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Biotechnology in China II

Chemicals, Energy and Environment

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Advances in Biochemical Engineering/Biotechnology reviews actual trends in modern biotechnology.

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Preface

The biochemical engineering and biotechnology is now becoming the most important industry all over the world. China, as a country that has more than 1.3 billion people, has become one of the fastest growing countries in the world during the last several decades. Both the Chinese government and companies pay more and more attention on the research and the application of biotechnology. In the 11th five-year plan (2006–2010), Chinese government unprecedented enhanced the support on the biotechnology in both policy and finance. Currently, the biotechnology gains the most R&D funding in China. With the great support and the increasingly frequent exchanges from abroad, the biotechnology in China becomes more and more important in the world.

In recognition of the enormous advances in biotechnology in China, we are pleased to present the second volume of Advances in Biochemical Engineering/Biotechnology: Biotechnology in China II, edited by P. K. Ouyang, J. Chen and G. T. Tsao, relatively soon after the introduction of the first volume of this multivolume comprehensive books. Since the previous volume was extremely well accepted by the scientific community, we have maintained the overall goal of creating a number of chapters, each devoted to a certain topic by several Chinese research groups working in the field, which provide scientists in academia and public institutions with a well-balanced and comprehensive overview of this growing field in China. We have fully revised the volume and expanded it from bioreaction, bioseparation and bioremediation to more extensive issues in order to cover all recent developments in China into account as much as possible.

The new volume of Advances in Biochemical Engineering/Biotechnology: Biotechnology in China II is a comprehensive description of the state-of-the-art in China, and a guide to the understanding the work of Chinese biochemical engineering and biotechnology researchers. It is specifically directed to microbiologists, biochemists, molecular biologists, bioengineers, chemical engineers, and food and pharmaceutical chemists, environmental engineers working in industry, at universities or at public institutions. The volume editors and the authors of the individual chapters have been chosen for their recognized expertise and their

contributions to the various fields of biotechnology. Their willingness to impart this knowledge to their colleagues forms the basis of the book and is gratefully acknowledged. Moreover, this work could not have been brought to fruition without the foresight and the constant and diligent support from the Springer.

The seven chapters are organized by more than 20 outstanding biotechnological groups in China. The first chapter reviews the general development history and the perspectives of the industrial biotechnology in China. The next two chapters consider the biotechnological production of organic chemicals and biofuels in China. The fourth chapter summarizes the development of bioreactors and bioseparation. The fifth chapter gives a profile on the current status of environmental biotechnology in China. Special attention is given here to traditional Chinese biotechnology. The last chapter describes the new biotechnology in China.

A carefully selected and distinguished Editorial Board stands behind the series. Its members come from key institutions representing scientific input from about 20 countries. We are grateful to Springer for publishing Advances in Biochemical Engineering/Biotechnology with their customary excellence. Special thanks are due to Editorial Board, without whose constant efforts the volumes could not be published. Finally, the editors wish to thank the Chinese researchers working in the field for their diligence, courage and wisdom, which greatly facilitate the development of Chinese biotechnology.

We believe that we have tried our best to draw a more comprehensive atlas for the development of biochemical engineering and biotechnology in China. However, due to the rapid development of both the researchers and research field, there are still many outstanding proceedings that were not included in this volume. Besides, most of the works included in this volume are still in development. In the following volumes, we wish there will be more Chinese scientists in the frontier of the area to share their exciting works.

Spring 2010

G.T. Tsao
Pingkai Ouyang
Jian Chen

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Past, Present, and Future Industrial Biotechnology in China

Zhenjiang Li, Xiaojun Ji, Suli Kan, Hongqun Qiao, Min Jiang, Dingqiang Lu, Jun Wang, He Huang, Honghua Jia, Pingkai Ouyuang, and Hanjie Ying

Abstract Fossil resources, i.e. concentrated carbon from biomass, have been irrecoverably exhausted through modern industrial civilization in the last two hundred years. Serious consequences including crises in resources, environment and energy, as well as the pressing need for direct and indirect exploitation of solar energy, pose challenges to the science and technology community of today. Bioenergy, bulk chemicals, and biomaterials could be produced from renewable biomass in a biorefinery via biocatalysis. These sustainable industries will match the global mass cycle, creating a new form of civilization with new industries and agriculture driven by solar energy. Industrial biotechnology is the dynamo of a bioeconomy, leading to a new protocol for production of energy, bulk chemicals, and materials. This new mode of innovation will place the industry at center stage supported by universities and research institutes. Creativity in industrial biotechnology will be promoted and China will successfully follow the road to green modernization. China's rapid economic development and its traditional capacity in fermentation will place it in an advantageous position in the industrial biotechnology revolution. The development and current status of industrial biotechnology in China are summarized herein.

Keywords Biotechnology, China, Industrial

Contents

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Abbreviations

β-Gase	β-Glucosidase
AFEX	Ammonia fiber explosion
BBCAG	BBCA Group Co. Ltd
CBP	Consolidated bioprocessing
CMCase	Carboxy methyl cellulase
CNPC	China National Petroleum Corporation
COFCO	China Oil and Food Corporation
CRC	China Resources (Holdings) Co. Ltd
ECUST	East China University of Science and Technology
ED	Entner–Doudoroff
FPase	Filter paper digesting activity

ICGOC	Information Center of Grain and Oil of China
ICI	Imperial Chemical Industries
IPE-CAS	Institute of Process Engineering, Chinese Academy of Science
MAC	Ministry of Agriculture of China
MOST	Ministry of Science and Technology
NBSC	National Bureau of Statistics of China
NDRC	National Development and Reform Commission
NJFU	Nanjing Forestry University
SD	Halcon Scientific Design Company
SEM	Scanning electron microscopy
SHF	Sequential hydrolysis and fermentation
SSCF	Simultaneous pentose and hexose cofermentation
SSF	Simultaneous saccharification and fermentation
TGG	Tianguan Group Co. Ltd
talB	Transaldolase
tktA	Transketolase
VOCs	Volatile organic compounds
XKS1	Xylulokinase
XRD	X-ray diffraction
xylA	Xylose isomerase
xylB	Xylulokinase
ZSBT	Zesheng Bioengineering Technology Co. Ltd

1 Introduction

The prototype of the biotechnology to produce soybean sauce, vinegar, and "brown wine" (Huang-Jiu) by fermentation originated in ancient China six thousands years ago. During the second half of the twentieth century, industrial biotechnology in its primary form in microbiology and fermentation, shifted from traditional commodity production of food ingredients and feed additives to much more prominent areas in the industries of energy, materials, environment, and resources. The rapidness of the emergence and expansion of the third wave in modern biotechnology, "industrial biotechnology" or "white biotechnology", in the last decade, is unprecedented when compared to the first two waves, viz., medicinal biotechnology and agricultural biotechnology.

Industrial biotechnology and related industries are one of the priorities of the Chinese Government. In the National Mid- and Long-Term Science and Technology Development Plan, and other national major R&D programs, industrial biotechnology is in the first tier. Although China ranks first in terms of fermentation volume in the world, and dozens of fermentation products led international markets in terms of output, the technologies employed were not up-to-date, and greater innovation and R&D support are badly needed.

Current research and development in industrial biotechnology is focused on biorefineries and biotransformation to produce bioenergy and bio-based products from biomass feedstock. One of our programs looks at integration of biogas production and oil algae cultivation with environmental remediation and protection by using rural and urban living wastes such as excrement combined with lignocelluloses as a feedstock. In the field of biopolymers and biomass-based materials and composites, we take advantage of monomers and polymers via bioprocesses on the one hand, and natural polymers or their modified substances on the other hand, to produce bulk biopolymers and a new generation of biocomposites. We are confident that industrial biotechnology will form the backbone of China's carbon budget, green house gas reduction, energy supply and security, environmental protection, and welfare of the people. Thus, industrial biotechnology is sure to take part in and play a significant role in every sector of national industry. This chapter partly reflects the endeavors of the Chinese R&D community in the fields of fine chemicals, bulk chemicals, biopolymers, bioethanol, and bioethylene as representative examples. Since fermentation is the most extensively applied process for the manufacture of bioproducts, one section is devoted to this technology and its application. The following sections summarize the development and current status of industrial biotechnology in the fields of fine chemicals, and production of biopolymers and monomers via bioprocesses. Bioethanol and bioethylene have been associated with lignocellulose feedstock according to the trend of using nonfood raw materials and agricultural waste.

2 Leading Fermentation Products from China

2.1 Vitamin C

Vitamin C (V_C), also known as L-ascorbic acid, plays an important role in cell metabolism and is an essential vitamin for humans. It is mainly used in food additives, cosmetics, nutritional agents, health care products, and pharmaceuticals. Demand for vitamin C has been increasing with the widening of its application area and its output reached 10.5 thousand tons in 2000.

Historical development of the industrial production process for vitamin C can be divided into three stages: concentration extraction, chemical synthesis, and fermentation [1]. Industrial production of vitamin C began in 1934, and it was from the 1950s that China began to produce vitamin C using chemical methods. Here chemical synthesis refers to the method which hydrogenates the raw material D-glucose to D-sorbitol, followed by utilization of fermentation and chemical synthesis techniques to obtain vitamin C. This method is named the "Reichstein Procedure." Because of good product quality and high yield, it is still the main process used in developed countries [2]. Manufacturers of vitamin C in China mainly employ a "two-step fermentation" process [3]. In this process, glucose is transformed into 2-keto-L-gulonic acid (2-KLG) by enzymes in microorganisms,

and then vitamin C is synthesized via chemical transformation. This method simplified the "Reichstein Procedure" production process, reduced investment in equipment, lowered production costs, decreased the utilization of a large quantity of organic solvents and other toxic substances, and reduced waste emission greatly. The process was awarded the China National Science and Technology Prize in 1980 [3], and it was granted patents in China, Europe, Japan, and the United States. This technology was successfully transferred to Hoffmann-La Roche Ltd., Switzerland in 1985, becoming the first large technology export item since 1949. The total yield of this process of synthesizing vitamin C from D-sorbitol exceeded 65% and the maximum was 72%. However, both the "Reichstein Procedure" and the "Two-step fermentation" procedure require D-sorbitol as a raw material which was chemically synthesized from glucose and H_2. Because of concerns in relation to future raw material scarcity, cost, and the environmental impact of the process, a great deal of attention has recently been focused on the biological production of 2-KLG by microorganisms or direct biosynthesis of V_C from glucose as an alternative method. New methods were developed for V_C production, such as a new two-step fermentation, one-step fermentation, etc. However, a certain gap remained in industrial production in terms of reaction conditions and cell utilization.

At present, the annual output of vitamin C in China is 100 thousand tons, which makes China the leading vitamin C exporter in the world; 70% of this amount is for the international market. Vitamin C produced by China represents most of the vitamin C world market and in the next few years it will remain in a leading position. However, the quality of domestic vitamin C needs to be improved in order to enter the international high-end consumer market, while a domestic Vitamin C market needs to be opened to exploit domestic consumption potential.

2.2 *Long-Chain Dicarboxylic Acids*

Long-chain dicarboxylic acid (DC) refers to an aliphatic terminal dicarboxylic acid containing more than 10 carbon atoms in the carbon chain. It is an important fine chemical product with wide application [4]. It is used as a raw material for chemical synthesis of musk perfume, high-performance nylon engineering plastics, high-grade nylon adhesives, high-temperature electrolyte, nylon powder coatings and paints, lubricants, cold resistance plasticizers, resins, poly anhydride, drugs, and pesticides.

At present, there are only two industrial-scale chemical plants that produce the long-chain dicarboxylic acid using a biological process in the world. The first is the Guangtong Chemical Limited Company in Shandong Province, China. Its production capacity reached 1,000 tons annually in 1999. The second plant of Cathy Holding Co. Ltd., which was built in early 2003 in Shandong Province, had a production capacity of 7,000 tons per year. The production technologies of both plants are patented in China. The technology was developed by the Microbiological Institute of the Chinese Academy of Sciences sponsored by the Ministry of Science and Technology of China within the "Eighth 5-Year Plan" and the "Ninth 5-Year

Plan" of the National Technological Programs of China. Products include C_{11} to C_{17} (mainly C_{12} to C_{15}) long-chain dicarboxylic acid, which were sold to the US, EU, and Japan with an annual value of 400–500 million RMB Yuan.

A mixture of long-chain dicarboxylic acids or one component long-chain dicarboxylic acid can be produced by the fermentation process. The processes are substantially the same for both purposes, but differ in the raw material formulations and in strains [5]. The production steps are as follows [6]:

1. Screening the production strain. To date, strains for long-chain dicarboxylic acid research and industrial production have all been screened by mutagenesis from wild strains of *Candida tropicalis*. Dicarboxylic acid levels are, however, low for wild strains, which produce a short carbon chain dicarboxylic acid mixture with less than 10 carbon atoms. It was reported that the Microbiological Institute of the Chinese Academy of Sciences used NTG, ultraviolet, and sodium nitrite to mutate the strain, and the *C. tropicalis* U3221 mutant was obtained with a higher yield of long-chain dicarboxylic acid.
2. Fermentation process. As an example, to produce DC13, which contains 13 carbon atoms, the bacteria are added to the fermentation tank with bulk C_{13} *n*-alkane substrate and a ventilation ratio of 1:1, and incubated under ventilated stirring at 30°C for about 6 days. As a result, the concentration of the dicarboxylic acid reached 150 g/L or above. The carbon source can be double substrate feedstock (DSF) consisting of *n*-alkane and sugar (such as glucose, sucrose, or molasses). Various inorganic and organic nitrogen-containing compounds can be used as the nitrogen source, for which urea was preferred. In addition, minerals and growth factors such as phosphate and magnesium sulfate were also required.
3. The downstream process. It was critical to develop an efficient downstream process for long-chain dicarboxylic acid production because the fermentation broth contained unused small particles, emulsion of oil and water, cells, and an "aqueous phase" with several medium components. As a heterogeneous, multicomponent complex dispersion system it was obviously different from other fermentation systems, leading to difficulties in separation. The main equipment in the downstream process includes a cell centrifuge, free acid centrifuges, granulators, production dryer, and the acid tank. The isolation and purification process consisted of solvent extraction, alkalization filtration, and solvent-free sections. Solvent extraction and alkaline complex filtration methods are complex and have poor product quality. A solvent-free separation process is very simple. Microporous filtration is employed at present.

The main applications [7] of long-chain dicarboxylic acid are as follows. DC13 is used to synthesize perfume. DC12 is used for production of nylon, especially nylon 1212 with a good market potential. As dicarboxylic acid derivatives have a tremendous potential for development, the demands for dicarboxylic acid will continue to increase. Demand for DC13 will be up to 300 tons, and DC12 demand reached 4,500 tons in 2008. The international market for DC15 is about 200–300 tons, but the market is underdeveloped. The current price of DC12 is

about 60,000 RMB Yuan/ton, DC13 76,900 Yuan/ton, and DC15 150,000–200,000 Yuan/ton. The price of nylon 1212 is about 94,000 Yuan/ton, and import high-grade nylon hot melt adhesive reaches 80,000–100,000 Yuan/ton. The export price of DC13 is 90,000 Yuan/ton. Comparing the domestic price with foreign products, the development of long-chain dicarboxylic acid and its derivative products is very competitive in the market.

2.3 Citric Acid

Citric acid, also known as 2-hydroxy-1,2,3-propanetricarboxylic acid, or 2-hydroxy-tricarballylic acid, is an important organic acid with wide applications in food, medicine, feed, cosmetics, detergents, building materials, plastics, and other industrial fields. It is also useful in environmental protection [8].

Citric acid was identified more than 200 years ago. As early as 1784, citric acid was extracted from lemon juice and obtained in its crystal form for the first time by Scheel. In 1917, Carrie succeeded in producing citric acid by fermentation with *Aspergillus niger*. From then on, large-scale industrial production of citric acid began [9]. In China, industrial production of citric acid started in the 1960s. In the 1960s and 1970s, there were only a few manufacturers, with production of not more than 1,000 tons. China mainly relied on imported citric acid at that time to meet domestic demand. The citric acid industry in China developed rapidly in the 1980s to 1990s. Output reached 300,000 tons by 1999, with an average annual growth of more than 30% [10]. The total output was more than 400,000 tons in 2000, and reached 450,000 tons in 2003 [11]. In 2005, output reached 630,000 tons, accounting for 44% of the world output, of which 485,100 tons were exported, representing 50% of the international citric acid trade [12]. There are about 100 citric acid manufacturers spread over every Province except for Tibet. Production scales vary from a few hundred tons to tens of thousands of tons. After 40 years of development, China has become the largest citric acid producer and exporter in the world, and citric acid is one of the leading chemical products by fermentation in China. At present, 80% of China's citric acid production is for export, accounting for 60% of citric acid trade on the world market.

There are three main methods for citric acid production: i.e. chemical synthesis, extraction from fruits, and microbial fermentation. Currently, nearly all citric acid is produced by fermentation, using sugar beet molasses, sugar cane molasses, glucose crystal liquor, starch, liquid paraffin, or fruit residues as feedstock, by *A. niger* or *Saccharomyces* [13].

In China, some less expensive and easily available raw materials, such as corn, molasses, and cassava, have been used in liquid submerged fermentation for the production of citric acid. The production process was simple and it was a leading technique in the world. The unique production strain of *A. niger* and special bioprocess technology introduced by Tianjin Research Institute of Industrial Microbiology (TRIIM) has been exported to Europe, the United States and other countries. In recent years, maize flour, rice flour, rice, and straw have been used

as raw materials. The production process was improved, resulting in a conversion rate exceeding 95%, fermentation period of less than 70 h, total yield raised by 5%, and significant cost reduction [14].

In the future, long-term short supply of raw materials, especially corn, will lead to an increasing price for citric acid. So, it is essential to develop new processes with low cost raw materials. With the expansion in citric acid application fields and the improvement of living standards, domestic and international demand for citric acid will continue to rise. At present, the per capita domestic consumption of citric acid in China is much lower than that at the international level. Therefore, there is a huge domestic market potential in China. Therefore, there are good prospects for development of the citric acid market in China.

2.4 Glutamic Acid

Glutamic acid has the largest share by tonnage of the current international amino acid market. It is widely distributed in the body of animals and plants. As the intermediate of monosodium glutamate (MSG), it is mainly used for cooking and food processing [15].

In China, glutamic acid production can be traced back to the early 1920s. Wu Yunchu, a chemist, first developed a method to produce glutamic acid by hydrolysis of gluten using sulfuric acid, and this was used to produce glutamic acid-based pharmaceuticals before the early 1950s. However, the process was low-yield and heavily polluting. In 1958, the Shanghai Tianchu MSG factory succeeded in glutamic acid production using Corynebacterium from starch.

With the development of fermentation technology, the output of glutamic acid was increased from 600 tons in the early 1950s to 6,000 tons in 1960. In the mid-1980s, the output of glutamic acid reached 80,000 tons, reaching 650,000 tons in the mid-1990s. In 2000, the output was one million tons. The total output of glutamic acid has reached 1.19 million tons in the last few years and China has assumed the top spot in terms of world glutamic acid production.

After decades of development of the domestic market and competition, the number of glutamic acid producers decreased continually from 300 to 200 in the 1980s, to 80 in 2006. Among these manufacturers, 17 have an output of more than 10,000 tons/year, and only seven or eight factories, including Henan Lianhua Group, Shandong Linghua Group, Shandong Snowflakes, Shenyang Hongmei Group, Jiangsu Juhua, and Hebei Meihua, have at least a 50,000 ton per year capacity. In addition, Wenzhou Kuailu and Zhejiang Yiwu Mifeng Group have grown rapidly in recent years. Presently, Henan Lianhua Group is the leading domestic enterprise for glutamic acid production, with a capacity of glutamic acid production by fermentation of 300,000 tons per year, and actual sales of 220,000 tons, of which the export volume accounted for 40%, about 90,000 tons in 2006. Presently, glutamic acid production of the Henan Lianhua Group accounts

for 40% of domestic market share, and it ranks first in glutamic acid productivity at home and second to Japan's Ajinomoto in the world.

Total output of sodium glutamate in China reached over 1.7 million tons in 2007, which was 23.75% higher than that in 2005, the total revenue of glutamic acid and sodium glutamic acid reached 17.79 billion RMB Yuan, and the profits reached 1.26 billion Yuan. Exports of Henan Lianhua Group represents 90% of the total export volume. Glutamic acid products from domestic factories led by Henan Lianhua Group have been sold to 57 countries, including Britain, France, Italy, Switzerland, and the United States. With the improvement in the quality of glutamic acid exported, its international market price has also increased. According to statistics of the foreign trade sector in China, the export price of glutamic acid and its salts has increased by 10% to 12% compared to 5 years ago.

Undoubtedly, sodium glutamic acid has now become one of the dominant amino acid products in terms of global sales share, its market growth rate is about 4%, and therefore there are good prospects for the development of glutamic acid. However, international production of glutamic acid by fermentation uses mainly sugarcane molasses as raw materials which are more reliable in terms of supply, and less expensive than corn starch feedstock [16]. The corn starch fermentation process developed in the 1980s is still used by the majority of domestic producers. Therefore, we must solve the problem of cost of production through the introduction of new strains and fermentation from sugarcane molasses [17].

2.5 *Validamycin*

Validamycin is a multicomponent aminoglycoside antibiotic widely used in Asia for control of sheath blight disease in rice plants and others as well as damping off diseases in vegetable seedlings, cotton, sugar beets, corn, and other plants caused by the fungus *Rhizoctonia solani* [18]. Validamycin is an excellent biopesticide, which is harmless to people, domestic animals, fish and plants, and has low dosage requirements and long-term efficiency [19].

In 1966, an antibiotic was extracted from *Streptomyces hygrosropicus* var. *Limoneus* isolated from soil in Japan. It was effective in controlling sheath blight disease in rice plants, and named validamycin. In 1972, an antibiotic, having the same effect as the validamycin isolated from Japan, was obtained from the fermentation broth of *S. hygrosropicus* var. *yingchengensis* isolated from rice soil by Huazhong Agricultural University in Yingcheng, Hubei Province, China, but the chemical potency of the antibiotic was low. *S. hygrosropicus* var. *jinggangsis Yen*, isolated from soil by Shanghai Pesticide Research Institute in Jinggang Mountain, Jiangxi province in 1973, was a superior validamycin producer [20]. After being mutagenized by ultraviolet, nitrogen mustard, NTG, and so on, the *S. hygrosropicus* var. *jinggangsis Yen* variants could produce large amounts of validamycin. Now *S. hygrosropicus* var. *jinggangsis Yen* has become the main producer for validamycin production in China, and the chemical potency of validamycin has been

enhanced from 10,000 μg/mL to 30,000 μg/mL [21]. In 2006, a validamycin biosynthetic gene cluster was cloned successfully and a novel biosynthetic model of validamycin was proposed by Shanghai Jiaotong University in cooperation with Oregon State University and the University of Washington. The work will play a key role in lowering production cost, improving production levels and activity, screening high producing strains, and production of further validamycin derivatives [22].

In China, validamycin was first produced by Shanghai 18th Pharmaceutical Plant, Yixing Bio-Pesticide, and Zhejiang Haining Pesticide in 1975. During the 1970s and 1980s, there was large numbers of factories producing validamycin but they were only small-scale and they only served the local area. However, five million tons of rice was saved every year through the use of validamycin at that time. Over the past 30 years, the scale of validamycin production has increased tremendously, and 90% of the sheath blight disease in rice has been controlled. Validamycin was used in China over an area of 12.5 million ha. The annual output of validamycin in China was stable at about 40,000 tons, which is the largest in world, and some of the product was exported to Japan, Korea, Thailand, and Singapore.

Because of its high efficiency, low toxicity, environmentally friendly characteristics and stable market, validamycin will still be a leading biopesticide for controlling sheath blight disease for years to come in China, and even in Southeast Asia.

3 Fine Chemicals, Active Pharmaceutical Ingredients, and Nutraceuticals

Fine chemicals are one of the most vigorous new fields in industrial chemicals. The annual growth rate is about 5–6% which is higher than that of the chemical industry by 2–3 percentage points. Fine chemical engineering is one of six priority developing fields in the 2005 to 2010 Plan in China. Some parts of the fine chemicals field in China are at a leading level in the world. China is an important producer and exporter of fine chemicals. The statistical report revealed that there are over 30,000 kinds of fine chemicals in China. The production capacity of fine chemicals in China is up to 135 million tons and output is up to 97 million tons annually [23].

In order to increase product competition, raise economic profile, and resolve the problems of energy shortage, resource shortage and environmental protection, development of new technology in fine chemical engineering should be a key requirement. Recently, biocatalysis has become a new breakthrough area in the fine chemical field. Biocatalysis has mild reaction conditions, saves energy, high transformation efficiency, and is environmentally friendly. Biocatalysis has various merits in synthesis of chiral compounds, bioactive complex macromolecular compounds, and biopolymers [24].

Today, humans are afflicted with severe diseases, such as tumors, vascular disease, and diabetes. New lead compounds are needed to resolve these problems. Optimization of molecular structure of lead compounds is the main task in new drug

discovery. Biocatalysis has been broadly employed in the field of new drug discovery. Because of the high selectivity, biocatalysis can be employed in reactions that are difficult to carry out by ordinary chemical synthesis. Biocatalysis, as an important supplementary method in chemical synthesis, has unique potential for modification of molecular structure. Through the diversity of biocatalysis, the abundance of molecular structures of active pharmaceutical ingredients in Chinese traditional medicines can be enhanced. Among those active compounds, lead compounds with better bioactivity and bioavailability should be easier to discover, and would be utilized in R&D of new drugs. Nowadays, including various natural product derivatives with better bioactivities, a great quantity of compounds with novel structures have been attained by biotransformation, which provides various lead compounds with high value in new drug discovery. Industrialization of biotransformation has created enormous economic profit. Active pharmaceutical ingredients in Chinese traditional medicines are the material base for treatment and prevention of diseases, and also provide the main source of the new active compounds sought by pharmaceutical chemists. With improvement in separation technology, thousands of active compounds have been isolated. Among those compounds, many have been successfully developed into new drugs, such as apoplon, digoxin, arteannuin, ephedrine, and taxinol. But because of low content, high toxicity and side effects, and poor water solubility, application of many active pharmaceutical ingredients in Chinese traditional medicines is limited. Through biotransformation of active pharmaceutical ingredients in Chinese traditional medicines, new compounds with improved activities and lower toxicity can be attained. The cheap pharmaceutical ingredients with low activity in Chinese traditional medicines are transformed into highly active pharmaceutical compounds with high added value at a large scale by industrial biotechnology [25].

Chiral compounds are important in the field of fine chemicals. Chiral compounds are precursors, intermediates and products of medicines, pesticides, and perfumes. Global sales of various chiral compounds were 88 billion dollars in 1997, and up to 372 billion dollars in 2004. Biocatalysis has unique priority in the synthesis of chiral compounds [26].

In total about 460 types of food additives are approved by China. The food additives produced by industrial biotechnology represented about 70% of 32 billion Yuan in 2004. Therefore, biotechnology has important effects on food additives. Industrial biotechnology has broad applications in the fields of fine chemicals, active pharmaceutical ingredients, and food additives. Some examples of biotechnology industrialization in China follow.

3.1 Acrylamide

Acrylamide is an important organic chemical used in many areas with great demand. More than 90% of the output of acrylamide is used to produce polyacrylamide and its derivatives as homopolymers and copolymers. Polyacrylamide is used extensively in the fields of oil exploitation, water treatment, textile dying,

papermaking, ore dressing, coal washing, medicine, sugar refining, building materials, and chemical engineering. The global total demand of polyacrylamide is over 650,000 tons/year. The domestic total demand of polyacrylamide is over 160,000 tons in China.

The catalysts used in the industrial production of acrylamide have varied from sulfuric acid to copper compounds and nitrile hydratase. Nitrile hydratase is found in many plants and microorganisms. Galzy et al. were the first to report on an enzymatic hydrolysis using the microorganism *Brevibacterium R312* to produce acrylamide. The Japanese company Nittoco was the first to produce acrylamide industrially by biocatalysis. Major work on producing acrylamide by biocatalysis in China was led by Prof. Sheng Yinchu with the Shanghai Pesticide Research Institute in 1984, and this work has attained great importance in the last few years. A high-yield nitrile hydratase-producing strain *Nocardia* sp. 86-163 was finally obtained successfully by extensive screening. In 1994, the first acrylamide-producing industrial plant with 1,500 tons annual output was set up, and a plant with 10,000 tons annual output was completed in 2000. The acrylamide produced by biocatalysis had a high purity of 99.9% and can be used to synthesize polyacrylamides with ultra-high molecular weight, which have played important roles in oil exploitation and water treatment. Prof. Shen Yinchu was awarded the Prize of the Second Award of National Science and Technology Progress in 1998 [27, 28].

3.2 Amino Acid

L-amino acid is an important physiologically active compound, and it is widely applied in food, medicine, and animal feed areas. The total output of sodium glutamate in China surpassed 1.7 million tons in 2007, which is about 70% of global production and China ranks first in the world. The export amount of glutamic acid exceeded 100,000 tons in 2006 [29]. As an important amino acid, methionine is an essential additive used in animal feed helpful in providing rapid animal growth over a short period of time, saving about 40% of the feed. Thus, consumption of methionine in feed additive is becoming the largest market in China. Methionine production capacity has reached over 1,000 tons and the annual output is about 700 tons in China. A new methionine production line with a total annual capacity of 140,000 tons has been designed and finalized by Bluestar and Adisseo in China, and it will be in full production in 2011 [30, 31]. In the past 10 years, production scale and output of lysine in the world have achieved sustained growth. Global lysine output was about 200,000 tons in 1993, up to 500,000 tons in 1999, 600,000 tons in 2001, and 700,000 tons currently. In the past 10 years, market demand for lysine as a feed additive has increased rapidly, which stimulated rapid development of production and output of lysine. The total production capacity for lysine was more than 10,000 tons in China in the early 1990s. By the mid-1990s, production capacity had expanded to 50,000 tons. It reached 100,000 tons at the beginning of the twenty-first century and is 350,000 tons at present [32, 33]. L-phenylalanine,

which cannot be synthesized by human beings and other animals themselves, is one of eight kinds of essential amino acids and an important raw material in the biological synthesis of tyrosine. The most important applications of L-phenylalanine is for synthesis of aspartame and for nutrition enhancers, amino acid infusion and the preparation of the amino acid components. In recent years, with the extensive production of aspartame and applications in drugs and amino acids, nutrition and health, the global demand of L-phenylalanine has amplified rapidly. According to statistics, annual demand of L-phenylalanine is more than 30,000 tons in the current global market [34]. In 2005, the domestic market in China for L-phenylalanine reached about 3,000 tons.

3.3 Citric Acid

Citric acid as an important sour taste additive is mainly used in food, medicine, and the chemical industry. At present, China has nearly one hundred citric acid manufacturing plants with a total production capacity of about 800,000 tons. China is the largest citric acid producing and exporting country in the world. In the last few years, the volume of exports of citric acid represented more than 90% of domestic output, and the proportion of global trade volume has already surpassed 60% [35].

Global demand for citric acid is about 130,000 tons and increased by 5% to 7%. In 2007, global demand for citric acid continued to increase at a rate of 3%. With the improvement in living standards in China, domestic demand for citric acid increased substantially. The major consumers of citric acid are in the food and beverage market, other consumer areas include pharmaceuticals, cosmetics, detergents, and industrial application. The food and beverages market accounted for about 70% and the others 30% of utilized citric acid.

3.4 L-Malic Acid

L-malic acid, the intermediate in the TCA (tricarboxylic acid) cycle, is tasted of natural fruit juice in flavor. Compared with citric acid, it lowers the heat, and tastes better. Therefore, it is widely utilized in alcohol, beverages, jams, chewing gum and other foods, and is gradually replacing citric acid. It is the world's most widely used organic acid in the food industry and has a bright future as one of the best organic acids. L-malic acid can be used for pharmaceutical preparations, tablets, syrups, and can be added to a solution of amino acids to improve their absorption rates. L-malic acid can also be used to treat liver disease, anemia, low immunity, uremia, hypertension, liver failure and other diseases and can reduce the toxic effect of anticancer drugs on normal cells and it can also be used for the preparation of synthetic insecticides, antitartar agents and so on. L-malic acid can also be used

in industrial cleaning agents, resin curing agents, synthetic material plasticizers, food additives etc.

The demand for L-malic acid has increased rapidly in recent years at an annual average rate of 10% on the international market. The major producers of L-malic acid are the United States, Canada, and Japan. The annual world production of malic acid reached 100,000 tons, of which L-malic acid production is about 40,000 tons. Moreover, Japan is an L-malic acid producing and exporting country. China domestic L-malic acid production reached 5,000 tons per year, with the Changmao Biochemical Engineering Co. Ltd. as the largest domestic producer. On the basis of coupling reaction and separation technology, the technical and economic indicators of L-malic acid have reached an internationally advanced level, and Nanjing University of Technology was awarded the Prize of the First Award of National Science and Technology Progress in 2001 [36].

3.5 7-APA and 7-ADCA

β-Lactam antibiotics including penicillin and cephalosporin-like antibiotics have the largest variety in the global pharmaceutical sales area. The rapid growth of amoxicillin, and other semisynthetic β-lactam antibiotics stimulated the growth in output of 6-APA (6-aminopenicillanic acid). In 2000, the output of 6-APA was 1,921.5 tons, which increased by 42.4% to 2,736.4 tons in 2001. And in the first quarter of 2002, the output of 6-APA was 751.6 tons, an increase of 83.1% compared with the same period of the previous year. At present, China's industrial penicillin salt represents 28,000 tons of fermentation production, accounting for 60% of the world market share.

6-APA is a raw material for semisynthetic penicillin. The industrial application is enzyme transformation, via immobilized cell technology to produce 6-APA. Penicillin acylase is a significant enzyme in the semisynthetic β-lactam antibiotics industry.

The Laboratory of Molecular Microbiology, belonging to the Institute of Plant Physiology and Ecology of the Chinese Academy of Sciences, has made great progress in the research of semisynthetic β-lactam antibiotic industrial enzymes. The researchers conducted a two-step enzymatic conversion study from cephalosporin C to 7-ACA. They constructed a polyploidy triangular yeast to produce D-amino acid oxidase and GL-7-ACA acylase, and developed many industrial strains such as host bacteria and genetic engineering bacteria used by gene cloning, in vitro gene passivation, recombinant DNA and cell fusion, and other biotechnologies. And then, they established a new technical process called "two steps for production of 7-ACA," including the fermentation of enzyme-producing strains, the enzymatic conversion of cephalosporin C, and the extraction and purification of products etc. Later on, the laboratory cooperated with Zhejiang Haizheng Pharmaceutical Co. Ltd, and carried out an expansion plan as part of the "Ninth 5-Year Plan" research programs. In the 150-L enzymatic reactor, when the refined solution

of the fermentation broth was 3% cephalosporin C, products were directly converted into 7-ACA by two-step enzymatic conversion without crystallization, and the total yield was 73.4%.

The cephalosporin C and a considerable part of the industrial penicillin salt were used in the manufacture of the corresponding 6-APA, 7-aminodesacetoxycephalosporanic acid (7-ADCA) and 7-aminocephalosporanic acid (7-ACA), and other intermediates, and thus made series of semisynthetic β-lactam antibiotics with a variety of therapeutic effects. Since development of cephalosporin intermediate raw material production in China was slow, cephalosporin drugs derived from 7-ACA and 7-ADCA were dependent on imports. This situation began to change in the late 1990s. The General Pharmaceutical Factory of Harbin Pharmaceutical Group took the lead in the production of important raw materials used for 6-APA, 7-ACA, and 7-ADCA. Domestic pharmaceutical companies built their own 7-ADCA production lines, such as the North China Pharmaceutical Factory, Fujian Fukang Pharmaceutical Co. Ltd., and Shandong Lukang Pharmaceutical Co. Ltd. In cooperation with Sichuan Institute of Antibiotics, Sichuan Changzheng Pharmaceutical Factory developed a 7-ADCA production line with an annual output of 100 tons. By 2006, domestic annual output of 7-ADCA had reached 1,000 tons. With the development of 3,000 tons of new production lines by the Shandong Lunan Pharmaceutical Group, China became the largest producer of cephalosporin. According to statistics from Chinese Chemical and Pharmaceutical Associations, China's 7-ADCA output was 589 tons in 2004 of which exports accounted for about 32%, 800 tons in 2005, and more than 1,000 tons in 2006, and it was estimated that the total output would be over 1,500 tons in 2007. Recently, China's 7-ADCA output exceeded 4,000 tons, and it will be over 5,000 tons with the launch of production lines by the Lunan Group and other pharmaceutical production companies [37, 38].

3.6 *p*-Hydroxyphenyl Glycine

As a pharmaceutical intermediate, *p*-hydroxyphenyl glycine is mainly used for half synthesis of β-lactam antibiotics, such as in amoxicillin, hydroxybenzyl cephalosporins, chlorobenzene cephalosporins (Pioneer IV), cefoperazone, cephalosporins Roach, hydroxylamine cephalosporins, cefaclor, cefradine (Pioneer VI), cephalosporins Qu triazine, which established broad-spectrum antibiotics. In the synthesis process, the D-*p*-hydroxyphenyl glycine side chain is essential. These antibiotics have excellent oral availability, and substituted the widespread use of penicillin G and are more extensively applied in clinical practice. Mass production of these antibiotics further promoted the development of the D-*p*-hydroxyphenyl glycine production process. In 1999, the global demand for D-*p*-hydroxyphenyl glycine for the production of amoxicillin amounted to 80,000 tons. The main producers of D-*p*-hydroxyphenyl glycine include companies such as Japanese Synthetic Chemistry, High Chem Co. Ltd., the Netherlands, and ANDN of Spain, and there

are manufacturers in South Korea, Sweden, India, Germany, and other countries. The development of production of D-*p*-hydroxyphenyl glycine started in the 1980s in China. Small-scale production began in the mid-1990s. In recent years, both domestic demand and production of amoxicillin increased rapidly. A number of production lines for antibiotics, semisynthetic antibiotics, and cephalosporins passed the qualification of the State Food and Drug Administration of China. North China Pharmaceutical, Hebei Pharmaceutical, Lukang Pharmaceutical Co. Ltd, Shan'xi Maite, Shanghai 4th Pharmaceuticals, and Livzon Pharmaceutical Group Inc. are the major producers. The output of amoxicillin was increased from 100 to 700 tons/year at an annual growth rate of 20–30%. The production output of D-*p*-hydroxyphenyl glycine exceeded the domestic market demand in 2002. Nearly 90% of the manufacturers of D-*p*-hydroxyphenyl glycine in China export some of their product currently.

The largest manufacturer of D-*p*-hydroxyphenyl glycine in China is Yangzi Pharmaceutical and Chemical Co. Ltd. at Taixing with 600 tons/year capacity. Nanjing University of Technology developed a new production process for D-*p*-hydroxyphenyl using a "one bacterium two enzyme in one-step" technique, in which hydantoin hydrolase and carbamoyl hydrolase produced from *Pseudomonas* No.1 were used in the transformation of 4-hydroxyphenyl hydantoin to D-*p*-hydroxyphenyl glycine in one-step through enzymatic hydrolysis. The technology integrated synthesis of the substrate 4-hydroxyphenyl hydantoin, culture of bacteria, fermentation process and enzyme production, and enzymatic transformation. The conversion rate was over 98%, and the yield reached 80% [39, 40].

3.7 Vitamin C

According to the statistics of the Chinese Chemical and Pharmaceutical Industry Associations and Feed Industry Association, total annual output capacity of various types of vitamin is about 200,000 tons in China. Of this figure the production of choline chloride is about 100,000 tons, accounting for 40% of global output; the production of vitamin C is about 48,700 tons, accounting for 40% of global output; the production of vitamin E is about 12,000 tons, accounting for 30% of global output; the production of vitamin A is 3,000 tons, accounting for 10% of global output; and other vitamins represent about 20,000 tons. Production and demand in China of feed-grade vitamins accounts for a significant amount of the global total production, of which domestic demand is about 120,000 tons, which represents 20% of the global market demand for feed-grade vitamins. China has become the world's largest market for vitamins.

The Shanghai Biological Technology Research Institute of the Chinese Academy of Science, invented a two-stage fermentation process for vitamin C production and were leaders in regard to this technology in the world. Roche improved its own vitamin C production technology after buying the Chinese Patent. Several producers in China have already emerged as powerful vitamin suppliers. The

annual production capacity of vitamin C for the Northeast Pharmaceutical Factory, North China Pharmaceutical Group, Jiangsu Aland Pharmaceutical, and Shijiazhuang Pharmaceutical Group accounted for more than 40% of global output. The export of vitamin C has exceeded 50,000 tons, which accounted for more than half of the total global consumption. The remarkable features of the current technology were the high yield strain from ion beam bombardment and mutation technology, and the giant tank of 300–1,000 m^3 for the fermentation [41, 42].

3.8 Nicotinamide

Nicotinamide is a component of coenzyme-I and coenzyme-II. Nicotinamide and nicotinic acid are forms of vitamin B3. The nicotinic acid can be transformed into nicotinamide in the animal body. A lack of nicotinamide and nicotinic acid leads to lesions of the skin and digestive tract, angular cheilitis and insane skin disease in animal. Therefore, nicotinamide and nicotinic acid have important applications in medicine, food, and feed. At present, the global market is 40,000 tons per year. China's domestic production cannot meet demand, and is partly dependent on imports. Traditional production methods for nicotinamide include nicotine acid ammonization and smoke sacrificial lye solution. Domestic factories mostly use the second method. But the technique is out of date. The production scale was small, the cost was high, and the total output was low.

Lonza is the main producer of nicotinamide with the largest global share. With the technology of Nittoco, Japan, Lonza has established the first industrial unit globally to produce nicotinamide by a microbiological method. Guangzhou Longsha Co. Ltd., which belonged to Lonza Corporation, has established nicotinamide industrial equipment using the microbiological method with an annual output of 3,400 tons. Subsequently, the Shanghai Agricultural Chemicals Research Institute has also developed production technology for nicotinamide using a microbiological method. Moreover, the industrial equipment was established in Zhejiang Xinchang in 2003. The characteristics of the microbiological method were simple operation, mild reaction conditions, little environmental pollution, easy separation, and high product purity [43].

3.9 D-Pantothenic Acid

D-pantothenic acid, also known as vitamin B_5, is a structural block of coenzyme A. D-pantothenic acid participates in the degradation of fatty acids, synthesis of fatty acid, citric acid cycle, and synthesis of antibodies. It is pivotal in metabolism of all kinds of nutritional components. Because the pantothenic acid is unstable upon exposure to heat, alkali, or acid, its commodity form is mainly the calcium D-pantothenate. The D-pantothenic acid is important in medicine, and in food and feed additives. Calcium D-pantothenate is widely applied with great market demand.

The key technology for producing D-pantothenic acid is chiral resolution of the racemic intermediate (DL-pantoic acid lactone). Formerly, chiral separation of DL-pantoic acid lactone mostly used chemical methods. Those methods were uncompetitive due to the high price of chiral reagent, difficulty in separation, environmental pollution, and toxic solvents. Therefore, development of enzymatic catalysis resolution was becoming more of a concern. In 1998, large-scale production of calcium D-pantothenate was realized by using an enzymatic process at Japanese Fuji Pharmaceuticals. In 2001, Prof. Sun Zhihao at Jiangnan University, China, reported the screening of a microorganism strain *Fusarium moniliforme Sheld Fusarium moniliforme* SW902, which could produce large quantity of DL-pantoic acid lactone hydrolytic enzyme with high stereospecificity. The advantages of this catalytic reaction using pantoic acid lactone hydrolytic enzyme are short conversion time (5–10 h), repeated use (180 or more times), and the high optical purity of the hydrolysate (above 99%). This process was industrialized by Hangzhou Xinfu Pharmaceutical Co. Ltd, and calcium D-pantothenate output reached 2,000 tons/year in 2002. In 2005 the output reached 5,000 tons/year. This technology was awarded the Second Prize of State Scientific and Technological Progress in 2003 [44].

3.10 Lycopene

Lycopene is the strongest singlet oxygen quenching agent and the strongest antioxidant among all carotenoids. After in-depth study of the biological activity of lycopene, especially epidemiological and clinical studies showed that lycopene is the strongest antioxidant. Its antioxidant activity is three times higher than that of β-carotene and is 100-times that of vitamin E. Lycopene has biological functions including antioxidation and mutation inhibition, thereby reducing DNA damage. It has antiaging, cancer and cardiovascular disease prevention, immunity enhancing, atherosclerosis amelioration, cosmetology, anti-ultraviolet radiation, and other functions. Lycopene can inhibit prostate, breast, and pancreatic cancers. Moreover, lycopene has beneficial effect on the angiosclerosis and coronary disease. Lycopene products are widely used in food additives, functional food, and pharmaceuticals. Lycopene products are one of the most popular functional food additives. In recent years, lycopene has become a new hot spot for research and development in the area of biotechnology. Israeli companies developed the first lycopene product and established a distribution agency in Europe and North America. Japanese companies also applied for a lot of related patents in the field. The United States and Europe approved the use of the product in 1988. The total sales of lycopene in 1990 were about $300 million. Now the annual production of lycopene is more than 1,000 tons. North China Pharmaceutical Group Corporation, Jiangsu Aland Pharmaceutical Corporation, Nanjing University of Technology and others used *Blakeslea trispora* to implement industrial production of lycopene. By means of a low-energy ion beam mutagenesis strain, mixed seed cultivation, metabolic path engineering, a new type of metabolism blocker, and optimal conditions for

fermentation, the fermentation level of lycopene has been significantly improved. The fermentation level of lycopene reached 1.5 g/L and the extraction yield of lycopene is more than 80% [45].

3.11 β-Carotene

Natural β-carotene from fermentation is a new product in the fermentation industry which was approved by the National Ministry of Health as a new food additive in 1998. It is widely used in food, beverage, and feed additives for its physiological functions in relation to vitamins and as a nutritional enhancer and coloring agent. In the international market, the quantity of β-carotene is about 1,200–1,500 tons per year, of which 95% is of synthetic origin. As the US FDA (Food and Drug Administration) declared that synthetic β-carotene cannot be used as a food and nutritional supplement, the production of natural β-carotene by microorganisms has been greatly promoted. *B. trispora* has been used to produce natural β-carotene under mixed culture with (+) and (−) strains. New techniques including mixed seed cultivation, air-lift fermentation, counter-flow extraction, new crystallization methods, and improvement of microcapsules, have led to a breakthrough in the production of β-carotene. All β-carotene products meet the requirements of international quality standards, and the purity was close to 100%. In a 10 m^3 fermentor, the fermentation level stably arrived at 3.0 g/L, and the extraction yield achieved 75% [46].

4 Bio-Based Bulk Chemicals and Polymers

4.1 ε-Poly-L-lysine

ε-Poly-L-lysine (ε-PL) consists of 25–35 lysine residues. The product is a light yellow powder and is slightly bitter. ε-Poly-L-lysine is a food preservative having superior antimicrobial performance, wide spectrum antimicrobial activity, and it can inhibit Gram-positive bacteria, Gram-negative bacteria and fungus. Its advantages include high safety, good water solubility, and high thermal stability. The ε-PL producing strains can be divided into two main categories, based on the degree of polymerization of the produced ε-poly-L-lysine. One is represented by strains producing high degrees of polymerization, for example, *Streptomyces albulus* subsp. *lysinopolymerus* No. 346 strain (Fermentation Research Institute, No. 3834), No.11011A-1 (Fermentation Research Institute, No. 1109), No. 50833 strain (Fermentation Research Institute, No. 1110), *S. albulus* strain no. 410 and the like; this type is commonly capable of producing ε-PL with a degree of polymerization between 25 and 35 on a large scale. The other is represented by strains producing middle to low degrees of polymerization of ε-PL, for example, *S. albulus*

subsp. sp-25 strain's (FERM P-17998) variant strain, *Streptomyces lavendulae* USE-53 strain (FERM P-18350), *Kitasatospora kifunense* strain MN-1 and the like; this type is commonly capable of producing ε-poly-L-lysine with a degree of polymerization between 10 and 19 in low yield [47].

A poly-L-lysine-producing strain was screened from soil, and it was preliminarily established to belong to *Kitasatospora*, and designated *Kitasatospora* sp. PL6-3, by combining morphological features, culture characteristics and chemical components of the cell wall of the strain and research results of the system development by 16S rDNA complete sequence analysis, with reference to physiological and biochemical properties. Using PL6-3 as the original strain, a S-AEC-resistant mutant *Kitasatospora* sp. MY5-36 with high yield was obtained by diethyl sulfate mutagenesis. The highest yield achieved by shake flask fermentation was 1.17 g/L, and the yield from fermenter culture was up to 13 g/L. Because of the problem of low content in the production of ε-PL, there are many difficulties with regard to isolation and purification to obtain a high purity product. A cationic ZH-5 resin was selected to separate ε-PL, which was high in adsorption capacity and selectivity for ε-PL. The adsorption capacity for ε-PL was 125.9 mg/g wet resin, and the purity and the yield of ε-PL obtained after the final isolation and purification were 88.3% and 87%, respectively. Finally, the crude product obtained after ion exchange was purified using Sephadex G-25 (81.2% yield), and the structure of the pure ε-PL product was then characterized by UV absorption spectrum, IR and ^{1}H NMR. As determined by SDS-PAGE electrophoresis and GPC, the relative molecular weights of ε-PL were 5.01 kDa and 5.05 kDa, respectively, and it was composed of 39–40 lysine monomers. Synthesis and degradation simultaneously occurred in the production of ε-PL, where the latter was caused by a ε-poly-L-lysine-degrading enzyme (PLD), so studies on the properties of PLD are very important to ensure the normal fermentation and high yield of ε-PL. The PLD enzyme was obtained by purification via three-step anionic ion exchange chromatography using DEAE-Sepharose, Source 15Q, and Mono Q, and was studied for its enzymological properties [48, 49].

4.2 D-Malic Acid and Poly-D-Malic Acid

D-malic acid (MLA) is a hydroxyl-containing dicarboxylic acid and has a molecular formula of $HOOCCH_2(OH)CH_2COOH$, which has high water solubility and is mainly involved in the TCA cycle in the body. Starting from maleic acid, MLA was prepared via enzymatic method with *Arthrobacter pascens*, which had a yield of 200 g/L, and a molar conversion rate of 93%. If calcium salt was used in the production, the yield was above 300 g/L, and the molar conversion rate was 99%, and the optical purity of the product was more than 97%. The calcium salt process can also be used in the production, to further increase the conversion rate and the optical purity [50].

Poly-D-malic acid (PMLA) is a homopolymer with MLA as the sole monomer, hydroxyl of one monomer and carboxyl of the other are linked by esterification to form a polymer compound in polymerization. Because MLA contains two carboxyl groups and one hydroxyl group, there are several products formed via esterification, and three PMLAs can be obtained through synthesis, i.e. α-, β-, and γ-types.

PMLA is a polyester-type polymer. Because of its special structure, biodegradability, and bioavailability, PMLA has promising applications in the pharmaceutical field, such as a pharmaceutical carrier and microcapsule material, as well as for biomedical material, such as surgical sutures, and bandages for wounds and burns treatment. In addition, PMLA is safe, nontoxic, and has high water solubility and water absorption properties, therefore it has important applications in the fields of food packing material, water absorbent material, and cosmetics. β-PMLA has a very attractive functional composition in relation to β-polyhydroxyl fatty acid ester, and can be used to produce superior degradable plastics. As a novel functional material and biological structural material, PMLA will surely have a promising future.

Commercial β-PMLA product is obtained mainly by chemical synthesis. However, chemical synthesis has high cost, severe operational conditions, and causes serious environmental pollution, so it conflicts with the currently developing tendency towards increasing awareness of environmental protection. In contrast, biosynthesis needs simple and readily available raw materials, produces high molecular weight and high purity product, and has mild reaction conditions. However, due to the stability and acid-producing capability of the strain, the fermentation process has not been industrialized.

Since 2004, the research group of Nanjing University of Technology, China, has carried out research on PMLA and has obtained an acid-producing strain of PMLA. The acid-producing conditions of the PMLA-producing strain were studied, the effects of carbon and nitrogen resources on acid production were investigated, and an optimized culture medium was obtained. The yield of PMLA reached 25 g/L by fed-batch culture. An isolation and extraction process for PMLA was also developed, and all of these have established a foundation for further research on application and properties of PMLA [51].

4.3 γ-Poly(glutamic acid)

γ-Polyglutamic acid (γ-PGA) is a water-soluble biological polymer. It has wide application potential in the fields of cosmetics, agriculture, pharmaceutics, and food etc., due to superior properties, such as nontoxicity, degradability, and high water absorption. γ-PGA, as a moisturizing agent, is used in cosmetics, whose moisturizing effect is 2–3-times that of hyaluronic acid; as a fertilizer synergist, γ-PGA can greatly reduce the dosage of fertilizer, decreasing environmental stress through fertilizer application, producing considerable economic benefit for farmers; high

water absorbent γ-PGA resin can be used in sanitary products, agriculture, forestry and horticulture, and sand fixing and vegetation materials.

A high yield γ-PGA strain, *Bacillus subtilis* CGMCC0833, was screened and systematically studied in relation to the metabolic mechanism, optimization of fermentation conditions, and scale-up of γ-PGA synthesis. In the fermentation production of γ-PGA in a 300-L fermentor, the average concentration of γ-PGA generated was 30.54 g/L, and the conversion rate of glutamic acid was 90%. Other types of PGA were developed such as γ-PGA (H^+-type polymer, food grade), γ-PGA (Na^+-type polymer, feedstuff/food/cosmetic grade), γ-PGA (Na^+-type oligomer, food/cosmetic grade), and γ-PGA (Na^+-type polymer hydrogel, industry grade). Preliminary studies on the application of γ-PGA in the fields of agriculture, water treatment, and cosmetics were also carried out [52–54].

4.4 Welan Gum

Welan gum is a microbial polysaccharide with excellent performance, whose properties are similar to those of Xanthan gum. It has a structural skeleton composed of repeat units of D-glucose, D-glucuronic acid, and L-rhamnose, and a side chain composed of single-chain L-mannose or single-chain L-rhamnose; the molecular weight is up to several millions. Welan gum can be widely used in petroleum, cement, and coating industries as a suspension agent, stabilizer, and thickener, and also in other industries as printing ink, food, textile and dye, pesticide, and pharmaceutics ingredients. A production strain, *Alcaligenes* sp. NX-3, was bred and developed, in which the batch fermentation yield was 25 g/L, the viscosity was above 9,000 *cp*, the fermentation time was 60 h, and the conversion rate was approximately 55%. In addition, the product can withstand a high temperature of 150°C, is stable at a pH ranging from 2.0 to 13.0 and has high salt resistance [55].

4.5 Lactic Acid and Polylactic Acid

Lactic acid, also known as α-hydroxyl propionic acid, has an asymmetric carbon atom in its molecule and thus is optically active, and it can be levorotatory, called L-lactic acid, dextrorotatory, called D-lactic acid, or racemic, called DL-lactic acid. Lactic acid is widely used as an acidic agent and preservative in the food industry due to its mild and stable acidity. L-lactic acid is harmless to humans and animals, and has a strong bactericidal effect, so it can be directly used as a disinfector in operating rooms, medical wards, laboratories, and industrial plants. Lactic acid can be polymerized to form a linear or cyclic polylactic acid (PLA). Poly-L-lactic acid is a nontoxic polymer, is biocompatible, and can be decomposed into L-lactic acid in the human body without the occurrence of allergic response. Poly-L-lactic acid plastic developed successfully in place of PVP and PP plastics can presently replace

the commonly used plastic products, so as to reduce the consumption of the nonrenewable resource petroleum, and PLA has been listed in a national key project.

There are more than 20 manufacturers of lactic acid in China with an annual production of about 30,000 tons. With regard to the production of L-lactic acid, progress has been made in all of the following technologies: *Rhizopus oryzae* and bacterial fermentation, continuous fermentation, and isolation of lactic acid from fermented solution by extraction, chromatography, and membrane technology, instead of the calcium salt process. With regard to the raw material, in addition to rice and corn, fermentation of corn starch and glucose has also been achieved. PLA, developed by Zhejiang Haizheng Group Co. Ltd., was reported to be in a pilot-scale stage with an annual output of 5,000 tons, and there was expectation to increase this to tens of thousands of tons or more.

The production strain *Bacillus coagulans* was employed and the acid production capability was up to 180–200 g/L, the feed conversion rate from glucose to acid was up to 95%, the fermentation cycle was 3–4 days, the extraction yield was 80–90%, and the optical purity of L-lactic acid was up to 99%, by anaerobic or microaerophilic fermentation at 50°C, with glucose from corn or tapioca starch as the raw material. Compared with other production methods of L-lactic acid, this method was characterized by the fact that *B. coagulans* was subjected to anaerobic fermentation, so lots of energy can be saved in contrast to aerobic fermentation (e.g. *R. oryzae*), and the apparatus for example the fermentor can be greater in dimension than that used for aerobic fermentation, facilitating large-scale production. The fermentation temperature of *B. coagulans* is 45–55°C (37°C for *R. oryzae*), at which temperature the fermentation medium can be directly fermented even without sterilization, so that the in situ extraction of lactic acid becomes more economical and feasible. In the process, glucose prepared from the starch of corn, tapioca, and rice etc., can be used in the fermentation production of L-lactic acid [56].

A D-lactic acid-producing strain with high optical purity of product was screened as the original strain, and a mutant with high sugar tolerance was obtained after multiple mutagenesis via nitrogen ion beam implantation. Starting from the main raw material glucose, *Sporolactobacillus* sp. was fermented to produce D-lactic acid with a yield of 140 g/L and an optical purity of above 99%. The conversion rate of glucose was above 98% [57].

4.6 L-Citramalic Acid

L-citramalic acid is a chiral building block, which can be used to synthesize pharmaceutical intermediates. Starting from itaconic acid, L-citramalic acid was prepared via enzymatic method with *Alcaligenes xylosoxydans*. The yield was up to 65 g/L, the molar conversion rate was up to 93%, and the optical purity was as high as 99%. D-citramalic acid could also be prepared via enzymatic method with *Arthrobacter pascens* starting from citraconic acid. The yield was up to 180 g/L, the molar conversion rate was up to 95%, and the optical purity was as high as 99% [58].

4.7 Propionic Acid

Propionic acid is an important fine chemical product and a basic chemical feedstock. As an important C_3-based compound, propionic acid and derivatives thereof are widely used in food, feedstuff, rubber, plastic, paint, coating, flavor, pharmaceutics, pesticide, and print ink. The biological process for preparing propionic acid has aroused more and more interest due to abundant raw materials and mild production conditions. In the long run, production of propionic acid as a commodity via biorefinery technologies is undoubtedly a route worth exploring.

A propionic acid-producing strain, *Propionibacterium freudenreichii* NX-4, was bred and developed. A novel fixed fiber-bed reactor was constructed and patents were filed. The production efficiency of propionic acid was up to 1 g/(L h), and the conversion rate of glucose was approximately 50% [52–54].

5 Lignocellulosic Bioethanol and Bioethylene

5.1 Introduction

Ethanol is an important solvent and intermediate in the chemical industry. It was first produced by carbohydrate fermentation thousands of years ago, and produced by catalytic hydration of ethylene industrially in the 1930s. The major applications for ethanol include as a solvent in pharmaceuticals, cosmetics, detergents, coatings, inks, etc. Ethanol is also used as a chemical intermediate for the manufacture of ethyl acetate, acetic acid, ethers, and ethylamine. However, a much larger and growing outlet for ethanol is as a liquid fuel. The petroleum price has driven the recent dramatic growth in ethanol production in the world. World fuel ethanol production was 33 billion L in 2005 [59].

Since the 1990s, China has developed high-speed growth in its economy. China was the fourth largest economic entity in the world with a GDP of 20.94 trillion yuan ($2.68 trillion) in 2006. Corresponding to the GDP, 2.525 billion tons coal equivalent was consumed. With the decreasing amount of fossil resources worldwide, China is facing an austere energy and resources crisis to satisfy its continuously increasing consumption based on its GDP. Therefore, it is extremely urgent for China to seek alternative energy and resources.

With many advantages, such as enhancing the octane value of gasoline, reducing emissions of hydrocarbons, nitrogen oxides, and VOCs, and no need for engine modifications, bioethanol is thought of as the most promising candidate. As an alternative fuel sourced from renewable materials, bioethanol has been used in many countries [60]. Nowadays, corn is a common feedstock for bioethanol production, along with grain, sugar beet, and molasses. In practice, bioethanol could ease not only the energy crisis as a liquid fuel, but also the resources crisis

by producing bioethylene by catalytic dehydration integrated into the modern petrochemical industry.

In April 2001, the Chinese government initiated an oil substitute project aimed at producing fuel ethanol from aged grain [61]. After 5 years, the total production capacity of the four dominant companies reached 1.24 Mt/year (Table 1). So far, China has become the third biggest fuel ethanol producer following Brazil and USA in the world. And fuel ethanol has been used as transportation fuel in nine provinces. Meanwhile, a project aimed at promoting industrialization of production of bioethylene from bioethanol was also initiated by the Ministry of Science and Technology of China in 2006.

It was declared that 10 Mt fuel ethanol will be produced in 2020 from a Chinese government project. However, the price of grain has sky-rocketed due to rising world food price and domestic high-speed economic growth, in addition to the overdeveloped grain deep processing in China. In view of the food security issue, the National Development and Reform Committee of China notes that new firms will not be given government approval to produce fuel ethanol. However, the government encourages companies to produce fuel ethanol from nonfood resources. In the short term, cassava, sweet potato, and sweet sorghum may be candidates. But in the long term, lignocellulosic bioethanol should become a future trend.

China is one of the biggest agricultural countries with the highest annual planted area and total production for some agricultural products in the world (Table 2). In China, 600 Mt/year agricultural straw can be produced, of which 300 Mt/year could be used to manufacture bioenergy. Meanwhile, 900 Mt/year forest residues can be produced by lopping and deforestation, of which 300 Mt/year could also be used to

Table 1 Production capacity of fuel ethanol from the dominant companies in China in 2006

Companies	Starting material	Production capacity (kt)
Jilin Fuel Alcohol Co. Ltd	Corn	500
BBCA Group Co. Ltd	Corn	440
Tianguan Group Co. Ltd	Wheat	300
China Resources (Holdings) Co. Ltd	Corn	100

Table 2 Planted area and total production of major crops in China in 2006

Crops	Total planted area (Mha)	Total production (Mt)
Rice	29.20	180.74
Wheat	23.27	102.97
Corn	27.05	142.00
Soybean	9.10	15.50
Rapeseed	6.74	12.20
Cotton	5.40	6.73
Sugarcane	15.0	99.25

produce bioenergy. As a result, China is a nation with plentiful improvable biomass resources. However, most of the lignocellulosic materials were disposed of or incinerated in China. This not only wastes the resources, but also pollutes the environment. If the lignocellulosic materials are converted into bioethanol, it could alleviate the resource and energy crisis. Meanwhile, it is helpful for solving problems relating to agriculture, countryside, and peasants in China.

There are still many technological bottlenecks that must be broken through for large-scale production of lignocellulosic bioethanol, such as the unavailability of a highly efficient lignocellulosic pretreatment technology, the high cost for cellulase to degrade cellulose to glucose, and the unavailability of strains that simultaneously convert pentose and hexose to bioethanol with high yield. Studies and industrialization relating to lignocellulosic bioethanol and its derivative bioethylene in China are introduced below.

5.2 Pretreatment Technology

The main challenges of bioethanol production from lignocellulosic materials are their low yield and the high cost of the hydrolysis process. So pretreatment is a key process in lignocellulosic conversion. In this step the feedstock, the recalcitrant lignocellulose complex, is chemically and physically broken down into more reactive structures. The ideal pretreatment process should include: (1) high hemicellulose and cellulose conversion; (2) avoidance of sugar decomposition; (3) reduction of byproducts; and (4) should be economic. Presently, steam explosion, dilute acid, hot water flow-through, AFEX (Ammonia Fiber Explosion), ammonia recycle percolation, lime, and organosolv processes have been attempted to carry out lignocellulosic pretreatment.

5.2.1 Steam Explosion

Among pretreatment technologies, steam explosion has been favored by researchers due to its low cost, lower energy consumption, and it being pollution-free. Many previous reports have shown it to be one of the most promising processes in the lignocellulosic bioethanol industry. Moreover, in order to decrease the treatment temperature in the steam explosion process, a series of modified processes, such as AFEX, carbon dioxide explosion, have also been used to destroy lignocellulose.

Steam explosion has been used in corn stover, bagasse, maize stalk, rice stalk, and lespedeza stalk pretreatments. Temperature, steam pressure, and acidity are the main factors that are usually investigated. On the basis of several reports, the steam pressure usually was 1.6–2.25 MPa, and the pressure-retaining time was 4–5 min in the process, and the yield of reducing sugar is obviously enhanced by steam explosion pretreatment [62]. Furthermore, in the steam explosion process, adding diluted acid can not only decrease the operating temperature, but also increase the

yield of sugar. Many characterization methods, such as SEM, IR, and XRD have been employed to probe the effect of steam explosion pretreatment on physical features, morphology, crystallinity, and composition of lignocellulose [63].

5.2.2 Acid Pretreatment

Acid pretreatments include concentrated acid and dilute acid processes [64]. In general, H_2SO_4 and HCl were used in these processes. Concentrated acids can hydrolyze cellulose, but they are toxic, corrosive, and hazardous, and must be recovered after hydrolysis to make the process economically feasible. The dilute acid pretreatment is thought of as a mature and effective method to remove hemicelluloses.

As an example, pine powder pretreatment by dilute hydrochloric acid yields maximum reductive sugar at a temperature of 120°C, a ratio of liquid to solid of 6:1, and a concentration of hydrochloric acid of 0.5%. In peracetic acid pretreatment of sugarcane bagasse, peracetic acid charge, liquid/solid ratio, temperature, and time show a very significant effect on the enzymatic conversion ratio of cellulose. More than 80% of the cellulose in bagasse treated under optimized conditions is converted to glucose by cellulase of 20 U FPase/g^{-1} cellulose. Compared with H_2SO_4 and NaOH pretreatments under the same mild conditions, peracetic acid pretreatment is the most effective for enhancement of enzymatic digestibility. It is suggested that peracetic acid pretreatment could greatly enhance the enzymatic digestibility of sugarcane bagasse by removing hemicelluloses and lignin, but removal of lignin is more helpful [65].

5.2.3 Alkaline Pretreatment

Alkaline pretreatment can remove lignin from the biomass and improve the reaction activity of the polysaccharide. The process can be conducted under normal temperature and pressure, but a long reaction time, for example several days, is generally required. The used alkaline compounds include NaOH, KOH, $Ca(OH)_2$, and ammonia.

The effect of treatment of cellulose with liquid anhydrous ammonia on the structure and lattice form has been explored. The results show that the lateral order distribution, crystallinity, crystallite size, and lattice form of cellulose are dramatically changed after the pretreatment. The crystallinity decreases by 21%, the lateral dimension of crystallite decreases by 20–40%, the lattice form converts from cellulose I to cellulose III, and the extent of conversion is up to 90% for 180 min. The pretreatment also causes a transition from the high order region to lower order and disorder fractions. The changed physical structure improves the accessibility of cellulose and the reactivity of cellulose in derivative formation [66]. Lignocellulosic pretreatment by sodium hydroxide indicates that the pretreatment has strong effects on lignocellulosic constitution. After the pretreatment, cellulose

is inflated with lower crystallinity characterized by IR spectra, and it is easier to be hydrolyzed by cellulase than the original lignocelluloses. In a process involving ammonia pretreatment of soybean straw, the combined effect of smash and ammonia pretreatment has a significantly positive influence on cellulose hydrolysis to produce sugars [67].

5.2.4 Liquid Hot Water

Compared with dilute acid pretreatment, no acid or chemical catalyst is needed in the liquid hot water pretreatment process. Meanwhile, liquid hot water appears to have the potential to generate reactive fiber, recover most of the xylose, and produce less fermentation inhibitors.

A corn stalk hot water pretreatment process has been investigated. The pH of corn stalk hydrolysate decreases more rapidly than that of corn leaf as the reaction time increases. On the contrary, the pH of the corn leaf hydrolysate increases more than that of corn stalk with dilute acid addition. Increasing temperature enhances the xylose decomposition rate and increases cellulose digestibility. Additionally, 0.1% sulfuric acid addition improves the xylan removal and the enzymatic hydrolysis of both corn leaf and corn stalk residue [68].

