(2) Nitrogenases containing vanadium and iron are abbreviated as VaFe nitrogenase, and are encoded by the gene *vnfDGK3*, whereas the transcription of these VaFe nitrogenase is induced in the absence of molybdenum.
(3) Nitrogenases containing only iron are abbreviated as FeFe nitrogenase, and are present in *Anabaena variabilis*, whereas they may be encoded by the gene *anf*.
(4) Nitrogenases relying on superoxide dismutase were isolated from *Streptomyces thermoautotrophicus*.

MoFe nitrogenase is the most common type of nitrogenase, whereas VaFe nitrogenase and FeFe nitrogenase can only be produced in certain nitrogen-fixing microorganisms and function under specific conditions. For example, in a media lacking molybdenum, nitrogen-fixing bacteria synthesize either VaFe nitrogenase or FeFe nitrogenase. The H_2 yielding capacities of the three nitrogenases in different nitrogen fixation bacteria cells are not the same. In *Rhodobacter capsulatus*, the H_2 yielding capacity of FeFe nitrogenase is greater than that of the MoFe nitrogenase. However, in *Anabaena variabilis*, the H_2 yielding capacities are found in the following descending order: vanadium-iron nitrogenase>MoFe nitrogenase>FeFe nitrogenase. The H_2 yielding capacity of VaFe nitrogenase is slightly higher than that of the MoFe nitrogenase, whereas the H_2 yielding capacity of FeFe nitrogenase is 40% that of the VaFe nitrogenase. Even in algae, which grow in a media lacking molybdenum and vanadium ions, its growth rate and nitrogenase activity are still very low.

1.2.2 Structure and Function of Nitrogenase

MoFe nitrogenase is the most common nitrogenase and is composed of two proteins. One is the MoFe protein, which is also known as the dinitrogenase or Component I, while the other is Fe protein, which is also called dinitrogenase reductase or Component II [23]. In order to distinguish the nitrogenase components from different sources, the first Latin letter of the generic and specific names are often used to represent microorganisms from different sources, while '1' and '2' are used to represent the MoFe protein and Fe protein, respectively. For example, Av1 refers to the MoFe protein of *Azotobacter vinelandii*, and Cp2 represents the Fe protein of *Clostrdium pasteurianum*.

The Fe protein is a homodimer (y_2) encoded by *nifH* gene having a molecular weight of 60–64 kD. The Fe protein contains two nucleotide binding sites (ATP-Mg or ADP-Mg) and one [4Fe-4S] cluster. The two nucleotide binding sites are separately embedded in two subunits, while the [4Fe-4S] cluster is located between the two subunits. The Fe protein mainly participates in ATP-Mg binding and hydrolysis.

The molecular weight of MoFe protein is 220–250kD and is composed of two different subunits ($\alpha_2\beta_2$), in which α is encoded by the gene *nifD*, while β is encoded by the gene *nifK*. Each αβ pair contains an iron molybdenum auxiliary factor (FeMoCo) and a P-cluster, both of which are present in αβ subunits, so that each MoFe protein contains two catalytic centers. FeMoCo is the catalytic center for substrate binding and reduction. Its elemental component is 7Fe-Mo-9S-homocitrate. One [4Fe-3S] cluster is connected to the [3Fe-Mo-3S] cluster by X atom and three bridge-S atoms [24]. The homocitrate coordinates with Mo through its hydroxyl and carboxyl groups. The identity of atom X is currently unknown. However, it is very likely to be C, N, or O as adjudged by the electronic cloud analysis. Furthermore, FeMoCo is anchored to Fe and Mo atoms by Cys_{275} and His_{442} of subunit α. The P-cluster is a [8Fe-7S] cluster. Two [4Fe-3S] cubanes are connected through a central S atom, and play an important role in the transfer of electron between the FeMoCo and the Fe protein.

Table 8.2 compares the composition and functionalities of Fe and MoFe proteins. The Fe and MoFe proteins must form a complex to perform normal functions. In fact, the two proteins catalyze nitrogen fixation in a mosaic pattern, where two Fe proteins are set at the two ends of one MoFe protein [25].

In addition to *nifH*, *nifD*, and *nifK*, many other genes are known to participate in the assembly and activity of nitrogenase. Table 8.3 shows the genes that have been identified so far and their functions.

1.2.3 Nitrogen Fixation Mechanism of Nitrogenase

Nitrogen fixation is achieved by two catalytic cycles of Fe protein and MoFe protein (Figure 8.3). There is an electron transfer between the two cycles of Fe protein and

Table 8.2: Comparison of two components of Molybdenum nitrogenase.

Item	Dinitrogenase reductase (Component II)	Dinitrogenase (Component I)
Number of protein subunit s	2(identical)	4(2 big 2 small)
Protein structure type	Homodimer	$\alpha_2\beta_2$ heterogeneous four polymers
Encoding gene	*nifH*	*nifD* and *nifK*
Relative molecular mass	67~70kD	220~240kD
Number of Fe atoms	4	30(24~32)
Number of unstable S atoms	4	28(20~32)
Number of Mo atoms	0	2
SH base of Cys	12	32~34
Active center	Electronic activation center(4Fe4S)	Iron molybdenum auxiliary factor (FeMoCo)
Function	Transfer electronic to component I	Complexation, activation, and reduction of N_2 and H^+
Oxygen sensitivity	Very sensitive	Relatively sensitive

Table 8.3: Genes encoding nitrogenase.[a]

Gene	Encoding product and its function
nifH	Fe protein
nifD	Subunit α of MoFe protein
nifK	Subunit β of MoFe protein
nifT	Unknown
nifY / nafY	The accompanying body of MoFe protein, can assist FeMoCo in the insertion of MoFe protein as the vector of FeMoCo
nifE	Form $\alpha_2\beta_2$ with the encoding product of *nifN*, which is necessary for the biosynthesis of FeMoCo, the possible function is acting as the molecular "Scaffolding" of biosynthesis of FeMoCo
nifN	Form $\alpha_2\beta_2$ with the encoding product of *knife*, which is necessary for the biosynthesis of FeMoCo, the possible function is acting as the molecular "Scaffolding" of biosynthesis of FeMoCo
nifX	Participate in the biosynthesis of FeMoCo, and is used to accumulate the precursor containing Fe-Mo-S
nifU	Participate in the formation of Fe-S cluster as the molecular "Scaffolding"
nifS	Participate in the movement of atom S in the formation and repair process of Fe-S cluster
nifV	Homocitrate synthase, participate in the biosynthesis of FeMoCo
nifW	Relevant to the stability of MoFe protein
nifZ	Unknown
nifM	Relevant to the maturity of Fe protein
nifF	Flavodoxin(Fld)
nifL	Negative regulator protein
nifA	Positive regulator protein
nifB	Necessary for the biosynthesis of FeMoCo, its metabolite NifB-Cois a special Fe and S donor of FeMoCo
fdxN	Ferredoxin(Fd), acts as electron donor for nitrogenase in *Rhodobacter capsulatus*
nifQ	Participates in the biosynthesis of FeMoCo; it likely participates in the early stage processing of MoO_4^{2-}
nifJ	Pyruvic acid: Flavodoxin(ferredoxin)oxidoreductase, act as electron donor of Fe protein in *Klebsiella pneumoniae*

[a] Rubio & Ludden (2005) [23].

MoFe protein, which is accomplished by P-cluster. The nitrogenase is provided with protons and electrons through aerobic respiration, anaerobic respiration, fermentation, and photosynthesis. The electrons are transferred to nitrogenase by ferredoxin (Fd) or flavodoxin (Fld) for reducing N_2. The energy source for the reaction of nitrogen fixation is the ATP-Mg. It is worth mentioning that one electron requires two molecules of ATP-Mg.

The Fe protein is involved in three enzymatic states. The [4Fe-4S] of Fe protein has +1 reduction state (Red state) and +2 oxidation state (Ox state), while the Fe protein is bound to two molecules of ADP-Mg or ATP-Mg. MoFe proteins are involved in eight states, and form one redox state ($MoFeP_1$, $MoFeP_2$, $MoFeP_8$) each time it accepts an

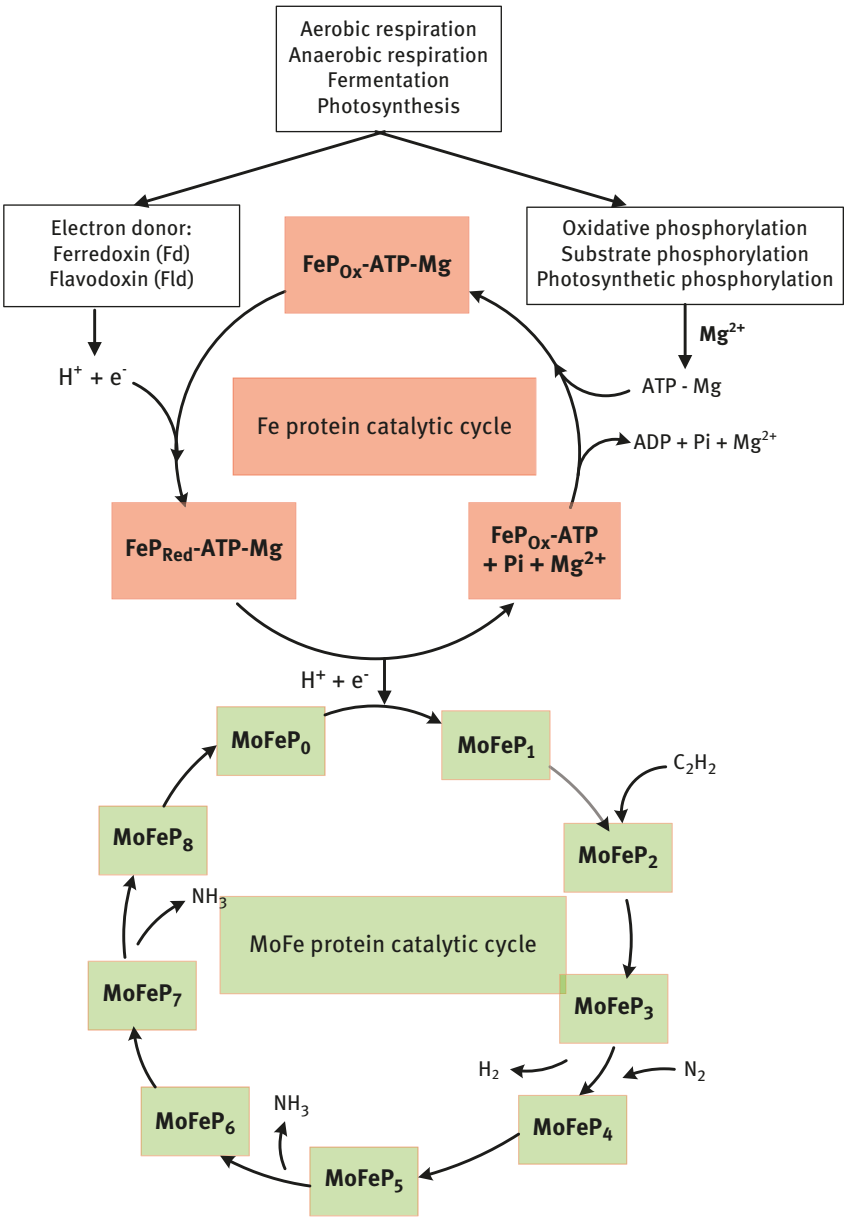

Figure 8.3: Catalytic cycle of Fe protein (FeP) and MoFe protein (MoFeP).
Source: Seefeldt et al. (2009) [24].

electron. In the MoFe protein cycle, C_2H_2 can bind to $MoFeP_2$ as a substrate, while N_2 can bind to $MoFeP_3$ and $MoFeP_4$ as a substrate. The binding of N_2 can replace H_2, whereas two NH_3 are released from $MoFeP_5$ and $MoFeP_7$.

Because N≡N has three covalent bonds, a large amount of energy is required to break this extremely stable molecule. Theoretically, the reduction of 1 mol of N_2 to two NH_3 requires 16 mol ATP. However, in practical situations, up to 24 mol ATP are required. These ATPs are provided through oxidative phosphorylation, substrate phosphorylation, and photosynthetic phosphorylation. The overall stoichiometric equation for biological nitrogen fixation is given by Equation (8.3):

$$N_2 + 8H^+ + 8e^- + 16\text{ATP-Mg} + 16\sim24H_2O \rightarrow 2NH_3 + H_2 + 16 \sim 24\text{ADP-Mg} + 16 \sim 24\text{Pi} \quad (8.3)$$

Nitrogenase catalyzes $N_2 \rightarrow NH_3$ and $2H^+ + 2e \rightarrow H_2$ at the same time. Notably, the biological nitrogen fixation is always accompanied by the reduction of H^+ to H_2. In the absence of N_2, nitrogenase uses all the electrons during the reduction of protons to produce H_2. Even in the presence of N_2, only 75% of the electrons are used to reduce N_2, while the other 25% are wasted in reducing protons to release H_2. At this point, the NiFe-uptake hydrogenase can recycle the reducing power and ATP, which are 'wasted' by nitrogenase to form H_2 (see the section regarding NiFe-uptake hydrogenase). Unlike the catalysis of reversible hydrogenase, which does not need ATP to produce H_2, nitrogenase requires ATP to produce H_2.

In fact, there are many substrates, which can bind to nitrogenase and include N_2, CH_3NC, $N{\equiv}CNH_2$, C_2H_2, C_2H_4, N_2O, COS, CO_2, CO, H_2, O_2, H^+, N_2H_2, $CH_3C{\equiv}CH$, $CH_2N{=}N$, C_2H_5CN, and $CH_3N{=}NCH_3$. Notably, some of these substrates can be reduced after binding to nitrogenase, while others can bind to nitrogenase without undergoing reduction (such as CO and O_2). Therefore, these two gases inhibit the biological nitrogen fixation. They can inhibit the transfer and reaction of H_2 by occupying either the molecular transfer pathway or active center binding sites.

1.2.4 Mechanism of Hydrogen Production with Nitrogenase

Nitrogenase can catalyze the following four reactions to release H_2 [26].

$$\text{Reaction I: } N_2 + 8\,(H^+ + e^-) \rightarrow 2NH_3 + H_2 \quad (8.4)$$

This reaction was originally proposed by Schrauzer et al. It is generally considered that H_2 is replaced during the complexation between N_2 and the reduced enzyme. One mol H_2 is released when 1 mol N_2 is reduced. This H_2-releasing reaction is inhibited by CO.

$$\text{Reaction II: } H_2 + 2H^+ + e^- \rightarrow 2H_2 \quad (8.5)$$

When N_2 is bound to nitrogenase, it is then reduced to E-N_2H_2. When there is H_2 in the reaction system, it can be degraded into N_2 and two H_2. At certain partial pressures of

nitrogen (pN_2), the amount of released H_2 increases with the increase in pN_2. However, when pN_2 increases to a certain level, N_2 inhibits the reaction. The reaction is completely inhibited when pN_2 becomes infinite. This reaction is also inhibited by CO.

$$\text{Reaction III: } 2H^+ + 2e^- \rightarrow 2H_2 \tag{8.6}$$

This is a hydrogen-releasing reaction that relies on ATP. It is inhibited by N_2, and not by CO.

Reaction IV: Release of H_2

The release of H_2 occurs in the pre-steady stage of the catalytic process of nitrogenase, which is the initial stage of the mixing of solid-state enzyme and reactants. The release of H_2 is equivalent to the number of Mo atoms in MoFe protein in the reaction system. It is possible that the release of H_2 is not a catalytic reaction, but rather an activation process.

Among the four hydrogen evolution reactions, Reactions I and III are the most significant. The amount of released hydrogen in Reactions II and IV is very small as compared to that in Reactions I and III. The relationship between the release of H_2 and the reduction of N_2 is relatively complicated. H^+ is a competitive inhibitor of N_2, while N_2 is the noncompetitive inhibitor of H^+. Under normal conditions, the amount of hydrogen released upon fixing 1 mol N_2 is greater than one mole. Furthermore, CO inhibits the reduction of N_2 and the release of H_2 in Reaction I. However, it does not inhibit the release of H_2 in Reaction III.

Section 2: Microbial Hydrogen Production by Photosynthesis

2.1 Phototrophic Microorganisms and Photosynthesis

Photosynthesis is a process used by plants and other organisms to convert light energy into chemical energy that can later be released to fuel the organisms' activities. Photosynthesis can be summarized as photosynthetic pigment in plants, algae, and bacteria under light, which use inorganic substances (CO_2, H_2O, and H_2S) to form synthetic organic compounds ($C_6H_{12}O_6$), during which they release O_2. It is a very important biological process in nature and uses the reduction force produced by photosynthetic electron transport (NADPH) and photophosphorylation energy (ATP) CO_2 fixation material synthetic cells. Some of the photosynthetic microorganisms include

eukaryotic algae, cyanobacteria, purple bacteria (purple sulfur bacteria and purple nonsulfur bacteria), green bacteria (green sulfur bacteria and green sulfur bacteria), and halophilic bacteria. In addition to the unique halophilic bacteria of chlorophyll or bacteria chlorophyll in purple membrane mediated photosynthesis, main types of photosynthesis, light nutrition microorganisms and their main characteristics are presented in Table 8.4.

2.1.1 Photosynthesis in Eukaryotic Algae

Chloroplast is the photosynthetic structural unit of eukaryotic algae and consists of outer membrane, thylakoid, and matrix.

(1) Outer membrane is permeable for nutrients, such as nucleoside, inorganic phosphorus, and sucrose, and can move freely in the cytoplasm into the inter-membrane space. CO_2, O_2, Pi, H_2O, glyceric acid, and phosphate can pass through the membrane, whereas ADP, ATP, sugar phosphate, glucose, and fructose can only pass slowly through the membrane. However, sucrose, $NADP^+$, and pyrophosphate cannot pass through the membrane, and need a special transporter to pass.

(2) The thylakoids are monolayer films of flat follicles, which are present along the long axis parallel to chloroplast. Many thylakoid fold together, for example in the form of a disk called grana, which are composed of grana thylakoid, and constitute a grana lamella membrane system. Grana are about 0.25–0.8 microns in diameter and are composed of 10–100 thylakoids. The combination of two or more thylakoids is called thylakoids matrix, which form the membrane system of substrate layer. Due to the adjacent grana thylakoid phase coupling matrix, all the thylakoid is essentially in the form of a closed system.

(3) The matrix for outer lining and the thylakoid part between the main ingredients include: CO_2 fixation related enzymes (such as RuBP carboxylase), which account for 60% of the total soluble protein matrix; the chloroplast DNA and protein synthesis system (such as ctDNA), all kinds of RNA, ribosome; other particulate components (such as starch grains, plastid balls and plant ferritin).

Thylakoid membranes contain photosynthetic pigments, which contain the electron transport chain, and react with light in the thylakoid membranes. Therefore, the thylakoid membranes are also called photosynthetic membranes. Thylakoid membrane's intrinsic protein mainly consists of cytochrome b6/f complex, plastoquinone (PQ), plastid blue pigment (PC), iron REDOX proteins, flavoprotein II and I, and optical system. The following section summarizes the role of thylakoid in photosynthesis reaction, and then introduces its role in CO_2 fixation reaction within the matrix.

Table 8.4: Phototrophic microorganisms and photosynthesis.

Photosynthesis types	**Anoxygenic photosynthesis**				**Oxygenic photosynthesis**	
Light energy microbial nutrition type	Procaryote microbiology					Eukaryotic organisms
	photosynthetic bacteria				Photosynthetic bacteria produce oxygen	algae
	Purple sulfur bacteria	Purple nonsulfur bacteria	Green sulfur bacteria	Green sulfur bacteria	cyanobacteria	Green algae, red algae, and brown algae
Photosynthetic apparatus	Cytoplasmic membrane	Cytoplasmic membrane	Green body	Green body	Cytoplasm of thylakoid and algal bravery	Chlorophyll a and b, carotenoids
Chlorophyll a and b, carotenoids	Chlorophyll a and b, carotenoids	Chlorophyll a and b, carotenoids	Chlorophyll a and b, carotenoids	Chlorophyll a and b, carotenoids	Chlorophyll a and b, carotenoids	Chlorophyll a and b, carotenoids
Photo system I (PSI)	Present	Present	Present	Present	Present	Present
Photo system II(PSII)	Absent	Absent	Absent	Absent	Absent	Absent
Photosynthesis of external electron donor and hydrogen donor	H_2 and H_2S, S	Mainly organic matter; The minority is H_2, S	H_2, H_2S, S	Light autotrophic donor: H_2 and H_2S H_2O	H_2O	H_2O
Sulfur deposition	Intracellular	extracellular	extracellular			
Generation of O_2	No	No	No	No	Yes	Yes
Basic metabolic type	Obligate anaerobic photoautotrophic	Usually anaerobic photoheterotrophic sometimes photoautotrophic or chemoheterotrophic (in the dark)	Obligate anaerobic photoautotrophic	Usually anaerobic photoheterotrophic, sometimes photoautotrophic or chemoheterotrophic (aerobic or in the dark)	Aerobic photoautotrophic	Aerobic photoautotrophic
Main carbon sources	CO_2	CO_2 or organics	CO_2	CO_2 or organics	CO_2	CO_2

2.1.1.1 Components of Photosynthetic Electron Transport Chain

(1) Photosynthetic pigments

Thylakoid contains two kinds of pigments, namely the chlorophyll and the carotenoid. Chlorophyll includes blue-green of chlorophyll a, yellow-green chlorophyll, and chlorophyll b c, d, and e. Chlorophyll a exists in all the photosynthetic organisms (including cyanobacteria) containing oxygen. Chlorophyll b is found in higher plants, and algae, in which the content of chlorophyll a and b is about 2:1, respectively. Chlorophyll d is found in red algae. Usually chlorophyll and carotenoid include orange yellow carotene and yellow lutein in the ratio of about 3:1, respectively. All the chlorophyll and almost all of the carotenoids are embedded in the thylakoid membrane, where they combine with proteins to form peptide. A peptide chain can combine with a number of pigment molecules. Furthermore, a fixed distance between consecutive pigment molecules and their orientation are conducive to energy transfer.

(2) Light harvesting complex (LHC)

Photosynthetic pigment molecules and proteins or peptides on the thylakoid membranes are organized as light harvesting complexes. Most of the pigment molecules are antenna pigment, which is responsible for the capture of light energy, and facilitate the light to induce resonance to the central pigment in the reaction. A handful of chlorophyll a pigment for reaction center is available, whereas the rest of the chlorophyll a and chlorophyll b are antenna pigments. Carotenoids act as ACTS as light energy capturing units and are called accessory pigment. The complex is the light system for antenna, such as the concentrated composite II (LHC II), which is photo system (PS) II antenna used for absorbing sunlight. Furthermore, the LHC II monomer's structure includes three transmembrane alpha helix segments and 14 pigment molecules (including seven chlorophyll a molecules, five chlorophyll b molecules, and two lutein molecules).

(3) Photo system II (PS II)

The LHC II reaction center in PS II pigment is affected by light near the wavelength of 680 nm, at which the electrons are transferred. The system is also called P_{680}. Photo system II contains multiple protein subunits (Figure 8.4). The core is a pair of inner membrane proteins, namely D1 (38 kD) and D2 (39 kD). It combines with P_{680} and magnesium chlorophyll (pheophytin; Pheo), plastoquinone A and B (PQA and PQB), LHC II, oxygen evolving complex (OEC) containing manganese atomic and cytochrome b559 on the protein.

After donating an electron, PS II or P_{680} transform to P_{680}+. It must return to ground state to capture the next photon. PS II forms a complex with oxygen in water. In addition, the electronic photolysis of two molecules of water needs four photon energy arguments (hν).

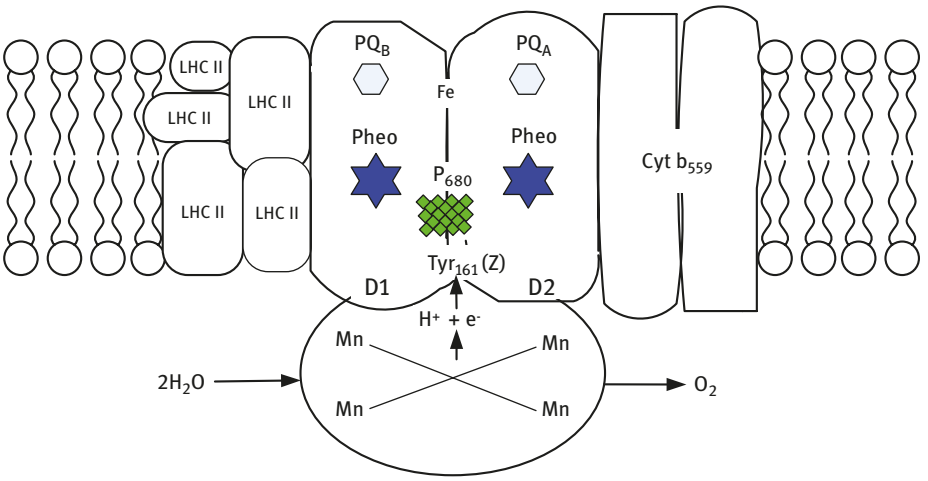

Figure 8.4: Structure of photo system II.

$$2H_2O + 4\,h\nu \rightarrow 4H^+ + 4e + O_2 \tag{8.7}$$

Four electrons are not directly obtained from water, and transfer to P_{680} +. P_{680}+ can only accept one electron; however, one oxygen complex can take four electrons. P_{680}+ acts as the direct electron donor (traditionally known as the primary electron donor) and constitutes the PS II reaction center of D1 protein Tyr161 residues (Z). After losing a proton and an electron, it changes to a neutral Z free radical (Z).

$$4\,P_{680}{}^+ + 4Z \rightarrow 4P_{680}+ 4Z\cdot + 4H^+ \tag{8.8}$$

Free radicals, Z, are produced due to oxidation in the Mn complex (including 4 Mn atoms) and regain the absence of electrons and protons. Mn complex loses one electron per 1 h and changes its state once. The absorption continues for 4 h, while its state changes from 0 to [Mn complex] and [Mn complex]^4 according to Equation (8.9):

$$4Z\cdot + 4\,h\nu + [\text{Mn complex}]^0 \rightarrow 4Z + [\text{Mn complex}]^4 \tag{8.9}$$

[Mn complex]^4 immediately releases one molecular oxygen and two molecules of water along with the regeneration of [Mn complex]^0 according to Equation (8.10):

$$[\text{Mn complex}]^4 + 2H_2O \rightarrow [\text{Mncomplex}]^0 + O_2 \tag{8.10}$$

(4) Cytochrome b6/f complex (Cyt b6 /f)

Cytochrome b6/f complex is also called the plasmids quinone alcohol:plasmid blue REDOX enzyme and is a polymer protein (210 kD) with 22–24 alpha helices across the

membrane. It is similar to the mitochondrial electron transport chain complex III, which contains two heme base (bH and bL) of cytochrome b6, an iron sulfur protein (20 kD), and a cytochrome f. Furthermore, it may also contain plastoquinone binding protein (Figure 8.5).

(5) Photo system I (PS I)

PS I (Figure 8.6) in the LHC of the I pigment reaction center could be near the wavelength of 700 nm light excitation of transferred electron, and therefore is called the P_{700}. PS I is made up of a reaction center and the LHC I. Three such composite

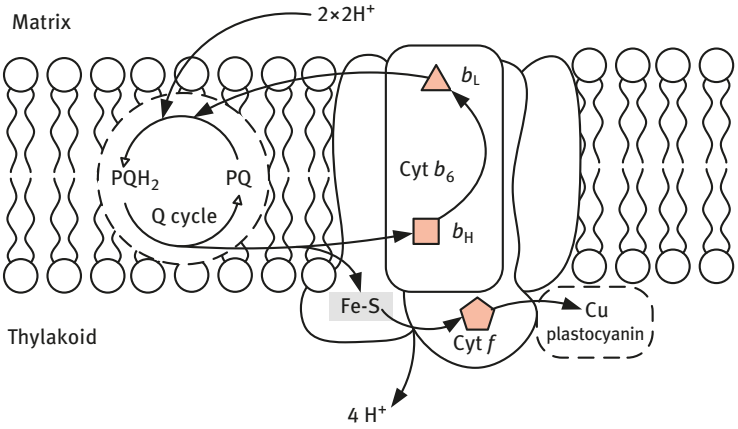

Figure 8.5: Cytochrome b6 /f complex.

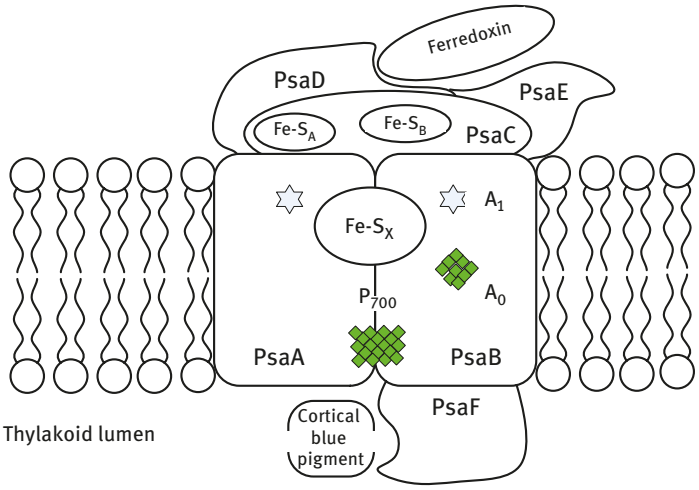

Figure 8.6: Major modules of photo system I.

PS I combine to form a trimer structure. Although PS I is composed of eleven different protein subunits, six of the subunits play the functional roles (except LHC I), as shown in Figure 8.6. PsaA and PsaB (83 kD) are the action centers of dimers. The presented structure is the common form of photosynthesis center. PS I has about 100 chlorophyll, which includes two of the P_{700} chlorophyll a molecules, while two others are present at about 1.6 nm from P_{700} chlorophyll a molecule. One is labelled A0 (its function is similar to that of the PS II magnesium chlorophyll). A0 is a P_{700} electron acceptor. Quinone to PSI combination form includes a labeled A1 vitamin k2 (phylloquinone) and forms the middle electron carrier. Fe-S forms the center, while PsaA and PsaB are bridged. The other two Fe-S centers (Fe-S_A and Fe-S_B) are embedded in the PsaC.

2.1.1.2 Electron Transfer

The photosynthetic electron transport is carried out by PS II, and cytochrome b6/f composite. The PS I completes three transmembrane complexes. Photosynthetic electron flow from the reaction center pigment P_{680} starts with the photon excitation by the ground state, due to which it goes into its excited state (P_{680} *). After that, P_{680} * becomes a good electron donor (E^0 is about 1 V) and gives off an electron to magnesium chlorophyll (Pheo, traditionally known as the original electron acceptor) within 20 ps, thus changing into a positively charged P_{680}^+. The magnesium chlorophyll ($Pheo^-$) will soon lose electrons and combine with D2 plastoquinone PQ_A. PQ_A and electronic magnesium chlorophyll (Pheo $^-$) change to D1 plastoquinone PQ_B (the equivalent of quinone Q) in the mitochondria. After the double transfer, PQ_B takes two electrons from PQ_A and two protons from the matrix and is fully restored to quinone alcohol form (hydroquinone, PQ_BH_2). PS II PQ_BH_2 leave plastoquinone and enter the membrane. Plastoquinone is a lipid, can swim inside the membrane, and constantly forms PS II, which is transferred to cytochrome b6/f complex. Followed by the cytochrome b6/f complex, the electronic plastid blue element passes on one side of the class capsule body cavity (Cu_{2+} in the plastocyanin, PC), and through the PC and PS I plastid blue pigment in the dock subunits (PsaF), where after electron transfer PS I forms P_{700} +. Plastoquinone exhibits two electron carrier effect and reduces hydroquinone REDOX alternately during the proton transfer. The protons are transferred from the substrate to class capsule body cavity. PS I P_{700} absorbs an exciton into excited P_{700} *, and then, immediately releases electrons to A_0, thus generating P_{700} + and A_0 -, respectively. P_{700} + is a strong oxidizer and can easily obtain an electron from PC. PC is a water-soluble electron carrier (10.4 kD) and can spread in the types of cystic cavity of shuttling between cytochrome b6/f complex and PS I transferred electrons. PC has a Cu atom. The change from Cu^+ to Cu^{2+} state involves electron transfer during the REDOX reaction. A0 is a strong reducing agent and transfers electron to vitamin k2 (A1), which in turn will give three electrons to PS I

Fe-S center (passing order is Fe-Sx, Fe-S_A and Fe-S_B). The electron transfer happens to the substrate side ferredoxin (Fd). Fd is combined with the membrane transport of sulfur protein, whereas soluble iron generally contains a Fe-2 S center. Fd is flavoprotein direct electron donor (Fp) and is also called ferredoxin Fp: NADP + REDOX enzyme. It contains flavin adenine dinucleotide (FAD) prosthetic group and has catalytic electrons from the reduction of ferredoxin (Fdred) transferred to the NADP + according to Equation (8.11):

$$2Fd_{red} + 2H^+ + NADP^+ \rightarrow 2Fd_{ox} + NADPH + H^+ \quad (8.11)$$

The generated NADPH usually serves as the reducing equivalent to fix CO_2.

The photosynthetic electron transfer process of oxygen is shown in Figure 8.7. The electron transfer process is a cyclic photosynthetic electron transport. Under certain conditions, such as in the absence of reducing NADPH, or insufficient electrical energy generated by the PS I to restore NADP +, photosynthetic microbes will cycle photosynthetic electron transport. In this case, the change from P_{700} * to the NADP + does not happen. Additionally, along the way of PS I Fe-S center (Fe-SA and Fe-SB), cytochrome b6/f complex and PC finally return to the P_{700} + dashed part. This cyclic photosynthetic electron flow does not involve the liberation of oxygen and NADP + light water reduction. However, it involves photophosphorylation ATP production.

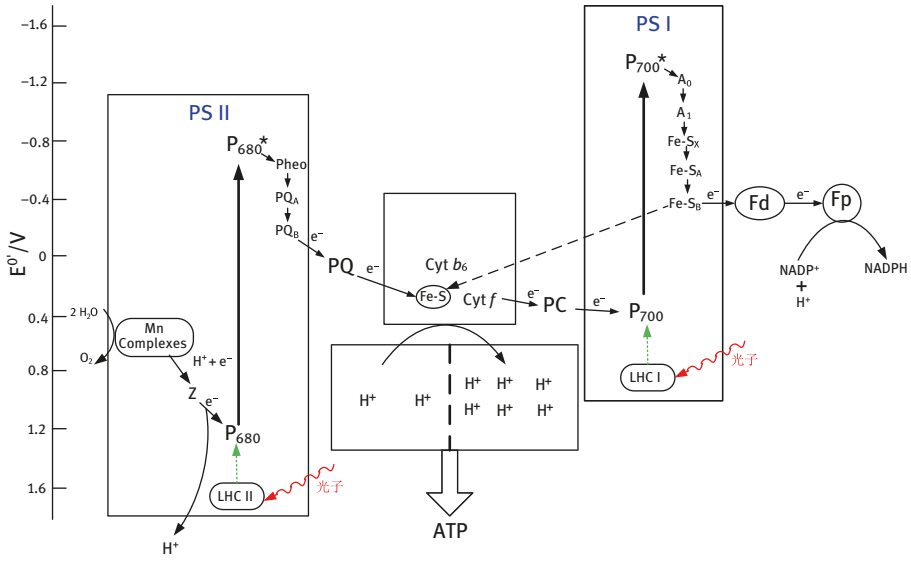

Figure 8.7: Photosynthetic electron transfer process.
Source: Wang et al. (2002) [27].

2.1.1.3 Photophosphorylation

Photosynthetic electron transport eventually forms NADPH resilience, and also results in pumping protons across the membrane from the matrix to class capsule body cavity, thus forming a proton gradient across the film. Photosynthetic electron transport results in proton gradient across the thylakoid membrane, and that is consistent with the proton gradient in respiratory electron transport chain caused by the mitochondrial membrane. They all can pass the ATP synthase catalytic ATP production. The former are usually called the photophosphorylation, while the latter are termed as oxidative phosphorylation.

According to the photosynthetic electron transport in a different way, photophosphorylation is divided into noncyclic photophosphorylation and cyclic photophosphorylation.

(1) Noncyclic photophosphorylation

Noncyclic photophosphorylation is caused by acyclic photosynthetic electron flow. Figure 8.8 shows the mechanism of noncyclic photophosphorylation process. The proton gradient across the membrane is shown at three locations. One is to put oxygen complexes, produces water photolysis of oxygen and protons (electronic) into the transport chain. Second is in the cytochrome b6/f complex, when electrons from PQ cycle move across the membrane and are caused by the REDOX reaction. It is flavoprotein (Fp). Its catalytic $NADP^+$ reduction takes place due to the protons from the substrate side.

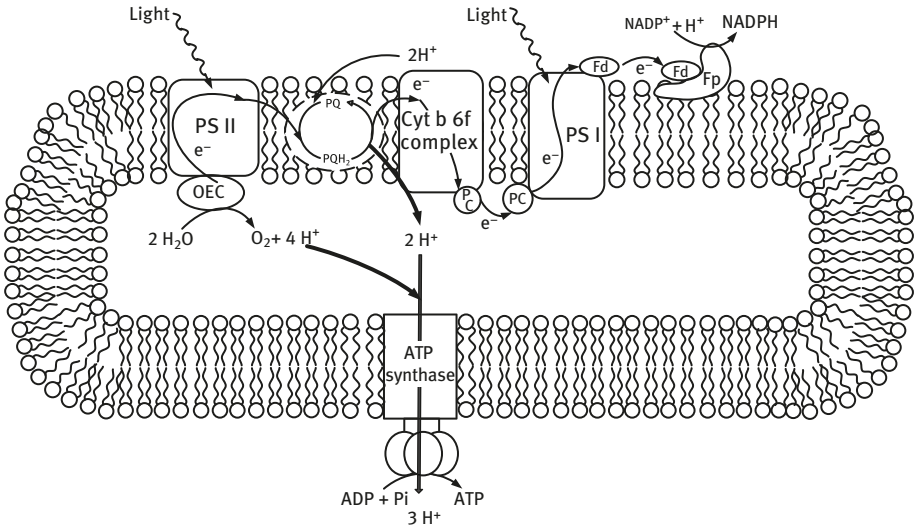

Figure 8.8: Mechanism of noncyclic photophosphorylation process.
Source: Wang et al. (2002) [27].

Every two pairs of electrons from H_2O transferred to $NADP^+$ (1 molecular O_2) add 12 H^+ in the class capsule body cavity. Four of them come from the oxygen complexes in the cystic cavity of H_2O photolysis, while eight PQ by cytochrome b6/f complex in circulation from the matrix transfer. Furthermore, according to the chemical permeation theory, the energy of the proton gradient across the membrane is equivalent to 260 kJ, which theoretically is equivalent to the synthesis of eight ATPs ($\Delta G^0 = 30.5$ kJ/mol). However, the experiments show that the release 1 mol of O_2 is almost equivalent to the synthesis of 3 mol ATP.

In PS I and PS II, the transfer of photons and electrons from H_2O to $NADP^+$ takes place. At the same time, a proton gradient is established, which drives the synthesis of ATP, and releases O_2. During the absorption of every two photons (PS II and PS I 1), and an electron transfer from H_2O to $NADP^+$, the formation of a molecular O_2 needs four of the electrons transferred. In total, eight photons are needed to be absorbed. Therefore, the overall equation for the noncyclic photophosphorylation is represented by Equation (8.12):

$$2H_2O + 8h\nu + 2NADP^+ + \sim 3ADP + \sim 3Pi \rightarrow O_2 + 2H^+ + 2NADP + \sim 3ATP + \sim 3H_2O \quad (8.12)$$

(2) Cyclic photophosphorylation

Cyclic photophosphorylation is caused by the electron flow in cycle photosynthetic, which does not need PS II and does not involve the liberation of oxygen and NADP + reduction. Its role is only for ATP and is represented by Equation (8.13):

$$ADP + Pi + h\nu \rightarrow ATP + H_2O \quad (8.13)$$

The maximum rate of cyclic photophosphorylation is less than 5% of the noncyclic photophosphorylation. Microbes can adjust the number of electrons that participate in NADP + reduction and circulation phosphorylation to adjust the photo-reaction of ATP and NADPH, which is needed to meet the needs of CO_2 fixation reaction. Usually, the CO_2 fixation requires the ATP to NADPH molar ratio of 3:2, respectively.

2.1.1.4 CO_2 Fixation Reaction

The reduction of photo-reaction force (NADPH) and energy (ATP) is mainly used in CO_2 fixation material, which produces sugars and other cells. The CO_2 fixation reaction does not need light and can take place under dark conditions. Therefore, it is also called the dark reaction. This was shown by Calvin and Bensen in 1950s using the circulation path, and therefore, is called the Calvin–Bensen circulation, while herein is referred to as the Calvin cycle. Calvin cycle contains the chloroplasts of eukaryotic

algae and has the enzymes necessary to participate in the Calvin cycle. The cycle includes three phases, which are briefly discussed as follows.

(1) Carboxylation of CO_2 and its receptor ribulose 1, 5-diphosphate (herein referred to as RuBP), in the ribulose 1, 5-bishosphate carboxylase (ribulose biphosphate carboxylase, RuBisCO) under the catalytic formation of 3-2 molecules of glyceric acid phosphate; CO_2 was fixed on the carboxyl.
(2) 3-Phosphoglyceric acid is reduced to glyceraldehyde-3-phosphate and the required reductant and energy are from NADPH and ATP formed in the light reaction. Therefore, this phase is the junction of light and dark reactions. Glyceraldehyde-3-phosphate is a kind of three C sugar. Once CO_2 is converted to glyceraldehyde-3-phosphate, the energy storage of photosynthesis is completed.
(3) Three carbon sugar RuBP is used and regenerated through a series of reactions, involving RuBP, CO_2 and acceptable for a cycle. This stage needs energy, which comes from the light reaction of ATP.

Through reaction (8.14), six molecular CO_2 are consumed to produce a glucose molecule. In fact, according to molecular glyceraldehyde, the production of 2-–3-phosphate are needed. Additionally, further synthesis of other cell materials, such as sugars, proteins and lipids, is also needed.

$$6CO_2 + 12\,NADPH + 12H^+ + 18ATP + 12H_2O \rightarrow C_6H_{12}O_6 + 12\,NADP^+ + 18ADP + 18Pi \quad (8.14)$$

2.1.2 Photosynthesis in Cyanobacteria

2.1.2.1 Cyanobacteria

Cyanobacteria is a kind of gram-negative bacteria with no flagella and includes chlorophyll a (but not form chloroplasts) and bile pigment. It is able to produce oxygen through large procaryote microbiology of photosynthesis. Since they are the same as the eukaryotic algae photosynthesis systems, cyanobacteria are also regarded as algae, and are called blue algae or blue green algae. Cyanobacteria were formed about 3.5 billion years ago, and changed the earth's atmosphere from anaerobic to aerobic conditions, which gave birth to the evolution and development of all aerobic organisms. Cyanobacteria are widespread and are common in fresh water, sea water, and soil. They can also grow in extreme conditions. They are blue due to gram-negative bacteria, whereas prokaryotic bacteria are similar to eutkaryotic cell chloroplast photosynthesis with regards to the cell wall structure and composition, as well as with regards to the functional and structural units. Therefore, the chloroplast in eukaryotic cells is likely to be evolved from cyanobacteria. Cyanobacteria have chlorophyll *a* and

the ability of oxygenic photosynthesis, which are different from other prokaryotic photosynthetic bacteria, such as the purple bacteria and algae. Besides photosynthesis, most cyanobacteria can react with the atmosphere of N_2 to produce NH_3, which is integral to entire biosphere.

Cyanobacteria are generally larger than bacteria with regards to individual cells and are usually 3–10 microns in diameter. At minimum, they are 0.5–1 microns, while the largest size is 60 μm (such as *Oscillatoria princeps*). According to the different cell forms, cyanobacteria could be divided into two types, namely the single cell and filaments. Single-celled groups are mostly spherical, ellipsoid and rod-shaped, single or aggregates, such as *Chroococcus, Microcystis* and *Dermocarpa*. There are many cells arranged orderly as filaments cyanobacteria, and include a heterocyst, such as the fishy cyanobacteria, and beads cyanobacteria, which have the same shape of the cell (such as fibrillation cyanobacteria). Another type is spiral cyanobacteria, which are in the form of branching. True branch cyanobacteria is an example of this kind. Figure 8.9 shows the typical cyanobacteria cell morphology.

The structure of cyanobacteria cells is similar to that of gram-negative bacteria. Cell walls have two layers (inner and outer). The outer layer is for lipopolysaccharide, while the inner layer is the peptide layer. The cell walls of most cyanobacteria can continuously produce adhesive material, which will keep them together as a group of cells or filaments, thus forming glue sugar or sheath. Most blue bacteria are without flagella. Cyanobacteria glycogen and polyphosphates were found in the cytoplasm of PHB, whereas the cyanobacteria peptide can store content and CO_2 fixation carboxylase. Air bubbles have been observed in a small number of cyanobacteria in water, due to which

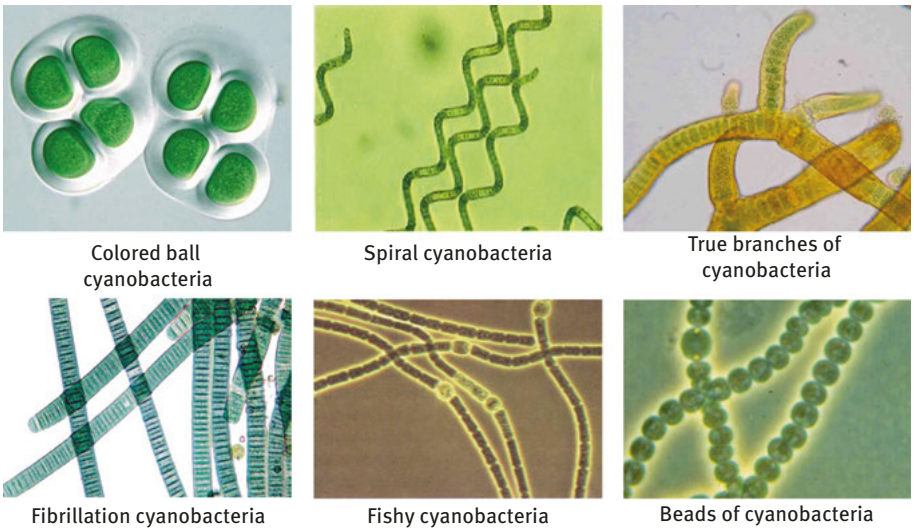

Figure 8.9: Typical cyanobacteria cell morphology.

they can float on the water surface and can accept sunlight for photosynthesis. Regarding the chemical composition, blue algae are the most unique bacteria containing two or more double bonds of unsaturated fatty acids. It is well known that bacteria are usually only saturated fatty acids or are unsaturated fatty acids of a double bond.

Cyanobacteria photosynthesis area includes thylakoid and phycoerythrin. Thylakoid formed by the cytoplasm membrane invagination, which are either parallel or medial curls, are arranged in the form of a plate-shaped membrane. Thylakoid contains chlorophyll, carotenoid, and a photosynthetic electron transport system. Phycoerythrin has granular structure and exists usually in pairs in thylakoid's outer side. It is a major set of cyanobacteria's light structure, which contains hundreds of subunits. These algae bile protein subunits are called phycobiliprotein, and include phycocyanin, phycoerythrin, and allophycocyanin. Combined with the pigment on these proteins, they are called phycocanobilin, phycoerythrin, and allophycocanobilin. These pigments are collectively referred to as phycobilin and play the auxiliary pigment role in photosynthesis. The ratio of algal blue to red element in algae is of two, which is due to the environmental conditions in which it grows, particularly the light conditions. Any change in these conditions would result in the change in blue color of bacteria. Most cyanobacteria cells have the algal blue edge. The cell shows special blue color, and therefore is called the cyanobacteria. While Phc or Phe accepts the photon, the energy is transferred to the aPhc, as shown in Figure 8.10.

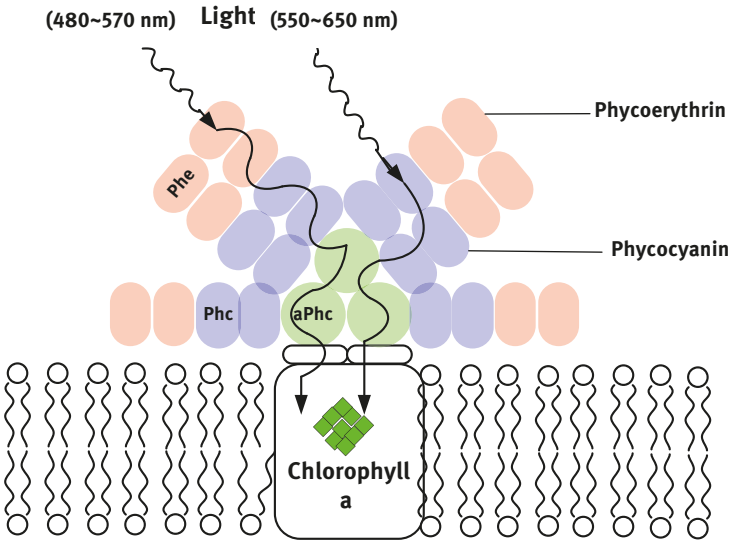

Figure 8.10: Phycocyanin, phycoerythrin, and allophycocyanin involved in the energy transfer process.

Source: Wang et al. (2002) [27].

Cyanobacteria often exist as a kind of specialized cells, which are called heterocyst. Heterocyst exists in the vegetative cell to secrete a layer within the original wall, and forms a shallow, slightly bigger body than a vegetative cell. In the middle or at the end of the chain of cells, the number of thick wall cells is small and uncertain. Generally, for the vegetative cell number of 5–10%, it can exchange information and nutrients with the vegetative cells. Additionally, it is able to differentiate into heterocyst filamentous cyanobacteria, such as *Anabaena* and *Nostoc*. Heterocyst thylakoid is gathered on both ends of the cell. The algal II is lost; however, the photosynthetic system retains chlorophyll a and photosynthetic system. Abnormity cell is the cell of cyanobacteria that deals with the nitrogen fixation. Therefore, it has heterocyst cyanobacteria that can fix nitrogen (some do not form heterocyst single-celled cyanobacteria that can fix nitrogen). Nitrogen fixation is done when organic compounds are provided. Adjacent vegetative cell heterocyst fixes nitrogen output in the form of glutamic acid and forms interchangeable products by cells connected through holes.

2.1.2.2 Photosynthesis in Cyanobacteria

Photosynthesis in cyanobacteria is similar to that in eukaryotic algae. However, there are some differences, two of which are as follows.

(1) The places where photosynthesis proceeds are different. This is because, the cyanobacteria are prokaryotes, which are not like a eukaryotic algae that has specialized carries on the photosynthesis of organelles, and chloroplast. Blue bacterial photosynthesis reaction occurs in the cytoplasm of thylakoid membrane, while the CO_2 fixation reaction occurs in the cytoplasm of carboxylase body.
(2) In addition to the chlorophyll, carotenoid, and algae bile pigment, it has photosynthetic pigment, algal blue pigment, algal red element, and other various algal blue pigments.

The process of photosynthetic electron transport of cyanobacteria is shown in Figure 8.11. The algal blue and red pigments in algae are used to absorb photons, produce different algal blue energy transfer elements. In addition, the energy transferred to the reaction center P_{680} chlorophyll is provided by the electronic water reduction plastoquinone (PQ), and then, through cytochrome b559, cytochrome f, plastid blue element to the PS I, electronic classics by cytochrome b559 to cytochrome f coupling ATP production. Low energy electron by chlorophyll a absorbs light energy of PS I electrons in the P_{700} that is motivated to restore Fe-S cluster. The light is absorbed again by soluble ferredoxin (Fd) and flavin protein (Fp), that is finally converted to NADP +, thus resulting in the reduction force of NADPH.

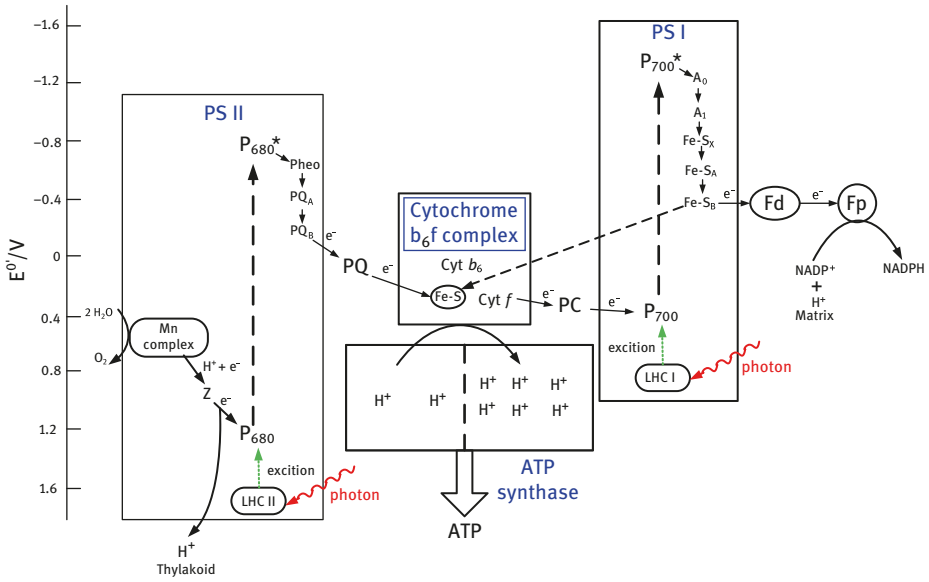

Figure 8.11: Noncyclic photo energy electron transport pathway in cyanobacteria.

Phc – algae blue pigment; Phe – algal red element; Aphc – different algal blue pigment; PQ – plastoquinone; PC – plastid blue pigment; Cyt – cytochrome; Fd – ferredoxin; Fp – flavoprotein

2.1.3 Photosynthesis in Photosynthetic Bacteria

2.1.3.1 Photosynthetic Bacteria

In addition to its existence in natural plants, the photosynthesis of oxygen does not take place in eukaryotic algae and cyanobacteria (oxygenic photosynthesis), and therefore are called as the photosynthetic bacteria. No oxygen bacteria, purple bacteria, and photosynthetic bacteria include green bacteria helix bacili, and bacteriachlorophyll aerobic bacteria. Aerobic bacteria containing Bchl contain photosynthetic membrane. However, they cannot rely on light energy for independent survival. Their energy mainly comes from the electron transfer process of oxidative phosphorylation aerobic respiration, and photosynthesis under the aerobic conditions. They only grow in a certain environment or under the condition of a kind of energy supplement and are not photosynthetic organisms in the true sense. Helix bacili contains Bchl; however, they lack the endometrium photosynthetic system. Their photosynthetic pigment is located in the cytoplasm membrane. They cannot use the square one sulfide and are close relatives of clostridium and bacillus. Therefore, the helix bacili belongs to the APB, though this is controversial. Green bacteria and Purple bacteria are considered the photosynthetic bacteria (Phototrophic

bacteria, PB) in traditional sense. The green bacteria are divided into green sulfur bacteria and green nonsulfur bacteria. Purple bacteria are divided into purple sulfur bacteria and purple nonsulfur bacteria. Purple sulfur bacteria and purple nonsulfur bacteria are also known as red sulfur bacteria and spiral bacteria, respectively.

Photosynthetic bacteria perform photosynthesis; however, the photosynthetic eukaryotic algae and cyanobacteria are different. The most significant differences are as follows.

(1) Photosynthetic bacteria are not thylakoid. The photosynthesis setups of purple bacteria and green bacteria lie in the cytoplasmic membrane and in the green cytoplasmic pigment, respectively.
(2) Pigment of photosynthetic bacteria is involved in light reaction for bacteria chlorophyll (bacteriochlorophyll, rather than chlorophyll). Purple bacteria have a bacterial reaction center, while the green pigment acts as the reaction center for bacteria chlorophyll a. The purple bacteria have an antenna for bacterial pigment chlorophyll and carotenoid, whereas the green bacteria also have an antenna pigment as the bacteria chlorophyll, c, d, e and carotenoids.
(3) Photosynthetic bacteria use only the light system to produce ATP and reduce power required. The light-driven reaction takes place by electronic transfer that completes the photophosphorylation ATP production and reduces the power from square one of H_2S, H_2, and organic matter (such as organic acids, alcohols, and sugars).
(4) The photosynthesis product of exogenous hydrogen donor and the electron donor is not water, due to which O_2 is not produced.

2.1.3.2 Light Reaction of Photosynthesis Bacteria

Purple bacteria and green bacteria differ slightly in the process of photosynthesis. The differences are as follows.

(1) Purple bacteria photosynthesis

Purple bacteria photosynthetic components consist of four parts, namely the optical system (P_{870}), Cytochrome b_{c1} complex (similar to mitochondrial respiratory electron transport chain complex III), Cytochrome b_{c2} (cytochrome c and mitochondrial respiratory electron transport chain), and ATP synthase, which is similar to the mitochondrial ATP synthase.

Optical system (P_{870}) is a large protein complex (145 kD) and includes four subunits (L-, M-, H-, and C-type cytochrome) and 13 cofactors. C-type cytochrome Cys subunits are produced by N-terminal amino acid residues on the diester-acyl glycerin fatty hydrocarbon chain, and anchor on the outer edge of the cytoplasmic membrane.

L and M contain two bacterial chlorophyll molecules (BChl) and one bacterial pheophytin (BPheo). The L-type has a quinone molecule combined with Q_A, while M quinone molecule contains a loose combination of Q_B. Both L and M types are present in nonheme ligand Fe atoms, while C-type cytochrome contains four hemes. The reaction center chlorophyll pigments are two bacteria, which are provided by L and M subunits. Its maximum light absorbing wavelength is 870 nm, due to which the light purple bacteria is called P_{870} reaction center.

In addition to containing cytochrome b, c_1, Cytochrome bc_1 complex also contains the center of the combination of Fe^{-2}'s iron sulfur protein, which includes cytochrome b that contains two hemes, b_{562} and b_{566}. In cytochrome bc_1 complex, Cytochrome b exists in a free form, while cytochrome c_1 has covalent bonds with protein. Cytochrome b, c_1 heme is the prosthetic group of Fe atoms, and during electron transfer reduces Fe^{2+} to Fe^{3+}.

Cytochrome c_2 is a water-soluble pigment cell with the molecular weight of 12000–14000. Its primary structure is very similar to mitochondrial cytochrome c and has C-type absorption spectrum, while the alpha maximum absorption peak appears at 550 nm. Cytochrome c_2 is present in optical systems, while the cytochrome bc_1 complex passes between the electrons.

Electron transfer: Photon is absorbed by molecular reaction centers around the antenna, while the energy is produced through the exciton and passes into the action center. The P_{870} goes into the excited state of P_{870}^{+}, and then releases electronic P_{870} +, after which it releases electrons through L of L subunits BPheo. BChl is produced, which forms BPheo, that is closely integrated with $BPheo^{-}$ and its electronic quinone Q_A. This promptly provides the electrons for the loose combination of quinone Q_B. After the transfer of two electrons, Q_B is produced, which reduces to quinone Q_BH_2 (protons from the cytoplasm). Then, Q_BH_2 leaves the light system and diffuse freely within the lipid bilayer. Q_BH_2 enters the quinone library and moves into the neighboring unit by fat double cytochrome bc_1 complex. In the complex, quinone (Q_BH_2) electrons are transferred in turn after $Cytb_L$, $Cytb_H$, fe^{-2} s center, and Cytc1. Finally, they are transferred to soluble $Cytc_2$. $Cytc_2$ on the cytoplasmic membrane performs lateral movement to the optical system of c-type cytochrome subunits, and through the subunits on four hemes to electron acceptor (P_{870}+). It lies between P_{870} and a hemoglobin L of a tyrosine residue (Tyr162), which is a direct electron donor to P_{870}. At this point, the electron transfer completes a cycle. P_{870} goes back to the ground state, while the aerial prepared from the surrounding molecules absorb another exciton packet, and starts the next cycle. Photophosphorylation: Similar to that through mitochondrial complex III, the flow of electrons through the cytochrome bcl complex involves a Q cycle. The cycle of protons from the inside of the cell membrane was transported to the outer membrane. Thus, a proton gradient across the membrane was formed. The ATP synthase uses the proton gradient for phosphorylation ATP production. At the same time, it will film the outside of the protons to cytoplasm.

(2) Green photosynthetic bacteria

Green photosynthetic components of bacteria and the electron transfer process are the same as in the purple bacteria. However, green bacterial photosynthetic apparatus is present in the green body, while the photosynthetic apparatus of purple bacteria is in the cytoplasmic membrane. Furthermore, other aspects also have some differences. Reaction center for P_{840} is the pigment green sulfur bacteria. In optical system and the transfer between cytochrome b_{c1} complex, electrons do not happen through cytochrome$_{c2}$, but through cytochrome c_{553}. Importantly, the green sulfur bacteria is present in the circulating electron flow of different circulating electron transport chain, which means that certain electrons flow from P_{840} * ferredoxin, and under the catalysis protein, pass to NAD^+, and form NADH. At this point, the electrons produced by the oxidation of H_2S from P_{840} consume S (and/or SO_4^{2-}) that is generated by the electrons to fill.

2.1.3.3 Reducing Force in Photosynthetic Bacteria (metabolic type)

Photosynthetic bacteria species and metabolic type are found more often, and include obligate anaerobic energy autotrophic, and anaerobic heterotrophic. The latter can function under aerobic or dark conditions. The energy source is the reduction force (NAD (P) H) which varies. H_2, H_2S, and organic matter (such as organic acids, alcohols, and sugars) are the exogenous hydrogen donors. Following are the main source of reducing power:

(1) Under the conditions of oxygen-free and illumination, the above-mentioned green sulfur bacteria produce NADH through non-cyclic electron transfer chain using H_2S as the exogenous hydrogen donor.
(2) Under the anaerobic light conditions, photosynthetic bacteria grow in hydrogen atmosphere, while under the effect of absorption hydrogenase catalytic, they directly use H_2 reduction of NAD (P) $^+$ to produce NAD (P) H.
(3) Under the anaerobic light conditions, when ATP (from photosynthetic bacteria circulating photosynthetic electron transport of phosphorus acidification) is provided, the exogenous hydrogen donor, such as H_2S and organic matter, can be produced by means of inverse electron transfer reducing power NAD (P) H. The process is similar as is observed in autotrophic organisms. The inorganic hydrogen donor adverse respiratory chain is the transmission way of reducing power.
(4) When there is no light in the aerobic or part of the photosynthetic bacteria (such as purple, nonsulfur bacteria and green sulfur bacteria), they can use enough organic matter and exogenous hydrogen donor, carbon and energy source for heterotrophic growth.

2.2 Microbial Hydrogen Production by Eukaryotic Algae

2.2.1 Hydrogen-Producing Eukaryotic Algae

Gaffron et al. [28] first discovered *Scenedesmus obliquus*, which absorbed both hydrogen and fixed carbon dioxide under anaerobic conditions. However, they also released H_2 under light conditions, the duration for which was of a few seconds to a few minutes. Several eukaryotic algae could produce photosynthetic hydrogen and have been reported in literature. These include green algae, red algae, and brown algae. Red algae and brown algae are relatively rare, whereas typical red algae are *Porphyra umbilicalis* and *Porphyridium cruentum*, and typical brown algae are *Ascophyllum nodosum*.

The main hydrogen-producing eukaryotic algae is green algae and is concentrated in Chlorophyceae, Volvocales, and Chlamydomonas, Platymonas, Scenedesmus, and Chlorella. Chlorococcum belongs to Chlorococcales and includes *Chlamydomonas reinhardtii*, *Chlamydomonas moewusii*, *Chlamydomonas noctigama*, *Chlorella fusca*, *Scenedesmus obliquus*, *Scenedesmus vacuolatus*, *Chlorococcum littorale*, *Chlorococcum submarinum*, *Lobochlamys segnis*, and *Platymonas subcordiformis*.

2.2.2 Mechanism of Microbial Hydrogen Release by Green Algae

FeFe-reversible hydrogenase mainly plays the role of hydrogen production in *Chlorella*. In addition, a few green algae contain NiFe-reversible hydrogenase under certain conditions. For example, both FeFe-reversible hydrogenase and NiFe-reversible hydrogenase can exist in *Scenedesmus obliquus*. NiFe-reversible hydrogenase exists in the form of a dimer, while FeFe-reversible hydrogenase exists in the form of a monomer. The activity of NiFe-reversible hydrogenase is 100 times higher than that of the FeFe-reversible hydrogenase. However, NiFe-reversible hydrogenase has a strong tolerance for O_2. Several three-dimensional structures of bacterial NiFe-reversible hydrogenase were analyzed, and a preliminary understanding of oxygen deactivation mechanisms was obtained. Therefore, further improvement in the ability of NiFe-reversible hydrogenase's oxygen tolerance is an important research direction for the green algae biohydrogen.

Green algae produce H_2 in two basic modes. First, reversible hydrogenase hydrogenation via enzyme-catalyze produced H_2 under anaerobic conditions. The process is directly competing electrons with CO_2 fixation process, while the electron was produced by the photolysis of water PS II. It is named direct biophotolysis process for hydrogen production. The second mode is under special conditions and resolves endogenous substrates that come from CO_2 fixation process to produce electrons.

The electrons are transferred to the hydrogenase through PS I, and then reduce protons to produce H_2. It is named indirect biophotolysis process for hydrogen production.

2.2.2.1 Direct Biophotolysis Process for Microbial Hydrogen Production

There is a reversible hydrogenase in the green algae chloroplast thylakoid membrane. With the help of nonheme iron-sulfur protein (Fe-S) and ferredoxin (Fd), the reversible hydrogenase is connected to photosynthetic transport chain, which may play a role in the deployment of electrons in flow photosynthetic transport chain. Under special conditions (such as anaerobic conditions or low pH) or excess electrons in photosynthetic transfer chain, the excessive electrons reach the reaction center of reversible hydrogenase, and catalyze the reduction matrix protons to H_2. This process eliminates the damage of electronic accumulation to cells, while the process does not require additional ATP. Instead, the energy is directly obtained from the photosystem, while H_2 comes directly from the biophotolysis of water (Figure 8.12). The reaction is represented by Equation (8.15):

$$2H_2O \xrightarrow{\text{Light energy}} 2H_2 + O_2 \tag{8.15}$$

In algae direct biophotolysis process for hydrogen production, a big problem is the production of oxygen at the same time. Oxygen is a strong inhibitor of hydrogenase, which not only inhibits the hydrogenase but also inhibits the positive gene expression of hydrogenase. When the oxygen concentration in the environment is close to 1.5%, the hydrogenase inactivates rapidly and immediately stops producing hydrogen. Therefore, the observed direct hydrogen production process is very short and lasts only a few seconds to a few minutes. For this reason, the reaction of hydrogen production can be continued while trying to reduce the concentration of O_2. The partial pressure of oxygen in the gas phase must be less than 0.1%, and the oxygen

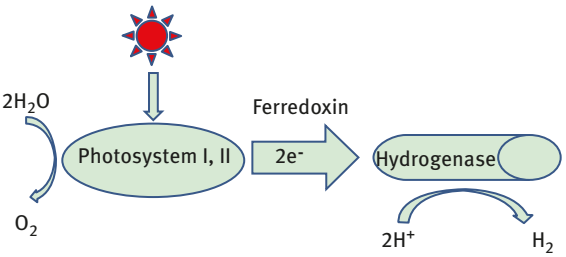

Figure 8.12: Green algae direct biophotolysis process for hydrogen production.

concentration in the liquid phase must be less than 1 μmol/L. In the experiment, gas diffusion is accelerated or the space is filled with inert gas to reduce the concentration of O_2 in the reaction system. Due to the high requirements of pumping equipment and extremely high energy consumption, this method is difficult to implement in practice. Utilization of O_2-regenerable sorbent can also reduce its concentration in the reaction system. However, the high cost of the sorbent is not suitable for large-scale applications.

2.2.2.2 Indirect Biophotolysis Process for Microbial Hydrogen Production

Happe et al. [9] identified *reinhardtii* which are able to produce hydrogen even after disrupting the photolysis activity of PS II. However, they cannot produce hydrogen when cytochrome b6/f complex is destroyed. Later, it is confirmed that the *reinhardtii* thylakoid membrane contains NAD (P) H–plastoquinone redox compound enzymes system. This system catalyzes endogenous substrates through catabolism to obtain electrons. Electrons are injected into the plastoquinone pool that lies between PS I and PS II. Electrons are passed to PS I by the cytochrome b6/f complex and plastocyanin. Afterward, the electrons are passed to Fe-S protein and ferredoxin, and ultimately, H_2 is synthesized by the reversible hydrogenase (Figure 8.13). Recent studies show that endogenous substrates may also perform in the absence of light conditions, and that too without the participation of PS I. They can produce hydrogen in dark-fermentation way (to be described later). However, the production efficiency is low.

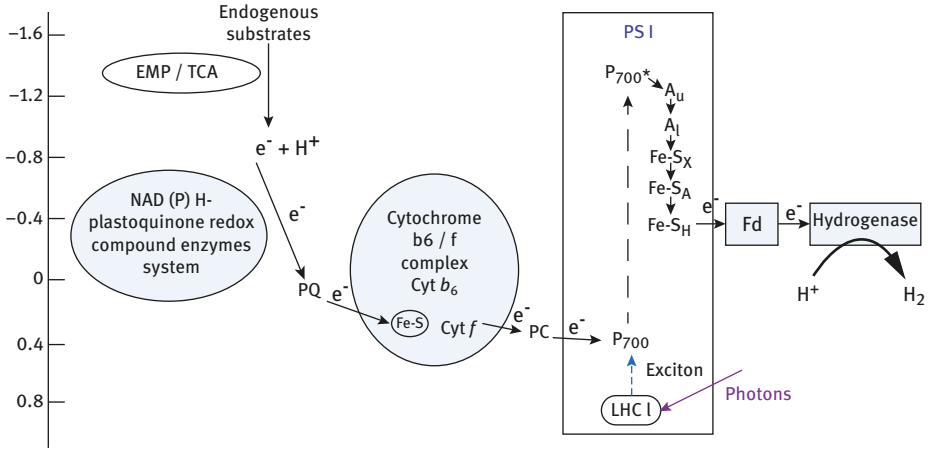

Figure 8.13: Electrons transfer process of green algae indirect biophotolysis process for hydrogen production.

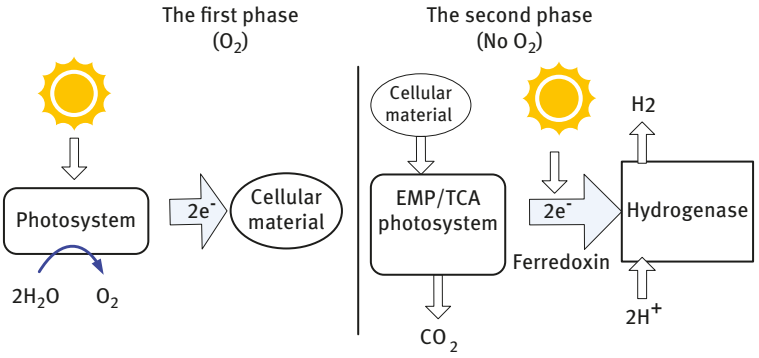

Figure 8.14: Green algae indirect biophotolysis process for hydrogen production.

Separate production of O_2 and H_2 in time and (or) space to avoid inhibition of the O_2 to hydrogenase develops a "two-stage hydrogen production". The first stage is completed in the aerobic environment, when green algae fix CO_2 through normal photosynthesis (PS II and PS I are involved). The synthesized cellular material contains hydrogen, while oxygen is released. The second phase is completed under anaerobic conditions. These cellular materials produce electrons through glycolysis (EMP) and citric acid cycle (TCA). Electrons are transmitted to PS I through PQ, cytochrome b6/f complex and PC. Afterward, the electrons are passed to hydrogenase for producing hydrogen by direct electron donor (ferredoxin). Glycolysis process of cellular material takes place along with the CO_2 production (Figure 8.14). The reaction is represented by the following equations:

$$12H_2O + 6CO_2 \xrightarrow{\text{Light energy}} C_6H_{12}O_6\,(\text{Cellular material}) + 6O_2 \qquad \text{First Phase}$$

$$C_6H_{12}O_6\,(\text{Cellular material}) + 12H_2O \xrightarrow{\text{Light energy}} 6CO_2 + 12H_2 \qquad \text{Second Phase}$$

$$12H_2O \xrightarrow{\text{Light energy}} 6CO_2 + 12H_2 \qquad \text{Overall Reaction ((glycolysis.wmf))}$$

Typically, the algae are placed in a medium without sulfur for the second phase. Consider *Chlamydomonas reinhardtii* as an example. When the algae is placed in a medium without sulfur, photosynthetic O_2 production and CO_2 fixation rate sharply decline within 24h, even under light. The reason is that D1 protein of PS II requires frequent replacement. However, its biosynthesis is hampered by the absence of inorganic sulfur. The polypeptide chain consists of massive sulfur-containing amino acids, such as cysteine and methionine. After a sharp decline in the capability of biophotolysis oxygen, respiration on mitochondria continues. Meanwhile, the amount of oxygen released by photosynthesis is less than the amount of oxygen consumed in respiration. After limiting sulfur for 22 h, medium under light conditions exhibits anaerobic conditions. In the absence of oxygen as a final electrons acceptor under

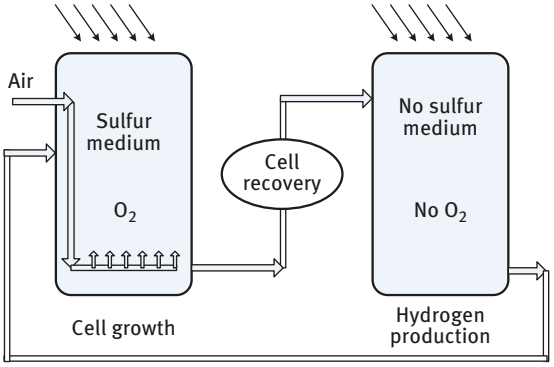

Figure 8.15: Indirect biophotolysis process for hydrogen production technology.

the circumstances, the electrons from EMP and TCA pathway are transmitted to the hydrogenase, and then combine with protons to produce H_2.

Figure 8.15 is a typical indirect biophotolysis process for hydrogen production technology. It divides the process into three steps: (1) Algae are cultivated in an open algae culture tank with sulfur. Carbohydrates are produced by photosynthesis and are stored in cells. (2) The cells are collected in the sedimentation tank. (3) Catabolism of sugars and production of hydrogen take place in a closed anaerobic tank, until algae deplete the intracellular materials. Then, the inorganic sulfur is replenished into the medium to restore normal photosynthesis. After a certain period time of fixing CO_2, the next cycle begins. By this method, Tolstygina et al. [29] studied the hydrogen production of *Chlamydomonas reinhardtii* and found that the hydrogen production rate was 0.07 mmol/(L • h). However, due to the high cost of this process, it still remains in the experimental stage. For example, the reaction system produces 1 GJ H_2 and will cost about 10$, whereas more than half of it is spent on the reactor.

2.2.3 Factors Influencing the Microbial Hydrogen Production by Green Algae

2.2.3.1 Sulfur

Sulfur in the medium plays a decisive role for photosynthetic cells and controls the production of H_2. However, recent studies have shown that, in the green algae culture, a set limit to supplement of sulfur can make cells have higher H_2 production yields than from that when sulfur is completely removed from the medium. Most of the sulfur in the medium is removed by centrifugation. However, this method produces a lot of waste, is time-consuming, and has high cost, due to which it is not conducive to large-scale hydrogen production. Some researchers believe that

the dilution for sulfur deprivation was better than centrifugation. However, this method makes hydrogen production cheaper, saves time, and reduces the microbial contamination.

2.2.3.2 Endogenous Substrates

Indirect biophotolysis process for hydrogen production is related not only to the activity of PS II but also to sugar, starch, protein, and other endogenous substrates. Intermediate metabolites, such as glucose and acetic acid, may provide electrons via respiration for hydrogen production. Heterotrophic growth of *P.subcordiformis* needs glucose, while the heterotrophic growth of *C.reinhardtii needs* acetic acid. Ghirardi et al. [30] noted that the anaerobic conditions were not enough to make hydrogenase gene expression and that the metabolism of starch was also an important factor. Starch degradation may affect the intracellular NAD (P) H and (or) PQ's oxidation level, both of which directly or indirectly affect the expression of hydrogenase gene. Many practices have proved that after the initial 24h when sulfur deprivation takes place, it needs to increase the level of intracellular starch.

2.2.3.3 Fe^{2+} and CO_2

When the algae are grown under atmospheric CO_2, Fe^{2+} does not reach saturation. However, when the algae are grown under 3% (v/v) CO_2, Fe^{2+} becomes saturated. Under 3% (v/v) CO_2 conditions, anaerobic H_2 production rate is three times faster than that under atmospheric CO_2. It also shows that the number of Fe^{2+} in the cells, CO_2 concentration, and anaerobic conditions are closely linked.

2.2.3.4 NaCl Concentration

Cells produce H_2 and ATP at the same time. ATP is needed by its own growth and will be more conducive to hydrogen production. NaCl concentration is an important factor affecting the consumption of ATP. Neale et al. studied the impact of medium's NaCI concentration for green algae hydrogen production. The results showed that the optimal salinity of green algae H_2 production was l0 mmol/L NaCl.

2.2.3.5 O_2 Concentration

Oxygen's inhibition of hydrogenation is mainly due to the binding of O_2 with hydrogenase's active sites, thereby preventing H^+ or H_2 binding with the hydrogenase.

Hydrogenase is very sensitive to O_2. When the oxygen concentration in the environment is close to 1.5%, the hydrogenase will lose its vitality. There are three solutions to solve this problem: (1) The use of molecular biology to obtain oxygen-resistant mutants, which hinder O_2 close to green algae's $FeFe^-$ hydrogenase catalytic site. (2) To partially inhibit the activity of PS II via sulfur-free regulation, which becomes intracellular physiological anaerobic, thereby preventing the expression of hydrogenase. (3) The reactor is filled with inert gas to dilute concentration of oxygen, which has been widely used in the past. Recent studies indicate that both the reversible hydrogenase and hydrogen metabolism's gene expression are irreversibly suppressed by O_2.