5.3 Cellulase

5.3.1 Cellulase-Producing Microorganisms

Cellulase is widely found in natural organisms. Initial calculations suggest that thousands of cellulose-producing strains in 53 genera have been recorded since the 1960s. However, microorganisms that have a relatively strong effect in relation to cellulose belong to the *Trichoderma*, *Aspergillus*, and *Penicillium* strains. Among them, *Trichoderma* strains account for a large number [69]. Moreover, yeasts can also produce cellulase. Currently, *Trichoderma reesei* is the strain most clearly probed.

Since cellulase was found about 100 years ago, researchers have constantly screened microorganisms degrading cellulose from soils, hot springs, oil wells, compost, and deadwood. Generally, the cellulase activity of microorganisms screened from nature is low and not suitable for industrial production. Improvement of cellulase-producing strains has received a great deal of attention from Chinese scientists. For example, an anticatabolite repression strain by mutation and capable of using 2-deoxyglucose as product inhibitor is obtained. The FPase and CMCase activities are 3.63 U/mL and 24.64 U/mL, respectively, which are four times higher than that of the original strain. Using UV mutation and agar plate screening techniques, a *Trichoderma pseudokoningii* mutant strain is selected successfully. The mutant strain produces high CMCase, FPase, and β-Gase activities.

The respective activities are 1,145.7 U/g, 55.6 U/g, and 24 U/g, which are respectively 2.4, 3.0, and 12.6 times higher than that of the original strain.

The genetic engineering of cellulase has been of interest since the end of the 1970s. With rapid development of molecular biology and genetic engineering, the recombinant cellulose-degrading microbes were studied widely in the world. Especially, great attention was paid to overexpression of *Trichoderma*, *B. subtilis*, and yeast. A cellulase from the ruminal fungus *Orpinomyces joyonii* was cloned and overexpressed in *E. coli*, and purified 88-fold by chromatography. The specific activity of the recombinant enzyme to barley β-glucan was 297 U/mg protein. The K_m values estimated for barley β-glucan and CMC were 0.32 and 0.50 mg/mL, respectively [70]. The *Pichia pastoris* expression system was applied to express the *T. reesei* EGIV gene. The EGIV gene was isolated from rice hull induced *T. reesei* culture through RT-PCR, and was ligated with the *Pichia* expression vector pPICZαA, resulting in the recombinant plasmid pPICZαA-EGIV. The recombinant plasmid pPICZαA-eg4 was transformed into *P. pastoris* GS115, and the EGIV gene was in frame integrated into the *Pichia* genome through homologous recombination, resulting in the recombinant strain *P. pastoris*-EGIV1. With methanol induction, the recombinant strain *P. pastoris*-EGIV1 expressed and secreted EGIV into the culture supernatant with CMC activity of 2.11 U/mL [71]. The complete sequence encoding the mature peptide of endo-1,4-glucanase II of *T. reesei* QM9414 was amplified. The gene was inserted into the *P. pastoris* vector pPIC9K, downstream of the α-factor signal peptide sequence. Then the recombinant plasmid pQY2025 was lineated by BglII digestion and introduced into the host *P. pastoris* GS115 by electroporation. After screening, the recombinant *P. pastoris* strain Gp2025 was obtained and fermented in 25-mL methylotrophic culture medium. The recombinant endoglucanase II activity could reach 1,573.00 U/mL.

Even now, there are still some limitations in recombinant cellulase. Not all the genes of cellulase can be overexpressed, and recombinant cellulase lack of combined protein cannot hydrolyze the crystal cellulose. Accordingly, the topic of how to use cellulase effectively will still be a challenge in the future.

5.3.2 Industrial Cellulase Production in China

Cellulase is generally produced by solid state fermentation and liquid state fermentation. To date, the production of cellulase has been widely investigated by submerged culture processes, but the relatively high cost of cellulase is not affordable by industry. Solid state fermentation, using abundant agricultural residues such as corn stover, wheat straw, rice straw, and bagasse as substrates in cellulase production, is an attractive process to produce cellulase economically due to lower capital investment and operational expense.

In the early 1960s, scientists began to study cellulase in China. Currently, cellulase is commonly produced by solid state fermentation in China. In Zaozhuang Jienuo Bio-enzyme Co. Ltd. and Shandong Longda Bio-products Co. Ltd., *T. reesei* mutants are used to produce acidic cellulase using submerged culture. The activities

of CMCase products are 20,000 U/g and 20,000 U/mL under pH 5.0 and 60°C by DNS (dinitrosalicylic acid) method, respectively. And the cellulase produced by Jiangyin Aidun Biotechnology Co. Ltd. has exoglucosidase and endoglucosidase activities of 600,000 and 2,000 U/g, respectively.

5.4 Bioethanol Fermentation and Product Recovery

5.4.1 Microorganisms

The ideal strains which can be used to produce bioethanol from lignocellulose must possess the following two properties: (1) Able to produce bioethanol by fermentation from different substrates, especially pentose hydrolyzed from hemicellulose; and (2) tolerate many inhibitors [72]. Traditionally, microorganisms used for bioethanol fermentation cannot metabolize pentose. So in the past xylose was not considered for use in alcohol fermentation. However, in the 1980s Wang and the others proposed that xylose can be metabolized to bioethanol by some microorganisms, and this resulted in a worldwide upsurge of strain studies for xylose bioethanol fermentation. So far more than 100 microorganisms have been found with the capacity of metabolizing xylose to bioethanol. On the basis of many previous studies, *S. cerevisiae* and *Zymomonas mobilis* are selected as good bioethanol-producing candidates.

S. cerevisiae is the perfect strain for industrial production of bioethanol. It not only has a high alcohol resistance, but also has a certain extent of tolerance to inhibitory factors in lignocellulose hydrolysate. So far, scientists have endeavored to reconstruct *S. cerevisiae* to convert xylose to bioethanol and improve the alcohol resistance. *S. cerevisiae* cannot utilize xylose, but it can metabolize the xylose isomer, xylulose to bioethanol by coding the xylulokinase gene *XKS1*. By changing the yeast xylose metabolic pathways, and cloning related genes to control metabolic pathways to change the metabolic carbon flux, the resulting recombinant *S. cerevisiae* can efficiently make use of xylose and glucose simultaneously.

To date, *Z. mobilis* is the only microorganism capable of anaerobic fermentation of glucose into bioethanol through the Entner–Doudoroff (ED) pathway. It is one of the preferred hosts to construct alcohol production strains because of its unique metabolic pathway. Zhang and colleagues have successfully introduced the key genes for xylose conversion, *xylA* (xylose isomerase), *xylB* (xylulokinase), *tktA* (transketolase), and *talB* (transaldolase) into *Z. mobilis*. The transformer CP4 (pZB5) is able to grow in the medium using xylose as the sole carbon source, and bioethanol yield reaches 86% of the theoretical value. To further enhance bioethanol yield, Zhang and colleagues have obtained recombinant strain ZM4 (pZB5) with higher alcohol resistance. After that, AX101, a chromosomal integration of the strain with seven necessary genes was developed and able to convert xylose and arabinose to bioethanol with high stability of external source genes without addition of antibiotics.

At present, mutant *S. cerevisiae* is the most popular industrial bioethanol production strain because of its high alcohol tolerance and requirement for mild operational conditions. However, recombinant strains are receiving much attention because of their broad substrate spectra. But rigid demand for growth conditions and security concerns are the major problems [73]. So far, whether by mutation or by genetic recombination, efficient strains have not been obtained which limits the industrialization of lignocellulosic bioethanol.

5.4.2 Fermentation Process

Of lignocellulosic bioethanol fermentations, sequential hydrolysis and fermentation (SHF) is the most popular process. It is divided into two steps: (1) Hydrolyze cellulosic materials to glucose; and (2) convert glucose to bioethanol under anaerobic conditions. At present, the production of lignocellulosic bioethanol using SHF is the dominant process in China. Jilin Tuopai Agricultural Development Company in collaboration with the Risø National Laboratory of Denmark has carried out bioethanol production using corn stalk pretreated by wet oxidation. The cellulose hydrolysis rate is 86.4%, and alcohol yield to sugar is about 48.2%. In a 10-L automatic bioreactor, the concentration of ethanol can accumulate to 6.2% (v/v) after 62 h. In another SHF process using acid-pretreated bagasse as the starting material, the concentration of glucose and bioethanol attained 65.3 g/L and 19.0 g/L, and the productivity of bioethanol is 0.34 g/g sugar [74]. By employing a fluidized bed and shaking table reactor in sorghum stalk bioethanol fermentation using immobilized yeast, environmental pollution can be reduced, and equipment utilization can be improved [75].

During the 1970s, the simultaneous saccharification and fermentation (SSF) process was developed. In the SSF process, cellulose hydrolysis by cellulase and bioethanol fermentation is carried out in one device to decrease the cost of production [76]. The State Key Lab of Biochemical Engineering of IPE-CAS developed a simultaneous saccharification and fermentation process. A coupled system for solid phase enzymolysis of cellulose and liquid fermentation of bioethanol was designed. In this system, the saccharification, fermentation and separation of bioethanol are operated in separated devices. Problems including the inconsistent temperature requirements for cellulase and fermentation, and the inhibitory action of sugar to cellulase can be solved. In a pilot experiment, the total solid concentration of acid pretreatment of corn cellulose is 20%, the load of cellulase is 10 U FPase/g cellulose, cellulose conversion is 81%, and 78.4% of glucose and 56.1% of xylose are converted to bioethanol by recombinant yeast LNH2ST over 4 days [77]. Coupled microwave- and alkali-pretreated rice straw and acid-pretreated corncob are also used as starting materials in the SSF process, and 25.8 g/L and 20 g/L bioethanol are achieved, respectively [78]. Although SSF technology can reduce the production time and enhance sugar conversion, the inconsistent saccharification and fermentation temperatures are a major problem.

With the development of recombinant technology, and simultaneous pentose- and hexose-utilizing microorganisms, cofermentation technology can be developed, which is called SSCF (Simultaneous Saccharification and CoFermentation). Since the 1980s, the production of cellulosic ethanol via SSCF has been a major focus in the world. For instance, the modified strain *Candida shehatae* R, can directly metabolize glucose and xylose to bioethanol. As a rule, it can consume 90% of the monosaccharides during fermentation. On the basis of recombination, yeast strain AS21190 is modified to strain GZ4-127. Although they can also carry out glucose and xylose cofermentation, the latter can consume xylose at twice the rate as the former. And the yield of bioethanol is increased by 12%. In a similar cellulosic hydrolysate fermentation process, the cofermentation of glucose and xylose can be achieved by *Pichia stipitis*. Sugar mixture conversion can attain 90.38%, and bioethanol concentration is 15.41 g/L [79]. Currently the main issues relating to cofermentation of glucose and xylose include two aspects: (1) The lower alcohol tolerance of strains in cofermentation; and (2) hysteresis of xylose metabolization.

The technology of consolidated bioprocessing (CBP) refers to the production of cellulase, cellulose hydrolysis, and fermentation of glucose and xylose in one bioreactor. And the cellulase production and bioethanol fermentation is carried out by a single microorganism or a microbial community. In nature, certain microorganisms, such as *Clostridium*, *Moniliar*, *Fusadu*, and *Neurospora*, have a capacity for direct conversion of biomass to bioethanol with low efficiency. Although CBP technology can substantially simplify the process and reduce the cost of production, the requirement for a highly efficient microorganism has not been fulfilled [80]. Therefore, reconstruction of the endogenous cellulose degradation pathway to modify microorganisms, especially anaerobic microorganisms to fulfill the requirements of the CBP process is very important.

5.4.3 Bioethanol Recovery

The bioethanol from lignocellulosic fermentation has low product concentration and efficiency. The resulting broth usually contains 4–5% by weight bioethanol in lignocellulosic fermentation. In general, distillation and rectification are employed to concentrate bioethanol from the dilute culture solution to an azeotropic mixture. Then, the feed can be further purified to anhydrous bioethanol using distillation with an entrainer, desiccants, or membrane pervaporation.

In recent years, membrane separation technology has become a hot topic in relation to bioethanol separation because of its energy savings and efficient equipment utilization. For example, a cross-linked polyamide composite membrane has been used to concentrate ethanol. The results show that cross-linked polyamide composite membranes possess good selectivity and high efficiency for low bioethanol concentration.

In the bioethanol fermentation process, the maximum concentration of ethanol tolerated by the microorganisms is about 10 wt% at 30°C, but this decreases with

increasing temperature. A self-made plate-type silicon rubber composite membrane has been developed for a system of continuous bioethanol fermentation and pervaporation. It was found that bioethanol can be selectively separated by a pervaporation membrane. Keeping membrane pervaporation in situ to maintain bioethanol concentration at about 40 g/L can weaken and eliminate the inhibitory effect on yeast of bioethanol. It can also obviously improve the growth of microorganisms, the bioethanol volume production rate, and the substrate consumption rate [81]. If the membrane can be made selectively permeable to bioethanol, a high concentration product can be achieved. Compared with the traditional distillation method, the energy consumption of membrane pervaporation is only 1/3 to 1/10 of that, and no wastes will be produced.

5.5 *Integrated Lignocellulosic Bioethanol Process*

Hereinabove, lignocellulosic pretreatment, cellulase production, cellulose hydrolysis, and bioethanol fermentation and recovery are included in the integrated lignocellulosic bioethanol process. To date, many universities, institutes, and corporations have widely studied lignocellulosic bioethanol production technology in China. As shown in Table 3, six pilot-scale studies have been carried out in companies. Acid and steam explosion pretreatments were employed, and half of them make use of cofermentation. On the whole, the level of bioethanol production from lignocellulose is not very high for unavailable of low cost cellulase, and lacking pentose and hexose cofermentation strains. In the future, a great deal of effort should be given to developing practical pretreatment technology, reducing the cost of cellulase, and decreasing the energy consumption in product recovery [87].

5.6 *Bioethylene*

Ethylene is the most significant raw material and primary product in the chemical industry. In 2005, the equivalent requirement for ethylene was 15 million tons in China. And it can be expected to reach 26 and 40 million tons in 2010 and 2020, respectively [88]. Currently, the great challenge incurred by the ever-increasing crisis of scarceness of fossil fuels must be faced by ethylene and its derivative industry which originates from the petrochemical industry. An old sustainable process to manufacture ethylene using dehydration of bioethanol has been evoked in order to alleviate the crisis. So the word bioethylene has been coined to represent the ethylene produced from dehydration of bioethanol.

The technology for converting bioethanol to bioethylene by dehydration could date back as much as 200 years. The first time ethylene was obtained by dehydration of bioethanol was in 1797. Before 1945, most of the ethylene was prepared

Table 3 The pilot-scale studies on lignocellulosic bioethanol in China

Items	ECUST	NJFU	ZSBT	TGG	BBCAG	CRC
Capacity (t/year)	600	–	3,000	300	300	500
Materials	Rice straw, wood chips	Corn stover	Corn stover	Corn stover	Corn stover, wheat straw	Corn stover
Pretreatment	Acid	Steam explosion	Steam explosion	Acid	Acid	Continuous steam explosion
Hydrolysis	Acid	Cellulase	Cellulase	Cellulase	Cellulase	Cellulase
Fermentation	Cofermentation of pentose and hexose	Cofermentation of pentose and hexose	Simultaneous saccharification fermentation	Hexose fermentation	Pentose fermentation and hexose fermentation	Cofermentation of pentose and hexose
Refs.	[82]	[83]	[84, 85]	[84]	[86]	[82]

from bioethanol dehydration in the world. Afterwards, ethylene production from oil feedstock was widely employed with the emergence of the petrochemical industry.

However, some developing countries, especially countries lacking oil, never stopped producing bioethylene by bioethanol dehydration. In the 1960s, Brazil, India, Pakistan, and Peru established bioethylene processes, of which the general annual output was 2,000 to 10,000 tons. Before the 1960s, ethylene was also mainly produced from bioethanol in China.

5.6.1 Catalysts for Bioethanol Dehydration

Catalysts for bioethanol dehydration mainly include activated clay, activated alumina, phosphoric acid, sulfuric acid, heteropolyacid, zeolites, Al_2O_3/SiO_2, Al_2O_3–MgO/SiO_2, etc [82, 83, 87]. The catalyst first used at industrial scale was clay loaded with phosphoric acid, which was first employed by ICI during the 1930s to 1940s. In the earlier 1960s, SD (Halcon Scientific Design Company) developed a multivariate oxide catalyst, that is, Al_2O_3–MgO/SiO_2. In 1981, a nonzeolite catalyst, Syndol catalyst, was introduced by SD to substitute the original Al_2O_3–MgO/SiO_2 catalyst. In China, Nankai University successfully developed the NKC-03A zeolite catalyst for bioethanol dehydration to bioethylene in industry in 1987.

The majority of bioethanol dehydration processes are carried out with oxide catalysts. Some binary or multivariate oxide catalysts have been developed. In China, there are also several reports about dehydrating bioethanol to bioethylene using Al_2O_3 as the catalyst. In a fixed bed reactor, the catalytic dehydration of bioethanol to bioethylene using a γ-Al_2O_3 catalyst was investigated and a selectivity of ethylene above 99.15% at 420°C was achieved [84]. According to the study on bioethylene production via catalytic dehydration of bioethanol with TiO_2/γ-Al_2O_3 catalysts in multimicrochannel reactors, results show the catalysts doped with TiO_2 have high bioethanol conversion and bioethylene selectivity, namely, 99.96% and 99.4%, respectively [85]. The advantages of oxide catalysts are high stability, good regenerated performance, resulting in high product purity. However, they also have some obvious disadvantages, such as high reaction temperature (above 350°C), low space velocity of feedstock (about 0.4/h), and high energy consumption.

For the purpose of avoiding high temperature, heteropolyacid catalysts were introduced. For example, the process of dehydration of bioethanol to bioethylene catalyzed by activated carbon loaded with heteropolyacid catalyst has been explored by scientists, and the effect of heteropolyacid catalysts are reported to be better than NKC-03A, Syndol catalyst, and γ-Al_2O_3 [86]. But the lifetime and hydrothermal stability of heteropolyacid catalysts have to be improved to fulfill industrial requirements.

Since the 1980s, research on dehydration reactions of alcohols has focused on using zeolites as the catalyst, where zeolites mainly refer to ZSM-5, SAPO-34, and mordenite. The activities of HZSM-5, alumina, silica, as well as trifluoromethanesulfonic acid-modified HZSM-5 and HY zeolite catalysts used in the dehydration

reaction of low concentration ethanol are compared. This showed trifluoromethanesulfonic acid-modified HZSM-5 has a better effect, bioethanol conversion reaches 100%, and bioethylene selectivity is more than 99% at 170°C.

A modified HZSM-5 catalyst with satisfactory dehydrating performance through hydrothermal treatment and with different metal oxides has been developed [89]. After modification using a transition metal, the HZSM-5 zeolite can attain a bioethanol conversion of more than 98% and bioethylene selectivity of 97%. In an operational experiment, the catalyst shows good stability over 2,000 h from 280 to 340°C. Another report shows that reducing Brønsted acid sites and increasing Lewis acid sites will be advantageous for ethanol dehydration to ethylene through a study on low concentration bioethanol dehydration to bioethylene with HZSM-5 catalyst modified by Zn and Mn through an impregnation method [90].

Like ZSM-5, SAPO-34 is another catalyst used to catalyze bioethanol to bioethylene, however with a 3D channel structure and smaller pore diameter than ZSM-5. The pore characteristics and the strength of acidic sites of SAPO-34 catalyst are similar to those of ZSM-5. It has been shown that SAPO-34 has a higher bioethylene selectivity than ZSM-5 at 267–297°C, i.e. more than 99.5%, and thermal stability was higher than ZSM-5 at 300°C, so it can be easily regenerated. However, the disadvantage is that the pores of the catalyst are very easily clogged because of their small diameter in practice.

The catalytic activity at a lower temperature and the lifetime of the catalyst are the key problems for industrialization. Worldwide many scientists are working on these problems.

5.6.2 Bioethanol to Bioethylene Processes

The bioethylene route has again received the attention of developed countries since the occurrence of the worldwide energy crisis in the 1970s. And up to 1981, Brazil had established three bioethylene production lines and produced 74,000 tons of bioethylene annually, while India had established four lines and produced 27,300 tons.

Multitubular reactors are firstly used to improve heat efficiency in the bioethanol to bioethylene by dehydration process. The Sichuan Vinylon Plant has employed isothermal multitubular packed-bed reactors designed by Shanghai Engineering Co. Ltd. and an activated alumina catalyst produced by the Shanghai Petrochemical Institute to perform bioethanol to bioethylene conversion by dehydration. Although bioethanol conversion is close to 100%, the bioethylene yield is only 89%. The main reason is ascribed to adoption of a noncirculatory molten-salt heating system, which results in a temperature fluctuation of 40 to 70°C, complex byproducts, and troublesome product separation. Because of the high cost, inconvenient catalyst loading/unloading, and complicated operation, this process will gradually be replaced. In the 1980s, BBCAG introduced parallel serial adiabatic reactor technology from the Petroleo Brasileiro Company of Brazil, which employed an advanced hot-air-circulating heating system. In this process, the temperature error

is only ±10°C, and the yield of bioethylene reaches 96%. Moreover, water vapor produced in the course was pumped back to prevent coking and improve the conversion as well as operational flexibility.

In 2007, in order to improve the straggling situation in Chinese bioethylene technology and upgrade international competitiveness, the following projects were all authorized as key bioethylene objectives: "Bio-refining Technology of Bio-based Chemicals" (Key Project of the High-tech Research and Development Program, "863" plan), "Development of Key Technology in Manufacturing Bulk Chemicals Through Nonpetroleum Route" (Key Project of the National Key Technology Research and Development Program-Eleventh 5-year Plan), and "High-tech in Bio-materials Industrialization" (Organized by the National Development and Reform Commission). At present, Nanjing University of Technology, in conjunction with the Institute of Biotechnology, Chinese Academy of Agriculture Science; Institute of Microbiology, Chinese Academy of Sciences; Dalian Institute of Chemical Physics, Chinese Academy of Sciences; Tsinghua University; Beijing University of Chemical Technology, and Jiangnan University, Shandong University, Tianjin University of Science and Technology, Hunan University, BBCAG, the Sichuan Vinylon Plant affiliated to Sinopec, is dedicated to solving this key technology bottleneck, and is trying to develop a green energy-saving and advanced bioethylene production process by 2010.

5.7 Conclusions

The inefficient utilization of resources, the excessive consumption of energy and resources, and the aggravated environmental pollution have become the main bottlenecks hampering the building of an energy-efficient and environmentally friendly society in China. Therefore, the green GDP project has been brought forward by the Chinese government as part of the Eleventh 5-year Plan period, to develop a circular economy, exploit renewable resources, improve integrated utilization of resources, and strengthen environmental protection. Alternative energy and resources are needed for transportation fuel and chemical feedstock in China, and lignocellulosic bioethanol and bioethylene could have obvious economic, environmental, and safety benefits.

Lignocellulose manufactured via photosynthesis by plants is the most abundant resource on earth. With the increasing scarcity of fossil resources, many countries have shifted their attention to the renewable green oil field of lignocellulose. Today, great attention is being paid to lignocellulosic bioethanol and bioethylene by many countries. Converting lignocellulosic feedstock to bioethanol can increase the income of peasants, reduce pollution, and ease dependence on overseas oil in China. But the high cost is the major problem.

In relation to studies and industrialization of lignocellulosic bioethanol and bioethylene in China, several bottleneck issues must be solved. Firstly, the collection of lignocellulosic feedstock is inconvenient. Although Chinese arable land can

produce plentiful biomass, they are dispersed through wide regions. The transportation cost will be enhanced to collect feedstock. This will restrict the location and scale of plants to produce bioethanol from lignocellulose. Secondly, low price cellulase is not available. In China, commercial cellulases derived from solid state fermentation are sold by several companies. In general, the microorganisms used to produce cellulase are screened by physical or chemical mutation. The low yield of cellulase by mutant microorganisms causes the high price. Although the overexpression of cellulase can be carried out by recombinant strains, there is no commercial recombinant cellulase for Chinese bioethanol industry. In the future, development of highly efficient recombinant microbes is feasible to reduce the price of cellulase. Thirdly, highly efficient microorganisms for pentose and hexose cofermentation are in short supply. Efforts must be made to reconstruct strains to utilize pentose and hexose simultaneously and resist the inhibitory effect of alcohol and inhibitors. On the basis of previous reports, *S. cerevisiae* and *Z. mobilis* are believed to be the best parental strains. Ideal producers can be obtained via metabolic pathway modification. Fourthly, membrane technology must be introduced to enhance product separation. In particular, in situ bioethanol separation can eliminate product inhibition, shorten the fermentation period, and decrease energy consumption. In relation to a green separation technology, a low price and high performance membrane is a major problem that needs to be solved. Finally, determining the perfect catalyst for bioethanol dehydration must be given great attention. Presently, many scientists are focused on developing a catalyst for bioethanol dehydration that has high performance, including low reaction temperature, long lifetime, and low cost. In addition, there are other factors affecting the industrialization of bioethanol and bioethylene, such as oil price, and policy.

Over the next 10 years, the Chinese government will focus on lignocellulosic bioethanol and bioethylene, and this should attract foreign technology and capital to the Chinese bioethanol and bioethylene market. Therefore, the industrialization of lignocellulosic bioethanol and bioethylene will be accelerated in China in the near future.

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Organic Chemicals from Bioprocesses in China

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Abstract Over the last 20 years, China has successfully established a modern biotechnology industry from almost nothing. Presently, China is a major producer of a vast array of products involving bioprocesses, for some China is even the world's top producer. The ever-increasing list of products includes organic acids, amino acids, antibiotics, solvents, chiral chemicals, biopesticides, and biopolymers. Herein, the research and development of bioprocesses in China will be reviewed briefly. We will concentrate on three categories of products: small molecules produced via fermentation, biopolymers produced via fermentation and small chemicals produced by enzyme-catalyzed reactions. In comparison with the traditional chemical process, in which, nonrenewable mineral resources are generally used, products in the first and second categories noted above can use renewable bioresources as raw materials. The bioprocesses are generally energy saving and environmentally benign. For products developed via the third category, although the raw materials still need to be obtained from mineral resources, the biocatalysts are more effective with higher selectivity and productivity, and the bioprocesses occur under ambient temperature and pressure, therefore, these are "green processes." Most of the products such as citric acid, xanthan and acrylamide etc., discussed in this paper have been in large-scale commercial production in China. Also introduced herein are three scientists, Prof. Shen Yinchu, Prof. Ouyang Pingkai and Prof. Chen Guoqiang, and six enterprises, Anhui Fengyuan Biochemical Co. Ltd., Shandong Hiland Biotechnology Co. Ltd., Shandong Fufeng Fermentation Co. Ltd., Shandong Bausch & Lomb-Freda Pharmaceutical Co. Ltd., Zhejiang Hangzhou Xinfu Pharmaceutical Co. Ltd., and Changzhou Changmao Biochemical Engineering Co. Ltd.; they have all contributed a great deal to research and development in the commercialization of bioprocesses.

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Contents

1 Introduction

China is a vast country with a population of 1.3 billion, rich natural resources and a long history of civilization. About 5,000 years ago, the Chinese had learnt to use microorganisms to make wine, to tan animal skin and to make various kinds of fermented food. However, China lagged the pace of development in biotechnology in modern history. From the 1980s onwards, the Chinese government made a decision to open the country to the outside world and to reform China's economic structure. Biotechnology gradually caught up with world trends. Universities, research institutes, and enterprises worked together in the research and development of biotechnology and bioprocesses. Especially in the area of industrial biotechnology, various comprehensive processes have been developed, and China has been a major producer of a significant number of organic chemicals such as citric acid, xanthan, glutamic acid, penicillin, and acrylamide for example.

Organic compounds are widely applied in various areas such as agriculture, energy, materials, environmental, medicines, commodity products and food etc. It could be said that no one can live without organic chemicals. It is interesting to note that the history of the production of organic chemicals represents a cycle as shown in Fig. 1. In ancient times, only natural mixed organic chemicals from plants, animals, and microorganisms (although unrecognized as such) were utilized as food additives, beverages, dyes, and medicines etc. In the nineteenth century, organic chemists found ways to extract pure organic chemicals from natural resources or to synthesize them mainly from coal, and the typical products were methane, furfural, benzene, phenol naphthalene, and acetylene from calcium carbide, etc. In the twentieth century, petroleum, instead of coal, became the main

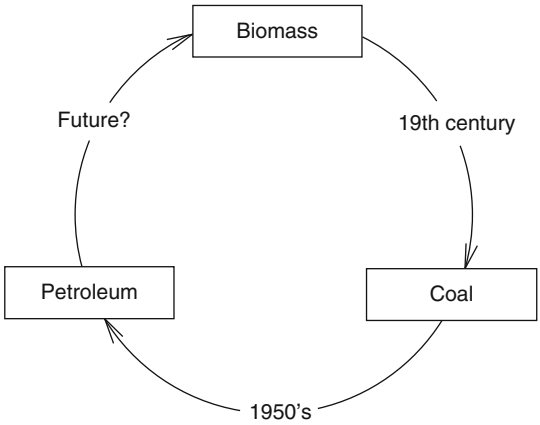


Fig. 1 Historic view of the production of organic chemicals

source of organic chemicals in much larger scale and over a broader product spectrum. Now, in the twenty-first century, the price of petroleum is rocketing and is no longer cheap and abundant. We therefore cannot help but to find alternative ways to produce organic chemicals. Should we go back to the biomass?

Biomass is synthesized by photosynthesis, which is renewable. In China alone, the total annual productivity of biomass is estimated at more than five billion tons. The main composition of biomass is carbohydrates and it is suitable for conversion to a variety of organic chemicals. If only 10% of the biomass is applied to the production of organic chemicals, it should be more than enough to satisfy current requirements. The conversion of biomass into organic chemicals can be carried out by gasification, high-temperature cracking or bioconversion. Bioconversion is performed at ambient temperature and pressure, the processes are energy efficient and environmentally benign. A great deal of effort has been paid to the development of new processes for the production of organic chemicals with biotechnology in China. Many review papers have been published on the research and development of industrial biotechnology in China [1–3].

Biomass-derived carbohydrates include starch, hemicelluloses, and cellulose. They are suitable to be applied as carbon sources for microorganism growth after proper pretreatment. The metabolites produced by the microorganisms can be divided into two categories: primary and secondary metabolites.

The primary metabolites are generally produced during energy metabolism. Taking glucose as an example of the carbon source, the primary metabolites are related to the EMP (Embden–Meyerhof–Parnas) pathway and tricarboxylic cycle. Various kinds of organic acids, alcohols, amino acids and nucleotides etc. belong to this category. Because of the carbohydrate nature of biomass, there are abundant oxygen atoms in the molecule of the carbon source. If the target primary metabolite is an oxygen-rich molecule, such as citric acid or lactic acid, the conversion ratio will be very high, whereas, it will be low if a hydrocarbon is predicted. The reductance degree [4] – which is defined as the number of equivalents of available electrons per gram atom carbon – is for carbon +4, hydrogen +1, oxygen −2, and nitrogen −3,

Table 1 Reductance degrees of carbon sources from biomass, and primary metabolites as well as their theoretical and practical productivities (glucose as carbon source)

	Name	Molecular formula	Reductance degree	Productivity, $Y_{P/S}$ (g/g)	
				Theoretical	Practical
Substrate	Glucose	CH_2O	+4.00		
	Xylose	CH_2O	+4.00		
	Glycerol	$CH_{8/3}O$	+4.66		
Product (glucose as substrate)	Methane	CH_4	+8.00	0.16	<0.1
	Butanol	$CH_{2.5}O_{0.25}$	+6.00	0.41	0.20
	Ethanol	$CH_3O_{0.5}$	+6.00	0.51	0.48
	Lactic acid	CH_2O	+4.00	1.0	0.90
	Citric acid	$CH_{8/6}O_{7/6}$	+3.33	1.42	1.20

and can be used as a criterion to estimate the yield of primary energy metabolites. The reductance degrees of several carbon sources from biomass, and primary metabolites as well as their theoretical and practical productivities are listed in Table 1.

The primary metabolites can be produced by either aerobic or anaerobic fermentation. The difference between aerobic and anaerobic fermentation is that the electron acceptor in aerobic growth of microbes is oxygen, whereas it is the organic substrate itself in anaerobic growth. Therefore, the reductance degree of primary metabolites in anaerobic fermentation is generally higher than that of the substrate. The higher reductance degree is desirable for a product such as alternative biofuel because of its higher heat of combustion, whereas, the product yield will be lower because more substrate must be consumed for reducing power.

The secondary metabolites, such as antibiotics, are generally produced by aerobic fermentation and not related to the cell growth, and their yields are relatively low. Herein, a brief review will be given of the organic chemicals produced from bioprocesses in China. The chemicals will be divided into three categories: small molecules, biopolymers and those produced by enzyme-catalyzed reactions.

2 Small Organic Chemicals Via Fermentation

2.1 Organic Acids from Bioprocesses in China

2.1.1 Citric Acid

In 1893, Wehmer discovered that *Penicillium* mold could produce citric acid from sugar. However, microbial production of citric acid did not become industrially important until World War I disrupted Italian citrus exports. In 1917, the American food chemist James Currie discovered that certain strains of the mold *Aspergillus niger* could be efficient citric acid producers, and Pfizer began industrial-level production using this technique 2 years later, followed by Citrique Belge in 1929, and this is still the major industrial route to citric acid used today.

In 2007, worldwide annual production of citric acid was approximately 1,700,000 Mt (Metric ton). More than 50% is used as acidulant in beverages and some 20% in other food applications. Twenty percent is used for detergent applications and 10% for other nonfood-related applications such as cosmetics, pharmaceuticals and in the chemical industry.

In China, citric acid fermentation began about 40 years ago on a small scale. Grounded corn powder or sweet potato was used as the carbon source, which is relatively cheap, however, it made the fermentation process and the citric acid separation from the broth difficult. During that period, the fermenter was about 20 m^3 and was made of carbon steel, therefore the *A. niger* for citric acid fermentation must be iron ion tolerant.

Although citric acid fermentation is a mature industry, studies on strain improvement [5], product separation process [6], and process optimization [7] are still under way in China.

Currently, more than 50% of citric acid production worldwide takes place in China. The largest citric acid producer in China is Anhui Fengyuan Biochemical Co., Ltd. Each fermenter has a volume of more than 100 m^3 and is made of stainless steel; in addition computer-controlled fermentation parameters are used. The main feature of the fermentation process is that a clear fermentation broth is used after low temperature liquefaction of corn starch. Because the manufacturer could be authorized to export citric acid only if the discharge of their wastewater meets the requirement, the wastewater treatment facilities including anaerobic and aerobic treatment have being operated properly in Fengyuan facility.

Anhui Fengyuan Biochemical Co. Ltd., which is a leading enterprise dealing with biological fermentation products in China, can produce 440,000 Mt of fuel ethanol, 220,000 Mt citric acid and its salts, 30,000 Mt of L-lactic acid (for epoxyethane production), and 60,000 Mt of lysine annually. The company developed "low-temperature starch liquefaction and clear broth fermentation" technology. The citric acid output is about 17% of world production.

2.1.2 Lactic Acid

Industrially, lactic acid fermentation is performed by *Lactobacillus* bacteria or *Rhizopus oryzae*. Traditionally, lactic acid is applied in the food industry as an acidifier. Today, lactic acid is used as a monomer for producing polylactic acid (PLA) which has applications as a biodegradable plastic, and is a good option for substitution of conventional plastic produced from petroleum because of the low emission of carbon dioxide and its biodegradability. An initial significant obstacle for PLA was the production cost. In 2002, Cargill started up the first of two polymer trains with a capacity of 150 million lb per year, and the price of PLA production dropped to $1.30/lb, which is competitive with plastics from the petroleum industry.

Lactic acid has gained importance in the detergents industry during the last decade. Being a good descaler, soap-scum remover, and a registered antibacterial agent an economically beneficial as well as environmentally beneficial trend towards safer and natural ingredients has also contributed to lactic acids importance. Another promising application of lactic acid is to produce acrylic acid via catalytic dehydration, by which 65% conversion ratio can be reached [8].

There is a long history of lactic acid fermentation in China. Qian et al. [9] reviewed current research and development of lactic acid fermentation in China. Jiang et al. [10] studied L-lactic acid fermentation kinetics of *R. oryzae*. Potato starch was used as the raw material for lactic acid fermentation [11]. Cell immobilization was also studied [12]. The separation and purification of lactic acid from the fermentation broth is a difficult task. Shi [13] and Wang [14] discussed the possibility for new separation technologies for the lactic acid separation process.

The worldwide annual production of lactic acid is more than 400,000 Mt; China alone represents about one-third of this figure. The main producers of lactic acid in China are Henan Jindan Lactic Acid Co. Ltd. and Anhui Fengyuan Biochemical Co. Ltd. The capacity of lactic acid production of Henan Jindan Lactic Acid Co. Ltd. is 100,000 Mt/year, which makes them the largest lactic acid manufacturer in China and Asia. The company has developed a high substrate concentration fermentation process. Also, they applied membrane technology for the separation and purification of lactic acid to reduce energy consumption. More than ten lactic acid production projects are currently under construction with a total capability of more than 100,000 Mt/year in China.

2.1.3 Itaconic Acid and Succinic Acid

Itaconic acid is an unsaturated diprotic acid. Characteristics of the plastic and coating, which is compounded by using 1–5% itaconic acid and styrene, include light color, easy to paint, easy separation, water-fast, and antisepsis; it can be used not only in the manufacture of high-strength enhanced plastic fiberglass, but also in the coating of carpets and book covers. *Aspergillus terreus* is generally applied for itaconic acid fermentation.

In China, several groups have been working on the improvement of itaconic acid fermentation, including a fermentation kinetic study [15], solid-state fermentation [16], strain improvement [17], and product separation [18]. Qingdao Kehai Biochemistry Company produces 10,000 Mt/year of itaconic acid, which is about 50% of the total production capability in China or 18% of worldwide production.

Succinate is a component of the citric acid cycle and is capable of donating electrons to the electron transport chain. It can yield acyl halides, anhydrides, esters, amides, and nitriles for drug applications, agriculture, and food products, and other industrial uses. Succinic acid producers include *Actinobacillus succinogenes*, *Anaerobiospirillum succiniciproducens*, *Mannheimia succiniciproducens*, and recombinant *Escherichia coli*.

On January 22, 2010, DNP Green Technology and Agro-industrie Recherches et Développements of France announced that the world's first succinic acid production by biotechnology was successfully put into operation with an annual output of 2,000 Mt in their joint venture Bioamner. In China succinic acid is currently produced by chemical process, and accounts for 25–30% of the global market production volume. However, research in the field of biological processes for succinic acid production is very active. Zhang [19] and Zhan [20] reviewed recent progress in succinic acid fermentation. Sun et al. [21, 22] screened a highly productive strain, *A. succinogenes* CGMCC 1593, and the succinic acid concentration in the fermentation broth was higher than 40 g/L. Kang et al. [23] constructed an engineering cell for succinic acid production under aerobic conditions. Li et al. [24] analyzed the fermentation process for succinic acid production from crop straw hydrolysate with a neural network method. A succinic acid production project of 30,000 Mt/year is planned in Dewei, Jilin Provence.

2.1.4 Long Carbon Chain Dicarboxylic Acids

The long carbon chain dicarboxylic acids ($n = 10–21$) are found in different plant lipids. It was shown that hyperthermophilic microorganisms specifically contained a large variety of dicarboxylic acids. China is the first country to commercially produce long carbon chain dicarboxylic acids by using biotechnology. In comparison with the chemical method, the biological oxidation reaction can be carried out at ambient temperature and pressure with less byproducts and lower cost. Researchers in Tsinghua University, Institute of Microbiology [25, 26], Chinese Academy of Sciences [27, 28], and the Fushun Research Institute of Petroleum and Petrochemicals [29, 30] have worked on research and development of the biological process for long carbon chain dicarboxylic acid production. After significant work in strain screening and breeding, process optimization, product separation and purification, one kg of long carbon chain dicarboxylic acid can be produced from 1.2 to 1.5 kg of alkane. Several commercial production facilities have been constructed, such as Shangdong Kaisai Biological Technology Material Co. Ltd. (16,000 Mt/year) and Shandong Hiland Biotechnology Co. Ltd. (10,000 Mt/year).

Shandong Hiland Biotechnology Co. Ltd., has finished first-phase construction with an annual production capacity of 10,000 Mt of long carbon chain dicarboxylic acid. The biological process, initially developed by the Institute of Microbiology, Chinese Academy of Sciences, uses a strain of *Candida* sp. to oxidize light gas oil to produce dicarboxylic acid. DC11–DC18 long carbon chain dicarboxylic acids and their derivatives are important intermediates of chemical synthesis and major raw materials for synthetic Musk-T, copolyamide, melt adhesives, high-end engineering plastic, etc., and also widely applied in pesticides, pharmaceuticals, liquid crystal materials, etc.

2.2 Alcohols from Bioprocesses

2.2.1 Acetone/Butanol/Ethanol Fermentation

ABE fermentation (to produce acetone, butanol, and ethanol) has a long history in the world as a successful industrial fermentation process. The earliest work on this method of fermentation was performed by Pasteur in 1882, who studied the production of butanol from lactic acid and calcium lactate [1]. Commercial production attracted interest in 1909 in England because of the possibility of making synthetic rubber via butandiene production from butanol, and DuPont invented nitrocellulose layers with butyl acetate as a solvent for the automobile industry. Acetone also found application in producing explosive cordite. Weizmann successfully developed the ABE fermentation process [31–33]. During 1940, over 45×10^6 and 90×10^6 kg of acetone and butanol, respectively, were produced worldwide. However, competition from the petrochemical industry caused a decrease of the use of ABE fermentation. During the 1960s, ABE fermentation facilities were shut down worldwide except for small-scale production in China and South Africa.

The first ABE fermentation facility in China began operation in the North China Pharmaceutical Factory in 1956. The butanol was applied to the production of butyl acetate, which was used in a penicillin separation process. Another enterprise to use ABE fermentation was the Shanghai Solvent Factory which began operation in the 1950s. The annual total solvent productivities were only a few thousand tons each. With the increasing requirement of solvent in both the chemical and pharmaceutical industry and the backward petrochemical industry, ABE fermentation facilities gradually expanded in the following years. In 1995, the total number of ABE fermentation facilities in China reached more than 50 with a total annual solvent productivity of more than 100 thousand tons. The largest facility was located in the North China Pharmaceutical Factory with an annual total solvent capacity of 20,000 tons. The hydrogen produced in ABE fermentation as a byproduct was recovered and used in the production of sorbitol from glucose. The facility was operated for less than half a year because of competition from the petrochemical industry. Apparently only one factory remained in operation in 2004.

New motivation for the use of ABE fermentation began after it was found that butanol can be used as an ingredient of diesel [34] and because of the sky-rocketing price for crude oil. Recent years have seen vigorous research and development programs in ABE fermentation in China [35–38]. Now, old facilities have been reopened for production and new ones are under construction in China. Henan Tianguan Group Co. Ltd. has signed a cooperation agreement with the Shanghai Institute of Life Science, Chinese Academy of Science, to develop a new acetone/butanol/ethanol fermentation process.

2.2.2 1,3-Propanediol

1,3-Propanediol (PDD) can be formulated into a variety of industrial products including composites, adhesives, laminates, coatings, moldings, aliphatic

polyesters, and copolyesters. It is also a solvent and used as an antifreeze and wood paint.

There are two routes involving a bioprocess with certain microorganisms: (1) Conversion from glucose affected by a genetically modified strain of *E. coli*, which was developed by DuPont Tate & Lyle Bioproducts. An estimated 120,000 tons were produced in 2007. According to DuPont, the Bio-PDD process uses 40% less energy than conventional chemical processes, and reduces greenhouse gas emissions by 20%. (2) Conversion from glycerol (a byproduct of lipid processing or biodiesel production) using *Clostridium diolis* bacteria. With the development of biodiesel production, more and more glycerol will be produced. It is necessary to find a way to comprehensively utilize glycerol. The biological conversion of glycerol into 1,3-PDD is a promising solution.

In 2000 Wang et al. [39] reviewed the production processes of 1,3-PDD and indicated guidelines for research and development of 1,3-PDD. Chen et al. studied 1,3-PDD fermentation with immobilized cells [40, 41]. Scientists in Tsinghua University [42, 43] and Dalian University of Technology [44] are working hard to develop new 1,3-PDD production lines in China. A group of scientists in Tsinghua University developed a two-step fermentation process to produce 1,3-PDD from low-grade starch such as cassava. In the first step, glucose is fermented into glycerol, then the fermentation broth is inoculated with *C. diolis* to convert glycerol into 1,3-PDD. In the separation and purification process, electrodialysis technology was adopted for desalting purposes in the presence of large amounts of organic acids. The purity and recovery of 1,3-PDD reached 99.92% and 80%, respectively. Pilot-scale facilities have been successfully operated, and a 25,000 Mt/year unit is under construction. Production of 1,3-PDD from glycerol by recombinant bacteria expressing recombinant diol dehydratase was also carried out successfully.

3 Biopolymers from Bioprocesses in China

The second category of fermentation products is biopolymers. Biopolymers are polymers produced by living organisms. Cellulose and starch, proteins and peptides, and DNA and RNA are all examples of biopolymers, in which the monomeric units, respectively, are sugars, amino acids, and nucleotides. They are very important and act as genetic materials, biocatalysts, cell membranes or cell walls as well as in energy storage. Some biopolymers, such as polyamino acid (PAA) and poly-3-hydroxybutyrate can be used as plastics, replacing the need for polystyrene or polyethylene-based plastics. Some polymers, such as xanthan and hyaluronic acid, find important applications in the food industry, pharmaceuticals, cosmetics, and even in the crude oil exploitation industry. Naturally occurring biopolymers are renewable, therefore, the use of biopolymers would create a sustainable industry. In contrast, the feedstocks for polymers derived from petrochemicals will eventually run out. In addition, biopolymers have the potential to cut carbon emissions and reduce CO_2 quantities in the atmosphere, because the CO_2 released when they degrade can be reabsorbed by crops grown to replace them. This makes them close

to carbon neutral. Some biopolymers are biodegradable: they are broken down into CO_2 and water by microorganisms. In this section, only those nonstructural biopolymers produced by microorganisms will be discussed [45].

3.1 Polyamino Acids

PAAs are of considerable commercial interest. As biodegradable polyanionic materials their applications range from slow release agents in agriculture, to detergents, surfactants, metal adsorbents, and cosmetics [46]. PAAs offer potential for other applications, such as in diagnostics, sustained-release matrices, microencapsulation, for plasma membrane isolation and chromosomal preparations, carriers for therapeutic protein conjugates and drug delivery systems [47]. Many drug delivery studies have explored their benefits for antitumor drug conjugates. A particularly useful PAA feature is that their *in vivo* degradation rate can be modulated by structural alterations, for example, the hydrophilicity of branch residues. Glycosylated poly(L-glutamic acid) has been proposed as a biodegradable carrier for liver-specific drug delivery. Particularly important are the very low toxicities and immunogenicities PAAs exhibit, for example, in comparison with the acute toxicity of poly(aspartic acid): *Mw* 1,500–3,000: LD_{50} >2,000 mg/L (rat, oral). These factors could result in reduced drug intake requirements, and hence more convenient administration and improved patient compliance. Certain PAAs may also offer therapeutic benefits based on their polyanionic nature.

Three kinds of poly amino acids, poly-γ-glutamic acid, poly(ε-L-lysine) and multi-L-arginyl-poly(L-aspartic acid) can be synthesized by bioprocesses independent of ribosomal protein biosynthesis pathways in microorganisms. These biosynthesized polymers have attracted more and more attention because of their unique properties and various applications. Only γ-PGA and poly(ε-L-lysine) will be discussed in this section.

3.1.1 γ-Polyglutamic Acid

γ-Polyglutamic acid (γ-PGA) is a high molecular weight (typically >1,000,000 Da), water soluble and biodegradable polymer elaborated from *Bacillus* sp. Ivanovics was the first scientist to find γ-PGA in *Bacillus anthracis* in 1937. The molecular structure of γ-PGA is shown in Fig. 2. The microorganisms, which are generally used for γ-PGA production, can be divided into two categories: glutamic acid dependent (such as *B. anthracis*, *B. licheniformis* ATCC 9945A, *B. subtilis* IFO 3335, *B. subtilis* F-2-01, *B. subtilis* CGMCC 0833), and glutamic acid independent (such as *B. subtilis* 5E, *B. subtilis* TAM-4, *B. licheniformis* A35). Glutamic acid must be added as a precursor for γ-PGA synthesis for glutamic acid-dependent strains. The reported yield of γ-PGA in the literature is in the range of 10–60 g/L in the fermentation broth [48, 49]. In this laboratory, a strain of *B. subtilis* was

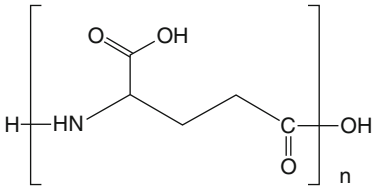


Fig. 2 Molecular structure of γ-PGA

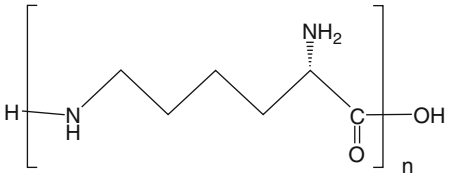


Fig. 3 Molecular structure of ε-PL

screened out from a Chinese traditional food: fermented bean curd. The productivity can be as high as 100 g/L and the molecular weight is about 1.3 MDa. A pilot-plant test has been successfully performed and a commercial production facility is under construction [50, 51].

3.1.2 ε-Polylysine

The molecular structure of ε-Polylysine (ε-PL) is shown in Fig. 3.

Production of polylysine by natural fermentation is only observed in *Streptomyces* strains. Shima et al. found ε-PL in the fermentation broth of *Streptomyces albulus* 346 in 1977 [52]. ε-Polylysine is a homo-polypeptide of approximately 25–30 L-lysine residues. The ε refers to the linkage of the lysine molecules. In contrast to a normal peptide bond that is linked by the α-carbon group, the lysine amino acids are molecularly linked by the ε amino group and the carboxyl group.

ε-Polylysine belongs to the group of cationic surfactants. In water, ε-polylysine contains a positively charged hydrophilic amino group and a hydrophobic methylene group. Cationic surface-active compounds have the ability to inhibit the growth of microorganisms. According to research, ε-polylysine is absorbed electrostatically to the cell surface of the bacteria, followed by a stripping of the outer membrane. This eventually leads to an abnormal distribution of the cytoplasm causing damage to the bacterial cell [53].

Because ε-PL can be adsorbed on the surface of microorganisms by the static electricity interaction, a powerful inhibitory effect on microbes is observed. For various kinds of microbes, the minimum inhibitory concentration (MIC) of ε-PL is as low as 100 μg/mL. The toxicological study showed that ε-PL is safe for human beings, and even at high concentration, no toxic effect or gene mutation is observed. Therefore, ε-PL has been widely applied as a food preservative. Another application of ε-PL is as a medicine carrier. Its cationic property can enhance the ability to penetrate cell membranes [54].

There has been active research and development of the production and application of ε-PL in China [55, 56]. Jia et al. [57, 58] studied the effects of stirring rate and pH value on ε-PL formation. And after mutation and screening, the ε-PL concentration in a fermentation broth of *S. albulus* 410 can reach 48.3 g/L with careful pH and glucose concentration control. Zhejiang Silver-Elephant Bioengineering Co. Ltd., Zhengzhou Bainafo Bioengineering Co. Ltd., and Chengdu Jinkai biology Engineering Co. Ltd. are currently the major ε-PL producers in China.

3.2 Microbial Polysaccharides

Studies on microbial polysaccharides were initiated during World War II, due to the discovery of dextran, which is able to reduce blood viscosity. After this, various kinds of polysaccharides and their important functions were identified. Currently, the output of microbial polysaccharides worldwide increases more than 10% annually.

Microbial polysaccharides can be divided into three types according to their location and function in the cells [59]: (1) Intracellular polysaccharides, which store carbon and energy resources for cell growth and metabolism. (2) Structural polysaccharides to form complete cell membranes and cell walls. (3) Extracellular polysaccharides to protect cells. In this section, only three kinds of important extracellular polysaccharides, namely: Xanthan, Gellan gum (Gellan), and Hyaluronic acid (HA) will be reviewed.

3.2.1 Xanthan

The molecular structure of xanthan is shown in Fig. 4. It is composed of three kinds of monomers, namely, D-glucose, D-mannose, and D-glucuronic acid with different pyruvyl or acetyl groups and with changing ratio. The average percent composition of xanthan produced by *Xanthomonas* bacteria, which is a plant-pathogenic type of bacteria, was listed in Table 2. Synthesis originates from glucose as the substrate for synthesis of the sugar nucleotide precursors UDP-glucose, UDP-glucuronate, and GDP-mannose that are required for building the pentasaccharide repeat unit. This links the synthesis of xanthan to central carbohydrate metabolism. The repeat units are built up at undecaprenylphosphate lipid carriers that are anchored in the cytoplasmic membrane. Specific glycosyltransferases sequentially transfer the sugar moieties of the nucleotide sugar xanthan precursors to the lipid carriers. Acetyl and pyruvyl residues are added as noncarbohydrate decorations. Mature repeat units are polymerized and exported in a way resembling the Wzy-dependent polysaccharide synthesis mechanism of *Enterobacteriaceae*. Products of the *gum* gene cluster drive synthesis, polymerization, and export of the repeat unit. Average molecular weight of xanthan is in the range of 2×10^6–20×10^6 Da [60].

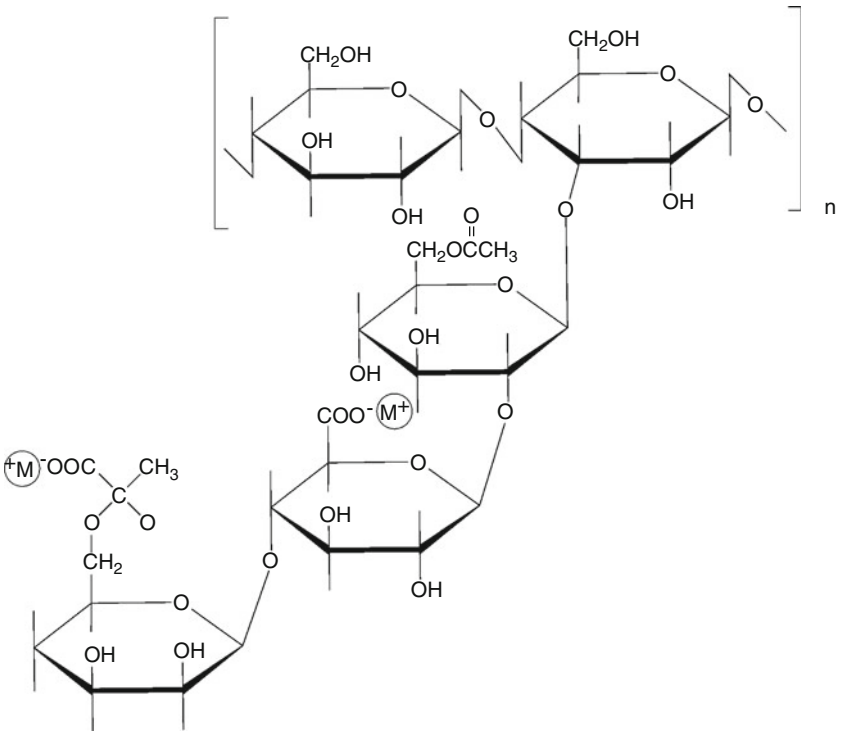


Fig. 4 Molecular structure of Xanthan

Table 2 Average percent compositions of xanthan produced by *Xanthomonas* bacteria

Bacteria	D-Glucose	D-Mannose	D-Glucuronic acid	Pyruvate	Acetate
X. campestris	30.1	27.3	14.9	7.1	6.5
X. fragaria 1822	24.6	26.1	14.0	4.9	5.5
X. gummisudans 2182	34.8	30.7	16.5	4.7	10.0
X. juglandis 411	33.2	30.2	16.8	6.9	6.4
X. phaseoli 1128	30.9	28.6	15.3	1.8	6.4
X. vasculorum 702	34.9	30.2	17.9	6.6	6.3

Xanthan was discovered by an extensive research effort by a group of scientists at the United States Department of Agriculture, and was brought into commercial production by the Kelco Company under the trade name Kelzan in the early 1960s [61]. It was approved for use in foods in 1968 and has been accepted as a safe food additive in many countries. One of the most remarkable properties of xanthan gum is its ability to produce a large increase in the viscosity of a liquid by adding a very small quantity of gum, on the order of one percent. In most foods, it is used at 0.5%, or even lower. The viscosity of xanthan gum solutions decreases with higher shear rates; this is called pseudoplasticity. Unlike other gums, it is very stable under a wide range of temperatures and pH because of its branched structure with a

cellulose-like backbone. In foods, xanthan gum is most often found in salad dressings and sauces. It helps to prevent oil separation by stabilizing the emulsion, and suspend solid particles, such as spices. Toothpaste often contains xanthan gum, where it serves as a binder to keep the product uniform. In the oil industry, xanthan gum is used in large quantities, usually to thicken drilling mud. In cosmetics, xanthan gum is used to prepare water gels. Xanthan gum is a common ingredient in fake blood recipes, and in gunge.

Shandong Fufeng Fermentation Co., Ltd is a company group with more than 60 years experience in producing various bio-fermentation products. The company has been engaging in the R&D, production and marketing of fermentation products, including glutamic acid (180,000 Mt/year), xanthan gum (50,000 Mt/year), monosodium glutamate (75,000 Mt), compound fertilizer (300,000 Mt/year) etc. Having the advantages of specific experience, technology, raw material and energy sources, the company has become the largest fermentation industrial base for producing glutamic acid and xanthan in China.

The research and development of xanthan fermentation in China began 20 years ago [62]. Because of its high viscosity nature, it is necessary to overcome difficulties caused by the high viscosity such as oxygen mass transfer, stirring, and fermenter design [63, 64]. After laborious research and development, Chinese scientists and manufacturers screened highly productive strains for xanthan gum production, such as Shanda-152 and L4, enhanced oxygen mass transfer in high viscosity fermentation, and modified xanthan gum separation and purification processes. Now, xanthan production has been successfully commercialized.

Although CP Kelco currently supplies 40% of the global market for xanthan gum, China is already the largest exporter. CP Kelco and Cargill Inc., world famous xanthan gum producers, have set up xanthan gum production facilities or formed joint ventures in China. Shandong province, China, has been the base for xanthan gum production. The annual output of xanthan gum from Shandong Deosen Corporation Ltd. and Shangdong Fufeng fermentation Co. Ltd., is over 40,000 Mt each. The progress of xanthan production in China is listed in Table 3.

Table 3 Progress of annual xanthan production in China

Year	Output (Mt/year)
1992	100
1998	1,000
2003	20,000
2005	40,000
2009	>100,000

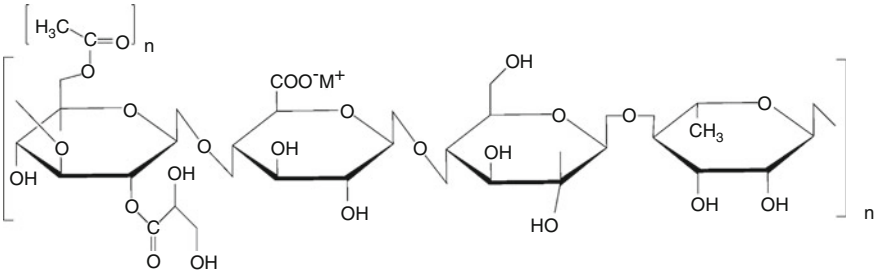


Fig. 5 Schematic diagram of Gellan molecular structure

3.2.2 Gellan Gum

The repeating unit of the gellan gum is a tetrasaccharide which consists of two residues of D-glucose and one of each residue of L-rhamnose and D-glucuronic acid [65]. The molecular structure of gellan gum is shown in Fig. 5. Compared with other colloids, gellan gum has many peculiar advantages such as: low dosage, excellent thermal and acid stability, good taste-releasing ability, high transparency, adjustable gel elasticity and rigidity and good combinability. Gellan gum, also known commercially as Phytagel or Gelrite, is used primarily as a gelling agent, an alternative to agar, in microbiological culture. It is able to withstand 120°C heat, making it especially useful in culturing thermophilic organisms. As a food additive, gellan gum is used as a thickener, emulsifier, and stabilizer. It is also used in soya milk to keep the soy protein suspended in the milk.

Gellan gum is a bacterial exopolysaccharide, prepared commercially by aerobic submerged fermentation from *Sphingomonas elodea* (previously called *Pseudomonas elodea*). It was found that inorganic nitrogen sources were favorable for cell growth and gellan production. The addition of ADP (1 mM) and tryptophan (0.05%) to the medium led to an increase in the yield of gellan up to 39.5 g/L [66].

The production and application of gellan gum in China has been reviewed [67, 68] and the fermentation process was optimized [69]. Commercial production of gellan gum has been performed in several enterprises on a small scale. The main producers of gellan gum in China are Zhejiang Zhongken Biotechnology Co. Ltd. and Shandong Anke Bioengineering Co. Ltd., with an annual output of a few hundred tons each.

3.2.3 Hyaluronic Acid

Hyaluronan (also called hyaluronic acid or hyaluronate) (HA) is an anionic, non-sulfated glycosaminoglycan distributed widely throughout connective, epithelial, and neural tissues. Hyaluronan is a polymer of disaccharides, composed of D-glucuronic acid and D-*N*-acetylglucosamine, linked together via alternating β-1,4 and β-1,3 glycosidic bonds. Its molecular structure is shown in Fig. 6. Hyaluronan

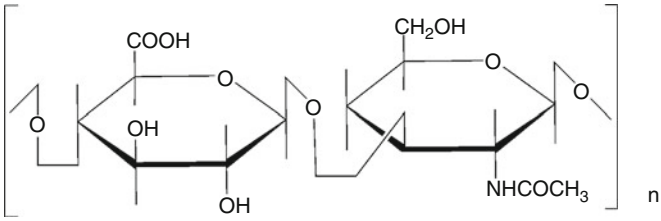


Fig. 6 HA molecular structure

can be 25,000 disaccharide repeats in length. Polymers of hyaluronan can range in size from 5,000 to 20,000,000 Da *in vivo*. The average molecular weight in human synovial fluid is three to four million Daltons. The first hyaluronan biomedical product, Healon, was developed in the 1970s by Pharmacia, and is approved for use in eye surgery. Hyaluronan is also used to treat osteoarthritis of the knee. Because of its high biocompatibility and its common presence in the extracellular matrix of tissues, hyaluronan is gaining popularity as a biomaterial scaffold in tissue engineering research. Hyaluronan is also a common ingredient in skin care products.

In 1937, Kendall et al. found that *Streptococcus haemolyticus* can biosynthesize HA [70]. Currently, HA is produced commercially by either extraction from animal tissue (i.e. rooster comb) or bacterial fermentation [71]. Increased concerns over the contamination of animal-derived products with infectious agents have made bacterial fermentation a more desirable production system to meet future demands. The high viscosity of HA dictates low titres of 5–10 g/L, a level readily achieved through batch fermentation of *Streptococci*. Strain and process development has focused on improving quality, in particular molecular weight. Little is known about what controls the molecular weight of β-polysaccharides such as HA. The HA synthase is responsible for all steps in polymerization and most likely also translocation. *In vitro* studies have identified several residues essential for high molecular weight and maximum molecular weight appears to be an intrinsic feature of the synthase. The actual molecular weight realized in fermentation, however, depends on fermentation conditions. In general, high molecular weight is observed under conditions with excess resources. Metabolic engineering and the recent advance in *omics* technologies are providing new opportunities. Heterologous hosts such as *B. subtilis*, *L. lactis*, and *E. coli* have been successfully engineered to produce HA and may prove more amenable to engineering high molecular weight HA [72].

In order to increase HA productivity, various strain improvement strategies have been adopted such as mutation by physical or chemical methods, plasma fusion and genetic engineering cell construction. After optimization of medium composition and fermentation conditions, the HA productivity increases 4.5 times compared to the original strain [73, 74]. Commercial production is successful in China. The largest manufacturers are Shandong C.P. Freda Pharmaceutical Co. Ltd. and QuFu GuangLong Biochemical Ltd.

Shandong Bausch & Lomb-Freda Pharmaceutical Co Ltd., is a joint venture of C.P. Pharmaceutical Group of Thailand, Freda International Inc. of America and Shandong Biochemical Pharmaceutical Co. of China. The company has been the leading manufacturer in the development and application of sodium hyaluronate series products. Bausch & Lomb spent $ 200 million in cash to acquire 55% of the pharmaceutical unit in 2008.

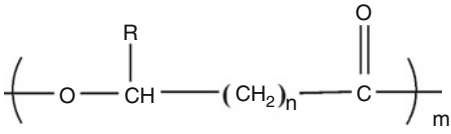
3.3 Polyhydroxyalkanoates

Polyhydroxyalkanoates (PHAs) are organic polyesters composed of (*R*)-3-hydroxy fatty acids which are synthesized by most bacteria as a carbon and energy storage material in times of unbalanced nutrient availability [75]. They are deposited intracellularly as insoluble spherical inclusions called PHA granules which consist of a polyester core surrounded by a phospholipid layer with attached proteins. One of these proteins is the PHA synthase, the key enzyme of PHA biosynthesis, which catalyzes polyester formation from different (*R*)-3-hydroxyacyl-CoA precursors. More than 150 different monomers can be combined within this family to give materials with extremely different properties. These plastics are biodegradable and are used in the production of bioplastics. The general chemical structure of PHAs is shown in Fig. 7.

To produce PHA, a culture of a microorganism such as *Alcaligenes eutrophus* is placed in a suitable medium and fed appropriate nutrients so that it multiplies rapidly. Once the population has reached a substantial level, the nutrient composition is changed to force the microorganism to synthesize PHA. Harvested amounts of PHA from the organism can be higher than 80% of the organism's dry weight. The biosynthesis of PHA is usually caused by certain deficiency conditions (e.g. lack of macro elements such as phosphorus, nitrogen, trace elements, or lack of oxygen) and the excess supply of carbon sources [76].

Polyesters are deposited in the form of highly refractive granules in the cells. Depending upon the microorganism and the cultivation conditions, homo- or copolyesters with different hydroxyalkanic acids are generated.

PHA synthases are the key enzymes of PHA biosynthesis. They use the coenzyme A thioester of (r)-hydroxy fatty acids as the substrate. The two classes of PHA synthases differ in the specific use of hydroxyl fatty acids of short or medium chain length. The resulting PHA is of two types: (1) Poly (HA SCL) from hydroxy fatty acids with short chain lengths including three to five carbon atoms are synthesized



n = 1, 2, 3, 4; m = 200-12000; R = H,CH_3,C_2H_5,C_3H_7,...

Fig. 7 The general chemical structure of PHAs

by numerous bacteria, including *Ralstonia eutropha* and *Alcaligenes latus* (PHB). (2) Poly (HA MCL) from hydroxy fatty acids with middle chain lengths including 6 to 14 carbon atoms, can be made, for example, by *Pseudomonas putida*.

A few bacteria, including *Aeromonas hydrophila* and *Thiococcus pfennigii*, synthesize copolyester, from the above two types of hydroxy fatty acids or at least possess enzymes. Another even large scale synthesis can be done with the help of soil organisms. When lacking nitrogen and phosphorus they produce a kilogram of PHA from three kilograms of sugar.

The simplest and most commonly occurring form of PHA is the fermentative production of polyhydroxybutyric acid, PHB. This consists of 1,000 to 30,000 hydroxy fatty acid units. After completion of the biosynthesis, the bacteria can be up to 80% polyester by weight.

In the industrial production of PHA, the polyester is extracted and purified from the bacteria by optimizing the conditions of microbial fermentation of sugar or glucose.

The British chemical company Imperial Chemical Industries (ICI), developed in the 1980s fermentatively created copolyester produced from 3-hydroxybutyrate and 3-hydroxyvalerianacid. It was sold under the name "Biopol."