2.2.3.6 Light Intensity

Light intensity plays an important role in the two-stage hydrogen production of green algae. Take *Chlamydomonas reinhardtii* as an example. Hydrogen requires light intensity in the range of 60–200 $\mu Em^{-2}s^{-1}$ after completely deprived of sulfur. Close to 200 $\mu Em^{-2}s^{-1}$ light intensity, the hydrogen production increases with the increase in light intensity. When the light intensity reaches 200 $\mu Em^{-2}s^{-1}$, the largest amount of hydrogen production is observed to be 2.6 times that at 60 $\mu Em^{-2}s^{-1}$. However, when the light intensity reaches 300 $\mu Em^{-2}s^{-1}$, the amount of hydrogen production drastically decreases.

2.2.3.7 pH

Proton is a substrate or producer of hydrogenase catalytic reaction. From the reaction equation, lower pH is conducive to hydrogen-desorption, while higher pH is conducive to hydrogen-absorption. However, some hydrogenases do not follow this rule. The best environment for its catalytic hydrogen-absorption is acidity. pH also affects the isoelectric point of an electron carrier, oxidation state, and functional groups' dissociation of the active center. Hydrogen production of *Platymonas subcordiformis* increases 14 times when the pH increases from 5 to 8. When the pH exceeds 8, the production of hydrogen decreases drastically. To Rhine-green algae, the maximum hydrogen production yield takes place at the pH of 7.7, when sulfur is removed. The yield will reduce when the pH becomes less than 6.5 or higher than 8.2.

2.2.3.8 Inhibitor

In 1933, it was found that a particular compound named 3-(3,4-dichloropHenyl)-1, 1-dimethylurea (DCMU) may specifically inhibit PS II. First, *Chlamydomonas reinhardtii* was placed in the environment that contained the absence of light and O_2. The DCMU was added for cultivation for a while. The dissolved oxygen in the

water was consumed through respiration, and then, the algae were cultivated with light. Since PS II was inhibited by DCMU, large amounts of hydrogen were produced. After the addition of PS II's inhibitors, hydrogen would be produced without removing sulfur-containing nutrient medium. Dalian Institute of Chemical Physics, Chinese Academy of Sciences found that one uncoupler named Carbonylcyanide-m-chlorophenylhydrazone (CCCP) could significantly reduce PS II's photochemical efficiency for *P. subcordiformis* and *Chlamydomonas reinhardtii*. CCCP would inhibit the evolution of photosynthetic oxygen, promote the expression of hydrogenase, and accelerate the hydrogen production under illumination process.

2.2.3.9 Immobilization

When the algae cells are immobilized, it will increase the photosynthetic hydrogen production time (up to 4 weeks), and the hydrogen production rate becomes similar to that when cells were not immobilized. Most algae produce hydrogen in the absence of sulfur conditions. The culture conditions will maintain the cycle of sulfur-deprivation and sulfur-filled, if the algae cells are not immobilized, while large energy input will be required during the centrifugation. Immobilization makes this process more simple, fast, and economical.

2.2.4 Green Algae Breeding for Microbial Hydrogen Production

2.2.4.1 Traditional Mutation Breeding

Melis et al. [31] put wild algae strains in different O_2 partial pressure environments for random chemical mutagenesis. Using this classic genetic mutagenesis method, they successfully screened two mutation algal strains whose oxygen resistances were 10 times higher than the wild algae strains. Since the regulation of green algae produce hydrogen and its photosynthesis is very complex, it is difficult to clarify regulatory mechanisms in near future. Therefore, the use of a classical genetic method for breeding algal strain, which are resistant to oxygen and produce high yield hydrogen, is an effective way in recent years.

2.2.4.2 Regulation of Oxygen Sensitive for FeFe-Reversible Hydrogenase

FeFe-hydrogenase has low redox potential, high catalytic activity and does not need to consume ATP. Compared with NiFe-hydrogenase and nitrogenase enzyme,

it has obvious advantages. However, its biggest problem is that it is extremely sensitive to O_2 and conflicts with the photosynthesis of green algae, which limits the development of green algae hydrogen production. FeFe-hydrogenase of algae is the same as that of bacteria. However, it is oxygen sensitive from reversible oxidation of catalytic center site [2Fe-2S]. Current research on the structure of bacterial hydrogenase has shown that O_2 may reach the catalytic site through a number of gas passages, which are formed by folded polypeptide. The passages consist of a number of hydrophobic amino acids (such as glycine, alanine, and valine), which are surrounded by two helical α_2 and twofold β_2. Simulation results using Viewer Lite software show that the green algae FeFe-hydrogenase's gas passage pore diameter is greater than the molecular radius of H_2 (2.8 Å) and O_2 (3.5 Å). King and others used site-directed mutagenesis technique and replaced corresponding amino of *reinhardtii* HydA1's gas passage, which reduced the pore diameter of the gas passage. It was found that the oxygen tolerance was increased to double, while the transformation of green algae hydrogenase could improve their resistance to oxygen.

2.2.4.3 Gene regulation of Chlorophyll's Antenna Pigment Molecular Size

The algae is cultured under direct bright light, and for that, the energy efficiency shall have reached maximum, but the reality is not so. Chloroplasts capture less than 10% of the radiated energy, while the remaining 90% of the energy dissipates in the form of fluorescence or heat. In general, Chlorella express antenna pigment molecules of large size automatically under low-density light, while small-size antenna pigment molecules act in a contrary way. In theory, smaller size pigment molecules can capture more energy, improve the light saturation, and reduce the shadowing effect of green algae on the surface to the deep cells. In addition, the unit area can gather more of such "antenna" that can ultimately improve the utilization of light energy. Melis et al. [31] used genetic engineering methods that included the insertion mutation *tlal* gene to express smaller size light-harvesting pigment molecules. The gene is a regulatory gene for chloroplast antenna pigment molecular size. Sizes of PS II and PS I are 50% and 65% (respectively) equivalent to wild algae strains. Mutant algae strains' light saturation increases, and so does the light use efficiency.

2.2.4.4 Sulfur Penetration *SulP* Gene of Chloroplast

C. reinhardtii's sulfur penetration *SulP* gene of chloroplast has been discovered. Its encoded protein is ABC type and is an important part of the complex. The complex is on the surface of chloroplast, plays an important role in the sulfur transportation

and enrichment. *SulP* mutations reduce the repairing capability of PS II. Therefore, sulfur penetration enzyme's activity is quantified by controlling the sulfur metabolic engineering. When microalgae cells are hermetically cultured, photosynthesis and respiration rate become equal (matching). Both of these ensure normal growth of microalgae and anaerobic environment of the whole system, and achieve sustained expression of active hydrogenase and efficient continuous production of hydrogen under light conditions. Currently, in *Sulp* inhibition experiments, cells are found to exhibit significant sulfur deficiency. Some of chlamydomonas have even achieved a sustained hydrogen production under light.

2.3 Microbial Hydrogen Production by Cyanobacteria

2.3.1 Hydrogen-producing Cyanobacteria

Cyanobacteria produce hydrogen and include filamentous heterocyst cyanobacteria, filamentous nonheterocyst cyanobacteria, single cells nonnitrogen fixation cyanobacteria, and other three categories. They include *Anabaena*, such as *Anabaena azollae*, *A. variabilis*, and *A.cylindrica*; *Nostoc*, such as *Nostoc muscorum* and *Nostoc spongiaeforme*; *Synechococcus*, such as *Synechococcus elongates*; *Oscillatoria*, such as *Oscillatoria limnetica*; *Synechocystis*; and other cyanobacteria, such as *Chlorogloea fritschii*, *Mastigocladus laminosus*, and *Westiellopsis prolifica*.

Current research is focused on heterocyst cyanobacteria, which belong to *Anabaena* and *Nostoc*, such as *A.cylindrica*, *A.variabili*, and *Nostoc* PCC 73102. It also includes some cyanobacteria of *Synechococcus* and *Synechocystis*. Heterocyst cyanobacteria grow in air and are measured in an argon atmosphere. Their typical hydrogen production rate is 0.17–4.21 μmol/ (mg chl a • h). Cyanobacteria hydrogen production is affected by many factors. Hydrogen production rate may differ by more than 10 times of one cyanobacterium under different growth conditions. *A.variabilis* ATCC 29413 grow under the light intensity of 90 μE/(m^2·s) and 25%N_2+2%CO_2+73%Ar conditions. Under the pressure of nitrogen nutrition (when N_2 decreases from 25% to 5%), the hydrogen production rate is 45.18 μmol/(mg chl a h).

Nonheterocyst nonnitrogen fixation cyanobacteria also have some desorption hydrogen capacity with reversible hydrogenase. *Gloeobacter* PCC 7421's hydrogen production rate is the same as those of some heterocyst cyanobacteria. Moreover, cyanobacteria under dark and anoxic conditions can also produce hydrogen through dark fermentation. However, the production rate is very low. Because of relatively perfect separation mechanisms of photosynthetic oxygen production and nitrogen fixation hydrogen production, filamentous heterocyst cyanobacteria have high hydrogen production rate. *Anabaena cyanobacteria* are very promising hydrogen-producing photosynthetic organisms.

2.3.2 Mechanism of Microbial Hydrogen Production by Cyanobacteria

Similar to eukaryotic algae, cyanobacteria have PS I and PS II. Protons and electrons, which are required by cyanobacteria for H_2 production, can also be derived from photolysis of H_2O. Cyanobacteria have hydrogenase and nitrogenase enzymes. In fact, enzymes involved in hydrogen metabolism of cyanobacteria include nitrogenase enzymes, absorption hydrogenase, and reversible hydrogenase. Filamentous heterocyst cyanobacteria's hydrogen production is affected by nitrogenase enzymes, absorption hydrogenase, and reversible hydrogenase. The hydrogen production of single cell nonnitrogen fixation cyanobacteria is mainly responsible for the reversible hydrogenase.

2.3.2.1 Hydrogen Production by Nitrogenase

O_2 is a potent inhibitor of nitrogenase. However, cyanobacteria have evolved a comprehensive protection mechanism of nitrogenase to counter the effect of O_2. The cyanobacteria take indirect biophotolysis process for hydrogen production. Depending on the species, indirect biophotolysis process for hydrogen production of cyanobacteria will separate O_2 and H_2 in the space or time. O_2 and H_2 come from photosynthetic, thereby avoiding the inhibition of photosynthetic production of O_2 to produce H_2.

(1) Space separation

For filamentous heterocyst cyanobacteria, spatially separate photosynthetic production of O_2 and H_2 takes place. Vegetative cells produce O_2 normally by photosynthesis. H_2 is only produced in the heterocyst. For example, *A.cylindrina* is a filamentous aerobic nitrogen-fixing cyanobacterium, which contains heterocyst and vegetative cells. Vegetative cells contain PS I and PS II, which can take photolysis H_2O and CO_2 reduction, produce reducing substances and O_2. Reducing substances can be transported through thick-walled tunnel to heterocyst as the hydrogen donor for nitrogenase hydrogen production. However, O_2 cannot pass through this thick-walled tunnel.

Volume of heterocyst is larger than the average nutrition bacteria. There is a thicker membrane layer that is composed of sheet-type glycolipid of cells. It is a barrier stopping O_2 from entering the cells. Heterocyst lacks PS II for oxygen production, and coupled with high activity of hydrogen-release enzymes and hydrogenase, it can maintain a strongly reduced state. The activity of superoxide dismutase (SOD) is high, due to which SOD has the function of releasing oxygen's toxicity. In addition, the respiration intensity in heterocyst is two times higher than that in the neighboring vegetative cells. The above conditions provide a partial anaerobic or low oxygen partial pressure environment to heterocyst, so that H_2 can be produced normally.

(2) Time separation

For nonheterocyst cells and filamentous cyanobacteria, separate photosynthetic produce O_2 and H_2 by time. O_2 is produced via photosynthesis under light, while H_2 is produced under dark and anaerobic conditions. In the absence of anti-oxygen protection agency, nitrogenase produces H_2 that can only occur in the case of alternating light and dark. Under light, cells fix CO_2, store polysaccharide, and release O_2. Under dark and anaerobic conditions, polysaccharides are degraded (EMP and TCA pathways), and then provide hydrogen donors, electrons, and ATPs to nitrogenase for producing H_2.

2.3.2.2 Hydrogen Production by Hydrogenase

Oscillatoria limnetica is nonheterocyst and facultative aerobic nitrogen-fixing filamentous cyanobacteria. Reversible hydrogenase catalyzes hydrogen production. Glycogen is accumulated at daytime by photosynthesis. In addition, hydrolyzate of glycogen is H_2 under illumination and argon gas conditions. *Spirulina platensis* produces hydrogen under dark and anaerobic conditions via reversible hydrogenase. In fact, nitrogenase or hydrogenation takes part in enzymatic H_2 production under dark and anaerobic conditions. However, H_2 production rate via hydrogenase is much lower than that of the nitrogenase.

2.3.3 Influencing Factors of Microbial Hydrogen Production by Cyanobacteria

The production of hydrogen by cyanobacteria requires appropriate environment, including light intensity, temperature, salinity, pH, atmosphere, culture conditions, and medium. For nitrogenase production of hydrogen, since nitrogenase's activity requires higher saturation light intensity than optimal growth requires, an appropriate increase in light intensity can often increase the production of hydrogen. Additionally, different cyanobacteria require different temperature, salinity, and pH conditions. Other major factors are as follows.

2.3.3.1 Atmosphere

For heterocyst cyanobacteria, N_2 and O_2 are inhibitors of nitrogenase. Absorption-hydrogenase consumes H_2 that is released by nitrogenase. O_2 will cause reversible hydrogenase inactivation. Therefore, cyanobacteria hardly produce any hydrogen under atmospheric conditions containing N_2 and O_2. The production of hydrogen by cyanobacteria is generally carried out in an argon atmosphere, or under conditions of

CO and C_2H_2 supplied along with nitrogen. CO and C_2H_2 are competitive gas inhibitors of nitrogenase and absorption-hydrogenase.

2.3.3.2 Culture Conditions

Immobilization can protect cyanobacterial cells and their enzymatic activity, reduce the inactivation caused by the environmental interference, and increase its hydrogen production rate and stability. Cyanobacteria's immobilized culture commonly uses carriers, such as agar gel, alginate gel, polyurethane foam, and polyethylene foam. Park and others fixed *A. azollae* with polyethylene foam in pillar bioreactors of light to achieve continuous production of hydrogen for six days. Markov and others fixed *A. variabilis* with hollow fiber, and achieved five months of continuous hydrogen production, whereas the production rate was 0.02–0.2 ml/(mg cell·h).

2.3.3.3 Medium

Heterocyst cyanobacteria release hydrogen, which is the result of nitrogenase hydrogen-release and absorption-hydrogenase hydrogen absorption. Enhancing the activity of nitrogenase or inhibit the activity of absorption-hydrogenase is conducive to increase the production of hydrogen. Vanadium and molybdenum-free culture can induce the expression of higher hydrogen discharge efficiency enzyme, which is vanadium nitrogenase to produce more hydrogen. Dawar found that the improved magnesium ion concentration and added fructose in the medium would create *Nostoc* sp. ARM 411' heterocyst increase the frequency by three times, due to which the activity of nitrogenase and hydrogen production increase. Cyanobacteria absorption-hydrogenase's cofactor contains Ni, which not only has a direct effect on the enzyme but also participates in encoding gene transcription regulation of absorption-hydrogenase. Ni restrictions can inhibit the activity of absorption-hydrogenase, and significantly facilitate the production of hydrogen. In addition, the medium for hydrogen production also plays a significant role. For example, *Nostoc* PCC73102's nitrogen fixation and hydrogen absorption capacity in light heterotrophic or chemical heterotrophic are stronger than those in light autotrophic conditions. The addition of glucose can inhibit the activity of absorption-hydrogenation. However, at the same time, it increases the hydrogen production yield of nitrogenase. Furthermore, the presence of exogenous $NH4^+$ will inhibit the nitrogenase synthesis and hydrogen production.

2.3.4 Cyanobacteria Breeding for Hydrogen Production

The utilization of cyanobacteria via photosynthesis to produce hydrogen raises three issues: (1) inhibition of O_2 to nitrogenase and reversible hydrogenation; (2)

absorption-hydrogenase's effect of absorbing hydrogen; (3) low overall hydrogen yield. Therefore, the work on cyanobacteria breeding has focused on strain breeding of high activity of nitrogenase and reversible hydrogenase, resistance to oxygen, and deficient absorption-hydrogenase.

It is well known that the nitrogen-fixing cyanobacteria have absorption- hydrogenase. Chemical mutagenesis or genetic mutations are used to obtain mutants, which lack absorption-hydrogenase gene. This technology improves the yield of hydrogen by cyanobacteria. Happe et al. [32] used NTG method to obtain two hydrogen metabolic defects mutants of *A. variabilis* named ATCC 29413 PK84 (absorption-hydrogenase and reversible hydrogenase double defects) and PK17R (absorption-hydrogenase defects). Their growth characteristics and heterocyst frequency were found to be same as those of the wild types. However, the hydrogen-release rates increased significantly. The capacity of hydrogen production by wild strains was 45.16 μmol/(mg chl a•h), while those for the mutants PK17R and PK84 were 59.18 μmol/(mg chl a•h) and 167.6 μmol/(mg chl a•h). Furthermore, the activities were found to be 1.4 times and 4.3 times of those of the wild strains. In addition, by outdoors test of mutant PK84 for hydrogen production, it was found that the hydrogen production occurred with continuous optical biological growth, whereas the highest hydrogen production activity was 0.11 μmol/(mg chl a•h). Lindberg et al. [33] inserted mutagenesis to N. punctiforme ATCC 29133, which lacked the reversible hydrogenase. The constructed hupL$^-$ mutant was named NHM5, and did not have any hydrogenase. NHM5 cultured under nitrogen fixation condition released hydrogen (14 μmol/(mg chl a•h)) under air, whereas the wild type did not release any hydrogen. Sakurai et al [34] constructed three mutants of *Anabaena sp.PCC* 7120 hydrogenase, namely the absorption-hydrogenase deficient strain hupL$^-$, reversible hydrogenase deficient strain hoxH$^-$, and double deficient strain hupL$^-$/hoxH$^-$. It was found that, hupU$^-$ and hupU$^-$/hoxH$^-$ mutant strains produced a considerable amount of hydrogen, while the maximum hydrogen production rate was – four to seven times higher than that of the wild types. The hydrogen production of hoxH$^-$ mutant strains was 15–33% lower than that of the wild type.

2.4 Microbial Hydrogen Production by Photosynthetic Bacteria

2.4.1 Photosynthetic Bacteria

Photosynthetic bacteria were observed to release hydrogen under dark conditions. *Rhodospirilum rubrum* was reported to produce hydrogen under light conditions. However, it was found to perform photosynthetic nitrogen fixation. Since then a large

number of studies have shown that the production of hydrogen under light or dark conditions is widespread in photosynthetic bacteria. It includes purple sulfur bacteria, purple nonsulfur bacteria, and green sulfur bacteria, which exist in almost all the purple nonsulfur bacteria. More research of photosynthetic hydrogen-producing bacteria is mainly focused on *Rhodopseudomonas*, *Rhodospirillum*, *Rhodomicrobium*, *Chromatium*, *Thiocapsa*, *Ectothiorhodospira*, *Chlorobium*, and seven genera of 21 strains, namely the *Rhodospirillum rubrum*, *Rhodospirillum tenue*, *Rhodomicrobium vannielii*, *Rhodobacter sphaeroides*, *Rhodopseudomonas capsulata*, *Rhodopseudomonas gelatinosa*, *Rhodopseudomonas acidophila*, *Rhodopseudomonas palustris*, *Rhodopseudomonas sphaeroides*, *Rhodopseudomonas sulfidophila*, *Chromatium* sp., *Chromatium minutissimum*, *Ectothiorhodospira mobilis*, *Ectothiorhodospira shaposhnikovii*, *Thiocapsa floridana*, *Thiocapsa roseopersicina*, *Chromatium sphaeroides*, *Chlorobium sphaeroides*, *Chlorobium limicola*, *Chlorobium thiosulfatephylum*, and *Chloropseudomonas ethylica*.

Photosynthetic bacteria have a variety of metabolic models, such as the aerobic respiration, anaerobic respiration, anaerobic fermentation, and optical autotrophy (Table 8.5). Anaerobic and optical heterotrophic model is the best model for hydrogen production. However, anaerobic and optical heterotrophic model is also the optimal growth model. In addition, under dark and anaerobic conditions, photosynthetic bacteria can produce hydrogen. However, the hydrogen production efficiency is much lower than that of the anaerobic and heterotrophic light model. Therefore, the exploitation of hydrogen production by photosynthetic bacteria and the process conditions should be controlled.

Table 8.5: Different growth models of photosynthetic bacteria

Model	Carbon source	Energy	Hydrogen production situation
Anaerobic and optical heterotrophic model	Organic carbon	Light	Optimum growth mode, the best hydrogen production mode
Anaerobic and optical autotrophic model	CO_2	Light	Growth model when lack of organic carbon, consume hydrogen
Aerobic and chemical heterotrophic (aerobic respiration)	Organic carbon	Organic carbon	Under Oxygen conditions, no hydrogen produced
Anaerobic and chemical heterotrophic(Anaerobic respiration)	Organic carbon	Organic carbon	Under anaerobic and low light intensity conditions; needs other electron acceptor except O_2 (e.g., N_2); no hydrogen produced
Anaerobic and dark fermentation	Organic carbon	Organic carbon	Under anaerobic and dark conditions; have some hydrogen production capacity

2.4.2 Mechanisms of Microbial Hydrogen Production by Photosynthetic Bacteria

2.4.2.1 Mechanisms of Microbial Photosynthetic Hydrogen Production

Under anaerobic, light, and nitrogen-deficient conditions, photosynthetic bacteria use acetic acid, lactic acid, malic acid, butyric acid, succinic acid, fumaric acid, glutamine, and glucose as substrates to produce hydrogen. Even alcohols, polysaccharides (e.g. starch), amino acids, and the aromatic compounds can also be used as substrates.

$$\text{Acetic acid: } C_2H_4O_2 + 2H_2O = 2CO_2 + 4H_2 \tag{8.16}$$

$$\text{Butyric acid: } C_4H_8O_2 + 6H_2O = 4CO_2 + 10H_2 \tag{8.17}$$

$$\text{Lactate: } C_3H_6O_3 + 3H_2O = 3CO_2 + 6H_2 \tag{8.18}$$

$$\text{Succinic acid: } C_4H_6O_4 + 4H_2O = 4CO_2 + 7H_2 \tag{8.19}$$

$$\text{Fumaric acid: } C_4H_4O_4 + 4H_2O = 4CO_2 + 6H_2 \tag{8.20}$$

$$\text{Malic acid: } C_4H_6O_5 + 3H_2O = 4CO_2 + 6H_2 \tag{8.21}$$

$$\text{Glutamine: } C_5H_9NO_4 + 6H_2O = 5CO_2 + NH_3 + 9H_2 \tag{8.22}$$

$$\text{Glucose: } C_6H_{12}O_6 + 2H_2O = 6CO_2 + 12H_2 \tag{8.23}$$

Various pathways for the photosynthetic bacteria to produce hydrogen are shown in Figure 8.16. These pathways are catalyzed by nitrogenase, require the provision of ATP and reducing power ($H^+ + e^-$). Different to cyanobacteria and eukaryotic algae,

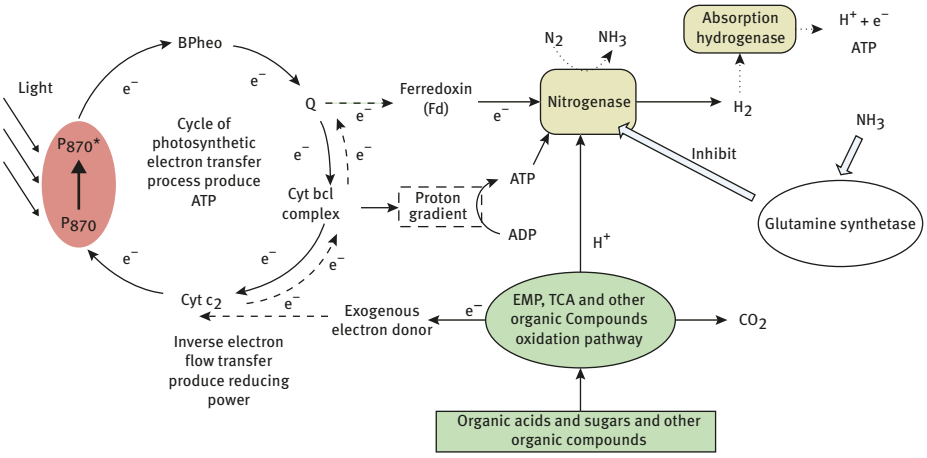

Figure 8.16: Photosynthetic hydrogen production pathways of photosynthetic bacteria.

anaerobic bacteria do not have photosystem II. They cannot use water as the electron donor and need electron donors whose reduction potentials are below that of the water. Photosynthesis can only provide ATP. In the cycle of photosynthetic electron transfer process, electrons pass Cyt bc1 complex and generate a proton gradient. ATP synthase uses proton gradient to produce ATP, which is required by nitrogen fixation and hydrogen production. Exogenous organic compounds generate protons, electrons, and CO_2 by EMP, TCA, and other organic carbon metabolic pathways. Electrons are transferred to ferredoxin through inverse electron stream, while ferredoxin transfers electrons to nitrogenase. The hydrogen produced by nitrogenase is reabsorbed by absorption-hydrogenase to generate the reducing power and ATP. In order to produce hydrogen, it needs to limit the presence of N_2. In addition, an effectively improved hydrogen yield inhibits the expression or activity of absorption-hydrogenase genes.

Similar to cyanobacteria, when there is NH_4^+, photosynthetic bacteria lose the ability to fix nitrogen and produce hydrogen. NH_4^+ reacts with glutamate to produce glutamine, while the reaction is catalyzed by glutamine synthetase. Glutamine combines with the product (a protein), which is genetic regulator of glutamine synthetase. The process leads to configuration changes of the product, which binds to the manipulation gene of glutamine synthetase and prevents the transcription of structural gene of glutamine synthetase. Glutamine synthetase cannot be synthesized. In this case, since there is no glutamine synthetase, RNA polymerase cannot transcribe the structural gene of nitrogenase. Therefore, the nitrogenase enzyme cannot be synthesized.

2.4.2.2 Mechanisms of Microbial Hydrogen Production Under Dark Conditions

Some photosynthetic bacteria not only have photosynthetic hydrogen capacity but also use glucose and organic acids (including formic acid) to produce H_2 and CO_2 by anaerobic fermentation under dark and anaerobic conditions. Dark fermentation reposes cells under dark, which have high activity. The hydrogen production activity decreases by about 25% under light conditions. In addition, CO inhibits the hydrogen production of fermentation repose cells. Furthermore, 20% CO can completely inhibit the production. Dark fermentation hydrogen production is catalyzed by reversible hydrogenase rather than the nitrogenase, while the mechanisms of hydrogen production may be similar to that of the dark fermentation hydrogen-producing bacteria.

2.4.3 Influencing Factors for the Microbial Hydrogen Production by Photosynthetic Bacteria

(1) Illumination

Light type and light intensity will affect the hydrogen production activity of photosynthetic bacteria, whereas the light intensity exhibits a greater impact on the conversion

efficiency than that of the type of light. Research on *R. sphaeroides* O.U.001 about hydrogen production and light intensity showed that the hydrogen production increases with light intensity. Maximum productivity is at 4000 lx. However, when the light intensity is continuously increased, it does not increase the amount of hydrogen produced.

(2) Temperature

Temperature has a significant impact on cell growth, and also on various biochemical reactions and metabolism within the cells. The optimal temperature of photosynthetic bacteria for growth and hydrogen production via metabolism are not the same. Photosynthetic bacteria could grow in the temperature range of 10–45 °C. However, the optimum temperature for photosynthetic hydrogen production is 30–40 °C.

(3) pH

Optimum pH value is generally about 7 for normal hydrogen production by photosynthetic bacteria. The optimum pH value is 7.29–7.31 when acetic acid, propionic acid, and butyric acid are used as the substrates.

(4) Inoculated concentration and the age of bacteria

Generally, higher concentration in the medium will produce a greater rate of hydrogen production. If the cell concentration is too high, its shading will affect the energy obtained by deep bacteria. Usually, the bacteria in logarithmic growth phase have larger hydrogen production capacity.

(5) Substrate

Different strains have their own preferences for substrates, while different concentrations of the same substrate will also affect the production of hydrogen. Therefore, varied strains are used in combination, which can increase hydrogen yield of multicomponent organic wastewater. Hydrogen production rate of *R. capsulata* Z-1 in the butyric acid and propionic acid system was 20–40 μl/(h•mg cell). However, in the DL-malic acid, DL-lactic acid, succinic acid, pyruvic system, it could reach 130 μl/(h•mg cell). Lactic acid is a better substrate for hydrogen production for *R. capsulata*. It also shows the effect of relieving ammonium salt's suppression. The best substrate *of R. aphaeroides* for hydrogen production is the acetic acid.

R. rubrum could use fruit acid, oxaloacetic, pyruvic acid, acetic acid, fumaric acid, succinic acid, and others to produce hydrogen. *R. rubrum* could use DL-phosphoric acid, DL-malic acid, glucose, succinic acid, pyruvic acid, fructose, sucrose, and glycerol to produce hydrogen.

(6) Cells immobilization

Cells immobilization provides a relatively stable living environment, prevents the danger of osmotic pressure to cells, avails continuous use of biological catalyst,

simplifies the culture and biological catalyst separation step, and exhibits a certain ease for inhibition. Photosynthetic bacteria commonly use sodium alginate, agar, PVA, and porous glass as the materials of immobilization. Compared with alginate and polyacrylamide, agar is nontoxic, low cost, has stable chemical properties, and simple immobilization process. However, it has poor physical strength, is easy to fracture, has poor matrix, and exhibits light permeability. Porous glass has good light permeability, and high mechanical strength. However, it has poor bacterial adsorption ability. Mitsui and others reported using agar embedding oceanic photosynthesis bacteria to produce hydrogen from the inhibition of oxygen. After 5 h aeration, bacteria retained 95% of the original activity of hydrogen production.

2.4.4 Bacterial Breeding for Microbial Photosynthetic Hydrogen Production

Strain screening and transformation are important means for improving the efficiency of photosynthetic bacteria to produce hydrogen. The goal is to obtain absorption-hydrogenase defects, glutamine nutrition deficiency, whereas the mutants can increase the conversion efficiency. Pierrard et al. [35] used *R. capsulata* B10 strains for chemical mutagenesis. They found absorption-hydrogenase defects mutants, which used L-malic acid, D-malic acid and DL-malic acid as the substrate for producing hydrogen. The capacities were 10–20%, 20–50%, and 70% higher than that of the wild strains, respectively. In comparison to wild strains, the absorption hydrogenase defects mutants of *R. sphaeroides* significantly increase H_2 production in acetic acid. Variants were isolated from *Rhodobacter sphaeroides*, *Rhodopseudomonas capsulate*, and *Rhodospirillum rubrum*, which exhibited tolerances for NH_4^+. Researchers tried to separate the variants that could not transmit NH_4^+ intracellularly. In this way, the recovery of H_2 production activity in the presence of NH_4^+ was likely going on under the premise of not changing metabolism of NH_4^+ system.

Section 3: Microbial Hydrogen Production by Anaerobic Dark Fermentation

3.1 Anaerobic Dark Fermentation for Microbial Hydrogen Production

In nature, a lot of bacteria produce hydrogen from anaerobic fermentation, including strict anaerobes, facultative anaerobes, and obligate aerobic bacteria. Strict anaerobes and facultative anaerobes were most widely used as inoculums for fermentative

hydrogen production, such as *Clostridium butyricum*, *C. pasteurianum*, *C.welchii*, *Enterobacter aerogenes*, and *Enterobacter cloacae*.

3.1.1 Strict Anaerobes

3.1.1.1 Clostridium

Clostridium is a large class of bacteria, which is a member of *Clostridia*, *Clostridiales*, and *Clostridiaceae*, and can mainly produce hydrogen through fermentation. *Clostridium* is widely found in soil, sewage sludge, human and animal intestines, and other environment and is strictly the anaerobic bacteria. These microorganisms can produce spores, whereas the environmental conditions have a strong tolerance. Due to this they are called *Clostridium* species. In early1960s, *C. butyricum* and *C. welchii* were applied for fermentative hydrogen production by Magna company. The research mainly focuses on *C. pasteurianum*, *C. butyricum*, *C. welchii*, and *C. beijerincki*. The hydrogen yield of glucose was 1.8–2.0 mol/mol with *C. beijerincki* AM21B [36], while the hydrogen production yield of xylose, arabinose, cellobiose, fructose, and other substrates for fermentation after 24 h process was around 15.7–19.0 mmol/g. The hydrogen production rate of xylose and arabinose with *Clostridium* sp.no.2 strains can reach up to 13.7 mmol/g and 14.6 mmol/g, while the hydrogen production rate of glucose was 11.1 mmol/g [36, 37]. *Clostridium* can be used for fermentation production of hydrogen in cellulose in biomass or hemicellulose. Since most *Clostridium* itself can synthesize cellulose enzymes, the use of clostridial fermentation of biomass can simultaneously produce hydrogen by the hydrolysis of biomass. Both the hydrogen production and facilitation of raw materials' hydrolysis can be carried out in the same reactor. *Clostridium* is the main microorganisms of anaerobic dark fermentation hydrogen production.

3.1.1.2 Methylotrophs

Methylotrophic bacteria are also known as the methyl-utilizing bacteria and are a kind of microorganisms which take advantage of carbon monoxide compounds (such as methane, methanol, formaldehyde, formic acid, and methyl amine) as the source of carbon and energy for bacterial growth. In 1983, Kawamula [38] found that the methylotrophic bacteria (*Methylomonas albus* BG8 and *Methylosinus trichosporium* OB3b) can produce hydrogen under anaerobic conditions to take advantage of methane, methanol, formaldehyde, formic acid, and pyruvic acid. In these substrates, formic acid is the best hydrogen-producing substrate. After five hours of fermentation, the hydrogen yield of formic acid substrates by *M. albus* and *M. trichosporium* was 2.45 and 0.61 mol/mol, respectively. The hydrogen produced

by methylotrophic bacteria is carried out using formic acid dehydrogenation and reversible hydrogenase enzyme.

3.1.1.3 Rumen Bacteria

In the family Lachnospiraceae, of *Ruminococcus albus* is a relatively common bacteria, which are able to hydrolyze cellulose and carbohydrates metabolism of acetic acid, ethanol, formic acid to produce hydrogen and carbon dioxide. In 1973, Innotti et al. found that the hydrogen yield of *R. albus*' continuous fermentation was 2.37 mol/mol glucose, which was accompanied by the production of 0.65 mol ethanol and 0.74 mol acetic acid [39].

3.1.1.4 Thermophiles

Bacteroidaceae, Clostridiaceae, and Thermoanaerobacteraceae have been used in high temperature conditions for hydrogen production. The hydrogen production rate of glucose by *Acetomicrobium flavidum* was 4.0 mol/mol. Some Clostridium family of *Thermoanaerobacter*, *Thermoanaerobacteroides,* and *Thermoanaerobacterium* were also used in fermentation hydrogen production. The hydrogen production rate of *T. thermosaccharolyticum* was 2.4 mol/mol.

Extreme thermophilic bacterium belongs to Archaea. *Pyrococcus furiosus* is the first extremely thermophilic bacteria that can produce hydrogen for which the optimum temperature is 100 °C. This kind of bacteria uses carbohydrates or peptides to generate organic acids, CO_2, and H_2. The maximum hydrogen production rate of glucose was 3 mol/mol. Many extreme thermophilic bacteria can break down cellulose to produce H_2, and include thermophilic bacteria of *Caldicellulosiruptor, Clostridium*, *Coprothermobacter*, *Dictyoglomus, Fervidobacterium, Pyrococcus, Acetomicrobium, Acetothermus, Thermotoga, and Thermoanaerobacter.*

The hydrogen production rate of extremely thermophilic bacteria is higher than middle temperature bacteria. The equivalent of the maximum theoretical hydrogen yield of glucose was 4 mol/mol by *Thermotoga maritima* under ultra-high temperature (80 °C). However, low cell concentrations (1.4×10^8/mL) and amount of glucose (1.6 mmol) are used. The maximum hydrogen production rate was 10 mmol/(L·h) [36]. *Themotoga elfi* and *Caldicellulosiruptor saccharolyticus* sucrose are used as substrates at 70 °C and 60 °C, respectively, for fermentation hydrogen production, whereas the hydrogen yield is 3.3 mol/mol glucose, which is the 83% of the maximum theoretical value. In addition, their maximum hydrogen production rates were 8.4 and 2.7 mmol/(L·h), respectively.

3.1.2 Facultative Anaerobes

The production of hydrogen by facultative anaerobes was mainly in Enterobacteriaceae, which included *Enterobacter, Escherichia, Citrobacter,* and *Klebsiella*. Among them, *Escherichia coli*, *Enterobacter aerogenes,* and *E. cloacae* are the most widely used facultative anaerobic hydrogen-producing bacteria.

3.1.2.1 Escherichia

Escherichia coli are a proven H_2– and CO_2– producing bacteria (from formic acid) under anoxic conditions. In early 1929–1933, Stickland [40], Yudkin [41], Nandi and Sengupta [42] carried out extensive research on generating H_2 and CO_2 from the anaerobic degradation of *E. coli* acid. *E. coli* can also produce hydrogen using carbohydrates, such as glucose, fructose, mannose, lactose, galactose, arabinose, glycerol, and mannitol. The hydrogen yield of the first three anaerobic degradations was similar to that of the formic acid yield. The hydrogen yield of other substrates is relatively low. *E. coli* glucose produces hydrogen through acidic pathway of the anaerobic process and has lower conversion efficiency. This is because of the formic acid, which is not the only product of glucose metabolism. Carbon balance analysis results showed that 1 mol of glucose produces 0.9 mol of ethanol, 0.9 mol H_2, 0.9 mol formic acid, 0.15 mol CO_2, and 0.15 mol of succinic acid during the production of hydrogen. Later research found that *E. coli* undertakes hydrogen production through pyruvic acid (derived from grape glycolysis) in the absence of external electron acceptors (such as nitrate or fumaric acid) conditions, which is completed in two steps. First, the pyruvate in the pyruvate-formate forms formic acid, and then through the next step, generates H_2 and CO_2.

3.1.2.2 Enterobacter

Enterobacter (Enterobacteriaceae) produce hydrogen, and exhibit a quick cell growth rate, are in widespread use for their advantages of a good carbon source. The *Enterobacter* spp. are formed under high hydrogen partial pressure. However, the hydrogen yield is usually lower than the strict anaerobes (e.g. Clostridium) of hydrogen yield. Tanisho et al. [43] reported discrete hydrogen production by *E. aerogenes* E.82005 from Mirabilis leaves. After 23 h of the batch anaerobic fermentation, the hydrogen yield and the maximum hydrogen production rate were 1 mol/mol glucose and 21 mmol/(L·h). Molasses is used as the substrate through a continuous hydrogen production process. The hydrogen yield and hydrogen production rate after 42 days were 1.5 mol/mol sucrose and 17 mmol/(L·h). Compared to batch fermentation, the

lactic acid is as a major metabolic by-product, whereas the butyric acid and acetic acid contents are relatively low. Despite the higher hydrogen partial pressure, hydrogen does not inhibit *Enterobacter*. However, in the medium with argon, the hydrogen yield can be significantly increased to 1.6 mol/mol glucose, which is due to the removal of CO_2. Yokoi et al. found strains producing gas and other acid-resistant *E. coli* isolated mutant (HO-39). The HO-39 bacteria can grow and metabolize hydrogen production at the pH of 4.5. When no pH adjustment of continuous culture is undertaken, the average hydrogen production rate after 26 days of anaerobic fermentation was 5 mmol/(L·h), whereas the hydrogen yield was 0.8 mol/mol glucose [44].

In order to improve the hydrogen production rate, *E. aerogenes* and *E. cloacae* mutants have been obtained. The two mutants will block other metabolic pathways, such as the production of macromolecular organic alcohol production pathway, which are generally two pathways in the hydrogen consumption process. For *E. aerogenes* double mutant, the metabolic process produces only a small amount of ethanol and butanediol, while mainly organic acids are produced. Compared to the original strain rate of hydrogen production, the hydrogen yield increased by one time. Kumar and Das isolated the strain of *E. cloacae* IIT-BT 08 from leaves, which can take advantage of a variety of carbon metabolism of hydrogen, glucose, sucrose, and cellobiose. The maximum hydrogen yield obtained through these substrates were 2.2, 6.0, and 5.4 mol/mol substrate, whereas the maximum hydrogen production rate was 35 mmol/(L·h) when sucrose was used as the substrate. Similarly, after the transformation of bacterial double mutation, blocking ethanol, butylene glycol, alcohols, such as lactic acid and butyric acid, and macromolecular organic acids, the hydrogen yield increased by 1.5-fold to 3.4 mol/mol glucose during continuous fermentation. The results showed that the bacterial cells flocculated. Even at high dilution conditions, the bacteria can remain in the reactor. The double mutant hydrogen obtained exhibited a yield of 1.1 mol/mol glucose at the dilution rate 0.67 h^{-1}. The maximum hydrogen production rate was 58 mmol/(L·h), which is equivalent to two times that of the wild strain.

3.1.3 Obligate Aerobic Bacteria

3.1.3.1 *Alcaligenes*

Some aerobic *Alcaligenes* are able to autotrophically metabolize and use H_2 and CO_2. They use them not only as the sole energy and carbon source but also to carry out heterotrophic growth. For example, *Alcaligenes eutrophus* take advantage of glucose or fructose acid heterotrophic growth, but once placed under anaerobic conditions, the bacteria can produce hydrogen using organic substrate. *A. eutrophus* contains

soluble reversible hydrogenase. When growing, they use H_2 and CO_2 as the substrate. *A. eutrophus* is able to directly reduce NAD^+ using H_2. When cells are in the body reducing power (NADH) excess and under anaerobic conditions, the enzyme transforms into consumption mode through desorption of excess reducing power. Klibanov studied the reversible reaction of formic acid cleavage using immobilized *A. entrophus*.

$$HCOOH \leftrightarrow H_2 + CO_2$$

However, in the formic acid degradation process, higher acid concentration (> 0.5 M) would inhibit the hydrogen-producing substrate.

3.1.3.2 *Bacillus*

Bacillus of the family Bacillaceae are also used in studies of fermentation hydrogen production. *Bacillus licheniformis* are typical hydrogen *Bacillus*. The hydrogen production rate of glucose that is used as the substrate was 0.58 mol/mol when subjected to 24 hours of batch fermentation. The hydrogen production rate of glucose increased to 1.38 times, and was 0.71 mol/mol after the *B. licheniformis* were fixed at a later brick debris and calcium alginate. Immobilized cells were incubated for 60 consecutive days, after which the average conversion rate was 1.5 mol H_2/mol glucose.

Different types of hydrogen-producing microorganisms use the same organic substrates. Generally speaking, hydrogen yield is higher than the strict anaerobic hydrogen production capacity of facultative anaerobes. In addition, the hydrogen yield is higher than the thermophilic bacteria and mesophilic bacteria.

3.2 Fundamentals of Dark Fermentation for Microbial Hydrogen Production

Microbial hydrogen-producing processes are closely related to energy of metabolism and electron flow. In the metabolism of aerobic bacteria, oxygen is used as the final electron acceptor that is released for oxidation of the substrate. In the metabolism of anaerobic substrates, the acceptor capable of accepting electrons includes nitrates, sulfates, carbon dioxide, and fumaric acid. In the absence of external electron acceptors, some organisms dispose of excess electrons generated. Meanwhile, the microbes use reducing protons to produce hydrogen under hydrogenation enzyme catalysis, which is a preferred choice to consume excess electrons. There are three main hydrogen-producing pathways using dark

fermentation hydrogen-producing bacteria [45]. These pathways are pyruvate decarboxylation pathway, formic acid cleavage pathway, and NADH/NAD^+ balance regulatory pathway.

3.2.1 Pyruvate Decarboxylation Pathway

Pyruvate decarboxylation pathway has been found in many strict anaerobes, such as *Clostridium*. The glucose generation of hydrogen by *Clostridium* pyruvate decarboxylation pathway is shown in Figure 8.17. The main enzyme involved in the

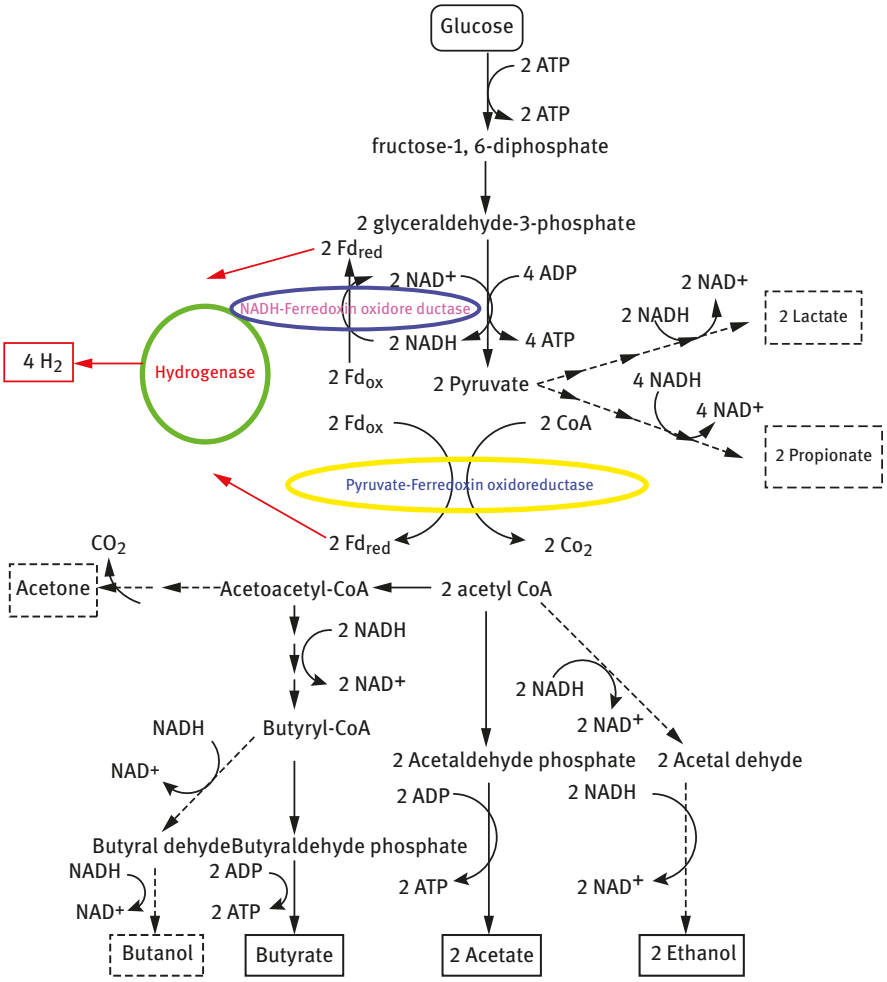

Figure 8.17: Generation of hydrogen by glucose through Clostridium pyruvate decarboxylation pathway.

production of hydrogen include, pyruvate-ferredoxin oxidoreductase (PFOR), NADH–ferredoxin oxidoreductase (NFOR), and hydrogenase. Due to the lack of participation of cytochrome oxidative phosphorylation, the ATP comes from substrate-level phosphorylation.

About 2 mol pyruvate, 2 mol ATP, and 2 mol NADH can be generated by the glycolytic pathway with 1 mol glucose. In this reaction, 2 mol NADH can generate 2 mol reduced ferredoxin (Fd_{red}) through NFOR catalysis. 2 mol pyruvate catalyzed by the enzyme PFOR can generate catalytic 2 mol reduction ferredoxin (Fd_{red}), 2 mol acetyl CoA, and 2 mol CO_2. According to this reaction, 4 mol of hydrogen catalyzed by the hydrogen enzyme can be generated during the co-generation of 4 mol Fdred. At the same time, 2 mol acetyl CoA catalyzed by the enzyme phosphate acetyltransferase and acetate kinase can successively generate 2 mol of acetic acid. In this reaction, which is called the "Thaure" limit, 1 mol glucose can produce 2 mol of acetic acid, 2 mol CO_2 and 4 mol H_2 (Equation (8.24)). Therefore, the maximum theoretical yield of biohydrogen from glucose fermentation is 4 mol H_2 per mol of consumed glucose. Acetoacetyl CoA generation by part strict anaerobes was 2 mol NADH, which is reduced to butyryl CoA, and then, in turn generates acid butyl catalyzed by phosphate acyltransferase and butyric acid kinases. The maximum theoretical yield for hydrogen becomes 2 H_2 per mol of consumed glucose (Equation (8.25)). In fact, acetic acid and butyric acid are the by-products to most of the strict anaerobes (Equation (8.26)). Under certain conditions, some *Clostridium* also metabolize ethanol (Equation (8.27)), propionic acid (Equation (8.28)), and even butanol, acetone, lactic acid, and the like molecules.

$$C_6H_{12}O_6 + 2H_2O \longrightarrow 2CH_3COOH + 2CO_2 + 4H_2 \quad \Delta G^{0'} = -184\ \text{kJ/mol} \tag{8.24}$$

$$C_6H_{12}O_6 \longrightarrow CH_3CH_2CH_2COOH + 2CO_2 + 2H_2 \quad \Delta G^{0'} = -257\ \text{kJ/mol} \tag{8.25}$$

$$4C_6H_{12}O_6 \longrightarrow 3CH_3CH_2CH_2COOH + 2CH_3COOH + 8CO_2 + 8H_2 \tag{8.26}$$

$$C_6H_{12}O_6 \longrightarrow 2CH_3CH_2OH + 2CO_2 \tag{8.27}$$

$$C_6H_{12}O_6 + 2H_2 \longrightarrow 2CH_3CH_2COOH + 2H_2O \quad \Delta G^{0'} = 357\text{kJ/mol} \tag{8.28}$$

Both the pH and hydrogen partial pressure of bacterial growth and fermentation system will affect the metabolic pathway, thereby affecting the yield of hydrogen. When the growing bacteria need ATP, the substrate-level phosphorylation produces acetic acid or butyric to get the ATP. With the production of acetic acid and butyric acid, the pH of the continuous fermentation system decreases, while the hydrogen partial pressure increases. Meanwhile, in order to avoid further reduction in the pH and the inhibition of cell metabolism, bacteria can produce acetic acid and butyric acid, which change the approach for producing alcohol pathway. In addition, the hydrogen partial pressure increases to contribute to the production of alcohol through hydrogen production pathway, which is not even a hydrogen-consuming metabolic pathway producing acid pathway. Therefore, in applications requiring hydrogen to

lower the hydrogen partial pressure, there is mainly vacuum extraction, which is filled using inert gas law.

3.2.2 Formic Acid Cleavage Pathway

Formic acid cleavage pathway (Figure 8.18) was mainly found in methylotrophic bacteria, facultative anaerobic and aerobic bacteria. Formic acid cleavage pathway enzymes are involved in hydrogen production, and mainly produce pyruvate-formate lyase (Pyruvate formate lyase, PFL) and formic acid-hydrogen lyase (formate hydrogen lyase, FHL). FHL is a membrane-bound multi-enzyme complex system, containing formic acid dehydrogenation enzyme (formate dehydrogenase, FDH)

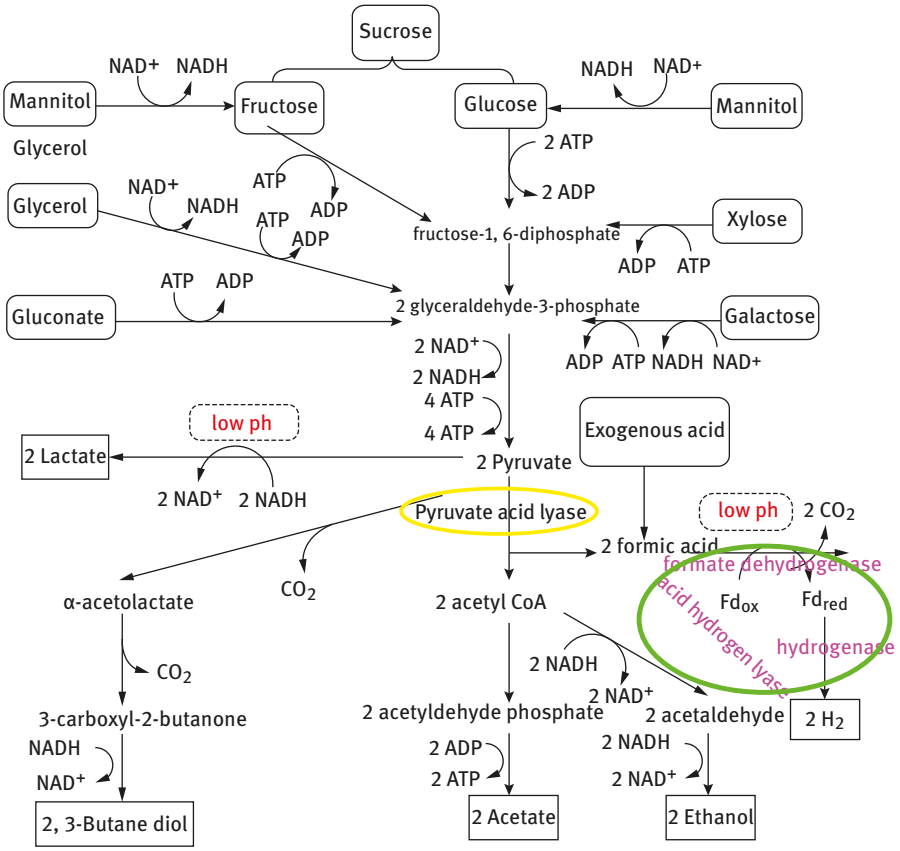

Figure 8.18: Generation of hydrogen using intestinal bacteria through formic acid cleavage pathway.

and hydrogenase, and through an intermediate electron carrier is connected to the two complexes. First, the removal of formic acid dehydrogenase catalyzes CO_2, and electron transfer to Fdox generates Fdred. Under the action of hydrogenase, using Fdred, the proton-electron reduction is used to produce hydrogen, and Fdox regeneration. FDH associated with hydrogen production uses only benzyl viologen (benzyl viologen, BV), while such electronic dyes exhibit activity. Unlike other FDH, this one is capable of reducing the methylene blue (methylene blue, MB). Therefore, usually FDH (BV) and FDH (MB) are used to distinguish between the two different FDH. When there are O_2, NO_3^-, fumaric acid, and the like MB suitable electron acceptor, FDH (BV) catalytic dehydrogenation reaction will be suppressed. Formic acid by the electron transport chain will reduce NO_3^- or fumaric acid to NO_2^- or succinic acid, whereas no electronic hydrogen is used for the reduction of protons. Therefore, to achieve sustained acid cleavage of hydrogen production, it is necessary to avoid the presence of other electron acceptors.

In fact, FHL is a reversible reaction catalyzed ($HCOOH \leftrightarrow H_2 + CO_2$); usually only at high enough concentrations of formic acid conditions, FHL catalytic acid can produce H_2 and CO_2. Whereas under acidic conditions, lactate dehydrogenase enzyme will be activated, so that the lactic glycolysis product, a corresponding reduction in the amount of pyruvate, thereby reduces the yield of hydrogen. In addition, other products of glycolysis (e.g. ethanol, butanediol) will reduce the hydrogen yield. Thus, for the production of hydrogen by microorganisms, formate lyase pathway may increase the yield of hydrogen production by blocking acid, ethanol, butanediol, and other means. It should be noted that formic acid can not only generate through pyruvate degradation catalyzed by PFL , but also can be added directly.

3.2.3 NADH/NAD$^+$ Balance Adjustment for Hydrogen Production

In a strict sense, NADH/NAD$^+$ balance adjustment is not a hydrogen production mechanism, because it does not involve the substrate hydrogen production. In fact, it is a microorganism to achieve redox balance of self-regulatory mechanisms. In glycolysis acid production process, NADH produced by EMP pathway is generally available through metabolic pathways, and produces butyric acid, propionic acid, ethanol or lactic acid, which are the re-oxidized forms of NAD$^+$, NADH, and NAD$^+$ to ensure the balance. However, NADH oxidation process is slower than the forming process. In order to avoid the accumulation of NADH, the biological organism will take other regulatory mechanisms, such as the role of reversible hydrogenase H_2 released, to keep the body of NADH/NAD$^+$ balanced (Figure 8.19). As can be seen from Equation (8.28), acidic conditions contribute to produce hydrogen.

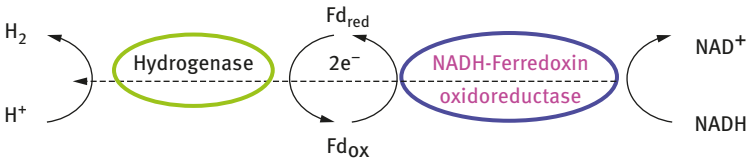

Figure 8.19: Generation of hydrogen with NADH/NAD+ balance adjustment.

3.3 Mixed Bacteria Dark Fermentation for Microbial Hydrogen Production

Anaerobic *Clostridium* has great potential for anaerobic fermentation. They are hydrogen-producing bacteria having the hydrogen yield similar to those of the other types of bacteria, but are strictly anaerobic bacteria. They are extremely sensitive to the presence of oxygen. Even in less than low oxygen concentration they cannot survive. Facultative anaerobes have a certain tolerance to O_2. However, the growth of these bacteria consumes oxygen rapidly, providing strict anaerobic environment, due to which the use of strict anaerobic aerobic bacteria and facultative anaerobic hydrogen production mix is a good choice for large-scale applications. In addition, some organic waste contains a wealth of indigenous microorganisms [46]. If pure culture requires sterilization of raw materials, it increases costs. In such a case, natural anaerobic sludge can be used as inoculum hydrogen mixed culture, which can reduce the running costs.

In natural anaerobic sludge [47, 48], in addition to the presence of hydrogen-producing bacteria, there is no hydrogen production using *Lactobacillus*, hydrogenotrophic methanogens, homoacetogenic bacteria, sulfate-reducing bacteria, and nitrate-reducing bacteria. Some of these bacteria will inhibit the production of hydrogen, while others will consume hydrogen. A previous study found that lactic acid bacteria will always suppress the generation of hydrogen by secreting bacteria, while other bacteria consume H_2 produced from methane, and generate acetic acid, nitrate reduction and sulfate reduction (Figure 8.20). Furthermore, batch fermentation hydrogen, that usually takes inoculum pretreated way to suppress the above-mentioned bacteria, does not produce hydrogen and hydrogen-consuming bacteria. The continuous hydrogen production using anaerobic fermentation is usually kinetically controlled to achieve the production of hydrogen.

3.3.1 Inoculums Pretreatment

According to the biological diversity of bacterial hydrogen production and hydrogen consumption, bacterial inoculum pre-treatment includes the following three methods.

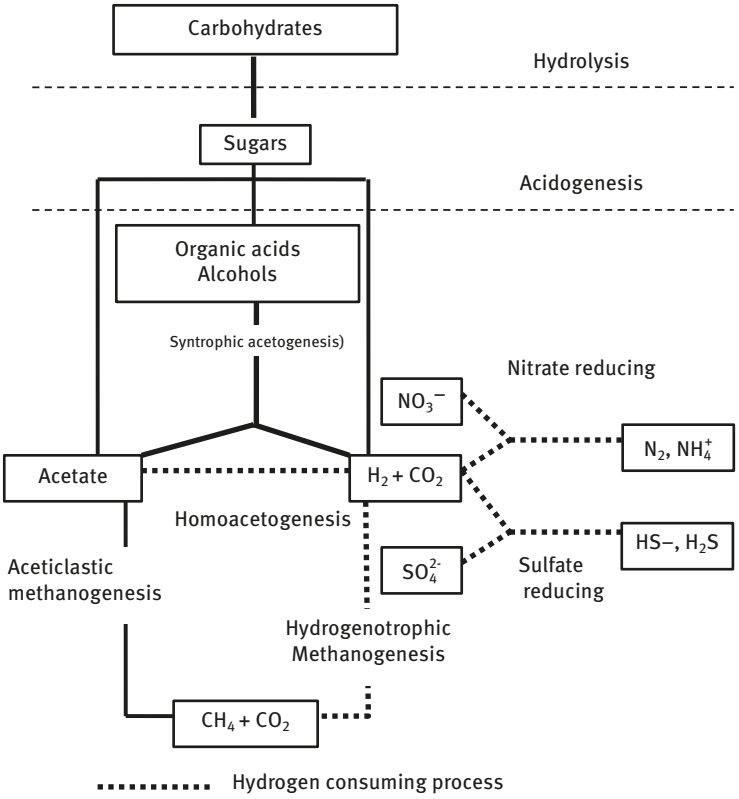

Figure 8.20: Hydrogen metabolism in a mixed culture.

3.3.1.1 Physico-chemical pre-treatment

In the bacterium, *Clostridium* suffers pressure (such as the extreme temperature, acid and alkali conditions, and radiation) [49], due to which it can form spores and go into hibernation and survive. On the contrary, the other bacteria (such as the hydrogen nutrition methanogens) cannot form spores, when subjected to pressure. These bacteria can be inhibited or killed. So usually heating, ultrasound, acids, bases, and freeze-thaw pretreatment are applied [50].

Heat treatment is the most common and an effective pretreatment method, that subjects the inoculums to 70–100 °C and humid conditions over 15 min/dry heat treatment. The treatment can effectively kill bacteria, which do not produce hydrogen and methanogenic archaea. Acid/alkali treatment method is used to retain the inoculum, which was placed under hydrogen-producing bacterial spores under strongly acidic (pH of 3) or alkaline (pH of 10) conditions [51]. Thawing the inoculum at –20 °C or keeping them frozen under the conditions of 10 °C for about 24 h is an effective method. After 30 °C, they are exposed to outer environment.

3.3.1.2 Adding Inhibitors of Methanogenic Archaea

These can inhibit the activity of methanogenic archaea inhibitors and include bromouracil acid (bromoethanesulfonic acid, BESA), acetylene, chloroform, iodopropane, and NO^{3-}. BESA is the methanogenic archaea-specific methyl coenzyme M (CH_3-CoM) analogs, which may hinder methyl coenzyme M generated methane, and are valid for all of the methanogenic archaea. Acetylene can decrease the adenosine triphosphate ATP concentration through methanogenic archaea cells, resulting in a pH gradient inside and outside the cell membrane, which cannot maintain and thus exhibits an inhibitory activity to the methane bacteria. Chloroform can effectively hinder methane production from acetic acid and the reduction of sulfate in the acetyl-CoA decomposition pathway. In addition, Chidthaisong and chloroform [52] may hinder the bacterial affect for hydrogen nutritional homoacetogenic. However, the methanogenic archaea may be resistant to effects of chloroform by acclimation. Kim et al. [53] pointed out that NO and N_2O generated in the denitrification process of NO_3^- can hinder methane production.

3.3.1.3 Aeration Process

Because hydrogen nutrition methanogens are strictly anaerobic bacteria, the use of aeration may inhibit or kill methanogens.

3.3.2 Dynamic Control

Inoculum pretreatment is generally applicable to small-scale batch fermentation. However, it is also applied to large-scale continuous fermentation, especially when dealing with complex organic waste. The raw material itself carries a large number of indigenous bacteria, which consume hydrogen not only for the inoculum process but also for the need to consume large amounts of energy or raw materials pre-treatment chemicals. Therefore, for the practical application of continuous fermentation, a kinetically controlled fermentation system is used to suppress the growth and metabolism of methane-producing bacteria. The so-called kinetic control is mainly under acidic conditions by controlling the anaerobic fermentation system or using shorter HRT to inhibit the growth and reproduction of methanogens.

3.3.2.1 Acidic Coonditions

Methanogenic bacteria usually grow in a narrow pH range (6.5–7.8). While maintaining the high organic loading conditions of the reactor, the accumulation of organic acid

by-product during the hydrogen production will inevitably lead to lower pH. When the pH is below the critical level of tolerance of methanogenic bacteria, they produce methane. Both the H_2 and CO_2 are produced as the major gaseous products. Therefore, low pH (<6.0) for continuous production of hydrogen is very effective. The equilibrium reactions of formic acid/hydrogen lyase catalyzed formic acid and hydrogen are as follows:

$$H_2 + HCO_3^- \longleftrightarrow HCOO^- + H_2O \ \Delta G^{0'} 391 = 1.3 \text{ kJ/mol}$$

Chemical equilibrium equation for this reaction is as follows:

$$\frac{[HCOO^-]}{H_{2aq}} = \frac{KK_{co2}pCO_2K_A[H^+]}{K_{H_2}} \tag{8.29}$$

Henry's constant for H_2, which represents the equilibrium constant, has the value of 1.31 at 35 °C. For the Henry's constant for CO_2, carbonate dissociation constant level is used. It can be seen from the following equation that the ratio of formic acid to hydrogen shows an exponential relationship with the pH. Therefore, acidic conditions are more conducive to producing hydrogen.

$$\frac{[HCOO^-]}{H_{2aq}} = \frac{KK_{co2}pCO_2K_A}{K_{H_2}} \times 10^{pH} \tag{8.30}$$

3.3.2.2 Short Hydraulic Retention Time

Hydraulic retention time (HRT) is defined as the ratio of the volume of feed of the reactor volume per unit time to that of the inverse of dilution rate (D). Only the growth rate is higher than the dilution rate ($\mu_{max} > D$), due to which the microorganisms are capable of staying inside the reactor. Higher dilution rate (or shorter HRT) can make totally "washed out" the methanogens due to the specific growth rate of methanogenic bacteria, which is far less than the growth rate of the hydrogen-producing bacteria.

Section 4: Microbial Hydrogen Production by Carboxydotrophic Bacteria

4.1 Carboxydotrophic Hydrogenogenic Bacteria

CO is a deadly toxic substance to most of the aerobic respiration creatures containing cytochrome. It binds to the cytochrome in the electronic transport chain, competitively inhibits the binding of molecular O_2 and cytochrome, thereby the

electron transfer and oxidative phosphorylation associated with electron transfer are inhibited, due to which the organism is ultimately killed. However, a class of naturally occurring bacteria can make use of not only CO but also of CO_2 and H_2 as the sole carbon, energy, and reducing power sources, respectively. They can fix CO and CO_2 through unique autotrophic Wood–Ljungdahl pathway, while the metabolic process will form CO intermediates, when CO_2 and H_2 are used as substrates. Therefore, such bacteria are categorized as *Carboxydotrophic hydrogenogenic bacteria*. Since these bacteria are able to make use of CO, CO_2, and H_2 as substrates to produce acetic acid, ethanol, butyric acid, butanol, hydrogen, and other important energy and chemical products, breeding and application of those bacteria is becoming a major research focus in the field of biotechnology in recent years.

The *carboxydotrophic bacteria*, which are able to utilize CO as the sole carbon, energy, and reducing power source, are used in the water-gas shift reaction for hydrogen production, which is shown in Equation (8.29). These bacteria are called the *carboxydotrophic hydrogenogenic bacteria*. Some of the *carboxydotrophic hydrogenogenic bacteria* are photosynthetic bacteria (mainly the *purple nonsulfur bacteria*), while some of them are nonphotosynthetic facultative anaerobic or strictly anaerobic bacteria. They can utilize CO as a substrate for cell constructive metabolism under bright or aerobic conditions. However, the water-gas shift reaction can be carried out only in the absence of light or under anaerobic conditions. Most of them can utilize saccharides for hydrogen production.

$$CO + H_2O \longrightarrow CO_2 + H_2 \quad \Delta G_0' = -20\text{kJ/mol} \tag{8.31}$$

Carboxydotrophic hydrogenogenic bacteria belong to *Rhodopseudomonas*, *Rhodospirillum*, *Rubrivivax*, *Citrobacter*, *Carboxydothermus*, *Methanosarcina*, *Carboxydocella*, and *Thermincola*. The carboxydotrophic hydrogenogenic bacteria, which are currently known to scientific community, are presented in Table 8.6. Some of them are strict chemolithoautotrophical, while some of them are photolithoautotrophical. However, most of them are able to grow in organotrophical conditions. All of the carboxydotrophic hydrogenogenic bacteria are not able to utilize CO for the synthesis of organic compounds (ethanol and acetic acid). They can only make use of CO for cell synthesis and carry out water-gas shift reaction for hydrogen production. Reports on carboxydotrophic hydrogenogenic bacteria are focused on *Rhodospirillum rubrum*, *Rhodopseudomonas gelatinosa*, and *Rubrivivax gelatinosus*. Most of these reports are from abroad, whereas the domestic reports are quite rare.

A *Citrobacter* sp.Y19 isolated from the sludge is a strain of facultative anaerobes. It has a faster growth rate under aerobic conditions, but only proceeds the water-gas shift reaction under anaerobic conditions. Its production rate of H_2 is 15 mmol/(L•h),

Table 8.6: Main physiological characteristics of carboxydotrophic hydrogenogenic bacteria.

Bacteria	Optimal growth pH	Optimal growth temperature (°C)	Generation time (h)
Carboxydocella sporoproducens sp.nov.	6.8	60	1
Carboxydocella thermoautotrophica	8.6	60	1.1
Thermincola carboxydiphila strain 2204	8	55	1.3
Thermincola ferriacetica strain Z-0001	7.0–7.2	57–60	/
Thermolithobacter carboxydivorans Strain JW/KA-2	7	70	8.3
Thermosinus carboxydivorans	7.5	50	1.2
Desulfotomaculum carboxydivorans	7	55	1.7
Thermococcus strain AM4	6.8	82	/
Rubrivivax gelatinosus	6.7–6.9	34	6.7
Rhodopseudomonas palustris P4	/	30	23
Rhodospirillum rubrum	6.8	30	8.4
Citrobacter sp. Y19	5.5–7.5	30–40	8.3
Methanosarcina barkeri	7.4	37	65
Moorella strain AMP	6.9	60–65	nr
Carboxydothermus hydrogenoformans	5.5	68	2
Carboxydibrachium pacificus	6.8–7.1	70	7.1

and the hydrogen yield is 1 mol/mol CO. *Rhodospirillum rubrum* has a high absorption rate and a higher CO conversion rate (close to the theoretical conversion rate), but its growth needs light, which is also relatively slow. In addition, the production process of H_2 is susceptible to be inhibited by the substrate CO. When the partial pressure of CO is lower than 0.2 atm, the hydrogen production will be inhibited. *Rubrivivax gelatinosus* utilizes CO as the sole substrate for cell growth under illuminated conditions, while the water-gas shift reaction is mainly carried out in dark. Water-gas shift reaction can also be carried out under illuminated conditions, but the hydrogenase will oxidize H_2 of formation in order to gain the reducing power for photosynthetic CO_2 fixation. The specific hydrogen production rate of CO of *Rubrivivax gelatinosus* is 0.8 mmol/(min·gcell) under the condition of low concentrations (OD660<0.2), high stirring speed (250 r/min), 20% CO contained in the gas phase, and no light. Since the culture conditions of cell growth and H_2 fermentation are different, a two-step process, which cultures the bacteria under illuminated or aerobic conditions for cell growth and under dark and anaerobic conditions for hydrogen production, is recommended.

4.2 Microbial Biosynthesis by Carboxydotrophic Hydrogenogenic Bacterium

According to the definition of Wilhelm Pfeffer, microorganisms which are capable of using carbon dioxide as the carbon source for cell growth are called autotrophic microorganisms. Since CO is also an inorganic carbon source, the microorganisms which use CO as the sole carbon source are also called the autotrophic microorganisms. Such microorganisms contain carbon monoxide dehydrogenation enzyme (CODH), which enables mutual conversion of CO and CO_2, and a series of CO_2 methylated enzymes. Then, the methyl and carbonyl under the use of acetyl CoA synthase can synthesize the precursors of acetyl (CoA). It is called the anaerobic pathway and is also known as the Wood–Ljungdahl pathway (Figure 8.21).

Wood–Ljungdahl pathway is an acyclic way, involves CODH/ACS complex, in which methyl, carbonyl group, and coenzyme A can form acetyl coenzyme A. CODH is a bifunctional enzyme that catalyzes the reversible conversion of CO and CO_2, when the CO_2/H_2 are used as the growth substrates. CODH reduces CO_2 to form CO which acts as the carbonyl source of acetyl CoA. When CO is used as the sole carbon and energy source, some of them directly use carbonyl source of acetyl CoA, while others

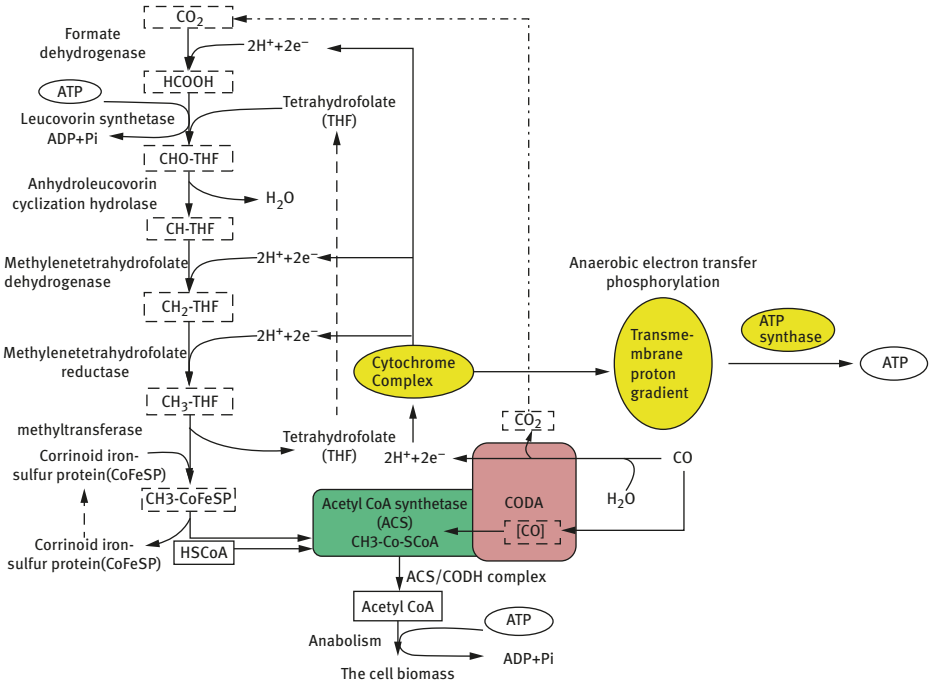

Figure 8.21: Biosynthetic pathway of carboxydotrophic hydrogenogenic bacterium.
Source: Wang et al. (2002) [27].

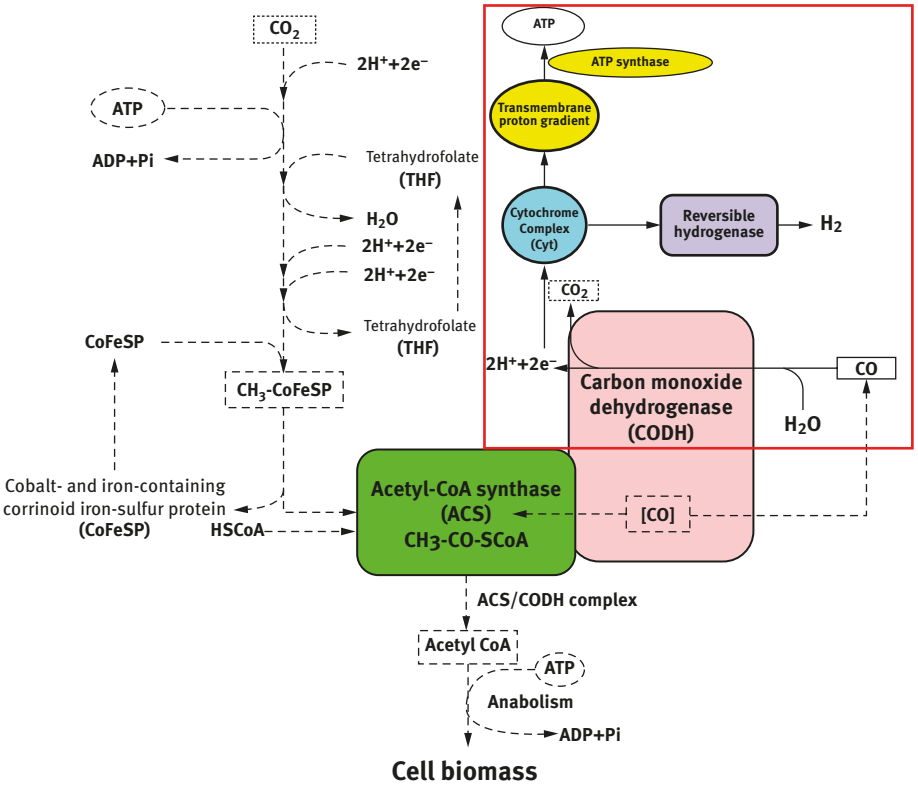

Figure 8.22: Hydrogen production pathway by water-gas shift reaction of carboxydotrophic hydrogenogenic bacteria.

Source: Wang et al. (2002) [27].

are catalyzed by CODH to CO_2 which is used as the methyl source of acetyl-CoA. CO_2 is formed by the formic acid hydrogenase (FDH), while the formation of formic acid group takes place due to the activation of ATP consumption. Finally, leucovorin (CHO-THF), methenyltetrahydrofolate (CH-THF), methylene group tetrahydrofolate (CH_2-THF) form methenyltetrahydrofolate (CH_3–THF). With the assistance of corrinoid iron-sulfur protein (CoFeSP), the methyl groups are transferred to the carbon monoxide dehydrogenation enzyme/acetyl coenzyme A synthetase (CODH / ACS) complex. The complex combines with CO and HSCoA, and eventually forms acetyl CoA [12].

In the Wood–Ljungdahl pathway, ATP, which is used for acid activation and cell forming, has three kinds of ways under different types of microorganism and different conditions. These three ways are as follows.