Research and development of PHA production in China can be traced back 20 years [77–80]. In the beginning, research interests were focused on strain screening and breeding as well as optimization of fermentation conditions to increase intracellular PHA yield [81]. In order to increase the productivity, high cell density cultivation was considered, however, oxygen mass transfer was found to be a limiting factor for high cell density cultivation. A group of scientists in Tsinghua University proposed a solution strategy. They constructed genetic engineering *E. coli* cells [82–84], in which, a PHB synthase gene (phbCAB), a *Vitreoscilla* hemoglobin gene (vgb) and a phage λ lysis gene (SRRz) were inserted into the chromosome of *E. coli*. By this method, during cell cultivation, hemoglobin was expressed to enhance the oxygen mass transfer to facilitate high cell density cultivation up to 200 gDW/L, PHB was synthesized in high yield (>90% of dried cell weight) and lyase was expressed at the end of fermentation, which was able to lyse the cell wall to facilitate the release of PHB. In this way, the production cost of PHB was reduced greatly. Several companies in China specialize in the production of various kinds of PHAs. For example, Ecomann Biotechnology Co. Ltd. set up a 5,000 MT/year facility in Shandong Province which was put into successful operation on July 29, 2009. Tianjin Green BioScience Ltd. (TGBS) is a biotech company dedicated to developing bioplastics and relevant products. By the 2008 Olympic Games, TGBS was capable of producing 10,000 tons annually.

Prof. Guo-Qiang Chen (Department of Biology Science and Technology, Tsinghua University) has focused his research on biopolyester polyhydroxyalkanoates (PHA) since 1986. He has been actively promoting the PHA-based bio- and material industries in China, including the use of bioplastics in the 2008 Beijing Olympic Games which were designated the Green Olympics,

(continued)

and the use of PHA-related products for applications such as biofuels and bulk chemicals. Prof. Chen has more than 20 years of R&D experience in PHA production and applications, and has published over 150 international peer-reviewed papers.

4 Products from Enzyme-Catalyzed Reactions in China

The third category of organic compounds produced by bioprocesses is those produced by enzyme-catalyzed reactions, which are traditionally produced by chemical-catalyzed reaction at high cost, with high energy consumption, low yield and severe environmental pollution. With enzyme-catalyzed reactions, the process can be operated at ambient temperature and pressure, with much higher efficiency and selectivity, and therefore it is a so-called "green process." A present trend is to find specific enzymes and design specific processes to replace the traditional chemical counterpart.

In this section, several successful industrial processes using the enzyme-catalyzed reaction in China will be introduced.

4.1 Acrylamide

Shen Yinchu, a member of the Chinese Academy of Engineering, is a well-known specialist in the field of biochemical technology. He graduated from the Department of Biochemistry of Fu Dan University in 1962. Since then, he has been working in the Shanghai Pesticide Research Institute, Shanghai Research Center of Bio-chemical Technology of the Ministry of Chemical Industry and Zhejiang University of Industry. He has long been engaged in the research and development of biochemical engineering and biopesticides, and has made important contributions. During the 1980s, Shen Yinchu and coworkers carried out difficult and creative research to produce acrylamide via the enzyme-catalyzed reaction instead of the traditional chemical method. Currently all acrylamide production units in China use their technology.

Acrylamide is a monomer of polyacrylamide, which is used in synthetic fibers, oil recovery and flocculating agents, paper manufacture, and textile sizers. The conventional process for acrylamide production is based on the hydrolysis of acrylonitrile using copper-based catalysts, which requires laborious preparation, difficult purification and isolation of reaction products, and high temperature conditions [85]. Several groups of bacteria such as *Nocardia*, *Bervibacterium*, *Arthrobacter*, *Rhodococcus*, and *Pseudomonas* are able to convert acrylonitrile to acrylamide. These bacteria are known to possess a diverse spectrum of nitrile

hydratase (NHase) activities. The NHase is the biocatalyst which has been used in industrial bioconversion of acrylontrile to acryamide.

In 1986, Shen and coworkers from the Shanghai Pesticide Research Institute screened a high-yielding NHase strain with an orange-red appearance, which was identified as *Nocardia* sp. and tentatively named *Nocardia* sp. 86-163. The NHase activity of the strain was significantly improved up to 5627.5 U/mL through strain improvement by mutagenesis and optimization of culture conditions. Also, they developed a comprehensive process to perform the enzyme-catalyzed reaction and product separation and purification [86]. In China, the first pilot-scale unit capable of producing 440 Mt/year of acrylamide began successful operation in 1993. The first generation of commercial facilities was constructed by the Hebei Wanquan Oilfield Chemical Company and Shandong Shengli Oilfield Group with a capacity of 1,000–2,000 Mt/year acrylamide. Presently acrylamide production using chemical processes is no longer carried out; instead, the annual output of more than 200,000 MT of acrylamide is solely produced by enzymatic processes in China.

Research on strain improvement is still under way [87, 88]. Various microorganisms such as *Micrococcus* sp., *Norcardia* sp., and *Rhodococcus* sp., were selected to screen for a high NHase activity strain. It was found that with a *Nocardia* sp., the RS strain, NHase activity was increased to 10,195 U/mL in the glucose–Co^{2+} coupling fed fermentation, which is the highest among all those reported in NHase production [89].

4.2 D-Panthenol and D-Pantolactone

Biocatalytic resolutions make use of the selectivity of enzymes for one of the enantiomers of a chiral molecule, whereby one enantiomer of a racemate remains virtually untouched and the other enantiomer is converted into the desired enantiomerically pure product/intermediate. Hydrolases are by far the most prominent group of enzymes used in production of fine chemicals by biocatalytic resolution.

D-Pantolactone is a key compound in the synthesis of D-calcium pantothenate and D-panthenol [90]. Both compounds are widely used as ingredients in pharmaceutical and cosmetic compositions, as well as in food and feed supplements. Panthenol is the alcohol analog of pantothenic acid (vitamin B5), which is an "antistress vitamin" and is part of the water-soluble B-vitamin group. In organisms it is quickly oxidized to pantothenate. Panthenol comes in two enantiomers, D and L. Only D-panthenol (*dexpanthenol*) is biologically active. In cosmetics, panthenol is a humectant, emollient, and moisturizer. It is the key precursor for coenzyme A (CoA) and acyl carrier protein. Biosynthesis of pantothenate takes place only in bacteria, fungi, and plants, while animals must obtain it from their diet. Pantothenate deficiency in humans can result in abdominal distress, vomiting, cramps, burning feet syndrome, fatigue, insomnia, and reduced immunity to

some infectious agents. In 2002, the global production of pantothenates exceeded 9,000 Mt.

In the 1990s the method of production still depended for the most part on chemical synthesis from bulk chemicals. The chemical synthesis process involves reactions yielding racemic pantolactone from isobutyraldehyde, formaldehyde, and cyanide, followed by optical resolution of the racemic pantolactone to D-pantolactone, and condensation of the D-pantolactone with β-alanine. However, this synthesis requires the optical resolution of racemic intermediates. In the resolution step, the use of an expensive alkaloid as a resolving agent is unavoidable. Therefore, a variety of routes have been assayed to improve its synthesis, including stereospecific reduction of ketopantolactone [91], ketopantoate [92] or 2′-ketopantothenate derivative [93], stereoselective inversion of L-pantolactone in a racemic mixture to D-pantolactone [94] and stereoselective hydrolysis of esterified pantolactone [95]. In 1995 one of the processes of D-pantothenate synthesis used the lactonohydrolase activity of *Fusarium oxysporum*, which catalyzes the stereospecific hydrolysis of chemically made D,L-pantolactone to generate D-pantolactone as a chiral building block for its further chemical conversion to D-pantothenate [96]. The reaction is stereospecific and only the D-enantiomer in the racemic mixture is hydrolyzed. *F. oxysporum* AKU 3702 showed high productivity of the enzyme and the whole cells containing the enzyme could be used repeatedly for this hydrolysis reaction. At 30°C and pH 6.8–7.2, 90–95% of the D-pantolactone was hydrolyzed after 21 h reaction with 90–97% optical purity. The immobilized mycelia retained more than 90% of their initial activity after 180 repeated reactions [97].

Calcium pantothenate used in the feed industry in China was dependent for a long time on imports. The Shanghai Fourth Pharmaceutical Factory was the first company to produce D-calcium pantothenate as a vitamin raw material in 1958. Until 1996, however, the production capacity of China's calcium pantothenate was less than 100 MT annually. In order to meet the need of feed additives, in the 1990s, China began to develop calcium pantothenate industries and built a number of 100-ton-scale D-calcium pantothenate production plants with two unique characteristics: the use of D-amine hydrochloride, which was the byproduct of chloramphenicol production, in splitting pantolactone; and the induced crystallization method to split DL-calcium pantothenate.

Sun et al. [98] isolated and identified a strain of *Fusarium moniliforme* SW-902 for producing D-lactonohydrolase in 2001. Using glycerol as the carbon source and peptone as the nitrogen source, in a 60 L fermentor under optimum conditions, about 7.18 g dry cell/L and 0.92 IU/g dry cell weight were obtained [99]. This technology has been applied to commercial production of D-calcium pantothenate and D-Panthenol in Zhejiang Hangzhou Xinfu Pharmaceutical Co. Ltd., China [100]. In comparison with the traditional chemical synthesis process, the biological method reduces raw material and energy consumptions by 69.2% and 12.7%, respectively. This means a higher atomic utilization ratio, low emissions, and higher profits. Now, Xinfu Pharmaceutical Co., Ltd. has become the largest producer of both Calcium D-Pantothenate and Panthenol in the world.

Zhejiang Hangzhou Xinfu Pharmaceutical Co. Ltd. is a globally leading manufacturer and supplier of Vitamin B5 (Calcium D-Pantothenate and Panthenol series products). The D-calcium pantothenate production capacity is 5,000 Mt/year, which is about 40% of the world market. Xinfu is also the only manufacturer of D-panthenol (Pro-Vitamin B5) in China.

4.3 Malic Acid and L-Tartaric Acid

Malic acid is a four-carbon dicarboxylic acid, and an intermediate of the tricarboxylic acid (TCA) cycle. Industrially, malic acid has been employed for the preparation of food additives and synthesis of various fine chemicals [101]. Malic acid is also applied in the pharmaceutical and cosmetic industries. Malic acid can be produced by various methods such as isolating it from natural fruit juices, enzymatic conversion and chemical synthesis. Commercially it is produced either by chemical synthesis via hydration of fumaric acid which results in a racemic mixture or by an enzymatic process which yields optical pure malic acid. L-malic acid is produced from fumarate by enantioselective hydration catalyzed by fumarase, using either whole cells or isolated enzyme [102, 103]. This process is a typical equilibrium reaction [104]. Malic acid can also be produced by direct fermentation with a wide range of microorganisms. Fermentation of *Aspergillus flavus* allows successful production of malic acid from renewable feedstocks, but the productivity of malic acid is low.

Brevibacterium ammoniagenses has high fumarase activity, and is suitable for malic acid production, either in free or immobilized whole cell systems. The disadvantage of this system was that byproducts like succinic acid were formed in considerable amounts. In the 1990s production of L-malic acid from fumaric acid using *Saccharomyces cerevisiae* cells was extensively studied. *S. cerevisiae* entrapped into polyacrylamide gel disks can produce malic acid from fumaric acid without formation of a byproduct. Malic acid concentration of 12 g/L was achieved in *S. cerevisiae* by overexpression of the cytosolic isoenzyme of malate dehydrogenase (Mdh2p) [105].

Ouyang et al. [106] found a strain of *S. cerevisiae*, which was high in maleate hydratase activity. A new process was developed based on the principle of the coupling reaction and separation process. They [107] studied the dissolution behaviors of the substrate calcium fumarate and product calcium malate. The kinetic equations for different temperatures were proposed and the effect of pH and temperature on the dissolution process was studied. On the basis of the above work, the free cells were used as the catalyst. The slightly soluble calcium fumarate was used as the substrate. The formation of malate was able to increase the solubility of calcium fumarate, which was favorable for the enzyme-catalyzed reaction. The produced excess calcium malate formed solid crystals again and was easily removed from the reaction system. By this process, the original reversible reaction becomes favorable to malate formation, and an almost 100% conversion ratio was

achieved. In 2004 a similar process in which D-malate was effectively produced from maleate by maleate hydratase of *Pimelobacter simplex* DM18 was developed by Ouyang et al. [108]. The conversion of a high concentration of Ca-maleate into Ca-malate was achieved by maleate hydratase, owing to the low solubility of both the Ca-maleate and Ca-malate complex. The coupling reaction and separation was beneficial to both product formation and downstream processing. After 36 h of reaction, 385 g/L of Ca-D-malate was produced with an optical purity and molar yield of 97.03% and 99%, respectively.

Prof. Ouyang Pingkai, a member of the Chinese Academy of Engineering, is a leading scientist in biochemical engineering. He originally presented the combinational method to construct and optimize bioprocesses, such as the combination of bioreactions, combination of bioreaction and biomembrane, combination of bioreaction and separation. These concepts have been applied in various bioprocesses for the production of FDP, L-Alanine, L-Phenylalanine, and L-malic acid etc. in China and the highest production levels in the world were achieved.

L-tartaric acid is also a four-carbon dicarboxylic acid and a well-known natural acid that is widely distributed in many kinds of fruits, especially grapes. A broad application spectrum has been found in food, pharmaceutical and cement industries. In addition, tartaric acid also usually serves as a starting substance for numerous chemical reactions, especially for chiral synthesis. Traditionally, L-tartaric acid is produced from crude potassium tartrate which is obtained during wine making. Currently, L-tartaric acid is produced biotechnologically from *cis*-epoxysuccinic acid [109].

Various microorganisms, such as *Gluconobacter suboxydans* [110, 111], *Corynebacterium* sp. [112], *Nocardia tartaricans* [113] and *Rhodococcus rubber* [114], have been used to produce *cis*-epoxysuccinate hydrolase (CES hydrolase), which is further applied for L-tartaric acid production from *cis*-epoxysuccinic acid. The activity of CES hydrolase of the abovementioned microorganisms is stimulated by the presence of *cis*-epoxysuccinic acid in the culture medium. Whole-cell immobilization has been investigated as a method to reuse the biocatalyst and increase the productivity of the CES biotransformation process.

In 1995, Sun et al. reported that immobilized *N. tartaricans* SW 13-57 cells showed high CES hydrolase activity [113]. The optimum pH of immobilized cells was pH 8.0–9.0. With a fixed-bed column packed with immobilized cells, the average molar conversion ratio reached 99.09% after being continuously operated for 53 days. In 2000, Zhang et al. [112] immobilized *Corynebacterium* sp. JZ-1 cells by entrapping in k-carrageenan gel beads. Satisfactory results were observed in repeated batch operation and continuous fixed-bed operation for 90 days. In 2004, Min et al. [114] isolated one strain of *R. rubber* with high CES hydrolase production ability.

Changzhou Changmao Biochemical Engineering Co. Ltd. The company specializes in manufacturing C-4 series organic acids and chiral products including fumaric acid, maleic acid, L-malic acid, D-malic acid, DL-malic acid, L(+)-tartaric acid, D(-)-tartaric acid, DL-tartaric acid, L-aspartic acid, and aspartame. The total annual output reaches 25,000 Mt. In D- and L-malic acid production, the company closely cooperated with Prof. Ouyang Pingkai and Prof. Sun Zhihao, and made great progress.

5 Future Perspectives

China is among the countries that have emerged out of the recent financial crisis and is developing at a rate higher than 8.7% annually. China is still a developing country and needs further development to improve the population's living standard and infrastructure. Also, China is a country facing a lot of challenges, including shortages of various kinds of resources, air and water pollution, CO_2 emission, and so on. Although the solution is not simple, the development of industrial biotechnology may help. Biomass is abundant and renewable. Besides satisfying requirements in daily life, biomass plays a unique role in oxygen and carbon recycling. The utilization of biomass as a raw material for the production of biofuels and a variety of important chemicals has made great progress in the past few decades both in the world and in China. Some key technologies will soon breakthrough, such as: pretreatment technology for lignocellulosic materials, high efficiency production of cellulase, discovery of new platform chemicals from biomass, high-throughput screening technology for microorganisms, application of genetic engineering to industrial microorganisms, and so on. In recent years, China has been catching up in the development of industrial biotechnology. Apart from a continuously expanded production scale, new products and new technologies are under development and are transferring into commercial production quickly.

The Chinese government is firmly supporting basic and applied research in industrial biotechnology through the National Natural Science Foundation Projects 973 and 863 at a previously unseen scale. The enterprises in China are eager to develop new products and new technologies, either by themselves or in cooperation with Research Institutes and Universities. Scientists have accumulated a great deal of experience during the past few decades and created various bioprocesses in China. With an emphasis on Intellectual Right Protection, more international cooperation treaties are under discussion and more foreign companies are investing heavily in industrial biotechnology in China. The solid research background, enormous human resources and sufficient financial support will guarantee the fast growth of industrial biotechnology in China in the future. The bioprocesses will contribute more and more to sustainable development of China.

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Biofuels in China

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Abstract The Chinese government is stimulating the biofuels development to replace partially fossil fuels in the transport sector, which can enhance energy security, reduce greenhouse gas emissions, and stimulate rural development. Bioethanol, biodiesel, biobutanol, biogas, and biohydrogen are the main biofuels developed in China. In this chapter, we mainly present the current status of biofuel development in China, and illustrate the issues of feedstocks, food security and conversion processes.

Keywords Bioethanol, Biodiesel, Biobutanol, Biogas, Biohydrogen, Cassava, Lignocellulose, Lipases, Sweet sorghum

Contents

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1 Bioethanol in China

1.1 Bioethanol

Bioethanol, unlike petroleum, is a form of renewable energy that can be produced from agricultural feedstocks. It can be made from very common crops such as sugar cane, potato, manioc, maize, and cellulose. According to the International Energy Agency, cellulosic ethanol could allow ethanol fuel to play a much bigger role in the future than previously thought [1].

Ethanol has many positive features as an alternative liquid fuel. First, ethanol is a renewable, relatively safe fuel that can be used with few engine modifications. Second, its energy density is higher than some other alternative fuels, such as methanol, which means less volume is required to go the same distance. The third benefit of ethanol is that it can improve agricultural economies by providing farmers with a stable market for certain crops, such as maize and sugar beets. Fourth, using ethanol increases national energy security because some use of foreign petroleum is averted. Another benefit is that using ethanol might decrease emissions of certain emissions. A 10% ethanol blend to ordinary gasoline results in a reduction of carbon dioxide emissions of 3–6%. It reduces the environmental impact from the transportation sector, one of the major contributors to the greenhouse gas emissions that are changing our climate. Toxic, ozone-forming compounds such as aromatics, olefins, and hydrocarbons are emitted during the combustion of gasoline; this emission would be eliminated with the use of ethanol [2].

Interest in alternative fuels began with the realization that the supply of nonrenewable fossil fuel is not infinite, a fact which has important energy security consequences for a country. For example, national dependence on foreign petroleum reserves creates economic vulnerabilities. In 2008, approximately 48% of the Chinese crude oil was imported. Back in the 1970s, when OPEC shut off the spigot, Brazil invested heavily in alcohol to replace gasoline. From the 1970s to the late 1990s, ethanol yields per acre had risen from 242 to 593 gallons. It is estimated that Brazil saved up to $55 billion (US dollars) on oil imports from 1975 to 2003 as a result of the national ethanol program. Nowadays, ethanol is widely used in Brazil and in the United States, and together two countries were responsible for 89% of the world's ethanol fuel production in 2008 (*Source: RFA, F.O. Licht 2008 Estimates*). Most cars on the road today in the US can run on blends of up to 10% ethanol, and the use of 10% ethanol gasoline is mandated in some US cities.

1.2 Background of Bioethanol Development in China

At the beginning of 1990, for the sake of national security, (the) PRC government advocated food self sufficiency and set a national grain reserve system. The lowest amount of reserved grain was 135 million tons, of which 20–30% was substituted by fresh grain every year. Annual crop outputs stabilized at 500 million tons from 1996 to 1999, and almost all grain barns of the reserve system across the country were full. It turned out to be a burden to the government, which spent 2.8 billion yuan to administer and maintain the grain reserve.

Concerned about excess old stocks in the grain reserve system, rural poverty, and the issues of rising dependency on oil imports and increasing greenhouse emissions, (the) PRC government began developing its plan for biofuels development, especially the fuel ethanol industry. In April 2001, standards for "Denatured Fuel Ethanol" (GB18350-2001) and "Bioethanol Gasoline for Automobiles" (GB18351-2001) were released. The China Petroleum and Chemical Corporation (also known as Sinopec) set up a series of enterprise standards on fuel ethanol's blending, storage, and delivery. In June 2001, according to the "State Scheme of Pilot Projects on Bioethanol Gasoline for Automobiles," three cities in Henan province (Zhengzhou, Luoyang, and Nanyang) and two cities in Heilongjiang province (Harbin and Zhaodong) launched pilot projects on mandatory utilization of fuel ethanol in all vehicles for a year. Sinopec was responsible for setting up a fuel ethanol blending facility in three cities in Henan province to blend and deliver fuel ethanol locally. The China National Petroleum Corporation (CNPC) was assigned to build blending facilities in the two cities in Heilongjiang province. The pilot projects achieved success in management of fuel ethanol's supply, delivery, marketing, and service. In the meantime, this helped consume a considerable amount of stale grain. For example, 1.05 million tons of stale grain in the reserve grain system was used to produce 30,000 tons of fuel ethanol in Henan. The consumption of 1.05 million tons of stale grain in the reserve grain system saved Henan province 200 million yuan in subsidies (Henan Tianguan Group) [3].

Given the success of the pilot project, on February 10th, 2004, the National Development and Reform Commission (NDRC), along with seven ministries, announced the "State Scheme of Extensive Pilot Projects on Bioethanol Gasoline for Automobiles" (hereafter referred to as SSEPP) in order to adjust further (the) PRC's national energy consumption structure, explore substitutes for crude oil, improve air quality, and promote agricultural production and sustainable development. According to the SSEPP, extensive pilot projects were launched in five provinces (Heilongjiang, Jilin, Liaoning, Henan, and Anhui), in nine cities in Hubei province, seven cities in Shandong, six cities in Hebei, and five cities in Jiangsu. Original #90, 93, 95, and 97 unleaded gasolines were mandated to switch to #90, 93, 95, and 97 bioethanol gasoline. Up to 2009, four ethanol plants have been authorized by the government to produce fuel ethanol from grain: Jilin Fuel Ethanol Co. Ltd., Anhui Fengyuan Biochemical Co. Ltd., Heilongjiang Zhaodong Biochemical Co. Ltd., and Henan Tianguan Group. A total of 1.56 million tons of

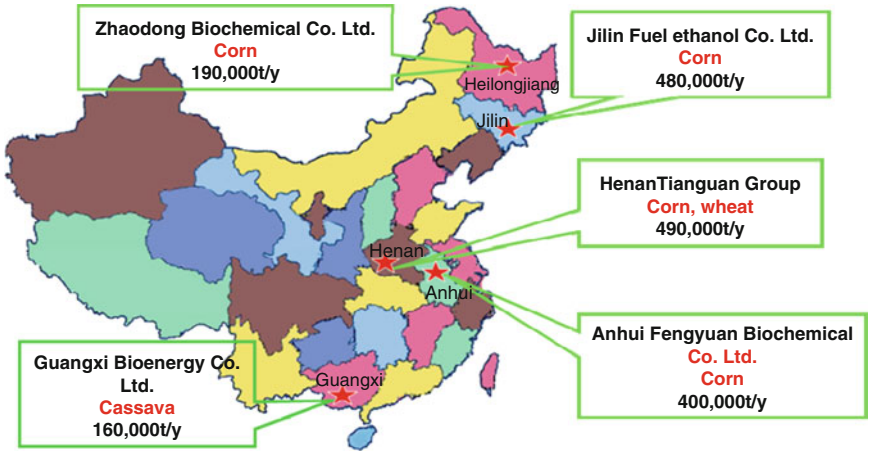

Fig. 1 (The) PRC's four existing government-sponsored ethanol plants based on corn or wheat and one bioethanol plant based on non-grain feedstock (cassava)

denatured fuel ethanol is involved, which is mainly based on grain (Fig. 1). It is estimated that 80% of production is corn-based and the remaining 20% is wheat or rice based. The fifth plant (in Guangxi Province), which started trial operations in December 2007, was designed to produce at an annual capacity of 200,000 tons, using only cassava as its feedstock. Its fuel ethanol production reached 160,000 tons in 2009 and, as prescribed in the E10 mandate (10% ethanol blended into fuel), the province has already implemented [3] its requirements.

In the twenty-first century, human society will face great obstacles of resource shortages and environmental pollution. After 28 years of rapid development, (the) PRC has become a huge oil consuming and pure oil importing country since 1993. More than 100 million tons of oil has been imported annually since 2003. In 2008, about 374 million tons of crude oil and oil products were consumed in (the) PRC, including 73 million tons of gasoline. This means that (the) PRC has become the second largest petroleum consumer in the world. The demand for imported petroleum will continue to increase, and the import ratio in the total Chinese petroleum demand will rise to 75% by 2030, which will have a serious impact on the national economic security of (the) PRC [4]. Next to industrial use, transportation use ranks second in petroleum utilization and is growing very fast because of the rapid growth in vehicle numbers. In Beijing alone, 1,000 cars are added to the city's roads every day [5]. As disposable incomes increase, more and more Chinese people are purchasing privately owned vehicles. Finding renewable substitutes, especially new liquid fuels, has become a pressing issue. Using ethanol from renewable biomass resources as fuel has strategic importance like reducing import of oil, reducing air pollution, and facilitating economic development and employment in rural areas.

1.3 Development of Non-Grain Ethanol

As (the) PRC has 1.3 billion people, the biggest population in the world, providing enough food for them is already a big challenge to its government. Producing more fuel ethanol from grain will endanger (the) PRC's food supply. In 2005, 0.7 million tons of reserved wheat and 2.87 million tons of reserved corn were used to produce fuel ethanol. The stale grain in the grain reserve was almost used up after several years of development of ethanol, and more and more new crops are being used [6]. About 30 million tons of grains have been imported to (the) PRC annually in recent years. In 2009, (the) PRC also began to import corn, the amount of which is predicted to reach 2.9 million tons in 2015 (Fig. 2) [7].

In December 2006, NDRC started to regulate corn-based bioethanol. The Chinese central government wants to expand bioethanol production, especially from non-grain stocks, instead of expanding corn-based bioethanol production. Technological innovation is a crucial issue for developing non-grain-based bioethanol production in (the) PRC. Sweet sorghum, sweet potato, and cassava have been chosen for ethanol production in (the) PRC, and this trend will develop rapidly with major support from the Chinese government.

1.3.1 Sweet Sorghum-Based Bioethanol

Sweet Sorghum

Sweet sorghum is a C4 crop in the grass family belonging to the genus *Sorghum bicolor L. Moench* which also includes grain and fiber sorghum and is characterized by a high photosynthetic efficiency. The primary advantages of sweet sorghum are:

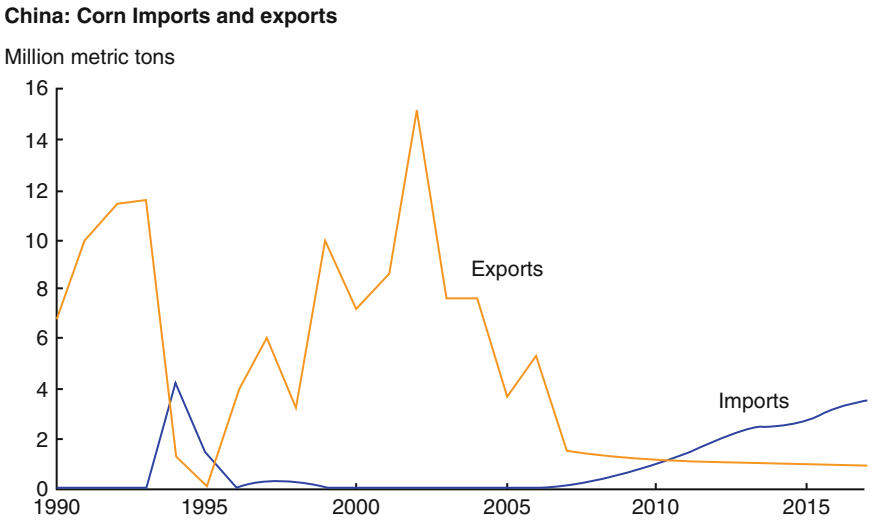

Fig. 2 Corn imports and exports of (the) PRC from 1990

1. Its high ethanol productivity, 3,500–5,600 L/ha/year. Sweet sorghum is a high biomass and sugar yielding crop. The plant grows to a height of from about 120 to above 400 cm, depending on the varieties and growing. The sugar content in the juice extracted from sweet sorghum varies from 16 to 23% Brix [8].
2. Its reduced need for nitrogen fertilizer and water when compared with more conventional crops such as corn. Sweet sorghum is often considered to be one of the most drought resistant agricultural crops as it has the capability of remaining dormant during the driest periods. Table 1 shows the annual water requirement, gasoline equivalent ethanol yield per hectare, and ethanol yield per unit of water input for select crops [9, 10].
3. Its adaptability to diverse climate zones and soil conditions. Like other sorghum types, sweet sorghum probably originated from East Africa and spread to other African regions, Southern Asia, Europe, Australia, and the United States. Although a native to the tropics, sweet sorghum is well adapted to temperate climates [8]. Figure 3 shows the potential planting area for sweet sorghum.

Table 1 Land and water intensity of potential sources for ethanol

Ethanol feedstock	Water required (mm/year) (low)	Water required (mm/year) (high)	Crop yield (tons/ha)	Ethanol conversion efficiency (L/ton)	Gasoline equivalent ethanol yield (L/ha)	Ethanol yield per mm of water	Growing season (months)
Sugarcane	1,500	2,500	70	70	3,300	1.65	10–12
Wheat	450	650	2.6	340	600	1.09	4–5
Maize	500	800	1.8	360	450	0.69	4–5
Sweet sorghum	450	650	40	70	1,900	3.45	4–5
Sugar beet	550	750	100	110	7,370	11.34	5–6

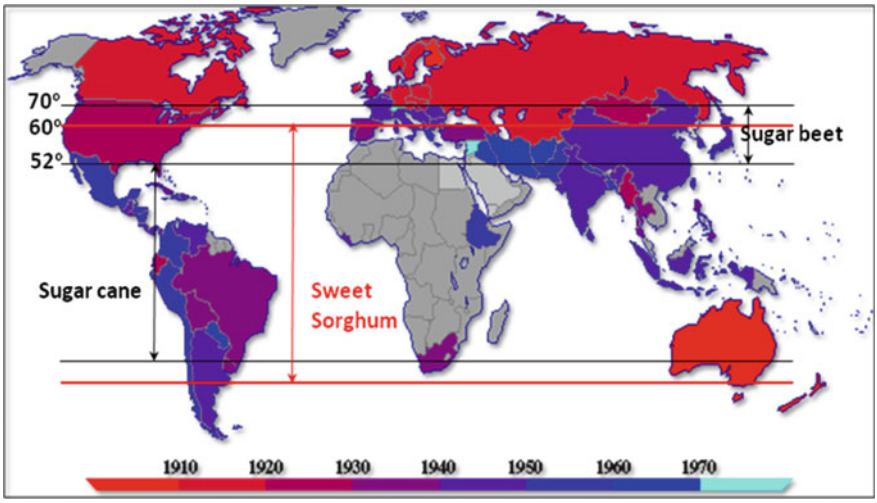

Fig. 3 The potential planting area for sweet sorghum

It has the potential of becoming a useful energy crop, which has been evaluated in several recent reports as an alcohol fuel crop with a promising future [8, 11].

Most saline soil located in North PRC, amounting to 23.6 million acres in the northern 13 provinces, can be used for development of sweet sorghum.

Bioethanol from Sweet Sorghum in China

Of the many crops currently being investigated for energy and industry in (the) PRC, sweet sorghum is one of the most promising, particularly for ethanol production. The stem juice of sweet sorghum is rich in fermentative sugar and is a desirable alcoholic fermentation material. Starting from 1983, research has been undertaken in (the) PRC to improve the yield of juice from sweet sorghum stem. For instance, the Shenyang Agriculture University bred new hybrids of sweet sorghum called Tianza No. 2, which has high yields of both grain and fermentable sugars (5.0 tons/ha grain yield and 16.1 °BX juice sugar degree). Several foreign varieties, like Rio and Bailey, are imported from the United States. Table 2 shows the Brix degree of juice in sweet sorghum stem of different varieties at different periods.

Most saline soil is located in North PRC. Amounting to 9.6 million hectares of saline-base land and 23.7 million hectares of waste grassland areas in Shandong, Hebei, Liaoning, Jilin, Heilongjiang, Inner Mongolia, Shandong, Shanxi, Gansu, Ningxia, Xinjian, and Qinghai provinces and Tianjin City can be used for development of sweet sorghum. In 2006, total production of sweet sorghum was 2.5 million tons grown on 570,000 ha [12].

In (the) PRC, ethanol production from sweet sorghum is currently out of commercialization. The major challenge for sweet sorghum as a feedstock to produce ethanol is stem collection and sugar storage. Because sweet sorghum can only be harvested once a year in most area of (the) PRC and the sugar content in the stem can be degraded very soon, sweet sorghum stem has to be collected and stored in a very short period. So, collection systems and storage techniques need to be established for industrial application. Another obstacle for large application is how to deal with the large amount of bagasse, which is produced after ethanol fermentation. High value by-products from bagasse, e.g., poly(lactic acid)/sweet sorghum fiber composites, should be available in the future.

On August 19th, 2008, the Ministry of Science and Technology (MOST) of the People's Republic of China signed an important agreement with the US Department

Table 2 Brix degree of sweet sorghum stem of different varieties at different periods

Variety	Date (day/month)						
	26/08	05/09	13/09	04/10	10/10	15/10	29/10
Rio	12.2	15.5	16.8	17.0	17.0	15.5	14.5
Shennong no.2	11.5	12.7	15.3	14.5	14.5	13.0	9.0
Jitian 2	10.1	12.8	15.8	15.5	14.0	14.0	12.2
Longshi 1	14.5	16.3	18.0	13.0	14.0	12.5	11.5

of Agriculture (USDA) to collaborate on biofuels research at the International Conference on Sorghum and other non-grain stocks for Biofuel. This collaboration will definitely improve the progress of industrialization of ethanol production from sweet sorghum.

Ethanol Conversion from Sweet Sorghum

The processes of ethanol production from sweet sorghum are the same as the traditional way for sugarcane molasses. Stem juice, containing most of the fermentable sugars from sweet sorghum, can easily be fermented into ethanol. To make ethanol an economically feasible biofuel, the process has to be optimized in terms of yield as well as ethanol production. Research in recent years has shown that the application of very high gravity (VHG) fermentation technology for industrial scale production of fuel alcohol is a distinct possibility. A research group from Beijing University of Chemical Technology achieves high ethanol concentration of 130 g/L using a novel yeast cell immobilization system (see Fig. 4). This system uses the bagasse as carriers for yeast cell immobilization. Comparing with other immobilization methods, it has many advantages, such as easier manipulation, cheaper carrier, and more steady.

The technique of solid state fermentation is considered more suitable for ethanol production from sweet sorghum stem (see Fig. 5). It avoids the need to isolate the sugars into a separate liquid phase before fermentation. It has many potential advantages especially for regions short of water: (1) less requirement for water (especially attractive in summer months when water is scarce), (2) less physical energy requirement, (3) less capital investment, (4) less liquid waste to be disposed of and hence less pollution problems. Chinese Academy of Agricultural Engineering has a pilot scale operation for ethanol production by solid state fermentation with a high ethanol yield of 10%.

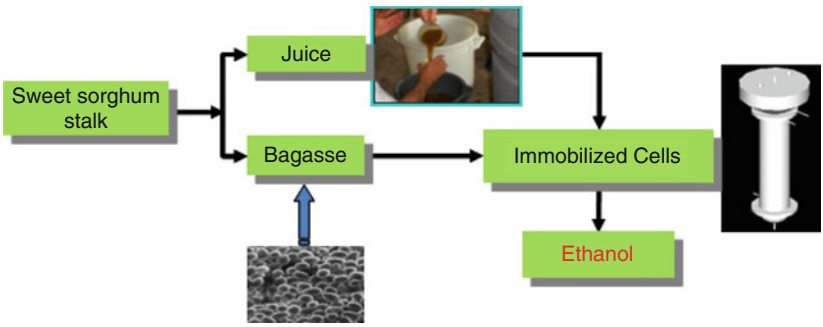

Fig. 4 Flowchart of ethanol production from concentrated sweet sorghum stem juice using novel immobilized cell system

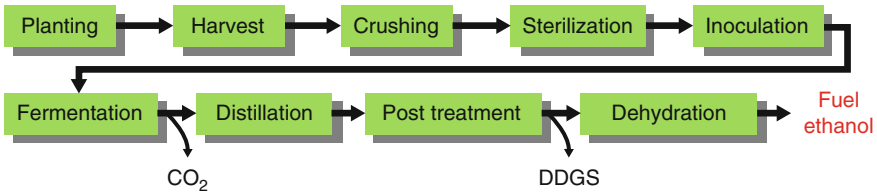

Fig. 5 Flowchart of ethanol production from sweet sorghum stem by solid state fermentation

1.3.2 Cassava-Based Bioethanol

Cassava

Cassava (*Manihot esculenta*), also called yuca or manioc, is a woody shrub of the Euphorbiaceae (spurge family) native to South America that is extensively cultivated as an annual crop in tropical and subtropical regions for its edible starchy tuberous root with up to 32% (fresh) starch content [13]. As feedstock for fuel ethanol, cassava has two advantages over other feedstocks [14]:

1. Its reduced need for nitrogen fertilizer and water. Cassava has excellent drought tolerance, and can be grown on marginal lands where other crops, such as corn, wheat, and sugarcane cannot grow well.
2. Its short growing period. Cassava can be planted and harvested all year round, so it can be served as a stable feedstock to the ethanol plant throughout the year.

Consequently, the cost of cassava starch is just half that of corn starch, which makes it a good non-grain feedstock for bioethanol production.

Bioethanol from Cassava in Guangxi, China

As biofuels are highlighted as substitute sources of energy to address the issues of economy, environment, and security, cassava has been considered as the most important non-grain energy feedstock. Based on the use of marginal lands, cassava production has no influence on the grain price. In fact, (the) PRC is one of the world's leading importers. In 1995, (the) PRC imported 300,000 tons of dry cassava, which accounted for 80% of the world trade. It was mainly used for starch and animal feed, and a small part was processed for the ethanol industry. This value has increased dramatically (see Table 3), a greater and greater percentage of which has contributed to ethanol production. Currently, cassava is planted in the south of (the) PRC (Guangxi, Guangdong, Yunnan, Hainan provinces, and so on) with about 440,000 ha yielding about 7.5 million tons of cassava in 2008.

Guangxi, with sufficient rainfall, adequate temperature, and plenty of marginal land, has the best natural condition for cassava growing. It has more than two million hectares of discontiguous margin lands around countless hills which are unsuitable for common crops such as corn, rice, wheat, and sugarcane. Research has

Table 3 Cassava production and import in (the) PRC during 2002–2004 (unit: tons)

Year	Production	Import	Total
2002	392	241	633
2003	401	327	728
2004	381	457	838

Fig. 6 High yield cassava variety
Source: Chinese Academy of Tropical Agricultural Sciences

been undertaken in Guangxi to improve the yield of cassava, for instance, breeding new hybrids and novel plantation techniques. In 2007, the cassava yield on each hectare was 27.05 tons, which was three times higher than that of 1990 [15]. It is much higher than the world average level of 12.23 tons/ha (see Fig. 6). Nowadays, Guangxi has more than ten plantation regions dispersed all over the province and produces about eight million tons of cassava annually. The production of cassava in Guangxi accounted for more than 70% of total production in (the) PRC.

In 2000, the local government of Guangxi launched a program of production of fuel ethanol from cassava, this ethanol then being blended with gasoline for cars. An ethanol plant based on cassava was set up in Qinzhou, Guangxi in 2005. As the PRC government called for the shift from grain based bioethanol to ethanol production from non-grain feedstocks, China National Cereals and Oils & Foodstuffs Corporation (COFCO) reached an agreement with the government of southern Guangxi Zhuang Autonomous Region, and set up a 200,000-ton cassava ethanol plant in the capital Nanning in 2009 (see Fig. 7). This plant would be (the) PRC's largest non-grain ethanol base over the next 5 years. However, because of the low total amount of cassava production, the capability of bioethanol production from cassava is just about 300,000 tons.

Several other energy crops, such as sweet potato and Jerusalem artichoke, can be used as feedstock for ethanol production. Like cassava and sweet sorghum, sweet potato can grow in marginal lands and poor soils, displaying useful properties such as drought resistance and saline-base tolerance.

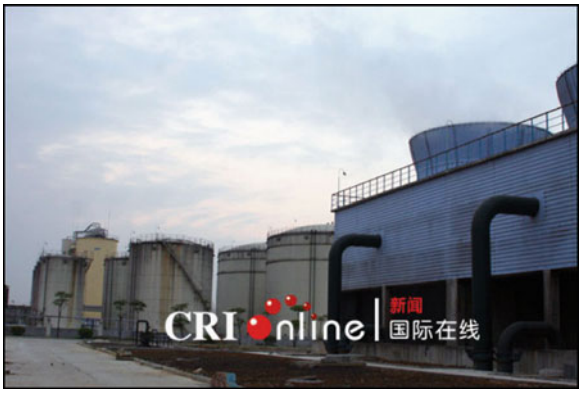

Fig. 7 A 200,000-ton cassava ethanol plant in Nanning, capital of Guangxi
Source: http://news.sina.com.cn/s/2008-11-19/233314754793s.shtml)

Table 4 The potential of ethanol production from non-grain feedstocks

Feedstock	Current production (million tons)	Region grown	Potential region	Potential acreage (million hectares)	Potential ethanol production (million tons)
Cassava	7.5	Tropical and sub tropical areas	Guangdong, Guangxi, Fujian, Hainan, and Yunnan	0.5	5
Sweet sorghum	2.5	Every province but mostly in the North	From Heilongjiang, to Hainan	8	30
Sweet potato	130	Tropical and sub tropical areas	Hainan to Northern Hebei Province	4.5	15
Jerusalem artichoke	0.1	Most of (the) PRC	Hunshandake, Inner Mongolia Jiangsu, Shandong, Hebei Province and Tianjin city	5	1

It is planted extensively from Hainan province to the northern Hebei province, with a production of about 130 million tons/year in 2008. Jerusalem artichoke can grow in both sandy land and tideland, and is resistant to wind and sandstorms. These characteristics make it the best candidate to be planted in Hunshandake and Inner Mongolia (sandy land), and in Jiangsu, Shandong, Hebei provinces, and Tianjin city (tideland). Table 4 summarizes the potential of ethanol production from these non-grain feedstocks [12].

Ethanol Conversion from Starchy Materials

Ethanol conversion from starchy materials, like corn and wheat, includes the processes of milling, mixing, liquefaction, saccharification, fermentation, distillation,

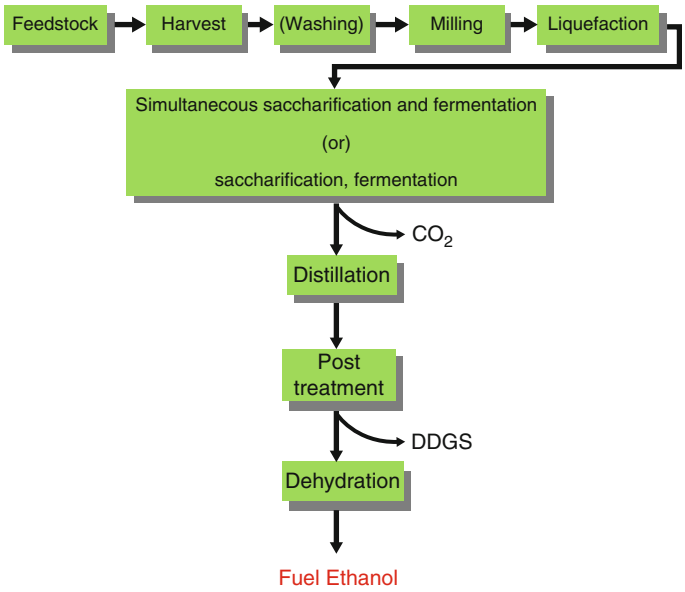

Fig. 8 Flowchart of ethanol production process from starchy feedstocks

and post treatment (Fig. 8). For cassava, washing before milling is sometimes necessary, depending on the sand content. Basically, ethanol is produced from glucose by microbial fermentation, so milling, mixing, liquefaction and saccharification are considered pretreatment processes to produce fermentable sugars. Traditionally, the liquefaction process is a high energy and time consuming process, the temperature of which is higher than 100 °C. With the application of new techniques, the energy input is dramatically decreased, allowing saccharification and fermentation processes to occur simultaneously. This consolidated process increased the saccharification efficiency which might be decreased by high sugar concentration, and lowered the possibility of contamination. Advances in the liquefaction process and the consolidated process of saccharification and fermentation are new techniques being used in (the) PRC.

Novel technologies are still being researched. For instance, with the VHG fermentation technology, the final ethanol concentration can reached to more than 20 vol%. It will save a large amount of energy on distillation. Minimizing the amount of glucoamylase, decreasing the fermentation time, as well as using advanced ethanol separation technique are under taken to make the process more commercial.

1.3.3 Cellulosic Ethanol

Lignocellulosic bioethanol production has been eagerly researched worldwide. Lignocellulosic or woody biomass is considered as a future alternative for the

agricultural products that are currently used as feedstock for bioethanol production, because of its large amount of potentially fermentable sugars. The US could convert 1.3 billion dry tons a year of biomass to 227 billion liters (60 billion gallons) a year of ethanol, which can displace 30% of the nation's transportation fuel [16]. In (the) PRC, nearly one billion tons/year agricultural and forest residuals are produced, most of which have not been fully used. If one fifth of them can be converted into ethanol, the annual ethanol output would be no less than 50 million tons.

Cellulosic Ethanol Production

Since the 1950s, (the) PRC has begun to produce ethanol from lignocellulose material, e.g., spent sulfite liquor being processed by the paper industry using an acid hydrolysis process introduced by the former USSR. With the support given by the PRC Government, many research projects on cellulosic ethanol have been carried out over the last 30 years, including lignocellulose pretreatment, ethanol fermentation from hydrolysis, cellulose production, and so on. For example, a research group from the East China University of Science and Technology developed the hydrochloric acid hydrolysis process and set up a pilot scale plant with a production capacity of 600 tons ethanol/year from agricultural residues, which was established at Jixian, a suburb of Shanghai. A group from Shandong University carried out research on cellulase production and enzymatic hydrolysis of lignocellulosics to investigate the biodegradation mechanism of cellulose. Many genetically engineered strains, such as bacteria and yeast, are constructed to coferment hexose and pentose from lignocellulose. As reported from Anhui in 2010, with the pretreatment of alkali, 1 ton of corn stover can yield more than 450 kg sugar. A new strain has also been constructed, which can produce high activity of cellulase (180 FPIU/g dry substrate). A pilot scale plant with capability of 3,000 tons cellulosic ethanol per year will be set up in Anhui. COFCO, Sinopec and Novozymes has finished research on a 1,000 tons per year cellulosic ethanol plant. They are planning to construct new facility which can produce three million gallons of bioethanol made from corn stover a year in China.

Currently, the production cost of cellulosic ethanol is still higher than that of corn ethanol, so cellulosic ethanol has not yet been produced commercially. However, intensive research and development is rapidly advancing the state of cellulosic ethanol technology, including biochemical and thermochemical conversion processes.

Ethanol Conversion from Lignocellulosic Material

Because cellulosic feedstocks are more difficult to break down into fermentable sugars than starch-based feedstocks, the cellulosic biochemical conversion process requires pretreatment and cellulose hydrolysis (see Fig. 9). During pretreatment, the hemicellulose part of the biomass is broken down into simple sugars and removed for fermentation. During cellulose hydrolysis, the cellulose part of the biomass is broken down into the simple sugar glucose. In Zhaodong (Heilongjiang Province),

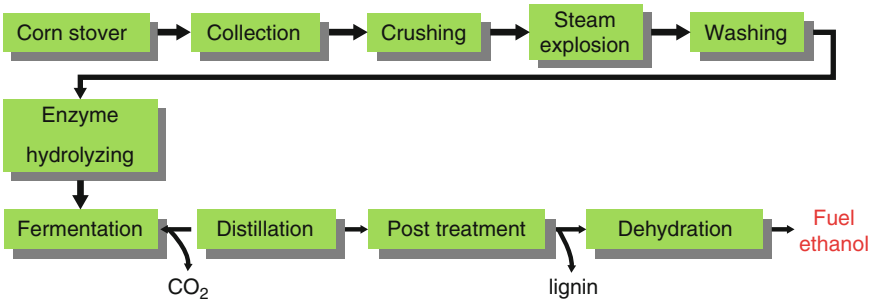

Fig. 9 Flowchart of pretreatment and fermentation processes of ethanol production from lignocellulosic feedstocks

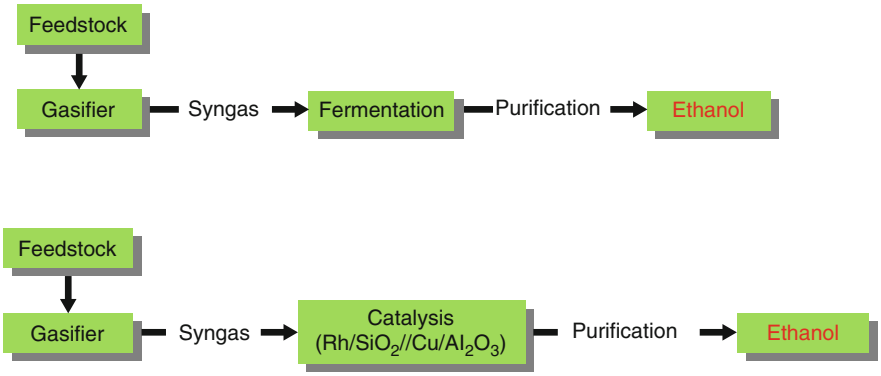

Fig. 10 Comparison of flowcharts of ethanol production through thermochemical processes, biological system (*upper*) and Fischer–Tropsch process (*lower*)

corn stover is pretreated by steam explosion, after which it is hydrolyzed by cellulase (Novozymes) until the sugar yield reaches 75–80% within about 48 h. After fermentation, ethanol concentration can reach 6–7% (w/v), with a yield of 0.43 g ethanol/g of the consumed sugars. The cellulose conversion by enzyme can be more than 90% [4].

Ethanol can also be produced using thermochemical processes. There are two ethanol production processes that currently employ thermochemical reaction in their processes (see Fig. 10). During the thermochemical reaction stage, heat and chemicals are used to break biomass into syngas (a mixture of carbon monoxide and hydrogen). The first system, called the biological system, introduces a microorganism into the syngas reactor under specific process conditions. This microorganism is capable of converting the synthesis gas to ethanol. The second system converts the syngas into fuel ethanol by thermochemical catalysts, such as in the Fischer–Tropsch process. In this process, biomass materials are first thermochemically gasified and then the synthesis gas is passed through a reactor containing catalysts, which cause the gas to be converted into ethanol. Dalian Institute of Chemical

Physics, the Chinese Academy of Sciences (CAS) has done lots of research on the second system, especially on developing novel catalysts. They set up a pilot scale plant in Sichuan with a capacity of 30 tons ethanol/year from syngas, which is produced from coal and natural gas. Currently, they achieved a new catalyst called $Rh/SiO_2//Cu/A1_2O_3$, which has two functions: first, syngas is catalyzed by Rh/SiO_2, and converted into a mixture of C2 compounds like acetone, ethanol, and acetates; second, under the catalysis of $Cu/A1_2O_3$, the mixture of carbon monoxide is deoxidized by hydrogen. The ethanol content of the final products can reach 92.7%.

1.4 The Problems and Prospects of Bioethanol Industry in China

It is reported that, as the number of motor vehicles in (the) PRC increases, the demand for fossil fuel oil will be 250 million tons, about 85 million tons of gasoline and 171 million tons of fossil diesel by 2030 [17]. A 10% ethanol blend to ordinary gasoline will result in a demand for 8.5 million tons of fuel ethanol. Development of fuel ethanol, which is used as an alternative fuel to replace fossil fuels in the transport sector, can also mitigate greenhouse gas emissions and improve energy security and rural development. It has been regarded as one of the solutions to meet the strategic goals in the twenty-first century. As a preliminary estimate, (the) PRC has about seven million hectares unused land potential for energy crops plantation. By using 50% of unused land together with improved yield, if substitute planting of crops is implemented and unit area yields are improved, potential production of bioethanol fuel will be 22 million tons in 2020 [18]. It is noteworthy that using this marginal and abandon land has no influence on agricultural development.

Although (the) PRC has made several significant initiatives for renewable energy development such as implementation of the new renewable energy law, establishment of renewable energy development plan, introduction of the financial incentives, etc., many issues arising during the bioethanol development process need to be overcome:

1. Most of the ethanol production is based on grain. Though (the) PRC has announced the intention to limit the production of grain based ethanol and promote the production of non-grain based ethanol, nearly 90% of the ethanol was produced from corn or wheat in 2009. Overuse of grain for ethanol has turned out to be a big issue for food security of (the) PRC these last few years. Owing to limitations of the market and investment, it's very difficult for small plants that produced ethanol from non-grain feedstock to survive. (The) PRC needs to set up new policies to support these plants, such as granting a loan for operation, introducing them into the consumer markets (currently most of the fuel ethanol is sold to the government owned wholesalers), providing technical support, etc.

2. (The) PRC has about seven million hectares unused land potential for energy crop plantations, but only a few of them are used to plant new energy crops. In this case, organizations should inform the local farmers of the demand for the right energy crops and set up new ethanol plants optimally to serve these feedstock area. This will stimulate the planting of energy crops on marginal land.

2 Biodiesel in China

2.1 Biodiesel

Biodiesel (fatty acid alkyl esters, FAAEs) is becoming the hot topic of every country's policy agenda due to the limited energy reserves and the increasing environmental pressure of exhaust gases coming from fossil fuels [19]. Biodiesel has drawn attention as a nontoxic, biodegradable and renewable source of energy with quite low exhaust emissions of particulate matter and green house gases such as CO, CO_2, and SO_x. Therefore biodiesel is environmentally friendly and shows great potential as an alternative energy [20]. Promotion of biodiesel in (the) PRC is promising because it can help not only reduce dependence on imported oil but also ease the problem of pollutions and global warming [21]. As a large agricultural country, (the) PRC biodiesel development can help to restore ecosystems especially in remote rural areas, promote rural economic development, and solve "three rural problems" which concern peasantry, rural areas, and agriculture.

2.2 Biodiesel Production by Various Methods

Biodiesel is produced by esterification of fatty acids or transesterification of oils and fats with short chain alcohols. Methanol is mostly used because of its lower cost compared with other alcohols, so FAAEs mainly refer to fatty acid methyl esters (FAMEs). One molecule of triglyceride reacts with three molecules of methanol, yielding three molecules of FAMEs and one molecule of glycerol as a by-product. The reaction can be shown as follows:

$$\text{Triglyceride} + 3CH_3OH \xleftrightarrow{\text{Catalyst}} \text{Glycerol} + 3RCOOCH_3$$

Currently, there are three main technologies for biodiesel production in (the) PRC: (1) chemical catalysis, (2) supercritical fluid, and (3) enzyme catalysis. Chemical catalysis is widely used in the Chinese biodiesel production industry. A low cost acid or base catalyst is used to achieve a high efficient oil conversion.

Ordinary acid catalysis requires H_2SO_4, H_3PO_4, HCl, and H_3SO_3, of which H_2SO_4 is most popular because of its low price and abundant occurrence. Ordinary alkaline catalysis requires NaOH, KOH, carbonate, and CH_3ONa, in which NaOH and KOH are most popular because of their lower cost. Transesterification with acid catalyst is much lower than that with base catalyst, and usually requires higher temperature. When using a base catalyst without strictly controlling the free fatty acid and water in oil, the pipeline may be blocked by soap which is created during the process due to too much alkali. The by-product glycerol is hard to recycle [22]. Acid catalyst erosion of equipment and pipelines is serious. Waste acid/alkaline water produced during the process affects the environment. Therefore, a lot of effort has been directed toward the development of environmentally friendly methods. Supercritical fluid systems perform with a quick chemical reaction rate and a high efficiency of conversion. But this technology always needs high temperatures and pressure so that the technical requirements of the equipment are much stricter. Hence much more research work has to be done before using on a larger scale. Comparisons of different chemical methods for biodiesel production are shown in Table 5.

Lipases can be used to catalyze the reaction in mild conditions and recently an increasing number of research projects on this subject have been reported [23, 24]. Enzyme methods are universal for almost all oils and fats and have no special requirement for the raw materials, which presents significant advantages in (the) PRC as the main raw materials for biodiesel production are waste cooking oil, acid oil and other cheap foots oil. One of the great bottlenecks for industrial application of lipases is the high cost of the biocatalyst. So immobilization methods have been introduced to improve lipase stability for repeated utilization [25, 26].

The immobilized lipases employed for biodiesel production are derived from different sources, such as *Candida antartica* [27–30], *Candida* sp. 99-125 [20, 24, 31–35], *Pseudomonas fluorescens* [25, 36, 37], *Pseudomonas cepacia* [38, 39], *Porcine pancreatic* [40], *Rhizomucor miehei*, and *Chromobacterium viscosum* [40–42]. The carriers used are acrylic resin, textile membrane, polypropylene, celite, and diatomaceous earth, etc. In general, all the biodiesel yields with immobilized lipases are higher than 80% with vegetable oil or waste cooking oil as feedstock. For all those lipase preparations, there are two kinds of lipase used most frequently, especially for large-scale industrialization. One is *Candida antartica* lipase immobilized on acrylic resin, which is known by its commercial name Novozym 435.

Table 5 Advantages and disadvantages of different chemical methods

	Alkali method	Acid method	Supercritical method
Advantages	Short reaction time; low cost	Suitable for oil with higher acid value	Higher yields at short time; clean and environmental friendly
Disadvantages	More methanol needed; not suitable for oil with high acid value; more saponification product and waste water produced	More methanol needed; longer reaction time	More methanol needed; high temperature and pressure needed

Table 6 Comparisons of immobilized *Candida antartica* and *Candida* sp. 99-125

Lipases	Carrier used	Substrate	Organic solvent	Effect of water on yield	Yield (%)	Stability	Cost	References
Candida antartica	Acrylic resin	Vegetable oil, waste cooking oil	Hydrophobic solvents, solvent free, *tert*-butanol	No water added	>90	500 h	high	[27–30, 43, 44]
Candida sp. 99-125	Textile membrane	Lard, waste oil, salad oil	Hydrophobic solvents, solvent free	10 wt% to the oil	>87	210 h	low	[20, 24, 31–35]

Novozym 435 can catalyze vegetable oil and cooking oil with yields higher than 90%, and the lipase shows quite excellent catalytic properties in *tert*-butanol solvent [43]. The other is *Candida* sp. 99-125 lipase immobilized on cheap textile membrane. This immobilized lipase textile can catalyze lard, waste oil and various vegetable oils with yields higher than 87%. Detailed comparisons between these two lipases are summarized in Table 6.

Recently, some novel catalysts such as acidic ionic liquids, cation-exchange resin, heteropolyacid, organic inorganic hybrid membrane, and even *Rhizopus oryzae* cells were developed for biodiesel production. Table 7 summarizes the technologies for biodiesel production in (the) PRC.

2.3 Feedstock for Biodiesel Production

Biodiesel is produced from the methanolysis of oils and fats from various sources. Therefore, theoretically, any form of oils and fats coming from animals, plants, or even microorganisms can be used as feedstock for biodiesel production. Currently the main biodiesel feedstocks are classified into three categories: (1) plant oils such as soybean oil [35, 65], Jatropha oil [42, 66], palm oil [67–69], cottonseed oil, and sunflower oil etc. [36, 70, 71]; (2) animal fats such as tallow [72], lard, and grease, etc. [73, 74]; (3) waste cooking oil and industrial waste oil [22, 71]. Structure of fatty acids and their distributions in raw materials of biodiesel are summarized in Table 8.

Every country uses its own particular advantages to develop biodiesel feedstock according to its national conditions [76]. The United States mainly uses genetically modified soybean oil as raw material, while the European Union and Canada use rapeseed oil to produce biodiesel. Some South-East Asian countries such as Malaysia and Indonesia have abundant palm oil, so these countries develop biodiesel from palm oil. Although (the) PRC is a large agricultural country, food supply is still a big problem facing the whole country. Observing the principle of never compromising the grain supply, (the) PRC's recent biodiesel development is based on waste cooking oil, acid oil, and other cheap foots oils. Jatropha oil will be another choice

Table 7 Current technologies for biodiesel production in (the) PRC

Reference	Catalyst	Feedstock	Yield	Reusability	Reactor beside stirred reactors
Acid or base catalyst					
[45]	H_2SO_4	Zanthoxylum bungeanum seed oil	98%	–	–
[46]	NaOH	In situ alkaline catalysis of cottonseed oil	98%	–	–
[47]	NaOH	Rapeseed oil leached by two-phase solvent extraction	98.2%	–	–
[48]	NaOH	Soybean oil	99.5%	–	Zigzag microchannel reactors
[49]	Calcium methoxide	Soybean oil	98%	–	–
[50]	CaO	Soybean oil	>95%	–	–
[51]	NaX zeolites loaded with KOH	Soybean oil	85.6%	–	–
Supercritical fluid					
[52]		Soybean oil	96%	–	A tube reactor
[53]		Soybean oil	>98%	–	–
Enzyme catalysis					
[29]	Novozyme 435 lipase	Soybean oil	92%	>100 batches	–
[54]	Lipozyme TL IM	Waste oils	93.7%	10 batches	–
[55]	Novozyme 435 lipase	Cottonseed oil with addition of ectoine	95%	–	–
[56]	Immobilized Novozyme 435 lipase	In situ catalysis of oilseeds *Jatropha curcas L*	95.9%	–	–
[31]	Immobilized *Candida* sp. 99-125 lipase	Waste oil	92%	>500 h	A continuous fixed bed reactor
[57]	Lipozyme TL IM and Novozyme 435 lipase	Soybean oil deodorizer distillate	97%	120 batches	–
Novel catalysts					

(*continued*)

Table 7 (continued)

Reference	Catalyst	Feedstock	Yield	Reusability	Reactor beside stirred reactors
[58]	A Brøsted acidic ionic liquid	Free fatty acids	93.6–95.3%	8 batches	–
[59]	Chloroaluminate ionic liquid	Soybean oil	98.5%	>6 batches	–
[60]	Active heterogeneous catalyst from eggshell	Soybean oil	>95%	17 batches	–
[61]	NKC-9 cation-exchange resin	Free fatty acids	90%	10 batches	–
[62]	Starch-derived catalyst	Waste cooking oils	93%	>50 batches	–
[63]	Organic inorganic hybrid membrane $Zr(SO_4)_2$/SPVA	Free fatty acid	94.5%	3 batches	–
[64]	A heteropolyacid (HPA) catalyst $Cs_{2.5}H_{0.5}PW_{12}O_{40}$	*Xanthoceras sorbifolia* Bunge oil	96.2%	–	A microwave-accelerated reactor

Table 8 Structure of fatty acids and their distributions in raw materials of biodiesel [75]

Fatty acid	Formula	Soybean	Cottonseed	Palm	Lard	Tallow	Coconut
Lauric	12:0	0.1	0.1	0.1	0.1	0.1	46.5
Myristic	14:0	0.1	0.7	1.0	1.4	2.8	19.2
Palmitic	16:0	10.2	20.1	42.8	23.6	23.3	9.8
Stearic	18:0	3.7	2.6	4.5	14.2	19.4	3.0
Oleic	18:1	22.8	19.2	40.5	44.2	42.4	6.9
Linoleic	18:2	53.7	55.2	10.1	10.7	2.9	2.2
Linolenic	18:3	8.6	0.6	0.2	0.4	0.9	0

for the following advantages: the Jatropha tree can grow in waste land with minimum water and fertilizers, and the oil is not edible because of the presence of some toxic compounds such as phorbol esters [19, 42].

Due to the lack of lipid source and the consequent need to exploit new lipid sources, other nonfood oil sources such as microalgae oil and microbial oil have great potential for they have short time production periods and they can be produced by fermentation from various sources, even CO_2 or waste water [77–79]. Lipid accumulations have been found in many microorganisms, such as algae, yeast, and fungi under some special cultivation conditions. Oleaginous microorganisms can accumulate 10–70% lipid varying between different species. Among these, yeasts and algae are more attractive because of the significant amounts of lipid accumulation. Some waste materials such as wastewater and lignocellulose could also be used as feedstocks for microbial lipid production. An excellent oleaginous microorganism, low cost, large feedstock quantities, and simple and inexpensive processes are the key points for biodiesel production by microbial cells.

2.4 The Status of the Biodiesel Industry in China

(The) PRC's biodiesel industry is still very much in its infancy. In 2004, total production in (the) PRC was only 50,000–60,000 tons while the total consumption of diesel was 95.5 million tons. With soaring oil prices and worsening pollution, the Chinese government has the ambitious target to promote low-polluting alternatives to foreign oil and biodiesel, which is viewed as the solution to offset increasing energy costs and pollution concerns. Systematic research started in the early 1990s, with the program of "Fuel Plants' Survey and Planting Technology Research" carried out by CAS, as one of the National Research Programs during the period of the eighth 5-year plan. From 1996 to 2000, another National Research Program of "Energy-Oriented End-Use Technologies with Plant Oil" was run during the ninth 5-year plan. In 2005, the Chinese Ministry of Science and Technology started a program for bioenergy, which aims at the development of industrial biodiesel production with a designed capacity of 50,000 tons/year. According to the 11th 5-year plan for renewable resources, the target production capacity of biodiesel is 200,000 tons/year.

Table 9 Main institutions involved in R&D relegated to biodiesel in China

Research institutions	Research field	Technologies
Guangzhou Institute of Energy Conversion, Chinese Academy of Sciences	Chemical catalyst, process	Biodiesel preparation by solid acid catalyst Continuous production process Biodiesel production by feedstock with high acid value
Beijing University of Chemical Technology	Enzyme, genetic modification, process	Lipase production by fermentation Lipase immobilization Continuous lipase catalysis process for biodiesel production Biodiesel production from waste oil
Tsinghua University	Enzyme, process, microbial oil	Continuous lipase catalysis process for biodiesel production Basic research on microbial oil
Jiangsu Polytechnic University	Chemical catalyst, process	Biodiesel preparation by solid base catalyst Biodiesel preparation in supercritical fluids
Sichuan University	Oily plants, process	Biodiesel production based on *Jatropha curcas* L. oil
China Academy of Forestry	Energy plant	Investigation of energy plant distribution and cultivation
Dalian Institute of Chemical Physics, Chinese Academy of Sciences	Microbial selection, microbial oil, genetic modification	Basic research of microbial oil for biodiesel production
Dalian University of Technology	Process	Biodiesel and 1,3-propanediol coproduction

The R&D on biodiesel in (the) PRC started late, but advanced rapidly. Research sectors include selection, genetic modification, cultivation of oily plants, enzymes technology, processing technology and equipment. Main research institutions and universities have made progress in biodiesel production technology (Table 9).

Table 10 lists the main companies for biodiesel production in (the) PRC. Recently lots of biodiesel factories with outputs of more than 10,000 tons have been established in (the) PRC. It is expected that the requirement of biodiesel in (the) PRC would be more than 200,000 tons by 2010.

2.5 The Problems and Prospects of Biodiesel Industry in China

Despite the rapid development of (the) PRC's biodiesel industry, most of its production companies are private enterprises with outputs of no more than 10,000 tons/year each. The main raw material employed is waste oil. For (the) PRC's current development of the biodiesel industry, there exist several problems which are as follows:

1. Raw material is the bottleneck for (the) PRC biodiesel development. With the principle of never competing with the grain, (the) PRC's recent biodiesel

Table 10 Main companies for biodiesel production in China

Companies	Start-up year	Feedstock	Technology	Output tons/year	By-product beside glycerol
Wuxi Huahong Biofuel Co., Ltd	2006	Waste oil, swill oil with high acid value	Chemical catalysis	2006:100,000	–
Longyan Zhuoyue New Energy Development Co., Ltd	2003 Ext. 2005 Ext. 2007	Waste oil	Chemical catalysis	2004:6,000–8,000 2006:15,000 2008:50,000	Bitumen
Xiamen Zhuoyue Biomass Energy Co., Ltd	2006	Waste oil	Chemical catalysis	2008:50,000	Bitumen
Sichuan Gushan Oil & Fat Chemical Ltd	2002 Ext. 2004 Ext. 2006	Waste oil	Chemical catalysis	2004:10,000 2005:30,000 2007:60,000	Bitumen
Wuan Zhenghe Energy Ltd	2002 Ext 2005	Chinese pistache seed oil	Chemical catalysis	2003:10,000 2006:30,000	Chinese pistache meal
Handan Gushan Oil & Fat Chemical Ltd	2003	Rapessed oil	Chemical catalysis	2004:20,000–30,000	–
Hunan Rivers Bioengineering Co., Ltd	2005	Waste oil	Enzyme catalysis developed by Tsinghua University	20,000 (designed capacity)	–
Shanghai Lvming Environmental Protection Technology Co., Ltd	2006	Waste oil	Enzyme catalysis developed by Beijing University of Chemical Technology	10, 000 (designed capacity)	–

development is based on waste cooking oil, acid oil, and other cheap foots oil. To meet the increasing demand for biodiesel fuel, we have to develop other lipid sources such as microalgae oil and microbial oils, and develop their corresponding processing techniques.