(1) For anaerobic energy autotrophic microorganisms, ATP is derived from the photosynthetic electron transport phosphorylation under light anaerobic conditions, such as *Rubrivivax gelatinosus*.

(2) For aerobic energy autotrophic microorganisms, ATP is derived from the respiratory electron transport phosphorylation under no light aerobic conditions, such as Citrobacter sp.Y19.
(3) ATP derived from the electron transport phosphorylation is different from that obtained from the photosynthetic or respiratory electron transport under no light anaerobic conditions. The main electron mediator for cytochrome complexes by menaquinone MK-7, cytochrome b554 and b559, and flavin proteins is the cytochrome electron transfer complexes, which are formed simultaneously in the transmembrane proton gradient. The ATP synthase uses the transmembrane proton gradient to produce ATP. This process is called the anaerobic electron transport phosphorylation (Figure 8.22). For example, the *Carboxydothermus hydrogenoformans*.

Methanogens' main function is to produce methane. However, these microbes also have CODH and hydrogenase. Furthermore, *Methanosarcina barkeri* can use water vapor transfer reaction to produce hydrogen when methanogenic pathway is inhibited (such as through the addition of BESA)

4.3 Water-gas Shift Mechanism for Microbial Hydrogen Production by Carboxydotrophic Hydrogenogenic Bacteria

Hydrogen production by water-gas shift reaction of carboxydotrophic hydrogenogenic bacteria is usually carried out under dark anaerobic conditions, as shown in Figure 8.22 (solid line). The reducing power ($2H^{+}+2e^{-}$) of carbon monoxide dehydrogenase in the dark anaerobic conditions cannot be effectively utilized due to the absence of ATP produced by the photosynthetic phosphorylation or oxidative phosphorylation for cell synthesis (dotted line). In order to maintain the intracellular redox balance and to avoid the damage caused by the release of excess electrons, electrons are transferred to reversible hydrogenase by the cytochrome complex, while the reversible hydrogenase releases excess electrons and reducing power through the production of hydrogen. The transfer of electrons by cytochrome complex generates a transmembrane proton gradient, and the ATP synthase uses energy from proton gradient to generate ATP, which is the process of anaerobic electron transport phosphorylation. This is the only source of ATP under dark anaerobic conditions, and its efficiency and productivity rates are very low, due to which it is mainly used to meet the basic cellular metabolic function, and only a small amount is used for cell anabolic, which is the reason for the slow rate of cell growth under dark anaerobic conditions.

Reversible hydrogenation of *Rubrivivax gelatinosus* CBS is connected to CO oxidation pathway directly and has a strong resistance to O_2. Its half-life can reach

up to 20h, when a whole-bacterial cell is placed in culture medium with stirring under an air atmosphere. The bacteria can acquire the theoretical yield of hydrogen under dark conditions, while the accumulation of hydrogen does not cause a product feedback inhibition in the reaction system. Based on the above two advantages, the synthesis gas may contain trace amounts of O_2. *Rubrivivax gelatinosus* CBS is the most ideal bacteria for biohydrogen production from carbon monoxide.

CODH and hydrogenase have optimal pH and temperature. Under alkaline conditions (pH>8), the catalytic activity of CODH is stronger, while the hydrogenase under neutral condition have the higher catalytic activity. This is consistent with their catalytic properties, because CODH release protons in the CO oxidation reaction, whereas hydrogenase consumes protons in the reaction of hydrogen production. CODH has a good thermal stability, and its optimum temperature is close to 50 °C. However, hydrogenase is sensitive to temperature, while the activity of hydrogenase declines or is lost when the temperature rises above 35 °C. Accordingly, the optimum temperature of hydrogen production by water-gas shift reaction should be below 35 °C.

Section 5: Application Prospects of Microbial Hydrogen Production

The biological hydrogen production technology is regarded as the national strategy in some countries. In order to realize the development plan of "clean hydrogen energy" through the establishment of "hydrogen micro algae farms", Canada has built a liquid hydrogen production plant with the production capacity of 10 t/d. Furthermore, Japan has laid more intentions on effective hydrogen production based on microalgae and photosynthetic bacteria. These biological hydrogen production technologies went hand in hand, and with their own advantages and disadvantages of these various microorganism-based hydrogen production methods (Table 8.7), no kind of microbial hydrogen production has been acknowledged as the best way for hydrogen production. In early 90s, under the support of biological hydrogen production research and development plan from Germany, Japan, and the United States, the study of photosynthetic hydrogen production by algae using water has been widely investigated. However, the total solar energy conversion efficiency of the whole process is still very low, while the dark fermentation bacteria and phototrophic bacteria could produce hydrogen from either the low-cost substrates or the organic waste, thus providing the advantages of clean energy production and effective organic waste treatment. Therefore, a couple of long-term research plans in this field have been carried out in some countries around the world in recent years.

Table 8.7: Hydrogen-producing microorganisms and their corresponding advantages and disadvantages.[a, b, c]

Microorganisms	Advantages	Disadvantages	Representative type
Green algae	Water substrate, 10 times higher energy conversion efficiency than plants	Light needed for hydrogen production, high cost of photosynthetic reactor, the inhibitory effect of by-product O_2 on hydrogen production system	*Scenedesmus obliquus Chlamydomonas reinhardtii Chlamydomonas moewusii*
Cyanobacteria	Water substrate, catalytic hydrogen production from nitrogenase	Light needed for hydrogen production, high cost of photosynthetic reactor, irreversible inhibition of by-products O_2 on nitrogenase	*Anabaena wariabilis Nostoc spongiaeforme Westiellopsis prolifica*
Photosynthetic bacteria	Coupling of hydrogen production with waste water treatment, utilization of a wide spectrum of sunlight, without O_2 production	Light needed for hydrogen production, high cost of photosynthetic reactor	*Rhodobacter sphaeroides Rhodopseudomonas capsulata Rhodopseudomonas gelatinosa Rhodospirillum rubrum*
Fermentative bacteria	Coupling of hydrogen production with waste treatment, Extensive utilization of all kinds of carbon sources such as sugar, cellulose, etc. continuously producing hydrogen	Incomplete energy recovery, formation of by-product acetic acid, butyric acid, propionic acid and ethanol, further utilization needed to fermented liquid for the largest extent energy recovery	*Clostridium butyricum Clostridium pasteurianum Enterobacter aerogenes Enterobacter cloacae Acetomicrobium flavidum*
Carboxydotrophic bacteria	Coupling of hydrogen production with waste treatment, indirect utilization of hard-biodegradable waste, such as lignin, etc	Low gas-liquid mass transfer rate, limiting the hydrogen production efficiency	*Rhodospirillum rubrum Rhodopseudomonas gelatinosa Rubrivivax gelatinosus Carboxydothermus hydrogenoformans*

[a] Mohan et al. (2008) [50].

[b] Dong et al. (2009) [54].

[c] Liu (2008) [55].

The research on hydrogen production by light green algae has also been conducted for more than 60 years. The initial direct light hydrolysis hydrogen has transformed into the "two-step" indirect light hydrolysis hydrogen production method, highlighting the journey the research has traversed throughout these years. Due to the most adequate resources from nature (sunlight and water) for hydrogen production and more than 100 times of the corresponding hydrogenase activity, the photosynthetic efficiency of green algae is much higher than that of the cyanobacteria and photosynthetic bacteria.

Therefore, in the assessment report of international energy agency (IEA) in 1988, the "two-step" indirect biological light hydrolysis hydrogen production (based on green algae reversible hydrogenase) was deemed as one of the most applicable methods for hydrogen production. However, the light energy conversion efficiency of the "two-step" method was still low (1%), with a significant difference from the standard of "practical industrial application value of light energy conversion efficiency of 10%".

The study on cyanobacteria hydrogen production has gone through 30 years since the discovery of the phenomenon of hydrogen production by anabaena cylindrical with illumination under the environment of argon in 1974. Cyanobacteria can convert solar energy into hydrogen though direct photocatalytic water splitting. Meanwhile, their growth and nutrient requirements are low, which include CO_2 and N_2 (as carbon and nitrogen sources, respectively), water (electronic and hydrogen donor), simple inorganic salt and light (energy). Hence, cyanobacteria hydrogen production has received great research attention. However, the hydrogen production based on cyanobacteria has not yet reached the requirements of practical application due to the low light energy conversion efficiency, which is still less than 1%.

Photosynthetic bacteria could utilize the organic product from hydrogen production under anaerobic conditions and without nitrogen. Additionally, the hydrogen purity and hydrogen production efficiency are higher than those of the green algae and cyanobacteria. Therefore, since the discovery of photosynthetic bacteria by Gest in 1949, many researches have been performed in Japan, American, Europe, China, and other countries. Attributing to the complexity and precision of the photosynthetic hydrogen release process, the researches are mainly focused on the screening or selection of highly active hydrogen-producing strains, and optimization and control of environmental conditions with the purpose of improving the hydrogen production. The process is conducted either on a laboratory or pilot scale. Among various kinds of hydrogen-producing microorganisms, photosynthetic bacteria have higher substrate conversion efficiency and can be combined with wastewater treatment and use of solar energy. Most people tend to utilize photosynthetic bacteria to produce hydrogen.

Since most of the efforts are focused on anaerobic fermentation hydrogen production from fermentation bacteria, researches on dark fermentation hydrogen production have been continued for 90 years. Compared with photo-hydrogen production, the dark fermentation hydrogen production technology has certain advantages. These advantages are as follows.

(1) Higher hydrogen production rate, which is around 100 times higher than the photosynthetic hydrogen production.
(2) Under anaerobic conditions, production without molecular oxygen and no oxygen inhibition.
(3) Hydrogen production through anaerobic decomposition of organic substrates without any light source.
(4) Continuously stable hydrogen production with simple reactor design, having handy operation and easy management.

(5) A wide range of available organic matter including various kinds of organic wastewater, solid waste, algae, and plants.

Therefore, the method of dark fermentation has shown more potential for hydrogen production among these biological hydrogen production methods. However, the problem of low energy recovery efficiency for the process still persists. Meanwhile, the process is accompanied by the generation of by-product, such as acetic acid, propionic acid, butyric acid, ethanol, and other organic acid or alcohol, which cannot make full use of the organic matter for converting it into hydrogen. To improve the efficiency of energy recovery, the following combination system for further use of organic acid or alcoholic by-products could be employed.

(1) Mixed cultivation on fermentation and photosynthetic bacteria for hydrogen production

    Without the strong ability for the degradation of macromolecules as fermentation bacteria, photosynthetic bacteria can effectively proceed with the photosynthetic hydrogen production over the utilization of small molecular organic acids or the by-product alcohols with the help of light energy and dark fermentation. Hence, the combination of these two microbial cultures is beneficial for the improvement of substrate's full-utilization and hydrogen production efficiency, such as the mixed cultivation of *Rhodobacter sphaeroides* and *Clostridium butyricum* for hydrogen production.

(2) Dark fermentation – photosynthetic fermentation combination for hydrogen production

    Due to the difference in optimal growth and hydrogen production conditions between the fermentation and photosynthetic bacteria, the hydrogen production process of the two kinds of microorganisms could be performed in different reactors, which could optimize the operating conditions of the fermentation systems.

(3) Two phase hydrogen-methane anaerobic fermentation system

    Based on the methanation originating from the organic waste fermentation, the system is actually the maximum energy recovery system for hydrogen and methane production through dynamic (pH and residence time, respectively) control of the conditions of producing hydrogen and methane in dark fermentation. The optimal substrate for the production of hydrogen in dark fermentation is carbohydrates, while the hydrogen production ability of proteins and lipids is extremely low. Therefore, in terms of the organic waste containing above three kinds of materials, the most probable means for hydrogen production is the two-phase hydrogen-methane anaerobic fermentation system, which is based on the utilization of not only the acetic acid, propionic acid, butyric acid, ethanol, and other organic acids or alcohols but also the protein and lipid produced by hydrogen bacteria in the process of methane production.

(4) Dark fermentation hydrogen production – microbial fuel cell combination system

Based on the full utilization of by-products from dark fermentation hydrogen production process (such as acetic acid, and butyric acid) to generate electricity, microbial fuel cells could further improve the efficiency of energy recovery; however, it still remains in the early stages of laboratory research.

The hydrogen-producing bacteria based on the nutrition of carbon monoxide was noticed as one kind of biological hydrogen production pathway until last decade. However, no relevant reports have been found in the domestic literature. Due to its ability of indirectly using biodegradable waste, such as lignin, this kind of hydrogen production pathway has attracted much attention in recent years. However, even though with the huge amounts of waste wood fiber on earth, the corresponding microbial degradation is hard to proceed due to the complex structure of wood fiber. The methods based on the acid/heat and enzyme hydrolysis could accelerate the separation of cellulose and hemicellulose from wood fiber. However, the residual lignin in raw materials cannot be effectively utilized, while the corresponding lignin contents account for about 30–40% of the wood fiber waste materials. Nevertheless, the problem can be solved through the transformation from CO to H_2 by carbon monoxide hydrogen-producing bacteria based on the gasification syngas (mainly including CO, H_2 and CO_2).

The advantages and disadvantages of hydrogen production with various kinds of microorganisms are listed in Table 8.7.

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Xiaoying Kong, Ying Li, Gaixiu Yang

9 Microbial Electricity Production

In early 1910, British botanist Potter discovered that the bacterial culture could produce electric current. Thus he successfully established the first microbial fuel cell (MFC), in which the platinum (Pt) electrode was placed into the inoculum including *Escherichia coli* (*E. coli*) and yeast. Since then, the research on the separation and the mechanism of electric conduction and electron transfer of electricity-generating microbial (electricigen) has not been interrupted. In 1991, after Habermann and Pommer first managed to treat wastewater using bacteria, synchronous technology of wastewater treatment and power generation by MFC has been studied and developed. Therefore, MFC provides an interesting technical platform for scientists to explore extracellular bacteria ecosystem.

Section 1: The Base of Microbial Fuel Cell

MFC is a high-efficiency device that converts chemical energy contained in organic waste to electrical energy using cell or enzyme as the catalyst by a bioelectrochemical approach.

As a power generation platform, the design, construction, and basic research of MFC have made significant progress.

1.1 The Technology Development of MFC

The technology development of the MFC is shown in Figure 9.1, exhibiting that the development of the MFC has passed through several research stages. The first stage involves the development of MFC concept and the second stage includes the breakthrough directions in MFC technology since 2002.

The first research direction: selection of electricigen used in the MFC, including: (1) the direct research result, for example, *Geobacter sulfurreducens* (*G. sulfurreducens*) microbe can be absorbed on the electrode and it stays active for a long time, thereby oxidizing the organic matter with quantitative transfer of produced electrons from one electrode to the other, this phenomenon could effectively enhance the efficiency of the MFC; (2) the choice of microbe for the MFC and the electron transfer mechanism.

Corresponding author: Xiaoying Kong, Email:kongxy@ms.giec.ac.cn
Ying Li, Gaixiu Yang, Guangzhou Institute of Energy Conversion, Chinese Academy of Sciences; CAS Key Laboratory of Renewable Energy; Guangdong Provincial Key Laboratory of New and Renewable Energy Research and Development, Guangzhou 510640, China

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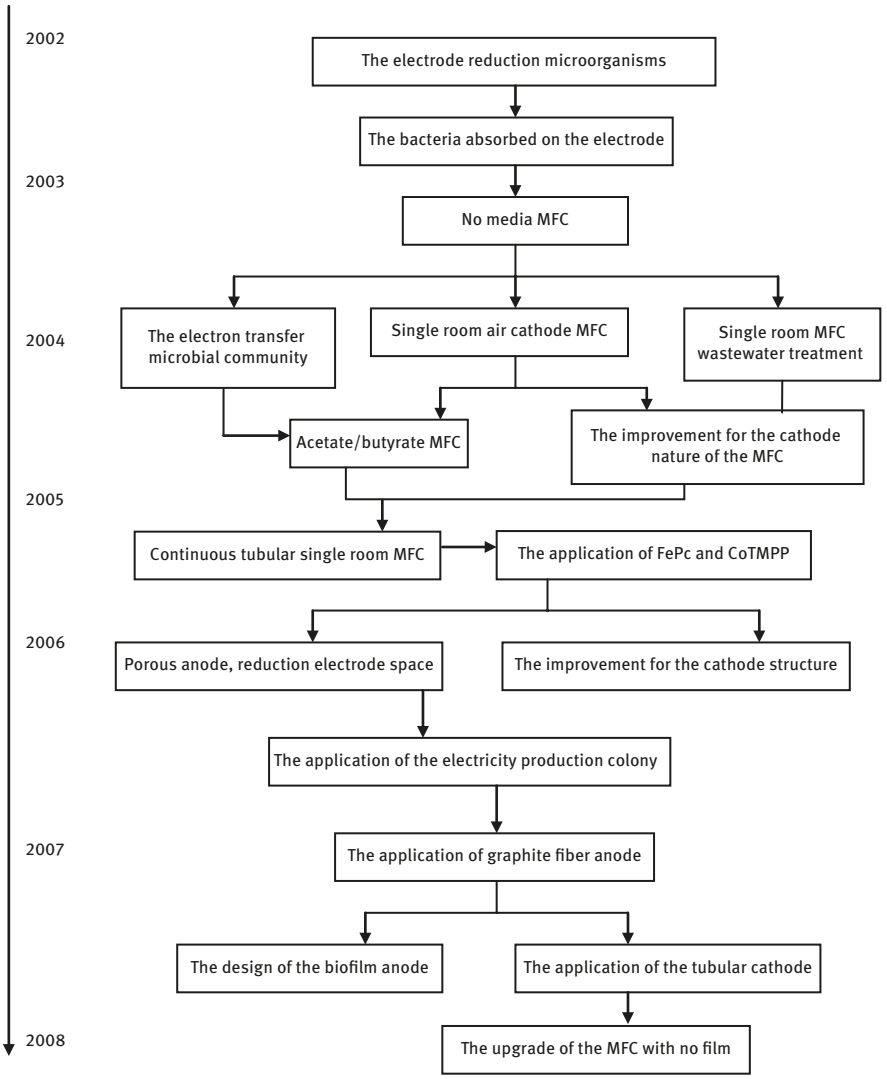

Figure 9.1: The technology development of the microbial fuel cell.

The important achievements include the research about the microbial community with self-adjusting electron transfer, adsorption, and suspended microbes, mainly to judge whether the bacteria community has the ability to output the high energy and is suitable for power generation [1]; and (3) for a summary of the electricity-producing bacteria community and their application in the MFC.

The second research direction: the improvement of the proton exchange membrane (PEM) design is mainly divided into the following two parts: (1) development of the low-cost PEM or the response system with no film; and (2) improvement of the system efficiency for proton transfer.

The third research direction: the material improvement of the cathode and anode and useful exploitation of the electrode space.

The fourth research direction: the cross application of MFC, which mainly includes the wastewater treatment and application of the industrial biotechnology byproducts. The electricigen is the most critical restraining factor in the MFC.

Throughout the development history of the MFC, the developing road is very zigzag. In the early stage, the fermentation products of the microbe, such as methane gas extracted from livestock manure, were used as fuel source in the fuel cell. Since the late 1960s, the researchers combined the process of microbial fermentation and power generation together into MFC. In the 1980s, the widespread application of the redox mediator made MFC more possible as a small power source, which promoted its extensive research and development.

After 2002, scientists found that the bacteria could lead to the direct electron transfer to the solid electron acceptor without the redox mediator, and the bacteria used could directly transfer the electron to the electrode. The MFCs have the advantage of providing stable power for a long time; therefore, they exhibit promising application potential in the special region such as the bottom of the deep sea. However, it has several disadvantages as well, including low fuel efficiency, low electron transfer rate, and numerous side reactions. These are the bottleneck problems of the application of MFC. Thus in the near future, searching for microorganism catalyst with high efficiency will be a hot spot.

1.2 The Working Principle of MFC

The partial principle of MFCs power generation is similar to that of the original battery. The main difference is that the catalyst present in the anode chamber includes the microorganisms or enzymes. Under the catalytic action of microorganism, MFC becomes a device that can transform the chemical energy to electrical energy. In general, the configuration of MFC contains the following two tanks: an anode chamber and a cathode chamber, which are separated by PEM. The anode chamber is anaerobic tank and the cathode chamber is aerobic tank. Following the microbial decomposition of the matrix in the anode chamber of MFC, protons and electrons are released. The electrons reach the cathode through the external circuit, protons start from the anode through the PEM, and then reach the cathode. Eventually, the electrons, protons, and oxygen combine to produce water at the surface of cathode. The principle of MFC and the battery capacity is substantially the same except for a slight difference in the generation and transmission electron paths.

Figure 9.2 shows the use of glucose as fuel source for the biochemical transformation in the MFC and the corresponding flow chart. First, wastewater containing glucose is injected into anode chamber. Subsequently, catalytic action of anaerobic microorganisms occurs, which leads to the decomposition of glucose to carbon dioxide, simultaneously producing hydrogen protons (H^+) and electrons (e^-), as represented by eq.

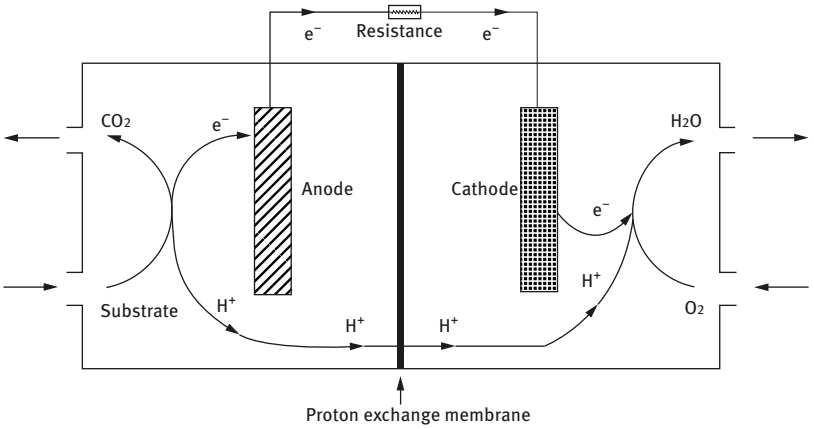

Figure 9.2: The working principle of MFC.

(9.1). The electrons generated during the reaction adhere to the microorganism membrane, and pass to the anode and then to the cathode through the conductor. However, H^+ passes through the PEM to reach the surface of the cathode. Under the action of the Pt catalyst, protons and electrons from the external circuit and an electron acceptor (such as oxygen as an electron acceptor) combine together to form water according to eq. (9.2). This work mechanism can be simply summarized as follows: glucose gets completely converted into carbon dioxide and water, and the process forms a close loop, releasing chemical energy and producing a current and an output voltage.

The overall redox reaction is shown in eq. (9.3):

$$\text{Anodic reaction: } C_6H_{12}O_6 + 6H_2O = 6CO_2 + 24H^+ + 24e^- \quad (9.1)$$

$$\text{Cathodic reaction: } 24H^+ + 24e^- + 6O_2 = 12H_2O \quad (9.2)$$

$$\text{Overall redox reaction: } C_6H_{12}O_6 + 6O_2 = 6CO_2 + 6H_2O + \text{Energy } (-2{,}840 \text{ kJ mol}^{-1}) \quad (9.3)$$

1.3 The Mechanism of Electron Transfer During Microbial Electricity Production

The electrode reaction of original battery is mainly the redox reaction, which has been studied well in electrochemistry. The microbial present in anode chamber of MFC decomposes organic matter and converts the chemical energy of organic matter into electrical energy. Nonetheless, the method to produce electricity from microbia is still the main research of MFC.

With the gradual deepening of research of MFC, scientists have discovered a series of strains with special metabolic processes. They can produce electrons through their own metabolism, and the electrons get directly or indirectly transferred to the outside

of cells, which plays a crucial role in the fuel cells. The electron transferred to the electrode must need a physical delivery system, in order to complete the electronic transfer to the outside of cells. This system can be the soluble electron shuttle body or the membrane-bound electron shuttle complex. So far, the following three methods of the bacteria metabolism involving the transference of the electron to the surface of electrode have been studied: the redox mediator transfer, direct electron transfer, and the nanowire transfer.

1.3.1 Redox Mediator Transfer

Electrons produced by metabolism of microbials such as *E. coli*, *Proteus vulgaris*, *Bacillus subtilis*, and *Klebsiella* are transferred to the electrode surface and the redox mediator. This cell is usually called the indirect MFC. For this approach, the bacteria can create reduced metabolic intermediates; however, the primary metabolic intermediate such as mercury (Hg) and HgS are also required. Schroder used *E. coli* K12 to produce hydrogen, reoxidized the catalytic electrode protected by polyaniline, and immersed it in a bioreactor. The current density of this experiment was as high as 1.5 mA cm^{-2}. Similarly, Straub and Schink employed *Sulfurospirillum deleyianum* to reduce sulfur and sulfide, and then oxidized it to intermediate with stronger oxidizing property than iron. Most of the currently available materials for redox mediators are toxic and easy to decompose, which seriously hampers the commercialization process of this type of MFC.

1.3.2 Direct Electron Transfer

Electrons produced by metabolism can be transferred directly to the surface of the electrode through the cell membrane, and this type of fuel cell is called the direct MFC. This approach is embodied in many bacterial species, such as *Shewanella putrefaciens* (*S. putrefaciens*) and *Pseudomonas aeruginosa* (*P. aeruginosa*). Recent studies have shown that the metabolic intermediate of these microbes usually affects the performance of MFC, and even obstructs the transfer of extracellular electrons. Silencing of the related genes of these metabolic intermediates in *P. aeruginosa* can separately reduce the current to 1/20 of the original. Oxidized metabolic intermediates produced by one bacterium can also be used by other bacteria in the process of electron transfer. Kim used a variety of metabolic inhibitors to establish the electron transport chain of the electrochemically active bacterial population in an MFC and found that the electron transport in an MFC shares an early part of the electron transport chain with aerobic bacteria.

Biological oxidation occurring in the microbial cells, in general, is divided into the following three steps: substrate dehydrogenation, hydrogen transfer, and receive

hydrogen (or electron). The [H] (or electron) produced by substrate under the action of the dehydrogenase needs to be transported to the final electron acceptor through a series of carriers in a specific order (electron transport chain), in order to obtain the energy and maintain the growth at the same time. However, the electrons can be transmitted to the acceptor on the outside of cells during the transfer process of the electricigens, and this special electron transfer mode provides the microorganisms with electrochemical activity.

1.3.3 Nanowires Transfer

Recently, a structure called nanowire was found in the process of extracellular electron transfer. It is a conductive tissue similar to cilia, and exists systematically in the *G. sulfurreducens* PCA, *S. oneidensis* MR-1, *Cyanobacterium synechocystis* PCC6803, and *Pelotomaculum thermopropionicum*. It is the fine wire produced by *Geobacter*, which can be used to transfer electrons. Currently, iron reducing bacteria are always used to research the transfer of microbial extracellular electron. The complete genome sequence of *S. oneidensis* MR-1 and *G. sulfurreducens* has been sequenced and the results indicate the presence of 37 and 100 encoding Cyt C gene [2], respectively. The study found that the two types of outer membrane protein Cyt C of *G. sulfurreducens*, namely OmcS and OmcE, participate in the transfer process of electrons to the electrode. The transcription level of OmcS increases by 19 times, and the level increases with the increase in the current. However, the OmcE can suppress the current produced, when the electrode as electron acceptors of *G. sulfurreducens*. Cytochrome proteins MtrC and OmcA of *S. oneidensis* MR-1 participate in nanowire pathway, and nanowires with gene defect strains are not conductive [3].

Section 2: Electricity-Producing Microorganisms

In recent years, a series of electricity-producing microorganisms including prokaryotic bacteria, algae, and eukaryotic yeast have been isolated by researchers. In the initial study of electricity-generating microbial, some intermediate mediators (neutral red, thionine, etc.) were required and added artificially for assisting the transfer of electrons from electricity-producing bacteria to the electrodes. However, the high cost and the toxic features of these intermediate mediators make electricity production application difficult in practice. Direct production of electricity from bacterium *S. putrefactions* IR-1 was discovered in 1999, and it was a significant turning point in the research of MFCs. It produces electric current in MFCs in the absence of chemical mediators. This strain is exoelectrogenic, indicating that it can transfer electrons outside the cell. From then

on, the researchers began to study the microorganisms directly generating electricity in mediator-less MFCs. Some isolated electrogenic strains are described in this section.

2.1 Classification of Electricity-Producing Microorganisms

Electricity-generating microbes are mostly isolated from the MFCs operating in a stable state. Most of these electricity-generating microbes are rod-shaped, Gram-negative, facultative anaerobe bacteria, and affiliate with the phylum Firmicutes and Acidobacteria. According to the type of terminal electron acceptor of anaerobic respiration, these strains with different functions can be classified into several types. For example, metal-reducing bacteria including the species from the genus *Shewanella*, *Geobacter*, *Geopsychrobacter*, and so on, which utilize Fe(III) or Mn(IV) as the terminal electron acceptor in the electron transport chain; sulfate-reducing bacteria including genus of *Desulfobulbus*, *Desulfovibrio*, and *Desulfuromonas*, which use sulfate as terminal electron acceptor; and nitrate-reducing bacteria including genus of *Pseudomonas*, *Ochrobactrum*, *Comamonas*, which use nitrate as the terminal electron acceptor in the electron transport chain. Moreover, *E. coli*, photosynthetic strain *Rhodopseudomonas* and green algae *Chlamydomonas* and yeast have ability to generate electricity in MFC as well. Specific classification of electricity-producing microorganisms is as follows (Table 9.1).

2.2 Characteristics and Electrochemical Activity of Electricity-Producing Microorganisms

The research on electricity-producing micro organisms is mostly focused on bacteria. The common electricity-producing bacteria in the MFC include some species affiliated with Proteobacteria, metal-reducing bacteria, *Clostridium*, and *E. coli*.

2.2.1 α-Proteobacteria

2.2.1.1 *Rhodopseudomonas palustris*

Rhodopseudomonas palustris (*R. palustris*) of Rhizobiales, Bradyrhizobiaceae, *Rhodopseudomonas* is a Gram-negative, rod- or oval-shaped anaerobic photoautotrophic bacterium and contains chlorophyll *a* or *b*, carotenoids, and no bubbles. It can use simple organic compounds for photosynthesis, molecular hydrogen as an electron

Table 9.1: Classification of electricity-producing microorganisms.

Classification			Genus and Species
Bacteria		α-Proteobacteria	*Rhodopseudomonas palustris*
			Ochrobactrum anthropi
			Rhodobacter sphaeroides
			Acidiphilium cryptum
		β-Proteobacteria	*Rhodoferax ferrireducens*
			Comamonas denitrificans
	Proteobacteria	γ-Proteobacteria	*Aeromonas hydrophila*
			Pseudomonas aeruginosa
			Shewanella putrefactions
			Shewanella oneidensis
			Escherichia coli
		δ-Proteobacteria	*Geobacter sulfurreducens*
			Geobacter metallireducens
			Geopsychrobacter electrodiphilus
			Desulfobulbus propionicus
			Desulfovibrio desulfuricans
			Desulfuromonas asacetoxidans
	Firmicutes		*Clostridium butyricum*
			Clostridium beijerinckii
			Clostridium cellulolyticum
			Thermincola sp. Jr
	Acidobacteria		*Geothrix fermentans*
Fungi			*Hansenula anomala*
Alga			*Chlamydomonas reinhardtii*

donor, and cannot utilize elemental sulfur as an electron donor for photosynthesis. During the process of photosynthesis no oxygen is released. The optimum temperature for growth is in the range of 30–37 °C. The best way to grow is the heterotrophic approach using light energy as organic carbon source.

A strain of *R. palustris* (DX-1) was the first photosynthetic bacterium found to generate electricity at high power densities in low-internal resistance MFC. The maximum power output density of DX-1 in MFC was up to 2,720 mW m^{-2}, which was higher than mixed cultures of microbes in the same conditions. Besides *R. palustris* DX-1 can produce significantly higher power densities, and also can use a wide range of substrates, including acetic acid, lactic acid, ethanol, valeric acid, yeast extract, fumaric

acid, glycerol, formic acid, butyric acid, and propionic acid in MFCs to produce electricity [4]. *R. palustris* has diverse metabolic pathways, broad carbon source, and relatively high electricity production capacity; therefore, it has raised interest in MFCs research community.

2.2.1.2 *Ochrobactrum anthropic*

Ochrobactrum anthropic (*O. anthropic*) strain is a rod-shaped, Gram-negative, non-pigmented, and motile by means of peritrichous flagella, obligate aerobe, possessing a strict respiratory type of metabolism with oxygen as the terminal electron acceptor. The optimum growth temperature is 20–37 °C. Colonies on nutrient agar are colorless, catalase-positive, oxidase-positive, and indole-negative; and cannot hydrolyze esculin, gelatin, and DNA. They are chemoorganotrophic, using various amino acids, organic acids, and carbohydrates as carbon sources and are both oxidase positive and catalase positive. They also reduce both nitrate and nitrite by assimilation.

O. anthropi YZ-1 was isolated by Zuo et al. by the method of diluting anodic liquid of an U-shaped MFC. YZ-1 is a Gram-negative bacterium with polar flagella, generating electricity using acetic acid, lactic acid, propionic acid, butyric acid, glucose, sucrose, cellobiose, glycerol, and ethanol as substrate. Both *O. anthropi* and *R. palustris* are the members of α-Proteobacteria, which use a wide range of substrates and have a variety of metabolic pathways.

2.2.2 β-Proteobacteria

2.2.2.1 *Rhodoferax ferrireducens*

Rhodoferax ferrireducens (*R. ferrireducens*) belonging to Proteobacteria, β-Proteobacteria, Burkholderiales, Comamonadaceae, Rhodoferax is a Gram-negative, facultative rod- or arc-shaped bacterium with a polar mobile flagellum. It is widely present in freshwater or sludge with cell size of 0.6–0.9 × 2–5 μm. The colonies on nutrient agar are light brown. Its optimum growth temperature is 25–30 °C. It can grow at a low temperature of 4 °C; however, the growth is inhibited when the temperature is higher than 37 °C. The pH for the optimal growth is in the range of 6.5 –7.0. *R. ferrireducens* can use glucose, fructose, sucrose, xylose, lactic acid, acetic acid, pyruvic acid, malic acid, succinic acids FeNTA, manganese oxide, fumaric acid, and nitrate as electron donors, while it cannot use carboxylic acid, butyric acid, ethanol, methanol, glycerol, hexanoate, isobutyrate, valerate, butanol, and other simple organic acids, hydrogen, iron citrate, uranium, chromium, sulfur, nitrite, sulfate, sulfite, and thiosulfate as electron acceptors. It is able to convert glucose to CO_2 for extracting energy to support growth through transporting electron to Fe^{3+}. The bacteria are

the first reported strain with the ability to generate electricity directly from oxidation of glucose in MFC. However, the electron donors of the most other iron-reducing bacteria can just use simple organic acids as electron donors. Oxidation of glucose exhibits an 81% coulombic efficiency in MFC [5]. This organism can utilize wide range of substrates with a higher efficiency, thus the current research is focused on developing the use of *R. ferrireducens* as a practical possibility in MFC.

2.2.2.2 *Comamonas denitrificans*

Comamonas denitrificans (*C. denitrificans*) belonging to bacteria, Proteobacteria, β-Proteobacteria, Burkholderiales, Comamonadaceae, and Comamonas is Gram-negative, facultative anaerobic, rod shaped, and mobile by means of polar flagella. Its colonies are white colored. The optimum growth temperature is 37 °C and the optimum pH is 7.5. It is capable of reducing nitrate to nitrogen, and utilizing fumaric acid, propionic acid, pyruvic acid, lactic acid, and acetic acid. The bacterium is a new species of the genus Comamonas reported in 2010, and is able to reduce nitrite and nitrate existing in wastewater to harmless nitrogen, which decreases the hazards posed by nitrite.

Strain *C. denitrificans* DX-4 was isolated by researchers from University of Pennsylvania based on dilution-to-extinction of biofilms on anode. Its performance of electricity production was investigated with strains *C. denitrificans* 110 as control. The results showed that both the strains DX-4 and 110 were able to produce electricity in the MFC with two chambers using $[Fe(CN)_6]$ as cathode catalyst, exhibiting almost similar maximum power density of 36 and 35 mW m^{-2}, respectively. However, two strains were not capable of producing electricity in single chamber MFC using air as the cathode electron acceptor. This might be attributed to the fact that the oxygen diffusing through cathode layer of MFC changed the metabolic pathway of strain DX-4. Moreover, nitrate was added to the anode chamber of the two-chamber MFC, which led to a decrease in the power output. This result revealed that the metabolism of denitrification is not associated with electricity producing process. The presence of denitrifying bacteria in electricity-producing community can help to maintain anaerobic environment in MFC in case of existence of nitrates.

2.2.3 γ-Proteobacteria

2.2.3.1 *Shewanella*

Several strains from genus of *Shewanella* such as *S. putrefactions* IR-1, *S. oneidensis* DSP10, and *S. oneidensis* MR-1 have been found capable of producing electricity in MFC. They all belong to the bacteria, Proteobacteria, γ-Proteobacteria, Alteromonadales,

and Shewanellaceae. Cells are straight or curved rod, Gram-negative, nonpigmented, mobile, and facultative anaerobic with the ability to reduce iron. Under aerobic conditions, they can completely oxidize both pyruvic acid and lactic acid to CO_2. Under anaerobic conditions, they use lactic acid, formic acid, pyruvic acid, amino acid, and hydrogen as an electron donor.

Cell size of *S. putrefactions* is 0.5–1.0 × 1.5–2.0 μm. On solid media, the colonies are round, opaque, yellowish brown, or pale pink. It is mesophilic strain, can grow at temperature in the range of 10–40 °C. The optimum growth temperature is 30–35 °C, capable of utilizing glucose producing acids rather than gas. It can use maltose, sucrose, and cellobiose as well. *S. putrefactions* IR-1 was isolated from the paddy soil by Kim, which is the first reported directly electricity-producing bacteria. It can transfer electrons to the electrode surface [6,7].

Cell size of *S. oneidensis* is 0.5–0.6 × 2–3 μm, and it is facultative anaerobic, iron-reducing bacterium. Its colonies on solid media are round and smooth and reddish-brown with rounded edges. They grow at the temperature of 4–40 °C, and the optimum growth temperature is 30 °C. Manganese oxides and sulfur are reduced to obtain energy to maintain growth. It can use glucose to produce simple acids. It is able to use lactose, galactose, succinate, fructose, glycerol, lactic acid, and malic acid.

S. oneidensis DSP10 is the first reported bacterium with the ability to produce electricity under aerobic conditions. Ringeisen et al. [8] and Biffinger [9] successively investigated the performance of DSP10 under aerobic conditions in mini-MFC. The results showed that *S. oneidensis* DSP10 was capable of oxidizing lactate to CO_2, thus producing electricity with the maximum power density of 500 W m^{-3}. *S. oneidensis* DSP10'll can also use glucose, fructose, and ascorbic acid (vitamin C) as an electron donor to generate electricity. When fructose is used as an electron donor, the highest power density of 350 W m^{-3} is obtained. These electricity-producing aerobic bacteria significantly broaden the range of substrates of MFC.