2. Green catalysts are also the key issue that constrains (the) PRC biodiesel development. In terms of enzymatic catalysts, although a group of Beijing University of Chemical Technology has developed a new lipase, *Candida* sp. 99-125, for enzymatic biodiesel synthesis, the catalyst cost is still high. As for solid acid–base catalysis, the solids are more sensitive to water and cannot meet the requirements for industrial application. So there is still a long way to go for the development of new green catalysts.
3. The additional value of product is low, and the economic effectiveness of the industry is less competitive. There are only two kinds of product in most companies, biodiesel and glycerol, so less comprehensive utilization is required. This single product structure, not the complete product chain of biodiesel and glycerol, and the weak industrial technology upgrading make the Chinese biodiesel industry less competitive.

So for further development of the biodiesel industry in (the) PRC, the prospects are focused on clean biodiesel production and corresponding chemicals coproduction. Specifically, the main technologies are as follows: development of microalgae and microorganisms to use low-quality raw materials to produce nonfood oils and fats; development of new catalysts and green catalytic process, including transesterification reaction, product separation and adjustment; development of biodiesel coproduction chemicals, including comprehensive utilization of microorganisms and algae biomass, and high value added of FAMEs and glycerol.

In a word, (the) PRC biodiesel industry has developed very quickly in recent years. In the near future, it will develop much faster and biodiesel will be an important substitute for fossil fuels in the Chinese energy consumption structure.

3 Other Biofuels in China

3.1 Biobutanol

3.1.1 Biobutanol

As the superior organic solvents and the essential chemical materials, acetone and butanol are widely applied in the chemical, plastics, and paint industries and organic synthesis. Biobutanol, mainly produced by biofermentation, largely relied on Clostridium acetobutylicum conversion under strictly anaerobic condition. In addition, the component of the product is virtually 60% butanol, 30% acetone, and 10% ethanol, which is known as AB (ABE) fermentation. ABE fermentation was once the second largest industry, following ethanol fermentation. However, since

the 1950s, ABE fermentation began to be adversely affected by the development of petroleum industry and gradually headed towards a recession [80]. Now, with the depletion of the oil resources and the deterioration of the environment caused by the greenhouse effect, more and more attention has been paid to the development of renewable resources. As the major product of ABE fermentation (above 60%), butanol has raised international concern again due to its excellent fuel performance [81]. Because of food shortages and the energy crisis, the conversion of lignocellulose to biofuels undoubtedly has a promising future and strong market prospects. Compared to ethanol, butanol has several advantages, for instance better compatibility when mixed with petroleum, higher energy density and combustion value, lower vapor pressure, few corrosion problems, lower water solubility, and so on. Moreover, its qualities are much closer to those of hydrocarbons, and it is more appropriate for transportation in petroleum pipe lines and mixing in refineries. Because of these advantages, biobutanol has become another research highlight. Since substrate cost plays an important role in the price of biobutanol production, international awareness has been focusing on exploration of lignocellulosic biomass conversion.

3.1.2 The Development of Biobutanol in China

(The) PRC has a long history of ABE fermentation. The established ABE producers already have the necessary technologies and rich experience, which have laid the foundation for cellulosic butanol. For example, both a Henan Tian Guang bioethanol incorporated company and a North China pharmaceutical company began to produce acetone and butanol again. The traditional fermentation method, using corn as a substrate for ABE production, had been used by the North China pharmaceutical company till the 1990s. At that time, the company had already reached the level of 20 g/L for the total solvent, with a ratio of acetone: butanol: ethanol = 6:3:1. The North China pharmaceutical company received general approval for the demonstration project of industrializing high-tech biobutanol production from agricultural nonfood products on August 23rd, 2008. After project completion, 100,000 tons of butanol will be generated per year. The total investment of this project is 71.5 billion yuan and the construction period is from March 1st, 2008 to September 1st, 2009.

Shanghai Institute of Plant Physiology of ACS, Institute of Microbiology of ACS, Institute of Process Engineering of ACS, Jiangnan University, Henan Agricultural University, Huazhong Agricultural University, Beijing University of Chemical Technology, Nanjing University of Technology, Guangxi University, Henan Tianguan College and North China Pharmaceutical Drugs College have been dedicated to the development of biobutanol production and, among these, Institute of Process Engineering of ACS, Henan Agricultural University, and Huazhong Agricultural University have attempted to convert lignocellulosic feedstocks into biobutanol, broadly supporting the industrialization of biobutanol production; additionally, Shanghai Institute of Plant Physiology of ACS has successfully selected high

butanol produced C*lostridium acetobutylicum* with two different novel methods – first improving the tolerance of butanol of *Clostridium acetobutylicum* itself, which could reach a ratio of 7:2:1; second blocking the action of *buk* and *solR* genes by employing the technique of introns interference, which leads to 44% and 37% higher butanol production than the control [82]. Shen [83] used *Escherichia coli* to convert glucose to butanol and acetone with the ratio 1:1 by metabolic regulation mechanism of amino acid; Wang [84] isolated a facultative anaerobic butanol producing bacteria named C2 from soil planted with rehmanniae, producing 17.17 g/L total solvent in a 7% corn mash and the butanol yield reached 11.2 g/L amounting to 65.2% of the total solvent. Using corn straw hydrolyzate as raw material (total sugar 25 g/L), 3.64 g/L total solvent was produced, among which 72.3% was butanol, reaching 2.63 g/L. All in all, China has achieved many successes in ABE fermentation using different techniques and methods and we look forward to further development in the future.

The development of cellulosic ethanol production has become increasingly technically sophisticated. The process and technologies of pretreatment and saccharification of cellulosic ethanol can be similarly directly applied to that of cellulosic butanol, and the revamped equipment used to produce ethanol can be used for butanol production. It is reported that the model bases of conversion from lignocellulose to biobutanol have been built. There will be assembly-lines in 3–5 years on a pilot scale and factories to produce butanol in 8–10 years, which means that the product will be put into commercial operation soon.

3.1.3 Lignocellulosic Materials for Biobutanol Production

(The) PRC, as a large agricultural country, produces nearly 800 million tons of straw whose power production is equivalent to 400 million tons of coals. Apart from straw, some quick growing grass, lignocellulosic materials or wasted cellulose can also be applied to the fermentation of biobutanol. Since it is impossible for solvent producing *Clostridium* to hydrolyze lignocellulosic biomass directly, the feedstocks have to first be pretreated (namely by physical, chemical, and biological methods) and then subjected to enzymolysis to monosaccharides which is equivalent to fermentation. Hydrolyzates contain 60% hexose (glucose and galactose) and pentose (xylose and arabinose) [85–87]. However, hydrolysis releases not only fermentable sugars but also some unwanted by-products such as acetic acid, phenolic compounds, and furans which become the major inhibitors of the growth of microorganisms and fermentation [88–92]. At present, many research achievements have been obtained, related to conversion of lignocellulose to biobutanol [93–95]. It should be noted that Chinese researchers have made great contributions too. Researchers of the Institute of Process Engineering of CAS used steam exploded corn stalk stover in a membrane reactor coupling enzymolysis reactor to produce biobutanol, achieving a yield of 0.14 g/g from cellulose and hemicellulose with the maximum productivity of 0.31 g/(L h). The conversion rates of cellulose and hemicellulose were 72% and 80% respectively, and 3.9 mg butanol was generated

per cellulase unit [96]. Chen [97] used rice straw enzymatic hydrolyzate (whose mass concentration of reducing sugars were 42.8 g/L) for fermentation by C1 acetobutylicum C 375, and obtained 12.8 g/L total solvent which contains 65.8% butanol.

3.2 Biogas

Biogas is another renewable source of energy, which can be produced by the anaerobic fermentation of livestock manure and agricultural residuals. As estimated, the total annual production of manure and night soil in rural China could theoretically generate about 130 billion m^3 of methane, equivalent to 93 million tons of coal [98]. It is a clean high-energy fuel for cooking and heating; meanwhile, the residual waste produced after digestion can be used as a fertilizer in agriculture. The major components of biogas are methane (CH_4, 60% or more by volume) and carbon dioxide (CO_2, about 35%) with small amounts of water vapor, hydrogen sulfide (H_2S), carbon monoxide (CO), and nitrogen (N_2) [99]. Owing to these characteristics, it is considered as an effective and rational use of natural resources in rural areas.

Rectangular digesters were invented in 1920 for biogas production by Luo GuoRui in Taiwan, and have been in use for nearly 100 years in (the) PRC. Between the late 1970s and early 1980s, with support from (the) PRC government some six million digesters were set up in China. In the twenty-first century, (the) PRC has increased the financial support on rural biogas development, with the purpose of "increasing energy supply, improving energy structure, guaranteeing energy safety, protecting the environment, and realizing the sustainable development of economy and society." By 2010, the programs will have invested 61 billion RMB to implement the rural biogas projects [100]. Nowadays, (the) PRC has the largest number of household biogas plants in the world. They are located throughout the country, mainly in the Yangtze River Basin. Specifically, Sichuan Province has the largest number at 2.94 million biogas plants. By 2007, there were 26.5 million biogas plants with a capacity of 10.5 billion m^3 (equivalent to more than 100 million tons of standard coal).

However, only about 19% of the biogas potential has been utilized in rural China [101]. Certain constraints need to be addressed for its full potential to be realized. New technologies are required for straw and cold fermentation, low comprehensive biogas utilization, and management of biogas digesters.

3.3 Biohydrogen

As a clean and renewable energy source, hydrogen is an attractive alternative to petroleum. It is carbon-neutral and innocuous to the social environment with its reaction product of water, with no or low emission of CO_2. It is sustainable and reliable, since it can be produced from various primary energies such as natural gas,

coal, biomass, solar energy, wind energy, etc. Since 2000, the number of projects supported by the National Natural Science Foundation has been increasing steadily, with focused areas of mechanism and materials in hydrogen storage, production, and fuel cells [102]. A lot of research has been done on hydrogen production from biomass using biological and photobiological approaches. A research group from Harbin Institute of Technology established a novel approach for biohydrogen production from organic waste water with a capacity of 368 Nm^3/day. However, this is still far from industrial scale production. Several obstacles need to be overcome: (1) the capability of hydrogen production by microorganisms is very low; (2) processes of hydrogen production and waste water treatment should be combined; (3) new and large-scale reactors are not available.

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Bioreactors and Bioseparation

Siliang Zhang, Xuejun Cao, Ju Chu, Jiangchao Qian, and Yingping Zhuang

Abstract Along with the rapid development of life science, great attention has been increasingly given to the biotechnological products of cell cultivation technology. In the course of industrialization, bioreactor and bioproduct separation techniques are the two essential technical platforms. In this chapter, the current situation and development prospects of bioreactor techniques in China are systematically discussed, starting with the elucidation of bioreactor processes and the principle of process optimization. Separation technology for biological products is also briefly introduced.

At present, a series of bioreactors made by Chinese enterprises have been widely used for laboratory microbial cultivation, process optimization studies, and large-scale production. In the course of bioprocess optimization studies, the complicated bioprocesses in a bioreactor could be resolved into different reaction processes on three scales, namely genetic, cellular, and bioreactor scales. The structural varieties and nonlinear features of various scales of bioprocess systems was discussed through considering the mutual effects of different scale events, namely material flux, energy flux, and information flux, and the optimization approach for bioprocesses was proposed by taking the analysis of metabolic flux and multiscale consideration as a core strategy.

In order to realize such an optimization approach, a bioreactor system based on association analysis of multiscale parameters was elaborated, and process optimization of many biological products were materialized, which resulted in great improvement in production efficiency. In designing and manufacturing large-scale bioreactors, the principle of scaling up a process incorporated with flow

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field study and physiological features in a bioreactor was suggested according to the criterion for the scale-up of cellular physiological and metabolic traits. The flow field features of a bioreactor were investigated through computational fluid dynamics (CFD), and the design of a bioreactor configuration was carried out depending on multiscale studies of parameters correlation in a bioprocess. With respect to the development trend of bioreactor studies in China, the significance of the development of microbioreactors for high throughput strain screening and large-scale bioreactors for animal cell cultivation were put forward. Finally, the importance of studies of systems biology for bioprocesses based on bioinformation processing was raised, and the necessity of establishing a bioprocess information database and local area network (LAN) were emphasized as well.

Bioseparation engineering plays a key role in biotechnology production. At present, many difficulties need to be resolved in the area. Scientists of China have made considerable progress in bioseparation engineering. This progress includes chromatography media, recycling aqueous two-phase systems (ATPS), affinity precipitation, molecular imprinting, renaturation and modification of proteins, protein fractionation using ultrafiltration (UF), ion liquid separation of bioproducts, reverse micellar extraction, etc. The preparation of bioseparation materials, as well as part of bioseparation process research development in the past 5 years, are introduced here.

Keywords Bioreactor, Metabolic flux analysis, Multiscale, Computational fluid dynamics, Bioseparation

Contents

1 Bioreactors

1.1 Overview: Development of Industrial Bioreactor for Cell Cultivation: Classification and Application of Bioreactors

Production of bioproducts and chemicals by using cell culture techniques is an important component of industrialization of biotechnology, involving the pharmaceutical, chemical, food, agriculture, ocean, and environment protection industries. The culture cell employed includes microbes, animal and plant cells, as well as algae. Great attention has been paid to the large-scale cultivation of these cells, and the prospect of its development is encouraging. The current constitution of energy and resources is proving difficult to support the target of further development for human society. The present economics based on hydrocarbon should be changed into the carbohydrates economy, and thus the development of biotechnology and industrialization of bioprocess become very crucial. Consequently, in addition to the acquisition of high yield strain, the development of novel bioreactors will be a key factor, which can lead to the development of equipments of high efficiency and energy saving during production.

In contrast to other manufacturing industries, bioreactors facilitate common and key techniques for reproduction and extends reproduction biotechnology, integrating various high-tech electromechanical equipment, which should not just be regarded as a simple combination of mechanical processing and instruments. Along with the development of computer software and hardware as well as transducers techniques, and the progress of streaming research skills and the production of novel components, bioreactor science has been constantly innovating and updating. That is why it can make such a great contribution to the industrialization of biotechnology. Consequently, development of a bioreactor commercial manufacture plant armed with various techniques and closely related to the industrialization of bioprocess, and the formation of supporting systems and corporate chains, are of indispensable critical benefit to the development of biotechnological industry.

According to the strain or biomaterial used, configuration, operation mode, and whether oxygen is employed, a bioreactor can be classified as microbial, animal or plant cell, or enzyme reactor, a CSTR (continuous stirred tank reactor) or airlift, immobilized, or fluidized bed bioreactor, a batch reactor or semicontinuous, continuous, or perfusion culture bioreactor, and as an aerobic or anaerobic fermenter. The design principle and deployment of a bioreactor are quite different depending on the usage.

1.2 The Status and Development of Bioreactor Industry in China

In China, with the development of biotechnology and its industrialization, the demand for commercial bioreactors has been continuously expanding in recent

Fig. 1 The 370,000-L bioreactor for erythromycin fermentation

years. Every year there is a lot of research work concerning laboratory, pilot-scale studies for optimization and scale-up of a bioprocess, using differing scales of bioreactors from shake flask, bench-top, to laboratory scale, and equipment renewal and maintenance are thus necessary. Since a couple of biotechnological engineering projects have now been launched, more industrial bioreactors are needed. Generally, the size of a conventional bioreactor is rather large (from tens to hundreds of tons; Fig. 1), whereas the capacity of bioreactors for modern bioprocesses is a bit smaller but with high-tech equipment, and there are increasing numbers of studies of bioprocesses using mammalian cells as pharmaceutical producers, needing high-performance bioreactors specially designed for the cultivation of animal cells. Furthermore, the innovation in traditional bioprocesses, such as those in the pharmaceutical industry, food industry, and light industry, also demands bioreactors equipped with modern facilities.

Matching the demand of the market, the bioreactor industry in China is developing in full swing. Tens of corporations are now engaged in the manufacture of bioreactors, most of them for the cultivation of microorganisms and some of them for optimization studies and large-scale production. However, due to the gap between the technological demand and the standard of processing for essential parts of apparatus, such as diaphragm valves to withstand high temperature sterilization and long term fatigue, most of the manufacturers are deficient in research experts for bioprocess studies and the integration of technology, engineering, and developing capacities. Therefore, they are in no position to develop the bioreactor in synchronization with developments in biotechnology, and to resolve appropriately the problems of in situ cleaning (CIP), automatic sterilization (SIP), enforcement of oxygen supply, fast rising temperatures, mechanical leak proofing, exit gas treatment, automatic sampling, and flow injection analysis (FIA) of culture broth sample. Hence, the apparatus, especially the whole set of equipment, which meets GMP demand for high-value added biotechnological products, is mainly imported from abroad [1–3]. For example, large-scale bioreactors for animal or plant cell culture which meets the GMP demand still cannot be produced in China so far.

1.3 Bioreactors Based on Metabolic Flux Analysis and the Multiscale Investigation Approach

1.3.1 Principles and Fundamentals of Bioreactor Engineering

Bioreactor engineering is a complicated systematic process aimed at biomaterial processing through cold and hot model experiments. Incorporated with the studies of macroscopic and microscopic kinetics, it can reveal the effects of structural and operational variables, and operation modes on a specific bioprocess and its product synthesis, so as to attain the purpose of strengthening and scale-up of reaction system. The essential content and development of biochemical engineering are as follows.

Studies on Microbial Growth and Reaction Processes

Whole analysis is carried out for the substrates from their entering the cells, intracellular reaction, to the excretion of metabolites, where the material and energy balances of intracellular metabolic reactions are first considered. Various studies on the theories, approaches, and experimental data of microbiological metabolic balances were set forth, namely, the material and energy relationships among catabolism, anabolism, and the synthesis of macromolecular substances *in vivo*.

Studies of Stoichiometry and Thermodynamics of Fermentation Processes

Once the substrate enters a cell, it will undertake hundreds of reactions before it turns into some metabolites and cellular constituents. We are still in no position to quantify the reactions individually. Generally, the cell can just be regarded as an opened black box system, the exchange of nutrient substances in and out of the cell is merely considered, and a reaction model based on cellular metabolism is suggested. Stoichiometry mainly demonstrates the rule of the changing of reaction components in the course of fermentation [4–6]. Considering the conservation of carbon, hydrogen, oxygen, and nitrogen, the activities of microbial cells can be represented by equations of chemical elements. In practice, the entire bioprocess can be treated macroscopically with general stoichiometric equations without knowing the details of every event. Thermodynamics merely emphasizes the initial and terminal status of a system, disclosing the maximal degree of a possible reaction. In fermentation engineering, yield coefficient $Y_{i/j}$ is always used to evaluate and quantify further the potential growth of cell and the biosynthesis of products from substrates.

Microbial Reaction Kinetics and Bioreaction Engineering

Microbial reaction kinetics is the core subject of process optimization, mainly studying the rate of bioreaction and the factors affecting it. The intrinsic kinetics usually will be considered first, i.e., the intrinsic reaction velocity of a microbial reaction without considering the effects of various engineering factors in transfer processes of a bioreactor. Microbial physiologists and biochemical engineers put forward many mathematical models related to microbial reaction kinetics [7–9], some of which are empirical models and others mechanical models.

In order to solve practical engineering problems, the study of microbial kinetics has been gradually developed into bioreaction engineering, which has become a subdiscipline of biochemical engineering. It involves two aspects: macroscopic and microscopic microbial reaction kinetics and bioreactor engineering.

1.3.2 Proposal and Principle of Multiscale Approach for the Study of Bioprocess

Plenty of difficulties are always confronted when applying such fundamental principles to solve practical problems of industrial fermentation processes. In reviewing the progress of chemical engineering and optimization techniques for fermentation processes, we have to ponder further upon process optimization and scale-up problems.

The operation and control of fermentation processes influence immediately the environment of microorganisms, which eventually affects the results of metabolism and production. For a long time, only extracellular operational factors of a bioreactor, such as optimal temperature, pH, dissolved oxygen (DO), ingredients of a medium, etc., as well as the mixing transfer studies related to those operational factors, were considered in terms of optimization studies of microbial process. Although the theories of chemical engineering and cybernetics are applied, there are still many restrictions.

Along with the development of molecular biology, the biology of the twentieth century has been experiencing development from macroscopic to microscopic processes, from the description of morphology and phenotype to the dissociation and demonstration of various molecules and functions of a cell for the understanding of the relationship among genome, proteome, and metabolome, which leads to the disclosure of intracellular reaction mechanisms. However, research depending on a pure physiological regulation mechanism often reveals the characteristics of local aspects for a certain period. It is always difficult to reach decisive conclusions on process optimization and scale-up processes if merely depending on highly diversified and scattered studies.

Consequently, how to incorporate biology into engineering, to resolve the relationships between local and whole cell, between time-varying and ultimate results, between the improvement of producing strain and process optimization are the paramount subjects of fundamental study.

Multiscale considerations in terms of bioreactors proposed by our research group are tentatively applied to the solution of the above systematic problems [10], i.e., the complicated bioprocess in a bioreactor is dissected into different levels of traits studies to demonstrate the relationships between several events at different scales. The event at every level has its own scientific principles and changing rules, where the changes are revealed from quantity to quality. Study is focused on the effect of these changes on the whole complicated system. In this way, we might be able to discover the relationship between the local and global systems in terms of the optimization of fermentation processes.

Multiscale phenomena are ubiquitous. However, when dealing with the classification of the scales of microbial reaction engineering, it can be classified according to the study of interest and the development of the relevant subject. For example, if a cell process is taken as a research object, then it may be dissected into molecule, molecular aggregate, cellular fine structure, and entire cell scales, and if mixing and transfer traits in a bioreactor are studied, then the system may be resolved into monogranule, cluster of particle, and reactor scales. When necessary, it may also be resolved into electron, atomic nucleus, and DNA macromolecule scales to reveal the fundamental rule of the transfer of genomic information into life processes.

After the traits of cells in a bioreactor have been studied, we suggest taking the cells in the fermentation process into multiscale account [10, 11] according to the demands of the research of bioreactor structure, performance, and production. Live cells, taken as a main object in the course of large-scale cultivation, can be classified as genetic molecular scale, cellular scale, and bioreactor scale, these being the relationships between input and output of a network status on different scales, which are reflected in the flows of information, substance and energy, possessing a wide range of spatial and time scales. The molecular level of genes belongs to the nanoscale and is generally below 10^{-9} m; the metabolism of a cell falls into the microscale range, i.e., between 10^{-8} and 10^{-4} m; and reactor engineering pertains to the macroscale, i.e., between 10^{-3} and 10^{2} m. Figure 2 shows these processes occurring in the different scales [12, 13].

In describing microbial cells at the enzymatic level (including the activation and inhibition of enzymes, the incorporation and dissociation of subunits, covalent modification and degradation), the time constant is controlled in the range of

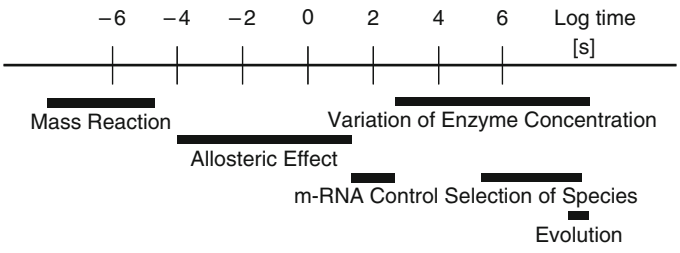

Fig. 2 Illustration of the time-scale events occurred in bioprocess

millisecond to second; for the regulation at genetic level (repression and derepression, induction, transcription), is in minutes, and for the selection of species and evolution level, days or greater time units are employed. Therefore, we have to decide at which time scale the study should be carried out dynamically or statically [14]. The optimization of a fermentation process cannot be carried out only on a single scale (genetic, cellular, or bioreactor). We should pay attention to the relationship between these levels and the problem of bottle-necks, where trans-scale observation and control are the keys to the success of the optimization of microbial processes. The optimization technique based on multiscale consideration and multiparameters correlation, and the scale-up technique depending on multi-parameters regulation in fermentation processes, are thus established.

The reactions occur on different scales, with huge differences in geometrical sizes, manifested both as a thermodynamic statistic relationship and a space-time series reactive relation exhibited specifically by life. It is reflected in the variation of information flux, substance flux, and energy flux, and it also exhibits multi-input and multi-output correlation of a network (Fig. 3). These network structures also display a reciprocating multi-input and multi-output correlation within various levels of the network, and the main stream of microbial metabolism is the core issue of such a network relationship. The mainstream microbial metabolism is

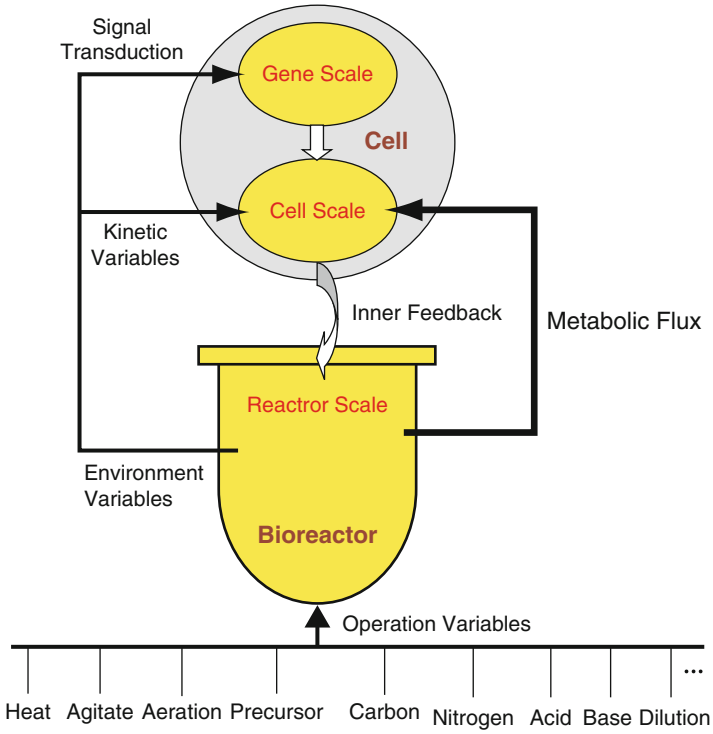

Fig. 3 Network relationship of multiscale reactions in a bioreactor

always changing: its direction and flow, as well as the pathway, may be changing as time elapses. This is exactly the fluctuation of microbial pivot metabolism and the selectivity of a metabolic pathway in a network shown by pivot metabolism. Such fluctuation and selectivity depends on the genetic substance of a microbial cell and the cause of selection is the change of environmental condition. Therefore, it is impossible and also unnecessary to investigate all of the loading pathways.

These facts provide us with an important approach and clues to investigate the multiscale problem of a bioreactor. The fundamental points are as follows:

1. Make a description qualitatively and quantitatively: the relativity of the changing between cellular metabolic flux and material flux within a bioreactor (dynamic or static) is an efficient approach for the research of multiscale relevant problems in a bioreactor.
2. Differentiate the characteristic of multiscale problems from the correlation between cellular metabolic flux (microscopic) and the material flux of a bioreactor (macroscopic) and analyze and deal with the problems with different scale methods; the research results of various individual scale studies are integrated before the optimization of a fermentation process can be achieved.

1.3.3 Trans-Scale Determination and Analysis

It is neither possible nor necessary to quantify all the microscopic details within a network where multiscale reactions occur in a bioreactor. Furthermore, in bioprocess engineering most of the complicated phenomena possess multicontrol factors characterized by nonlinearity and nonequilibrium, so it is difficult to find a universal stability criterion for nonlinearity and nonequilibrium systems. Hence, it is a hard task to find the optimum result of a bioreactor system by merely using a static kinetic approach. This reflects the difficulty in establishing the relativity of various systems, and also in the identity and processing of boundary conditions, especially when confronted with the instability generated from the structural changing of certain systems, which leads to difficulty in developing the criterion of stability for the whole system.

Industrial production of microbial metabolite or recombinant pharmaceuticals can only be realized through large-scale cultivation in a bioreactor, and the microscopic conditions of a biological reaction can be regulated with the change of operation condition or the structural alteration of a bioreactor. In such a case, different flow fields in a reactor might form, which would affect the hierarchical metabolic reaction, so this is an extremely complicated system. Just as with other industrial processes, the essential characteristics of a complicated system with multihierarchical structure simply possesses a "changing configuration," and leads to the intricate variation of inner structure in a reaction system and also substance transformed performance along with the change of scales. In addition, the events observed may change substantially at some levels.

Therefore, when the structure of a bioreactor or operation conditions are changing, the resulting change, which is not a change of substance status, cannot simply

be described by a linear relation or statistical approach. The cause of the change of a process is attributed to linear or dynamic factors, and structural mutation of a system also occurs frequently. Although such a structural mutation is taken as dynamic behavior in nature, it will lead to a variety of system structure or serious deviations in optimization processing, which is due to the difficulties in stipulating the boundary conditions on various scales.

In spite of these difficulties, the stability condition or relevant characteristics of many bioprocesses can be found through scale and process resolution, and the problems can thus be solved. So it is worthwhile to pay attention to the acquisition of the phenomenon characteristics related to the change of cellular metabolic flux distribution, and cellular pivotal metabolism, its direction and flux, even the pathway it goes through, may also be subject to variation. Therefore we must find the phenomena of clues related to such a variation of metabolic flux before we can understand the intrinsic rule of a complicated process.

However, the experimental data obtained from bioreactors reflect only a single-scale problem. For an example, the DO determined is actually the concentration (activity) of DO in microbial culture broth; air flow rate is the amount of sterile air entering into a bioreactor; and the measurement of temperature is the Euler's average temperature relative to the temperature gage components scale in the culture broth environment. To this end, how should we analyze the relativity of data to really reflect the intrinsic behavior of metabolic flux. For instance, when pH is adjusted with ammonia, we are usually concerned about the optimal pH and neglect the relationship between the amount of ammonia added and other parameters; when DO is determined and controlled, people usually only care about the optimal and critical values and ignore the oxygen consumption rate during fermentation; the changing of cellular metabolism and morphological traits are seldom considered. This sort of static operational approach using optimal operational control point as a criterion is actually the extent of the concept of macroscopic kinetics of chemical engineering to fermentation engineering, and its application to the fermentation optimization with live cell metabolism is restricted. Thus we must pay great attention to the metabolic flux existing in the cell.

Consequently, trans-scale operation is a difficult problem. To analyze trans-scale problems we have to rely on interdiscipline or intertechnique measures. If one wants to control a monoscale phenomenon, usually it is necessary to find out an operable solution method of another scale. Each application has its own special main scale but, to solve a problem, other relative subscales should be matched and comprehensive consideration should be undertaken, hence suggesting new demands for traditional chemical engineering concepts. In terms of the process theory of bioreactor engineering, it is unfavorable to rely only on traditional concepts; we must focus our research on the relativity from different scales, and observe a process nature of a single scale through observing another scale phenomenon.

The association analysis of cellular metabolic flux and material flux in a bioreactor is an essential view of the research of bioreactor engineering with multi-scale consideration and the realization of process optimization and scale-up.

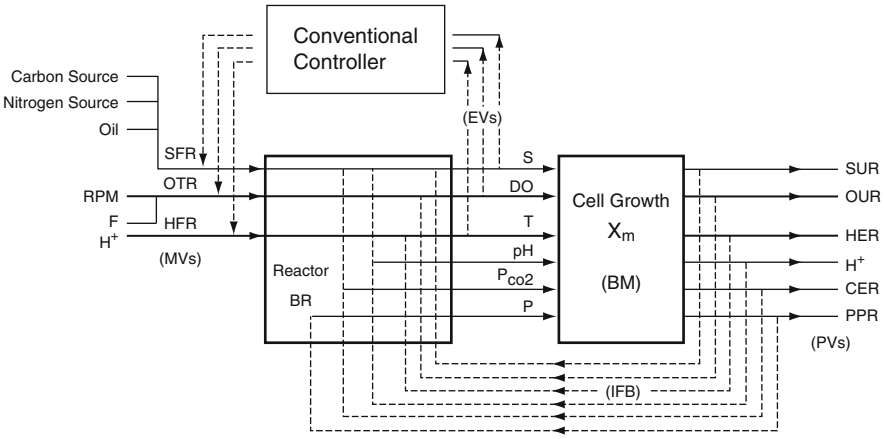

Fig. 4 The relationship between the material flux in the bioreactor and cellular metabolic flux

Konsstantlinov and Stephanopoulos [15] have studied the material flux and control module trait in a bioreactor. Figure 4 illustrates the relativity of material flux and cellular metabolic flux. The bioreactor (BR) is a relatively simple physical system. The reactor is conducted through the manipulation variables (*MVs*). The status of a reactor can be described with the use of environmental variables (*EVs*). Cell growth (BM) is a complicated system. Its inputs are various environmental variables, so its status can only be partially expressed by physiological variables (*PVs*).

Since the growth of cells occurred in a bioreactor, the consumption of various substrates and the release of metabolites into a bioreactor are bound to lead to a change in the material flux of a bioprocess, which is symbolized by internal feedback (IFB). For example, the DO in a bioreactor is influenced by the agitation and aeration (OTR) and also the consumption of oxygen by cells (OUR). Such a change of material flux provides important clues about the cellular metabolic flux status. Some material balances can be set up for the quantitative investigation of DO, oxygen transfer rate (OTR), and oxygen uptake rate (OUR) kinetics, so as to reveal the phenomena and intrinsic natures of the bioprocess, which is unlikely to be realized when conventional methods are employed.

However, the conventional control method employed currently can be realized only for the closed loop control for *MVs* and *EVs* in BR. The cell growth or metabolism (BM) is excluded in the closed loop; it can only affect the process indirectly through IFB, and sometimes it is treated as a noise; from this we can understand why the conventional optimization control method is limited. For this reason, Stephanopoulos pointed out that, in spite of the monitoring device of a bioreactor being increasingly sophisticated, there are still a lot of physiological phenomena which cannot be detected with current transducers and control systems. The utilization of fermentation analytical data is a complicated problem related to many aspects. From the information determined from a bioreactor, physiological information can be extracted. The indirect variables derived are more useful than

the original variables in terms of the disclosure of the intrinsic nature of a bioprocess, and thus the classification of variables of a bioprocess into an input and output structure of a bioreactor, instead of using the originally physical, chemical and biochemical variables, is suggested, in which much attention is now paid to the acquisition of physiological variables.

Stephanopoulos has suggested an important approach for the observation of a phenomenon, since physiological variables are capable of reflecting the change in cellular metabolic network scale directly related to the synthesis of a product. However, in practice, it is unlikely to obtain an ideal physiological variable with current limited technical conditions. Therefore, it is unwise to explore the physiological traits only from related derived variables extracted. It is advisable to pay much more attention to the growth status of BM and its effect on the interior feedback. There are two reasons that we must emphasize. First, the synthesis of products in a bioprocess is related to cellular metabolic flux. It is obviously restrictive to realize the optimization and scale-up of a fermentation process without knowing the change and shift of metabolic flux relevant to the production, and merely rely on the environmental state variables (*EVs*) or operational variables (*MVs*). Second, the data correlation generated through IFB facilitates an important clue to realize trans-scale analysis of and operation of a process. We should obtain as much as possible the analyzed information of practical values from *PVs* and establish further the relationship between *PVs*, *EVs*, and *MVs*, distinguishing the problems of different scales from the characteristics of such relationship before realizing effectively the theory and methods regarding optimization of a bioprocess.

We should also pay attention to the establishment of optimization theory and approach for a bioprocess that possess practical value, and to replenish them continuously in terms of theory, methods, and analytical techniques, along with the development of biotechnology, since the expression of genome and the regulation of metabolism are so complicated that *PVs* can only describe partial cellular status.

In addition, we should take care of the variation of a time scale when relevant analysis of metabolic flux and material flux in a reactor is performed. From the first section we already understand that there is a large time span in bioprocesses, and these physiological variables may occur in milliseconds such as in enzyme activation, or in days such as in the growth of microorganisms, and it may also be a quick process, where enzyme allosterism may take place. We might distinguish the static or dynamic behavior of a process with the comparison of the time scale of the material flux of a bioreactor. A nonstructural model is in no position to reflect the intrinsic mechanism, and a number of adjustable empirical parameters should be introduced. It is also unable to describe the structural characteristics of a system, and it is hard to quantify such a complicated scale effect.

Consequently, the comprehensive and high nonlinearity of a bioprocess and some other factors, as well as the multicapacity nature of a process, renders the system with a dynamic property and is hard to predict. The mathematical model using a linear or quasilinear relation can only roughly reflect the status of a process,

which is far from meeting the response to the perturbation factor necessary for the optimization of a bioprocess. The prediction capability of the model is so poor that it is difficult to solve the scale-up and regulation problems quantitatively.

1.3.4 Data-Driven Approach

In conducting data processing and investigation of a bioprocess, the approach of establishing a mathematic model is currently applied. However, in terms of practical application of the optimization, it is very difficult to materialize due to the limitation of computation and monitoring technique, an especially the understanding of the complicated bioprocess.

We deem that a multiscale approach is the most appropriate method so far, since it can reflect the most essential "structural" feature of a complicated system, which is a simple but efficient way of characterization. The so-called "structural" here implies various discipline contents and cross meaning, which embodies the latest progress of various disciplines on a different scale. Our primary task is to study the association of different scale phenomena, i.e., to correlate a phenomenon of one scale to another one, which leads to the disclosure of the intrinsic nature of a phenomenon, and makes it possible to provide the theoretical basis of scaling up and regulation of a biological reaction process.

For instance, we can incorporate the most efficient unit operation into the study of a reactor engineering feature, even the study of a transfer mixing feature with a computation hydrodynamics approach to perform the simulation experiment by computer. It is also possible for us to correlate the metabolic traits with genetic engineering features of different strains in a single scale, and to relate the morphological feature of a strain with rheological property, physiological property, or productivity. In studying the high-density cell culture and highly efficient expression of a genetic engineering strain, we can resolve and correlate an effective vector component, the metabolic features of a host cell with the operation traits of a bioreactor.

A big difficulty in implementing the investigation approach is to establish the boundary value condition of a multicontrol structure, which reflects the difficulty of establishing the dynamic relationship among various reaction mechanisms, and also the recognition and treatment of the boundary conditions of various events, especially the instability occurring due to the structural change of a system. It is very difficult to seek a ubiquitous extreme value condition. Therefore, it is not easy for us to attempt to establish a mathematical expression with a differential equation, and further set up the extreme value condition. As a result, we have to seek an approach not only clear and operable but also capable of revealing the intrinsic rule of a complicated process, i.e., the so-called data-driven method.

The data-driven method is defined first and foremost to acquire the online and offline parameters with time in a bioreactor by using a transducer technique or analyzing manually in a lab, and to display in a computer the profile of a parameter

through a data acquisition system; then, from the relative features of these time course variables, the rules of a process may be demonstrated. Consequently, the so-called data-driven method, i.e., the optimization and scale-up technique of a fermentation process, based on the association of parameters and multiscale considerations and on the regulation of multiparameters, is proposed [16].

1.3.5 Data Acquisition and Control System of a Computer, Based on Multiscale Consideration and Parameters Correlation in a Bioreactor

In a bioreactor multiscale system, the study of the association of parameters with trans-scale observation and operation is the key procedure of process optimization. Such relativity mainly manifests itself in the correlation among multiparameters in a time course mode, and from the tendency of metabolic curves, the diversity, time-varying property, relative coupling, and uncertainty of the parameters monitored can be observed. Hence, we should pay attention to the cooperation and the whole target in terms of the design of applicable software to meet the demand of the precision and drifting of the sensor system.

The study and observation focusing on the genetic information flux, material flux, energy flux, or metabolic flux are the initiating points of fermentation process optimization, relying heavily on the experimental data determined. However, the data analyzed is so deficient on account of the restriction of transducer techniques that it is necessary not only to explore the novel measuring principle of transducer technique but also to pay much attention to the development and application of software monitoring technique, based on currently available measures. In addition to the conventional manual determination of parameters in the lab, the determination of enzyme activity, regulatory protein responsible for the expression of gene as well as RNA and DNA, and omic-data should be carried out through offline analysis to serve as an important tool to perform association analysis through data acquisition systems.

In software determination techniques, in addition to the indirect parameters, which represent cellular physiological activity or reactor engineering features, a stoichiometric approach used for the determination of metabolic flux distribution is applied to implement trans-scale element balance calculation based on current online and offline data determined in the lab. Thus, all the techniques mentioned above have to possess strictness, the continuity of time sequencing close to a real time system, which can be used for qualitative observation and further used for quantitative analysis.

In data acquisition systems we must also pay attention to the time scale problem or the response within different scales. For instance, “typical time constant” in a bioprocess is always, in error, looked at as the same as the whole cultivation duration, and false results are thus obtained from the study. Therefore, we must analyze a bioprocess with different time scales according to the demand for the deployment of hardware and software of a monitoring system.

1.3.6 Application of Optimization Based on Parameters Association with Multiscale Consideration

Along with the development of transducer and computer techniques, a novel concept of fermentation apparatus, Model FUS-50L (A) for research into bioprocesses by using a multiscale concept, has been designed and manufactured by the Shanghai Guo Qiang Company Ltd for Biochemical Engineering Equipments (Fig. 5). This bioreactor system possesses not only conventional sensors, namely, temperature, stirrer speed, pH, foam detector, DO but also broth actual volume, precise feeding rate (substrate, precursor, oil, and acid/alkali), as well as the determination and the control of precise air flow rate, back pressure, and is equipped with exhaust O_2 and CO_2 content analyzers. The whole system possesses 14 in situ sensors/online analytical instruments for the monitoring and control of variables/parameters, and the data monitored can be processed and analyzed by computer.

For the monitoring and control of variables/parameters, a sophisticated software package, integrated with various process and control theories, has been developed for the technological analysis and optimization of a fermentation process. From this, indirect parameters such as OUR, carbon dioxide evolution rate (CER), respiratory quotient (RQ), volumetric oxygen transfer coefficient ($K_L a$), specific growth rate (μ), etc., which reflect the metabolic flux features and engineering traits

Fig. 5 The novel fermentation apparatus manufactured by Shanghai Guo Qiang Company Ltd for Biochemical Engineering Equipments

and are necessary for the optimization and scale-up of a fermentation process have been obtained. On the basis of apparatus research, a commercial laboratory bioreactor was developed. It has been used for the fermentation of guanosine, from which the shift of cellular metabolic flux through the profile of parameters has been discovered, and eventually led to the optimization of the bioprocess [17]. The apparatus has also been successfully applied to the fermentation of many products, such as, penicillin, erythromycin, feed grade aureomycin, streptomycin, flavomycin, tylosin, clavulanic acid, inosine, engineered albumin, engineered malaria vaccine, engineered phytase, porcine insulin precursor (PIP), engineered biotechmycin, etc. The titers of all these fermentations have been raised significantly, and the processes have been scaled up from tens of liters to more than 100 cubic meters.

1.3.7 Study on Bioreactor Process by Integration of the Flow Field of a Bioreactor and Physiological Properties of Microbes

Computational fluid dynamics (CFD), a booming discipline, is a major branch of hydrodynamics. On the basis of theoretical hydrodynamics, it is an applied science that makes use of the high-speed property of modern electronic computers to solve the mass conservation equation, energy conservation equation, and momentum conservation equation for the acquisition of flow field information. In the early stage of the development of CFD it was mostly applied to aerospace research and national defense, but with continuous improvement of the technique, it has since been applied to the study of fluid dynamics in various industries.

The computation simulation of the flow field in a CSTR was initiated in the early 1970s. Patterson [18] was the first to apply a "black box" model to characterize an impeller through the determination of the flow field surrounding the impeller, and take it as an input of boundary conditions to calculate eventually the field flow in a reactor by solving a set of fluid dynamic equations. However, such a method is still reliant on the experimental determination of flow field. It has been gradually developed into miscellaneous methods, namely "momentum source approach," "interior extra-iterative method," "multireference coordinate system," and "slippage grid approach." These approaches no longer rely on the experimental determination of flow field.

An interior extra-iterative method was suggested by Brucato [19], who divided the whole reactor flow field into interior and exterior overlapped regions. The inner region includes rotating impeller and data exchange is carried out in each iteration at the overlapped region; in this way, processing of data is huge. Luo [20] suggested a steady-state simulation approach, i.e., "multireference coordinate system approach." This method also divides the flow field into two nonoverlapped regions. The interior region is calculated by rotating coordinate system and the exterior region by static coordinate system. The interface area of these two regions is calculated by interpolating approach to carry out data exchange, ended in the accomplishment of the simulation of the whole region. Since the flow field of CSTR possesses periodically varied asymmetry, the above steady-state simulation approaches are incapable of reflecting the status.

Luo [21] in 1993 was the first to suggest the "slippage grid approach" for the simulation of the transient state flow field of CSTR. Results acquired in this way coincide with the rule of the periodical change of flow field in CSTR; however, this method requires more computational sources and storage space. The researcher of the Department of Chemistry in Villanova University suggested a method of simulating the degradation of biological macromolecule in a CSTR with the use of CFD [22]. The method can be applied to calculate the size of a "high shear stress" region, where the breakdown of biological macromolecule has occurred. Such a model and experimental method is helpful for the study of the scale-up of a bioprocess.

In industrial production, the function of a biosystem depends on the joint effects of the stimulus of the exo-environment and intracellular functional genes [23]. The effect of the exo-environment on cell function occurs in the following ways. First, the effect of environmental conditions (such as temperature, pH) on the cellular enzyme or immediately on metabolic rate. Second, changes occurring due to the deficient or excess of substrate caused by mixing and transfer status of a bioreactor (oxygen supply is affected by agitation and in terms of other carbohydrate substrate, the homogeneity of substrate distribution is influenced only by mixing transfer). Third, the response of signal transmission system in a cell is affected by environmental conditions (including the insufficient or excess supply of substrate), resulting in the change of cellular transcription expression system, and eventually leading to the change of cellular metabolic network.

Consequently, it is necessary to consider the environmental condition and physiological feature systematically before we can realize the optimization and scale-up of a bioprocess. The practical industrial production is performed in submerged cultivation, and hence the study of extracellular environment can be summed up as the study of flow fields in a bioreactor, and hence different temperature fields, concentration fields, and shear stress fields are attained. The behavior of a cell is expressed through the physiological traits of a cell. The study of interaction rules through the integration of these two features would lead to a brand new scientific idea for the optimization and scale-up of a bioprocess.

For example, the analysis of mycelial morphological parameters and carbon and nitrogen metabolism of avermectin producing strain, *Streptomyces avermitilis* under different impellers combination were studied in our research group. Figure 6 shows the CFD simulation of different impeller combinations, which can characterize the magnitude of local shear strength. The closer the blue exhibits in the region, the lower the shear strength and the closer the red displays in the region, the higher the shear strength (Fig. 6). It can be seen clearly from Fig. 6 that no red region exists at the first set of impeller combinations, where the shear strengths of three layers are fairly even, while near the top two layers of 6-arrowy-blade disk turbines, there appears a large red region, and near the bottom layer, the situation is almost the same as in the first set. Near the third set of impeller combinations, all three layers show red regions, indicating that the shear strength is intensive there. From the figure it can be seen that intensive shear stress is concentrated at the location near the impeller. For the second and third sets with different impellers combination, their distributions of shear strength are uneven.

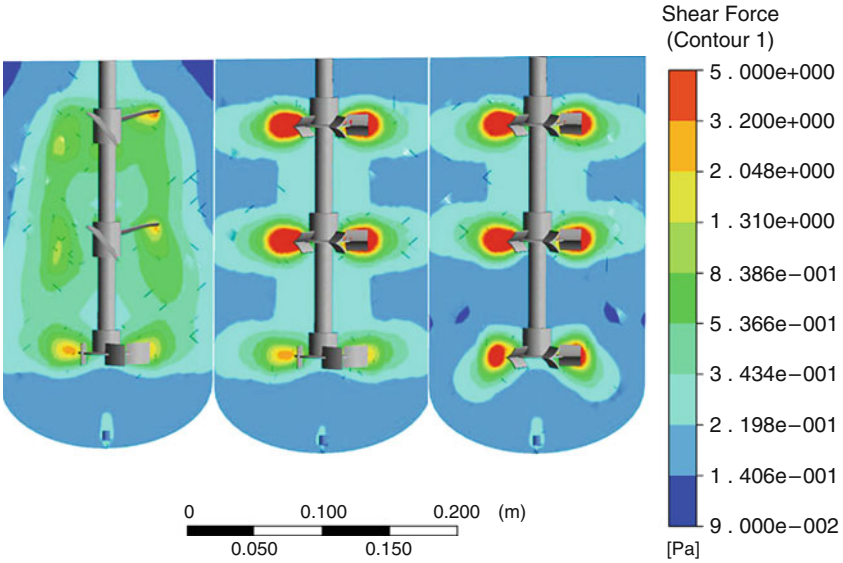

Fig. 6 Turbulent shear force distribution of different impeller combination

After CFD simulation and analysis of mycelial morphology and metabolic feature, the following conclusions can be drawn:

1. For the first bioreactor, two down-pump propellers (DPP, located at the top and middle of the bioreactor) and a six-curved-blade disk turbine (6CBDT, located at bottom) were combined. The power of agitator consumed was the lowest, and its mean shear strength was also the smallest, gas content was high, mean OTR was the highest, and its mixing performance was the best one among three impeller combinations, possessing both axial and radial circulations. For two 6-arrowy-blade disk turbine (6ABDT, located at the top and middle) combined with a 6-curved-blade disk turbine (6CBDT, located at the bottom) in the second bioreactor, the power of agitator consumed was high, and its mean shear strength was also high, but gas content was low, and mean OTR was the lowest. Three 6-arrowy-blade disk turbines (6ABDT) were combined in the third bioreactor. A dead zone exists between the bottom impeller and the bottom of the fermenter, suggesting that the 6-arrowy-blade disk turbine as the bottom impeller was inappropriate, and under the operational condition its power of agitator consumed was the highest, and under the same area of impeller blade, with different impeller combinations, their performances were also different.
2. For the combination with highest shear strength, the size of mycelial pellet was the smallest, i.e., the surface area, perimeter, and size of pellet were all smaller than that of the other two combinations; uneven mixing led to the retardation of mass transfer, and also the variation of mycelial morphology, which resulted in the unwinding of pellets. During the initial phase of fermentation the growth of

periphery mycelia was also affected by shear stress, and hence lower shear stress was favorable to the growth of periphery mycelia.

The final fermentation titer using two down-pump propellers (DPP, located at the top and middle of the bioreactor) and a 6-curved-blade disk turbine (6CBDT, located at the bottom) was obviously higher than that of the other two combinations. Since its shear stress was the smallest, the size of the pellet could keep constant for a longer period of time. This is in accord with the conclusion of the study described above, i.e., to keep the size of the pellet constant is favorable to the production of avermectin. However, for an impeller combination using three 6-arrowy-blade disk turbines (6ABDT), the size of the pellet was rather big during the initial phase, and in the later phase autolysis occurred earlier than with the other two combinations. Accordingly, it is concluded that in the initial phase the bigger size of pellet is unfavorable to the synthesis of avermectin in the production phase.

1.4 Design of a Large-Scale Bioreactor and the Study of Manufacture Technique

1.4.1 Principle of Scale-Up and Design of a Large-Scale Bioreactor

Along with the fast development of the fermentation industry in the last few decades, there has been a tendency for fermentation engineering to increase the size, efficiency, and automation functions of installations. With respect to traditional biotechnological products, like amino acids, antibiotics, or light chemical fermentation products, the volumes of their bioreactors are developed from tens to hundreds of cubic meters. Some of the smaller old plants have moved to the new developing area, and have asked universally for a bigger size of bioreactor. An exception is the production of genetic engineering products on account of their high-value, as smaller size bioreactors can still meet the demand of production. However, along with the progress of the production technology of recombinant enzymes, like phytase, and the great demand for feed additives, it is imperative to study the large-scale bioreactor for high-density cell cultivation and high expression of genetic engineering product. Especially, as fossil energy is becoming exhausted, the utilization of biomass to produce fuel ethanol is again put on the agenda for strategic decision. Application of high efficiency and energy saving large-scale bioreactor is the indispensable key technique for the reduction of production cost, for example, the fermentation equipment used in US and Brazil possesses up to 2,000 M^3 capacity.

The utilization of large-scale installation also raises new technical problems. At present, the scale-up of a fermentation process in China mainly depends on experience, such as equality in power input or airflow rate per unit volume, or using the same type of impeller. It is rather hard to materialize in practice. Then the cold simulation experiment of chemical engineering was introduced, and intensive study of the flow pattern in a bioreactor was carried out; in the end the design of

a large-scale of bioreactor was according to the mixing and transfer features of the apparatus. Sometimes deviation from practical situation is still rather obvious.

The cause of the difficulty in scaling up of a fermentation process is that it is unlikely to simulate simultaneously in geometry, fluid kinetics, and fluid dynamics. In spite of the similarity of fluid movement and fluid dynamic, it is likely that an essential factor, which affects the productivity, may not be observed during the laboratory study, and, if this happens to be the key factor, then the entire fermentation process is doomed to failure. Therefore, in the study of the scale-up of a fermentation process, we suggested a feasible approach, taking metabolic flux analysis and control as a core, to acquire some state parameters or physiological parameters that are useful to the scale-up of a process. Whenever changing the profile of metabolic flux, physiological data in the bioreactor investigated are exactly the same as in laboratory studies, and it is very likely that we may overcome the problem of scaling up; the scale-up technique based on the regulation of parameters during fermentation process is thus suggested.

However, the above studies still pertain to the scale-up principle of resembling physiological metabolic parameters, and are unlikely to substitute the decision of designable parameters in terms of geometric structure and momentum structure of a large-scale fermenter; in another words, in scale-up studies, the transformation of state parameters into operational and designable parameters requires more time to accumulate working experience and further upgrade into theory or an approach. For instance, the following studies are necessary: the estimation of agitation power for different size of fermenter according to OUR, $K_L a$ and the trait of impeller; selection of different type, speed and structure of impeller for different size of fermenters according to OUR, and the resistance capacity to shear stress by producing strain; application of CFD; processing and momentum balance of a large stirrer with high power supplied, and design of fermenter structure and driving device; estimation of fermentation heat and heat transfer area required according to OUR, and the cost of different heat transfer efficiency, intensity, and sterile capability; the design and investigation of computer-controlled compensation with scale-up effect, and the data acquisition and control system of a computer with compensatory and control capability, as well as the development of data base and the promotion and application of the above studies.

Hence, to realize the design to attain the purpose of operating bioreactors of different sizes under the same parameters of metabolic flux needs the coordinated efforts of different disciplines and techniques, namely, biology, chemical engineering, mechanical manufacture, transducer technology, as well as computer-controlled techniques.

1.4.2 Design of Agitation System in a Large-Scale Bioreactor and the Study of Its Flow Field Feature

Application of computational fluid dynamic to the study of the traits of a reactor flow field may render beneficial guidance to the design of an agitation system for a

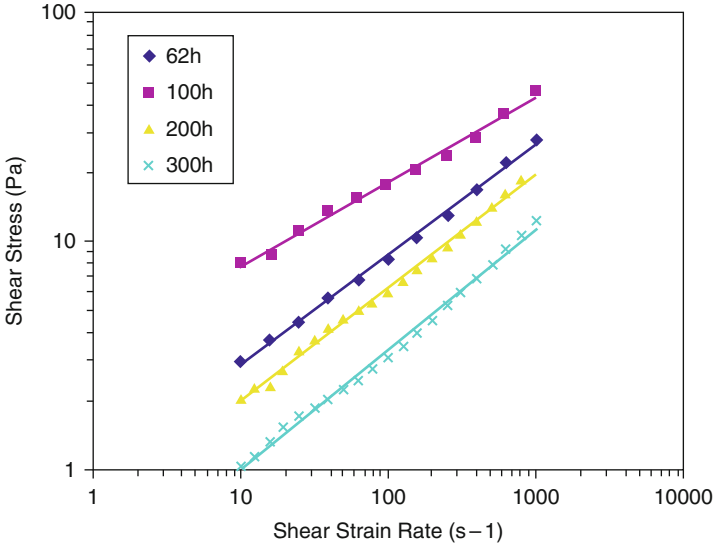

Fig. 7 Non-Newtonian property during 60 ton griseofulvin fermentation

large-scale bioreactor. In the course of scaling up griseofulvin from 30 L to 60 tons, a "gas column" raised above the surface of the fermentation broth was encountered frequently during the midphase of fermentations, and in some batches the deficiency of DO in the mid- and late-phases, which led to broth dilution and turning to red, eventually resulted in the decrease of the fermentation titer. Being analyzed, it was found that the fermentation broth of griseofulvin belongs to the pseudo-plastic fluids, its non-Newtonian property changes incessantly, and exhibits from low to high and from high to low levels of non-Newtonian property during fermentation, as seen in Fig. 7.

Through the analysis of the CFD before and after the alteration of the diameter of the impeller installed at the bottom of the fermenter and fermentation being carried out for 100 h, significant improvement of the distribution of gas was observed, which was favorable to the oxygen transfer between gas and liquid; a particular simulation result is shown in Fig. 8.

1.5 Development Trend of Bioreactor

1.5.1 Research and Development of Large-Scale Bioreactor for Animal Cell Culture

Due to defects in transcription and modification aspects of the expression system of prokaryotic cells, like bacteria, and the necessity of protein glycosilation for many valuable proteins, particularly genetic engineering drugs, vaccines, antibodies, etc.,

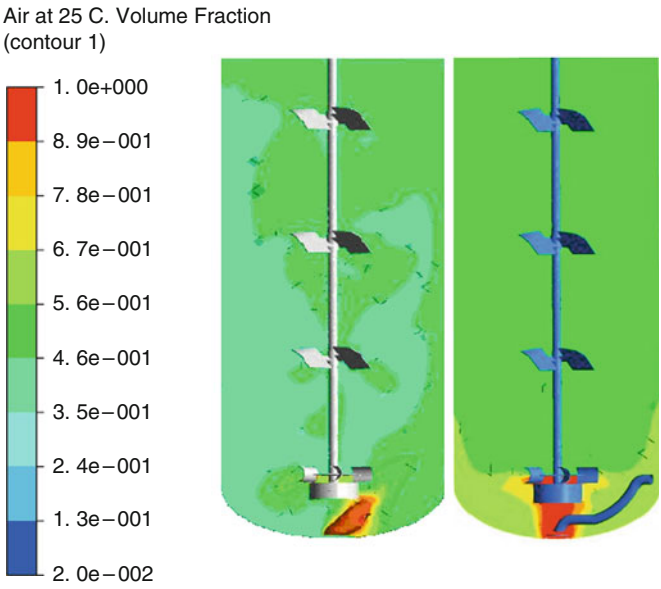

Fig. 8 The simulation results of impeller combinations before and after change

a mammalian cell expression system becomes a more suitable tool for such a purpose. Therefore, great attention is paid to the mammalian cell expression system, and a biopharmaceutical industry based on large-scale cultivation techniques has been developed in western countries like the US. Tens of these products have been launched onto the market, and huge economic and social benefits have been achieved [24–26].

Due to the lack of a cell wall, animal cells have strict demands on the nutrition, and hence whether or not the cell is anchored and grows at the surface of a solid or semisolid (anchorage-dependent growth), the use of serum in culture medium, the accumulation of inhibition factor, osmotic pressure and so on are always the key factors of large-scale cultivation of animal cells. Besides, animal cells are highly sensitive to environmental conditions such as pH, DO, temperature, shear stress, etc. The demand for resistance to contamination is strict. Consequently, the scale-up of a bioreactor, automation, and refinement are the problems concerned [27].

In terms of reactor models, most animal cell reactors adopted anchorage-dependent cultivation reactor functions in the early days. However, suspension cultivation reactors possessing various models are used especially for the cultivation of blood, lymphatic tissue, tumor cells (including hybridoma), and other transformed cell lines, in which the packed bed bioreactor manufactured by NBS Co. US is universally employed, but generally they were used only on a laboratory scale. Some companies, namely BIO of Sweden and B Braun of Germany, are also developing novel suspension reactors of newer fashions which are suitable for industrial scale production.

From the developmental trend point of view, suspension cell and serum-free cultivation are currently the development direction of most biotech companies in the world for the industrial production of biopharmaceuticals. Promotion of productivity during large-scale suspension cell, serum-free cultivation and realization of high-density cell cultivation, high-level expression, simplification of production technology, reduction of production cost, and assurance of product quality during large-scale production all play important roles in the industrialization of a bioprocess. These are leading edge topics that many companies were concerned about and explored, and they required various companies to establish technological platforms for industrialized production as required by FDA of US [28].

An animal cell bioreactor used for production has the tendency to grow larger (the largest one attained ton-level), possessing a computer control system with multiparameters monitoring and control devices. It is highly automated to adapt the high sensitivity of the animal cell to environmental factors and this kind of bioreactor has been elaborated and commercialized [28, 29]. From 1986 to 1995, the studies of bioreactors for animal cell culture had been carried out in China, which has led to fairly good progress, and also trial-production of bioreactors designated for research work. Nonetheless, there is demand for control precision from cultivation process of mammalian cell, and high quality materials should be employed. Manufacturing of essential parts is still very tough. That is why no commercialized products made in China are available at present.

Moreover, from the analysis of metabolic flux, the strict demand for nutrition in animal cell culture is related to intracellular metabolism [30–35]. The ratio of various nutrients consumed via different pathways is, in fact, the result of the shift of cellular metabolic status. Particularly, in recent years, there have been a great deal of scientific reports concerning the distribution of metabolic flux, and the regulation of metabolism at genetic and cellular levels [36, 37]. Therefore, there is likely to be a substantial breakthrough for the realization of the optimization of an animal cell cultivation process with regulation and control of relevant parameters through the study of multiparameters transducer technology and the data acquisition system of a computer [38, 39]; taking the metabolic flux determination as a target, we may achieve such a great breakthrough in the optimization of bioprocess.

1.5.2 Development and Application of Microreactor for the High Throughput Screening of Producing Strain

The construction and screening technique of high yield industrial producing strains is the major task of research, but currently in China, random breeding, including natural breeding, mutation breeding, and hybridization presented by protoplast fusion, are still the main ways of obtaining high yield industrial producing strains, which is time consuming, tedious, and requires a great deal of screening work. This is attributed to the fact that productivity is decided by multigenes, and it is

quite hard to increase greatly with only a single mutation. Usually, multiple cycles of mutation and screening (in each cycle a lot of labor is required) are needed before a stable and high yield single colony culture (strain) can be achieved.

Classic primary screening and secondary screening are generally conducted in shake flasks or test tubes without monitoring any parameters in situ. The strain passed through primary screening is subjected to laboratory testing in a bench-top fermenter, and further to pilot scale fermentation for optimization and scale-up. The whole process of screening and bioprocess optimization requires a lot of labor and investment, which is time consuming, and also manifests itself as a static feature. There are great differences in the cultivation environment between culture screening and industrial fermentation. Generally, missing some high yields strains may happen during a screening process. This static screening approach is characterized by separation of these two aspects.

Consequently, in the field of strain breeding and screening, particularly along with the universal employment of metabolic engineering for strain breeding, characterized by the study of systems biology, the screening technique and methodology research for high throughput screening incorporated with the fermentation process and multiparameters monitoring is currently an urgent requirement.

Bioreactors with the performance of high throughput, miniaturization, automation, and instrumentation are currently the main trend for the development of a bioreactor. The three types of microbioreactor – microtiter plate bioreactor [40], microfluid-based lab-on-a-chip [41], and miniature bioreactor (MBR) [42, 43] – which are miniaturized according to the prototype of a shake flask or bioreactor. While there are various types of MBRs, they all possess the following functions and features: online multiparameter monitoring functions, capable of determining pH, DO, pCO_2, and OD, etc. Generally, a set of MBR composed of 6, 12, 24, 48 or 96 microreactors [40, 43–45], whose capacity is smaller than 100 mL, even down to 5 μL [46], and for the microfluid-based lab-on-a-chip its volume is nL-grade. Besides, it also has the advantage of low cost, low consumption of high costs material, and low intensity of labor force.

Miniaturization of bioreactors is mainly based on successful application of photochemistry transducer techniques to fermentation processes. The principle of photochemistry is that excited light with a special wavelength irradiates on a particular fluorescence dye indicator, which is added to a medium in advance, or on a patch stuck to the wall of a bioreactor (i.e., chemical sensor), then the light emission or absorption resulting can be detected. The fluorescence intensity detected depends upon the content of oxygen and CO_2 or the pH of the broth. Accordingly, the concentration of DO, pCO_2, and pH can be monitored.

In contrast to previous sensors based on electrochemical principles, a photochemical transducer is a noninvasive sensor, and hence it can minimize the interference during online monitoring of the fermentation status, and is insensitive to electromagnetic disturbance, while solving the contamination problem [47]. These sensors possess outstanding antidisturbance properties. According to [46] it is still stable after being subjected 80 times to sterilization at 121°C. It requires only one calibration during the experiment. Furthermore, the cost of a photochemical sensor

is low and its operation expenditure is 10–20 times lower than that of traditional electrochemical electrodes.

1.5.3 Study on Systems Biology of Bioprocess and Bioreactor

Years ago, research on bioprocesses as represented by microbes was based on the engineering concept derived from classical dynamics, and on the biological concept founded on classical stoichiometry and thermodynamics. Along with the development of process engineering technology and biotechnology, the recognition of a process system from macroscopic to microscopic and from reductive theory to integrative view, modern engineering and omics technology should be introduced, especially when we confront the important problems regarding the notion of how to deal collectively with the achievements of genetics and physiology in terms of process optimization and scale-up of a bioprocess. In current bioprocess research, strain breeding is usually carried our first, and follows the subsequent optimization of fermentation conditions; the change of genome and proteome information flux is always omitted. In other words, the study of the improvement of strain production behavior is isolated from the study of the optimization of fermentation. In the course of a study, we often notice that the conclusion made from a single physiological regulation research alone is lack of integrity. It reveals only a local part of physiological regulation in a certain period, and therefore is unlikely to play the key role of regulating and optimizing the whole process.

Consequently, we deem that it is worth considering the importance of applying systems biology methodology to research on fermentation processes. Systems biology is different from the experimental biology that concerns only individual genes and proteins, it studies the genome, proteome, and metabolome and their correlation, and is thus characterized as an important science aimed at global study of the whole process. The concept of industrial microbial processes is to treat the bioreactor system as an ecosystem that is relatively closed. Systems biology is usually applied to the study of the physiological activity in a microbial cell, the interactions among microorganisms, and the correlation between microorganisms and exo-environment conditions. Such a macrocosmic study is likely to disclose the regulation genes responsible for microbial product biosynthesis, and offers the theoretical basis of strain improvement with metabolic engineering, reconstruction of microbial genome and expression regulation system, and reveals the molecular mechanism for the optimization of the parameters of fermentation process, so as to lay the theoretical foundation of the optimizing the parameters of the fermentation process.

With regards to research methodology, macroscopic variance analysis approach is emphasized. Such a research method possessing the background of systems biology can correlate directly the metabolite/pathway to transcriptomes, and establish a screening platform with high throughput. This may generate pedigrees different from those generated by conventional strain selection programs. Moreover, the integration of strain breeding with the exploitation of

the fermentation process lays the research foundation, which can be applied to the industrially produced strain, and speed up the screening of high yield strain and process development.