S. oneidensis MR-1 is capable of reducing Fe(III) and Mn(IV), and is the main type of strain for studying the electron transfer mechanism of bacteria during the process of electricity production. Its genome sequence has been obtained. The mutations of *S. oneidensis* MR-1 are often used to investigate which enzymes are related to extracellular electron transfer. It has been found that *S. oneidensis* MR-1 has about 37 genes coding of Cyt C [2], which is considered to be the transmembrane channel of electron transfer [10].

2.2.3.2 *Aeromonas hydrophila*

Aeromonas hydrophila (*A. hydrophila*) belongs to Proteobacteria, γ-Proteobacteria, Aeromonadales, Aeromonadaceae, Aeromonas. It is Gram-negative, rod-shaped bacterium with size of 0.5–0.7 × 2.3–2.5 μm. Colonies on nutrient agar are buttery

round projections with neat edges. The optimum growth temperature is 22–28 °C. It can be grown at 37 °C. *A. hydrophila* are capable of using glucose and other sugars for producing acids and gas. They can reduce nitrate. The vast majority of species can ferment maltose, D-galactose, and trehalose, and they grow in fresh water and sewage.

Strain *A. hydrophila* PA3 was isolated from the mediator-less MFC fed with artificial wastewater containing acetate by Pham et al. It has been proved that it can be made electrochemically active by the method of cyclic voltammetry. Strain *A. hydrophila* PA3 produced low current using yeast extract as carbon source. Moreover, *A. hydrophila* is widely considered as a major fish and amphibian pathogen, thus it seems to be inappropriate for application in the MFC [11].

2.2.3.3 *Pseudomonas aeruginosa*

P. aeruginosa belongs to Proteobacteria, γ-Proteobacteria, Pseudomonadales, Pseudomonadaceae. It is Gram-negative and facultative aerobic bacteria. *P. aeruginosa* is the first reported microorganism which is capable of producing electricity with mediator shuttling. Rabaey et al. [1] found that *P. aeruginosa* separated from MFC produced pyocyanin which was used as mediator for itself or other bacteria to transfer electrons to the electrodes. This was a new discovery related to the electron transfer mechanism of electricity-producing bacteria. Pyocyanin is as toxic as other artificially added electron transfer mediators; therefore, *P. aeruginosa* is not considered to be an ideal electricity-producing bacterium for MFC, although its pure bacterium and mixed bacteria in MFC exhibit a good electricity-producing performance.

2.2.4 δ-Proteobacteria

2.2.4.1 *Geobacter sulfurreducens*

G. sulfurreducens belong to bacteria, Proteobacteria, δ-Proteobacteria, Desulfuromonadales, Geobacteraceae, Geobacter. It is Gram-negative and obligate anaerobic bacterium. It can use less as electron donors for anaerobic respiration, and only acetic acid and hydrogen. Fe(III), S, Co-EDTA, fumaric acid, and malic acid are used as electron acceptors for anaerobic respiration.

G. sulfurreducens is the first reported strain with the ability to completely oxidize electron donors using Fe(III) as the terminal electron acceptor for anaerobic respiration under anaerobic conditions. It can attach to electrodes and remain viable for long periods, while completely oxidizing organic substrates with quantitative

transfer of electrons to an electrode. French researcher Dumas prepared the anode biofilm and cathode biofilm covered with *G. sulfurreducens* with stainless steel as the sole electron acceptor (acetic acid as electron donor) and electron donor (fumaric acid as electron acceptor), respectively. They investigated the electrochemical activity of the biofilm by cyclic voltammetry, which confirmed the presence of electrochemical activity in the biofilm with the maximum current output density 2.4 and 24.2 A m^{-2}.

2.2.4.2 *Geopsychrobacter electrodiphilus*

Geopsychrobacter electrodiphilus (*G. electrodiphilus*) belonging to Proteobacteria, δ-Proteobacteria, Desulfuromonadales, Geobacteraceae, Geopsychrobacter is Gram-negative strain. Its optimum growth temperature is 22 °C, and it even survives at low temperature of 4 °C. *G. electrodiphilus* can reduce a variety of soluble and insoluble form of Fe(III), and oxidize organic acids, amino acids, long-chain fatty acids, and aromatic compounds using soluble Fe(III) as the electron acceptor. It is capable of completely oxidizing several acids in MFC for electricity generation, including acetic acid, malic acid, fumaric acid, and citric acid. It offers the advantage of growth at low-temperature environment; therefore, it is considered to be more suitable for marine sediment MFC (SMFC).

2.2.4.3 *Desulfobulbus propionicus*

Desulfobulbus propionicus (*D. propionicus*) belongs to Proteobacteria, δ-Proteobacteria, Desulfobacterales, Desulfobulbaceae, Desulfobulbus. *D. propionicus* is Gram-negative, rod-shaped bacterium. The optimum growth temperature is 39 °C. This strain is capable of using hydrogen, ethanol, lactic acid, and pyruvic as an electron donor or a carbon source for anaerobic respiration. It can also use sulfate, sulfite, thiosulfate, nitrate, and Fe(III) as electron acceptor and obtain the energy for growth by reducing the insoluble Fe(III).The main application of *D. propionicus* is in marine SMFC. It is able to produce electricity using lactic acid, propionic acid, pyruvic acid, or hydrogen as an electron donor. However, the electronics recovery efficiency MFC derived from *D. propionicus* is low, and it cannot utilize acetate as an electron donor.

2.2.4.4 *Desulfuromonas asacetoxidans*

D. asacetoxidans (*D. asacetoxidans*) belonging to Desulfuromonadales, Desulfuromonadaceae, *Desulfuromonas* is capable of using ethanol, propanol, butanol, and

pyruvate as an electron donor to reduce the Fe. It can obtain energy for growth by using sulfur, L-malic acid, and fumaric acid as an electron acceptor to convert sulfur to hydrogen sulfide.

The ability of electricity production of *D. asacetoxidans* was found in SMFC. Bond et al. [12] simulated an SMFC in laboratory. They found *D. asacetoxidans* after analyzing the microbial community attached on the graphite anodes buried in the sediment, and then inoculated it into two-chamber MFC. *D. asacetoxidans* produce a current of about 0.3 mA with sodium acetate as carbon source.

2.2.4.5 *Desulfovibrio desulfuricans*

Desulfovibrio desulfuricans (*D. desulfuricans)* belonging to Proteobacteria, δ-Proteobacteria, Desulfovibrionales, Desulfovibrionaceae, Desulfovibrio, is a gram-negative, rod-shaped, spiral or curved, facultative anaerobic bacterium, which is mobile with flagella. The optimum growth temperature is 25–35 °C, and the maximum temperature is up to 44 °C. *D. desulfuricans* is a sulfate-reducing bacterium, which reduces sulfuric acid, sulfur, nitrate, and nitrite to hydrogen sulfide. It is capable of reducing toxic metals, such as uranium(VI), chromium(VI), and iron(III). *D. desulfuricans* can be found in soil, freshwater, brackish, and animal intestines. These species are commonly used in desulfurization process of coal and oil.

D. desulfuricans bacterium exhibits the ability to combine together the sul fae-re ducing and electricity-generation processes on the anode of MFC. This approach is based on an in situ anodic oxidative depletion of sulfide produced by *D. desulfuricans*. Sulfate was removed from a bacterial suspension, which represented 99% removal. A maximum power density of 0.51 mW cm^{-2} was obtained with a one-chamber, air breathing cathode, and continuous-flow MFC operated in batch mode at 22 °C [13]. The in situ oxidation of sulfide and the microbial product of sulfate metabolism, in an MFC anode chamber have high potential for sulfate removal from wastewaters compared to traditional methods, where sulfate bioreduction pathways are inhibited by the sulfide end product, the accumulation of which led to an almost complete loss of bacterial sulfate reduction activity.

2.2.5 Firmicutes and Acidobacteria

2.2.5.1 *Clostridium*

Clostridium belonging to Firmicutes, Clostridia, Clostridiales, Clostridiaceae are Gram-positive, obligate anaerobes capable of producing endospores. Individual cells are rod-shaped. These species are present extensively in soils and also in microbiota of humans and animals. Most of the clostridia can hydrolyze carbohydrates and protein

and they can also produce toxin. *C. butyricum* is the typical strain of the genus. Three species from genus of Clostridium are capable of generating electricity. These species include *C. butyricum* and *C. beijerinckii* with the ability to degrade starch, and *C. cellulolyticum* capable of utilizing cellulose.

The growth temperature of *C. butyricum* falls in the range of 15–42 °C, with its optimum at 37 °C and pH required for growth ranges from 5.5 to 7.4, with the optimum pH from 6.8 to 7.4. They can use cellobiose, fructose, glycerol, galactose, lactose, maltose, raffinose, ribose, starch, sucrose and trehalose, but cannot use mannitol, melibiose, rhamnose, and sorbitol. *C. butyricum* is capable of oxidizing glucose or acts as combining reduction of $Fe(OH)_3$ and oxidation of glucose producing lactic acid, butyric acid, formic acid, acetic acid, CO_2, and H_2.

C. beijerinckii are obligate anaerobic microbes, capable of using a variety of pentoses, hexoses, and starch, with the optimum growth temperature of 37 °C. They are mostly applied in butanol, acetone, or isopropanol production industry.

C. cellulolyticum are capable of fermenting various carbohydrates, such as cellobiose, glucose, fructose, xylose, and cellulose. The main products of the fermentation of cellulose are CO_2, H_2, ethanol, acetic acid, lactic acid, and formic acid. The growth temperature of *C. cellulolyticum* is in the range of 25–45 °C, and the optimum growth temperature is 32–35 °C.

C. butyricum EG3 is the first reported Gram-positive, motile, and rod-shaped strain capable of generating electricity. Park et al. confirmed the electrochemical activity of *C. butyricum* EG3 by the cyclic voltammetry method. It was inoculated into MFC using glucose as the electron donor. The highest current of 0.22 mA was observed at 10 h after the inoculation, which dropped rapidly to around 0.08 mA at the beginning of the exponential growth phase. The current did not decrease after the glucose was completely consumed [14].

Niessen et al. [15] investigated the performance of several strains from genus *Clostridium*. They demonstrated that by combining specially designed anodes, consisting of Pt covered with poly(tetrafluoroaniline) and living cells of the biocatalyst *C. butyricum* or *C. beijerinckii*, electricity can be generated from a variety of substrates, including starch, one of the major biomass constituents. Current densities were between 1 and 1.3 mA cm^{-2}. *C. cellulolyticum* generated a lower current density of 0.13 mA cm^{-2} using cellulose.

Noteworthy, there are two significantly important features of the species of *Clostridium* that need significant attention. First, they are Gram-positive bacteria, which make them different from other electricity-producing bacteria. The mechanism of electron transfer between Gram-positive bacterial cells and the electrodes demands further systematic explorations. Second, the genus of these species can degrade a variety of complex organic compounds to generate electricity, which provides a broad prospect in starch wastewater and other organic wastewater treatment and cellulose degradation fields.

2.2.5.2 *Thermincola* sp.

Thermincola sp. Jr belonging to bacteria, Firmicutes, Clostridia, Clostridiales, Peptococcaceae is a Gram-positive anaerobic thermophilic bacterium. It was isolated from thermophilic H-cell MFC operated at a high temperature of 55 °C by US researchers. In pure culture electricity producing experiments, Jr produced current of 0.42 mA with sodium acetate as carbon source, and Coulombic efficiency was 91% [16]. The study reported the domestication of thermophilic electricity-producing microbial communities and isolated direct electricity-generating strains. High temperatures can reduce the amount of dissolved oxygen in the MFC which helps maintain anaerobic conditions. Moreover, high-temperature conditions can also inhibit the growth of pathogenic bacteria.

2.2.5.3 *Geothrix fermentans*

Geothrix fermentans (*G. fermentans*) belonging to Fibrobacteres, Holophagae, Holophagales, Holophagaceae, Geoghrix is a rod-shaped, Gram-negative anaerobic bacterium, capable of using Fe(III) as the electron acceptor and a variety of simple organic molecules such as acetic acid, lactic acid, pyruvic acid, and fumaric acid as electron donor to maintain growth. It can ferment citric acid and fumaric acid in the absence of an electron acceptor. Fermentation products are acetic acid and citric acid.

G. fermentans are obligate anaerobic microbes isolated from contaminated aquifer under the Fe(III) reduction environment, capable of using Fe(III) as the sole electron acceptor to complete the oxidation of palmitic acid and a variety of simple organic acids, such as succinic acid, malic acid, lactic acid, and propionic acid. The electron recovery was up to 94% when it generated current with acetic acid as electron donor; however, the current output was relatively low.

2.2.6 Fungi and Algae

2.2.6.1 *Hansenula anomala*

Hansenula anomala (*H. anomala*) belongs to fungi, Ascomycetes, Endomycetales, Mucoraceae. Cells are spherical, oval, or cylindrical, often forming false hyphae, hat-shaped or spherical spores, and homogeneous colonies. The optimum temperature for growth ranges from 25–28 °C. It is capable of using glucose, maltose, sucrose, raffinose, and soluble starch-synthesized vitamins.

H. anomala is first reported yeast capable of generating electricity directly, and without intermediate mediator which transfers electrons to the electrode. Prasad et al.

demonstrated the direct electron transfer by *H. anomala* using the electrochemical technique cyclic voltammetry by immobilizing the microorganisms by two different methods viz., physical adsorption and covalent linkage [17]. Furthermore, they also analyzed the redox enzymes present in the outer membrane of the microorganisms. They indicated that yeast cells with redox enzymes present in their outer membrane were capable of communicating directly with the electrode surface and contributed to current generation in mediatorless biofuel cells. The efficiency of current generation was evaluated using three anode materials, which were 0.69, 2.34, and 2.9 W m^{-3}, respectively. Compared to other electricity-producing bacteria, electricity produced by yeast is still relatively low, needing further research to improve its ability of electricity generation.

2.2.6.2 Algae *Chlamydomonas reinhardtii*

Chlamydomonas reinhardtii (*C. reinhardtii*) belong to the green algae, Chlorophyta, Chlorophyceae, Chlamydomonadales, Chlamydomonadaceae, *Chlamydomonas*. Cells are spherical or oval, with flagellum. *C. reinhardtii* is model strain in laboratory as it grows very rapidly. Its growth generation time is just 5 h. *C. reinhardtii* algae are widely distributed in the natural environment, such as fresh water and moist soil, capable of using acetic acid, and growing in complete darkness condition.

Rosenbaum et al. [18] employed living solar cells of the green alga *C. reinhardtii* to demonstrate the possibility of direct electricity generation from microbial photosynthetic activity, which was similar to MFC. Sealed and illuminated glass jar was used as the reaction vessel with a working electrode coated with catalytic polymer, graphite rod auxiliary electrode, and Ag/AgCl reference electrode inside. The PEM spaced graphite electrode from chlorella liquid. To measure the electricity generation, a permanent potential of 0.2 V was applied to the working electrode, the cell began to produce electric current after running for 30 h, and the current increased rather quickly to a maximum value of 9 mA. Thus the concept was based on process of producing electricity based on an in situ oxidative depletion of hydrogen, photosynthetically produced by *C. reinhardtii* under sulfur-deprived conditions, by polymer-coated electrocatalytic electrodes.

Section 3: Isolation and Culture of Electricity-Producing Microorganisms

Theoretically, all microorganisms can produce electricity; however, both the experimental and theoretical studies indicate that the main electricity-producing microorganisms (bacteria) are the Fe-reducing bacteria and photosynthetic bacteria. In this research, the corresponding microfloras enriched in the selected medium were the subject of study. Cyclic voltammetry was used to determine the electrochemical

activity and electricity-producing capacity of these bacteria. Subsequently, a single strain of electricity-producing bacteria was isolated and applied in MFCs, in order to enhance the conversion efficiency of the wastes in the MFCs and improve their electricity-producing capacity.

3.1 Medium for Electrogenesis Microorganism

Electrogenesis microorganisms are mostly anaerobic or facultative anaerobic bacteria. In general, they use simple organic acids or sugars as carbon source, with the sludge, wastewater etc. being employed as inoculum. Moreover, the carbon source can also be adopted according to the utilization such as cellulose, starch, and so on. Special electrogenesis bacterium can be enriched by controlling temperature, pH, or inoculum concentration.

Culture medium with sodium acetate as carbon source was used and the composition of medium was (1 L): NaAc (1 g), NH_4Cl (0.31 g), KCl (0.13 g); trace elements solution (10 mL), with the composition as follows: nitriloacetate (1.5 g) (500 mL distilled water, pH 6.5 adjusted by KOH), $MgSO_4 \cdot 7H_2O$ (3 g), $MnSO_4 \cdot H_2O$ (0.5 g), NaCl (1 g), $FeSO_4 \cdot 7H_2O$ (0.1 g), $CoCl_2 \cdot 6H_2O$ (0.1 g), $CaCl_2 \cdot 2H_2O$ (0.1 g), $ZnSO_4 \cdot 7H_2O$ (0.1 g), $CuSO_4 \cdot 5H_2O$ (0.01 g), $AlK(SO_4)_2 \cdot 12H_2O$ (0.01 g), H_3BO_3 (0.01 g), $Na_2MoO_4 \cdot 2H_2O$ (0.01 g), $NiSO_4 \cdot 6H_2O$ (0.03 g), Na_2SeO_3 (0.02 g), and $Na_2WO_4 \cdot 2H_2O$ (0.02 g).

Composition of culture medium with glucose as carbon source: NH_4Cl (0.5 g L^{-1}), KH_2PO_4 (0.25 g L^{-1}), K_2HPO_4 (0.25 g L^{-1}), $MgCl_2$ (0.3 g L^{-1}), $CoCl_2$ (25 mg L^{-1}), $ZnCl_2$ (11.5 mg L^{-1}), $CuCl_2$ (10.5 mg L^{-1}), $CaCl_2$ (5 mg L^{-1}), $MnCl_2$ (5 mg L^{-1}), and glucose (3 g L^{-1}, pH 5.5).

Composition for culture medium for Fe-reducing bacteria (1 L): $NaHCO_3$ (2.5 g), KCl (0.1 g), NH_4Cl (1.5 g), NaH_2PO_4 (0.6 g), NaAc (or sodium lactate, sodium citrate) (20 mM), yeast extract (0.5 g); trace elements solution (10 mL): nitriloacetate (1.5 g), (500 mL distilled water, pH 6.5 adjusted by KOH), $MgSO_4 \cdot 7H_2O$ (3 g), $MnSO_4 \cdot H_2O$ (0.5 g), NaCl (1 g), $FeSO_4 \cdot 7H_2O$ (0.1 g), $CoCl_2 \cdot 6H_2O$ (0.1 g), $CaCl_2 \cdot 2H_2O$ (0.1 g), $ZnSO_4 \cdot 7H_2O$ (0.1 g), $CuSO_4 \cdot 5H_2O$ (0.01 g), $AlK(SO_4)_2 \cdot 12H_2O$ (0.01 g), H_3BO_3 (0.01 g), $Na_2MoO_4 \cdot 2H_2O$ (0.01 g), $NiSO_4 \cdot 6H_2O$ (0.03 g), Na_2SeO_3 (0.02 g), $Na_2WO_4 \cdot 2H_2O$ (0.02 g), ferric citrate (20 mM), agar (20 g, pH 6.7). A 100 mL anaerobic bottle was radiosterilized at temperature below 115 °C for 30 min.

3.2 Isolation of Electricity-Producing Microorganisms

Hungate roll tube can be used to separate the obligatory anaerobic electricity-producing bacteria. Plate screening technique or streak plate method in an anaerobic

chamber is used to isolate facultative anaerobic electricity-producing bacteria. The process of obtaining only one type or a particular strain of microorganism from mixed microbial populations is called isolation and purification of microorganisms. The commonly used purification methods to achieve this objective are single cell picking method, streak plate method, and dilution-pour plate technique. In this study, dilution-pour plate method and streak plate method were used. The basic principle of dilution-pour plate method involves the initial dilution of the multi-species bacterial suspension, which is followed by inoculation into the medium-coated plates. Subsequently, the cultivation is conducted under particular conditions, aiming to promote the growth of different types of bacteria to form their own colonies on their own substrates. Thus, single strain colonies are formed by the aggregation of single-cell proliferation.

Composition of culture medium with sodium acetate as carbon source adopting hungate roll tube method (L): sodium acetate (1 g) as electron donor, ferric citrate (5 mmol) as electron acceptor, NH_4Cl (0.31 g), KCl (0.13 g); trace elements solution (10 mL): $MgSO_4.7H_2O$ (3 g), $MnSO_4.H_2O$ (0.5 g), NaCl (1 g), $FeSO_4.7H_2O$ (0.1 g), $CoCl_2.6H_2O$ (0.1 g), $CaCl_2.2H_2O$ (0.1 g), $ZnSO_4.7H_2O$ (0.1 g), $CuSO_4.5H_2O$ (0.01 g), $AlK(SO_4)_2.12H_2O$ (0.01 g), H_3BO_3 (0.01 g), $Na_2MoO_4.2H_2O$ (0.01 g), $NiSO_4.6H_2O$ (0.03 g), Na_2SeO_3 (0.02 g), and $Na_2WO_4.2H_2O$ (0.02 g).

Culture medium with glucose as carbon source adopting plate screening method: (L): glucose (3 mmol) as electron donor, ferric citrate (5 mmol) as electron acceptor, Na_2HPO_4 (4.0896 g), NaH_2PO_4 (2.544 g), NH_4Cl (0.31 g), KCl (0.13 g), and a small amount of vitamins and trace elements ($MgSO_4 \cdot 7H_2O$ (3 g), $MnSO_4 \cdot H_2O$ (0.5 g), NaCl (1 g), $FeSO_4 \cdot 7H_2O$ (0.1 g), $CoCl_2 \cdot 6H_2O$ (0.1 g), CaCl2 · $2H_2O$ (0.1 g), $ZnSO_4 \cdot 7H_2O$ (0.1 g), $CuSO_4 \cdot 5H_2O$ (0.01 g), $AlK(SO_4)_2 \cdot 12H_2O$ (0.01 g), H_3BO_3 (0.01 g), $Na_2MoO_4 \cdot 2H_2O$ (0.01 g), $NiSO_4 \cdot 6H_2O$ (0.03 g), Na_2SeO_3 (0.02 g), and $Na_2WO_4 \cdot 2H_2O$ (0.02 g)), 1.5–2% agar, pH 7.0.

Culture medium with sodium acetate as carbon source adopting plate screening method: (L): NaAc (1 g), ferric citrate (5 mmol) as electron acceptor, NH_4Cl (0.31 g), KCl (0.13 g), trace elements solution 10 mL (nitriloacetate (1.5 g), $MgSO_4 \cdot 7H_2O$ (3 g), $MnSO_4 \cdot H_2O$ (0.5 g), NaCl (1 g), $FeSO_4 \cdot 7H_2O$ (0.1 g), $CoCl_2 \cdot 6H_2O$ (0.1 g), $CaCl_2 \cdot 2H_2O$ (0.1 g), $ZnSO_4 \cdot 7H_2O$ (0.1 g), $CuSO_4 \cdot 5H_2O$ (0.01 g), $AlK(SO_4)_2 \cdot 12H_2O$ (0.01 g), H_3BO_3 (0.01 g), $Na_2MoO_4 \cdot 2H_2O$ (0.01 g), $NiSO_4 \cdot 6H_2O$ (0.03 g), Na_2SeO_3 (0.02 g), $Na_2WO_4 \cdot 2H_2O$ (0.02 g)), 1.5–2% agar, pH 7.0.

3.3 Investigation of Electricity Production by Bacterial Suspension

After the bacteria are isolated, the cyclic voltammetry is performed to detect whether there is electrochemical activity in mixed bacteria or single bacteria. Further, a

preliminary comparison of the electrochemical activities is carried out, in order to determine the electricity-producing bacteria that can be applied in MFCs for the purpose of sewage treatment.

Cyclic voltammetry refers to a linear scanning voltage applied to electrodes at a constant scanning speed. When the previously set ending potential is reached, the scan is reversed to return to the initial potential. Figure 9.3 shows the relationship between time and potential in cyclic voltammetry. The electrode reaction is $O + e^- \rightarrow R$. There is only particle O present in the solution before the reaction, and both O and R can be dissolved in solution. The initial potential for the positive scan is controlled to start at the initial potential φ^i, which is much more positive than the standard equilibrium potential φ^0 of the system, and the current response curve is shown in Figure 9.4. When the potential of the electrode gradually shifts more negatively close to φ^0, the reduction of O starts to take place on the electrode, and Faradaic current

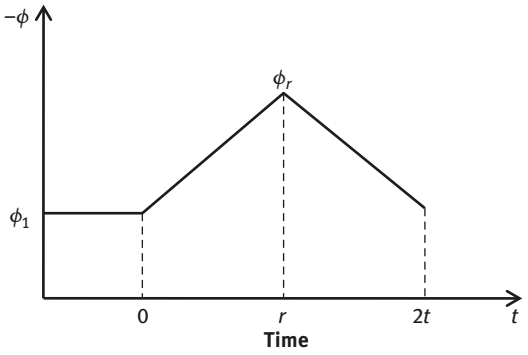

Figure 9.3: Cyclic voltammetry potential versus time.

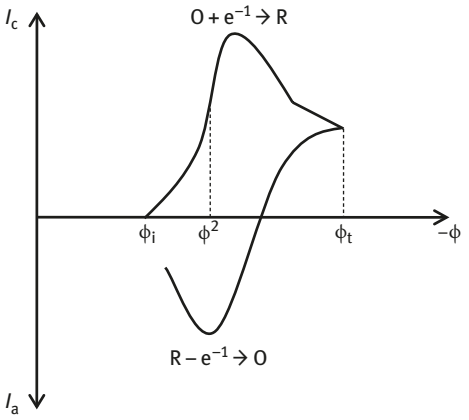

Figure 9.4: Current response curve.

flows through the electrode. The potential becomes more and more negative and the concentration of electrode surface reactant O decreases gradually, thus the particles flow to the electrode surface and current is increased. When the surface concentration of O drops to near zero, the current increases to the maximum value I_{pc}, followed by a gradual decline. When the potential reaches φ^r, the potential scan is reversed. When the electrode potential gradually becomes positive, the concentration of oxidizable particles R near the electrode reaches a relatively high level. When the potential approaches and passes through φ^0, the electrochemical equilibrium on the surface should move toward the direction favorable for the generation of R. Consequently, the oxidation of R begins to take place, and the oxidation current increases to the peak current I_{pa}. Subsequently, the current drops due to the significant consumption of R. Therefore, the entire curve is called "cyclic voltammogram" and the process is "cyclic voltammetry." The redox peak may appear if the bacterial solution is electrochemically active, and the microflora (bacterium) can then be preliminarily determined to be the electricity-producing colony.

3.3.1 Electrochemical Activity of Mixed Microfloras

X. Y. Kong [19] studied electrochemical activity of mixed bacterial strains. The cyclic voltammograms of the bacterial solutions in the sewage sludge samples collected from different locations are shown in Figure 9.5. The figure exhibits the five closed

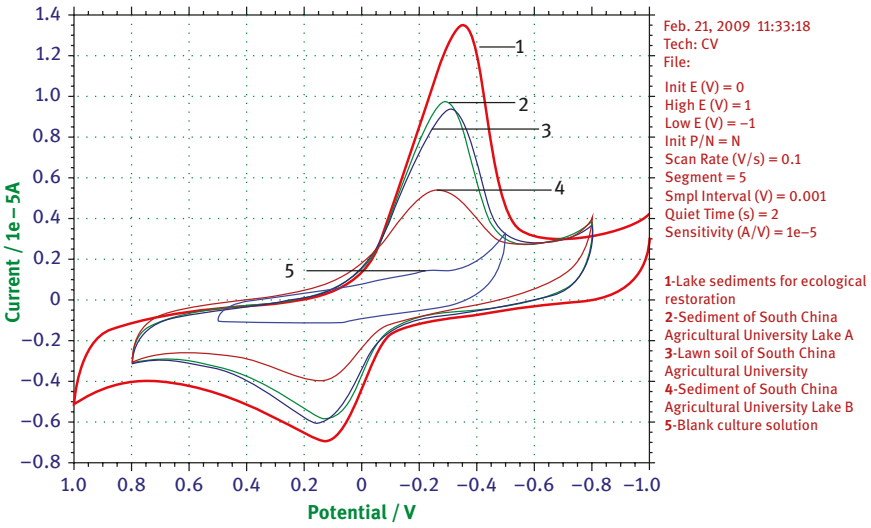

Figure 9.5: Cyclic voltammograms of the microfloras from different sediments.

curves, among which sample number 1 was collected from ecologically restored sediment, that is, the sediment from Yudai River in Panyu (a district of Guangzhou city). The first was collected from cesspool sediments; the second was from lawn soil from lake sediments; and the third sample was collected from empty substrate as a control unit. Compared to the control unit, the other four bacterial solutions showed oxidation peak and reduction peak. The most prominent oxidation peak and reduction peak were found in the restored sediment sample (number 1), indicating the presence of multiple types of bacteria with electrochemical activity in the restored sediment.

Figure 9.5 shows the absence of redox peak for sample number 5 (empty substrate), and the other four microfloras exhibit oxidation peaks and significant reduction peaks (top). However, the peak values varied, and oxidation and reduction processes were irreversible.

3.3.2 Electrochemical Activities of the Single Bacterial Strain and Microflora

Figure 9.6 presents the cyclic voltammograms of the single bacterial strains isolated from the anaerobic sludge and the corresponding microfloras. The numbers in the figure represent different microfloras, and the curves with letters are the cyclic voltammograms of single bacterial strains. The figure clearly indicates that single bacterial strains exhibit significant reduction peaks, while weak oxidation peaks. Nonetheless, microfloras exhibit symmetrical oxidation and reduction peaks. This indicates that microfloras have stronger redox activities due to the complementarity of a variety of bacteria.

Section 4: Community Analysis of Microbes at Microbial Fuel Cell Anodes

Studies on the microbial communities in MFCs, whether related to those that are electrochemically active themselves, or those that can transfer electrons between different species, are still in the early stage. Current studies on the molecular characteristics of the microbial communities in the biofilms of MFCs provide an insufficient understanding of electrochemically active microbes and their interactions in the biofilms. In some microbial communities of MFCs, *Geobacter* [20] or *Shewanella* [21] is a dominant genus. However, some studies have indicated that the microbial communities in MFCs

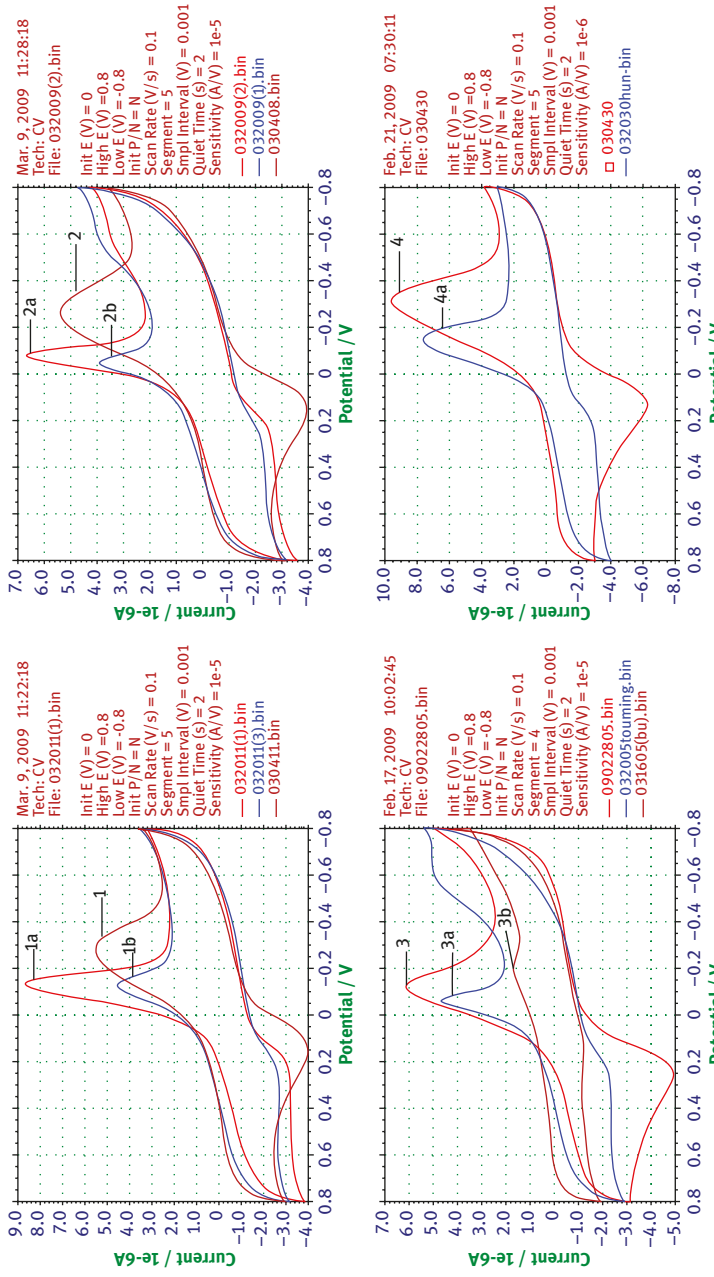

Figure 9.6: Cyclic voltammograms of the single bacterial strains and the corresponding microfloras.

have greater diversity. Studies have clearly shown that the microbial communities in the biofilms of MFCs obtained either through continuous transfer or culture still can differ significantly.

4.1 Community Analysis of the Electricigens Cultured from Sodium Acetate

In a study on the microbial community of an alcohol-based two-chamber MFC, most microbes (83%) had a 16R sDNA similar to that of β-proteobacteria; the rest of them mostly had a 16R sDNA similar to that of *Dechloromonas*, *Azoarcus*, and *Desulfuromonas*; a tiny minority of them had a 16R sDNA similar to that of δ-*proteobacteria*; only one microbe had a 16R sDNA similar to that of *Geobacter*; and none of them had a 16R sDNA similar to that of *Shewanella*.

Figure 9.7 shows the phylogenetic tree of the microbes in a sodium acetate-based MFC [22]. The figure clearly shows that the microbial bacteria existing in the anode chamber of the MFC mainly include *α*-proteobacteria, γ-proteobacteria, *δ*-proteobacteria, and β-proteobacteria, *Clostridia*, *Bacteroides*, *Flavobacteria*, and Sphingobacteria, with *δ*-proteobacteria accounting for 21%.

4.2 Community Analysis of the Microbes at the Anode of a Glucose-Based Microbial Fuel Cell

Molecular cloning of the microbes was conducted in a glucose-based MFC. The phylogenetic tree constructed from the obtained results is shown in Figure 9.8 [23]. The obtained 40 operational taxonomic units (OTUs, a sequence-based approximation of bacterial species) included 64 bacterial clones. Comparison with the corresponding gene sequence on the website of the National Center for Biotechnology Information (NCBI) revealed that most of these clones (over 98%) were primarily included in the following six groups: high G + C Gram-positive bacteria, low G + C Gram-positive bacteria, β-proteobacteria, γ-proteobacteria, δ-proteobacteria, and Green nonsulfur bacterium. The G + C Gram-positive bacteria and γ-proteobacteria were dominant bacterial groups, accounting for 34.8% and 37.5%, respectively. The high G + C Gram-positive bacteria primarily included a species of bacteria in the *Enterococcus* genus and a species of filamentous bacteria, and the subclasses of γ-proteobacteria were predominantly comprised of *Klebsiella oxytoca*.

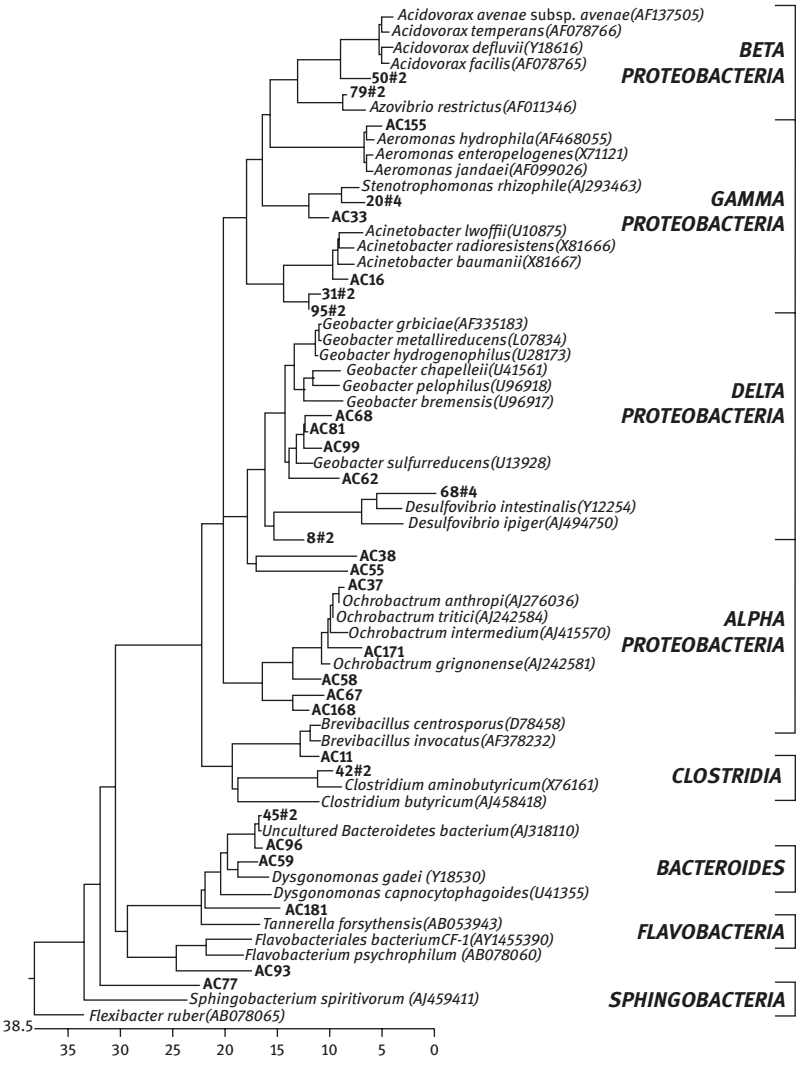

Figure 9.7: Phylogenetic tree of the bacteria in an acetate-based MFC.
Source: Lee et al. (2003) [22].

4.3 Community Analysis of the Microbes in Synthetic Wastewater in a Microbial Fuel Cell

Figure 9.9 shows the phylogenetic tree of the microbial community in the anode chamber of an MFC enriched with the synthetic wastewater prepared with glucose and glutamic acid [24]. The main bacteria in the community are α-proteobacteria

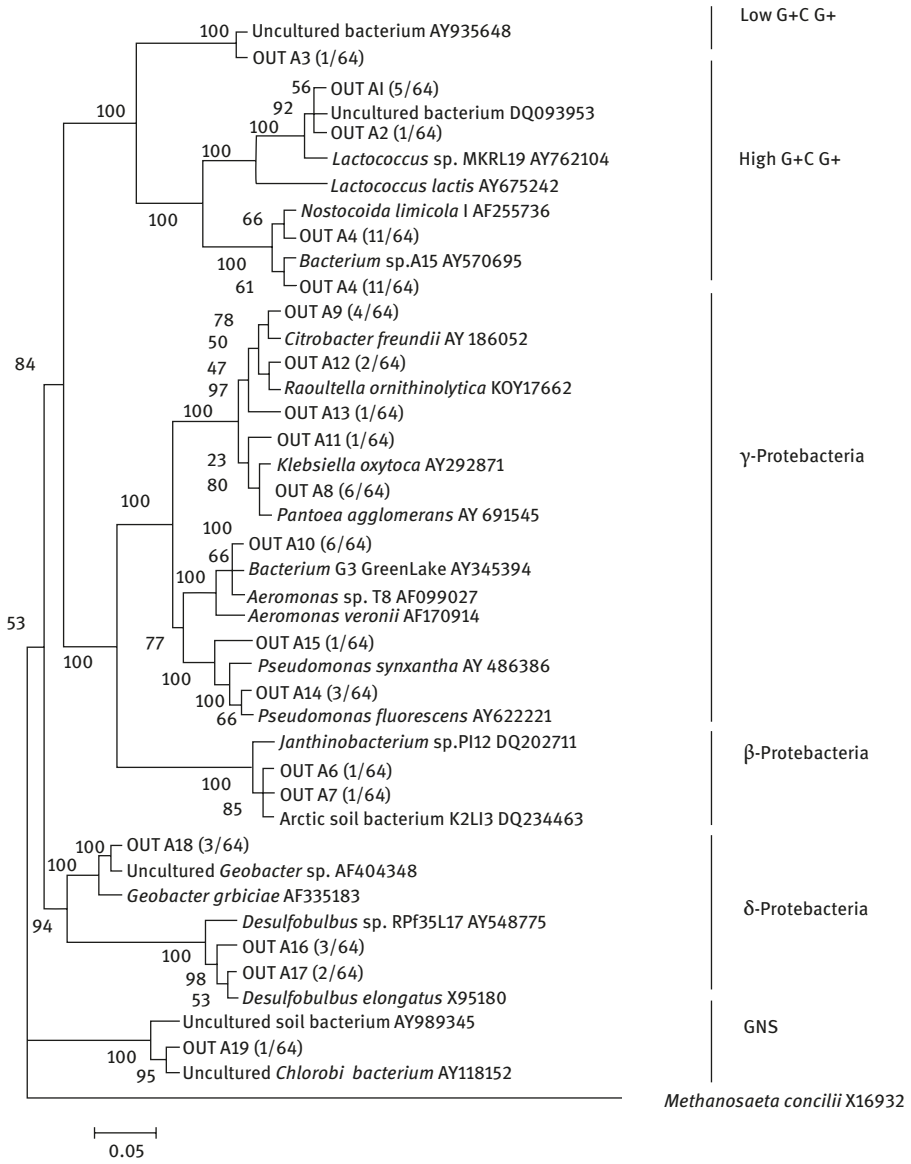

Figure 9.8: Phylogenetic tree of the microbial community in a glucose-based MFC.
Source: Sun et al. (2008) [23].

(64.5%), β-proteobacteria (21.1%), γ-proteobacteria (3.3%), Bacteroidetes (7.8%), and Actinobacteria (3.3%), indicating that α-proteobacteria is the primary microbe in the MFC that is enriched with glucose and amino acid as carbon sources.

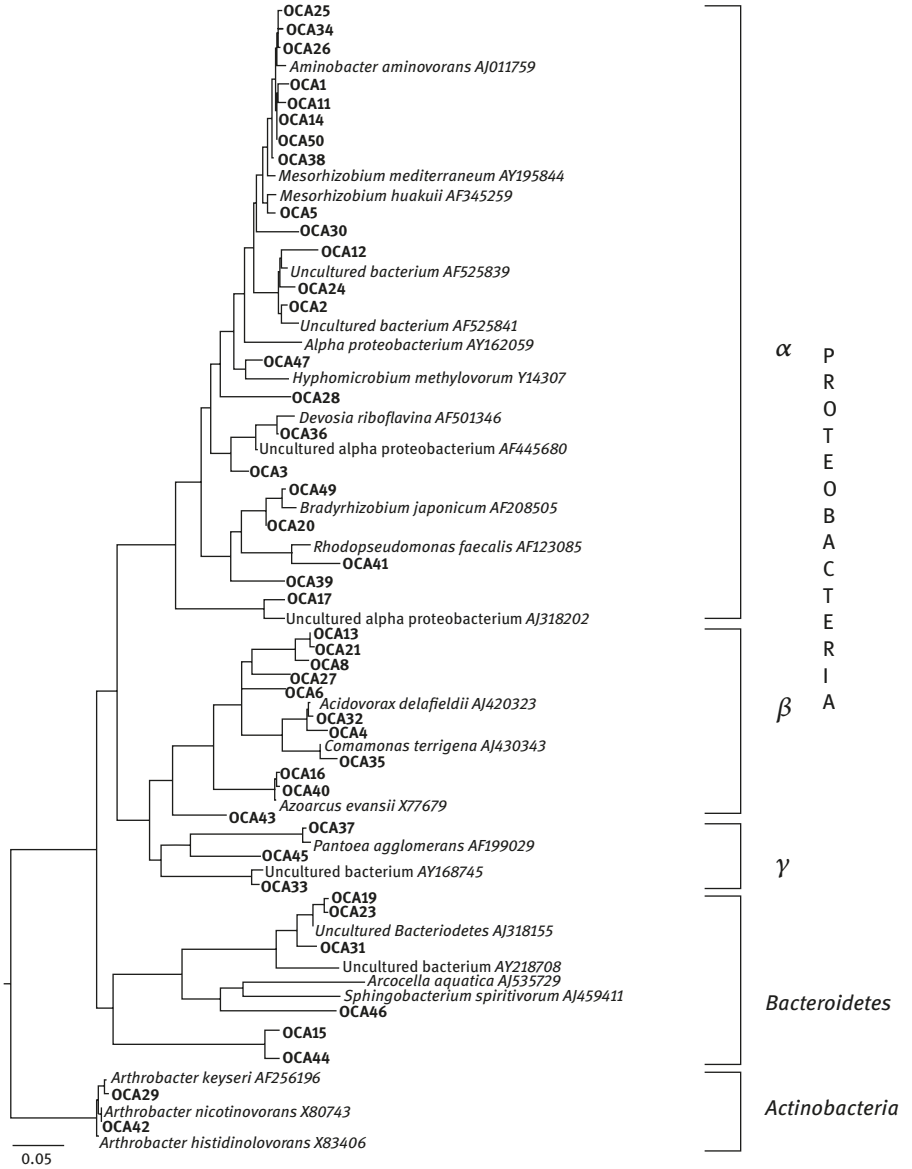

Figure 9.9: Phylogenetic tree based on 16S rDNA sequence analysis of the microbial community in the anode chamber of an MFC present in the synthetic wastewater prepared with glucose and glutamic acid.

Source: Phung et al. (2004) [24].

4.4 Community Analysis of the Microbes at the Anode of a River Water-Based Microbial Fuel Cell

The microbial bacteria enriched in a river water-based MFC are significantly more diverse than and different from those enriched in synthetic wastewater. Figure 9.10 shows the phylogenetic tree of the microbes enriched in a river water-based MFC. The bacteria mainly include β-proteobacteria (46.2%), γ-proteobacteria, and δ-proteobacteria (12.9% in total), α-proteobacteria (10.8%), Bacteroidetes (8.6%), Acidobacteria (5.4%), Verrucomicrobia (1.1%), and Chloroflexi (2.1%). The bacteria in a river water-based MFC are obviously more diverse than those in synthetic wastewater.

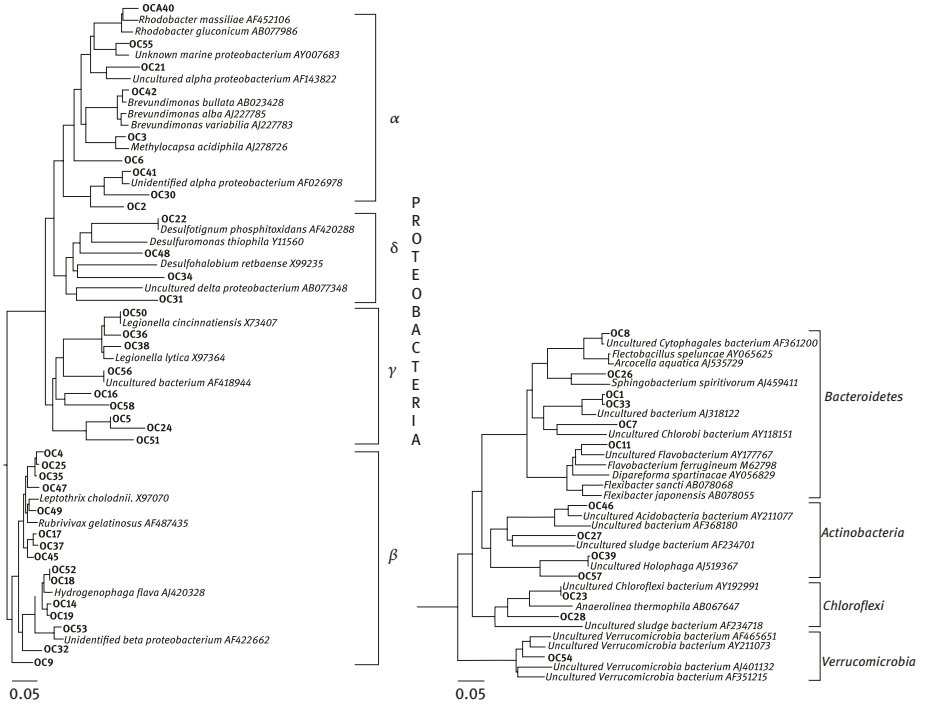

Figure 9.10: Phylogenetic tree based on 16S rDNA sequence analysis of the microbial community recovered from a river water-based MFC.

Source: Phung et al. (2004) [24].

4.5 Community Analysis of the Microbes at the Anode of an Anaerobic Effluent-Based Microbial Fuel Cell

Figure 9.11 shows the phylogenetic tree of the bacteria cultured from anaerobic effluent obtained by molecular cloning. The obtained 22 OTUs included 64 clones.

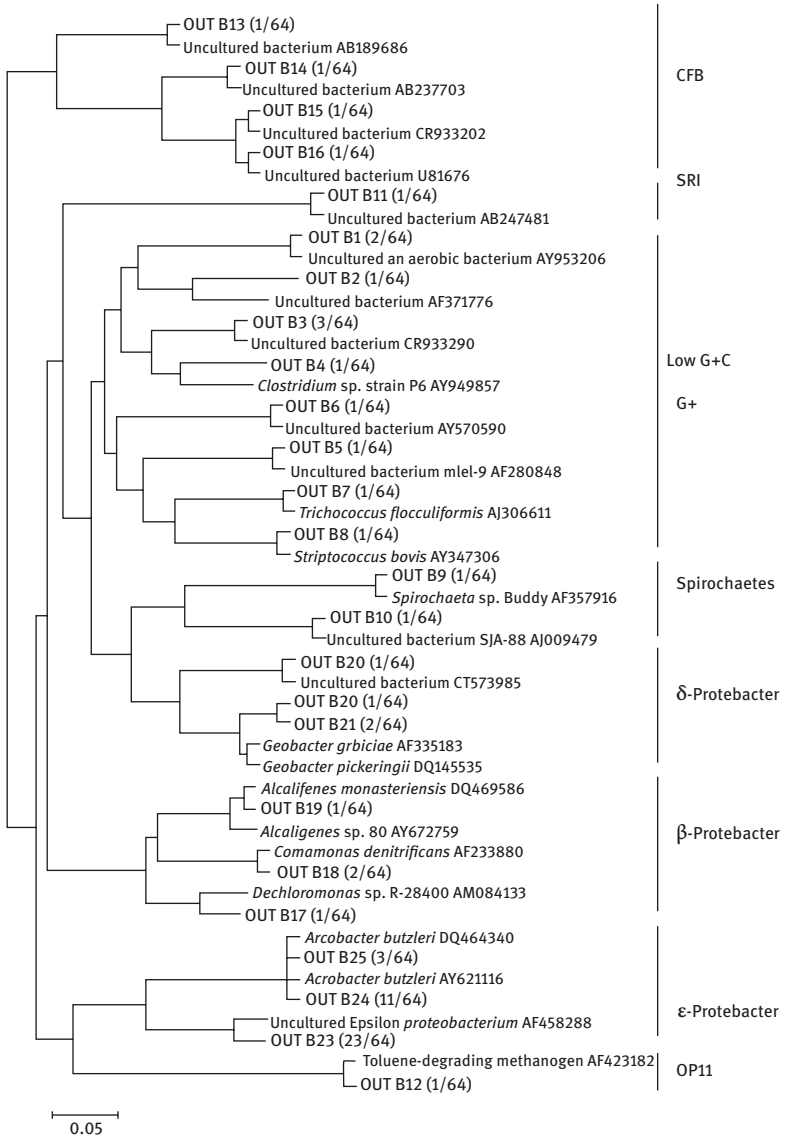

Figure 9.11: Phylogenetic tree of the microbial community in an anaerobic effluent-based MFC. Source: Sun et al. (2008) [23].

Comparison with the corresponding gene sequence on the website of the NCBI revealed that these bacteria were primarily included in the following eight groups: low G + C Gram-positive bacteria, Spirochaetes, SRI, Op II, CFB Bacteroidetes, β-proteobacteria, γ-proteobacteria, and ε-proteobacteria, which are significantly different from the range of groups of the microbes in a glucose-based MFC. ε-Proteobacteria and low G + C Gram-positive bacteria are absolutely dominant in the microbes in an anaerobic effluent-based MFC, accounting for 57.81 and 17.91%, respectively. The low G + C Gram-positive bacteria primarily include acid-producing bacteria *Trichococcus* and *Clostridium*.

It has been found that the species of the electricity-producing bacteria on the surface of anode of a MFC using different substrates differ significantly, which makes it difficult to study the effects of some other factors on the microbial communities in MFCs. However, the following bacterial groups are derived from most substrates, such as low G + C Gram-positive bacteria, *β*-proteobacteria, and *y*-proteobacteria, which are perhaps the auxiliary bacteria related to electricity production.

Table 9.2 summarizes the analysis results of the microbial communities in five different culture systems. Evidently, α-proteobacteria, β-proteobacteria, and/or γ-proteobacteria were dominant bacteria in four of the culture systems, and no dominant microbes were identified in the remaining culture systems. Kim et al. found that most of the bacteria cultured from a dual-chamber MFC incubated with anaerobic sludge and based on the wastewater flowing out from a starch processing plant belonged to a β-proteobacteria-dominant microbial community. Another

Table 9.2: Community analysis of the microbes in different MFCs using oxygen as the cathodic electron acceptor.

Inoculums	Substrates	Communities
Riverbed sediment [24]	Glucose + glutamic acid	α-Protecteria (65%, mostly Actinobacteria), β-protecteria (21%), γ-protecteria (3%), Bacteroidetes (8%), others (3%)
Riverbed sediment [13]	River water	α-Proteobacteria (11%), β-proteobacteria (46%, mostly *Leptothrix* spp.), γ-proteobacteria (13%), δ-proteobacteria (13%), Bacteroidetes (9%), others (8%)
Seafloor sediment [21]	Cysteine	α-Proteobacteria (*Shewanella affinis* KMM, accounting for 40%), Vibrio spp., and *Pseudoalteromonas* spp.
Wastewater [22]	Acetic acid	α-Proteobacteria (24%), β-proteobacteria (7%), γ-proteobacteria (21%), δ-proteobacteria (21%), others (27%)
Wastewater [25]	Starch	Unidentified bacteria (36%), β-proteobacteria (25%), α-proteobacteria (20%), Cytophaga, Flexibacter, and Bacteroides (19% in total)

MFC incubated with riverbed sediment and based on low concentrations of glucose and glutamate produced an α-proteobacteria-dominant microbial community [24]. A study by Lee et al. [22] on a system incubated with activated sludge and based on acetate indicated that the quantities of α-protecbacteria, γ-protecbacteria, and δ-protecbacteria were almost equal in the microbial community cultured from the system.

The above-mentioned analysis reveals that Fe-reducing bacteria are a major contributor to energy production in MFCs. Many characteristics of other bacteria remain to be further discovered and explored. The microbial sources with which many MFCs are incubated come from the untreated domestic sewage, the wastewater in wastewater treatment reactors, or the sludge in wastewater treatment systems. One of the above mentioned studies used microautoradiography (MAR), a method that utilizes isotopes to label acetic acid. In this study, Fe(III) ions were used as the sole electron acceptor to investigate the abundance of Fe-reducing bacteria in the wastewater treatment reactor of an activated sludge (AS) process. In the experiment, sodium molybdate and bromoethanesulfonate were used to inhibit the reactions involving sulfate reduction and methane generation. The results indicated that Fe-reducing bacteria accounted for 3% of the AS. Fluorescence in situ hybridization was coupled with MAR for testing. The results revealed that all MAR-positive bacteria could be hybridized with bacteria-specific probes; however, 70% of the cells could not be hybridized with proteobacteria-specific probes. The hybridization results of the probes specific to the subclasses of proteobacteria indicated that 20% of these metal-reducing bacteria belonged to γ-proteobacteria, and 10% of them belonged to δ-proteobacteria. Therefore, compared to the status quo of studies on *Shewanella* (γ-protecbacteria) or *Geobacter* (δ-proteobacteria), our knowledge of other metal-reducing bacteria is still very limited.

High coulombic efficiencies (greater than 50%) were determined in some MFCs using pure compounds as the substrate, indicating that most of the substrates were involved in cell respiration and electricity production. Although the proportion of the substrate that is being converted into biomass by electricigens is still unclear, the highest coulombic efficiency could reach 85%, indicating the conversion of about 15% of the substrate into biomass. Till date, the proportion of electrochemically active bacteria in the bacterial strains that grow on the anode surface of MFCs has not been determined. Therefore, it is essential to explain and analyze the communities in MFCs with great caution. In the study, in order to investigate the bacteria that grow on the anode surface of a MFC, it was essential to obtain and analyze the samples of the microbial community at the anode; compare them with the microbial community in the solution, on the film, and at the cathode; and study the electricity production mechanisms and genes of the electricigens in order to better understand the evolution of the electricigens in the system.

4.6 Electricity-Producing Capabilities of Electricigens in Microbial Fuel Cells

4.6.1 Electricity-Producing Performance of Electricigens from Mediatorless Microbial Fuel Cells

Electricity producing capabilities of different electricigens in MFCs differ significantly. Some literature reports have stated the use of two-chamber, U-tube, and mini-MFCs to study the electricity-producing capabilities of single bacteria. Moreover, the anode materials and substrates used by microbes in MFCs also affect the output of electricity, as shown in Table 9.3.

Currently, most of the isolated electricigens are Fe-reducing bacteria, and the understanding of mechanism for the electricity production by Fe-reducing bacteria is still preliminary. Therefore, the MFCs based on Fe-reducing bacteria are still a research hot spot. Furthermore, other types of electricity-producing single bacteria have also been gradually discovered, such as the photosynthetic bacterium *R. palustris*, whose ability to produce electricity is greater than that of the community in which it is a member. This perspective indicates that the selection of efficient electricity-producing single bacteria will be an interesting trend in the studies on the electricigens in MFCs. Compared to the electricity production process of mixed bacteria, single type of bacteria enables more effective control over the operation of MFCs such as fuel selection and the environments for growth and metabolism of electricigens. The substrates used by electricity-producing single bacteria can be roughly divided into the following two categories: saccharides

Table 9.3: Comparison of the electricity-producing performance of electricigens in MFCs.

Electricigens	Substrates	Anode materials	MFC type	Maximum power density
Rhodopseudomonas palustris [1]	–	Graphite brush	Dual chamber	2720 mW m^{-2}
Ochrobactrum anthropic [20]	Acetic acid	Carbon cloth	U-tube	89 mW m^{-2}
Rhodobacter sphaeroides [21]	Lactic acid	Pt-PEDOT	Dual chamber	7.3 mW L^{-1}
Comamonas denitrificans [22]	Acetic acid	Graphite brush	Dual chamber	36 mW m^{-2}
Shewanella oneidensis [9]	Lactic acid	Graphite felt	Mini	3000 mW m^{-2}
Escherichia coli [14]	Glucose	PTFE graphite	Single chamber	600 mW m^{-2}
Pseudomonas aeruginosa [15]	Glucose	Carbon rod	Dual chamber	28 mW m^{-2}
Geobacter sulfurreducens [17]	Acetic acid	Graphite rod	Dual chamber	1.9 mW m^{-2}
Desulfovibrio desulfuricans [26]	Lactic acid	Activated carbon cloth	Single chamber	0.51 mW cm^{-2}

and simple organic acids. Most electricigens cannot directly oxidize saccharides. They need to rely on fermentative microbes to convert saccharides into their required micromolecular organic acids, such as acetic acid, lactic acid, pyruvic acid, and formic acid. Whether electricigens can completely oxidize substrates (electron donors) is crucial to their electricity-producing capability. For example, *R. ferrireducens* can completely oxidize glucose, and *G. Sulfurreducens* can completely oxidize acetic acid.

4.6.2 Electricity-Producing Performance of Electricigens Using Mediators

For the direct MFCs to realize mediator-less electron transfer, microbial cells must form direct contact with the electrode surface, which is a basic condition for the completion of this transfer process. Some microbes transfer electrons directly to the electrode via oxidoreductases, which are the electrochemically active components on the surface of their cell membrane, so as to produce electricity in the absence of mediators (Table 9.4). These microbes include *A. hydrophila*, *G. metallireducens*, and *Rhodoferax ferrireducens, etc.* Moreover, *C. butyricum* in the fermentative bacterial genus can also utilize hydrogenase to transfer electrons directly to the electrode. Therefore, this

Table 9.4: Electricigens using mediators for electron transfer and anodic electrochemical reactions.

Microbes	Substrates	Mediators	UFC voltage	Current or current density
Pseudomonas methanica	CH4	1-Naphthol-2-sulfonate indo-2, 6-dichlorophenol	0.5–0.6 V(OC)	2.8 μA cm^{-2} (0.35 V)
Escherichia coli	Glucose	(Alkaline) methylene blue (15)	0.625 V(OC)	
Proteus vulgaris	Glucose	Thionin	0.64 V(OC)	0.8 mA (560)
Proteus vulgaris	Glucose	Sulfur, thionin	350 mV (OC)	3.5 mA (100)
Proteus vulgaris	Sucrose	Sulfur, thionin	350 mV (OC)	3.5 mA (100)
Escherichia coli	Glucose	Sulfur, thionin	390 mV (560)	0.7 mA (560)
Lactobacillus plantarum	Glucose	Fe(III)EDTA	0.2 V (OC)	90 μA (560)
Erwinia dissolvens	Glucose	Fe(III)EDTA	0.5 V (OC)	0.7 mA (560)
Proteus vulgaris	Glucose	2-Hydroxyl-1, 4-naphthoquinone	0.75 V (OC)	0.45 mA (560)
Escherichia coli	Acetate	Neutralizer (1)	0.25 V (OC)	1.4 μA cm^{-2} (sc)
Escherichia coli	Glucose	Neutralizer (1)	0.85 V (OC)	17.7 mA (sc)
Escherichia coli	Glucose	2-Hydroxyl-1, 4-naphthoquinone	0.53 V (OC)	0.18 mA cm^{-2} (sc)

microbe, when growing on the electrode surface and forming a biofilm, can improve the rate at which electrons are transferred to the anode.

Section 5: Applications of Microbial Electricity Production

Previous research has shown that MFCs act as significantly important candidates with potential value-added novel applications in the following areas: novel wastewater treatment technology, alternative energy, sensor, bioremediation, water desalination, and the use of the special environment of MFCs to enrich uncultured bacteria.

5.1 Novel Technology for Water Treatment

The main objective of MFC research involves the recycling and reuse of the chemical energy of the organic matters in wastewater with organic waste as fuel. The experimental results of MFC-treated wastewater's quality in the study have triggered resear chers' deep interests to develop novel wastewater treatment techniques based on the operating principles of MFCs.

In the early stage of research in this field, researchers isolated the bacteria that could remove Fe^{3+} ion contaminant present in sewage, and those were found to be genetically close to *C. butyricum* [14] and A. hydrophila [11] from MFCs. They also reviewed the current situation (at that time) and prospects of utilizing industrial and agricultural wastewater. In later studies, the application of MFCs in wastewater treatment has been extended to the sulfide removal, synchronous organic waste removal that does not rely on H_2 generation, and biological denitrification.

Jang et al. [26] found that with MFCs, the removal rate could reach 90% for synthetic dichromate chemical oxygen demand (COD_{Cr}) sewage with a concentration of 300 mg L^{-1}, and the COD_{Cr} was based on glucose and glutamic acid. Logan et al. directly used air as the cathode for MFCs to treat domestic sewage, and COD_{Cr} removal rate reached 80%. Jang et al. [27] used a plug-flow MFC with serpentine pipes to treat the sewage containing different substrates. As a result, they achieved continuous sewage treatment and continuous electricity generation. Noteworthy, during the anaerobic degradation of organic matters by the MFC, the pH of sewage remained neutral. Moreover, the CH4, H2, and other substances that were produced in the conventional anaerobic fermentation were not found in the solution by this method of treatment. Therefore, MFC can be used as an alternative technique for sewage treatment, and a COD_{Cr} removal rate similar to that of general anaerobic processes can be achieved. Furthermore, MFC exhibits a very promising prospect for further development, due to the facts that it does not acidify the water or produce hazardous explosive gases.

5.2 Electricity-Producing Capacity

The electricity-generating capacity of bacteria can be effectively measured only after the metabolic pathways controlling the electron and proton flow in microorganisms are completely understood. Other than the effect of the substrate, the anode potential of MFCs also affects the metabolism of bacteria. The increase in MFC current leads to a decrease in the anode potential, causing bacteria to transfer electrons to more reductive complexes. Therefore, the anode potential not only determines the redox potential for the final electron shuttling in bacteria, but also the types of metabolism. In the case of a high anode potential, the bacteria can use the respiratory chain during the oxidative metabolism. Electron transfer and the concomitant proton transfer are conducted via NADH dehydrogenase, ubiquinone, coenzyme Q, or cytochrome. Kim et al. [6] studied the utilization of this pathway, and they observed that the MFC current generation could be blocked by a variety of respiratory chain inhibitors. In the used MFC, electronic transfer system utilized NADH dehydrogenase, iron/sulfur (Fe/S) proteins, and quinone as electron carriers, without the use of the No. 2 site of the electron transport chain or the terminal oxidase. Moreover, common observations have shown that the transfer process of MFCs requires the use of oxidative phosphorylation, resulting in an energy conversion efficiency of 65%. The common examples include *Pseudomonas*, *Enterococcus,* and *R. ferrireducens*.

5.3 Biosensor

Five-day biochemical oxygen demand (BOD_5) has been widely used to evaluate the content of biodegradable organic matters in wastewater. Since it takes 5 days to measure BOD by the conventional method, numerous studies are being conducted on BOD sensor to decrease the measuring time, and the one based on MFC operating principles is the focus of researchers. The key information to develop new BOD sensors based on MFC operating principles is as follows: a positive linear relationship between the concentration of pollutants and the current or charge generated by cells, a rapid response rate of cell current to the concentration of sewage, and a reliable repeatability.

Currently, all the MFC sensors being studied have PEM in a dual chamber. The cathode of the cell is also mostly made of phosphate buffer solution with dissolved oxygen, and the anode is made of an aqueous solution await to be tested. When Kim et al. [9] used the self-designed BOD sensor to measure the BOD concentration of the solution in a batch mode, the transferred charge of the cell exhibited a strong linear relationship with the sewage concentration. Such correlation had a coefficient of 0.99, and a standard deviation of 3–12%. The response time of the cell at low concentration was less than 30 min; when the solutions with BOD concentration of less than 100 mg L^{-1} were continuously monitored. The current and concentration displayed a linear

relationship, and the differences among three current measurements were less than 10%. Moreover, when the MFC anode was in a "starvation" state and it was followed by the addition of fresh sewage, MFC current could be restored. When the concentration of the sewage in the cell changed, it took one hour for the current to get stabilized. At the same time, the current response time was shortened by 5 min via changing the sewage flow rate and cell anode volume.

The presence of nitrates, sulfates, and other electron acceptors with high redox potential in real sewage can reduce the MFC current response signal. Azides, cyanides, and other respiratory inhibitors can be added to the anode chamber to eliminate the effects of nitrates and sulfates. The results revealed that via the addition of respiratory inhibitors, the MFC-type BOD sensors could be used to accurately measure the BOD concentration of oxygen- and nitrate-containing oligotrophic surface water.

Moreover, the main obstacle for adopting MFCs as the sensor cell for oligotrophic water bodies (such as surface water and effluent from sewage treatment plant) is the high diffusion rate of O2 through the cathode and the PEM, and the low reduction rate of the cathode. These factors lead to a very weak signal of cell output current. Kang et al. improved the MFC cathode, which in particular significantly increased the reproducibility and signal-to-noise ratio of MFC current output.

5.4 Bioremediation

High cost is associated with the transportation of sediments for centralized treatment. Direct in situ bioremediation of sediments using the electricity-generating principle of electricity-producing microorganisms can save high transportation costs and reduce the environmental interference caused by the transportation processes. As a result, incorporation of microorganism technology into this process could be a better approach to treat the contaminated sediments. For the sediments enriched with organic pollutants, in situ microbial decomposition would be an ideal approach. Although some microorganisms can decompose most of *polycyclic aromatic hydrocarbons*, polychlorobiphenyls, and other organic matters after pure culturing process, the result is still suboptimal in the area of preparing the product that can actively perform in situ decomposition of organic matters. In situ processing requires addition of nutrients and microorganisms with a highly efficient degradation capacity, and sometimes electron accepting or oxygen releasing agents are also needed. However, the externally added microorganisms or other substances are susceptible to the strong influence of hydraulic conditions, indigenous microorganisms, and other factors; thus, it is difficult to achieve the desired effect. Therefore, a new in situ remediation technology, namely SMFC technology, was developed to improve this situation.

The operating principles of SMFCs are similar to those of regular MFCs; however, the structure of reactor structure is much simpler. In the SMFCs, the anode electrode

is buried in the sediments, and the cathode electrode is suspended in the water above the anode. Compared to the traditional in situ bioremediation, addition of electron acceptors or oxygen releasing agents is not required. The electrode is used as an electron acceptor, and certain amount of electricity can be produced during the oxidation of organic matters. Therefore, the characteristics of SMFCs include mobility, high in situ remediation efficiency, and electricity-producing capacity. Consequently, there is a positive prospect for the application of SMFCs. In recent years, foreign researchers have started to apply this technique in remediation of water bodies; nonetheless, the domestic studies on the related area have been rarely reported.

5.5 Hydrogen Production by Microbial Fuel Cells

Hydrogen produced from biomass is considered as an important source for the future hydrogen fuel cells. The commonality between MFC and hydrogen collected from biomass paves the way for the adoption of biomass as a raw material. However, during the process of hydrogen production from biomass, a considerable part of hydrogen in the glucose and other biomasses remains unused. Moreover, biomass produces hydrogen gas as an intermediate product in the pathway to obtain energy, whereas MFCs directly consume and convert all the hydrogen in glucose into H_2O, exhibiting a high conversion efficiency.

Recently, the Pennsylvania State University has reported the production of clean hydrogen fuels by bacteria living in vinegar and wastewater, when they were conducted to electricity for a short amount of time, and such hydrogen fuels were able to power cars the same way as gasoline does. This "MFC" could convert almost any biodegradable organic materials into hydrogen gas with zero-carbon emission. When acetic acid was extracted from vinegar and then put into an electrolyzer, the bacteria generated a voltage of 0.3 V while consuming acetic acid, and releasing both electrons and protons. When another small voltage was applied externally, the hydrogen was observed to be released from the solution, and the energy required for the electrolysis of acetic acid was only one-tenth of that for the hydrolysis. Bacteria performed a large amount of electrolysis including decomposing organic matters into subatomic particles, thus the external electrons could easily recombine the released particles into hydrogen gas to power vehicles. Furthermore, the electrolysis process of MFCs could be carried out in cellulose, glucose, acetate, or other volatile acids, and water was the only product of electrolysis.

The above-mentioned results indicate that the major improvement required for the development of MFCs as a new energy source involves the further increase in the output power density of the cell and the efficiency of transferring electrode electrons. With further studies, MFC may be able to provide energy to remote control devices that only need a small amount of electrical power.

5.6 Water Desalination

Desalination of saline water can be achieved by changing the electricity-producing conditions of microorganisms. The researchers first collect samples from a pond or other natural water sources. Among millions of microorganisms in the sample, some bacteria whose types scientists have not yet determined could automatically generate electrons and protons in their cells, and transport them to the outside of the cells. Other bacteria could absorb these electrons and protons, and they use these electrons and protons as "fuels" for the production of energy substances, including hydrogen, methane, and other chemicals.

Currently, all the applications of MFCs including the saline water desalination, electricity production, and the productions of hydrogen, methane, or other gases are limited to the laboratory environment. With further research on this hot topic, MFC is expected to reach pilot scale. Logan and his colleagues installed a large-scale MFC near a winery, and planned to convert the wastewater from the winery into hydrogen. The researchers found that the energy produced by these microorganisms could be utilized by two pieces of special plastic films. This film could separate electrons, ions, or gases produced by microorganisms and allow them to flow to the cathode or anode. A cathode, an anode, and a film were assembled in a transparent plastic box of the size of a tissue box. After a cup of pond water was added between the films, the bacteria began to work, and ultimately water with 90% purity was produced. The purity of the water could be adjusted according to scientific or commercial needs and even drinking water standards could be reached. MFCs can remove most of the salts in water. This process can reduce power consumption, thus consequently, the cost of desalination can be reduced.

5.7 Summary

The twenty-first century will be the era of rapid development for the biotechnology. As a possible solution to the energy crisis and to achieve sustainable development, MFCs will become a focus of the bioenergy research. Compared to other bioenergy technologies, MFCs have several advantages. First of all, a variety of organic and inorganic substances can be used as a fuel in theory, which indicates that even photosynthesis and wastewater can be utilized to meet our demands for energy. Second, organic matters can be converted into electricity with an extremely high efficiency. Third, the fact that MFCs can be operated at room temperature or lower temperature reduces the cost of cell maintenance, and it is also safer at the same time. Fourth, the products of MFCs are mainly CO_2, and no post-treatments are required to process it. Fifth, this technology has a strong biocompatibility, as it can use glucose and oxygen in human body to produce energy as a power source for artificial organs. Finally, this technology is capable of producing a biosensor.

In summary, utilization of electricity-producing microorganism is a new technology that combines new ways of energy generation and solutions to environ-

mental problems. This technology exhibits significant potential for people to completely utilize the industrial and agricultural wastes, municipal solid wastes, and other biomass resources for residential electricity supply in the future. Electricity-producing microorganisms have promising prospects for the applications in alternative energy development, microbial sensor research, and development of water treatment technology. However, in-depth explorations are undeniably required to improve the electrochemical performance of electrodes, enhance the output power density of the cell, and reduce the cell cost. With the continuous study on the electrical conduction mechanism of electricity-producing microorganisms, industrial applications of electricity-producing microorganisms will be possible in near future.

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Jingliang Xu, Ying Guo, Minchao He, Yu Zhang

10 Microbial Fossil Energy Conversion

In 2010, China's oil imports increased significantly, with 200 million tons of net imports of crude oil, more than 55% of which was foreign dependence. Our fossil energy consumption is currently as high as 92.2% of the total energy consumption, in which coal accounts for 70.4%, oil accounts for 17.9%, and gas accounts for 3.9%. Such an energy consumption structure has brought great risks for the economic development, social stability, and environmental security. The development of efficient means to utilize fossil energy is significant to alleviate the current pressure of energy supply and to enhance the utilization of green energy.

Microbial-enhanced oil recovery (MORE) refers to the injection of microorganisms or bacteria into oil reservoirs to activate the microbial nutrient solution. This process will improve the physical properties of crude oil or its metabolites and thus enhance oil recovery. MORE has advantages such as wide range, simple process, low cost, good economic returns, and being pollution-free. The global oil surplus is significantly dwindling, and the utilization of MORE to improve oil recovery becomes increasingly attractive.

Coal microbial transformation refers to the use of fungi, bacteria, and actinomycetes to improve the coal liquefaction and gasification process, and coal can be converted into water-soluble hydrocarbon substances. Furthermore, a range of important chemicals could be obtained during the microbial conversion process of coal such as clean fuel, industrial additives, and promoting agents for crop production. China's coal resources are very rich, and the conventional source of energy consumption is still coal-based; therefore, microbial conversion and utilization of coal would open a path for the efficient use of coal.

This chapter describes MORE and microbial conversion of coal development including the history of the technology, classification, mechanism (biochemistry course), breeding methods, and finally an overview of typical cases is provided.

Section 1: Microbial Oil Recovery

Currently, the world's recoverable oil reserves have been estimated as $1{,}272 \times 10^8$ m^3, and heavy oil, extra-heavy oil, and about 1, 510×10^8 m^3 of bitumen can be mined, which exceeds that of conventional crude. Heavy crude oil is difficult to explore due

Corresponding author: Jingliang Xu, E-mail: xjl@ms.giec.ac.cn
Ying Guo, Minchao He, Yu Zhang, Guangzhou Institute of Energy Conversion, Chinese Academy of Sciences; CAS Key Laboratory of Renewable Energy; Guangdong Provincial Key Laboratory of New and Renewable Energy Research and Development, Guangzhou 510640, China

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to its high viscosity and high-density. Conventional thermal and chemical oil extraction methods are limited due to economic reasons and for environmental protection. Therefore, the development of economical and efficient heavy oil recovery technologies is significant.

1.1 Development of Microbial Enhanced Oil Recovery Technology

Conventional enhanced oil recovery (EOR) technologies include the approaches of thermal flooding, chemical flooding, and polymer flooding. MEOR is a method that utilizes microbial metabolic activity to improve oil recovery. MEOR technology can effectively utilize microbial products from metabolism such as polymer flooding, surfactant, carbon dioxide, and organic solvents, and unlike other EOR technologies, the driving force for MEOR technology is the microorganism medium [1, 2]. Compared to other conventional technologies, MEOR technology is simple, easy to apply, offers wide application, and is economic and green.

The idea of using microorganisms to improve the efficiency of heavy oil recovery dates back to the 1920s, equipping it with 90 years of development history [3]. From the early use of microbes to reduce water and land pollution, to the development of complicated microbial applications such as oilfield wax, single well stimulation, viscosity reduction, selective blocking formation, and enhanced water flooding, the development process of MEOR can be divided into three stages.

1.1.1 Basic Research Phase (1926–1975)

In 1926, the US scientist Beckman noticed that bacteria can improve oil exploration, and he was the first to propose the idea of "microbial enhanced oil recovery in reservoirs" [4]. In 1940, the US marine biologist Zobell discovered that bacteria can release oil from the reservoir sediments and proved the chemical similarity of EOR metabolic substances. In 1946, Zobell won the first MORE patent [5]. In the same year, Updegraff and Wren applied for a patent for the use of symbiotic *Desulfovibrio* bacteria for MORE [6]. Unfortunately, these two patents were not tested in the field. In 1954, Coty conducted the first pilot test with MORE technology in the United States. Then, MEOR technology for field test was conducted in Poland, the Czech Republic, Romania, the former Soviet Union, Canada, and Australia.