The improvement of theoretical study and the progress of the technical approach can be reflected in the change of structure and function of a bioreactor. In particular, we put forward a strategy that is based on the development of the feedback control of transducer technology, the monitoring and analysis of a bioprocess depending on the processing of information. The detection system is capable of providing a great deal of data detected in a process, which is not the same as a pH or DO probe, providing only a single estimated variable. It offers a multivariable estimation approach, which relies on spectrum analysis and many variables can be monitored simultaneously. The spectrum analyzer includes various types of mass spectrometer (MS), comprising a gas mass spectrometer for exhaust gas analysis during fermentation and pyrolytic cracking mass spectrometer (PyMS), various types of infrared (IR) spectrum analyzers, including near infrared (NIR) for the estimation of nutrient consumption and product formation, and medium infrared (MIR) based on the application of infrared fingerprint region, Raman spectroscopy for the analysis of biological cell and bioorganics, and dielectric spectrum analysis for the study of polarizability of an electrode in a biological system. In processing the huge amount of data obtained from these spectra figures, multicomponent stoichiometry possessing powerful functions, a straightforward high dimension rapid analyzer may be employed.

In addition, with respect to the research method for industrial microbial processing using systems biology, at transcription level, transcriptome can be obtained with a microarray chip; at translation level, proteome can be analyzed with 2D electrophoresis or time-flying mass spectrometry; and all the regulation loci for transcription in a regulation network are analyzed by using a chromosome immunosegmentation approach. Thus, it can be seen that the information obtained from analysis by life science techniques and analytical instruments has already served as important bioprocess monitoring parameters, and how to solve the relationship between the processing of the information acquired from a bioprocess and control system is worthy of consideration.

1.5.4 Development of a Computer Network System Based on the Information and Investigation of a Bioprocess

In research on a bioprocesses a huge amount of data is confronted, and we can neither study the cases one by one nor ignore them all. It is rational to adopt the efficient engineering method of finding out the characteristic changes of interest over time and then extracting the information we need from relevant data in due course. Therefore it is pivotal to construct a data information center for bioprocess engineering, and various professional databases concerning bioprocess engineering subjects, whose functions should be as follows:

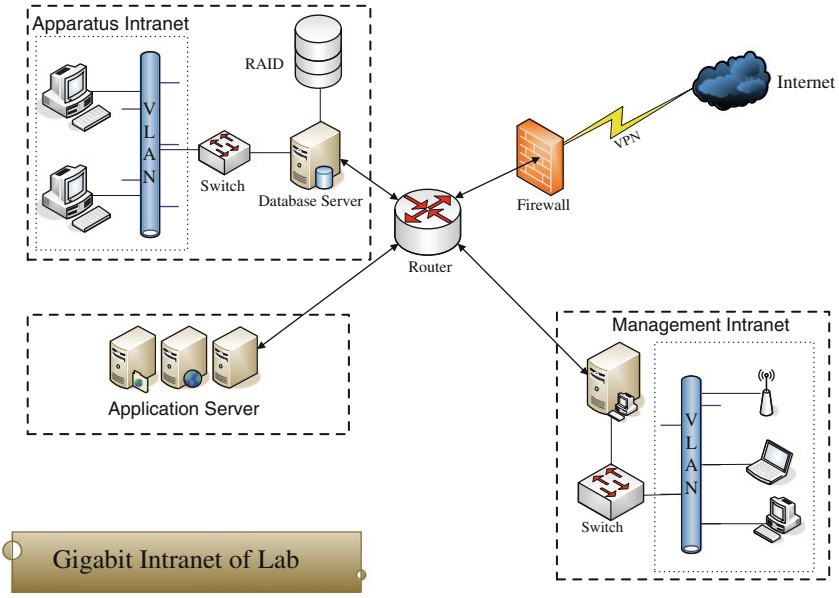

Fig. 9 Data processing system on laboratory scale

1. Theme data base for bioprocess: mainly for online monitoring of fermentation variables, including industrial microbial strain, genetic engineered strain and vector for bioprocess and microbial or cell genome, transcriptome, proteome, metabolome, and metabolic network related to bioprocess
2. Software analysis platform: including software tools for modeling, simulation, and analysis of metabolic networks relative to bioprocess studies, and analysis of process image monitoring, real time metabolic flux, as well as omics data relevant analysis
3. Management system for data acquisition and standardization, centralized storage, and remote access: the construction should be favorable to assure the implementation of an experiment project by using ordered, retraceable, and full range monitoring and control (Fig. 9)

2 Bioseparation in China

Bioseparation processes play a key role in biotechnology. Separation of bioproducts often needs entirely new processes to handle material with unusual properties. The desired product might be a single component present at low concentration that must be separated from bulk water and other soluble components. The concept of using biological pathways to produce a wide variety of valuable compounds offers many opportunities for innovative products. The bioseparation challenge for the future

will be to develop and improve current technologies or to create innovative separation technologies to meet the bioprocess requirements [48].

In the past 5 years, the scientists involved with bioseparation in China have made considerable progress. Their research has focused on the following subjects: chromatography media, recycling aqueous two-phase systems (ATPS), affinity precipitation, molecular imprinting, renaturation and modification of proteins, protein fractionation using ultrafiltration (UF), ion liquid separation of bioproducts, reverse micellar extraction, etc. In the following, major aspects of these subjects will be introduced, the cited references being taken mostly from related international journals in English and a few from Chinese journals.

2.1 Chromatography Media

Liquid chromatography plays a major role in typical downstream processes producing biomolecules such as recombinant proteins. Column packings of uniform microspheres with nanoporous structures usually show excellent resolution capacity due to less eddy diffusion and larger interaction area. Microspheres with large pores in the range of submicrons to microns can be used to separate large molecules such as proteins. The Shirasu Porous Glass (SPG) membrane emulsification technique is a promising technique which was first proposed by Nakashima et al. to prepare uniform-sized emulsion droplets and later used by Omi and Ma et al. to prepare uniform microspheres by polymerizing uniform monomer droplets. Microspheres are usually prepared from various types of emulsions, such as O/W, W/O, and W/O/W.

The Ma research group has done much work in the field [49–51]. Relatively uniform biodegradable polylactide microspheres were prepared by employing an SPG membrane emulsification technique (Fig. 10). Polylactide dissolved in hydrophobic dichloromethane (DCM) with a cosurfactant was used as the dispersed oil phase. An aqueous phase containing polyvinyl alcohol (PVA) was used, with sodium lauryl sulfate (SLS) as the continuous phase. The oil phase permeated through the uniform pores of the SPG membrane into the continuous phase by pressured nitrogen gas to form the droplets. Then, the solid polymer microspheres were obtained by simply evaporating DCM at room temperature for 24 h. A relatively uniform spherical PLA microsphere was obtained successfully.

The Sun group reported a novel approach for the fabrication of macroporous poly(glycidyl methacrylate–ethylene glycol dimethacrylate) monolith (Fig. 11) [52]. The method involved the use of sodium sulfate granules and organic solvents as coporogens. Photography of the prepared monolith is shown in Fig. 12. Compared with the conventional monoliths [ML-(1-3)] using organic solvents only as a porogen, the new monoliths [MLS-(1-3)] showed not only higher column efficiency and dynamic binding capacity (DBC) for protein (BSA) but also higher column permeability and lower back pressure. It is considered that the superpores introduced by the solid granules played an important role for the improvement of the

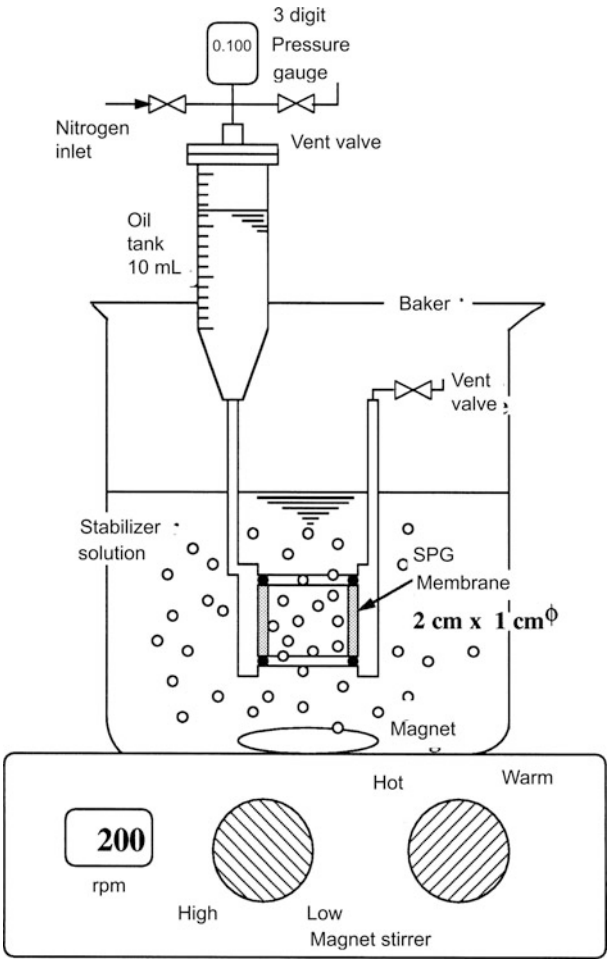

Fig. 10 Schematic diagram of a miniature kit for SPG emulsification

monolith performance. Moreover, poly(glycidyl methacrylate–diethylamine) tentacles were grafted onto the pore surface of MLS-3 monolith. This has further increased the DBC of bovine serum albumin (BSA) to 74.7 mg/mL, about three times higher than that of the monoliths without the grafted tentacles. This grafting does not obviously decrease the column permeability. The Sun group is currently focusing on preparation of perfusion chromatography media.

Recently, the Tan group reported immobilization of oligo-β-CD onto several polyacrylate beads and evaluated the chromatographic separation characteristics of puerarin on three novel coupled media [53]. They provided an available experimental foundation for choice and synthesis of polymeric supports for separating puerarin in preparative scale. The isoflavonoid puerarin, a well-known bioactive constituent in traditional Chinese medicine, can be separated from other

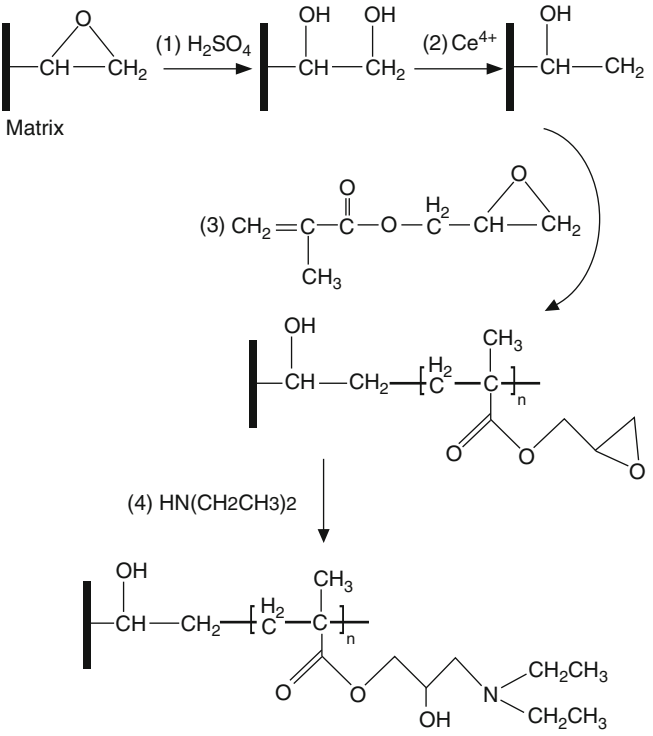

Fig. 11 Graft polymerization of glycidyl methacrylate and modification with diethylamine on the surface of poly(GMA–EDMA) monolith

isoflavonoids in the extracts of Radix puerariae (root of the plant *Pueraria lobata*) by using oligo-β-cyclodextrin-coupled D152 beads. The fine-sized coupled microspheres packing displayed a high resolving ability and efficiency. This study provided a novel alternative support for the separation of puerarin.

Among the various industrial chromatography techniques developed in recent years, expanded bed adsorption (EBA) is perhaps the most successfully used in the biotechnological industry. However, a robust EBA process primarily depends on the availability of a suitable adsorbent media, demanding a sufficiently high density and an appropriate distribution of particle sizes. Some of the other physicochemical properties, such as shape, porosity, hydrophilicity, and chemical modifiability, are also important. Cellulose, one of the early-developed natural polymers, has been widely used to manufacture hydrophilic and porous beads as column packing in liquid chromatography of bioproducts. To obtain improved performance of protein separation by the EBA process, the Yao and Liu research groups focused on preparation of expand bed adsorption media. The two groups have prepared a lot of new media such as cellulose/TiO_2 and TiO_2/macroporous copolymer from glycidyl methacrylate (GMA) and ethylene dimethacrylate (EDMA), cellulose-stainless steel powder composite matrix etc. [54–59]. These media have been

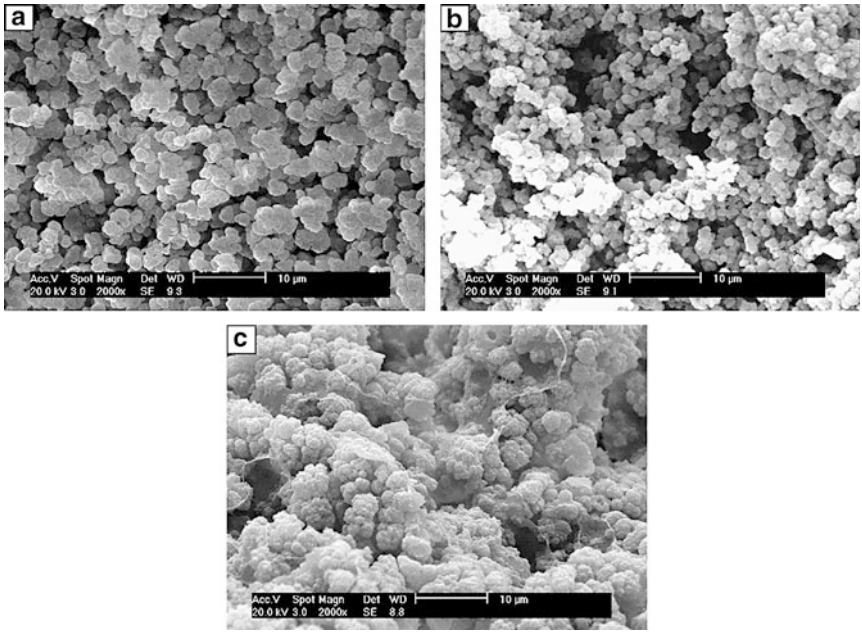

Fig. 12 SEM images of: (**a**) ML-3; (**b**) MLS-3; and (**c**) MLS-3T

used for purification of D-2-chloropropionic acid dehalogenase, lysozyme, BSA, and interferon-Con-l. The results indicated that the proposed adsorbent exhibits stable expansion properties and good adsorption behavior. For example, TiO_2/cellulose composite microspheres had an adsorption capacity for lysozyme of 98.7 g/L adsorbent. The protein breakthrough behavior in expanded bed was similar to that in packed bed with a dynamic adsorption capacity of lysozyme at 10% breakthrough reaching 69.3 g/L adsorbent. TiO_2-densified cellulose composite beads with diethylamine were used as the matrix for EBA to purify D-2-chloropropionic acid dehalogenase directly from the unclarified homogenate of *Pseudomon* sp. NT21. The binding capacity of the dehalogenase was found to be 8.54 U m/L adsorbent, and two active peaks were eluted respectively at 0.15 mol/L and 0.3 mol/L $(NH_4)_2SO_4$. The result indicated that the overall enzyme yield was 68%, with a purification factor of 22. In comparison to other recovery processes, the yield of the expanded bed process was higher, and operation time and cost were less.

2.2 Recycling ATPS

ATPS were discovered 50 years ago. Their applications, such as protein purification, separation of antibiotics, amino acids, organic acids, and natural products, and bioconversion have been developed since the 1980s. However, one key problem is

that copolymers forming ATPS could not be effectively recycled in use, thereby resulting in high cost and environment pollution. In recent years, researchers have focused on this problem and have attempted to find new ways of recycling copolymers of ATPS. Thermosensitive ethylene oxide–propylene oxide copolymers (EO–PO) appeared in some reports since the 1990s. Johansson et al. synthesized a hydrophobically modified random copolymer of EO and PO with aliphatic $C_{14}H_{29}$-groups coupled at the end of the polymer chain (HM-EOPO) [60]. ATPS with aqua micelles could be formed by single polymer HM-EOPO and H_2O. The top phase of ATPS contains 100 wt% water and the bottom phase contains 5–7 wt% HM-EOPO. The polymer could be recycled by temperature-inducing phase separation. Afterward, Persson et al. presented thermoseparating ATPS with $EO_{50}PO_{50}$ and HM-EOPO. In the systems used, 73% $EO_{50}PO_{50}$ and 97.5% HM-EOPO could be recycled, respectively [61]. Asrof and co-workers synthesized an anion polymer with *N*,*N*-diallyl-*N*-carboethoxymethylammonium chloride as a monomer to form ATPS. The polymer was pH-sensitive, and would precipitate in 0.1 M HCl solution. It could form ATPS with PEG-35000, and could be recycled by shifting the pH of the solution [62]. In China, the Cao group synthesized a visible light-sensitive polymer P_{NBC} which was copolymerized by using *N*-isopropylacrylamide (NIPA), *n*-butyl acrylate (BMA), and chlorophyllin sodium copper salt (CHL) as monomers (Fig. 13). The ATPS were formed by the P_{NBC} and DEX20000 [63]. Over 98% of the P_{NBC} could be recycled by using light radiation at 488 nm. The recovery experiments were repeated five times. The recoveries were fairly stably maintained. It was also found that the polymer could be recovered at more than 95% by using thermoseparation. Trypsin can constantly maintain a relatively high activity and stability in the ATPS. CAO group also synthesized a novel pH-sensitive polymer (P_{ABC}), which was copolymerized by using 2-(dimethylamino) ethyl methacrylate (DMAEMA), *tert*-butyl methacrylate (tBMA), and methyl methacrylate (MMA) [64]. ATPS was formed by 5 wt% P_{ABC} and 10 wt% PEG-20000. The partition coefficient *K* of lysozyme was 6.8 and that of BSA could reach 12.5 in the ATPS.

Further, the group reported a novel light-sensitive copolymer (P_{NNC}) forming ATPS with another pH-sensitive copolymer (P_{ADB}) [65]. The copolymer (P_{NNC}) was synthesized by using *N*-isopropylacrylamide (NIPA), *N*-vinyl-2-pyrrolidone (NVP), and chlorophyllin sodium copper salt (CHL) as monomers, and 2,2′-azobis-isobutyronitrile (AIBN) as initiator. The P_{NNC} could be recycled through light radiation at 488 nm, and the copolymer P_{ADB} could be recovered by adjusting the isoelectric point (PI) to 4.1, with a recovery of 97%. BSA and Tyr were partitioned in the P_{NNC}–P_{ADB} ATPS, showing that the partition coefficients of BSA and L-Tyr could reach 4.1 and 0.12, respectively.

The polymer P_{ADB} can also form ATPS together with the reported light-sensitive polymer P_{NBC} [66]. The top phase polymer P_{NBC} can be recycled through light precipitation, and it needs to absorb 4.5 kJ light energy to precipitate 1 g of polymer P_{NBC}, and the P_{NBC} and P_{ADB} recovery can still be kept at a higher level, reaching 98.6% and 97.9% respectively after five recycles.

The partition coefficient of BSA, lysozyme, and citric acid were studied in P_{NBC}/P_{ADB} ATPS. The result showed that this system could make a better partition effect

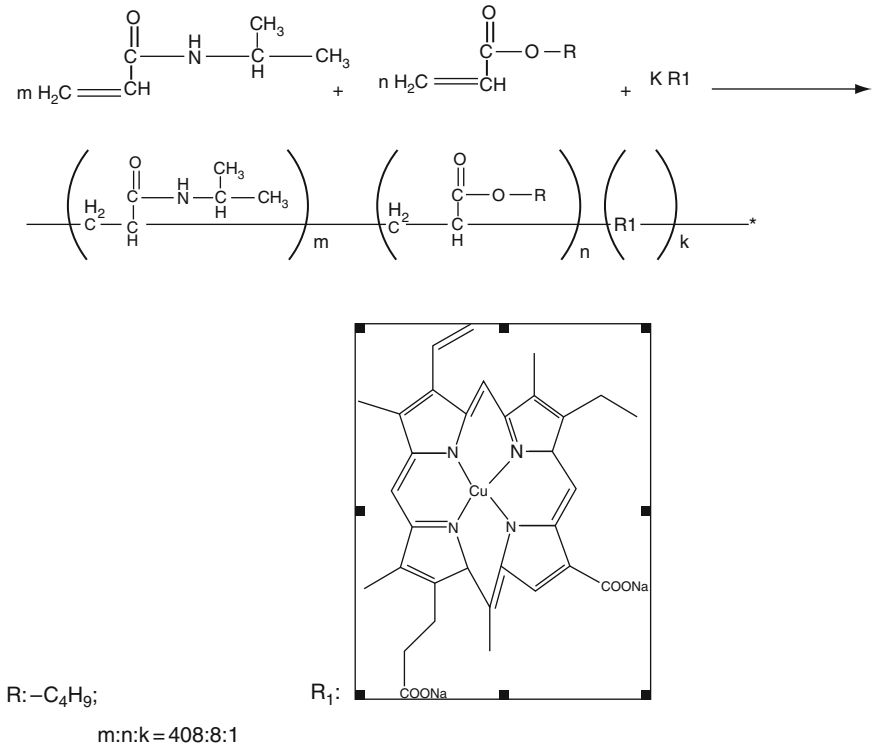

Fig. 13 Synthesis of light-sensitive polymer P_{NBC}

to BSA. The partition coefficient of BSA can reach 27.5 by the addition of 30 mM Na_2SO_4. The Cao group is now continuing the work of design and synthesis of more light–pH, light–thermo, thermo–pH, light–light, pH–pH, and thermo–thermo total recycling ATPS. It is expected that more than ten sets of recycling ATPS with high recovery will be available in the near future.

2.3 Affinity Precipitation

Affinity precipitation is a potential bioseparation method in industry. The method is suitable for the initial stages of separation and purification of bioproducts. It could significantly reduce the volume of the starting material and improves the purification efficiency.

Affinity macroligand polymers have to be recycled for economic reasons and to minimize environmental pollution. Until now, recovery of the affinity polymers is not high, resulting in hindrance of its application. It is necessary to design and synthesize cheap, effective polymers to realize the recycling of the polymer by simply changing the environmental conditions at low cost. In China, in recent years,

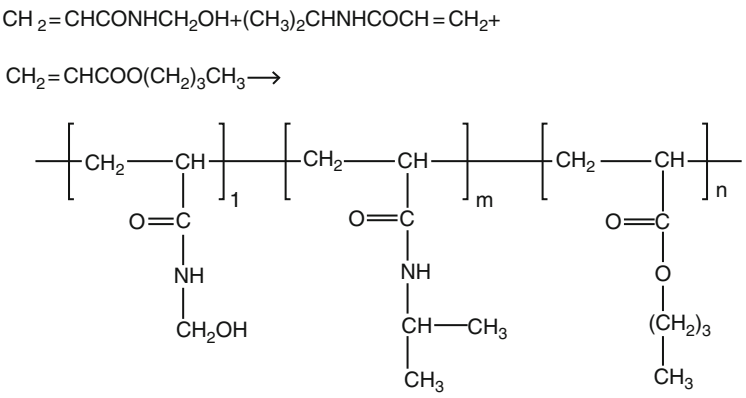

Fig. 14 Thermosensitive three-block copolymer consisting of *N*-hydroxymethyl acrylamide/*N*-isopropyl acrylamide/butyl acrylate

considerable research interest has been devoted to developing environmentally sensitive copolymers in bioseparation engineering. Responding to changing environmental conditions, the conformation of polymer can transform reversibly, resulting in shift between dissolution and precipitation of copolymer in aqueous solution.

The Cao research group developed a new thermosensitive three-block copolymer consisting of *N*-hydroxymethyl acrylamide (NHMA)/*N*-isopropyl acrylamide (NIPA)/butyl acrylate (BA) [67]. The structure is schematically shown in Fig. 14.

Among the three blocks, NIPA provides the thermosensitive character to the copolymer and NHMA brings hydroxyl groups to the polymer for the immobilization of ligands, while hydrophobic monomer BA helps to control the lower critical solution temperature. Recycle ability of the polymer was investigated and then coupled with a ligand; Cibacron Blue F3GA was the polymer, used for the purification of lysozyme. Lower critical solution temperature (LCST) of the thermosensitive *N*-alkyl substituted polyacrylamide polymer was controlled at 28°C. The thermosensitive recovery of polymer was over 95% in the presence of 0.05 M $NaClO_4$. Cibacron Blue F3GA was covalently immobilized onto the polymer via the nucleophilic reaction between the active chlorine atom of its triazine ring and the hydroxyl group of the polymer. The ligand density was 30 μmol/g polymer. The adsorption capacity for lysozyme of the polymer was 3.4 mg/g polymer in an affinity precipitation process. Over 90% of adsorbed lysozyme was eluted by 0.5 M KSCN at pH 8.0. When the affinity polymer was applied in the purification of lysozyme from egg white, the purification factor was 28 and the yield was about 80%.

Additionally, a novel light-sensitive copolymer (P_{NBCC}) was also designed by the Cao research group [68], consisting of four functional monomers. Its dissolution and precipitation was able to be shifted reversibly by light irradiation. The copolymer P_{NBCC} was applied to the purification of lysozyme.

The Sun research group prepared a magnetic affinity precipitation support by oxidization–precipitation with $FeCl_2$ and H_2O_2 in PVA solution [69]. Cross-linked directly with glutaraldehyde, a support with magnetic particles entrapped by cross-linked PVA gel was produced. Cibacron Blue 3GA (CB) was immobilized to the magnetic support to prepare the magnetic affinity support (MAS). The TEM showed that the MAS ranged from 1 to 10 μm and consisted of nanometer-sized colloidal magnetite particles. The adsorption kinetics and equilibrium behavior of BSA showed rapid adsorption and large capacity of the MAS. The equilibrium of BSA adsorption was reached in 5 min. At a CB coupling density of 23 μmol/g, the adsorption capacity of the MAS was 35 mg/g at an aqueous phase concentration of 0.1 mg/mL. Lysozyme was also used as a model protein to test the adsorption properties of the MAS. The adsorption equilibrium of lysozyme onto the MAS was described by the Langmuir-type isotherm. The capacity for lysozyme adsorption was more than 70 mg/g MAS (wet weight) at a relatively low CB coupling density (3–5 μmol/g). In addition, 1.0 M NaCl solution could be used to dissociate the adsorbed lysozyme. Finally, the MAS was recycled for the purification of alcohol dehydrogenase (ADH) from clarified yeast homogenates. Under proper conditions, the magnetic separation yielded better than fivefold purification of the enzyme with 60% recovery of the enzyme activity.

Sun research group immobilized soybean trypsin inhibitor (STI) on polymerized liposome of pentacose-10,12-diene-1-ol-phospho ethanolamine by diglycolic anhydride, glutaraldehyde and 1,4-butanediol diglycidyl ether. It shows PL-STI can be used as the affinity precipitation adsorbent of enzyme.

2.4 Molecular Imprinting

Molecularly imprinted polymer (MIP) is a new kind of tailor-made material, which has high affinity and selectivity for its template. Because of its high selectivity and easy preparation, MIP has gained more and more attention. This is confirmed by the exponential growth of molecular imprinting literature. In China, a vast number of papers have been published to describe MIPs prepared by using new template structures, monomers, and polymerization techniques, and have presented new application areas for these materials. However, less effort has been directed towards the molecular level events underlying the imprinting process and subsequent recognition mechanisms. What rules govern imprint formation? Can we use such rules to design MIP rationally? With the development of computer technology and computational chemistry, computer-aided design of MIP has also merged in China. The Wang and Tan group used the density functional theory (DFT) method to calculate the binding energy, ΔE, between a template molecule and monomers as a measure of their interaction, which facilitated the selection of monomers for the synthesis of MIP [70]. These works indicate that the computer-aided MIP synthesis, analysis, and evaluation are a safe and economic methodology. The Tan group established model was used to give an insight into the properties of MIP at the molecular level using a force field, which could be easily scaled-up for rapid

screening molecular imprinted systems. The group gave more attention to paracetamol-imprinted MIP. Paracetamol (acetaminophen) is well recognized as an analgesic and antipyretic agent, taken instead of aspirin. MIP is promising to be used for the quantification of drugs in real samples using competitive binding assay. The experimental study of paracetamol MIP has been reported. This group studied the affinity and selectivity of the MIP theoretically and predicted the possible best monomer for paracetamol imprinting in a fast and no-reagent-consuming way (Fig. 15). Moreover, the Su and Tan group prepared surface molecular imprinting adsorbent with Ni^{2+}[71, 72].

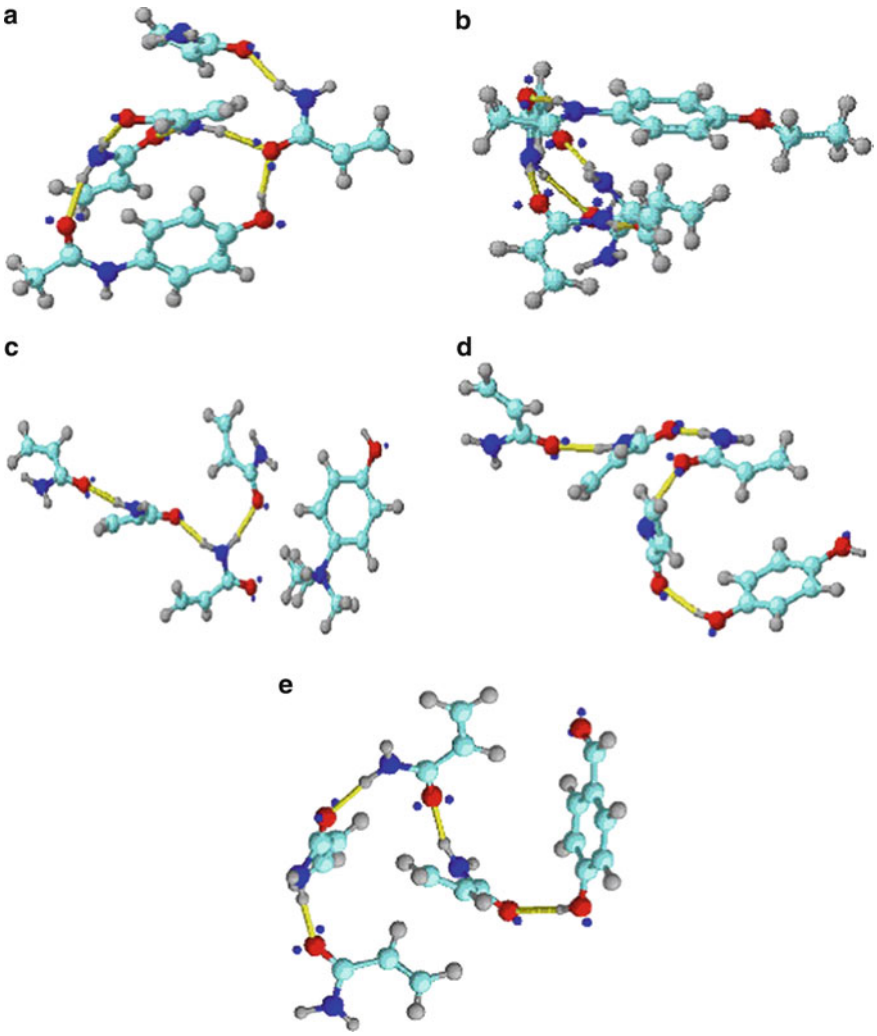

Fig. 15 Some typical conformations of paracetamol or analogs interacting with AAM: (**a**) paracetamol; (**b**) phenacetin; (**c**) *p*-tertiary butylphenol; (**d**) *p*-benzenediol; (**e**) 4-hydroxybenzaldehyde

2.5 Renaturation and Modification of Proteins

Overexpression of functional proteins in *Escherichia coli* usually leads to the formation of inactive inclusion bodies. Refolding therefore becomes a crucial step in protein production. Aggregation and misfolding are often responsible for low refolding yield. Dilution is a conventionally used refolding method, but the protein concentration has to be carefully controlled as low as possible to prevent aggregation or misfolding. As a consequence, this approach generally requires large volumes of buffers, huge tanks, and lengthy experiments. Therefore, the challenge is to achieve satisfactory active protein yield at high protein concentration from the inactive inclusion bodies. A number of chromatographic processes, including size exclusion chromatography (SEC), ion-exchange chromatography (IEC), and immobilized metal affinity chromatography (IMAC), have been developed to improve the refolding efficiency. In China, the Su group refolded protein by HIC using commercially available conventional media [73]. HIC is an efficient tool for protein separation. The mechanism involves hydrophobic interaction between immobilized hydrophobic ligands and the proteins in solution. Considering that hydrophobic interactions are the dominant forces in protein folding and structure stabilization, the research group believes that HIC can be an artificial chaperone system through its interaction with the denatured protein. Furthermore, HIC can directly deal with guanidine hydrochloride (guanidine-HCl) denatured proteins, which is an advantage over ion-exchange chromatographic refolding that cannot work in high-salt solution. However, HIC also has the possibility of hindering protein refolding. The strong interaction between the hydrophobic core of the denatured protein and the ligand on the matrix might stop the further folding of the peptide chain, leading to irreversible adsorption. To prevent this happening, the Su group purposefully selected polyethylene glycol (PEG) as an additive to regulate the hydrophobic interaction in the chromatographic refolding process. It is an attractive challenge to explore novel refolding methods that can effectively refold C-IFN at high concentrations. The group also developed a dual-gradient elution mode, consisting of decreasing guanidine-HCl concentration and increasing PEG concentration along the column. This provides a gradually changed solution environment, allowing the protein to refold gradually. The group has successfully refolded some proteins, such as Hepatitis B virus surface antigen (HBsAg), Consensus interferon (C-IFN), etc. [74, 75].

2.6 Protein Fractionation Using UF

Protein fractionation using UF has attracted significant interest in recent years. UF exhibits the advantage of a high throughput and easy scale-up. Fractionation processes are strongly influenced by operating and physicochemical parameters such as pH, salt concentration, permeate flux, and system hydrodynamics. Their effects have to be understood and used to control the process precisely in order to

achieve the desired levels of fractionation. This normally involves extensive experimentation, which is both time-consuming and expensive. Moreover, the results of such exercises are difficult to translate into practice when using conventional modes of UF. Therefore, the development of an efficient technique for UF process optimization and high-resolution UF process is required. To achieve the rapid identification of operating conditions which will be optimal for protein fractionation using UF, a new experimental technique, parameter scanning UF, has been developed. With this technique, the time taken, experimentation, and protein consumption can be greatly reduced. To ensure that the optimized conditions will be maintained throughout the entire operation, a new mode of UF operation, termed carrier phase ultrafiltration (CPUF), has been proposed by Ghosh [76]. The effectiveness and usefulness of parameter scanning UF and CPUF have been demonstrated in the fractionation of human plasma proteins, and monoclonal antibody alemtuzumab and BSA. By combining the parameter scanning UF with CPUF, an experimental methodology has recently been developed for rapid process optimization and high-resolution protein fractionation. This can be summarized in the following operations: (1) selection of appropriate buffer solution (e.g., buffer type and concentration); (2) pH and salt scanning UF for single protein to identify the suitable conditions for fractionation process; (3) further controlling operating conditions if necessary; (4) validation of the pH and salt concentration identified by fractionating protein mixture using CPUF; (5) optimization of system hydrodynamics and permeate flux using CPUF; and (6) fractionation of protein mixture at optimized conditions using CPUF. Unlike conventional cross-flow filtration, all these experiments are performed at a constant permeate flux. Although more and more recent studies have demonstrated the feasibility of using UF for fractionating proteins with very similar molecular sizes, most of those studies only dealt with simulated mixtures composed of pure model proteins, whereas experimental studies with real biological multicomponent streams are much more limited. It has been shown that the purity and yield of product obtained in a real mixture could be significantly lower than those obtained with model mixtures. Therefore, much more dedicated experimental work with real biological streams is essential in promoting the wide acceptance of UF in fractionation of proteins. It is also very clear that the usefulness and effectiveness of the methodology needs to be examined using real protein mixtures. As a case study for fractionation of real biological mixtures, the proposed methodology was applied to the separation of lysozyme from natural chicken egg white (CEW) solutions. Using a methodology based on the combination of recently developed parameter scanning UF with CPUF, Wan research group separated lysozyme directly from natural CEW solutions with 30-kDa membranes (Fig. 16) [77]. The suitability of two membranes, i.e., Biomax 30 kDa polyethersulfone and Ultracel Amicon YM 30 kDa regenerated cellulose membranes, was rapidly assessed and the optimal physicochemical conditions were identified by parameter scanning UF. Under suitable conditions, with the Biomax 30 kDa membrane, lysozyme transmission from the CEW solution could be more than 80% and the purity obtained in permeate was greater than 94% after a one stage UF process.

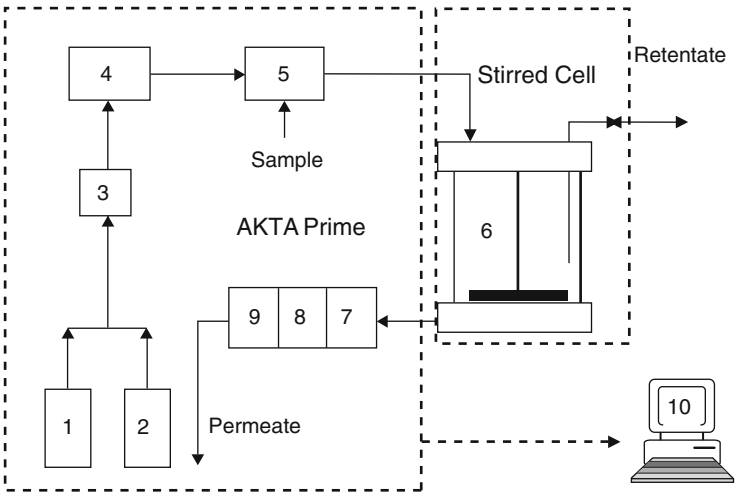

Fig. 16 Experimental setup for ultrafiltration experiments: (1) buffer reservoir a; (2) buffer reservoir b; (3) pump; (4) buffer mixer; (5) sample injector; (6) stirred cell module; (7) UV monitor; (8) conductivity monitor; (9) pH monitor; and (10) computer for data logging and processing

The effects of pH and ionic strength (salt concentration) on transmission of single protein human serum albumin (HSA) and human immunoglobulins (H&G) through a 100 kDa molecular weight cutoff (MWCO) polyethersulfone membrane have been studied by Wan and Cui group using a pulsed sample injection technique [78]. Experimental results clearly demonstrated the effectiveness of changing solution pH and ionic strength optimization for selective transmission of HSA or HIgG. The results also demonstrated the usefulness of pulsed injection experimental technique for the optimization of UF processes. A pulse input CPUF was employed to fractionate a binary protein mixture of HSA and HIgG at low NaCl concentration, showing that it is feasible to separate the binary protein at optimized conditions.

However, the problems of membrane fouling and concentration polarization remain and widespread application was impeded. By using a pulsed sample injection technique, requiring a very small amount of pure protein, it allows rapid determination of sieving coefficient and online monitoring of pressure profiles during UF, showing great potential in the analysis of membrane fouling and concentration polarization. The sieving coefficients of lysozyme and BSA, their pressure profiles (thus concentration polarization), and fouling behavior were examined with totally and partially rejecting UF membranes under different conditions [79].

Affinity membrane chromatography is a new type of isolation method with the combination of membrane and affinity chromatography, and it offers some apparent advantages over conventional bead-packed column chromatography in bioseparation, such as higher flow rate, shorter diffusion path, lower pressure drop, and simple scale-up. Membrane matrices have been increasingly studied in recent years

as alternative supports for affinity adsorption. The improvement of available membrane support is one of the most important factors in the development of membrane chromatography. Correct choice of membrane support and covalent coupling between membrane and ligand are essential for the success of the chromatographic separation. The Chen research group developed a novel amino acid affinity membrane by coating HEC on PVDF hollow fibers to increase the membrane hydrophilicity and then bound them with 1,6-hexanediamine (HDA) as the spacer arm [80]. The nonspecific binding to the modified membranes were investigated using BSA as the object protein. Capacity of the affinity membranes were investigated in a batch system. The adsorption phenomena appeared to follow a typical Langmuir adsorption isotherm under the optimal condition, where the maximum adsorption capacity (qm) of L-Phe affinity membrane for globulin was 0.318 mg/cm^2 membrane area and the equilibrium constant (Kd) value was found to be 0.453 mg/mL solution from the Scatchard plot. Forty hollow fibers with a membrane area of 0.0188 m^2 in the membrane module adsorbed 21 mg HGG from 10 mL human plasma with a purity of 83.9% in a single-pass mode, and the affinity membrane showed good stability throughout repeated adsorption–elution cycles.

2.7 Reverse Micellar Extraction

In the 1970s, a reverse micelles system was firstly used for extraction of protein by Luisi. Surfactants can spontaneously form spherical aggregates in organic solvent. The polar heads of surfactant form polar water pools. These aggregates are called as "reverse micelles." Proteins and other large biological molecules could be dissolved in the water pool and maintained good stability. Additionally, the cost of reverse micellar extraction process is low and the system can be used in recycle.

The separation of proteins using reverse micelles is rather easy to scale-up and can be operated continuously. It is well known that the backward transfer of protein from reverse micelles to the aqueous solution is relatively slow due to high interfacial resistance in mass transfer. Conditions of high ionic strength and pH within a certain range could not completely recover proteins solubilized in the forward extraction. A variety of alternative backward transfer methods has been studied. How to improve the recovery of proteins in back extraction? The scientists of bioseparation have done much research work. The Liu research group used a mixed reverse micellar system of CTAB and alkyl halides to improve forward and backward extraction of BSA [81]. The mixed reverse micelles formed with CTAB and alkyl halides exhibited excellent backward extraction behavior for BSA. The mixed reverse micelles formed with CTAB and R_4I can realize the recovery of BSA effectively in a wide range of pH up to or higher than the isoelectric point of BSA. The mixed reverse micelles formed with CTAB and R_4Cl, R_8Br or R_4I can obviously enhance the BSA backward transfer at low ionic strength with addition of KBr or KCl as electrolyte. The mixed reverse micellar system indicated that it required less time to reach mass transfer equilibrium in comparison with the reverse

micellar system with CTAB only. The mechanism of backward extraction proposed that, with the addition of alkyl halides to CTAB reverse micelles, the hydrophobic interaction between the reverse micelles decreased. The Liu research group purified Nattokinase from fermentation broth with AOT/isooctane micellar solution as the extractant [82, 83]. Many factors, including pH, surfactant concentration, addition of isopropanol, and contact time, were examined. By using the reversed micelles of AOT/isooctane to perform a full extraction cycle, it was found that about 80% of the total activity of nattokinase in the fermentation broth could be recovered and the purification factor was about 2.7.

The Sun research group modified Sorbitan trioleate with Cibacron Blue F-3GA (CB) to create an affinity surfactant and to form affinity-based reverse micelles in *n*-hexane [84]. The partition equilibrium and the extraction kinetics of lysozyme and BSA were then examined. The solubilization capacity of the reverse micellar system for lysozyme increased linearly with increasing the CB concentration from 0.1 to 0.5 mmol/L. In contrast, the capacity for BSA at 0.5 mmol/L of coupled CB was only about one-fifth that for lysozyme. It indicated a strong steric hindrance effect of the micelles for the high molecular mass protein. The overall volumetric mass transfer coefficient of lysozyme in the forward extraction increased from 0.43×10^{-3}/s to 1.25×10^{-3}/s with increasing CB concentration from 0.1 mmol/L to 0.5 mmol/L. Due to the high molecular mass of BSA, its volumetric mass transfer coefficient in the forward extraction was only one-sixth that of lysozyme. The ratio of the coefficient in the back extraction to that in the forward extraction was less than 0.03, much lower than that in other micellar systems. It indicated that the interfacial resistance in this system was more severe than in others.

In the process of AOT/isooctane reverse micellar extraction of proteins, a big problem is inactivation of interesting proteins when ionic surfactant AOT was used to form reverse micelles. The Cao research group found that adding ethanol could stabilize the activity of protein, and it greatly improved extraction recovery of trypsin [85]. When trypsin was extracted from crude material of pig pancreas by the using AOT/isooctane reverse micellar system, almost 90 and 100% of activity recovery of trypsin could be obtained in forward and backward extraction, respectively. The effects of ethanol concentration, organic salt types, AOT concentration, pH, and temperature on recovery of trypsin were investigated and extraction process were optimized. Finally, about 88% of total yield was obtained, and the specific activity of trypsin purified was increased from 300 U/mg to over 1,800 U/mg proteins with a purification factor five times higher.

2.8 Ionic Liquids and Their Applications in Bioseparation

An ionic liquid is a salt melting at about room temperature, which consists of organic cation and inorganic or organic anion. Ionic liquids, as a class of novel environmental friendly "green solvents," have remarkable properties and attractive applications in many fields. The applications of ionic liquids in bioseparation are of

interest. In China, a large number of papers have been published in recent years. Some of them are involved with bioseparation.

ATPS based on ionic liquids are gaining wide recognition as novel "greener" separation systems. ATPS based on ionic liquids have some unique advantages, such as lower viscosity, very quick phase separation, difficult emulsifiability, as well as good extractability for bioproducts. The Liu research group investigated the phase behavior of ionic liquid aqueous two-phase systems (ILATPS) [86]. It was found that the additive salt had little effect on the phase equilibrium of the aqueous two-phase. The effect of anions mainly obeyed the Hofmeister rule, and the formation of ILATPS could be promoted by the increase of the hydrophobicity of anions. The formation of ILATPS could also be promoted by the increase of polarity of the head group, and by the increase of the side-chain length of ionic groups, but the effect was weaker for the anion. An increase in temperature would inhibit the formation of ILATPS for short-chain ionic liquids, but would promote the formation of ILATPS for long-chain ionic liquids.

The Mei research group studied the resolution of *R*,*S*)-4-hydroxy-3-methyl-2-(2-propenyl)-2-cyclopenten-1-one ((*R*,*S*)-HMPC) using ionic liquids or vinyl acetate as the reaction medium [87]. The effects of the reaction medium, water activity, temperature, pH value, and the cosolvent on the resolution were investigated. The results showed that the ionic liquid [bmim]PF6 was suitable for the reaction, and the initial rate of lipase in [bmim]PF6 was 18.48 μmol/(g min), which was considerably higher than that in vinyl acetate (9.18 μmol/(g min)). The half-life of lipase in the ionic liquid was 74.53 h, which was higher than that in vinyl acetate (64.29 h), but the conversion of (*R*,*S*)-HMPC in ionic liquids was lower than that in vinyl acetate. The addition of an acryl donor could improve the conversion of (*R*,*S*)-HMPC in ionic liquids. The optimum reaction conditions in [bmim]PF6 were water activity 0.17, 40°C, and pH 7, the same as those in vinyl acetate. When the cosolvent was added to the reaction system, the conversion in [bmim]PF6 decreased, whereas that in vinyl acetate increased, which might be attributed to the different properties of the reaction media.

The Jiang research group prepared a new and effective stationary phase based on ionic liquid-modified silica and used it for the separation of ephedrines in high-performance liquid chromatography (HPLC) [88, 89]. The separation results indicated the high efficiency and reproducibility of the stationary phase. The effective separation was considered to be attributed to the electrostatic interaction, ion-exchange interaction between the solutes, and the stationary phase. Moreover, the free silanols on the surface of the silica are effectively masked by the immobilized ionic liquid. As a result, nonspecific absorption was decreased.

3 Conclusion

From the foregoing, we can see some aspects of bioseparation research in China. More than ten groups are undertaking important research. Some works have been completed, while others are still being carried out. It is believed that China's

scientists will produce important innovation in future years. Probably, these innovations will be in these areas: preparation of chromatography media, recycling polymers forming ATPS, applicable molecular imprinting polymer and ion liquids, etc.

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Environmental Biotechnology in China

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Abstract Environmental biotechnology has emerged as an important measure to tackle the environmental pollution as China experiences great economic success. Over the past decade, much emphasis has been paid to the following fields in environmental biotechnology: microbial degradation of toxic and organic chemicals, bio-treatment of wastewater, waste recycling. The Chinese researchers have done a lot of work to understand the natural degradation processes for organic and toxic compounds and finally to clean these compounds from polluted environments. For the treatment of wastewater, many new processes were proposed and optimized to meet the more strict effluent standards in China. Finally, more and more attention has been paid to the reuse of discharged wastes. In this chapter we review the development in the above fields.

Keywords Biodegradation, Bio-treatment, Environmental biotechnology, Organic pollutants, Renewable resources, Reuse, Waste, Wastewater

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Contents

1 Introduction

In the past three decades, China has experienced economic success. However, environment deterioration almost neutralized this economic achievement. According to data released by the State Environmental Protection Administration of China, annually 8.3 million tons of industrial wastes have been produced of which 3 million tons were not treated, and 100 million hectares of arable land was polluted by industrial wastes. Many of these industrial wastes are toxic and organic compounds.

Microbial degradation is one of the major processes that can completely minimize toxic and organic compounds in the environment. In this field, Chinese researchers have done a lot of work aiming to understand the natural degradation processes for organic and toxic compounds and finally to clean these compounds from polluted environments. In addition, China has made great efforts to improve wastewater treatment capability and to reduce water pollution. Environmental biotechnology has been developed rapidly and become one of the most important technologies to treat industrial and municipal wastewater in China.

In this chapter, we review the major progress of environmental biotechnology in three fields including microbial degradation of pollutants, wastewater bio-treatment and reuse of wastes.

2 Microbial Degradation of Toxic and Organic Chemicals

2.1 *Microorganisms Isolated for Degrading Various Toxic and Organic Compounds*

Extensive work has been done in the past few years to obtain microorganisms that can degrade various toxic and organic compounds. Table 1 summarizes the microbial isolates reported during 2004–2007 (published in Chinese scientific journals). These and other lab-stored bacterial strains are key resources for many bioprocesses to remove toxic and organic compounds from environments.

Table 1 Microbial isolates from China reported for degradation of organic and toxic compounds

Compounds	Strains	Investigators and time*
Aromatic compounds		
Bisphenol A	*Achromobacter xylosoxidans* B-16	[36]
Nitrophenols	*Achromobacter xylosoxidans* NS12	[24–26]
Phenol	*Acinetobacter calcoaceticus*	(Xu et al. 2000)
	Bacillus cereus Jp-A	[36]
	Candida sp. P5	(Hu et al. 2007)
	Raoultella sp. PS1	[41, 42]
Di-*n*-butyl phthalate	*Acinetobacter calcoaceticus* TS2H	(Duan et al. 2007)
2,6-Di-tert-butylphenol	*Alcaligenes* sp.	(Fang et al. 2004)
Phthalate esters	*Burkholderia pickettii*	[17–21]
DBP	*Rhodococcus ruber* CQ0302	[54–56]
DEHP	*Cellulomonas* sp.	(Qin et al. 2005)
p-Chloronitrobenzene	*Comamonas* sp. CNB-1	[2]
3-Clorobenzoate	*Rhodococcus erythropolis*	[50]
3,5-Dinitrobenzoic acid	*Comamonas testosteroni* A3	[41, 42]
p-Chloroaniline	*Diaphorobacter* sp. PCA039	(Ren et al. 2005)
Nitrobenzene	*Pseudomonas putida* NB1	[41, 42]
	Rhodococcus sp. Ns	[24–26]
Biphenyl	*Rhodococcus pyridinovorans*	(Sun and Qian 2004)
PAHs		
Phenanthrene	*Acinetobacter* sp. L2	(Zhu et al. 2005)
	Agrobacterium sp. Phx1	(Zhang and Yuan 2005)
	Pseudomonas sp. GF2	[47]
	Sphingomonas sp. GY2B	(Tao et al. 2006)
Benzo[*a*]pyrene	*Azomonas* sp. JL14	(Sheng et al. 2005)
Pesticide		
Methamidophos	*Acinetobacter* sp. HS-A32	(Zheng et al. 2006)
Chlorpyrifos	*Alcaligenes faecalis*	(Yang et al. 2005)
Atrazine	*Arthrobacter* sp. AG1	(Dai et al. 2007)
	Burkholderia glumae	[10–14]
	Exiguobacterium sp. BTAH1	(Hu et al. 2004)
	Micrococcus luteus sp. AD3	(Wen et al. 2005)
	Pseudomonas sp. SA1	(Dai et al. 2007)

(continued)

Table 1 (continued)

Compounds	Strains	Investigators and time*
Methylparathion	*Arthrobacter* sp. X4	[130]
Carbendazim	*Bacillus pumilus* sp. NY97-1	(Zhang et al. 2006)
	Ralstonia sp.	(Zhang et al. 2004)
Triazophos	*Klebsiella* sp.	[63, 64]
Mefenacet	*Sphingobacterium multivolum* Y1	(Ye et al. 2004)
Fenpropathrin	*Sphingomonas* sp. JQL4-5	(Hong et al. 2006)
Chloro-derivatives		
1,2,4-Trichlorobenzene	*Bordetella* sp.	[24–26]
	Pseudomonas nitroreducens J5-1	(Song et al. 2007)
DDT	*Sphingomonas* sp. BD-1	[95]
	Brevunmdimonas sp. W-1	(Gu et al. 2007)
α-Hexachlorocyclohexane	*Sphingomonas* sp. BHC-A	[76, 77]
Bromoamine acid	*Sphingomonas xenophaga*	(Qu et al. 2005)
Alkane		
SDS	*Ochrobactrum anthropi* WZR-A	(Wu 2006)
Oil	*Burkholderia cepacia* X4	(Qing et al. 2007)
Others		
Quinoline	*Burkholderia pickettii*	[58]
MTBE	*Chryseobacterium* sp. A3	[17–21]
Cyclohexanone	*Micrococcus* sp. CN1	(Li and Shao 2007)
Nicotine	*Ochrobactrum intermedium* DN2	(Yuan et al. 2005)
Polyacrylamide	*Pseudomonas* sp. PD 1	(Li et al. 2004)
Biosurfactants releasing	*Pseudomonas* sp. XD-1	(Yin et al. 2005)
Polyvinyl alcohol	*Rhodococcus* sp. J-5	(Li et al. 2004)
17α-Ethynylestradiol	*Sphingobacterium* sp. JCR5	(Ren et al. 2006)

*References for these isolates are not listed but the names of the main investigator and the publish time are provided (*right column*). *Abbreviations*: DBP, dibutyl phthalate; DEHP, di-(2-ethylhexyl) phthalate; PAHs, polyaromatic hydrocarbons; DDT, dichloro-diphenyl-trichloroethane; SDS, sodium dodecyl sulfate; MTBE, methyl tertiary-butyl ether

Microorganisms have tremendous capacity to metabolize various compounds. They can also quickly adapt to environments where they evolve new metabolic abilities to grow. Thus, the metabolic capacity of microorganisms is even larger when their fast evolving and adaptive nature is considered. Table 1 also provides a glimpse of the diversities of microbial degraders of the toxic and organic compounds. In the following paragraphs, microbial degradation of some selected compounds (chloronitrobenzenes, parathion and nitrophenols, carbazole and dibenzothiones) are discussed in more detail. A special paragraph focusing on aromatic degradation with *Corynebacterium glutamicum* is included, because this work was mainly conducted by Chinese scientists and reflects the efforts to understand better the degradation processes of aromatic compounds by the widely distributed and environmentally important Gram-positive bacteria.

2.2 Chloronitrobenzenes

Three isomers of chloronitrobenzenes (CNBs), namely 2-chloronitrobenzene (2CNB), 3-chloronitrobenzene (3CNB), and 4-chloronitrobenzene (4CNB) (Fig. 1), are chemically synthesized. They are important intermediates for commercial production of various dyes and drugs. China is the major producer of CNBs and the annual production in the year of 2006 was over 500,000 tons, accounting for approximately 65% of the global production. 4CNB can be metabolized in human body and the major metabolites are mercapturic acid *N*-acetyl-*S*-(4-nitrophenyl)-l-cysteine. Long term exposure to high doses of 4CNB causes damage to the liver and spleen and affects hematopoiesis.

Of the three CNB isomers, microbial degradations were reported for 2CNB [1], 3CNB and 4CNB [1–4]. Degradation of 3CNB and 4CNB by a co-culture of two bacterial strains was also reported. In other examples, 2-, 3- or 4CNB was degraded via co-metabolism. For example, *Pseudomonas acidovorans* strain CA50 reduced CNBs to their corresponding monochloroanilines in the presence of additional carbon and nitrogen sources [5].

Although the genetics and metabolic pathway(s) for 2- and 3CNB degradation are still not clear, the genes involved in 4CNB degradation and the metabolic pathway of *Comamonas* sp. strain CNB-1 that uses 4CNB as carbon and nitrogen sources have been extensively studied [6–14]. Genes encoding enzymes for the degradation of 4CNB were located on a large plasmid pCNB1 from *Comamonas* sp. strain CNB-1, and this pCNB1 was fully sequenced (NCBI GenBank database under accession no. EF079106) [15]. A similar plasmid involved in 4CNB degradation was detected in *Pseudomonas putida* strain ZWL73 [16]. In both strains CNB-1 and ZWL73, the initial step of 4CNB degradation was the partial reduction of the nitro- group to a hydroxylamino- group, which converted 4CNB into 2-hydroxylamino-4-chlorobenzene and this product was subsequently rearranged to 2-amino-4-chlorophenol [7–9]. The genetic organization and reaction steps for 4CNB degradation with strain CNB-1 are shown in Fig. 2.

Sequence analysis of pCNB1 suggested that gene deletion and acquisition as well as genetic rearrangement of DNA molecules happened during the evolution of 4CNB degradation pathway [15]. A novel deaminase was identified that is not

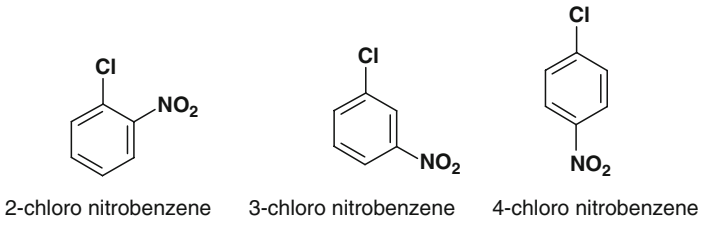

Fig. 1 Three isomers of chloronitrobenzene

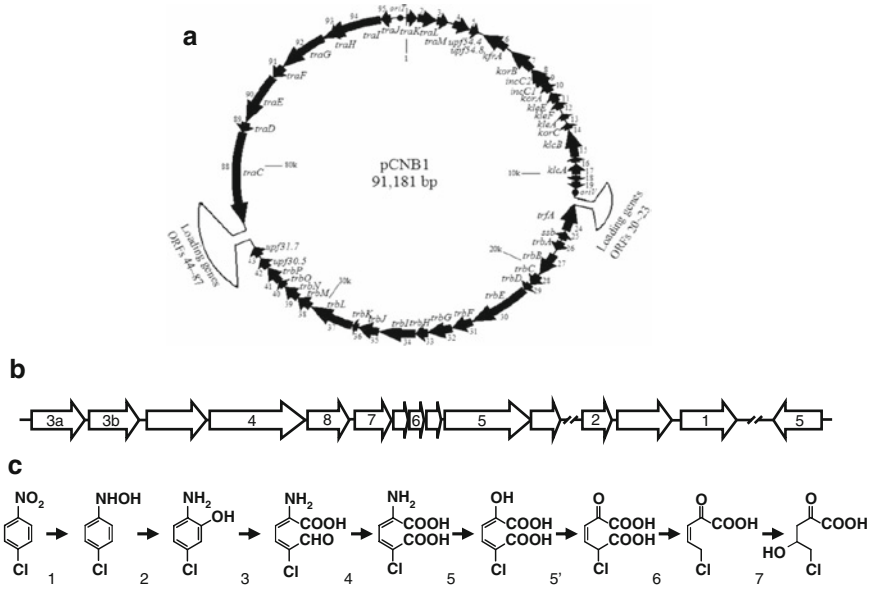

Fig. 2a–c Genetic organization of pCNB-1 from *Comamonas* sp. strain CNB-1 (**a**), organization of 4-chloronitroenzene metabolic (*cnb*) genes in ORFs 44–87 of transposon Tn*CNB1* (**b**) and putative metabolic pathways for 4CNB (**c**)

phylogenetically closely related to any known deaminases and catalyzes 2-amino-5-chloromuconate to 2-hydroxy-5-chloromuconate [10–14] and its encoding gene was located distantly to other 4CNB-degradative genes but associated with arsenate resistance genes [17–21]. Biochemical studies further revealed that the 4CNB-degradative enzymes such as 4CNB nitroreductase and 2-amino-5-chlorophenol 1,6-dioxygenase were more adapted, as indicated by their high affinities to 4CNB and its degradative intermediates [6, 9].

Microbial degradation of 4CNB is of interest to both the bioremediation of 4CNB-polluted sites and the understanding of the evolution of 4CNB degradation. A plant-microorganism system consisting of alfalfa and strain CNB-1 was used for remediation of 4CNB-polluted soil. Results showed that strain CNB-1 successfully colonized the rhizosphere of alfalfa roots and released the toxicity of 4CNB. Meanwhile, 4CNB at concentrations between 50 and 200 mg kg^{-1} of soil was completely degraded within 2 days [10–14].

2.3 Parathion and Nitrophenols

Methyl/ethyl parathion is an organophosphorus insecticide that was first synthesized in the 1940s. It is relatively insoluble in water, poorly soluble in petroleum

ether and mineral oils, and readily soluble in most organic solvents. It is thermally unstable and undergoes fast decomposition above pH 8. Since it was invented, parathion has been widely used as insecticides in agriculture as well as for domestic animals. Due to its high toxicity, parathion is one of the insecticides that have been restricted for application since 2004 in China. However, a large amount of parathion is being produced. During 2003–2006, more than 50,000 tons of methyl parathion was produced in China.

Animals can degrade parathion and eliminate the degradation products within a very short time. By far the most important route for the environmental degradation of methyl parathion is a microbial processes [22–27]. Cui et al. [28] systematically investigated microbial degradation of parathion by *Plesiomonas* sp. strain M6. The strain M6 hydrolyzes methyl parathion into 4-nitrophenol (4NP), and the gene (*mph*) encoding methyl parathion hydroxylase was cloned and functionally expressed in *Escherichia coli*. Although this report is more than 10 years later than the reports for the *Flavobacterium* and *Pseudomonas* species, it is interesting that the *opd* (generally for "organophosphate degradation") genes encoding parathion hydrolases in *Plesionmonas* M6 are absolutely different from those in *Flavobacterium* or *Pseudomonas* [28]. The *mph* gene encoding methyl parathion hydroxylase in strain M6 was used for generation of engineered bacterial strains for bioremediation of polluted soil [23, 29]. Biochemically, the hydroxylase hydrolyzes methyl parathion to dimethyl phosphorothinate and 4NP. A similar methyl parathion hydroxylase was also found in *Pseudomonas* sp. strain WBC-3 [3]. This methyl parathion hydroxylase was purified from strain WBC-3, crystallized [30, 31], and its structure was revealed at 2.4 Å resolution. Structural information revealed that the methyl parathion hydrolase from WBC-3 is homologous with other metallo-β-lactamases but does not show any similarity to phosphotriesterase that can also catalyze the degradation of methyl parathion with lower rate, despite the lack of sequence homology [32].

The strain WBC-3 is different from the strain M6 that could not degrade 4NP further. In contrast, strain WBC-3 uses methyl parathion or 4NP as the sole source of carbon, nitrogen, and energy [33]. This property made strain WBC-3 unique, because many other bacterial strains with parathion degradation ability isolated from diverse geographical regions lead to the production of 4NP and it is still commonly accepted that mixed-cultures or co-metabolisms or engineered organisms are the major microbial processes for the complete detoxification of parathion and methyl parathion.

Although there has been no further report on how 4NP is degraded by strain WBC-3, it is presumed that strain WBC-3 has a similar 4NP-degrading pathway via hydroxyquinone as intermediate to that of *Moraxella* species. Alternatively, 4NP could be degraded via 1,3,4-trihydroxybenzene as intermediate, as proposed for 4NP degradation in a bioreactor [10–14]. Microbial degradation of 3-nitrophenol (3NP) by *Alcaligenes* sp. strain NyZ215 has been investigated at genetic level. This 3NP is degraded by *Alcaligenes* sp. strain NyZ215 via catechol as intermediate, and three genes involved in the degradative pathway were cloned [34].

2.4 Carbazole, Dibenzothiophene and Biodesulfurization

Carbazole is a heterocyclic aromatic compound and is produced during coal gasification. Coal tar produced at high temperature contains an average of 1.5% carbazole. Several thousand tons of carbazole are produced each year from coal tar and crude oil. Carbazole is widely used in synthesis of dyes, pharmaceuticals, and plastics and is a suspected carcinogen. Carbazole is degraded via a meta-cleavage pathway and the genes involved in carbazole degradation were first identified in *Pseudomonas* sp. strain CA10 (by Japanese Scientists Ouchiyama et al. in the year of 1993). During the last few years, there have been growing interests in microbial degradation and transformation of carbazole. Bacterial strains of *Sphingomonas* [35] and *Pseudomonas* [36] were obtained that degrade carbazole by a similar pathway, in which carbazole is initially attacked at the angular position by dioxygenation, followed by spontaneous conversion of the dihydroxylated intermediate to 2-aminobiphenyl-2,3-diol. Moreover, genetically engineered microorganisms that degrade carbazole were constructed [37–39].

Dibenzothiophene (DBT) is structurally an analog to carbazole, and was regarded as a model compound for organic sulfur in fossil fuels. Microorganisms attack on DBT via different routes and can completely degrade DBT to CO_2 and energy for cell growth. Alternatively, microorganisms can selectively remove the sulfur atom from DBT and keep the carbon skeleton of DBT unchanged (Fig. 3). This latter route was exploited for developing a green process of desulfurization in the last decade. A recent review summarized the progresses in China on DBT degradation and biodesulfurization [40]. More recent developments in biodesulfurization include the improvement of expression of desulfurization enzymes [41, 42] and cloning the hemoglobin gene in *Rhodococcus* species for stimulating desulfurization [43].

2.5 Degradation of Aromatic Compounds with Corynebacterium glutamicum

C. glutamicum has been used for the mass production of amino acids, such as L-lysine (560,000 tons per year) and l-glutamate (1,000,000 tons per year). Very recently, the ability to metabolize various aromatic compounds by this bacterium has been disclosed (Fig. 4). Not only were the diverse metabolic pathways found, but also genes and enzymes involving in aromatic catabolism have been reported [24–26, 44]. Moreover, a novel glutathione (GSH)-independent gentisate pathway was described [45]. Although the potential applications of this robust ability to degrade aromatic compounds by *C. glutamicum* still needs to be explored, these discoveries are certainly helpful to improve the knowledge of degrading aromatic compounds in phylegentically closely related bacteria such as species of *Rhodococcus* of environmental importance. An MSH-depended maleylpyruvate isomerase involved in the gentisate pathway was discovered in *C. glutamicum* [44]

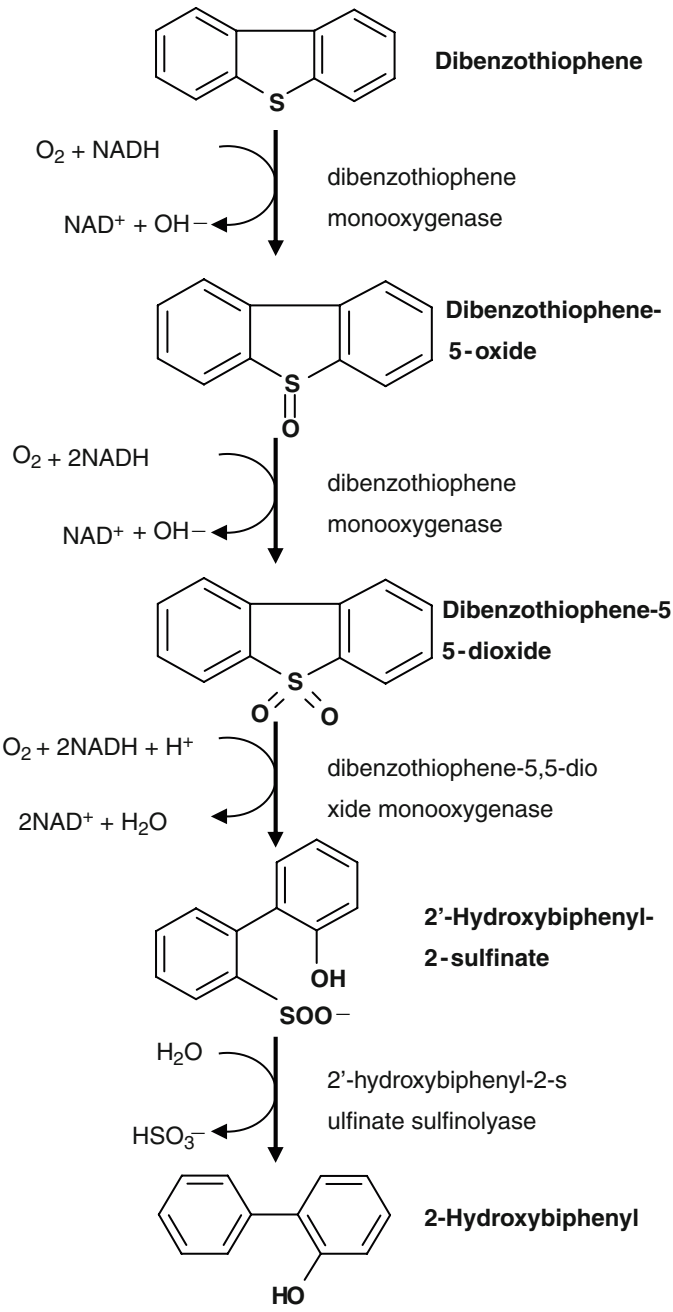

Fig. 3 Selective removal of sulfur from dibenzothiophene, which is exploited for biodesulfurization

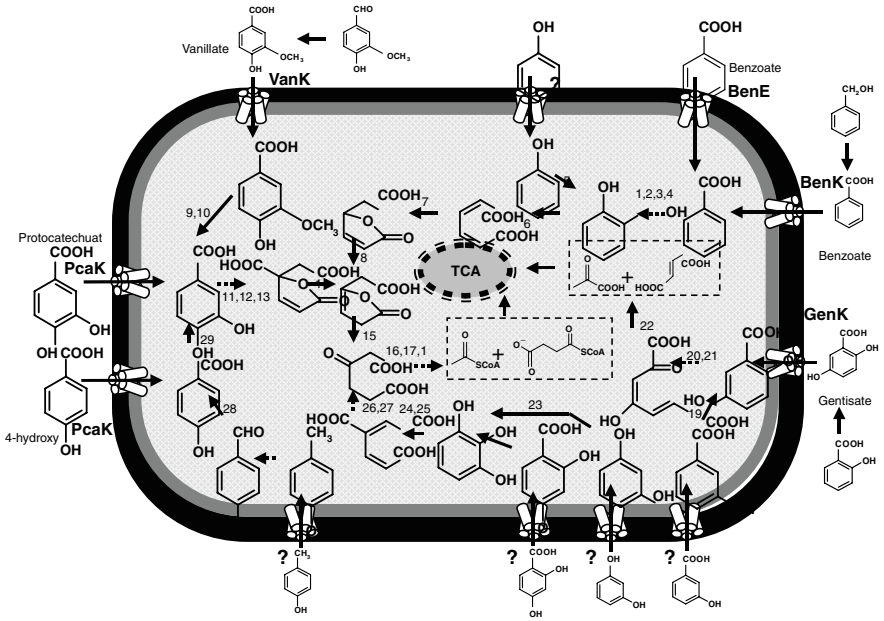

Fig. 4 Multiple metabolic pathways for aromatic compounds in *C. glutamicum* after membrane transport through specific transporters

and a similar maleylpyruvate isomerase was detected in *Rhodococcus* species (unpublished data from Liu et al.). Recently, aromatic acid transporters have been characterized in *C. glutamicum* [46].

C. glutamicum can use the following compounds as sole carbon source for growth: phenol, benzoate [47], vanillate, vanillin, protocatechuate, 4-hydroxybenzoate [48], 3-hydroxybenzoate, gentisate [45], resorcinol, 2,4-dihydroxybenzoate, 3,5-dihydroxytoluene [49], 4-cresol, and benzyl alcohol [50]. Several genetic segm-ents on the *C. glutamicum* genome were annotated for aromatic compound degradation. A unique 30 kb (approximately 1% of the whole genome) catabolic island that channels the degradation of various aromatic compounds was mapped at positions 2,525–2,555 kb of *C. glutamicum* genome. For degrading a wide range of aromatic compounds, *C. glutamicum* operates three degradative pathways, i.e. the gentisate pathway, the hydroxyquinol pathway, and the -ketoadipate pathway that includes the catechol branch and the protocatechuate branch. In the gentisate pathway, maleylpyruvate is produced following the aromatic ring cleavage catalyzed by gentisate 1,2-dioxygenase. The isomerization of maleylpyruvate to fumarylpyruvate is catalyzed by an MSH-independent maleylpyruvate isomerase recently identified in *C. glutamicum* [44, 45]. In *C. glutamicum*, resorcinol and 2,4-dihydroxybenzoate are degraded through the hydroxyquinol pathway. Interestingly, there are two sets of genes (*ncgl2950-ncgl2953* and *ncgl1110-ncgl1113*) occurring at the genome of *C. glutamicum*. All genes at the genetic cluster *ncgl1110-ncgl1113* were involved in resorcinol assimilation, but the genes at genetic cluster *ncgl2950-ncgl2953* were not necessary for growth on resorcinol [49].

As in many other bacteria, the entire -ketoadipate pathway in *C. glutamicum* is composed of the catechol branch, the protocatechuate branch and the -ketoadipate central pathway. Aromatic compounds such as 4-cresol, vanillin, 4-hydroxybenzoate, benzoate, and phenol are degraded through the -ketoadipate pathway. Benzoate and phenol are representative compounds that are degraded through the catechol branch of the -ketoadipate pathway. The genes involved in the catechol branch are organized in a single cluster (*ncgl2317-ncgl2319*), and this gene order is conserved in Gram-positive bacteria *R. opacus* and *C. glutamicum* but not the Gram-negative bacteria such as *P. putida*. When analyzed according to sequence identity, the genes involved in the protocatechuate branch of β-ketoadipate pathway are generally more similar to their Gram-positive bacteria counterparts such as *Streptomyces* sp. and *R. opacus*. However, significant differences of gene structure and organization were also found among *C. glutamicum* and *Streptomyces* sp. and *R. opacus*. A single gene, *pcaL* (encoding γ-carboxymuconolactone decarboxylase/β-ketoadipate enol-lactone hydrolase) in *Streptomyces* sp. strain 2065 and *R. opacus* replaced the hypothetical *ncgl2312* (encoding γ-carboxymuconolactone decarboxylase) and *ncgl2310* (encoding β-ketoadipate enol-lactone hydrolase) of *C. glutamicum*. Sequence analysis indicated that NCgl2310 shared 50 and 31% identity with the N-terminal of PcaL in *R. opacus* and *Streptomyces* sp. strain 2065, respectively. Ncg12312 showed significant identities of 78 and 46%, to the C-terminals of *R. opacus* and *Streptomyces* sp. strain 2065, respectively. Thus, it was proposed that the *pcaL* of *R. opacus* and *Streptomyces* sp. strain 2065 originated from the fusion of independent genes such as *ncgl2310* and *ncgl2312*. The catechol and protocatechuate branches converge at the intermediate β-ketoadipate enol-lactone (Fig. 4, step 8 and 14) and flow into the β-ketoadipate central pathway, which starts by conversion of β-ketoadipate to β-ketoadipyl-CoA by means of a putative β-ketoadipyl-CoA thiolase (NCgl2307/NCgl2307). The genes (*ncgl2306/ncgl2307*) encoding this putative thiolase were located on a large catabolic island (ca. 33 kb at 2,524–2,557 kb) in the *C. glutamicum* chromosome. To our knowledge, direct linkage of genes involved in the two branches of the β-ketoadipate pathway like in the *C. glutamicum* chromosome have not been found in other Gram-positive or Gram-negative bacteria, and this well-organized catabolic island (contributing 1% of the entire genome) is also a unique feature of the *C. glutamicum* chromosome.

3 Biological Treatment of Wastewater

3.1 Biosorption Technology for Removal of Heavy Metals and Persistent Organic Pollutants (POPs) from Wastewater

Heavy metal pollution has become one of the most serious environmental problems today. Biosorption, with biomaterials such as bacteria, fungi, yeast and algae as biosorbent, is regarded as a cost-effective biotechnology for the treatment of high volume wastewaters containing low concentration heavy metal(s) ranging from 1 to

100 mg L^{-1}. In addition, although the biodegradation is a removal process for organic compounds in wastewater, some persistent organic pollutants are difficult to be removed by biodegradation process. As a result, organic pollutants that are not biodegradable can still be removed from the wastewater by the microbial biomass via the process of biosorption.

During the last decades, biosorption technology for removal of heavy metal ions and persistent organic pollutant also attracted more attentions in China due to the obvious advantages of biosorbent, namely its biodegradability, without recontamination and lower cost. The researches focused on the screen and preparation of highly efficient biosorbents, clarification of biosorption mechanism, modification of biosorbent to enhance biosorption capability, and optimization of biosorption process.

3.1.1 Highly Efficient Biosorption Materials

In China, many highly efficient biosorption materials, such as microbial cells, or biosynthetic materials were found and obtained during the past decades, and their biosorption capabilities and characteristics for heavy metal ions and POPs were assessed. Zhou et al. [51] found a bacterium *Gordona amarae* from industrial wastewater with higher capability of adsorbing heavy metal ions. The bacterial cell could absorb heavy metals from aqueous solution with about 94% recovery ratio under suiTable operation conditions. Xiao et al. [52] isolated a sorption strain HX with excellent ability of adsorption-decolorization for anthraquinone dye and azo dyes from the sludge of biochemical treatment pond of certain printing and dyeing plant in Guangzhou. The strain exhibited excellent ability of adsorbing property and decolored completely the KN-R with concentration of 250 mg L^{-1} within 48 h.

Wang et al. [53] obtained a cell envelope material from *Pseudonomos. putida* 5-x cell, and found its heavy-metal ion adsorption capability was three times higher than intact *P. putida* 5-x cell. Spatial obstacle may be the main reasons of lower biosorption capability of the intact cell. In addition, Li et al. [54–56] extracted a novel adsorption-type bioflocculant ZL5-2 from penicillium and actinomycetes cultures successfully. It has higher adsorption and flocculation capability, and can effectively reduce chemical oxygen demand (COD) and biological oxygen demand (BOD) in print-works and refinery wastewater. Furthermore, a new chitosan molecular imprinted adsorbent was prepared from mycelium of waste biomass. The adsorption capacity for Ni^{2+}, Cr^{3+}, Cu^{2+} of the adsorbent increased considerably [57]. These highly efficient biosorption materials can effectively remove heavy metal ions and POPs from wastewater.

3.1.2 Biosorption Mechanism and Kinetics

For modifying biosorbent to enhance biosorption capability further, biosorption components and groups in biosorbents were widely studied. Infrared spectroscopy analysis showed that the acidic groups, for example carboxylate groups, –OH

groups in the biomass such as brewer's yeast and *Trichoderma sp* play an important part in heavy metal biosorption. The esterification of carboxylate functions presented in the cell walls of yeast results in a marked decrease in lead uptake [58, 59]. Wang et al. [60, 61] found that all cell surface components of Gram-negative bacteria, such as peptidoglycan layer, outer membrane and inner membrane, contributed to heavy metal ions adsorption, but phospholipids and lipopolysaccharides in outer and inter membrane play an important role in heavy metal biosorption by cell envelope of Gram-negative bacteria.

For designing and optimizing biosorption process, the biosorption kinetics was also studied in the past decade in China. Gao and Wang [62] studied the biosorption characteristics and kinetics of Ni^{2+} by *Saccharomyces cerevisia*, and indicated that the process of Ni^{2+} biosorption onto the biomass of *Saccharomyces cerevisia* could be divided into two stages. The first stage was a physical sorption and reached equilibrium very quickly (within 10 min). The biosorption kinetics could be described by the pseudo second-order equation quite well ($R^2 = 0.999$). The equilibrium isotherm could be fitted by the *Langmuir* and *Freundlich* models. Zhou et al. [51] investigated the sorption of Cr^{6+} from aqueous solution in a batch system with dead cells of *Bacillus licheniformis* isolated from metal polluted soils. The biomass exhibited the highest Cr^{6+} uptake capacity at 50 °C, pH 2.5 and Ci = 300 mg L^{-1}. The *Langmuir* model fitted this experimental data well and the sorption system was better described by the pseudo-second kinetic model.

3.1.3 Modification of Biosorbent for Enhancing Biosorption Capability

Many studies have shown that biosorption capacity of biosorbent correlates with cell surface structural, component and groups. For further enhancing biosorption capability, the surface component and structure of microorganism cells was modified either by genetic modification, or by cell surface pre-treatment.

Zhang et al. [17–21] isolated a 1,053 bp of the nickel/cobalt transferase gene (NiCoT gene) from *Staphylococcus aureus* ATCC6538, and constructed a recombined plasmid pET23c, then transferred the plasmid into *E. coli* BL21. The Ni^{2+} accumulation of the genetically engineered *E. coli* BL21 was 11.33 mg g^{-1}, which was three times higher than that of the original strain. Wang et al. [63, 64] found that pre-treatment with dilute HCl can increase the biosorption capacity of *P. putida* cell by 25–30%. Transmission electron microscopy analysis indicated that enhanced adsorption of heavy metals by dilute HCl pretreated cells was relative to the degradation of a loose superficial layer outside the fresh cell.

These researches indicated that biosorption capability of biomass to heavy metal ions and POPs can be further improved by physical–chemical or biological methods.

3.1.4 Optimization of the Biosorption Process

For effective biosorption in more rigorous industrial application, immobilization technology of biosorbent was developed. Wang and Hu [65] developed a biosorption

system with immobilized *Aspergillus fumigatus* immobilized by sodium carboxymethylcellulose (Na_2CMC)] to remove anthraquinone dye, namely reactive brilliant blue KN_2R from wastewater. The biosorption efficiencies using the immobilized beads with bead diameter 2.0–3.85 mm could remove above 90% of KN_2R from wastewater within 24 h under a variety of operating conditions. Wang et al. [66] developed a magnetite immobilized cell system to remove heavy metal ions from industrial waste effluent. The cell pre-treated with diluted HCl was immobilized by magnetite and used as biosorbent in a semi-continuous biosorption system to remove and recover Cu^{2+} from electroplate effluent. The removal and recovery efficiency of Cu^{2+} reached 96 and 95%, and the immobilized biosorbent could be effectively reused more than five times. In addition, some reports indicated that biosorption combined with active sludge process and bioflocculent could effectively treated industrial and municipal wastewater [60, 61, 67].

3.2 *Bioflocculation and its Application in Water and Wastewater Treatment*

Bioflocculation means using bio-flocculent to flocculate, settle and then remove particles, suspended solids and color in wastewater. Generally, bio-flocculent is a metabolite of some microorganisms under special culture conditions. Bio-flocculent are non-toxic, biodegradable, and easily operative in water and wastewater treatment. Since the early 1990s there have been many reports in China about microbial bioflocculent-producers, bioflocculation mechanism, optimization of production conditions and the application of microbial flocculent (MBF) on water and wastewater treatment.

3.2.1 Bioflocculents and Bioflocculation Mechanism

Chinese researchers have isolated many bioflocculent-producing microorganisms from different sources (soil, activated sludge, river sediment, etc.). The species of bioflocculent-producing microorganisms includes bacteria, moulds and antinomycetes [59].

Sun et al. [68] isolated a microbial flocculant-producing strain (X1), a *Bacillus. Sp*. from soil, which is of good flocculation activity. The flocculant produced by this strain had a significant flocculation effect on chrome black T wastewater and lysine liquid waste. Liu et al. [69, 70] studied *Penicillium sp.* HHE-P7 isolated from municipal wastewater sludge. Using the source wastewater as culture medium, the bioflocculant MBF7 was produced by this strain. Wang et al. [71] studied the effects of carbon source, nitrogen source, initial pH and cultivation time at 30 °C on the yield and activity of bioflocculant produced by *Agrobacterium sp.* LG5-1. Under optimal cultivation conditions, produced bloflocculant possesses good stability and flocculating activity of 76.3%, and can be deposited for 200 days at low temperature.

To lower the cost of bioflocculant, easily-obtained materials are selected and used as medium for production MBF ; for example starch wastewater [24–26] and brewery wastewater were used as substrate for bioflocculent production. In addition, Bai and Wang [72] studied the optimal conditions of immobilized cells to produce bioflocculent in a semi-successive system using lacunal polyester as immobilized carrier. Compared with the suspended version, the production efficiency increased by 77.78%.

The flocculation mechanism of microbial flocculent has been widely studied in many laboratories. The chemical compositions of many MBFs have been analyzed. The polysaccharide is one of the important compositions in MBF [73] and the flocculation process is based on the bridging mechanism.

3.2.2 Application of Microbial Flocculent to Wastewater Treatment

The application of microbial flocculent on treatment of different kinds of wastewater has been widely studied during the past decades, including the treatment of wastewater with high concentration of organic compounds, the removal of turbidity, and the decoloration the dewatering of sludge. Instead of the single species of flocculent-producing microorganisms, the compound microbial flocculent (CMF) has been used to treat the wastewater. Because of the different species of microorganisms and their functions, the CMF has higher efficiency [74].

Zhang and Lin [75] obtained a microbial flocculent from multiple microorganisms using brewery wastewater as carbon and energy source. It was applied to treat indigotin printing and dyeing wastewater. The effect of pH, dosage of MBF and 1% $CaCl_2$ on the removals of COD and color was tested, and two processes for removals of COD and color were developed.

Although there have been many reports about the research of MBF and its application on wastewater treatment in China, most studies were on a lab scale. Few successful applications on industrial operations were reported. However, the flocculation mechanism of MBF and the biological and molecular background deserve more studies in the future.

3.3 Bio-Removal of Nitrogen and Phosphorus in Wastewater

The discharge of nutritional ingredients such as nitrogen and phosphorus into the aquatic environment resulted in the eutrophication of lake and river and occurrence of algal blooms in China. For remediation of aquatic environment and avoiding occurrence of algal blooms, many technologies to remove N and P from wastewater have been developed during the past few decades. Among these, bio-removal of nitrogen and phosphorus attracted more attention due to the low cost and freedom from recontamination. In China, much research focused on the mechanism and process optimization of bio-removal of N and P.

3.3.1 Denitrification

Recently, one of the focuses on nutrition removal is short-cut denitrification via nitrite. Most of the studies were carried out with a sequencing batch reactor. Fast realization of nitrosofication is the result of multi-factors such as temperature, dissolved oxygen (DO), pH, SRT and influent ammonium concentration, of which DO and pH are the most important. When ammonium concentration in influent is within 120–240 mg L^{-1} and SRT is about 23 days, it is easy to realize nitrosofication at 30 and 35 °C under the condition of low DO (0.5–1.0 mg L^{-1}) and appropriate pH (7.5–7.8). At room temperature (21–25 °C) with controlling lower DO (0.5–0.6 mg L^{-1}) and higher pH (8.0) it is also easy to implement nitrosofication [76, 77]. Zhou et al. [78] observed that the optimum DO for simultaneous nitrification and denitrification was 0.5–0.6 mg L^{-1}. The nitrogen removal must be via the short-cut route since a high nitrite concentration was accumulated in the reactor.

In a pilot-scale pre-denitrification process at normal temperature [7, 8], stable nitrite accumulation was realized when treating domestic wastewater at DO of 0.5 mg L^{-1}. However, the accumulation vanished when the DO increased to 1.5 mg L^{-1}; if DO was lowered to 0.5 mg L^{-1} again, nitrite reappeared.

DO, pH and oxygen reduced potential (ORP) can also be used for traditional nitrification and denitrification. In a continuous anaerobic/oxic process, for example, the variation of pH in anoxic zones could be classified into “descending type” and “rising type”, which indicated whether the denitrification extent and nitrate recirculation flow were sufficient. The ORP value and nitrate concentration at the end of anoxic zone also had good correlation. The DO concentration in the first aerobic zone could indicate the influent ammonia load. The variation of pH in aerobic zones could also be classified into “descending type” and “rising type”, clearly indicating the extent of nitrification, aeration and alkalinity. The experimental results also showed good correlation of ORP values in the last aerobic zone with effluent ammonia and nitrate concentrations. An online system to make an integrated use of these signals for online control of aeration, nitrate recirculation flow and external carbon dosage was presented and demonstrated with promising results [79].

Simultaneous nitrification and denitrification with aerobic granular sludge was another hot topic. High ratios of COD/TN (25), COD/TP (58), temperature (22 °C) and low sludge retention time (SRT) (10 days) are beneficial to the cultivation of the aerobic granular sludge. The proper TN/TP rate (2.36), DO and selection of anaerobic seed sludge are basic requirements and the most important factors to guarantee such aerobic granules in the process [80]. The gases produced in simultaneous nitrification and denitrification were investigated by Wang et al. [63, 64]. They found that the emitted NO_2 amount was less than the background value. However, emitted NO and N_2O were 10 times more than the background. Less N_2O was emitted under low DO and high pH conditions. When DO mass concentration was 1.5–3.0 mg L^{-1}, about 0.58 and 6.53% of total lost nitrogen was emitted as NO and N_2O, respectively. When DO concentration was 2.5–4.0 mg L^{-1}, 0.48 and 39.34% of total lost nitrogen was emitted as NO and N_2O, respectively.

3.3.2 Phosphorus Removal

In the literature most studies on the effect of pH on enhanced biological phosphorus removal were conducted with acetate wastewater, and the pH was controlled during the entire anaerobic and aerobic stages. Liu et al. [10–14] investigated the influence of anaerobic initial pH control, which is more practical than the entire process pH control strategy, on enhanced biological phosphorus removal from wastewater containing acetic and propionic acids. In batch experiments, the optimal initial pH for higher soluble ortho-phosphorus (SOP) removal efficiency should be controlled between 6.4 and 7.2. However, when phosphorus assimilating organisms (PAO) were cultured for a long time under different pH conditions, the best pH range for phosphorus removal was 7.6–8.0 [17–21]. In the PAO system, with the increase of pH, the phosphorus removal efficiency was improved greatly, and a phosphorus removal efficiency of 100% was achieved at 8.0. With the comparison between the non- and long-term cultured enhanced biological phosphorus removal (EBPR), it was concluded that the higher phosphorus removal efficiency at higher pH was mainly caused by a biological effect instead of chemical one, which could be reflected by the higher PAO growth rate at about pH 7.6 [81]. Liu et al. [69, 70] observed that the polymer hydroxybutyric (PHB) and polymer hydroxyvalerate (PHV) formed during enhanced biological phosphorus removal (EBPR) process affected the phosphorus(P) release, uptake and removal.

In order to find a sustainable carbon source for improving EBPR performance, Tong and Chen [82] used the alkaline fermentative short-chain fatty acids (SCFAs) which were produced from waste activated sludge as the carbon sources of EBPR microorganisms [83]. The phosphorus removal efficiency was around 98% with the fermentative SCFAs, and the toxicity of fermentation SCFAs to EBPR microorganisms was not observed.

Glycogen accumulating organisms (GAO) are thought to be the potential competitors of PAO for the often limiting carbon sources in wastewater; thus the study of the GAO mechanism is of great importance in order to restrain GAO growth. Yao et al. [84] found that, when cultivated with high propionic/acetic acid ratio, GAO consumed less glycogen and synthesized less PHA in the anaerobic phase, and in the aerobic phase accumulated less glycogen and degraded less PHA, and at the same time the microbial growth was lower. When the carbon mole of acetic acid equaled that of propionic acid in the influent, GAO utilized acetic acid faster than propionic acid.

3.3.3 Simultaneous Nitrogen and Phosphorus Removal

Recently, biological phosphorus removal with nitrate/nitrite as electron acceptors has attracted much interest, since this kind of simultaneous nitrogen and phosphorus removal strategy is both time and money saving. Zou et al. [85] found that PAO could use nitrate as electron acceptor for biological phosphorus removal instead of oxygen, but it would be inhibited if COD was presented. The rate of taking up phosphate in

anoxic stage was related to the concentration of nitrate: the higher the concentration of nitrate, the higher the rate of taking up phosphate. The continuous and steady addition of nitrate is of benefit to phosphate removal. The efficiency of PHA digestion in the system using nitrate as electron acceptor is lower than using oxygen [86].

Liu et al. [10–14] observed that the anoxic phosphate uptake of DPB was rarely influenced by the concentration of nitrate with adequate nitrate as electron acceptor. It takes 1 mg PO_3^{4-}–P when the consumption of NO_3^-–N is 1 mg. The nitrite could be regarded as the electron acceptor to participate into the activities of denitrifying phosphorus removal. Compared with the nitrate, the phosphorus uptake rate of DPB with nitrite was rather higher at low concentration (NO_2^-–N with the concentration range of 5–20 mg L^{-1}). Furthermore, the rate of anoxic phosphorus uptake increased with decreased concentration of NO_2^-–N. The restraining effects related to anoxic phosphorus uptake of DPB was increased as the increase of nitrite concentration, and DPB was entirely inhibited when the concentration of NO_2^-–N was higher than 35 mg L^{-1}, which was little different to the threshold concentration obtained by Huang et al. (25 mg L^{-1}) [87, 88].

In addition to denitrifying phosphorus removal, simultaneous nitrogen and phosphorus removal under low-DO conditions provide another way for both saving energy and improving effluent quality. Li et al. [41, 42] investigated the influences of addition of propionic acid on two lab-scale SBRs under conditions of anaerobic/low-DO (0.15–0.45 mg L^{-1}). The results showed that the simultaneous nitrification, denitrification and phosphorus removal (SNDPR) occurred in both SBR1 (acetic and propionic acid as mixed carbon source with the carbon molar ratio of 1.5/1) and SBR2 (acetic acid as sole carbon source), and ammonia was completely oxidized during the aerobic period without substantive nitrite accumulation.

3.3.4 New Processes for Biological Nutrient Removal

Based on traditional and new theories for nitrogen and phosphorus removal, many novel processes were developed in the last 10 years, among which the most representative processes include A_2N process, reversed A_2/O process and integrated A_mO_n process.

A_2N Process

After making a thorough study of the mechanism of nitrogen and phosphorus removal, it was found that biological removal of nitrogen and phosphorus is too independent of and overlaps biological processes. The overlap represents that denitrification, phosphorus uptake and nitrogen removal occurs under anoxic condition due to the contribution of PAO. On the basis of this concept, a new two-sludge treatment system [i.e. A_2N process consists of three reactors, an anaerobic (A) reactor, an anoxic (A) reactor and a nitrification; see Fig. 1] was developed for biological denitrification and dephosphoration [89]. It gives a successful solution to the different requirement of

nitrifying bacteria and PAO on sludge age and the contradiction between denitrification and anaerobic phosphorus release from PAO. The process has the advantages of steady operation and good treatment effect, and is particularly suiTable for treatment of wastewater with low COD/TP ratio. The simultaneous presence of carbon and nitrate would be detrimental to phosphorus removal. The two-sludge system was beneficial to improving the system's efficiency and stability (Fig. 5).

Reversed A^2/O Process

The typical layout of a traditional A^2/O process is in an anaerobic/anoxic/oxic mode (Fig. 2). However, this type of disposal is unfavorable to neither denitrification or phosphorus release/uptake. Its phosphorus and nitrogen removal rates are markedly higher than that of conventional A^2/O process, whereas the COD removal rates are about equal. The reversed A^2/O process has been applied widely in China and shown good COD, N and P removal performance (Fig. 6).

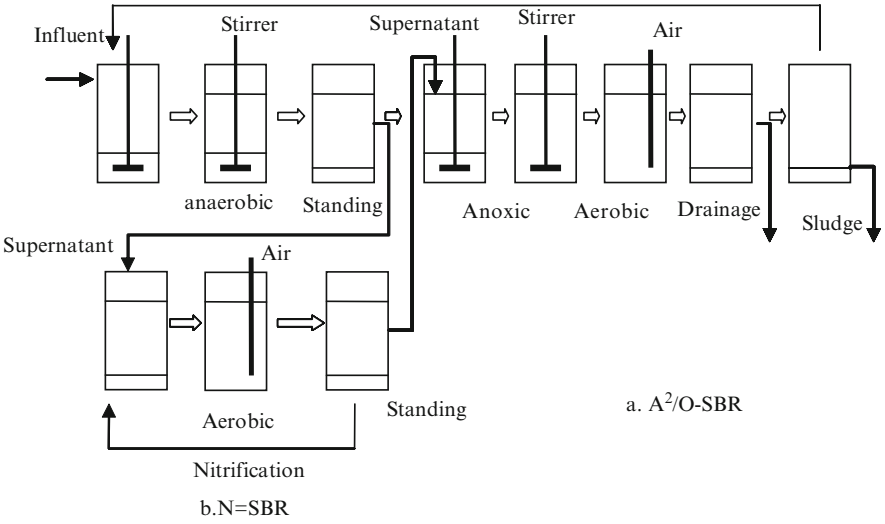

Fig. 5 Flow chart of A_2N process

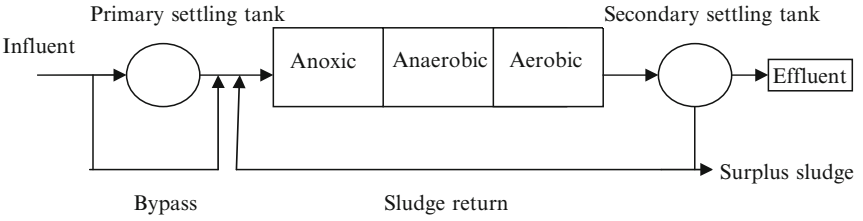

Fig. 6 Flow chart of reversed A2/O process

Integrated A_mO_n Process

With a reduced area of land occupation and simplified process flow, the integrated A_mO_n process breaks through the limitations of the traditional wastewater treatment process both in spatial and temporal arrangement. In the abbreviation A_mO_n, "A" represents anaerobic or anoxic while "O" symbolizes oxic, and "*m*" and "*n*" describes the treatment degree of anaerobic (or anoxic) and oxic, which can be easily controlled by changing the process running mode. It is hard to quantify how much wastewater was treated in the anaerobic/anoxic or in the oxic zone because m and n is a fuzzy concept, but it can be confirmed that the influent passes through several anaerobic-anoxic-oxic circulations. Experiments showed that the suiTable HRT, SRT and DO were 8.5 h, 10 days and 2.5–4.5 mg L^{-1}, respectively, which resulted in the optimum contamination removal efficiency [69, 70].

3.4 Novel Bio/Eco-treatment Process for Wastewater

During the last few decades efficient, low-cost bio-treatment of wastewater has become important, leading to examination of the enhancing biodegradation capability of microorganisms, Novel bioreactors and bio-processes have also been widely studied and applied for treating industrial and municipal wastewater, such as constructed wetland, expended granular sludge bed and membrane biological reactors.

3.4.1 Constructed Wetland Technology for Wastewater Treatment

Constructed wetlands are a promising alternative to techniques of wastewater treatment, especially for developing countries due to the low investment and operation costs [90]. It is composed of one or more treatment cells designed and constructed to provide many types of wastewater treatment at different levels [91]. In recent years, domestic wastewater, agricultural non-point wastewater, mine drainage water and contaminated river water are treated by constructed wetlands in China [92]. Figure 4 shows the constructed wetlands for controlling storm runoff in Dian Lake. Constructed wetlands are ecological systems that combine physical, chemical, and biological processes in an engineered and managed system. There are numerous different technological variants in terms of design, but constructed wetlands mainly comprise two types of systems of free water surface constructed wetlands and subsurface flow constructed wetlands. The removal efficiency of SS, COD, and BOD_5 are generally high; however nutrients removal efficiency is usually variable. In China, excessive nutrients loading from various sources is commonly related to eutrophication of water bodies and, therefore, researches on the removal of nutrients by constructed wetland have gained much attention in decades (Fig. 7).

In the study of agricultural non-point wastewater treatment using surface constructed wetland, Zhang et al. [93, 94] it was found that the amount of TN removed

Fig. 7 Constructed wetland built in Dian Lake to control storm runoff

by harvesting *Zizania caduciflora* and *Phragmitas communis* are about 440 and 700 kg N ha^{-1}·year. The effect of plants on nitrogen and phosphorus removal was further studied in pilot-scale in subsurface constructed wetland [95]. The amount of N and P removed by plant harvesting is about 5% of the total removed nutrients, and the best harvesting periods is 9–10 month every year. Plant could play an important role to maintain the micro-organism around the roots, and plant harvest was observed to affect fluctuation of effluent quality. Additionally, the plant roots were observed to extend hydraulic retention time of the system by decreasing dead area of 5–10%.

Li et al. [54–56] indicated that gravel wetland with soil layer above has the highest phosphorus removal rate of 70% due to the P sorption on the substrate. During recent years, the application of horizontal subsurface-flow constructed wetlands for the treatment of the contaminated rivers has been increasing [96]. The composition of the dissolved organic carbon could undergo a considerable shift in composition, and non-labile aromatic hydrocarbons and alkyl hydrocarbons in the effluent were significant portions compared with labile alcoholic and alkenes in the influent. Moreover, researches on microbial community in complex wetland systems were being conducted in China.

3.4.2 Another Combined Bioprocess for Treating High Concentration Organic Wastewater

EGSB is an advanced form based on UASB. Compared with UASB, the features are its high ratio of height and diameter and its effluent recycling system. Ren [97] has investigated the removal of streptomycin, a kind of antibiotics wastewater by EGSB technology. Figure 8 shows the processing technology. The result of the experiment shows that the combination of EGSB and contact oxidation process technology has an effective removal function for the wastewater. The removal rate

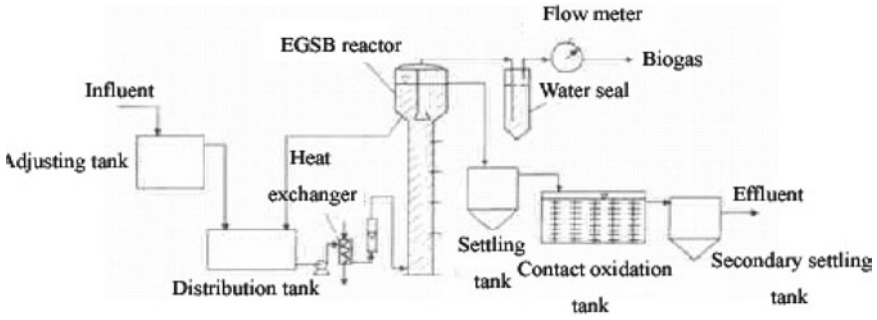

Fig. 8 Processing technology for what? in a streptomycin plant

of COD and SO_4^{2-} was 75 and 60%. Granular sludge was also obtained during the operation. In this facility, the upflow velocity is controlled at 4.3–4.7 m h^{-1} to reach a better removal effect.

Membrane biological reactor (MBR) is an advanced system developed by the combination of membrane separation and biological system. This technology can prevent the microbes from leaking out of the reactor to increase the sludge concentration. In this way, the sludge retention time could be infinite theoretically to raise the removal rate of organic compounds that are difficult to degrade.

4 Bioprocesses for Recycling of Organic Wastes

Organic waste is a large category pollutant in China, and usually includes the following pollutants: municipal solid waste, sewage sludge, manure, agricultural biomass and food waste. According to the relative data, the amount of discharged organic waste reached to 4 billion tons in 2002 and with a growth rate at 8–10% annually. Because of the high content of organic matter in the organic wastes, it is estimated that there is about 1.2 billion tons of crude organic biomass in these organic solid waste. Table 2 lists the current generation amount, features, main treatment or disposal methods and the environmental problems of several types of organic wastes.

Generally, there are many biotechnological methods for the recycling of organic waste. However, the main research fields were focused on the following fields: (1) anaerobic compost or aerobic compost; (2) methane production through anaerobic digestion; (3) hydrogen production by fermentation; (4) microbial fuel cell; and (5) biochemical production.

Overall, all these methods have different advantages and disadvantages. The application of these methods depends on their detailed scale and conditions. Among these techniques, the compost and methane production were mature and developed technologies and have been widely used in waste treatment and disposal. As for the

Table 2 Several types of organic wastes and its treatment methods in China

Types of pollutants	Generation amount (10^9 tons per year)	Content of organic matters	Treatment methods	Environmental problems
Sewage sludge	0.2	Relative high	Landfill, combustion	Land occupation, secondary pollution
Manure	27.0	High	Compost	Water pollution, odor, distribution of pathogenic bacteria
Agriculture waste	13.0	High	Disposal	Low efficient for use
Food wastes	0.035	High	Compost, reuse	Secondary pollution

Table 3 Comparison of the five bioprocessing strategies for waste treatment

Bioprocess strategy	Level of maturity	Separation of products	Culture	Value added
Anaerobic digestion	Mature, operational	Easy, gas	Mixed	Low
Hydrogen Fermentation	Laboratory phase	Easy, gas	Mixed	Low to medium
Microbial fuel cell	Laboratory phase	Easy, electricity	Mixed	Low
Biochemical production	Scale-up phase	Hard, soluble products	Pure or co-culture	Medium to high

hydrogen production and microbial fuel cell from organic waste, they are promising technologies and most of these research activities were at laboratory or pilot scale. Finally, production of biochemicals from organic waste by using fermentation represents a new trend because the produced biochemicals are usually highly valuable products. For example, the acetate, one of the main intermediate during the anaerobic fermentation process, has four times the value of traditional biogas as the source of energy. Table 3 lists the features of the five bioprocessing strategies for waste treatment.

Here we will review the recent developments of the five bioprocessing strategies.

4.1 Methane Production

4.1.1 Progress on the Methanogens Study

Methanogen is an important class of environmental microorganism. It not only produces methane from the biomass during the anaerobic fermentation but also plays a pivotal role in the global carbon cycle. Methanogens are difficult to isolate as some members require long incubation period for growth and some are sometimes difficult to separate from their syntrophic partners. In addition, a large majority

of microbes including methanogens have evaded isolation as they are not amenable to laboratory cultivation due to the limited knowledge of their growth requirements. Up to 2000, only 83 species of methanogens were separated and described [98]. In China, new species has now been isolated. In 2005, two methanogenic strains, $8Ac^T$ and 6Ac, were isolated from an upflow anaerobic sludge blanket reactor treating beer-manufacture wastewater in Beijing, China. The two strains used acetate exclusively for growth and methane production. Based on the phylogenetic and phenotypic analyses, the novel species *Methanosaeta harundinacea* sp. nov. was proposed, with strain $8Ac^T$(=JCM 13211^T=CGMCC 1.5026^T) as the typical strain. In 2006, two strains, $8–2^T$ and 4–1, with rod-shaped (0.4–0.5 × 3–5 mm), non-motile cells, sometimes observed in chains, were also isolated from two anaerobic digesters in Beijing. The two methanogenic strains used H_2/CO_2 and formate for growth and produced methane. Based on the phylogenetic analysis and phenotypic characteristics, the novel species *Methanobacterium beijingense* sp. nov. was also proposed, with the typical strain $8–2^T$ (=DSM 15999^T=CGMCC 1.5011^T) [76, 77].

Psychrophilic methanogen has now attracted more and more research interests. However, only a few species have been isolated. The application of psychrophilic methanogens in anaerobic biotreatment process could essentially break through the bottleneck of anaerobic technology at lower temperature, greatly extending the application fields of anaerobic technology, and reduce the operational cost of wastewater treatment [99, 100]. Therefore, the search for psychrophilic methanogens and the application of them in extreme environments will still be a research hotspot.

4.1.2 Enhancement of Methane Production

Currently, most anaerobic digestion plants run around the world can be divided into single phase, two-phase, and batch style plants. Batch reactors have economic advantages in developing countries, but their organic load rate is much lower than continuous feed systems and the reactors take up a larger area. Two-phase anaerobic digestion reactors have good shock load tolerance for the separation of acidification and methanogenesis processes, but the technique is complex and relatively expensive. Single-phase reactors provide an accepTable result at less cost. How to improve methane production in single phase reactor has attracted more attention.

Addition of Trace Metals

The effect of the addition of trace metals on the performance of bioreactors was an important study field in anaerobic processes, as metals are involved in the enzymatic activities of acidogenesis and methanogenesis. Xu et al. [101] investigated the effects of application of zero valence Fe on the anaerobic digestion of sewage. The addition of Fe(0) significantly increased the CH_4 yield by 8.7% and decreased the effluent COD concentration by 21.0% compared with the control reactor. Li and

Yang [102] also found that a supplement of trace metals to the anaerobic system could obviously shorten the digestion period. At the same time, the substrate degradation rate and the gas production rate could be improved. The addition of a blended promoter comprised of five metal ions of K^{+}, Mg^{2+}, Fe^{2+}, Co^{2+}, Ni^{2+} was also found to stimulate anaerobic digestion in a two-phase anaerobic system [103]. Xia et al. [104] investigated the effects of the rare earth ions La^{3+} and Ce^{3+} on the activity of anaerobic granular sludge and the kinetics of anaerobic digestion. The results showed that both of the ions can promote the specific methanogenic activity (SMA) at concentrations from 0.01 to 0.1 mg L^{-1} with a maximum promotion of 10.35% for La^{3+} and 20.79% for Ce^{3+}.

Addition of Enzymes

In order to improve the conversion of organic matters, the organic waste sometimes required to be pretreated to increase the methane yield during the anaerobic digestion process. Enzyme pretreatment breaks down the complex organic structure into simpler molecules which are then more susceptible to microbial degradation. For example, cellulose can improve the methane yield and the degradation rate of cellulose in anaerobic digestion of distillery wastewater. Zhang et al. [93, 94] also found that biogas production from anaerobic digestion of pig manure was enhanced by 27% after addition of hydrolases The total solid (TS) degradation rate can be increased by 8.69–15.53% and that of VS by 22.23–47.05%. Dr He's research group investigated the how these important factors influencing the performances and its mechanisms from the conversion of municipal solid wastes [17–21].

Innovations of Digester Designs

The bioreactor design significantly influences the treatment efficiency. For example, the upflow anaerobic filter (AF) process has been widely used for the treatment of a variety of types and strengths of organic wastewaters. However, the anaerobic microorganisms are not evenly distributed along the height of the filter. This results in the low COD removal efficiency of its upper part. Yu et al. [37–39] examined the effectiveness of a multi-fed upflow anaerobic filter process for the methane production from a rice winery effluent at ambient temperatures. Compared with the single-fed AF, the multi-fed upflow anaerobic filter was proved to be more efficient than the single-fed reactor in terms of COD removal efficiency and stability against hydraulic loading shocks. Guan and Zheng [105] modified the traditional UASB reactor by adding a recycling water pipe under the three-phase-separation system. The modified UASB reactor is beneficial to raising the COD loading and developing appliances and has been applied to treat calcium alginate wastewater under normal temperature [106]. The first, second and third generation anaerobic reactors had been developed and the new innovations may still be an attractive research field.

4.1.3 New Processes for Methane Fermentation

Simultaneous Denitrification/Methanogenesis

A new process which was called anaerobic simultaneous denitrification/methanogenesis has now been investigated in China. The denitrification and methanogenesis can be accomplished in a single reactor. According to the reality of Chinese situation, the anaerobic simultaneous denitrification/methanogenesis process is the developing direction in the future for high strength wastewater treatment containing organic nitrogen. Denitrification and methanogenesis of a synthetic wastewater were obtained in a single-stage process using anaerobic suspended granular sludge reactor. During a steady stage at a loading of 0.75 kg NO_3–N·m^3 day^{-1} and 14.1 kg CODm^{-3} day^{-1}, nitrate removal of over 99.5% and carbon removal of 90.1% were achieved [107].

In recent years, a new direction to integrate methanogenesis with simultaneous anaerobic ammonium oxidation and denitrification in their preferred micro-ecological symbiosis environment by using special reactors has also been put forward [63, 64, 108]. It could change the organic compounds into a clean energy source, and at the same time remove nitrogen in wastewater treatment.

Methanogenesis in Microaerobic Conditions

It is also reckoned that oxygen is detrimental to methanogens. However, some research also indicated that methanogens can survive in the microaerobic conditions, even showing higher methanogenic activity. The amphimicrobe coexisted with methanogens can maintain adequate low ORP for MPB. In the microaerobic conditions, the intermediate produced in anaerobic metabolism will be degraded instantly by aerobic microbes, thus decreasing the accumulation of toxic intermediates and the anaerobic reactors should run more stably. Dong and Lu [109] operated an EGSB rector under microaerobic conditions and the results showed that supplement of limited oxygen could increase the COD removal efficiency and decrease the effluent VFA concentration. Supplement of low level oxygen was not harmful to the methanogens. The microaerobic EGSB reactor had very strong ability of resisting pH, temperature and loads shock.

4.2 Hydrogen Production

4.2.1 Mechanisms of Microbial Hydrogen Production

Hydrogen is a clean, environmentally “friendly” fuel that produces water instead of greenhouse gases when combusted. Furthermore, hydrogen has a high-energy yield (142.35 kJ g^{-1}) that is about 2.75 times that of hydrocarbon fuels. Hydrogen produced directly from organic materials and water by bacteria has considerable potential in defining hydrogen’s future use. Microbial hydrogen production from organic

wastewater or sewage sludge has been one of the most important research fields in China. Many researchers focused on the mechanisms of microbial hydrogen production. Li et al. reported there are three pathways for hydrogen production: butyric dominated fermentation, ethanol dominated fermentation and fission of formate by the mixed microbial community [54–56]. In addition, Ren et al. [110] proposed that four mechanisms existed for hydrogen production in anaerobic bioreactors. They are pathway of decarboxyl from pyruvate, balance adjustment of reduce and oxygen of NADH, hydrogen generation by syntrophic bacteria and adjustment of NADH and NADPH. Some other researchers believed that mixed acids fermentation, butyric acid type fermentation and NADH conversion are the three basic pathways for hydrogen generation. It is reported that ethanol type fermentation in the bioreactor will be beneficial for the high hydrogen production. On the other hand, the reduced NADH I (NADH + H^+) from carbohydrate by EMP pathway can be coupled with proper proportion of propionate, butyric acid, ethanol or lactic fermentation to ensure the equivalent of NADH + H^+/NAD^+ (Fig. 9).

4.2.2 Microorganisms for Hydrogen Production

Many microbial strains have been isolated and investigated for hydrogen production. As for bacterial strains, there are more than 20 genera which have been found to having the ability of hydrogen production. Cai and Liu [111] divided the hydrogen microorganisms into four divisions. (1) Anaerobic heterotrophic microbes

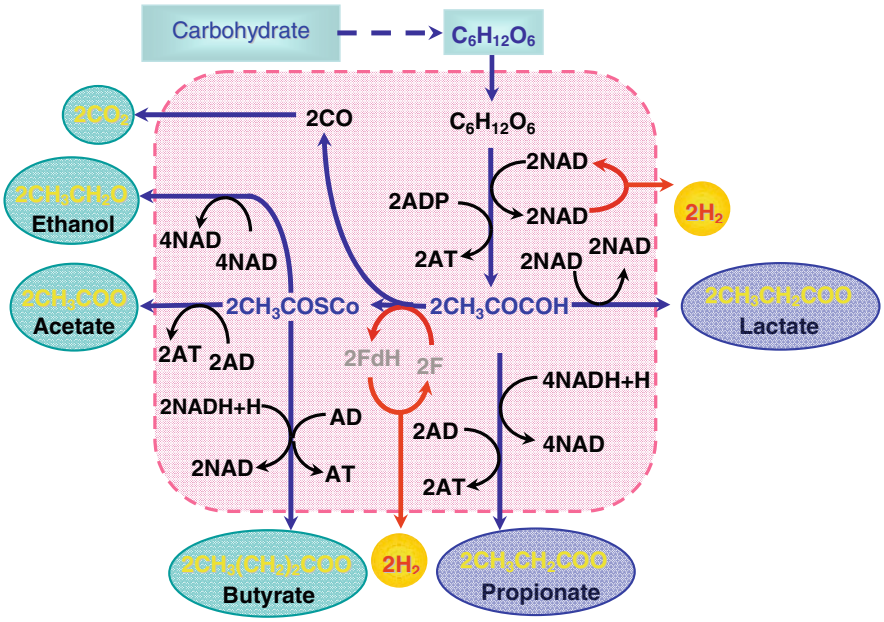

Fig. 9 Metabolic pathway of carbohydrate in hydrogen producing bacteria

– these microbes, which generate hydrogen through pyruvate pathway and they don't have cytochrome system, include Clostridium, Methylotrophs, Methanogenic bacteria, Rumen bacteria and Archaea. *Desulfovibrio desulfuricans* is the only one kind of anaerobic bacteria strain with cytochrome system. (2) Facultative anaerobic – these bacterial strains contain cytochrome system , and can degrade formate to produce hydrogen. *Escherichia coli* and *Enterobacter* are classified in this division. (3) Aerobes – this division includes *Alcaligenes* and Bacillus. (4) Photosynthetic bacteria. Among the above microbes, the anaerobic bacteria and facultative anaerobic are the two main hydrogen bacteria strains. Till now, the Clostridium genus, such as *Clostridium butyricum* and *Clostridium pasteuria-hum*, and the *Enterobacter*, such as *Enterobacter aerogenes* and *Enterobacter cloacae*, are the representative species which are widely studied by Chinese scholars. The researchers in Harbin Institute of Technology developed the suiTable culture media and condition for the isolation of hydrogen producing bacteria. Using these techniques, they isolated and investigated more than 550 anaerobic bacterial strains.

4.2.3 Process Development

Pretreatment of Raw Material

When the fundamental fields were explored widely, the aspects of process development also attracted much more attention. The raw materials usually used for hydrogen production were straw, municipal sewage sludge or high strength wastewater, e.g. molasses wastewater [112]. In order to improve the conversion efficiency of the organic matters, many researchers are trying to use various methods to pre-treat the raw waste. Various methods for sludge pretreatment have been reported, such as mechanical treatment, chemical treatment, thermo-alkaline treatment, oxidative treatment and radiation treatment. Other methods, such as thermo-acid and ultrasonic-alkaline were also evaluated. The effects of these pretreatment methods on the solubilization of sludge and further methane production have been investigated. Among all of these methods, thermo-alkaline, ultrasonic-alkaline and thermo-acid pretreatments were reported as relatively effective. More than 85.4% COD of the sludge was solubilized after thermo-alkaline pretreatment, 50% of VS was solubilized after thermo-acid pretreatment, and 89.3% COD of the sludge became soluble after ultrasonic-alkaline pretreatment. Cai et al. [113] reported that alkaline pretreatment can improve the production of hydrogen from sludge without any sludge inoculums. When the pH was 11.0, the hydrogen production rate can reach the maximum at 14.4 mg g^{-1} VS.

Process Parameters

Some bioreactors such as fluid bed, expanded bed, immobilized bed and stirred reactors have been reported for hydrogen production. Ren et al. developed a kind of CSTR reactor to realize the continuous hydrogen production from molasses wastewater (Fig. 10). Some of the most important parameters influencing hydrogen

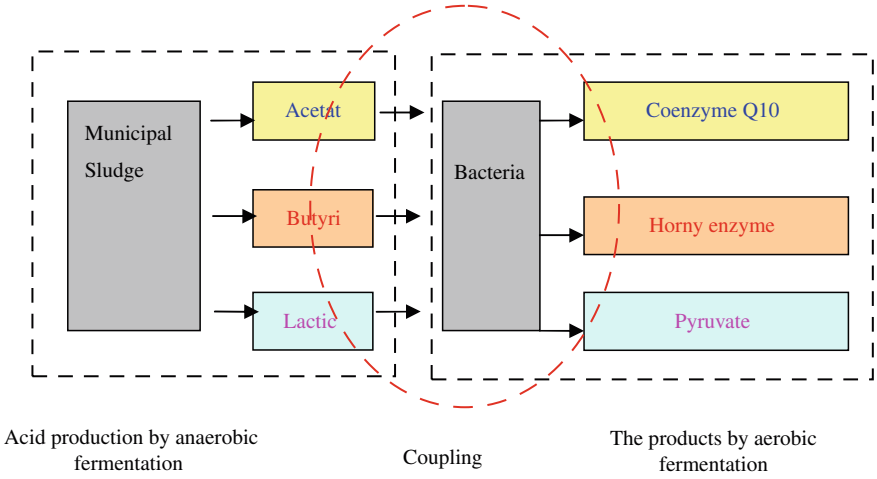

Fig. 10 Scheme diagram of CSTR reactor for continuous production of hydrogen from wastewater

production have been investigated by many Chinese researchers [114]. For example, because of the pH value in the bioreactor, which can influence the balance of the NADH/NAD, the group led by Dr. Ren (Sun et al. 2005) studied the relationship between the pH and the fermentation type in detailed and found a new kind of fermentation type–ethanol type fermentation. Beside the pH, other parameters, such as temperature, ORP and C/N were also studied by many researchers. Some researchers found that the addition of trace metal can improve the hydrogen production. For instance, the Fe, Ni and Mg can improve the average hydrogen production rate of bacterial strain B49 ranged from 2.24 times to 4.42 times.

4.3 Biochemicals Production

4.3.1 Process of Two-Stage Fermentation for Utilization of Sewage Sludge

Many volatile fatty acids (VFA) produced during anaerobic fermentation, e.g. acetic acid, butyric acid, lactic acid, etc. can not only be converted to methane by methanogens, but can also be used as raw materials to produce higher value-added products by fermentation industries. For example, acetate and butyric acid can be used as substrates to produce enzyme by specific microorganisms. Moreover, acetate is the desired substrate for the microbial fuel cell. Because of the higher value than methane production from the conversion of VFA, the production of valuable biochemicals from organic wastes represents a new strategy for the reuse of organic wastes.

Nie et al. [115] have proposed that municipal sludge could be converted to high value products by a new process called two-stage fermentation strategy. At the first step of anaerobic fermentation, sludge can be converted to a few kinds of volatile fatty acids (VFA) products by mixed anaerobic microbial flora. At the second stage,

using organic acids which were produced in the former step as carbon sources, high value biochemical products can be produced by specific and pure bacterial strains through aerobic fermentation. Between the two steps, the acids were separated by appropriate methods or by using coupling techniques to combine the two units. Thus, the municipal sludge can be reused to generate crude products in the former step (organic acids) and then produces fine biochemicals (enzyme or other biochemicals) in the later step (Fig. 11).

To realize the two-stage strategy, the technical problems involved in the three parts should be tackled: (1) the controlled and high efficiency of anaerobic acidification from municipal sludge; (2) the fermentation technology of production high value-added products using organic acids as substrate; (3) the coupling technology of sludge anaerobic acidification and target products fermentation.

4.3.2 Factors Influencing the Acidification

For the sludge acidification, many studies focused on the impact of process parameters on the acids production. These process parameters include sludge retention time (SRT), volatile organic load (VOL), and microbial growth conditions, such as temperature, pH, oxidation–reduction potential (ORP), microbial nutrients composition ratio, and so on. The pH value is one of the most important factors controlling the anaerobic fermentation. For example, it can influence the composition of anaerobic bacterial community and the proportion of VFAs [116]. Another important factor is the structure of the anaerobic microbial community. Because the acid production from organic compounds is dependent on the anaerobic bacterial community, it is very necessary to understand the population composition and shift to illustrate the mechanism of acid production during the anaerobic fermentation acids accumulation. Since the late 1990s, microbial molecular methods have been used to identify the diversity and dynamic change inside

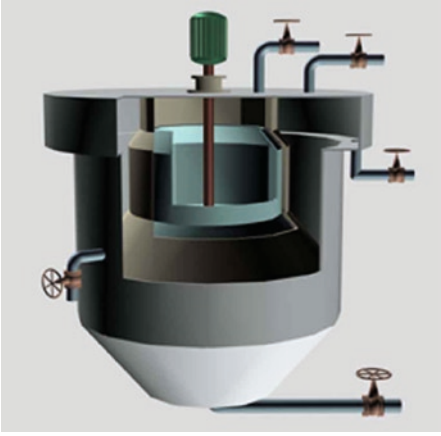

Fig. 11 Two-stage fermentation strategy to produce biological activity products from sludge

the reactor and to investigate the relationship between the change and the performance of methane and acid production. Nie et al. [115] reported a novel process to realize highly efficient acetate production by a syntrophic acidogenesis/acetogenesis process based on the relationship of hydrogen transfer and conversion of H_2/CO_2 by acetogens in the anaerobic bioreactor. The mechanisms involved in the improvement of acetate production include the following: (1) the removal of inhibition of syntrophic acidogenesis by hydrogen; (2) the realization of homoacetogenic acid production; (3) other products being transformed into acetic acid.

As for the coupling technology, Du and Yu [117] reported a coupling system to realize lactic acid production from food waste and PHA production from the lactic acid produced. The content of PHA could reach 72.6 wt%, which is the highest value ever reported, and could be equivalent to the use of glucose as raw material. This study confirmed the practical feasibility of the strategy of "anaerobic acidification, coupled fermentation to produce high-valuable products". Currently, some techniques such as membrane separation, two-phase-system technology and electrodialysis, etc. are promising technologies to couple the acid production and high value products generation.

One of the most important steps of biochemical production is fermentation by pure microbial strains. This field is involved in the screening of specific microorganisms which can use organic acids to produce useful products, the optimization of the fermentation process and the separation of the products, etc. The researchers at Jiangnan University have done much work in the field of production of polyhydroxyalkanoates by *Ralstonia eutropha* from food waste or high strength wastewater [118–122]. Yan et al. [123–125] optimized the fermentation conditions of polyhydroxyalkanoates production by *Ralstonia eutropha.* Apart from the PHAs, another useful enzyme – cutinase – was produced by *Thermobifida fusca* by using VFA as material. Table 4 summarizes the study of microorganisms that use VFA as

Table 4 Biochemistry commodities produced by sludge acidification

	Microorganism	Biochemical products	Product types
VFA	*Alcaligenes eutrophus*	PHA	Biodegradable plastics
Acetate	*Pseudomonas putida BH*	Biosurfactant	Biosurfactant
Acetate	*Corynebacterium glutamicum*	Microbial flocculant	Microbial
Acetate, glucose	*White-rot fungi*	Mn-peroxidases	Enzyme preparation
Acetate	*Rhodopseudomonas capsulata*	Isocitrate lyase	Enzyme preparation
Acetate	*Candida tropicalis*	Citric acid	Organic acid
Acetate	*Phycomyces blakesleeanus*	Coenzyme Q10	Medicine and health protection
VFA glucose	*Mucor circinelloides CBS*	γ -Linolenic acid	Medicine and health protection
Lactic acid	*Hansenula polymorpha*	Pyruvate	Organic acid
Lactic acid	*Pichia pastoris*	Pyruvate	Organic acid
Butyric acid	*Thermobifida fusca*	Cutinase	Textile enzyme preparation

carbon and energy sources to synthesize or convert into target metabolites (bio-plastics, bio-surfactant, enzyme preparations, organic acid, amino acid).

4.4 Microbial Fuel Cell

The microbial fuel cell (MFC) provides a waste resource recovery technology which is becoming very important and has become a very attractive research field for the environmental microbiologist. The principle of power generation by MFC does not involve combustion of fuel (such as carbohydrate in wastewater, etc.), but the production of elec trons (produced from microorganism respiration) from the fuel molecule and then transferring the electrons to oxygen by a predetermined approach (electrodes). The energy originally used for oxidative phosphorylation will be transformed into electricity. MFC has several advantages such as (1) wide raw material sources; (2) environmental protection process; (3) mild reaction conditions; (4) low operation costs, etc. At present, the medium of MFC was mainly organic waste water and the research is still at the initial stage.

Recently, a microbial fuel cell has been constructed by using new methylene blue as the electron mediator and *E. coli* as the biocatalyst to explore the performance of the new mediator. The results show that the MFC which uses new methylene blue as the electron mediator has a lower open circuit voltage and higher steady short circuit current than those MFC which use neutral red as electron mediator. When the discharge current density is larger than 114 mA/cm^2, the former has a higher power density and better stability than the latter [126]. On the other hand, Huang et al. [87, 88] believed that mediator-less MFCs may be a very promising trend. In that critical review, aspects including electricigens and the structure of MFCs were discussed. The electricigens, including the main species, parameters affecting the electricity production, and electron transfer mechanisms, were described. The influences of the anode and anode chamber, the cathode and cathode chamber, the spreader, configuration and operation modes on MFC's electricity production capacity were discussed.

5 Conclusion and Perspectives

Environmental protection will be a major challenge for Chinese economy development in the following years. Obviously, environmental biotechnology would be one of the most important technologies for the pollutants biodegradation, wastewater treatment and waste reuse and resource. However, although many biotechnologies have been developed in China for accumulation of more microbial resources and for understanding the microbial degradation of toxic and organic compounds and wastewater treatment during recent years, most of these are only used on laboratory and pilot scales. Future work should be focused on field studies and application of

the obtained bacterial strains and knowledge of biodegradation to develop practical processes to clean up toxic and organic pollutants from various environments. It becomes evident that the combined effort of microbiologists, chemists and engineers is necessary to improve the development of modern biotechnology such as design, operation and control of bioreactors and wastewater treatment systems. Fortunately, more and more microbiologists, chemists and engineers are showing interest in applying these new biotechnologies to practical operation combined with other technologies, such as physical and chemical processes in China. In addition, the original technical innovation has recently been emphasized. This would be helpful to the rapid development and application of biotechnology in pollution control and waste reuse in the near future. Certainly, biotechnology will provide some major contributions to wastewater treatment in the years to come in China.

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Traditional Chinese Biotechnology

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Abstract The earliest industrial biotechnology originated in ancient China and developed into a vibrant industry in traditional Chinese liquor, rice wine, soy sauce, and vinegar. It is now a significant component of the Chinese economy valued annually at about 150 billion RMB. Although the production methods had existed and remained basically unchanged for centuries, modern developments in biotechnology and related fields in the last decades have greatly impacted on these industries and led to numerous technological innovations. In this chapter, the main biochemical processes and related technological innovations in traditional Chinese biotechnology are illustrated with recent advances in functional microbiology, microbial ecology, solid-state fermentation, enzymology, chemistry of impact flavor compounds, and improvements made to relevant traditional industrial facilities. Recent biotechnological advances in making Chinese liquor, rice wine, soy sauce, and vinegar are reviewed.

Keywords Enzyme, Functional microorganism, Liquor, Qu (koji), Rice wine, Solid-state fermentation, Soy sauce, Vinegar

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1 Chinese Liquor

1.1 Introduction

1.1.1 Classification

Chinese liquor is one of the oldest distillates in the world. Compared with other spirits such as vodka, whiskey, and brandy, Chinese liquor has a higher ethanol content (normally 40–55 vol.%). Although the trend today is toward a lower alcoholic strength, most are in the 46–52% range, with some as high as 60%. This strong alcoholic beverage is normally consumed straight up or "neat," not mixed with water, soda, fruit juice, or other liquid. The Chinese presently consume approximately 4 billion liters annually.

Sichuan, Guizhou, Shandong, Jiangsu, Anhui, and Henan are the main producing regions, commanding more than 90% of the total Chinese liquor output. The famous brandy Chinese liquors, Moutai, Wuliangye, Jiannanchun, Fenjiu, and Yanghe daqu, are distilled and bottled in these regions.

Because of differences in manufacturing practices, the aroma profiles of various Chinese liquors are quite different. On the basis of aroma characteristics, Chinese liquor can be classified into five categories: strong aroma type, light aroma type, soy sauce aroma type, sweet honey type, and miscellaneous type. Of these, the strong aroma type accounts for about 70% of the total liquor production. Strong aroma type liquors typically have strong pineapple- and banana-like fruity aromas [1]. Within this category, Wuliangye and Jiannanchun are two of the most famous brands, followed by Yanghe daqu and a few others. Chinese Moutai, a most famous spirit with the distinction of "the national liquor," belongs to the family of soy sauce aroma type liquors.

According to alcoholic content, Chinese liquors can be classified into three types: high alcoholic level (50–60%), middle alcoholic level (40–50%), and low alcoholic level (18–40%). Twenty years ago, most Chinese liquors had a high alcoholic content. Since the 1980s, Chinese liquors of low alcoholic content have been studied, because of changes in the sight, smell, taste, and the tactile or sense of touch quality when adding water to high alcoholic liquors. Presently, about 40% of the Chinese liquors produced belong to the low alcoholic category.

Chinese liquors are typically fermented and distilled in solid-state, and most of the premium Chinese liquors such as Moutai, Wuliangye, and Jiannanchun must be manufactured in solid-state. The major steps in Chinese liquors production are solid-state saccharification, fermentation, and distillation in the presence of fermenting grains (mash) with a 60% moisture content.

1.1.2 Production Processes

Most Chinese liquors are a traditional distillate fermented from grains. After fermentation, the fresh spirit is distilled out and subsequently aged under controlled conditions. The aged distillate is adjusted to a target ethanol concentration and blended to ensure the quality of the finished product and to maintain brand consistency [2]. The raw materials for making Chinese liquors vary depending upon availability and cost. In general, Chinese liquors are made from sorghum or a mixture of sorghum, wheat, corn, rice, and sticky rice. Rice hull is typically used as a fermentation aid [2, 3].

The saccharifying and fermentation starter cultures for making Chinese liquors are daqu, xiaoqu, or other enzyme preparations [4]. Daqu, which is basically moldy grains, is the most widely used starter culture. It is made from wheat or a mixture of wheat, barley, and pea. The raw materials of daqu are typically milled and pressed in a mold of different sizes depending on the manufacturer. The daqu starter bricks are placed in a room for fermentation under controlled conditions. On the basis of the maximum temperature at which daqu is incubated, it can be classified into low-temperature (<45 °C), moderate-temperature (45–60 °C), and high-temperature (<60 °C). Daqu is rich in an assortment of microorganisms including bacteria, yeast, and fungi [2]. In addition, a complex enzyme system forms in the finished daqu [5].

The grains for liquor fermentation are first milled, cooked, and subsequently mixed with the husk. Hot water (about 95 °C) is added to the cooked grains to adjust the moisture content to 55 wt%. The cooked grains are cooled to 13–16 °C and mixed with the daqu powder. The mixture is fermented in a special fermentor (3.4 m length, 1.8 m width, and 2.0 m height), which is coated inside with a layer of fermentation mud made of clay, spent grain, bean cake powder, and fermentation bacteria (*Clostridium* sp.). Fermentation is typically carried out at 28–32 °C for 60 days under anaerobic conditions in solid-state. After fermentation, the liquor is distilled out with steam and aged in sealed pottery jars to develop a balanced aroma. While most of the liquors are aged for about 1 year, some of them are aged for more than 3 years. The aged liquor is diluted with water and blended to yield an ethanol content of 40–55 vol.% for consistent quality in the finished product.

1.2 Functional Microorganisms and E nzymes

1.2.1 Microorganisms and Enzymes of Daqu

During Chinese daqu fermentation, an assortment of enzymes are established, including liquefying amylase, saccharifying amylase, acid protease, cellulase, lipase, and esterase. Fan and coworkers [6–8] researched these enzymes in Chinese daqu. Table 1 lists their findings. Liquefying amylases in the daqu are mainly produced by *Aspergillus*, *Rhizopus*, and *Endomycopsis*, whereas, saccharifying amylases are yielded by *Rhizopus*, *Aspergillus*, *Monascus,* and others and acidic proteases are made by *Aspergillus*, *Rhizopus*, *Rhizomucor*, and other molds. The cellulase producing microorganisms include *Trichoderma (T.)*, *Aspergillus*, and *Mucor.* Microorganisms such as *T. reesei*, *Furium oxysporum*, *T. viride*, and *T. koningii* are all well known prodigious producers of cellulases. It is reported that some bacteria can also produce cellulase [9]. Lipases naturally present in the daqu are produced by bacteria and epiphytes. Esterases naturally present in the daqu are produced by *Rhizopus*, *Saccharomyces*, and *Monascus*. In general, molds are the major microorganisms that yield liquefying amylases, saccharifying amylases, acid proteases, cellulases, lipases, and esterases.

Molds, being aerobic microorganisms, are influenced greatly by the oxygen environment. Under aerobic conditions, molds proliferate and produce large amounts of enzymes. During fermentation of the daqu, more oxygen is dissolved near the surface of the daqu than in the interior. The number of microorganisms that exist on the suface of the daqu is higher than that in the center by almost a magnitude. As can be seen in Table 1, different parts of the daqu show quite different activities of the hydrolyzing enzymes, including liquefying enzymes, saccharifying amylases, acidic proteases, cellulases, and lipases. Hydrolyzing enzymes produced near the surface of the daqu possess the highest activities, followed next by those from the corners, and followed lastly by those from the center of the daqu. The activities of these enzymes closely correlate with the number of microorganisms. Furthermore, at the same part of the daqu, activities of different enzymes differ widely. The activity of liquefying amylases near the surface of the daqu is three times of that from the center, and saccharifying amylases exhibit a similar pattern; whereas, the activities of acidic proteases and cellulases are only twice as high. On the other hand, the activities of lipases from the surface and from the center of the daqu are not as different as other hydrolyzing enzymes.