In early 1955, China also began to study the MEOR technology. From the 1960s to the mid-1970s, biological surfactants and petroleum microbial screening were conducted [7]. In 1966, the Xinjiang Petroleum Administration began to explore the use of microbial oil dewaxing technology. Studies during this period provided the theoretical basis for MORE.

1.1.2 The Rapid Developmental Phase (1975–1990)

From the 1970s, the MEOR technology has been widely utilized in the United States, the former Soviet Union, and other countries. In 1973, the oil crisis attracted considerable research focus and the MEOR technology, an economical and environmentally friendly technology, is still attracting a significant research interest.

During this period, the United States, Canada, United Kingdom, Romania, the former German Democratic Republic, the former Soviet Union, and Australia have conducted numerous experimental studies both in theory and in the field; however, the applications were mainly single wells. In 1982, 34 countries participated in the first "World Conference on Microbial Enhanced Oil Recovery" in the United States. The conference aided the exchange of research, and decided to convene an international conference every 2 years.

The US Department of the Petroleum and Energy Research Institute conducted a MORE economic evaluation on the Phoenix Oilfield and the Mink Unit Oilfield in Oklahoma in 1986 and 1990, respectively. The test succeeded in increasing the oil production by 13% and 19.6%, respectively. From 1985, Russian researchers also chose their fields and injected the nutrient material and air contained in water into a reservoir, which could activate microbes in the oil reservoir and subsequently improve the production of the reservoir. In 1986, the US Micro-BAC International and National Parakleen Company jointly developed a series of field-specific microbes, which greatly improved MEOR technology.

Many studies on microbial growth in a reservoir have been conducted in China. During the late 1980s, the Daqing Oilfield first simultaneously performed a field trial in two single wells. From the late 1980s to the early 1990s, the Chinese Academy of Sciences (CAS) cooperated with the Jilin Oilfield to conduct single well stimulation technology research and testing. Then the CAS, the Nankai University, and the Shandong University also cooperated in the oil field to develop laboratory and field strategies for the applications of MEOR.

1.1.3 In-Depth Research and Field Application Stage (1990–Present)

During the 1990s, with the development of the technology, genetically engineered bacteria, mathematical models, and numerical simulation processes were introduced into MEOR. Currently, MEOR technology has developed an ideal assessment system, which includes strain screening and strain evaluation, oil displacement test evaluation, reservoir screening, evaluation of experimental design and microbial field trials [8]. During this period, a large number of patented technologies and pilot project have emerged. The number of patented technologies authorized in the United States associated with MORE reached 133 up to 1990, and the number of conducted field trials has surpassed 400 up to 2003 [1].

During this period, the Chinese MEOR technology has also been rapidly developed and field tests were conducted in the Daqing, Shengli, Dagang, Xinjiang, and Liaohe oil fields. Daqing oil field has introduced the MORE from the United States and Canada, and conducted a large number of corresponding studies. The Jilin Oilfield conducted microbial tests in 197 wells, and the average single-well oil production increase between around 65.9 tons up to 1997 [9, 10]. In addition, Chinese scientists performed microbial tests for the wax-proofing of microorganisms, contamination elimination, and aquifer blocking during this period.

1.2 Classification of Microorganisms for Oil Exploration

Microbial oil recovery refers to the injection of microorganism solution and nutrient solution or the sole nutrient solution into the oil layer so that microorganisms could grow and reproduce and hence metabolic products could be produced, which would increase the yield of oil recovery. Microorganisms improve the yield of oil recovery by directly influencing the properties of the oil. Microorganisms utilized in oil recovery can be divided into two groups according to their source. Oil recovery by native microorganisms is achieved via mere injection of nutrient solution to improve the oil displacement efficiency. Furthermore, the method of heterogeneous microorganisms refers to the pouring of microorganism solution and nutrient solution into the oil layer.

1.2.1 Native Microorganisms

Native microorganisms refer to a specific stable microbial community formed over a period in an internal reservoir. Such microbes are mostly developed and preserved through the adaptive growth of microorganisms in the oil field, and only a smaller share of bacteria existed before reservoir formation. The native microbe formations grow with a state of limited growth and metabolism, and the majority of native microorganisms were in dormancy. Therefore, when provided with the right amount of nutrients, native microorganisms can be activated and improve oil recovery through their metabolic activity.

1.2.1.1 Aerobic and Native Microorganisms

The major representative of native aerobic microorganisms is petroleum hydrocarbon-degrading bacteria. Hydrocarbon-degrading microorganisms could utilize

petroleum hydrocarbon as a substrate for growth. These bacteria are abundantly available around injection wells and the wellbore. Hydrocarbon degrading microbes produce metabolic substances such as enzymes, surfactants, polymers, organic acids, alcohols, and carbon dioxide, which can be used to split heavy hydrocarbons and paraffinic oil, reduce oil viscosity, and improve the liquidity of oil.

Petroleum hydrocarbon degrading bacteria are mostly aerobic bacteria, namely hydrocarbon oxidizing bacteria. Representative strains include *Micrococcus*, *Arthrobacter*, *Rhodococcus*, *Halobacterium*, and *Bacillus*.

1.2.1.2 Native Anaerobic Microorganisms

Native anaerobic microorganisms are present in the oilfield wastewater reinjection system and the oil hypoxic environment. These strains include fermentation bacteria, sulfate-reducing bacteria (SRB), nitrate-reducing bacteria, and methane-producing bacteria [11].

(1) Fermentation bacteria

Since most oil fields have high-temperature characteristics, thermophilic fer menta tion bacteria are far more prevalent than mesophilic fermentation bacteria. In addition, some fermentation strains need to adapt to the high salt and anaerobic environments and other extreme environments in reservoirs. Currently, isolated strains are mainly thermophilic bacteria, extremely thermophilic bacteria, and halophilic bacteria. Thermophilic microorganisms mainly include *Thermotoga elfii*, *Thermotoga subterranean*, and *Thermotoga hypogea* while hot *Lactococcus* include *Thermococcus celer* and *Thermococcus littoralis*. Hydrogen sulfide thermal hot anaerobic bacteria include *Thermoanaerobacter thermohydrosulfuricus* and *Pyrococcus lithotrophicus*. Anaerobic microorganisms separated from oilfield water with halophilic properties are mainly *Haloanaerobium acetoethlicum*, *Haloanaerobium congolense*, *Haloanaerobium congolense*, *Haloanaerobium salsugo*, and *Spirochaeta smaradinae* [12].

(2) Sulfate-reducing bacteria

In oilfield water reinjection systems with anoxic environments, SRB can produce acidic metabolic H_2S gas, which will increase the pressure, dissolve carbonate rock, promote the release of crude oil, and enhance the permeability of the formation. At the same time, some SRB can also degrade oil components, thus improving the mobility of crude oil and enhancing the recovery of crude oil. It is worth noting that H_2S and other acidic gases are produced and may cause equipment corrosion. The SRB strains isolated from the reservoir include *Desulfovibrio*, *Desulfotomaculum*, *Desulfacinum*, and *Desulfomicrobium*. The genus *Desulfovibrio* was the most abundant SRB.

(3) Methanogens

Anaerobic degradation of hydrocarbons to methane includes two steps. First, hydrocarbon is degraded into small organic mole cules which are eventually converted to methane. Alkali species (*Alcanivorax* sp.), Desulfurizationbacteria (*Desulfoglaeba alkanexedens*), and fermentation bacteria (*Thauera* sp., *Pseudomonas* sp., *Thermotogae*, and *Clostridia*) participate in the first part of the anaerobic process, where they degrade petroleum hydrocarbons into small organic molecules. *Methanogenic archaea* will finally be converted into methane. *Methanogenic archaea* are divided into mainly three categories: (1) acetic acid nutrition *methanogens* (*Methanosaeta*): utilization of acetic acid to produce methane; (2) hydrogen nutrition *methanogens* (*Methanospirillum* and *Methanoculleus*): utilization of H_2 and CO_2 to produce methane; oxidative; (3) acid bacteria, oxidized acetic acid to H_2 and CO_2. *Methanogens* convert H_2 and CO_2 to methane.

1.2.1.3 Heterologous Microorganisms

Heterologous microorganisms are known as exogenous microbes, which are screened and injected into culture. Heterologous microbes can multiply and grow at extreme environmental conditions, and can produce beneficial metabolites to increase oil recovery and reduce environmental pollution.

These microorganisms are mainly *Pseudomonas* bacteria heterologous, *Bacillus*, *Micrococcus*, *Corynebacterium*, *Mycobacterium*, *Arthrobacter*, *Clostridium*, methane bacteria, *Bacteroides*, as well as others. These are anaerobic or facultative and can have metabolic substances such as gas (hydrogen and methane), organic acids (formic acid, acetic acid, propionic acid, and lactic acid), surfactants, biopolymers, and organic solvent (methanol, ethanol, propanol, and acetone). The main strains and their effect are shown in Table 10.1 [13].

Table 10.1: Classification of microorganisms and their function [13].

Strain type	Metabolite	Function
Bacillus, Xanthomonas, Leuconostoc	Biomass	Selectively increasing viscosity or humidity
Bacillus, Arthrobacter, Acinetobacter, Pseudomonas	Surfactant	Emulsification or de-emulsification
Bacillus, Brevibacterium, Leuconostoc, Xanthomonas	Polymer	Selectively increasing viscosity
Clostridium, Klebsiella, Zymomonas	Solvent	Dissolve rock
Enterobacter, Clostridium, Mixed acidogens	Acids	Increase of permeability
Clostridium, Enterobacter, Methanobacterium	Gas	Increase of pressure and decrease of viscosity

1.3 Mechanisms of MORE

During recent years, MORE has been developed from the laboratory to field test with remarkable achievements. The progress of biotechnology and production technology continues to extend the field of MORE research. Although early studies suggest that microbes can survive in the reservoir and would aid crude oil recovery, the specific mechanism still needs to be further investigated.

The current view on MORE technology is that the microbial community could grow in the reservoir by inoculating microorganisms or nutrients. Their metabolic substances contain biosurfactants, organic acids, biopolymers, and gas. These microorganisms or their metabolites acted on crude oil and could reduce the viscosity of crude oil, increase the flow properties of crude oil, and drive crude oil from wells. The classification of microorganisms is shown in Table 10.1.

1.3.1 Microbes Can Change the Composition of Crude Oil and Decrease Viscosity of Oil

Microbes can reduce the viscosity of crude oil based on the following principles:

(1) Enzymes are produced by microorganisms that can decompose heavy oil polymer material such as converting asphaltenes or resin acids into low molecular compounds, splitting carbon strands, depolymerizing chains into low-carbon–carbon chains, and thus reducing heavy components and increasing the availability of light components. The viscosity of crude oil will significantly decrease and the quality of crude oil will be improved.
(2) Bacteria can utilize *n*-alkanes in the heavy oil as a carbon source. The metabolic activity in the process will produce biosurfactants, and the heavy oil is dispersed or emulsified into water-in-oil emulsions, which will reduce their viscosity.

1.3.2 Microbes Can Change the Oil Displacement Environment

1.3.2.1 Biosurfactants

Microbially active ingredients mainly include palmitic acid, daturic acid, and stearic acid. On the one hand, these agents can reduce the pressure of the oil-water interface, the water flooding capillary force, and the flooding capillary number. On the other hand, bio-surfactants will alter the reservoir rock from lipotropy to hydrophily and exfoliate the film adsorbed on the surface of the falling rock, which will decrease residual oil saturation and hence improve recovery efficiency.

1.3.2.2 Biogas

The vast majority of microorganisms in the metabolic process will produce gases such as carbon dioxide, hydrogen, and methane. These gases can reduce the viscosity of crude oil and increase the flow velocity of crude oil. Second, the produced gas will increase the formation pressure, which aids the driving force.

1.3.2.3 Acids and Organic Solvents

Microbes can produce acids, esters, alcohols, and other solvents via their metabolism. Microorganisms mainly produce organic acids such as relatively low molecular weight ingredients (formic acid, acetic acid, and propionic acid) as well as some inorganic acids (sulfuric acid). Those acids can dissolve carbonates, increase their porosity and permeability, increase reservoir pressure, reduce oil viscosity, and improve oil flowability by releasing carbon dioxide. Organic esters produced by microorganisms, can change the surface properties of the rock and the physical properties of the crude oil. Crude oil adsorbed on the surface of the porous rock will be released, and is then easy to drive. Alcohols produced by microorganisms are helpful for improving the viscosity of crude oil and increase the porosity of the rock, which is similar to a mild acidification process.

1.3.2.4 Biopolymers

The metabolites of the microorganism may work with heavy metals to form a polymer. These metabolites have an efficient water shutoff effect and can selectively block large pores, increase the sweep coefficient, and reduce the water–oil ratio. Thus, a polymer in zones of high permeability reservoirs can significantly increase oil production and recovery. However, the high penetration of biopolymers will block strips and reduce water injection. Oil-producing strains and the properties of biopolymers are shown in Tables 10.2 and 10.3.

1.3.2.5 Direct Action of Microorganisms

Microorganism can adhere to the rock surface, grow on the surface of the rock particles, and occupy pore spaces. The oil film can be pushed out, and finally, the oil can be discharged from the film. The rate of closure of pure bacteria may reach 99%.

1.3.2.6 Combined Effect

Combined effects of metabolites are listed in Table 10.4.

Table 10.2: Microorganisms and their biopolymers.

Strain type	Classification of biopolymer	Discoverer
Bacillus sp.	Levan	Akit et al.
Xanthomonas sp.	Biogum	Pollock and Thorne
Aureobasidium sp.	Amylose	Cho et al.
Alcaligenes sp.	Curdlan	Buller and Vossoughi
Leuconostoc sp.	Glucan	Kim and Fogler
Sclerotium sp.	Scleroglucan	Sandford

Source: Zhao et al. (2008) [13].

Table 10.3: Comparison of polymers.

Properties	Polyacrylamide	Xanthan gum	Glucan
Shear stability	Irreversible	Reversible	Reversible
Heat stability	70 °C	80 °C	125 °C
Hydrolysis property	Hydrolyze easily	Hydrolysis at high temperature	Hydrolysis at high temperature
Biodegradability	Attacked by bacterium and yeast	Degraded easily in aerobic condition	Degraded easily in aerobic condition
Multivalention	Cross-link easily	Cross-link easily	Cross-linkable

Source: Park et al. (2001) [14].

Table 10.4: Function of microbial metabolites on the oil layer.

Microbial metabolite	Function mechanism of the oil layer
Biomass	Selectively or randomly blocking, emulsifying hydrocarbon, wetting capability, oil degradation, lowering the viscosity, solidifying points of oil, and desulfurization of oil
Polymer	Controlling of fluidity, selectively or randomly blocking, improve the efficiency of displacement, lowering the ratio between water and oil
Surfactant	Lowering the surface tension, emulsification
Acids	Increasing the permeability by dissolving the carbonate among rock, corrosion surface of carbonate, increasing the porosity, and lowering the viscosity of oil by releasing carbon dioxide
Gas	Recovery of oil pressure, lowering viscosity, and dissolving the sulfur substance in mineral
Solvent	Dissolve the oil in rock, lowering the viscosity in oil

Source: Park et al. (2001) [14].

1.4 Microbial Oil Extraction Methods and Techniques

1.4.1 Screening of the Oil Reservoir

Only when the parameters of the reservoir including depth, pressure, temperature, formation water content, salinity, pH, and oil density are within an appropriate range, MEOR can be applied. Therefore, as part of the MEOR process, we need to determine whether the conditions are suitable or not. Reservoir parameters of the MOER technology are shown in Tables 10.5 and 10.6, developed by the American Petroleum Energy Research Institute [14].

Table 10.5: Reservoir screening criteria and function of microbial metabolite on oil layer.

Parameters	Ranges of assessing
Temperature	<76.67 °C
Depth	<2438 m
Salinity	Content of sodium chloride <10%
Microelement contents	Content of arsenic, mercury, nickel, selenium <10–15 mg/L
Permeability	>0.05 μm2
Intrinsic microorganism	Suitability with the engineering microorganism
Saturability of residual oil	>25%
Area of an single well	<16,974.4 m2

Table 10.6: Type of microorganism oil recovery.

Process	Difficulties in the production	Types of microorganisms
Stimulation treatment	Weak pressure, difficulties in injection, irreducible oil	Capable of producing surfactant, gas, acid, and alcohol
Well flushing	Wax precipitation	Capable of secreting emulsification, surfactant, and acid
Water displacement	Irreducible oil	Able to produce surfactant, gas, acid, and alcohol
Improving permeability	Inferior spreading	Able to secrete surfactant, gas and, acid
Biopolymer displacement	A dash of injection, poor fluidization	Yielding of biopolymer
Water plugging	High ratio between water and oil	Production of a large amount of biomass or biopolymer

1.4.2 Desired Microorganisms and Selection of Nutrient Solution

To study MORE technology, we need to have a detailed knowledge of the microbial community, nutrient solution, bacteria physiological characteristics, and their influence of formation fluid to evaluate the expected results of experiments in the oil field. For MORE technology, suitable strains need to be selected according to the formation environment and engineering purposes. Ground conditions including salinity, permeability, pH, and formation water composition need to be considered. The formation fluid and microorganisms need to be tested for compatibility. Microbial treatment can either choose a single species or more than one species thereof. The nutrients required for injection into the reservoir can be determined when microbial compatibility tests are conducted.

1.4.3 Injection of Microorganisms or Nutrient Solution

Single well stimulation tests can be conducted after the initial oilfield selection investigation. During the single well stimulation test, diluted microbial broth or nutrient solution is pumped into the tank via pipe with stable string and ground equipment.

1.5 Modern Technology Applied in the MORE

Some modern technologies or strategies have been utilized in MORE. Hence, these advanced methods or high-efficiency strategies could improve the development of MORE.

1.5.1 Biolog Method

The Biolog method was developed by the United States in 1989 to identify microorganisms, which has so far identified more than 2,000 strains of pathogenic and environmental microorganisms including bacteria, yeasts, and molds. Currently, Biolog technology has been widely used to study the microbial community. Techniques for Biolog community analysis can be applied to identify the microbial community for MORE.

1.5.2 Molecular Biology Techniques

Molecular biotechnology is helpful for microbial breeding and composition for reservoir environment. Technology such as polymerase chain reaction and DNA chip can accurately evaluate the ability of microbial species such as their adaptability,

underground transport capacity, proliferation, and increasing mining capacity in MORE technology [15]. Denaturing gradient gel electrophoresis (DGGE) and temperature gradient gel electrophoresis (TGGE) technology can also be used to analyze the microbial community in environments treated by MORE.

1.5.3 Tracer Technology

Tracer technology has been applied to oilfield water injection to qualitatively study the underground fluid movement. During the late 1970s, tracer technology has been applied to study the reservoir heterogeneity and to guide the rational development of oil fields. Currently, this technology is mainly used to study the residual oil saturation of the flooded reservoir. In 1994, treated tritiated water was used as tracer to study the role of microorganisms for the entire reservoir.

1.5.4 Visualization Technology

Visualization is an important method to improve MORE technology. The methods include the microscopic glass beads transparent model, which works with magnetic resonance imaging or computer tomography. The former is used to study the surface of solid particles, bacterial bio-film formation, and ways of pore clogging, while the latter is used to study the effect of the microbes on reservoir cores.

The characteristics of visualization technologies are as follows: firstly, visibility, the process can be directly observed in MORE to test the flooding mechanism; secondly, simulation, the natural structure of the reservoir geometry and displacement process can be simulated via their characteristics.

1.5.5 The Mathematical Model

During the late 1980s, a mathematical model for MORE has been established to conduct a numerical simulation study and typical models included Islam, Zhang, and Chang models. The model proposed by Zhang can describe the formation of microbial activity, which was better than that obtained via the Islam model, however, limitations of the Zhang model were difficult to simulate a one-dimensional scene. The Chang model can describe the behavior of microorganisms and the nutrients in the formation; however, it cannot describe the mechanism of reservoir stimulation. In addition, Gu Jianwei introduced a three-dimensional two-phase multi-component mathematical model to describe the MORE process, including a continuity equation and a behavior equation for the process of microbes, nutrients, and end-products

such as convection diffusion, adsorption, growth, death, and consumption. Using this model can dynamically determine the actual site, optimize site construction parameters, and forecast yield.

Section 2: Microbial Coal Conversion

Abundant coal resources are available in China, which account for over 70% of the Chinese energy composition. Nevertheless, the reserves of low-rank coal are tremendous (130.3 billion tons of explored lignite) and it could reach 13% of the overall coal storage. The disadvantages of low-rank coal, such as low thermal efficiency of direct combustion, poor value of industrial application, and long-term of required air storage not only lead to energy waste but also results in environmental pollution. Hence, it is very important to reasonably exploit and utilize low-rank coal. Coal could introduce numerous problems in the traditional application process; however, the modern biotechnology applied in the clean treatment could bring introduce advantages including simplicity of process, low energy expenditure, and the fact that it is completely pollution free. Therefore, it is critical to study microorganisms related to coal bioconversion.

Coal biotechnological research mainly includes two directions: one is the biological cleaning of coal, which removes sulfur from the coal through microbial bacteria (mainly) and the other is the bioconversion of coal. The biological cleaning was conducted earlier and many related reports have been published; consequently, the massive scale and industrial application of biological cleaning are promising. The definition of bioconversion of coal indicates that the coal was dissolved and liquefied by the function of some microorganisms (fungi, bacteria, and actinomyces). Then, some special and valuable chemicals, clean fuel, industrial additives, and vegetative accelerants after the coal were converted into soluble matters. This technology was first conducted by Fakoussa and Faison who observed that untreated lignite could be dissolved by specific fungi [16]. The bioconversion of coal can be conducted under mild pressure, which will reduce the cost of the equipment. Therefore, focus is increasing to explore microbial degradation coal technology. The role of microorganisms associated with the bioconversion of coal is discussed in this section.

2.1 The Formation of Coal and Its Microbial Conversion

After a series of long-term biological, chemical, and physical reactions, ancient plants turned into a macromolecular solid mixture composed of polycyclic aromatic hydrocarbons with a complex structure, and the solid mixture was termed coal. The raw coal structure and the bridged bond have undergone two stages, one was a peatification stage

and the other was a coalification stage. During the peatification stage, many reactions happened such as cellulose was broken into monosaccharide, lignin was oxidized to complex humus acid and soluble ramification of benzene ring, and plant debris degraded into humus. Then, the peatification stage was completed and the bio-chemical reaction gradually decreased and halted when other sediments covered the formed peat. During the coalification stage, peat successively converted into lignite, sub-bituminous coal, and soft coal under physical and chemical treatment (Figure 10.1).

Microbial coal conversion is an inverse process of coal formation, in which the macromolecular of coal was depolymerized by the oxidation of enzymes or microorganisms so that coal dissolution, coal liquefaction, and coal gasification could be achieved. It could be defined as bio-degradation or biotic dissolution. The action of fungi, bacteria, and actinomyces were required for the microbial conversion of coal. The majority of the coal microbiological conversion production resulted in soluble substance or hydrocarbon gas.

The initial study on the liquefaction of coal could be dated back to the early twentieth century; however, systematic research of the coal dissolution under the function of microorganism had begun in the 1980s. Fakoussa and Hofrichter [18] and Cohen and Garbiele [19] reported that some microorganisms were capable of growing on coal

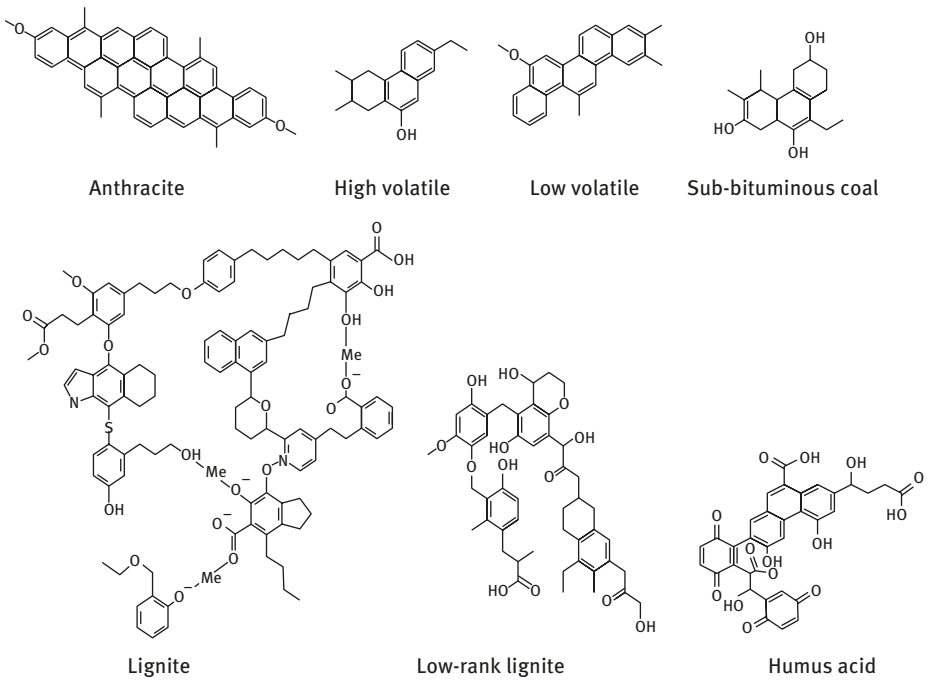

Figure 10.1: Typical structure of various rank coals.
Source: Prasad et al. (2007) [17].

Table 10.7: Advances of coal microbiology.

Year	Microorganism and its action	Researchers
1981	Hard coals degraded by *Pseudomonas*	Fakoussa
1982	Lignite degraded into droplets on agar plates by fungi	Cohen and Gbriele
1987–1989	Dissolution by the synergism of fungi and bacteria	Quigley
1988–1990	Chelating produced by fungi	Cohen and Quigley
1991	Improved analysis methods, such as ^{13}C-NMR, determination of MW, and ultrafiltration	Ralph, Catcheside, Henning, et al.
1994–1997	Enzymatic hydrolysis	Ralph and Catcheside
1996	Preferentially polymerization of humus acids without the effect of the fungus.	Willmann, Frost, et al.
1997	Bioconversion of humus acid into bioplastics by pure cultures	Steinbuchel and Fchtenbusch

and that coal could be transformed into black liquid. Hence, a large group of researchers around the world embarked on the exploration of the biodegradation of coal using microorganisms. It was observed that several types of bacteria, fungi, and actinomyces grow well on dainty and coal piled up in the air for a long term, and some low molecular organic matter and hydrocarbon gas could be produced during this process.

A transformation study of inferior coal into humus acid had been conducted in the Soviet Union in 1988. Coal ashes were mingled with water in special devices and degraded into suspending humus by a certain bacterium obtained from activated sludge. The end-products could be directly sprayed into an agricultural field with no associated waste. The composition of humus acid had been altered after lignite degradation with some fungi and bacteria in the China agriculture university, in which the content of humus was higher (25%) than that of lignite without prior treatment [20]. An obvious difference could be observed on the pot culture in which the suspension of humus was fertilized. The research progress about micorbila coal conversion is shown in Table 10.7.

2.2 Classification of Coal Conversion Microorganisms

2.2.1 Types of Convertible Coal

Microorganisms could degrade the majority of coals, which includes weathered and unweathered lignite, sub-bituminous coal, and bitumite. The low-rank coal could be easily converted and utilized by microorganisms due to the low degree of coalification, abundant side chains, and bridged bond in coal molecules as well as the high content of activated functional groups.

A close correlation relationship has been reported between the degradation degree of coal by microorganism and the degree of coal oxidation. As for the same coal, the degrading capability of microorganism to coal can be ordered as dainty > coal exposed in air > coal without access to air. Therefore, the oxidation of coal was an important factor affecting the effectiveness of the coal bioconversion. A variety of agents such as nitric acid oxidation, air oxidation, ultrasonic, microwave, ultraviolet, and ozone were employed by several researchers to improve the degradation effectiveness and to shorten the experimental period. These strategies enhanced the oxygen content of coal so that it could facilitate the bond breakage interrupted by oxidase, esterase, and chelating agents, and could boost the dissolving ability of microorganisms.

2.2.2 Classification of Coal Biodegradation Microorganisms

The screening and isolation of microorganisms with the ability of dissolving coal was according to these criteria if the secreted metabolite could attack some component and structure within coal. Due to the analogous lignin and aromatic ring abundance in coal, both *Phanerochaete chrysosporium* (capable of degrading lignin) and *Pseudomonas* (capable to degrade aromatic rings) could be suitable for the biodegradation of coal. Furthermore, the desired microorganism could be isolated from coal, which was exposed in the air for a long time. Microorganisms capable of degrading coal include bacterium, fungi, and actinomyces (Table 10.8).

Table 10.8: Classification of coal microorganisms.

Microorganisms		Genus and species
Bacteria	Bacillus	*Bacillus subtilis, B. pumilus, B. cereus, B. amyloliquefaciens*
	Other	*Arthrobacter* sp., *Pseudomonas fluorescens, P. cepacia*
Actinomycetes	*Streptomyces*	*Streptomyces viridosporus, Streptomyces setonii, Strep tomyces badius, Streptomyces flavovirens*
	Other	*Actinosynnema* sp., *Nocardia* sp.
Fungi	Basidiomycetes	*Phanerochaete, Chrysosporium, Trametes/Polyporus/ Coriolus versicolor (Poria placenta)*
	Penicillium	*Paecilomyces* sp., *Penicillium* sp.
	Trichoderma	*Trichoderma viride, Trichoderma atroviride*
	Aspergillus	*Aspergillus terricola, Aspergillus ochraceus*
	Pleurotus	*Pleurotus florida, Pleurotus sajur caju, Pleurotus eryngii*
	Yeast	*Candida* sp.
	Other	*Mucor* sp., *Cunninghamella* sp., *Stropharia* sp., *Fusarium oxysporum, Doratomyces* sp.

Source: Rosenbaum et al. (2005) [18].

2.3 Mechanism of Microbial Coal Conversion

Low-rank coals such as lignite are composed of aromatic rings in which the salt bridge and the aliphatic chain are interweaved, thus forming a macromolecular net structure that cannot diffuse into the microorganism cell. This is possibly because the diameter of macromolecular is bigger than the diameter of cell channel. Therefore, various active matters secreted by different microorganisms can degrade lignite. Overall, three major mechanisms have been studied including alkali mechanism, biological chelating agent mechanism, and the enzymatic hydrolysis mechanism.

2.3.1 Enzymatic Action Mechanism

The majority of enzymes could degrade the low-rank coal because the lignin and low-rank coal have a similar macromolecular structure. Lignin-degrading enzymes were mostly produced by white-rot fungi and those enzymes mainly include lignin peroxidase and manganese peroxidase.

In 1987, Cohen et al. [21] reported that filter liquor of the *Polyporus versicolor* liquid culture had the activity to liquefy coal. The degrading activity was related to the culture time of fungi because proteins were tested in the filter liquor and the liquefaction activity reduced after protein degeneration via acid treatment. This observation could provide solid evidence that the essence of coal degradation by microorganisms was a type of enzymatic hydrolysis process. In the same year, Pyne et al. [22] isolated and purified a protein that markedly reduced liquefaction activity after heating to 60 °C for 30 min. In 1999, Yuan et al. [23] observed that *Pseudomonas* had the capability of degrading coal. After that, numerous hydrolases, oxidases, and reductases could partially degrade lignite and the phenol oxidase even had a more powerful ability for coal degradation. In additional, both *Streptomyces viridosporus* belonging to actinomyces and *Pseudomonas* belonging to bacterium have stronger activity for coal degradation.

These degrading enzymes could oxidize the macromolecular substrate using the active center and the action has three major properties, (1) the degrading enzyme is being beneficial for the penetration of macromoles into cell walls and cell membranes, (2) the substrate specificity of degrading enzyme is poor and the range of catalyzed substrate could be expanded with the aid of small molecules, which play a role as medium between the reducing agents and the degrading enzyme, and (3) the degrading enzyme had a higher redox potential and could oxidize a series of aromatic compounds. The classification of lignite-degradation enzyme is shown in Table 10.9.

Table 10.9: Properties of different oxidative enzymes for the depolymerization of lignite.

	Laccase	Lignin peroxidase	Mn peroxidase
Redox potential (V)	0.4–0.96	1.2–1.5	1.1
Demand of H_2O_2	–	+	+
Specificity	Broad	Broad	Specific
C–C bond rupture	Rarely reported	Often reported	Often reported
Stability	Very high	Low	Very high
Molecular weight	53–110	38–47	38–50
pH value range	2.0–8.5	2.0–5.0	2.0–6.0
Optimum pH value	3.5–7.0	2.5–3.0	4.0–4.5
pIs or isoenzymes	2.6–4.5	3.2–4.7	2.9–7.0
Structure	Mono-, di-, or tetramer, glycoprotein	Monomer, glycoprotein	Monomer, glycoprotein

Source: Rosenbaum et al. (2005) [18].

2.3.2 Alkali-Dissolving Mechanism

Several researchers found the catalysis from alkali to dissolve. These types of catalysts are produced by some microorganisms such as fungi, actinomyces, and single-cell microorganisms and these catalysts of alkali nitrogenous matters include ammonia, bioamine, polypeptide, and associated derivatives. The alkali-dissolving ability of microorganisms was different due to the distinction between type and quantity.

In 1987, it was reported that a type of extracellular substance produced by actinomyces could liquefy coal into a type of black liquor, which was thermally stable and had anti-protease activity with a small molecular weight. It could be inferred that this type of active matter did not consist of enzymatic proteins produced by microorganisms. After that, it was observed that the amount of coal liquidation increased with the pH value of the medium and the degree of pH value increased related to the content of polypeptide and polyamine. In 1989, it was finally confirmed that fungi could produce the alkali catalysis in synthetic medium which could promote the ionization of the acid group of low-rank coal and enhanced the hydrophily of coal.

2.3.3 Chelation Mechanism

Some researchers proposed a type of chelation mechanism. A sort of chelating agents produced by fungi could form metal chelates with the metals in coal. Subsequently, the metals were removed from the coal and the structure of the coal was destroyed so that the coal could be transformed into soluble matter in water.

Polyvalent metal cations existed in lignite, such as Ca^{2+}, Fe^{3+}, and Al^{3+}, which play a bridging role among the carboxyl groups. After removal of the polyvalent metals, lignite could have several beneficial characters, such as being more soluble to alkali, enhanced biological dissolving force, and reduced oxidation reaction. Cohen and Gabriele [19] observed that the degradation degree of lignite coal was associated with oxalic acid salt because oxalate can chelate multivalent metal ions in lignite, especially Ca^{2+}, Fe^{3+}, and Mg^{2+}. The degradability of coal was improved after its metal ions were chelated with oxalate.

Alkali action and chelate action could not disrupt the C-C bond of the coal so that both of them could not lower the molecular weight of coal. In addition, the metabolite of a sole microorganism was not a type of pure substance. Different microorganisms will produce different metabolic products. Moreover, the same microorganism will produce various products in different culture media. Therefore, the bioconversion of coal is complicated. It is still inclusive that a single mechanism or several mechanisms play a role in the process of coal dissolution. When the mechanism of coal dissolution with microorganisms has been clarified, the process of coal dissolving reaction could be neatly controlled so that a single and higher value-added fuel or chemicals could be obtained.

2.4 Microbial Breeding for Coal Conversion

In 1981, Fakoussa [16] reported that microorganisms such as bacteria, fungi, and actinomyces could utilize coal as energy and carbon source. However, a microorganism with properties of efficiency and adaptability has still not been obtained. The massive scale application of the microorganism for coal bioconversion remains impossible due to its high cost.

Microorganisms capable of dissolving coal could produce various active substances such as enzymes and chelating agents, which could destroy the structure of organic compounds in coal or degrade macromolecules. Therefore, there are three types of screening methods according to the difference of action mode of microbial productions.

(1) Coals possess an analogous structure with lignin; therefore, it can be degraded with lignin-degrading microorganisms such as *Trametes versicolor* to conduct research on the microbial solubilization of coal.
(2) Coal is an aromatic compound with aromatic ring structure; therefore, it can be used to study the degradation of aromatic rings of bacteria such as *Pseudomonas* sp.
(3) Another obtaining method for coal solubilizing microorganisms is from growth obtained from long-term exposure to nature of isolated coal microorganisms, such as from mining areas of coal slime.

Due to the development of modern biotechnology, new technologies and new methods are emerging, which provide technical support for the modification of coal degradation microorganisms. Presently, this has attracted the wide attention of researchers to improve the ability of dissolving coal by using molecular manipulation. According to a report, researchers from the United States transformed four genes from *Pseudomonas* into the same host bacteria, to cultivate a special ability to degrade oil, with a very fast degrading speed of oil, only requiring a few hours to break down two-thirds of hydrocarbons in oil. Coal and oil have an analogous structure and composition in many respects, which means that the era of the genetic engineering of bacteria to convert coal is soon to emerge. In addition, other conventional breeding methods have also achieved good results in the selection of soluble coal microbes. Yuan et al. [23] mutated an initial strain via UV mutation treatment for 5 min and the mortality rate was 99.99%. Hence, 28 strains were selected on the lignite culture medium. Nine strains of their study were significantly improved. The strain with the highest efficiency could be used to produce black liquid when directly cultured on the lignite for 36 h, and the culture solution still had the capability to degrade after three days.

Enzymes capable of dissolving coal are mainly encoded by degradable plasmids with a huge molecular weight, which is commonly distributed in the bacterial cytoplasm. Plasmids from a bacterial donor could transfer into a recipient bacterium through the contact between both bacteria, whereas the donor bacterium is insurable to the plasmid by copying. The discovery of the degradable plasmid and its transfer as well as the construction of the super engineered strain introduces a promising prospect for the biological transformation of coal. Of course, the road is still very long, and it requires microbiologists and chemists in the coal industry to work together. With regard to the new species, we should try to construct efficient gene engineering bacteria by using gene-engineering methods. More endeavors should be put into these research fields, such as the discovery of new microorganism species, separation, purification, domestication, and breeding of microorganism, especially the construction of super engineering strains with a gene manipulation strategy.

2.5 The Application of Microbial Coal Conversion

The approach for the biodegradation of coal is green, clean, and sustainable. Microbial coal conversion does not require high temperature or high pressure and can thus reduce construction and operational coast.

The product of microbial coal conversion has wide applications. For instance, microorganisms can convert coal into low cyclic aromatic compounds which can then be further converted into water-soluble liquid products such as methanol and ethanol, all of which are excellent liquid fuels. Coal can then be converted to other low molecular aromatic substances. As we can see, coal treated with lignin microorganisms could

be used as antioxidants, surface active agents, adhesive composites, ion exchange resin, adsorbent, soil conditioners, and immune adjuvants. Other low-molecular aromatics with a considerable amount of oxygen containing functional groups could be developed into high value-added chemicals.

Microbial coal conversion is a complicated and challenging process due to the complicity of the coal ingredients. However, the bioconversion of coal by microorganisms achieved satisfactory success in some respects and offers a promising prospect.

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