Due to diverse environmental conditions, such as humidity, temperature, and air composition, molds proliferate differently in different parts of the daqu. The high enzyme activity on the daqu surface results from the high density of mold proliferating at that part of the daqu. In this case, the quantities of liquefying amylases, saccharifying amylases, acid proteases, cellulases, lipases, and esterases produced by mold correlate closely with mold growth.

Through a combined strategy to screen for lipolysis and esterification abilities, a fungal strain capable of synthesizing ethyl esters of short-chain fatty acids has

Table 1 Activities of enzymes and microorganisms in Chinese daqu

	Surface	Center	Corner	Whole
Activities of enzymes				
Liquefying amylase (g soluble starch/g dry daqu·h)	3.949	0.905	2.056	1.342
Saccharifying amylase (mg glucose/g dry daqu·h)	201.54	66.51	179.82	109.89
Acid protease (μg tyrosine/g dry daqu·min)	100.21	50.33	80.56	61.06
Cellulase (mg glucose/g dry daqu·h)	68.25	35.67	45.48	39.90
Lipase (mg xylose/g dry daqu·h)	180.24	120.55	145.51	128.43
Esterase (decrease hexanoic acid, %)	66.64	42.39	–	–
Microorganisms				
Bacteria (cfu/g dry daqu)	6.25×10^5	1.12×10^5	–	–
Yeast (cfu/g dry daqu)	2.88×10^5	1.35×10^4		
Mold (cfu/g dry daqu)	3.21×10^6	2.55×10^5	–	–

Table 2 Effect of lipase sources on the synthesis of ethyl hexanoate in heptane

Lipase source[a]	Molar conversion (%)	Relative activity (%)
RCL (whole-cell)	96.5	100.0
RCL (cell-free)	84.9	88.0
RML	91.5	94.8
CRL	75.4	78.1
PPL	87.7	90.9
CLL	32.6	33.8
CAL	61.2	63.4
PNL	18.2	18.9
MJL	16.0	16.6
RAL	80.0	82.9
ANL	18.5	19.2
PSL	87.1	90.3

[a]Reaction condition: 0.5 m ethanol, 0.5 m hexanoic acid, 6 g L^{-1} enzyme, 150 rpm and 30 °C for 72 h

been isolated from daqu samples and was identified to be *Rhizopus chinesis* CCTCCM201021 [10, 11]. Compared with the other ten commercial lipases isolated in the screening process, the whole-cell lipase of *R. chinesis* CCTCCM201021 (RC lipase) shows the highest ability to synthesize ethyl hexanoate with a maximum yield of 96.5% after 72 h conversion starting with 0.5 M equal molar substrate concentration (Table 2). Thereafter, the effect of important reaction parameters on enhancing ester formation by whole-cell RC lipase was investigated. The whole-cell RC lipase achieved high esterification (>90%) of carboxylic acids with chain length C2–C8. RC Lipase in solvents with high partition coefficient (>100) gave high conversion

(>88.8%). Better esterification was achieved at 30–40 °C. Increasing substrate concentration (ethanol and acid) from 0.2 to 1.4 M suppressed molar conversion of ethyl hexanoate from 96 to 85%. At 0.6 M acid, changing the molar ratio of acid/ethanol from 1:1 to 1:3 maximized conversion at 98.5%. Better esterification was achieved with initial water activity ranging from 0.66 to 0.97. Based on its effect on esterification reaction, an enzyme concentration of 6 g L^{-1} was chosen for further studies. In batch esterification reaction of different flavor esters, the whole-cell RC lipase was stable, with a half-life of 840–975 h for ethyl flavor esters.

1.2.2 Bacteria for Producing Hexanoic Acid

Hexanoic acid and ethyl hexanoate play a very important role in the aroma and flavor of Chinese liquors. Ethyl hexanoate is the key aroma in Chinese strong aroma type liquors [12]. Improving the hexanoic acid and ethyl hexanoate contents can enhance the quality of Chinese strong aroma type liquors.

Since the 1960s, many researchers have studied the relationship between Chinese strong aroma type liquors and the microorganisms that reside in the clay that lines the inside of the fermentor wall. They find that the clay is full of many types of anaerobic microorganisms that play an important role in producing hexanoic acid. Based on research conducted by Moutai distillery, hexanoic acid and ethyl hexanoate are the primary active flavor/aroma components in Chinese strong aroma type liquors. There is no doubt that the microorganism producing hexanoic acid, *Clostridium* sp., features significantly in developing aroma. Early studies in the 1970s reported on the isolation, cultivation, and application of *Clostridium* sp. in developing strong aroma type liquors. Liang and coworkers isolated three strains from different origins [13] (Table 3). Table 4 reveals that adding ethanol and sodium acetate affect the concentration of hexanoic acid [14]. Until now, naturally occurring *Clostridium* sp. has been used in the production of strong aroma type liquors.

Clostridium sp., a facultative anaerobe, can convert ethanol to hexanoic acid in the metabolic process. The starting substrates ethanol and CO_2 first yield methane and acetic acid as products [2, 15]:

$$2CH_3CH_2OH + CO_2 \rightarrow 2CH_3COOH + CH_4$$

From the reaction between ethanol and acetic acid, butanoic acid can be obtained. Finally, from the reaction between butanoic acid and ethanol, the product of the process, hexanoic acid, is produced:

$$CH_3CH_2OH + CH_3COOH \rightarrow CH_3CH_2CH_2COOH + H_2O$$

$$CH_3CH_2CH_2COOH + CH_3CH_2OH \rightarrow CH_3(CH_2)_4COOH + H_2O$$

The quantity of hexanoic acid is greatly increased when 0.3% acetic acid and butanoic acid are added. The fermentation condition must be carefully controlled because the pH for hexanoic acid formation must be above 4.5 in an industrial production process.

Table 3 Comparison of some strains that produces hexanoic acid

		Strain K_{21}	Strain W_1	Strain M_2
Origin		Freshwater and sludge	Clay inside of fermentor	Sludge in anaerobic reactor
Colony morphology		Circular, edge neat or villous, gray, smooth, pico protrude, diameter: 1–3 mm	Circular, edge neat or villous, cream color, opaque, diameter: 1.5–3 mm	Circular, edge neat, gray, smooth, pico protrude, diameter: 1–3 mm
Bacteria form		Rod, (0.9–1.1 mm) × (3–11) mm	Rod, (0.6–0.7 mm) × (3.5–4.6) mm	Rod, (0.9–1.0 μm) × (4–9) μm
Gram stain		Positive	Negative	Positive, indeterminate
Substrate utilization	Ethanol + acetic acid	Yes	Yes	Yes
	Glucose	No	Yes	Yes
Growth conditions	pH	6.0–7.5	–	5.4–7.9
	Optimum pH	6.8	6.5–7.5	6.5–7.9
	Temperature (°C)	19–37	20–45	20–46
	Optimum temperature (°C)	34	34	35–36
Anaerobic conditions		Strictly anaerobic	Oxygen-resistant	Strictly anaerobic
CO_2 requirement		Yes	No	Yes

Table 4 Effect ethanol and sodium acetate levels on hexanoic acid formation

Ethanol concentration (%)	Sodium acetate concentration (%)	hexanoic acid concentration (mg/L)
1	1	652
2	0.5	512
2	1	799
2	2	1,010
2	3	987
3	1	787
4	1	573
5	1	456
6	1	215

1.2.3 Microbial Molecular Ecology

In making Chinese liquors, mixed microbial fermentation in solid-state is carried out both in the daqu and in fermented grains. The complex microbial community in them is affected by many factors, such as production techniques, raw materials, and the

environment. In the 1960s, Chinese microbiologists, through traditional culture methods, examined the microorganisms in the daqu, the clay coated inside the fermentor, and fermented grains. It is believed that only 0.1–10% of the total microorganisms can be cultured based on present knowledge. Furthermore, the primordial microbial community is altered during cultivation, enrichment, and isolation; thus, the true mutual relationship between the microorganisms and the environment cannot be analyzed [16–18]. In recent years, molecular ecological methods have been developed and applied to analyzing the microbial community in the daqu and fermented grains.

Garland and Mills [19] first reported Biolog microplates (Biolog, USA) for classifying and characterizing microbial community on the basis of sole-carbon-source utilization. Xu and coworkers [20], using ECO and GN microplates, analyzed several types of daqu produced in different liquor factories. They found that the microbial community in the daqu was closely related to the types of liquor. Based on microbial community, the daqu for making strong aroma type liquors was classified into one group; whereas, that for making soy sauce aroma type liquors was classified into another group. Moreover, the microbial community in the daqu was affected by the production environment, techniques, and storage time. Hu and coworkers [21] extracted total DNA directly from the daqu of strong aroma type liquor and amplified and sequenced the 16S rRNA genes. The bacterial composition in the daqu was identified through Genbank BLAST, and the phylogenetic tree was further established. The results revealed that the main bacteria of the daqu belonged to *Delftia*, *Dysgonomonas*, *Nocardiopsis*, *Pseudomona*, and *Arthrobacter*, and indicated that the daqu contained high bacterial diversity.

The evolution of the microbial community that developed in the fermented grains was closely examined as fermentation progressed. PCR-DGGE and 16S rRNA gene library analysis were carried out to follow the bacterial community in the fermented grains during fermentation of different aroma type liquors of different geographical regions [22, 23]. In the fermented grains, bacterial diversity diminished with fermentation time, and eventually culminated in only one bright band in the final gel. Through DGGE fragment sequencing and Genbank BLAST, the predominant species at the end of fermentation was identified as *Lactobacillus acetotolerans*, which can tolerate the extreme environment in the fermentor. This is a common characteristic of the strong aroma type liquors and roasted-sesame-like aroma type liquors. In the fermented grains of the strong aroma type produced from the Sichuan region, the bacterial diversity narrowed more quickly than that from the Jiangsu region because the higher fermentation temperature characteristic of the Sichuan region accelerated oxygen utilization, propagation, and death of the microorganisms. In the fermented grains of strong aroma type liquors and roasted-sesame-like aroma type liquors, the different upstream production techniques, fermentors, and fermentation cycle methods resulted in different DGGE profiles, especially in the middle of fermentation. Genbank BLAST of the clone sequences reveals that only three genera (*Acetobacter*, *Bacillus*, and *Lactobacillus*) are present, and these have not been reported previously. The roles of these newly identified bacteria in fermentation need to be further investigated.

Zhang and coworkers [24] in 2007 analyzed with DGGE and 18S rRNA gene library the fungal community in the fermented grains during the fermentation of a strong aroma type liquor. DGGE showed a wide microbial diversity developing with time in the fermented grains. However, further information on community diversity required optimized electrophoresis conditions. A clone library was constructed from DNA extracted from samples collected at different layers and at different fermentation times. The library clones belonged to ten fungal genera: *Torulaspora*, *Talaromyces*, *Issatchenkia*, *Saccharomycopsis*, *Trichosporon*, *Eurotium*, *Saccharomyces*, *Aspergillus*, *Zygosaccharomyces*, and *Fomitopsis*. It was previously assumed that molds (including *Rhizopus* and *Aspergillus*) and yeasts (including *Hansenula* and *Saccharomyces*) existed in greater amounts in the fermented grains. However, this study failed to detect *Rhizopus* and *Hansenula* and identified *Saccharomyces* only in a sample collected from the middle-edge layer after 1 week of fermentation. These results disagree with those from the traditional methods and need to be further confirmed.

Raw materials, production techniques, and the external environment from different producing regions all contribute to diverse microbial populations in the daqu, on the clay coated on the interior fermentor wall, and in the different types of fermented grains. Microbial community in the daqu and fermented grains can be characterized with a combination of modern DNA fingerprint methods and traditional culture/isolation methods. Identifying critical process parameters in solid-state fermentation and characterizing key aroma and flavor components in liquors will help isolate important relevant bacteria and direct improvement of production techniques.

1.2.4 Aroma Compounds in Chinese Liquors

With the introduction of gas chromatography (GC) in the 1960s, it became possible to study trace volatile compounds in Chinese liquors. In the 1980s, GC was widely applied to the analysis of volatiles in Chinese liquors. Recently, new techniques, such as aroma extract dilution analysis (AEDA) and other time-intensive methods [4, 12, 25, 26], have been developed to evaluate the relevance of detected volatiles to odor perception in Chinese liquors. Acids seem to play an important role in Chinese liquors; these include hexanoic, butanoic, 3-methylbutanoic, pentanoic, acetic, 2-methylpropanoic, octanoic, heptanoic, propanoic, 4-methylpentanoic, and nonanoic acids. Volatile esters introduce fruity flavors and are considered highly positive flavor attributes in Chinese liquors. Important esters in Chinese liquors include ethyl acetate, ethyl propanoate, ethyl butanoate, ethyl pentanoate, ethyl hexanoate, ethyl octanoate, ethyl nonanoate, ethyl decanoate, ethyl 2-methylpropanoate, ethyl 2-methylbutanoate, ethyl 3-methylbutanoate, methyl hexanoate, propyl hexanoate, butyl hexanoate, pentyl hexanoate, hexyl acetate, hexyl butanoate, hexyl hexanoate, hexyl octanoate, 2-methylpropyl acetate, 2-methylpropyl hexanoate, 3-methylbutyl butanoate, 3-methylbutyl hexanoate, 3-methylbutyl octanoate, ethyl 2-hydroxyhexanoate, ethyl 2-hydroxypropanoate, ethyl 2-hydroxy-3-methylbutanoate, diethyl

butanedioate, and ethyl cyclohexanecarboxylate. Ethyl hexanoate has a fruity, floral, and sweet aroma. The Osme value of this compound is very high among the aroma compounds identified in Chinese liquors, especially in strong aroma type liquors [27]. In Chinese strong aroma type liquors, important alcohols consist of 2-methylpropanol, 3-methylbutanol, 2-ethyl-1-hexanol, 1-butanol, 1-pentanol, 1-hexanol, 1-heptanol, 1-octanol, 2-butanol, 2-pentanol, 2-heptanol, and 2-octanol. In Chinese liquors, guaiacol (2-methoxyphenol), 4-methylguaiacol (4-methyl-2-methoxyphenol), and 4-ethylguaiacol (4-ethyl-2-methoxyphenol) could be important to the aroma based on their high Osme values or flavor dilution (FD) values determined with a DB-Wax column [12]. These compounds contribute strong clove, spicy, and smoky odors. Phenol, *o*-cresol (2-methylphenol), *p*-cresol (4-methylphenol), 4-ethylphenol, and 4-vinylphenol are also identified in Chinese liquors. These compounds contribute medicine and animal odors. Some acetals are identified in the neutral/basic fraction. 1,1-Diethoxy ethane has a strong fruity aroma. 1,1-Diethoxy-3-methylbutane gives fruity aroma and has an extremely high FD value. 1,1-Diethoxynonane, 1,1,3-triethoxypropane, 1,1-diethoxy-2-methylpropane, 1,1-diethoxy-2-methylbutane, and 1,1-diethoxy-2-phenylethane are also identified in Chinese liquors. They contribute a fruity aroma. Two sulfur compounds, dimethyl disulfide and dimethyl trisulfide, are detected in Chinese liquors. Aldehydes are detected in liquors, including acetaldehyde, 2-methylbutanal, 3-methylbutanal, 1-pentanal, and others. Several aromatic esters are identified, including ethyl benzoate, ethyl 2-penylacetate, ethyl 3-phenylpropanoate, 2-phenylethyl acetate, 2-phenylethyl butanoate, and 2-phenylethyl hexanoate.

In 2007, Xu and coworkers identified the pyrazines of Chinese liquors using liquid–liquid extraction followed by headspace solid-phase microextraction (SPME) coupled with a GC–flame thermionic detector [27]. A total of 27 pyrazines, mostly alkyl- and acetylpyrazines, were identified in Chinese liquors. 2,6-Dimethylpyrazine was detected in all samples and ranged from 20 to 1,057 μg L^{-1}. Several pyrazines were found in most of the samples: 2-methylpyrazine (39–1,011 μg L^{-1}), 2,5-dimethylpyrazine (0.63–182 μg L^{-1}), 2-ethylpyrazine (20–101 μg L^{-1}), 2-ethyl-3-methylpyrazine (47–898 μg L^{-1}), 2,3,5-trimethylpyrazine (0.41–2,328 μg L^{-1}), 2,6-diethylpyrazine (84–1,621 μg L^{-1}), 3,5-diethyl-2-methylpyrazine (41–546 μg L^{-1}), 2-methyl-6-propenylpyrazine (42–451 μg L^{-1}), and 2-acetyl-3,5-dimethylpyrazine (39–338 μg L^{-1}).

2 Chinese Rice Wine

2.1 Introduction

Chinese rice wine, like beer and wine, is one of the oldest drinks in the world. It is a popular alcoholic beverage with an annual consumption of more than 2 million kiloliters in China, especially in the south [28]. Chinese rice wine is mainly produced

in the southern part of China. Shaoxing city in Zhejiang province, Shanghai city, and Jiangsu province are the most famous regions, and their Chinese rice wines have representative aroma and flavors.

Compared to sake, which is typically described as having "caramel," "burnt," "heavy," and "complicated" characteristics [29], Chinese rice wine has yellow, sweet aromatic and nutritious characteristics. Based on their sugar concentration, Chinese rice wines are classified into four categories: dry type (sugar content <15 g L^{-1}), semi-dry type (sugar content 15–40 g L^{-1}), semi-sweet type (sugar content 40–100 g L^{-1}), and sweet type (sugar content >100 g L^{-1}). Chinese rice wine is saccharified in solid-state, but fermented in a semi-liquid state or in a submerged liquid-state.

Chinese rice wine is typically fermented from sticky rice with "wheat qu" as a saccharifying agent and yeast as a fermenting agent. Wheat qu, similar to the *koji* of sake, is made from wheat. First, the wheat, the raw material for making wheat qu, is typically milled and pressed into a mold of different sizes with appropriate natural water. Then, it is incubated at 28–30 °C for 48 h in a special room, and dried at 45 °C to a moisture level of less than 12 wt%. The fermented wheat qu is rich in various microorganisms, including bacteria, yeast, and mold. In addition, a complex enzyme system is established inside wheat qu during this process.

Like making sake, sticky rice is first polished before it is used in making Chinese rice wine. Subsequently, the polished sticky rice is washed with natural water at room temperature and immerged in the same water at 12–15 °C for 40 h. After that, the steeped sticky rice is steam-cooked in a steaming machine to gelatinize the starch contained in the rice kernel. Finally, the steamed rice is cooled with cold water to 30 °C.

Preparation of seed mash is a special process in Chinese rice wine fermentation. The steamed sticky rice is mixed with wheat qu and spring water at 25 °C at a weight ratio of rice:qu:water = 10:1:20. After the pH of the fermenting mash is adjusted to 4 with lactic acid, 3 wt% selected pure yeast is added to the mixture. Subsequently, the mixture is cultured at about 25 °C for 48 h.

In the main fermentation, steamed sticky rice is mixed with 10 wt% wheat qu, 6 wt% seed mash, and 150 wt% spring water in pottery vats. A noteworthy characteristic of Chinese rice wine fermentation is that the process is simultaneously saccharifying and fermenting. The main fermentation is typically carried out at 28–30 °C for 7 days in a semi-solid state, and the post-fermentation is further carried out at room temperature for 15 days. After post-fermentation, the rice wine mash is first filtered with a presser, and the slightly turbid rice wine filtrate is pumped to refining tanks for clarification. Caramel, which determines the color of Chinese rice wine, is added to clarified rice wine. Subsequently, the fresh rice wine is cooked with steam at 88–90 °C for 3 min. Finally, the cooked rice wine is aged in sealed pottery jars to develop a balanced aroma. Most Chinese rice wines are aged for 1–3 years, but some are aged for 3–5 years or longer. The aged rice wine is generally blended to yield an ethanol content of 14–17 vol.% for a consistent quality in the finished product.

2.2 Wheat Qu and Yeast for Making Rice Wine

2.2.1 Microorganisms and Enzymes of Wheat Qu

A characteristic in Chinese rice wine brewing is the use of several types of wheat qu. Wheat qu is mainly a culture of mold and yeast grown on and within ground rice meal. Wheat qu, which is comparable to *koji* used in sake brewing, is used for liquefaction and saccharification of the starch contained in the rice. Wheat qu also contributes to aroma and flavor formation. Without wheat qu, the rice wine would lose its characteristic aroma and flavor.

In a study aiming at the molecular identification and classification of the main molds from wheat qu [30], 18 molds were isolated from a wheat starter by plating on potato agar, wort agar, and Candida diagnostic agar (CDA). Of these, the five main strains (with $>10^5$ cfu g^{-1}) were *Absidia corymbifera*, *Rhizomucor pusillus*, *Aspergillus orgyze*, *Aspergillus fumigatus*, and *Rhizopus oryzae*. They were identified through sequencing and sequence contrast (DNA extraction by benzyl chloride followed by amplification of the whole ITS sequence including ITS1 and ITS2 with ITS1 and ITS4 primers and ITS rDNA). See Table 5.

Table 5 Sequence of ITS of *Absidia corymbifera* in wheat qu (813 bp)

No.	Sequence (813 bp)					
1	GGATCA TTAC	TGAGAGG TCT	AAAAAAC CAC	TAGTTGG GGG	TCTCTTC CCC	TCTAGTAGTT
61	CCTCAC AGTT	TTGTGCA AAG	TCGGGTC AAC	TTGGTTG GCT	CTGCCTT GTG	CCTTTGATCT
121	CTAGTT GGCA	TAGAGAC CCT	CCTTTTA GGA	GACTTGT GCC	ACTAAGA TCT	AGGCTGCTTG
181	AAAAGA GACG	TATGGAC CCT	TCTTTCA GGA	GACCTAT GTC	TCGAGTC AAA	GCAAGCAAGG
241	CATGAT GTAC	GGGCTCT AGT	ACTAACT ATC	CCCAAAG GTG	TTTATTC TTC	TCGTGTAAAC
301	CATGAT GTAC	GAAAAAA GTT	AGTTGTT AAC	TTAAAAA CAA	CTCTKGG CAA	KGRWYYTCTT
361	GGTTCT CGCA	TCGATGA AGA	GCGTAGC AAA	GTGCGAT AAT	TATTGCG ACT	TGCATTCATA
421	GCGAAT CATC	GAGTTCT CSA	ACGCATC TTG	CGCCTAG TAG	TCAATCT ACT	AGGCACAGTT
481	GTTTCA GTAT	CTGCAAC TAC	CAATCAG TTC	AACTTGG TTC	TTTGAAC CTA	AGCGAACTGG
541	AAATGG GCTT	GTGTTGAT GG	CATTCAG TTG	CTGTCAT GGC	CTTAAAT ACA	TTTAGTCCTT
601	AGGCAA TTGG	CTTTAGT CAT	TTGCCGG ATG	TAGACTC TAG	AGTGCCT GAG	GAGCAACGAC
661	TTGGTT AGTG	AGTTCATT AAT	TCCAAGT CAA	TCAGTCT TCT	CTTGAAC TAG	GTCTTAATCT
721	TTATGG ACTA	GTGAGAG GAT	CTAACTT GGG	TCTTCTA TTA	AAACAAA CTC	ACATCTAGAT
781	CTGAAA TCAA	CTGAGAT CAC	CCGCTGA ACT	TAA	–	–

The α-amylase from wheat qu of Chinese rice wine has been studied by Fang and co-workers. From wheat qu of Chinese rice wine, an α-amylase producing strain was isolated and identified as *Aspergillus oryzae* by molecular and sequence alignment methods. α-Amylase from wheat qu of Shaoxing rice wine and that produced by *A. oryzae* were purified via ion-exchange chromatography and gel filtration chromatography and further characterized. (Fig. 1 and Table 6).

2.2.2 Yeast

Yeast converts sugar to ethanol and carbon dioxide. Different yeast strains produce different products, such as esters, alcohols, acids, and other chemical compounds that affect the nuance of aroma and flavor in rice wine. These compounds are present in various levels, depending on the choice of yeast and the fermentation process parameters. Fermentation temperature greatly affects the distribution of products, for example, fatty acids esters, high alcohols, and other compounds.

Several types of yeast, including rice-sprinkling yeast, fast-fermenting yeast, saccharifying yeast, and active dry yeast, have been employed in the production of different rice wines.

Tables 7–9 compare the properties of major Chinese rice wine yeasts (HJ1, HJ2, HJ3, and HJ4) and sake yeast (K-7) to tolerate high sugar concentration, high/low temperatures, and low pH. There exist statistically significant differences among different yeasts. HJ4 rice wine yeast can tolerate high sugar concentration; whereas

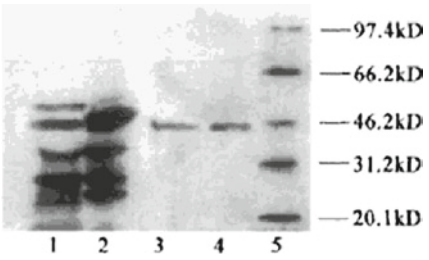


Fig. 1 SDS-PAGE analysis of α-amylase. Lane 1, crude α-amylase of wheat qu; 2, crude α-amylase from Aspergillus oryzae in solid-state fermentation; 3, purified α-amylase from wheat Qu; 4, purified α-amylase; 5, protein marker

Table 6 Basic properties of α-amylases

	From wheat qu	Purified α-amylase
Molecular weight (kD)	46	46
Optimum pH	4.6	4.6
Optimum temperature (°C)	60	60
K_m (g/L)	1.12	1.09
Hydrolysis product	Glucose and maltose	glucose and maltose

Table 7 Effect of sugar concentration on generation time of rice wine yeasts (HJ) and sake yeast (K)

	15%	20%	25%	30%	35%	40%
HJ1	3.35 ± 0.11	4.22 ± 0.10	5.86 ± 0.10	7.04 ± 0.07	14.15 ± 0.55	32.35 ± 2.41
HJ2	3.88 ± 0.09	4.80 ± 0.13	6.34 ± 0.08	7.23 ± 0.11	8.76 ± 0.13	9.80 ± 0.27
HJ3	4.08 ± 0.06	4.28 ± 0.05	4.46 ± 0.05	4.74 ± 0.06	7.18 ± 0.04	9.02 ± 0.34
HJ4	4.25 ± 0.07	4.60 ± 0.06	4.84 ± 0.07	5.27 ± 0.10	6.98 ± 0.17	7.47 ± 0.23
K-7	4.36 ± 0.06	4.96 ± 0.06	6.76 ± 0.06	7.52 ± 0.07	9.64 ± 0.13	10.77 ± 0.20

Table 8 Effect of temperature on δOD_{600} of rice wine yeasts (HJ) and sake yeast (K)

	Low temperature					High temperature			
Yeast	12 °C	15 °C	18 °C	21 °C	24 °C	35 °C	38 °C	41 °C	44 °C
HJ1	1.04 ± 0.06	1.30 ± 0.05	1.52 ± 0.07	1.70 ± 0.08	1.85 ± 0.09	1.13 ± 0.09	0.90 ± 0.02	0.66 ± 0.03	–
HJ2	0.50 ± 0.05	0.81 ± 0.03	1.09 ± 0.07	1.28 ± 0.07	1.72 ± 0.08	1.35 ± 0.08	0.75 ± 0.07	0.34 ± 0.03	–
HJ3	0.92 ± 0.04	1.30 ± 0.06	1.61 ± 0.04	1.81 ± 0.07	2.09 ± 0.10	1.68 ± 0.11	1.16 ± 0.02	0.50 ± 0.03	–
HJ4	1.06 ± 0.05	1.35 ± 0.05	1.53 ± 0.04	1.74 ± 0.04	1.92 ± 0.10	1.48 ± 0.02	0.87 ± 0.05	0.46 ± 0.05	–
K-7	1.18 ± 0.04	1.37 ± 0.03	1.45 ± 0.11	1.63 ± 0.07	1.88 ± 0.03	1.19 ± 0.08	1.07 ± 0.05	0.90 ± 0.04	

Table 9 Effect of pH on ΔOD_{600} of rice wixne yeasts (HJ) and sake yeast (K)

Yeast	pH 1.5	pH 2.0	pH 2.5	pH 3.0	pH 3.5
HJ1	–	–	0.44 ± 0.02	0.64 ± 0.03	0.72 ± 0.04
HJ2	–	–	0.40 ± 0.03	0.63 ± 0.03	0.65 ± 0.03
HJ3	–	–	0.54 ± 0.03	0.82 ± 0.03	0.85 ± 0.02
HJ4	–	–	0.67 ± 0.07	0.82 ± 0.01	0.87 ± 0.06
K-7	–	–	0.56 ± 0.08	0.75 ± 0.04	0.79 ± 0.02

HJ3 rice wine yeast has the highest fermentation efficiency at 40% sugar. K-7 sake yeast tolerates temperature variations; whereas HJ4 rice wine yeast tolerates low pH better than other yeasts.

2.3 New Technology Evolution

2.3.1 Liquefaction Method of Fermentation

There have been studies on liquefaction and nonstarter processes of making Chinese rice wine [31]. Mixing glutinous rice flour with amylase at a ratio of rice:amylase = 1 g:4U followed by liquefaction at 80–95 °C for 20 min yields a

liquefied mash with good fluidity. When the liquefied mash is cooled to 60 °C, its pH is adjusted to 5.8 by adding lactic acid. After saccharifying enzyme and other enzymes are added to the mash, it is held at 40–60 °C for 10 min. This saccharified mash is fermented in a stainless steel tank at 28 °C for 15 days. The finished Chinese rice wine should have a pleasant aroma and taste, and it should meet applicable quality standards. A rice wine that results from submerged fermentation of corn has been studied [32]. In this study, corn is milled, added water, liquefied with a-amylase, and saccharified with saccharification amylases of *Rhizopus* and *Aspergillus*. To the mash, 0.15% acidic protease is added to help decompose the protein. Active dry yeast is added to initiate fermentation in submerged liquid-state in a stainless steel tank at 30 °C then finished at 28 °C. Alternating high and low temperatures during the aging process helps develop the bouquet aroma of corn wine.

2.3.2 Extrusion Method of Fermentation

Recently, to minimize water pollution and energy consumption, the extrusion method of fermentation is tested to brew Chinese rice wine [33]. During extrusion, the physical cellular structure of rice changes from granules to flake-like. There are also changes in the chemical characteristics: starch becomes gelatinized and decomposed; starch content decreases while reducing sugar increases; protein decreases due to denaturation and decomposition while amino acids rise; and lipid decreases due to formation of protein–lipid and starch–lipid complexes and auto-decomposition. These changes in starch, protein, and lipid composition favor improved rice wine quality with lighter characteristics.

3 Soy Sauce

3.1 Introduction

3.1.1 History of Soy Sauce in China

Soy sauce, also called chiang-yu, is one of the world's oldest condiments and has existed in China for more than 2,500 years. Historically and culturally since the Han and Tang dynasties, Chinese soy sauce has referred to a salty liquid seasoning that is made mainly from soybean and wheat by employing any of the brewing techniques of "making qu from all materials" and "caihuang by nature."

Soy sauce was discovered about 2,000 years ago during the Zhou dynasty as the liquid collected at the bottom of casks in the process of producing fermented soybean paste. Its sweet, sour, salty, and bitter tastes add interest to flat-tasting processed foods. The flavor enhancing properties, or umami, of the soy extract help blend and

balance taste. The condiment also possesses functional preservative aspects in that its acid, alcohol, and salt content help prevent food spoilage. Once a home-made staple, it has become an important industrial product. Industrialization has altered the production process, changed the raw materials, standardized the products, and modified somewhat their characteristics in the process.

Today, Chinese soy sauce has become not only an absolutely necessary condiment in Chinese family life, but also increasingly favored and accepted by people all over the world. Most of the Chinese factories rely on fermentation processes instead chemical processes because soy sauce generated through cell-free hydrolytic reactions and seasoned with corn syrup and salt and colored with caramel lack the savory flavor of naturally brewed soy and often taste metallic.

3.1.2 Soy Sauce Industry in China

The total world output of soy sauce reached about 8 million tons in 2006, and the demand continues to grow at an annual rate of higher than 10%. The Chinese market share is over half of the world's. The Chinese soy sauce output is 5 million tons presently, up from only 1 million tons in 1975.

The fermentation process takes up to 6 months to complete and results in a transparent, delicately colored broth with a balanced flavor and aroma. In comparison, nonbrewed sauces take only 2 days to make and are often opaque with a harsh flavor and chemical aroma.

Most producers of soy sauce in China continue to resort to traditional fermentation methods. The factories are located mainly in southern China, which in turn can be divided into three regions.

The first region is Guangdong where Haitian and Jammychai are representative producers. Hai Tian Soy Sauce Factory, which boasts over 200 years of history dating back to the Qianlong period during the Qing Dynasty, has become the largest Chinese flavoring enterprise.

The second region is Shanghai, which is home to several famous producers including Lao Cai and Amoy (Ajinomoto Japan).

The third includes a collection of famous regional brands of soy sauce such as Wangzhihe (Beijing) Zhenji (Shijiazhuang), and JiaJia (Changsha). Factories operated by foreign soy sauce enterprises such as Amoy (Ajinomoto Japan), Kikkoman (Japan), Lee Kum Kee (Hong Kong), JiaJia (Hong Kong), and Wadakan (Japan) account for 50% of the soy sauce output in China.

Most of the soy sauce producers in China employ the low-salt solid-state fermentation process that yields a medium quality product. The high quality soy sauce, which accounts for less than 10%, is prepared either with the traditional fermentation process or with the high-salt diluted-state fermentation process developed by the Japanese. The former is preferred primarily by factories located in Guangdong Province, while the latter is found mainly in factories near Shanghai and Beijing.

3.2 Fundamentals of Soy Sauce Production

As we know, soy sauce is a dark brown salty liquid with a distinctive aroma and is used to season oriental foods. The main ingredients of soy sauce are soybean or defatted soybean meal, wheat or wheat bran, salt, and water. The manufacturing process is a complex fermentation process where carbohydrates are fermented to alcohol and lactic acid, and proteins are broken down to peptides and amino acids. A brown color appears during the course of the 6–8 month maturation process. The process of manufacturing soy sauce can be divided into three stages: making qu (koji) from raw materials, mash fermentation, and product refining.

Figure 2 outlines the basics of a representative soy sauce manufacturing process. After mixing with roasted broken wheat and wheat bran, protein materials are steam-cooked in a continuous pressure-cooker. The seed qu, which is inoculated with *Aspergillus orizae* and incubated in shallow vats with perforated bottoms, is added to the medium to make qu. After several days of incubation under controlled conditions of temperature and moisture, mold growth covers the entire mass, which turns greenish from sporulation. The dry mash, called koji, is an essential ingredient in most oriental fermented products because it is the source of amylolytic and proteolytic enzymes for the decomposition of starch and protein.

Subsequently, the qu is mixed with brine and transferred to deep fermentation tanks. The high salt concentration effectively inhibits the growth of undesirable wild microorganisms. The starch is transformed to sugars, which are fermented to lactic acid and alcohol. The fermenting mash is held in fermentation tanks for 6–8 months.

In the refining stage, the fermented mash is pressed with layers of filtration cloth to separate the sauce from the cake of wheat and soy residues. The sauce is filtered, clarified and heated to 70–8 °C. Heating is necessary to pasteurize the sauce and to develop the characteristic color and aroma. After the final clarification step, the sauce is bottled.

3.2.1 Raw Materials

Whole soybean or more commonly defatted soybean meal constitutes a raw protein source in soy sauce production. In China, soybean is referred to as the “King of Legumes” because of its valuable nutritional properties, such as being rich in minerals and vitamin B. Wheat or wheat flour, which supplies starch material for fermenTable reduced sugars, is also a good source of amino acids. Table 10 [34] lists the amino acid composition of common raw materials for soy sauce production and that of the final soy sauce product. All proteins in these raw materials contain glutamic acid, which is an important taste ingredient of soy sauce.

Salt is added at the beginning of fermentation to help establish a proper chemical environment for desirable lactic acid bacteria and yeast to thrive and simultaneously protect the finished product from spoilage by undesirable microorganisms.

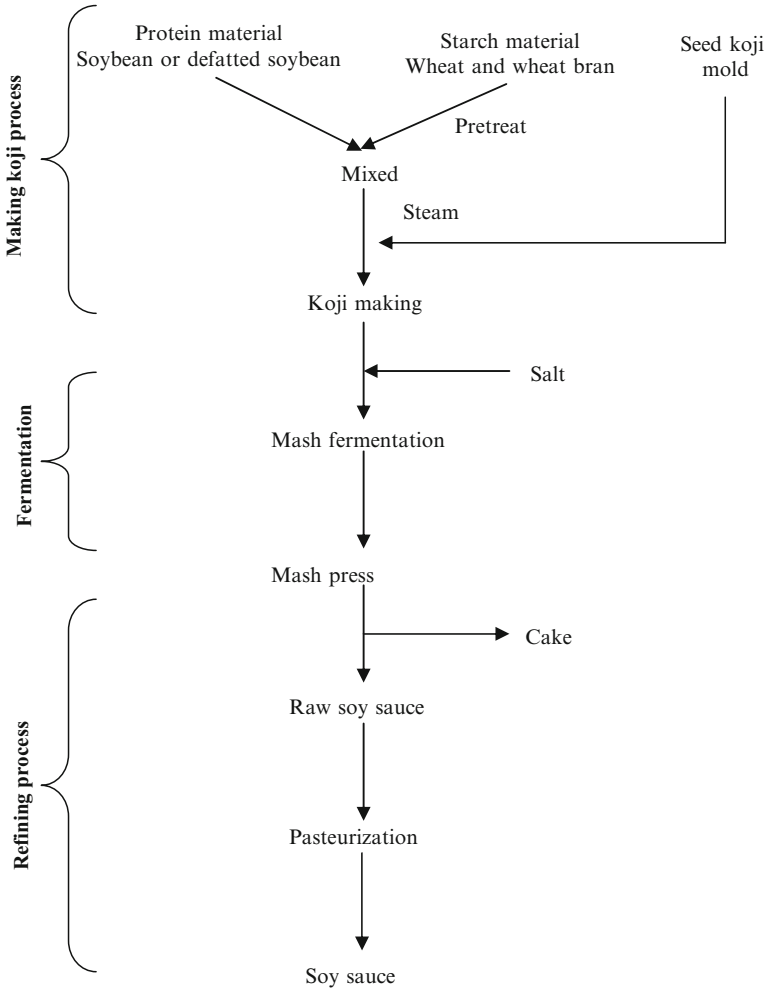


Fig. 2 Main process of soy sauce production

3.2.2 Raw Material Treatment

The protein in raw soybean must first be denatured to become digestible by the proteases present in the qu. In ancient times, soybean was boiled in water at atmospheric pressure, and this tradition does not bode well with modern automation practices. Nowadays, soybean is autoclaved under pressure with steam and becomes highly enzymatically digestible.

Table 10 Amino acid composition of materials and final soy sauce product [34]

Amino acid	Soybean g/100 g	Soybean meal	Wheat	Wheat flour	Wheat bran	Soy sauce g/100 mL
Arginine	3.01	3.5	0.524	0.368	0.950	0.258
Histidine	0.91	1.25	0.236	0.190	0.319	0.096
Lysine	2.46	3.05	0.298	0.194	0.542	0.420
Tyrosine	1.40	1.2	0.355	0.323	0.406	0.094
Tryptophan	0.46	0.56	0.126	0.091	0.227	–
Phenylalanine	1.79	2.4	0.493	0.471	0.515	0.403
Cysteine	0.57	0.46	0.200	0.174	0.219	–
Methionine	0.56	0.65	0.194	0.171	0.213	0.231
Serine	1.99	2.48	0.581	0.533	0.659	0.643
Threonine	1.54	2.05	0.307	0.261	0.411	0.393
Leucine	2.76	3.78	0.725	0.654	0.793	0.718
Isoleucine	1.83	2.21	0.421	0.386	0.473	0.407
Valine	1.93	2.35	0.522	0.426	0.675	0.470
Glutamic acid	7.52	9.8	3.260	3.327	2.331	1.420
Aspatic acid	4.30	5.4	0.540	0.385	0.955	0.554
Glycine	1.62	2.14	0.438	0.292	0.737	0.295
Alanine	1.61	2.24	0.375	0.264	0.669	0.419
Proline	2.25	2.08	1.106	1.154	0.879	0.767

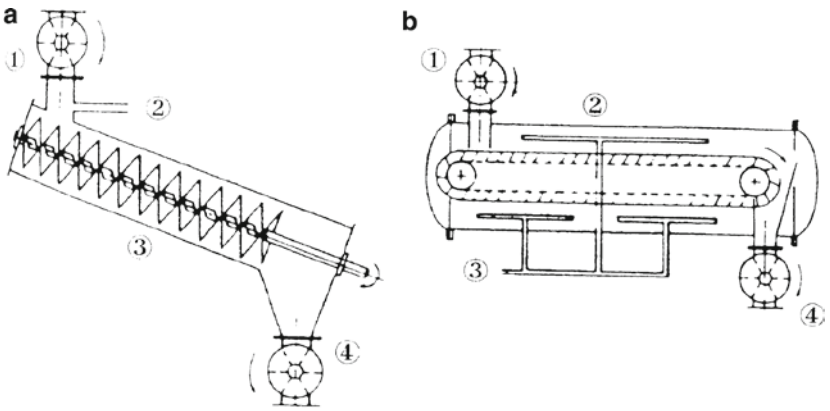


Fig. 3 Continuous soybean cooker. 1, rotary valve (charge); 2, steam; 3, cooker; 4, rotary valve (discharge)

Continuous Cooking Process

Soaked soybean or soybean meal is cooked in a rotary cooker (Fig. 3) [35] at high pressure for several hours and cooled immediately in a jet condenser. Table 11 compares the results of two different ways of treating raw materials. Presently, computer-controlled continuous cookers have already been developed and practiced in the soy sauce industry [36].

Table 11 Continuous cooking of soybean as compared with conventional method

Cooking method	Digestibility of protein	Ratio of formal N to total N	Ratio of glutamic N to total N
Conventional	68.7%	49.4%	5.5%
Continuous	73.1%	53.8%	7.3%

Table 12 Ingredients of protein materials with extrusion process

Sample	Water	Ash	Lipid	Starch	Protein	Soluble protein
Soybean	10.2	4.25	ND	23.95	33.67	26.17
Extruded soybean	3.76	5.05	ND	18.90	28.77	21.54
Soybean meal	11.3	5.62	1.32	46.71	49.82	ND
Extruded Soybean meal	7.1	5.99	1.26	40.16	45.01	ND

ND: not determined

Extrusion Processing

Extrusion processing is now widely adopted in the food industry, including soy sauce. The raw ingredients are transformed into modified intermediates and finished products. Table 12 lists alterations in the physical and chemical properties of soybean components during the extrusion process [37–39].

The soy sauce qu prepared with extruded raw materials exhibits higher hydrolytic enzyme activities and the product contains higher pyrazines and ethanol than the traditional counterpart. The soy sauce product fermented from expanded soybean has a higher protein utilization rate and is nutritionally richer with special flavors (Fig. 4).

Enzymatic Treatment of Raw Materials

Starch raw material is very important in soy sauce brewing. Likewise, enzymes play a critical role in soy sauce brewing. Amylases help form reducing sugars and dextrins from starch by providing the necessary substrates for alcohols and organic acids, and in the process they release solid matters in the soy sauce. According to Table 13, a production process that employs amylase for liquefaction can save 40% in starch raw materials, increase the solid content without salt, and enhance the cohesiveness of soy sauce [40, 41]. Proteases hydrolyze bean protein into protein hydrolysates such as peptone, conjugated protein, polypeptide, and amino acids, which contribute positively to the nutritional value and impart a wonderful flavor in soy sauce. Cellulases hydrolyze cellulose into dextrose and break down the plant cell wall. The industry practices the enzyme preparation processes (Fig. 5) [42] to improve the productivity and flavor of soy sauce.

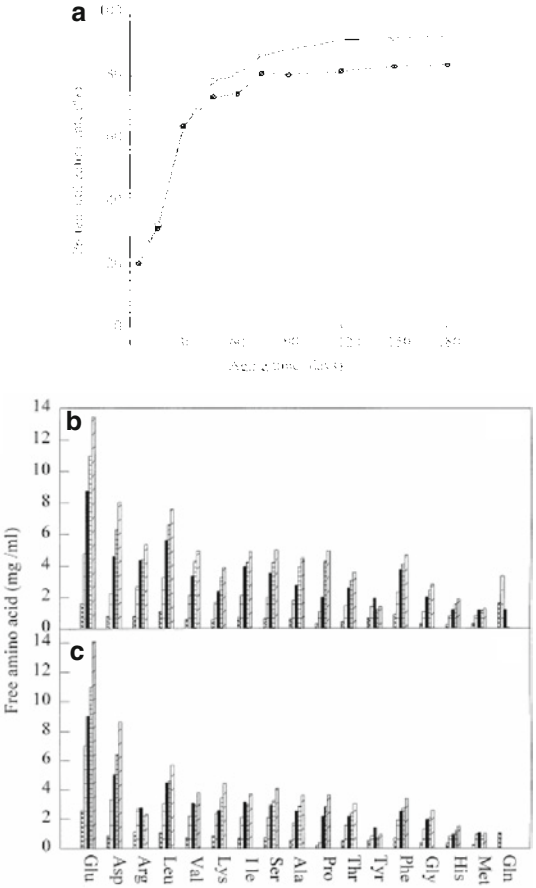


Fig. 4 (**a**) Changes in protein utilization rate during soy sauce fermentation. *Open circle*: soy sauce prepared with extruded substrate; *filled circle*: soy sauce prepared with traditional substrate. **b,c** Free amino acid composition in raw soy sauce at various stages of aging; soy sauce prepared with extruded substrate (**b**); soy sauce prepared with traditional substrate (**c**). The aging times of different column are 5, 30, 60, 120, and 180 days

Table 13 Comparison of soy sauce made from traditional qu and with added enzyme

Sample	Total nitrogen (g L^{-1})	Amino acid nitrogen (g L^{-1})	Soluble solid content (g L^{-1})	Output (kg)
Control[a]	7.4	4.1	86.2	4,233
Added enzyme[b]	7.8	4.3	90.9	4,250

[a]Average of three batches
[b]Average of four batches

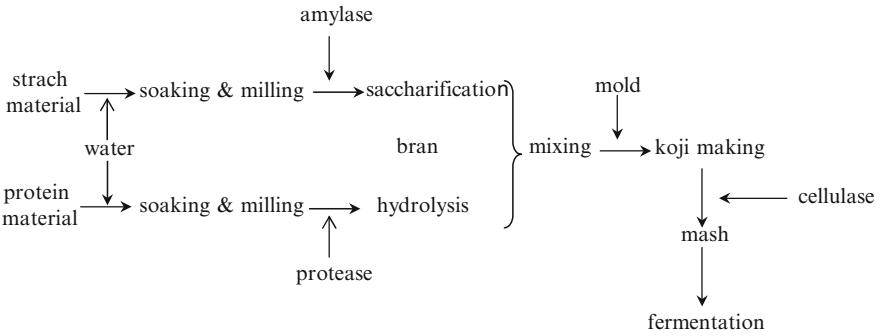


Fig. 5 Soy sauce production with enzymatic treatment of raw materials

3.2.3 Qu Making

The quality of qu is very important in soy sauce production. The traditional qu making process is performed in an open system. After steam-cooking, the mixture of soybean and wheat cools to 27 °C and is inoculated with the qu seed culture, which is a light green mold of the *Aspergillus* group. The inoculated mixture is incubated in a vessel where temperature and humidity are carefully controlled and monitored. Here the spores germinate, appearing initially as small white spots on the surface of the beans and wheat. During its growth, mold produces enzymes that convert proteins, fats, and starches to simpler and more easily fermenTable substances.

With increasingly stringent requirement in large scale production of soy sauce, several new qu making processes have been developed to shorten the cycle of qu making and improve the activities of qu.

Submerged Qu Making Process

It is easier to control the condition of mold growth and protease expression with submerged fermentation of *Aspergillus* to achieve higher activities. The submerged method allows not only preparation in a continuous and mechanized manner but also reduces the cost. Figure 6 [40] shows the submerged qu fermentation process.

Qu Making with Double Molds

There is evidence that a mixed culture of *Aspergillus oryzae* and other molds increases the utilization of nitrogen in protein materials. Table 14 shows that total nitrogen, amino acid nitrogen, and glutamic acid all increased when *Aspergillus oryzae* 3.042, the most common strain in soy sauce production in China, was cocultured with a second mold [43]. Modern qu making utilizes mixed cultures of strains of *Aspergillus*, *Rhizopus*, *Cephalosporium*, and *Mucor*.

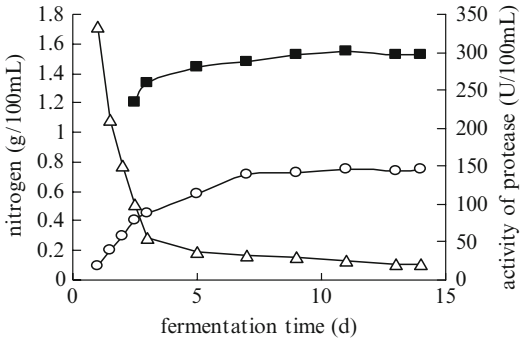


Fig. 6 Overall fermentation process that utilizes submerged qu making. *Open square*, activity of protease; *filled square*, total nitrogen; *open circle*, amino acid nitrogen

Table 14 Nitrogen analysis of soy sauce prepared by mixing cultured qu with different molds

Microorganism	Total nitrogen g L^{-1}	Amino acid nitrogen	Glutamic acid
Aspergillusoryzae 3.042 (control)	6.93	3.35	2.9
Aspergillus tamari	7.14	3.61	3.5
Aspergillus flavus	7.35	3.61	3.5
Aspergillus niger	7.42	4.25	5.2
Rhizopus sp	7.42	3.42	3.3
Cephalosporium 3.170	7.21	3.69	4.1
Mucor 3.13	7.42	3.73	3.2

Qu Making with Multiple Strains

Numerous bacteria and yeasts, such as wild yeast, *Micrococcus,* and *Bacillus*, can be isolated from qu making in an opening system [44, 45]. These microorganisms derived from the qu impart the flavors into soy sauce. These microbes in a pure culture form fail to produce flavor components during the overall fermentation. They must be co-inoculated at the beginning of the qu making process and do their magic in concert. Figure 7 shows the changes in amino acid nitrogen, total acids (based on lactic acid), and reducing sugars (based on glucose) during the overall fermentation by three different types of qu making molds.

3.2.4 Fermentation

Traditional Fermentation Process (Shining and Exposed Fermentation Process)
The heart of the manufacturing process is complex fermentation whereby the carbohydrates are fermented to alcohol and lactic acid and the proteins are broken down to peptides and amino acids. Reactions between the original components and the fermentation products create the color and aroma of soy sauce. The entire traditional Chinese brew process of soy sauce from beginning to end is lengthy.

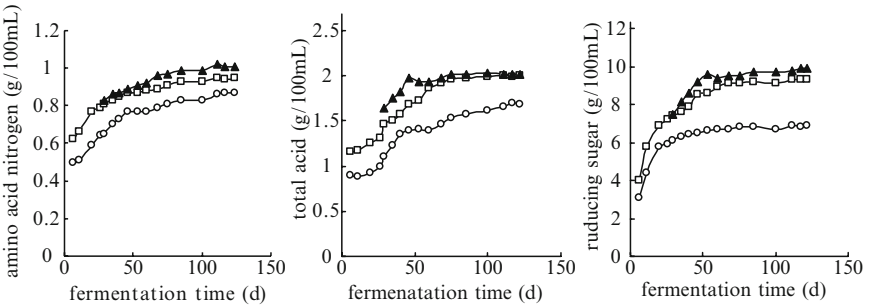


Fig. 7 Changes in amino acid nitrogen, total acids (based on lactic acid), and reducing sugars (based on glucose) during the overall fermentation process. *Filled triangle*, qu making with multiple strains; open square, qu making with double molds; *open circle*, qu making with *Aspergillus oryzae*

The mature qu is mixed with brine containing 18 Be or more salt at a weight ratio of 1:1.5 and transferred to porcelain jars or kegs to create a mash for fermentation. The mash must be exposed to open air day and night for 6–12 months until full flavor is achieved, during which time the soy-wheat paste turns into a semi-liquid, reddish-brown mature mash. This fermentation process creates over 200 different flavor compounds. The jars remain uncovered throughout the whole fermentation, and their contents are stirred regularly to maintain active fermentation. Stirring helps break up sedimentation and lumps as well as aerate the bacteria's environment. Fermentation is most active during the warm summer months, and traditional craftsmen consider aging through a second summer essential to the quality and flavor of the finished product.

High-salt Diluted-state Fermentation Process

This process is similar to the traditional one and starts by mixing mature qu with brine containing about 18 Be salt at room temperature [46]. The difference is that the ratio of brine is increased to 1:2.5 and the mash is fermented in large scale tanks. The aging time can be deduced to 3–6 months.

High-salt Diluted-state Cooled Fermentation Process

This process is popular in the factories in Zhenji and Wadakan in China. It is derived from the high-salt diluted-state fermentation but operated with cooling to produce high quality soy sauce. The fermentation temperature is kept at nearly 15 °C for optimum glutaminase synthesis, and the pH is maintained at neutral for optimum protease activity. The temperature is raised to 30 °C after nearly 1 month when all amino nitrogen becomes dissolved. This shift-up in the fermentation temperature aids the esterification activities of lactic acid bacteria in enhancing soy sauce flavor.

Low-salt Solid-state Fermentation Process

This process, originally developed during the 1950s, is presently practiced by most of the producers in China. In this process, the starch material is mainly wheat brine. After the mash is mixed with salt water of about 13 Be, it is fermented at 40–45 °C [47, 48]. The entire fermentation process lasts for only 1 month. The extract of mash in the fermentor, but not the solid matter due to difficulties in transferring solids, is recycled back to the mash to improve the flavor.

Sequential Solid-liquid Fermentation Process

Compared with the soy sauce fermented via either high-salt liquid-state and low-salt solid-state, that fermented via solid–liquid state has a shorter production period and better flavor [49]. Thus, solid–liquid means is now widely practiced in many fermentation factories. With equipment identical to low-salt solid–state fermentation and by following the low-temperature qu production and low-temperature fermentation techniques, a strong soy sauce is made that is similar to the Guangdong type soy sauce in both color and flavor. This process was developed in the 1980s to overcome the shortcomings of the low-salt solid-state fermentation process. After fermentation in solid-state for 15 days, salt water is added again at a ratio similar to the diluted-state fermentation process, and fermentation resumes at 30–35 °C after the mash extract is recycled back.

3.2.5 Refining

The fully aged mash is poured onto cloths, folded, and pressed. The filtrate emerges as the raw soy sauce, leaving behind the press cake, which is a valuable byproduct as livestock feed. The raw soy sauce is subsequently refined and pasteurized or ultrafiltered. Samples of the refined soy sauce are carefully analyzed to ensure the finest quality. The raw soy sauce is settled and cleared for about one month, during which time oil is skimmed off of the surface, remaining solid proteins are broken down, and coarser sediments are drawn off from the bottom.

Pasteurization Process

Heating the soy sauce is a traditional practice handed down over many generations. The raw soy sauce is heated to 70–80 °C, a temperature range that is low enough not to deactivate the valuable enzymes and retains other nutritional properties of the finished product, but high enough to kill the working bacteria to assure a long shelf life of the finished product. Pasteurization will be performed at nearly 110 °C for several seconds. The high temperature increases the acidity, color, and clarity while

decreasing the reducing sugars and amino acids. Pasteurization also changes the flavor of soy sauce due to the loss of volatile compounds.

Ultrafiltration Membrane Separation Process [50]

To avoid the shortcomings of the pasteurization process, new technologies have been developed. Figure 8 shows a typical ultrafiltration process, which is popular in soy sauce factories in China [51].

Wang and coworkers [52] successfully ultrafiltered raw soy sauce to remove secondary sediments in soy sauce with 50 nm pore-sized inorganic ceramic membrane tubes at 40 °C. Table 15 compares raw soy sauce and ultrafiltered soy sauce.

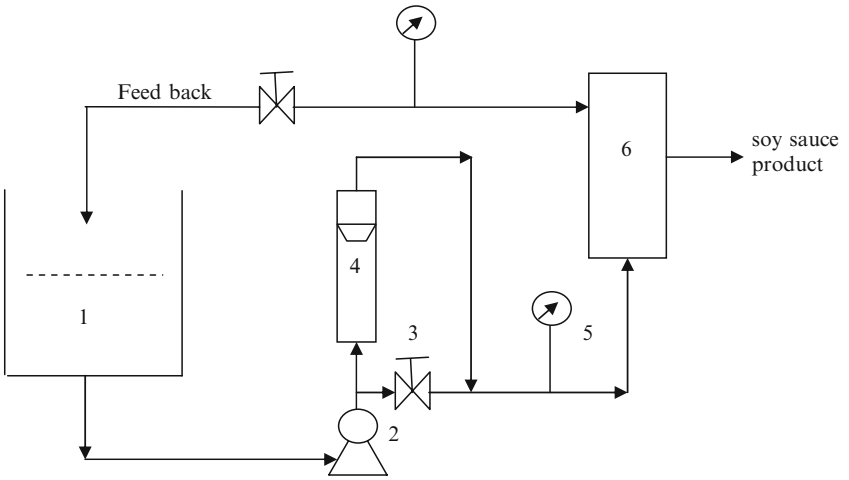


Fig. 8 Typical ultrafiltration process: 1, raw soy sauce storage tank; 2, pump; 3, control valve; 4, flow meter; 5, pressure meter; 6, ultrafiltration membrane system

Table 15 Comparison between three batches of raw soy sauce (control) and ultra-filtered soy sauce

		Amino acid N	Total N	Soluble solid without salt	Salt	Bacteria
Soy sauce		g L^{-1}				cfu mL^{-1}
Control	1	6.4	14.6	184.5	185.5	2.1×10^6
	2	6.4	14.4	172.5	184.5	1.0×10^6
	3	4.5	9.7	115.0	160.0	4.1×10^6
Ultrafiltered	1¢	6.2	11.9	184.1	171.9	3.3×10^2
	2¢	6.0	13.0	150.1	185.7	5.0×10^2
	3¢	4.3	8.6	101.6	160.0	0.5×10^2

3.3 Advances in Soy Sauce Production

3.3.1 Microorganisms in Soy Sauce Production

Soy sauce fermentation is a complex process involving molds, yeasts, and lactic acid bacteria, and the density and the ratio change as fermentation progresses (Table 16). The most important microorganisms are molds, especially the strains of *Aspergillus. Aspergillus oryzae* 3042, originally isolated by Shanghai Niangzao Institute, is now commercially available for soy sauce production in China. *Aspergillus sojae*, which produces both protease and glutaminase and is popular in Japan, is seldom used in China. *Monascus* and *Aspergillus niger*, which produce higher amounts of alcohols and esters, are usually used to improve raw material utilization.

In order to enhance *Aspergillus* mold's properties, especially utilization efficiency of nitrogen, Pan obtained strains with improved neutral protease through UV treatment [53] and used these strains as candidates of gene shuffling library for multiparental inactivated protoplasts electroporation [54] .He also developed RAPD (Random Amplified Polymorph DNA) to analyze soy sauce strains isolated from a commercial soy sauce qu .A phylogenetic tree of these strains was constructed according to their RAPD PCR patterns, and it basically matched the tree constructed from traditional morphological toxicology (Fig. 9) .

Table 16 Changes in microbial population (in cfu mL^{-1}) during soy sauce fermentation

	Fermentation time		
Microorganism	30 days	40 days	50 days
Total bacteria	5.7×10^4	8.8×10^4	9.5×10^4
Enteric bacilli	1.0×10^2	1.0×10^2	1.0×10^2
Bacillus	1.0×10^3	5.0×10^2	3.2×10^3
lactic acid bacteria	2.5×10^5	1.5×10^5	3.5×10^5
Anaerobic microorganisms	2.3×10^5	1.1×10^5	1.0×10^5
Yeast	5.6×10^3	3.4×10^3	2.1×10^2

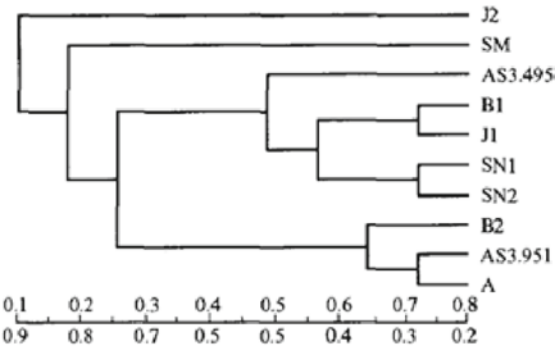


Fig. 9 Phylogenetic tree of soy sauce strains isolated from commercial soy sauce qu

Yeasts and bacteria are also very important in soy sauce production. Xie and coworkers identified yeasts and bacteria in high-salt diluted-state fermentation based on a systematic analysis. *Torulopsis, Pichia, Trichosporon, Rhodotorula, Candida,* and *Saccharomyces* are familiar yeasts and the number of these yeasts is helpful in judging the fermentation status during soy sauce production (Table 17 [55])**.**

Zhang observed variations in the microflora in different low-salt solid-state fermentation processes [56, 57]. At the end of fermentation, the total numbers of bacteria, yeast, and *Aspergillus* were 2.8×10^5, 5.8×10^5, and 5.2×10^5 cfu g^{-1}, respectively.

The soy sauce industry has improved traditional production technology and adopted a more complex multistrain qu making process [58]. It now better understands the actions of enzymes secreted by these microorganisms and employs a few commercial enzymes [42, 59].

3.3.2 Aroma and Flavor in Soy Sauce and Immobilization Technology

In soy sauce production, molds are mainly responsible for digestion of starch and protein raw materials into umami, sweet amino acids, and peptides (Table 18), while yeast and bacteria take important roles in flavor formation. Ethanol, higher alcohols, aromatic fusels, esters, and 4-hydroxy-furanone are the main components of flavor in soy sauce [60]. Cao [61] analyzed Chinese soy sauce samples for soluble salt-less solid components, total nitrogen, and amino nitrogen. Their results suggest that ethanol and acetic acid may improve the flavor of soy sauce.

Table 17 Amount of yeast (in cfu mL^{-1}) in soybean sauce liquor

	Abnormal fermentation			Normal fermentation		
Process	30 days	40 days	50 days	30 days	40 days	50 days
High salt	1.0×10^5	1.7×10^5	1.8×10^4	5.6×10^3	3.4×10^3	2.1×10^2
6% NaCl	1.5×10^5	1.4×10^5	4.6×10^4	2.7×10^3	2.9×10^3	2.9×10^2
6% NaCl	1.4×10^5	1.8×10^5	5.6×10^4	3.1×10^3	$2.7v10^3$	2.2×10^2

Table 18 Acidic and alkaline peptides in soy sauce

Acidic peptides		Alkaline peptides	
Gly–Asp	Asp–Glu	Gly–Glu	Ser–Ser
Gly–Glu	Glu–Gly	Gly–Thr	Ser–Pro
Als–Asp	Glu–Ala	Gly–Ser	Ser–X
Ala–Glu	Glu–Asp	Gly–Gln	Thr–Thr
Asp–Ala	Glu–Glu	Gly–Pro	Thr–Pro
Asp–Asp	Glu–(Thr/ser)	Gly–X	Thr–X
Asp–(Thr/ser)	Glu–Glu-Glu		Ala–Pro

X: unidentified amino acid

We now know that *Zygosaccharomyces rouxi, Candida etchellsii, Candida versatilis*, and other salt-tolerant yeasts are important in enhancing the flavors attributed to alcohols, esters, and 4-hydroxyl-2(5)-ethyl-5(2)-methyl-3(*h*)-furanone (HEMF) in soy sauce fermentation [62]. Figure 10 shows the metabolic pathways of these compounds in *Zygosaccharomyces rouxi*.

The buildings and the jars where the mash is made represent important variable environmental factors. Different bacteria appear during the various stages of fermentation. In addition, certain geographical regions have different bacterial cultures that contribute certain distinct characteristics to the flavor. During the last few decades there has been significant progress on understanding the action of flavor substances and the biochemical mechanisms of flavor formation in immobilized cells (Table 19) or immobilized enzymes from yeasts and bacteria [63, 64] .The microorganism secreted protease, peptidase, amylase, glutaminase, pectinase, cellulase,

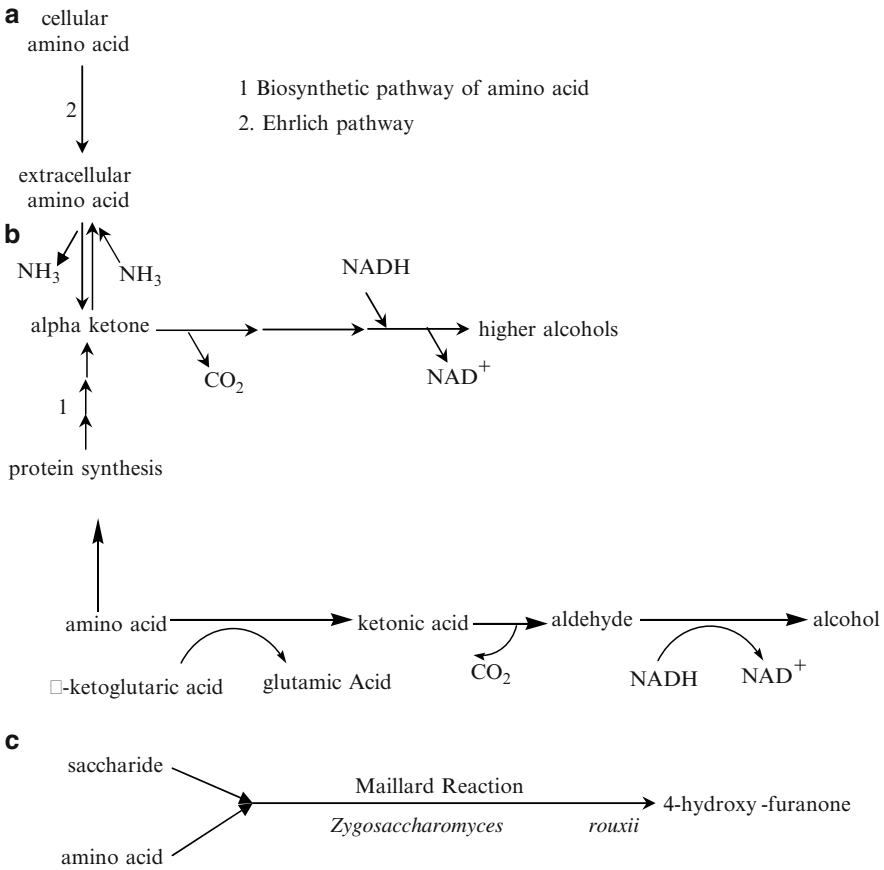


Fig. 10a–c Metabolic pathways of important flavor components from *Zygosaccharomyces* rouxi. **a** Higher alcohols. **b** Ethanol. **c** 4-Hydroxy-furanone

Table 19 Soy sauce production by immobilized cells

Batch	Alcohol (vol. %)		Reducing sugar (wt%)		Amino aicd N (% w/v)		NaCl (% w/v)		Soluble solid without salt (% w/v)		Total acid (% w/v)	
1	0.1	0.31	5.3	4.8	0.88	0.81	16.4	16.4	20.1	20.1	1.95	1.84
2	0.04	0.43	4.8	3.9	0.78	0.8	15.5	15.5	19.5	18.5	2.01	2.01
3	0.09	0.42	4.6	3.7	0.81	0.78	15.5	15.3	18.5	18.5	2.01	1.77
4	0.07	0.37	5.1	4.0	0.79	0.8	15.2	15.2	19.8	19.1	2.04	1.83
5	0.08	0.5	5.1	3.7	0.76	0.75	15.4	15.5	18.8	18	2.24	1.97
6	0.06	0.43	4.7	3.7	0.72	0.72	16.7	16.6	18.8	18.5	2.33	2.15
7	0.13	0.48	4.5	4.1	0.74	0.72	17.3	17.3	17.7	17.3	1.92	1.84
8	0.05	0.41	5.1	3.5	0.67	0.65	17.2	17.3	18.3	17.7	2.15	2.15
9	0.03	0.67	4.1	3.4	0.77	0.76	14.9	15.1	20.9	19.7	2.34	2.28
10	0.06	0.64	5.1	3.8	0.65	0.66	16.8	16.8	18.4	17.9	2.26	2.22
11	0.05	0.57	4.6	3.6	0.66	0.67	16.7	17	18.3	17	2.22	2.24
12	0.08	0.62	4.9	3.6	0.62	0.62	17.2	16.9	17.3	17.1	2.19	2.15

and hemicellulase into the mash during the qu making process. All these enzymes have been commercially applied.

3.3.3 Health and Safety Issues of Soy Sauce

Soy sauce not only improves consumer appetite but is also highly nutritional. Soy sauce is made from soybean, which provides the Chinese people a healthy and inexpensive source of protein. Two pounds of soy flour contains approximately as much protein as does five pounds of meat. Soy sauce contains some trace compounds that can reduce the cholesterol level, and researchers in Singapore have recently identified a natural compound in soy sauce that possesses antioxidant activity [65].

Soy sauce also possesses antimicrobial activities against bacteria and contains an antihypertensive component, specifically an angiotensin I converting enzyme inhibitor. The active compound has been identified as nicotinamine, which comes from soybean. The important flavor component of soy sauce, 4-hydroxy-2-(5)-ethyl-5(2)-methyl-3(2*h*)-furanone (HEMF), also exhibits antioxidant and anticarcinogenic properties. Fermented soy sauce contains three tartaric isoflavone derivatives called shoyuflavones. (Note that "shoyu" is a Japanese term for soy sauce.) These shoyuflavones have inhibitory activities against histidine decarboxylase, which produces histamine, a mediator of inflammation, allergy, and gastric acid secretion. In soy sauce, beta-carbolines are the active compounds that exhibit antiplatelet activities.

Presently, there are numerous new products that alter the flavor and nutrition of soy sauce. In China, about 25% of children and 20% of adult male suffer from iron-deficiency anemia [66]. Without changing the cooking methods and its flavor, soy sauce fortified with NaFeEDTA can be easily absorbed by the body, thereby help alleviate anemia symptoms. Savvy consumers increasingly pay attention to iron-deficiency problems. HACCP (hazard analysis and critical control point) system was established for the safety of soy sauce [67].

4 Chinese Traditional Vinegar

4.1 Introduction

4.1.1 History of Chinese Vinegar

Vinegar, defined directly and simply as a sour liquid, is an impure dilute solution of acetic acid produced by a two-stage fermentation process and commonly used as a condiment and preservative. Presently, Chinese vinegar is usually made by the submerged liquid fermentation of fruit juices. In contrast, the traditional Chinese vinegar or c*u* was made mainly from grains such as barley, millet, and rice by solid-state fermentation.

Chinese have been making vinegar from rice for more than 3,000 years. The first appearance of this sour liquid might had been in the Zhou Dynasty (12th century bc to 221 bc) as the seasoning *liu* described in such texts as *Zhou Li*, *Zuo Zhuan*, and *Mo Ti*. *Liu* is usually interpreted as "vinegar." It is also called *kujiu*, meaning bitter wine, which suggests that vinegar was once made from wine or alcohol. The professional workshop producing vinegar appeared in the Chunqiu period (770–476 bc). Between 369 and 404 ad, the Chinese techniques for making rice vinegar were introduced to Japan. In the Northern Wei Dynasty (386–534 ad), Sixie Jia described in detail in his book *Qi Min Yao Shu* 23 methods of vinegar brewing. In the Tang Dynasty (618–907 ad), vinegar was used as a preservative. Chinese vinegars are slightly sweeter than the Western white or cider vinegars. Its flavor is sometimes enhanced with the addition of spices. The average acidity is about 5–6% with some residual sugar remaining from the fermentation process. Nowadays in China, there are a variety of vinegar products as condiments, and the market demand for vinegar is expanding. With higher productivity made possible by the advancement of technology, a few traditional trademark brands dominate the Chinese vinegar market.

4.1.2 Fundamentals of Vinegar Production

Although some of the Chinese traditional vinegars are made by liquid fermentation, most manufacturing processes rely on solid-state fermentation, which combines the techniques for making grain (rice and sorghum) wine and soy sauce. As a result of the differences in the raw materials, saccharifying agents, and manufacturing techniques, there exist a variety of Chinese traditional vinegars. However, the underlying fundamentals are almost identical. Figure 11 outlines Chinese traditional vinegar

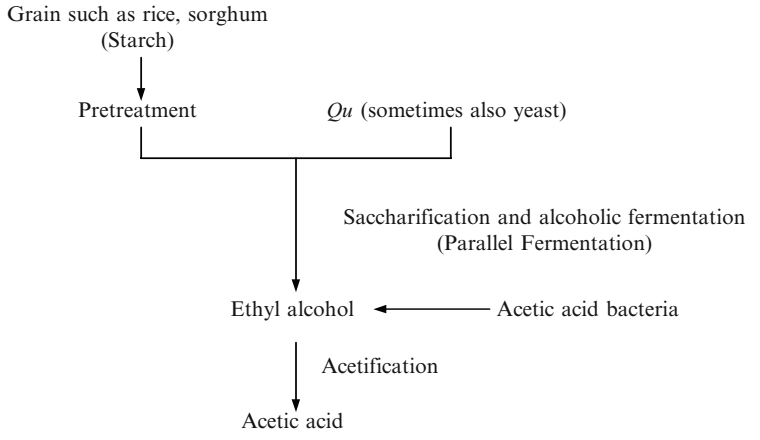


Fig. 11 Schematic of Chinese traditional vinegar production

production. Qu (koji in Japanese), molded cereal containing various microorganisms and enzymes as the starter or inoculum used to initiate various types of fermentations described earlier in this chapter, functions as saccharifying and fermenting agents in vinegar fermentation. "Parallel fermentation" combines progressive saccharification of starch with slow alcohol fermentation.

4.1.3 Vinegar Production and Use in China

As a condiment, fermented vinegar accounts for about a quarter of the Chinese seasoning production. The production of vinegar in China was more than 2 billion liters in 2005. There are many vinegar manufactories, and some of them operate under traditional trademarks, especially in Shanxi and Jiangsu provinces. The term *"Famous Four Chinese Vinegars"* applies to Shanxi aged vinegar, Zhenjiang aromatic vinegar, Sichuan's Baoning herbal vinegar, and lastly either Zhejiang rice vinegar or Fujian red rice vinegar. These vinegars start with different raw materials, are manufactured with different techniques, and finish with different flavors and properties. The factory with the highest capacity belongs to the Hengshun group that produces the Zhenjiang aromatic vinegar; its recent annual yield has been about 100 million liters.

Throughout Chinese history, fermented vinegar was added to food. Besides use as a condiment, vinegar is also a good solvent for the essential oils of herbs and spices. During the Song Dynasty (960–1,279), sushi made with rice, vinegar, oil, and various meat or raw fish was popular in China, but eventually became better known as a Japanese specialty. Vinegar also functions as a preservative due to its acetic acid content, which inhibits the growth of most spoilage and food-poison microorganisms. Pickling food in vinegar is one of the traditional techniques of food preservation, and the presence of salt (sodium chloride) in vinegar frequently enhances the preservative effect.

Vinegar's role in health has become more important with time. There exist Chinese records dating back to third century bc that describe the use of vinegar to cure various wounds. Later ancient Chinese medical literature describes a number of vinegar's other utilities. It is believed that vinegar could reduce blood pressure, cure colds, and prevent other epidemic diseases. According to Chinese medical theory, vinegar's flavor is sour and bitter and its nature or temperature is warm. It enters the liver and stomach channels, and its functions are to scatter stasis, stop bleeding, resolve toxins, and kill parasitic worms. In Chinese folk medicine, vinegar is a medicinal in its own right, capable of treating a myriad of disorders, including internal medicine, gynecological, dermatological, and traumatological conditions. The bio-functions and bioactive compounds of vinegar are attracting increasingly more attentions from the scientific community.

Although there are also ancient Chinese records that document vinegar's other nonfood uses such as fire-setting, the main use of Chinese vinegar remains with food.

4.2 Techniques of Chinese Vinegar Production

4.2.1 Raw Materials

Vinegar can be made from any nontoxic raw material that furnishes fermen Table sugars. In Chinese traditional vinegar production, the raw materials include principal materials, adjuvant materials, stuffing, and additives. The principal materials furnish abundant starch or sugars. The common principal materials are amylaceous grains or cereals (such as broomcorn millet, rice, maize, and sweet potato) that can be converted into fermenTable sugars. Millet, wheat, barley, and other grains also can be used as principal materials. For example, Shanxi aged vinegar is made chiefly from broomcorn millet (sorghum vulgare), and Zhenjiang aromatic vinegar is made from glutinous rice.

Adjuvant (supplementary) materials frequently are wheat bran or rice meal, which supply important nutritional substrates and hydrolytic enzymes (such as b-amylase) for vinegar production. Stuffing is provided by wheat bran, but can also be corncob and hulls of peanut, rice, broomcorn, or other grains. Their functions are to absorb water, loosen the c*u pei* (fermented grain), store air, and facilitate acetic acid fermentation.

Additives such as salt, sugar, parched rice and spices modify the color, enhance the flavor, and alter the taste of vinegar. The appearance of vinegar is usually improved by adding coloring agents produced from rice browned by parching. Salt can also inhibit the growth of microorganisms and acetic acid degradation.

Qu itself can also be regarded as a raw material. In some recipes, the ratio of qu to principal material can be up to 0.6:1. The use of different qu depends on the type of vinegar. Qu not only provides microorganisms and enzymes for vinegar fermentation, but also contributes various complex flavors and nutrient components.

The main chemical components of the raw materials include carbohydrate, cellulose, hemicellulose, and protein. Carbohydrate provides the primary carbon and energy source needed in vinegar production; cellulose and hemicellulose contribute to the stuffing; and amino acids produced by protein hydrolysis improve vinegar's nutrition, appearance, and flavor.

4.2.2 Microorganisms in Chinese Vinegar Production

Chinese traditional vinegar, just like traditional Chinese liquor and rice wine, is produced via a process of mixed culture fermentation. However, few researchers have studied the microorganisms present in Chinese vinegar production.

Various microorganisms have distinct functions in different phases of vinegar production. In grain saccharification, microorganisms, especially molds, from qu produce amylolytic enzymes and proteases. The dominant molds depend on the types of qu. These molds include *Aspergillus* sp (*A. usamii, A. batatae, A. niger,*

A.oryzae, etc.), *Rhizopus* sp, *Mucor* sp, and *Absidia* sp. The production of Fujian red rice (ang-kak) vinegar calls for ang-kak (meaning red yeast/fermented rice) on which *Monascus* sp grows and produces amylolytic enzymes.

The microorganism used in alcoholic fermentation is normally a yeast strain of *Saccharomyces cerevisiae*, which converts the sugars produced from saccharification into ethanol. *Hansenula anomala* and *Candida berkhout* are found to produce alcohol during vinegar production. After that, acetification (acetic acid fermentation) is carried out by acetic acid bacteria. Acetification is the oxidation of ethanol by bacteria to produce acetic acid and water. Acetic acid bacteria include *Acetobacter* sp and *Gluconacetobacter* sp, which come naturally from air, materials, appliances, and the environment in traditional vinegar production. This process also attracts other microorganisms such as *Lactobacillus* sp, *Staphylococcus* sp, *Enterobacter* sp, *Pseudomonas* sp, *Flavobacterium* sp, and *Sinorhizobium* sp. It is believed that they contribute to the generation of vinegar's flavor.

4.2.3 Vinegar Production by Solid-State Fermentation Process

Chinese vinegar is traditionally produced by solid-state fermentation. This process, called "parallel fermentation," starts at a low temperature and combines progressive saccharification of starch with slow alcohol fermentation. Glucose is first gradually liberated from starch in pretreated grains by the action of glucoamylase and is then slowly fermented into alcohol by yeast. Subsequently, secondary fermentation to vinegar acid is performed by acetic acid bacteria mixed with wheat bran or rice meal. Vinegar is extracted, followed by aging and posttreatment before bottling.

Depending on the saccharifying agents, raw materials, processes, and flavor, Chinese traditional vinegars by solid-state processes can be categorized as daqu vinegar, xiaoqu vinegar and fuqu vinegar. The saccharifying agents, qu, are the same as those jiuqu used in Chinese liquor making.

Daqu Vinegar

A representative of daqu vinegar is Shanxi aged (5–8 years old) vinegar popular in north China. It uses sorghum as the raw material and daqu (made from barley, wheat and peas, and *Mucor* sp. and *Rhizopus* sp. as the dominant microorganisms) as the saccharifying and fermenting agent. The process involves 6–7 months of soaking, steaming, and cooling of the chosen grain, primary fermentation and secondary fermentation (in urns, jars, or fermenting pools), sunning during daytime, and exposure to the cool night air for aging, followed by straining and pasteurization before bottling. Sunning and icing during aging greatly concentrate the various components of the vinegar except the volatile acids. Its vapor possesses a distinctive smoky fragrance. Preparation of Shanxi aged vinegar is very complex and is schematically shown in Fig. 12.

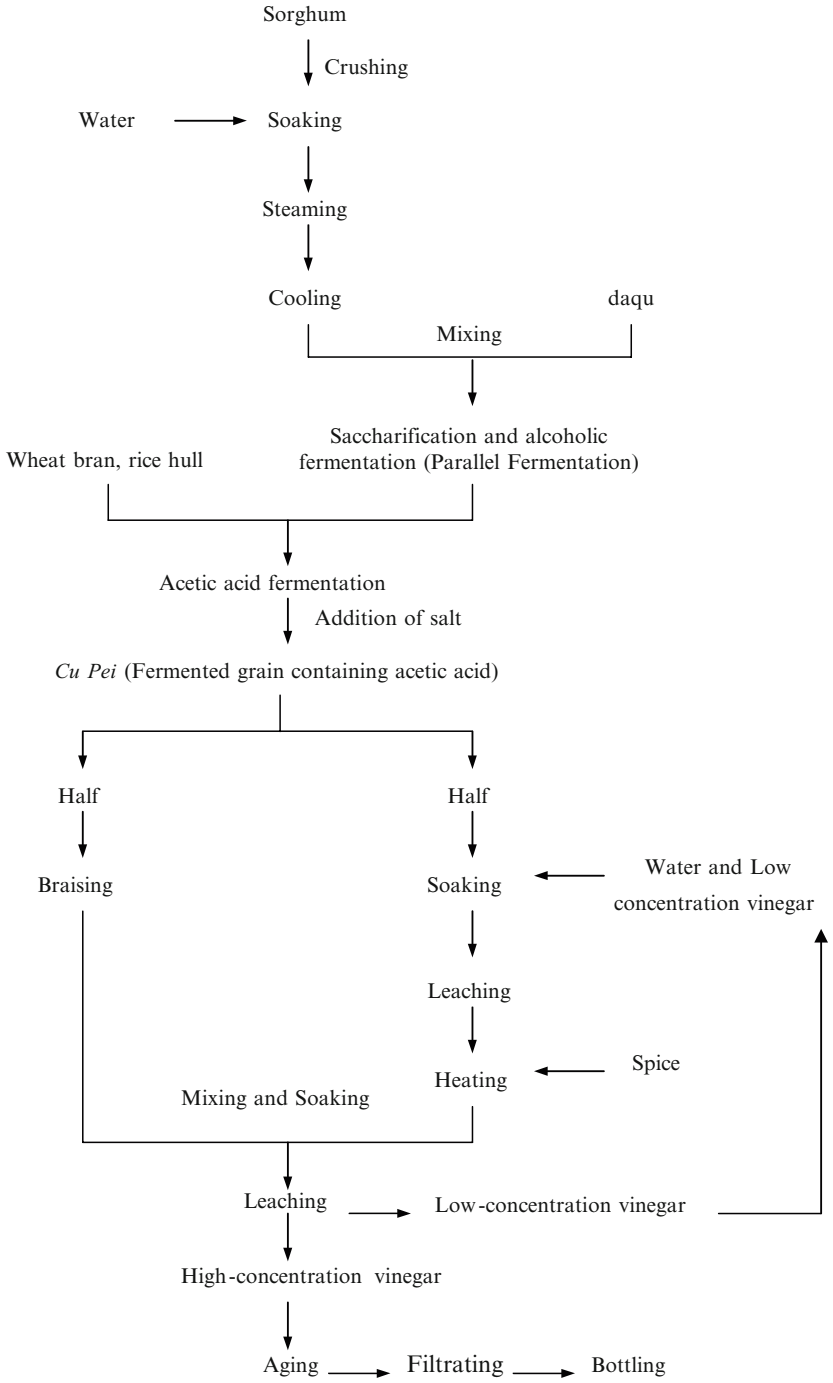


Fig. 12 Preparation of Shanxi aged vinegar

Xiaoqu Vinegar

The process of xiaoqu vinegar, which originated in south China, is similar to daqu vinegar, except that it uses sticky rice as the raw material and xiaoqu (usually made from mixing rice and *Aspergillus* sp., the dominant microorganism) as the saccharifying and fermenting agent. After the alcohol fermentation step, the lao (fermented mash) is mixed with wheat bran or rice hull and a portion of the cu pei (fermented grain) from the previous batch, which is rich in acetic acid bacteria. During the solid-state acetic acid fermentation phase, new cu pei needs to be continually turned to expose fresh surface. Subsequently, cu pei is soaked and fresh vinegar leached out. Finally, aging is carried out in hermetic urns or jar fermentors (Fig. 13). The Zhenjiang aromatic vinegar is a typical xiaoqu vinegar, which is produced from sticky rice through surface acetic acid fermentation. It gives off the aroma of esters such as ethyl acetate without smelling smoky. It tastes smooth, sour, and a bit sweet.

Since xiaoqu vinegar is made from sticky rice and the process of alcoholic fermentation is similar to making rice wine, modern techniques and equipment for rice wine or alcoholic industry have been introduced in vinegar production.

Fuqu Vinegar

The fuqu vinegar uses bran as the raw material and fuqu, which is a mold cultured in a solid medium, as the saccharifying and fermenting agent. *Aspergillus* sp. and yeasts are grown as separate pure cultures and inoculated to the above mixture. The production process of fuqu vinegar is characterized by short fermentation time and low production cost; thus, it has become popular in China, although its flavor is not as well developed as daqu and xiaoqu vinegars. Fuqu vinegar production, like

Fig. 13 Aging of Chinese vinegar in hermetic jar fermentors

xiaoqu vinegar production, consists of two types each having two phases: (1) solid-state alcohol fermentation followed by solid-state acetic acid fermentation and (2) liquid-state alcohol fermentation followed by solid-state acetic acid fermentation. Acetic acid fermentation usually occurs in a fermenting pool (Fig. 14). The underlying production principles are similar to those mentioned above.

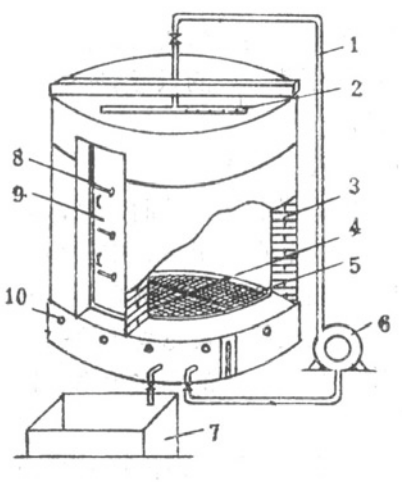
4.2.4 Vinegar Production by Liquid-State Fermentation Process

In China, as in Europe, some famous vinegars, such as Zhejiang rice vinegar and Fujian red rice (ang-kak) vinegar, are produced via acetic acid fermentation in either liquid-state or semi-liquid-state, which is less complicated than solid-state. The raw material, usually rice, is first converted into alcohol, and vinegar is subsequently produced. Certain vinegars start directly with Chinese liquor as the raw material.

Vinegar production via liquid-state fermentation employs several techniques, including surface fermentation (surface culture), sparged fermentation (quick vinegar process), and submerged fermentation. These techniques have been described by Adams [68]. In these processes, adjuvant materials are unnecessary.

4.3 Development of Chinese Vinegar

Although vinegar has a long history in China, the science and technology related to vinegar started to develop in recent decades in the areas of microbiology, bioengineering, health care and medicinal functions of Chinese vinegar.



1- External recycleloop
2- Sparger
3- Pool wall
4- Support
5- False bottom
6- Pump
7- Storage
8- Thermometer
9- Discharge
10- Air holes

Fig. 14 Acetic acid fermenting pool

4.3.1 Microecology in Chinese Vinegar Production

The production of Chinese traditional vinegar hinges on fermentation by multiple microorganisms, and relevant microbiology is attracting researchers' attention. Microecology in Chinese vinegar production has been investigated recently. Xu et al. constructed the 16S rDNA library via PCR and analyzed the bacterial communities in aerobic solid-state fermentation of Zhenjiang Hengshun vinegar [69]. Based on a phylogenetic analysis (Fig. 15), the microorganisms in Hengshun vinegar fermentation were found to include *Lactobacillus*, *Acetobacter*, *Gluconacetobacter*, *Staphylococcus*, *Enterobacter*, *Pseudomonas*, *Flavobacterium*, and *Sinorhizobium*.

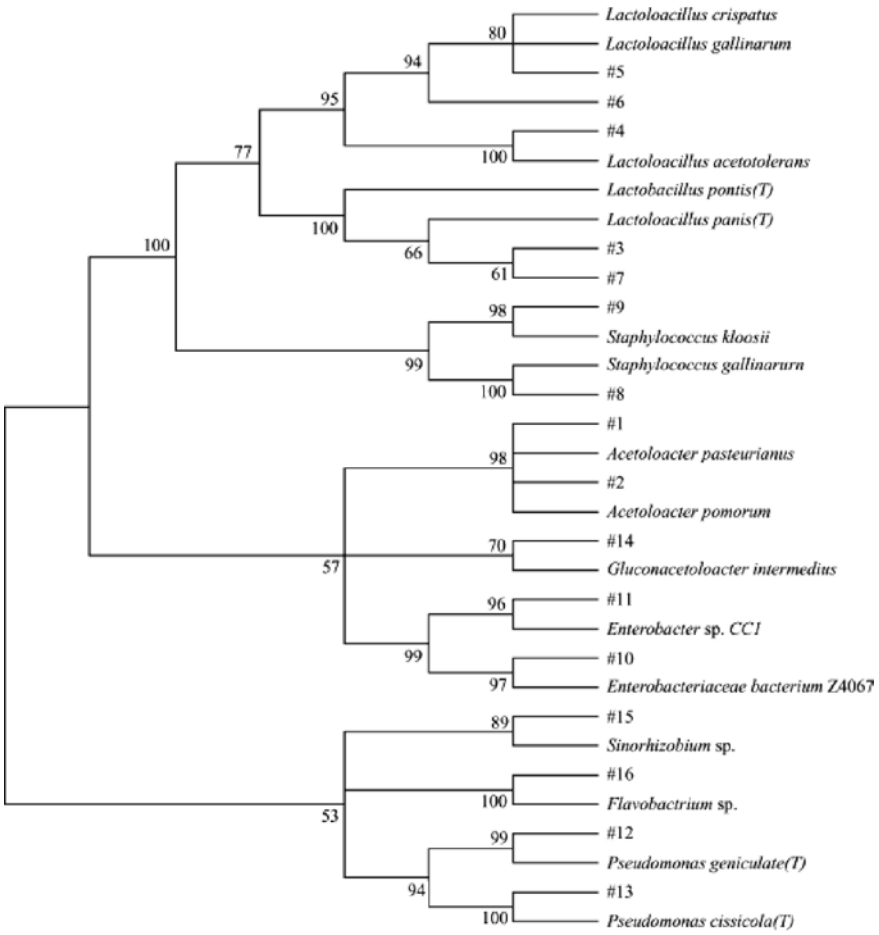


Fig. 15 Phylogenetic tree based on representative sequences of 16S rDNA from 16 bacterial communities in Zhenjiang Hengshun vinegar fermentation

Because of the multiple microorganisms involved in the production of Chinese vinegar, the metabolites present in vinegar are complex, and product properties are varied. For example, based on our unpublished data, Hengshun aromatic vinegar contains acetic acid at levels 20–40 g L^{-1}, lactic acid at 10–20 g L^{-1}, and a variety of other organic acid such as citric acid, succinic acid, malic acid, pyruvic acid, and tartaric acid. Hence, vinegar's sourness comes from not just acetic acid, and its flavor is affected by an assortment of compounds as well.

4.3.2 Modification of Techniques and Equipment for Chinese Vinegar Production

In recent years, a growing vinegar market demands matched growth in vinegar production. The combined vinegar production process of liquid-state alcohol fermentation followed by solid-state acetic acid fermentation is well suited to scale-up. To increase vinegar output, many manufactories have adopted techniques and equipment of modern alcohol and rice wine fermentation.

Traditionally, alcohol and acetic acid fermentation of Chinese vinegar are performed in jar fermentors (Fig. 16). Nowadays, vinegar is produced in large-scale modern fermentors or fermentation pools. Lessons learned in mature rice wine or alcohol industry ease transitioning to large-scale fermentors for vinegar production in recent years (Fig. 17a). For example, Hengshun Vinegar Co. has newly installed 340-M^3 fermentors. The use of large-scale fermentors improves conversion of raw materials, decreases production costs, facilitates process control, and greatly increases productivity.

Large-scale acetic acid fermentation pools (Fig. 17b) have replaced traditional jar fermentors. Technically designed upturners can automatically turn the surface of cu pei (Fig. 18), ensuring proper oxygen supply for acetic acid fermentation.

Fig. 16 Production of Chinese traditional vinegar in jar fermentors

Fig. 17a,b Vinegar production with large-scale alcohol fermentors (**a**) and acetic acid fermentation pools (**b**)

Fig. 18 The machine upturner automatically turns cu pei in acetic acid fermentation pool

This equipment reduces labor intensity and increases production efficiency in vinegar production.

Other bioprocess innovations have appeared in vinegar production. Some examples are the newly developed acetic acid fermentation agents and liquid-state acetic acid fermentation operated in a continuous mode with immobilized microorganisms.

4.3.3 Health Care and Medicinal Uses of Chinese Vinegar

Vinegar has a list of health benefits and a long history of medicinal uses. Vinegar, commonly used as a food condiment, has high nutritional contents and special pharmacological benefits, such as promoting recovery from exhaustion [70], regulating blood glucose [71] and blood pressure [72], aiding digestion [73], stimulating appetite [74], promoting calcium absorption [74], and antioxidation [75]. Vinegar capsule has been produced and marketed as a health supplement in China.

Table 20 Tetramethylpyrazine content in Zhenjiang aromatic vinegar during aging

	Tetramethylpyrazine ($\mu g\ mL^{-1}$)	Acid ($g\ L^{-1}$)	Tetramethylpyrazine/acid ($\times 10^{-4}$)
Fresh vinegar	<3.0	44.7	<0.67
Mature vinegar	5.8	57.6	1.01
Storage 5 days	21.9	57.2	3.83
Storage 10 days	24.0	57.0	4.21
Storage 15 days	22.3	57.4	3.89
Heating	35.5	53.6	6.62
Aging 2 months	77.0	55.0	14.0
Aging 2 years	>500	65.5	>76.3

The bio-regulatory functions of vinegar are related with its various compounds. Vinegar made from grain is rich in polysaccharides, phenolic compounds, and proteins. It undergoes profound molecular changes during fermentation, extraction/concentration, storage and aging. Xu et al. showed that total phenolic compounds and total flavones could be indicators of antioxidant activities of Chinese rice vinegar [75]. Melanoidins, the brown polymers formed through Maillard reaction in the Zhenjiang aromatic vinegar process, can promote health [76]. Tetramethylpyrazine, which form from Maillard reaction products in vinegar, has antioxidant property [77]. Zhenjiang aromatic vinegar has a high tetramethylpyrazine content, which increases with aging (Table 20) [78].

Analysis of the compositions of Chinese vinegar shows an abundance of organic acids and amino acids. For example, Hengshun aromatic vinegar contains 2 $g\ L^{-1}$ arginine and 0.8 $g\ L^{-1}$ glutamic acid. Many other compounds are also being found gradually. Further investigations into the bio-active compounds in vinegar are warranted.

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Modern Biotechnology in China

Qing-Zhao Wang and Xue-Ming Zhao

Abstract In recent years, with the booming economy, the Chinese government has increased its financial input to biotechnology research, which has led to remarkable achievements by China in modern biotechnology. As one of the key parts of modern biotechnology, industrial biotechnology will be crucial for China's sustainable development in this century. This review presents an overview of Chinese industrial biotechnology in last 10 years. Modern biotechnology had been classified into metabolic engineering and systems biology framework. Metabolic engineering is a field of broad fundamental and practical concept so we integrated the related technology achievements into the real practices of many metabolic engineering cases, such as biobased products production, environmental control and others. Now metabolic engineering is developing towards the systems level. Chinese researchers have also embraced this concept and have contributed invaluable things in genomics, transcriptomics, proteomics and related bioinformatics. A series of advanced laboratories or centers were established which will represent Chinese modern biotechnology development in the near future. At the end of this review, metabolic network research advances have also been mentioned.

Keywords Bioinformatics, China, Flux analysis, Genomics, Metabolic engineering, Metabolic network, Metabolomics, Modern biotechnology, Pathway analysis, Proteomics, Transcriptomics

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Contents

1 Introduction

Since the late 1970s, China's economy has developed so fast that it has attracted world attention. However, the present development still relies on huge consumption of resources, especially detrimental to the environment. To maintain quick and environmentally friendly development, China had already identified biotechnology as one of the key topics for the new century at least 5 years ago. As expected, biotechnology will be the key impetus for agriculture and food, industrial biotechnology, environmental control and public health related categories. Currently, there are nearly 200 key national laboratories with more than 20,000 researchers. China also adopted many projects to attract talented researchers back from overseas.

Thanks to the efforts of all the biotechnology related researchers, China has made many remarkable achievements in this field such as with the rice genome [1] and stem cells [2]. In this review we do not intend to list all the achievements of the Chinese biotechnology researchers; instead we just focus on the categories of metabolic engineering and systems biology, especially in the microorganisms related biorefinery category. FIRST, we begin with the definition of traditional and modern biotechnology. Then, the detailed research findings and methods are analyzed in the field of metabolic engineering. Finally, we try to list some important achievements in the framework of systems biology. This review is definitely not a complete picture of Chinese biotechnology developments in recent years. We just want to help you understand more about modern Chinese biotechnology, especially regarding fundamental metabolic engineering development.

2 From Traditional Biotechnology to Modern Biotechnology

China is one of the prime centers for the rise of human civilization. In the very long history of civilization, China had grasped many complicated techniques, especially in applying knowledge of life and traditional biotechnology. According to the research findings on ancient organics absorbed into pottery jars from the early Neolithic village of Jiahu in Henan province of China, they could produce one kind of mixed fermented beverage of rice, honey, and fruit as early as the seventh millennium before Christ [3]. Numerous clues have shown that traditional Chinese biotechnology developed proficiently. At the present time China is still the world top producer of vinegar, soy sauce, and drinking alcohol. These are all traditional Chinese foods. Almost all the traditional biotechnology processes are based on spontaneous reactions so it is usually categorized into traditional biotechnology or classical biotechnology. In fact, any attempt to explain the term "modern biotechnology" and to distinguish it from the classical biotechnology soon reveals that the boundary is anything but distinct and frequently disappears in a terminological fog [4]. We also do not want to try to define a clear difference between classical and modern technology. In this review, all rational use of microorganisms or other cells are considered to be our topics.

The first steps in the rational use of microbes can be traced back to the nineteenth century when Pasteur introduced pure cultures. In the 1940s antibiotics were produced and the productions of penicillin reached thousands of tons as early as 1948. China soon adopted the technology and processes; the current output of antibiotics, vaccines and vitamins is ranked number one in the world. For some kinds of bulk chemicals, such as citric acid, China has innovated different fermentation substances, organisms and specialized processes techniques. It is already the world's top producer and exporter of citric acid. However, in the new century, researchers have realized more and more the importance of recombined DNA and related techniques. With these techniques, people can rationally design and implement many changes in genetic levels. They are faster than the old mutation techniques and more reasonable in themselves. These directed modification methods are currently called metabolic engineering. Although the exact meaning varies from author to author, the general definition is "the directed improvement of product formation or cellular properties through the modification of specific biochemical reactions or the introduction of new ones with the use of recombinant DNA (metabolic engineering)." It generally includes analysis and synthesis steps and metabolic network and pathway analysis is the core of metabolic engineering.

Since the first sequencing organism, *Haemophilus influenzae,* in 1995, genome sequencing initiatives have been performed in over 200 organisms, including 23 archeal, 236 bacterial and 39 eukaryal genomes. The huge amount of data catalyzed the development of systems biology. Systems biology is the study of an organism viewed as an integrated and interacting network of genes, proteins and biochemical reactions which give rise to life. The metabolic engineering founder, Bailey, twice illustrated the development trends of metabolic engineering towards systems biology [5, 6].

The general accepted framework of systems biology had been illustrated in Fig. 1. Together with the bioinformatics, genomics, transcriptomics, proteomics and metabolomics, it plays a key role in modern biotechnology. In our review, we will classify modern biotechnology development in China based on this framework and introduce the advances made under that heading.

With the development of metabolic engineering and systems biology, the Chinese government also increased input to it. In 2007, the Institute of Microbiology, Chinese Academy of Sciences initiated the state key development project (2007CB707800): Microbial cell factories for biorefinery; Beijing University of Chemical Technology started the project of "The basic science of bioprocess in industrial biotechnology." The Chinese government also applied great resources to modern biotechnology development through the National Natural Science

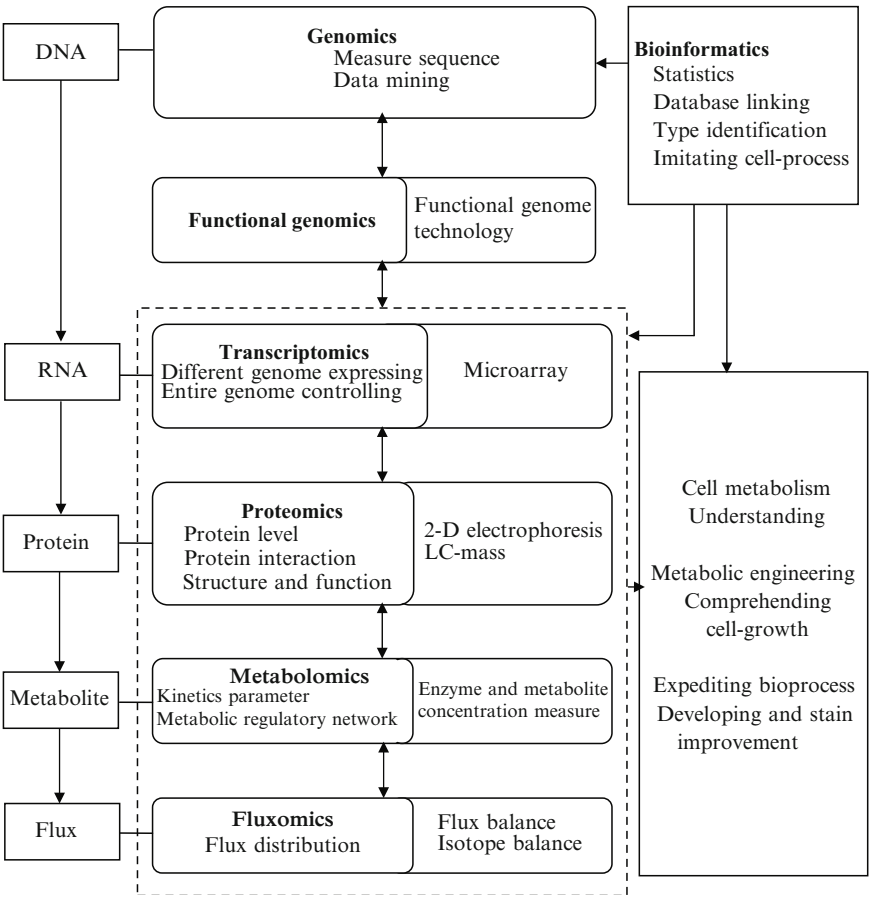

Fig. 1 Systems biology framework representation [110]

Table 1 The National Basic Research Program of China supported metabolic engineering projects in bioindustry

Projects	Number	Implementing units
The basic science of microbial cell factories for biorefinery	2007CB707800	Institute of microbiology, Chinese Academy of Sciences
The basic science of bioprocess in industrial biotechnology	2007CB714300	Beijing University of Chemical Technology
The basic science of transforming biomass to high level biofuel	2007CB210200	Zhejiang University and University of Science and Technology of China
The basic science of biocatalyzing and biotranforming	2003CB716000	Tsinghua University
The basic science of microbial metallurgy	2004CB619200	Ministry of Education, Zhongnan University
The basic science of chemical and microbial methods to improve the oil yield	2005CB221300	PetroChina Company Limited of China
The basic science of extremophiles and the applications of its function	2004CB719600	Chinese Academy of Sciences
Agricultural microbial functional gene discovery and mechanism research	2003CB114200	Chinese Academy of agricultural Sciences

Foundation and State 863 projects. Table 1 lists some important government supported research projects in China.

3 Metabolic Engineering in China

Metabolic engineering is a highly multidisciplinary field and has broad fundamental and practical importance to modern biotechnology. According to the Scopus searching results, the published papers and patents with metabolic engineering as key words by Chinese teams increased steadily from 2000 to the present (see Fig. 2).

The papers increased sharply in the periods 2000–2001 and 2004–2005. These two periods reflected that Chinese researchers had accepted the metabolic engineering concept and applied it in particular problems. In this review, based on the slightly modified classification of Cameron and Tong [7], we organized the hot metabolic engineering applications in China into five basic groups, i.e., (a) yield and productivity improvement of biobased products, (b) expansion of the range of the substances, (c) cellular properties improvement, (d) environmental related pollutants or xenobiotic degradation, and (e) specialties and novel products.

3.1 Yield and Productivity Improvement of Biobased Products

Yield and productivity are the key indexes for biobased products industrialization, especially large volume and low cost bulk chemicals. In the past few years, Chinese

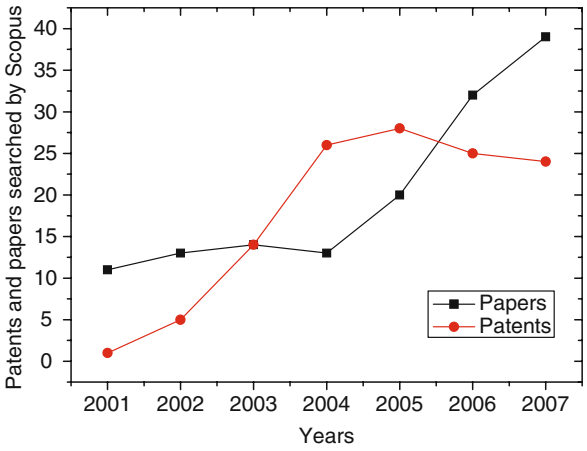

Fig. 2 Published papers and patents searched by Scopus with metabolic engineering as the key words

researchers have put much effort into this and research findings cover almost all the important products including ethanol, 1,3 propanediol (1,3-PD), polyhydroxyalkanoates (PHA), organic acids, amino acids, vitamins and some speciality products.

Ethanol is an important biofuel to replace fossil fuels. To improve ethanol production and reduction of glycerol in *Saccharomyces cerevisiae*, many mutants were constructed, which involved GPD1 and GPD2 gene deletion, which encodes a glycerol 3-phosphate dehydrogenase to synthesize glycerol, and the glutamate synthase encoded GLT1 over-expression [8, 9]. The FPS1, which encodes the glycerol transport enzyme, was also knocked out. Compared with the wild type, the ethanol yield of the *fps1D* mutant was improved by 10 ± 2% and glycerol yield decreased by 18.8 ± 2% [10]. Combined with the above manipulations, the recombined strains can produce 12.24% and 10.42% higher ethanol and 39.72% and 31.03% lower glycerol yield during anaerobic batch fermentations, respectively. Meanwhile, dramatic reduction of formation of acetate and pyruvic acid was observed in all the mutants compared to the wild type [11] (Fig. 3).

1,3-PD is an important bulk chemical which can be used as a monomer to synthesize a new type of polyester, polytrimethylene terephthalate (PTT). Currently, there are two different strategies for 1,3-PD fermentation. One is from glucose with *E. coli* and another is from glycerol with *Klebsiella pneumoniae.* Chinese researchers mainly focused on the second one. Zhang et al. found the enzyme, aldehyde dehydrogenase (ALDH) competes with 1,3-PD oxidoreductase for the cofactor NADH, so inactivation of ALDH resulted in close to zero ethanol formation but a significantly improved 1,3-PD production [12]. This report describes a novel redox potential (oxidoreduction potential [ORP])-based screening strategy for the isolation of mutants of *Klebsiella pneumoniae* which have an increased ability to produce 1,3-PD [13]. *Klebsiella oxytoca* M5al is an excellent 1,3-PD producer, but the excessive yield of lactic acid greatly reduced the fermentation efficiency for 1,3-PD. To counteract the disadvantage, four lactate deficient mutants

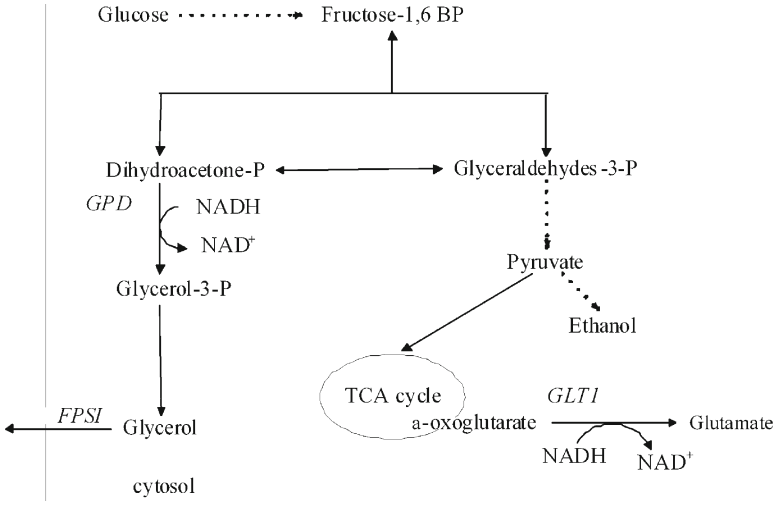

Fig. 3 Schematic representation of ethanol and glycerol metabolisms in yeast [11]

were obtained by knocking out the *ldhA* gene of lactate dehydrogenase (LDH) of *K. oxytoca* M5al. In fed-batch fermentation with sucrose as cosubstrate under microaerobic conditions, the 1,3-PD concentration, conversion, and productivity were improved significantly to 83.56 g L^{-1}, 0.62 mol mol^{-1}, and 1.61 g L^{-1} h^{-1}, respectively [14]. The structural gene *yqhD* from a wild-type *Escherichia coli* encoding 1, 3-propanediol oxidoreductase isoenzyme and the structural gene *dhaB* and *dhaT* from *Citrobacter freundii* encoding glycerol dehydratase were amplified and expressed in *E. coli*. [15–17], but the production level is still not competitive compared with *Klebsiella pneumoniae*. The process has been scaled up to 1,000 and 5,000 L [18] respectively. The best results in the 5,000 L fermentation were achieved, leading to 58.8 g L^{-1} 1,3-propanediol with a yield of 0.53 mol mol^{-1} glycerol and productivity of 0.92 g L^{-1} h^{-1}. The final 1,3-PD concentration led to a molar yield and volumetric productivity of 72 g L^{-1}, 57% and 2.1 g L^{-1} h^{-1}, respectively on a 1,000 L scale [19].

PHA, the natural polyester that many microorganisms accumulate to store carbon and reducing equivalents, has been considered as a future alternative to traditional plastic due to its special properties. Particularly, using metabolic engineering approaches, PHA structures and contents can be manipulated to achieve controllable monomer and PHA cellular contents (copolyesters consisting of 3HB and 3HHx). The main contribution comes from Tsinghua University, Professor Chen's group [20] (Table 2).

Key genes *phbA* and *phbB*, which encode β-ketothiolase and acetoacetyl-CoA reductase, respectively, from various bacteria have been identified and functionally expressed in *E. coli* [30]. To produce polyhydroxyalkanoates (PHAs) efficiently from substrate mixtures containing various carbon sources, *E. coli* phosphotransferase system (PTS) mutants were constructed, in which the carbon catabolite repression in the presence of glucose was released. When cultivated in the medium containing

Table 2 Metabolic engineering of microorganisms for production of copoplyesters (3HB and 3HA) from Chen's group

Organism	Gene manipulated	Substrates	Monomers composition	Wild type PHA	Recombinant PHA (%)	References
Aeromonas hydrophila 4AK4	*phaA, phaB, phaJ, phaC, phaP, vgb, Fad, tesA*	Lauratet and gluconate	70–97 mol% 3HB + 3HA	30–40%	50–70	[21–26]
Ralstonia eutropha PHB-4	P_{lac}, *phaC2Ps*	Gluconatetoc-tanoate	80 mol% 3HB + 3HA	0	34.6	[27, 28]
Pseudomonas putida KTOY01 (KT2442 *pha* deleted mutant)	P_{lac}, $phaC2_{Ps}$, *phbA, phbB*	Glucose or fatty acids	30 mol% 3HB + 3HA	0	10.2	[29]

P_{lac} lac promoter; P_{Re} promoter of *phbCAB* operon of *Ralstonia eutropha*; P_{Ps} promoter of *pha* operon of *Pseudomonas* sp 61-3; $phaG_{Ps}$ from *Pseudomonas* sp 61-3; $phaC1_{Ps}$ *phaC1* of *Pseudomonas* sp 61-3; $phaC2_{Ps}$ *phaC2* of *Pseudomonas stutzeri* 1317; *phbA, phbB* from *R. eutropha*; $phaJ_{Pa}$ *phaJ* from *Pseudomonas aeruginosa*

glucose and xylose, *E. coli* LR1010, harboring *phaCRe* and *phaAB Re* genes from *Ralstronia eutropha*, could simultaneously consume glucose and xylose and accumulate short-chain-length PHA, whereas *E. coli* LR1120 and LR1110, harboring *phaC1* gene from *Pseudomonas aeruginosa*, could accumulate medium-chain-length PHA. At the same time, LR1110 could simultaneously utilize a mixture of glucose and fatty acids. Semiquantitative reverse transcription polymerase chain reaction analysis indicated that disrupting phosphoenolpyruvate: sugar PTS in this strain released the repression on *fad* genes [31].

In order to increase the yield of pyruvate in *Torulopsis glabrata*, pyruvate decarboxylase (PDC, EC 4.1.1.1) that degrades pyruvate to acetaldehyde and ultimately to ethanol, was disrupted. Under favorable conditions in jar fermentation, the disruptants accumulated 82.2 g L^{-1} of pyruvate in 52 h [32, 33]. They also identified the manipulation of cofactor, such as thiamine, biotin, availability of NAD^+ and Ca^{2+} levels, as a potential tool to redistribute carbon flux in *Torulopsis glabrata* [34, 35].

Succinic acid is another potential biobased bulk chemical. Wang et al. found the expression of galactose permease and pyruvate carboxylase in *Escherichia coli ptsG* mutant increases the growth rate and succinate yield [36]. They also combined the dry experiments (genome-scale *E. coli* stoichiometric model flux balance analysis) and wet experiments very well. The final strain TUQ19/pQZ6 had a high yield of 1.29 mol succinate per mol glucose and high productivity [37]. Wu et al. implemented the two stage fermentation with *pflB, ldhA* double mutant, the overall yield and concentration of succinic acid reached 1.13 mol mol^{-1} glucose and 28.2 g L^{-1}, respectively in 5-L fermentors [38].

3.2 Expansion of the Range of the Substances

Gene bgl1 encoding β-glucosidase was cloned from *Saccharomycopsis fibuligera* and over-expressed in *S. cerevisiae*. The recombinant yeast strain could grow well on cellobiose as a sole carbon source and could be used for ethanol production through simultaneous saccharification and fermentation (SSF) of cellulose substrate [39].

The xylulokinase gene XKS1 was cloned from *S. cerevisiae* and expressed in pre-constructed recombinant yeast strain, which contains XYL1 and XYL2 genes from *Pichia stipitis*. Xylose consumption and ethanol production were respectively 120.9% and 36% higher than in the parent strain. Furthermore, the by-product xylitol was 0.7 g L^{-1}, a decrease of 84.9% [40].

3.3 Cellular Properties Improvement

Ethanol tolerance limit of *S. cerevisiae* is one key barrier for ethanol over-production. In order to increase these properties, Wei et al. proposed an effective, simple, and convenient method to improve yeast's multiple-stress tolerance with nine cycles of freeze-thaw [41]. Lei et al. indicated that the average size distribution of the floc populations of the self-flocculating yeast exerted great influence on the physiological status of yeast cells during the ethanol production process, leading to changes in plasma membrane composition [42].

For pyruvate production by *Torulopsis glabrata*, a high osmotic stress resistance mutant was selected and pyruvate concentration by the mutant strain RS23 reached 94.3 g L^{-1} at 82 h (yield on glucose 0.635 g^{-1}) in a 7-L fermentor with 150 g L^{-1} glucose as carbon source [43].

To conquer the oxygen fluctuation and hypoxic environments in fermentation, cloning of the hemoglobin gene from the *Vitreoscilla* is used widely. It has been used in *Pseudomonas putida* to biotransform aromatic compounds [44], in bacterium, *Rhodococcus erythropolis* for desulfurization [45] and for many other antibiotics and aerobic fermentation processes. There are at least 20 applications of hemoglobin gene expression.

3.4 Environmental Related Pollutants or Xenobiotic Degradation

The gene cassette (***camA*$^+$ *camB*$^+$ *camC***) encoding a cytochrome P-450cam variant was integrated into the nonessential gene *pcpM* of the pentachlorophenol degrader *Sphingobium chlorophenolicum* ATCC 39723 by homologous recombination. The recombinant strain could degrade hexachlorobenzene at a rate of 0.67 nmol mg (dry weight)$^{-1}$ h^{-1}, and intermediate pentachlorophenol was also identified [46]. The high

molecular weight polycyclic aromatic hydrocarbons-benzo pyrene, toluene, ethylbenzene and xylene were also targeted and reviewed by Chinese researchers [47, 48].

3.5 Speciality and Novel Products

The metabolic engineering techniques had also been used to produce a series of secondary metabolites such as astaxanthin [49], chitin oligosaccharides [50] and ubiquinone [51]. Most of the important secondary metabolites come from the plant cell. Although it is difficult to identify the exact biosynthetic pathways or rate limiting pathways of some secondary metabolites, there are still some attempts being made in China. Li et al. over-expressed the *Saussurea medusa* chalcone isomerase gene in *S. involucrata* hairy root cultures to enhance their biosynthesis of apigenin [52].

Zhao's group from Tianjin University completed one series of metabolic engineering attempts with riboflavin-producing *Bacillus subtilis*. *B. subtilis* is not a natural over-producer of riboflavin, but deregulation of its purine and riboflavin biosynthesis by means of classical mutagenesis resulted in riboflavin accumulation. The further metabolic designs of *B. subtilis* have turned it into an excellent producer, which can give a high riboflavin titer exceeding 20 g L^{-1}. The major metabolic designs methods are the following: (1) over-expression of riboflavin biosynthetic genes (riboflavin operon) and *ribA* gene; (2) redirection of electron flow to high coupling efficiency of terminal oxidase by knockout *bd* oxidase; (3) increase of precursor supply by over-expression of *gdh* gene and *purF* gene [53–56].

4 Genome Sequencing and Bioinformatics

China initiated its human genome project (HGP) in 1993 and Chinese scientists proposed a "two-one percentage" strategy to contribute to the international HGP, i.e., large-scale sequencing for 30 Mb of human genomic DNA (1% of the human genome) and cloning of 500–1,000 full-length human cDNAs for previously undefined genes based on gene expression profile analysis. China had established a series of high level genome research centers such as the Chinese National Human Genome Center at Beijing and at Shanghai, respectively. Genomic Information Research Center of Chinese Science Academy at Hangzhou. Chinese colleagues had made such great breakthroughs in so short a time that genomic research had attracted world attention. Nature and Science magazine all reported the "take off of China genome research" [57]. Here we will just review the important developments in microorganisms sequencing and genomic research.

4.1 Genome Sequencing and Genomic Research in China

China's genome sequencing work on microorganisms is outstanding, and contributes about 10% of the world reported sequenced microorganisms. The most remarkable

work is about the genome sequence and genomic studies of many virulent strains. Together with many other research centers, the Chinese National Human Genome Center at Shanghai sequenced *Leptospira interrogans*, which can cause flu-like episodes with frequent severe renal and hepatic damage. The genome information revealed a series of genes possibly related to adhesion, invasion and the hematological changes that characterize leptospirosis; this has provided clues about how an environmental organism might evolve into an important human pathogen [58]. *Xanthomonas campestris* pathovar *campestris* (*Xcc*) is the causative agent of crucifer black rot disease, which causes severe losses in agricultural yield world-wide. Qian et al. sequenced and annotated the complete genome of *Xcc* 8004, and compared it with that of *Xcc* ATCC 33913. Among the identified pathogenicity-related genes, three of unknown function were found in *Xcc* 8004-specific chromosomal segments. Their work may offer novel insight toward the development of efficient methods for prevention of this important plant disease [59] sequencing the genome of *S. cerevisiae* strain YJM789, which was derived from a yeast isolated from the lung of an AIDS patient with pneumonia were also sequenced [60]. The other important pathogen related microorganisms also include *Shigella flexneri* 2a and 5b [61, 62], *Salmonella enterica serovar Choleraesuis* [63], *Staphylococcus epidermidis* ATCC 12228 [64] and *Yersinia pestis* [65].

In the field of industrial microorganisms there has also been some important work. Feng et al. sequenced *Geobacillus thermodenitrificans* NG80-2, a thermophilic bacillus isolated from a deep oil reservoir in Northern China. Combined with proteomics, many long-chain alkanes degraded genes were identified. The NG80-2 genome provides an excellent platform for further improvement of this organism for oil bioremediation and other biotechnological applications [66]. Vitamin C is one of the most important bulk chemicals, which is usually fermented by Gram-negative facultatively anaerobic strain, *Ketogulonigenium* sp. One industrial scale vitamin C producing *Ketogulonigenium* sp. WB0104 was sequenced by State Key Laboratory for Molecular Virology and Genetic Engineering [67]. They identified L-sorbosone dehydrogenase, L- sorbose dehydrogenase and three other enzymes corresponding to the synthesis of 2-keto-L-gulonic acids. This complete sequence is a new platform for new metabolic engineering manipulations to improve vitamin C production, which is very crucial for China to maintain its position as the world's biggest vitamin C producer. In 2002, the Institute of Genetics and Development Biology, Chinese Academy of Sciences sequenced *Thermoanaerobacter tengconggensis*, which was isolated from a fresh water hot spring in Tengcong, China [68]. This sequence promotes a series of molecular and proteomic analysis, which will help us understand the mechanisms of hot tolerance.

4.2 Bioinformatics in Genome Research

Bioinformatics is an indispensable tool for us to understand the huge amount of genome information. The detailed research interests in genome informatics include gene-finding algorithms, database construction and comparative genomics.

Tianjin University Bioinformatics Center (Tubic) is one of the outstanding centers in genome informatics (http://tubic.tju.edu.cn(. Since 1998 when Tubic was founded, more than 50 papers have been published with more than 400 SCI citations. They proposed one new system, ZCURVE, for genes finding in yeast, bacterial and archeal genomes [69]. Dr Janet Thornton, Director of EBI, evaluated this paper as one of the F1000 papers, and made her comments on its value. Microbial gene-finding system, ZCURVE 1.0, has been used at 14 domestic and 46 foreign research institutes currently, and has been considered as one of the most accurate microbial gene finders.

Based on the Z-curve method, they also determined the first isochore map for the human genome and plant, *A. thaliana* [70]. They also use ZCURVE to predict replication origins (oriCs) of 520 bacterial genomes and genomic islands identification [71, 72]. Center for Bioinformatics of Peking University proposed one user friendly KO-Based Annotation System server for gene annotation and pathway identification [73]. Chen et al. also designed one software tool, GenomeComp, for summarizing, parsing and visualizing the genome sequences comparison results [74].

5 Transcriptomics, Proteomics and Metabolomics

5.1 Transcriptomics

Transcriptome analysis has been proven to be essential for functional genomics research and it is expected that, together with the efforts of proteomics studies, this discipline is laying down the foundation for future development of systems biology. There has been significant progress of transcriptome analysis in hematopoiesis, neuroendocrine systems, pathological characteristics and medicine in China. For human and animal transcriptome research progress, the reader should refer to the detailed review by Han et al. [75]. In this review we will focus on the real applications in microorganisms and the continuous technology improvement in its technology efficiency, accuracy and reliability.

Microbial microarray analysis has been used mainly in medical related fields. The research group of the Chinese Academy of Military and Medical Science published one series of *Yersinia pestis* transcriptome results. They used a whole genome DNA microarray, which contains 4,005 annotated genes of *Y. pestis*, to investigate the gene expression profile of the bacterium in responses to different stresses or conditions, such as Mg^{2+} limitation, polymyxin treatment and oxidative stress [76, 77]. Their studies provide useful targets for the new drug investigations and the cluster analysis of *Y. pestis* can lead to the identification of virulence factors and regulation mechanisms. Some of the other clinically important microorganism, such as drug resistant *Candida albicans* [78] and *Trichophyton rubrum* [79], are also studied in detail. All of the research will contribute greatly to our understanding of the relationship between microbes and their human host.

Another hot topic of transcriptome analysis is about the model organism *Saccharomyces cerevisiae*. Liu et al. compared the transcriptome difference of bromodomain factor 1 in the salt stress response of yeast. Genetic analysis indicated that the salt sensitivity of the BDF1 deletion mutant was suppressed by increased gene dosage of its homologous gene BDF2. Furthermore, comparative transcriptome analysis revealed that the differences in transcriptional response between the wild type and the bdf1 Delta mutant in the presence of salt stress were mainly related to cell wall biosynthesis, the mitochondria, and several unknown genes [80]. Zhang et al. studied the genome wide expression profiling of the response of *S. cerevisiae* to 5-fluorocytosine, amphotericin B and nystatin, plant isoflavone-wighteone using a DNA microarray [81, 82]. These results are very important for us to understand cancer chemotherapy. Gu et al. studied rapid evolution of expression and regulatory divergences after yeast gene duplication [83]. Relative expression rate tests suggest that the expression of duplicate genes tends to evolve asymmetrically, that is, the expression of one copy evolves rapidly, whereas the other largely maintains the ancestral expression profile. Combined with yeast genome sequences, microarrays, and transcriptional regulatory networks, their study highlights the crucial role of early rapid evolution after gene/genome duplication for continuously increasing the complexity of the yeast regulatory network.

Although there are many other specific examples for transcriptome analysis in microorganisms, we cannot find the real cases used for industrial strain improvement purposes. The cost issue will be one of the main barriers for that. With the development of transcriptome analysis technology, many of microorganism's standard microchips are commercially available, and we would like to see industrial applications in coming years. Some of the groups in China, such as the metabolic engineering group of Tianjin University, are dealing with *B. subtilis* microarray analysis.

Regarding the technology platform development of transcriptome, the representative research results focused on the data mining of the huge gene expression data. Expressed Sequence Tags (ESTs) data sets constitute valuable resources for comparative transcriptomics. However, methods are not available yet to process EST-derived transcription maps to enable gene ontology based global functional profiling for comparative transcriptomics in a high throughput manner. Chen et al. proposed one GO-Diff software, which integrates EST profiles with GO knowledge databases to mine functional differentiation between biological systems [84]

5.2 Proteomics

The concept of proteome is more than 10 years old with the first paper published in 1995 [85]. Now it has developed into one proficient technology, which is widely used in the life science and biotechnology fields. In fact, over 4,000 proteomics research and review articles were published in 2005 (10 years of the proteome). In China, it is also one hot research spot. From 2000 to 2007, there have been at least 400 papers published in international journals among the thousands of papers published in mainland of China. In this review we will coordinate the conceptual breakthroughs, technical

advances and real applications achieved in protein separation, mass spectrometry and proteomic bioinformatics, which have been achieved by Chinese researchers.

In protein separation and extraction technology, Xi et al. found polyethylene glycol fractionation improved detection of low-abundant proteins by two-dimensional electrophoresis analysis of plant proteomes [86]. Zhang et al. presented an improved method for sample preparation for MALDI-MS and MS/MS using AnchorChip™ targets. The method, termed the SMW method (sample matrix wash), results in better sensitivity for peptide mass fingerprinting as well as for sequencing by MS/MS than previously published methods in liver proteome [87]. Jiang et al. found a new high efficiency method for extraction from bone tissues. The bone tissue was first demineralized by HCI solution and then extracted using three different lysis buffers [88]. They also established the protocol for protein extraction from formalin-fixed tissues. Incubation of tissue in a lysis buffer containing 6 M guanidine hydrochloride at high temperature led to the highest protein yield and the largest number of proteins identified [89]. Some other kinds of new separation methods are also reported. For example, Jiang et al. proposed an approach to automate sample introduction for nanoflow LC-MS/MS (μLC-MS/MS) analysis using a strong cation exchange (SCX) trap column [90]; Gu et al. used capillary array reversed-phase liquid chromatography-based multidimensional separation systems [91]; Xie et al. proposed an octadecylated silica monolith capillary column with integrated nanoelectrospray ionization emitter for highly efficient proteome analysis [92].

In the field of two-dimensional (2D) gel electrophoresis, mass spectrometry and proteomic bioinformatics, Zhang et al. compared two methods for studying the proteome of human lens fibers. In the first method, the total proteins were digested with trypsin, and the derived peptides were analyzed by strong cation exchange (SCX) coupled with reverse phase liquid chromatography tandem mass spectrometry (RPLC-MS/MS). In the other method, proteins were first resolved by sodium dodecyl sulfate PAGE (SDS–PAGE) and then in-gel digested with trypsin, and the peptides were analyzed by RPLC/MS/MS. Their data clearly indicated that the SDS–PAGE–RPLC-MS/MS method was more suitable than the SCX-RPLC-MS/MS method for analyzing lens fiber proteome [93]. Recently, the matrix-assisted laser desorption ionization (MALDI) technique has been shown to be complementary to electrospray ionization (ESI) with respect to the population of peptides and proteins that can be detected. Zhang et al. tried to hyphenate MALDI-TOF-TOF-MS and ESI-QUADRUPOLE-TOF-MS with a single 2D liquid chromatography for complicated protein sample analysis with good results [94]. Yan et al. developed and optimized a 2D gel electrophoresis condition for *Candida albicans* proteome analysis [95]. Wang et al. also developed a more sensitive and convenient Coomassie Brilliant Blue (CBB) staining method for visualizing proteins [96]. Jiang et al. implemented a machine learning approach known as predictive genetic algorithm (GA) for the optimization of filtering criteria to maximize the number of identified peptides for SEQUEST database searching to improve proteome coverage in shotgun proteomics [97]. Zhang et al. reported the development of PCAS (ProteinCentric Annotation System) as an online resource of pre-computed proteome annotation data; PCAS gives better annotation coverage for model proteomes by employing a

wider collection of available algorithms [98]. Based on array CIEF (ACIEF) and a novel whole column imaging detection (WCID), Mao et al. proposed a comprehensive 2D system with laser-induced fluorescence for protein mapping [99].

The proteome applications of China mainly focused on the biomedical field such as the human plasma and liver. Proteome Research Center of Fudan University, Beijing Proteome Research Center and Dalian Institute of Chemical Physics of Chinese Academy of Sciences are very active research groups in this area. There are already many detailed reviews targeting this. Here we will briefly introduce the real applications of proteome in microbial and industrial biotechnology field. Feng et al. sequenced the genome of *G. thermodenitrificans* NG80-2, which is the only thermophilic bacterial strain described that degrades long-chain alkanes up to at least C36. Further proteome analysis reveals the presence of a long-chain alkane degradation pathway. This is a very important finding which can be used for the treatment of environmental oil pollutions and in other biocatalytic processes [66].

Another kind of thermophilic organism, *thermoanaerobacter tengcongensis*, can survive harsh living conditions in temperatures ranging from 50 to 80 °C. The proteomic can expedite our understanding of the molecular mechanisms with regard to how thermophilic bacteria adapt to the alterations in living environment [100]. Wang et al. use the idea of proteomics to study the antibiotic biosynthesis pathway of *Streptomyces luteogriseus* Strain 103 and find that maituolaimysin is synthesized through a polyketide pathway. Their research provided a feasible proteomic way to investigate the antibiotic biosynthesis pathway and had the potential for improved antibiotic production [101]. The proteomic approach had also been used to study the responses of *Taxus cuspidata* cells to local microenvironments in different zones of immobilized support matrices [102].

In the model organism of *E. coli*, Wang et al. used proteomic methodologies to compare the alkaline proteins expressions in an overgrowth-mediated growth arrest and cell death of *Escherichia coli*. They identified six alkaline proteins as key ones in an overgrowth-mediated growth cycle of *E. coli* [103]. Huang et al. studied the sub-proteome of *E. coli* cell envelope and revealed a number of previously unrecognized membrane-interacting protein complexes such as the complex consisting of OmpW and fumarate reductase [104]. The above findings shed light on the understanding of the complete protein functions of *E. coli*. The proteome of different organisms under different stress conditions, such as salt shock [105, 106], were also studied.

There are also many important research findings in the biomedical microbial field, involving such as the *Helicobacter pylori* [107], *Vibrio cholerae* [108] and *Pseudomonas aeruginosa* [109]. The targets are usually to understand the disease related mechanisms and drug development through comparative proteomics.

5.3 Metabolomics

Metabolomics, as the complement to transcriptomics and proteomics, has its own advantages. One of the major advantages of metabolomics is that there are fewer

metabolite types than genes or proteins. Such advantages reduce the processing complexities. Moreover, with the improved combined analytical platforms, the individual metabolites in the complex mixtures can be identified with certainty. Such improvements will make the metabolic comparatives analysis more precise and efficient, since the raw data of genome and proteome must be explained based on the homology or homeology. Finally, the dynamic metabolites levels in organisms must reflect the exact metabolic phenotypes under different cultural and genetic conditions fundamentally [110]. With the development of new analytical platforms and data mining methods, metabolomics are becoming an important part of systems biology and playing key roles in new drug discovery, clinical diagnosis and metabolism understanding. Many Chinese research groups are very active in this field. For example, Dalian Institute of Chemical Physics, the Chinese Academy of Sciences is very strong in analytical platform of mass spectrometry; Wuhan Magnetic Resonance Centre, Wuhan Institute of Physics and Mathematics, Chinese Academy of Sciences is very strong in NMR based metabolomics. Shanghai Institute for System Biology, Shanghai Jiao Tong University had carried out much metabolomics related work for Chinese traditional medicine.

In the metabolomics field, Yang et al. employed a comparative lipidomics approach to investigate the changes in membrane phospholipids during the procession of cellular development and apoptosis of two plant cell lines, *Taxus cuspidata* and *Taxus chinensis* var. mairei. Through measuring and comparing more than 100 membrane glycerophospholipids between different cells, it was suggested that the alternation of membrane phospholipids may regulate apoptosis, triggering an increase in taxol production of *T. chinensis* var. mairei cells [111]. They also applied Fourier Transformation Infrared Spectroscopy to measure the metabolites of antibiotic producing *Streptomyces lydicus*. Based on PCA and Hierachical Cluster Analysis, their experiments successfully distinguished high producing mutants. So it has potential to be used for high-throughput mutant screening [112].

The organic acid metabolite pools' fluctuation of *E. coli* and *Pseudomonas aeruginosa* under different antibiotic stimuli were measured and clustered distributions of different antibiotics for each strain could be easily distinguished [113]. A 2D separation system of coupling chromatography to electrophoresis was developed for profiling *E. coli* metabolites [114]. Metabolomics has also been used in rapid on-line detection of metabolic markers in complex biological samples. It has the potential to be used in food regulation, homeland security, in vivo metabolomics, and clinical diagnosis [115].

Regarding the data processing methods, Xia et al. attempted to integrate the wavelet transform method with principal components analysis and ANN artificial neural network to extract information from metabolomics data [116]. Li et al. proposed to exclude unstable metabolites and the data should be properly scaled before further processing in principal components analysis of metabolome data [117].

Hundreds of papers have been published on metabolomics research in China. Many of them studied the profiling of urine samples [118] or other tissue samples [119] with standard protocols for biomarker discovery. However, it is not the key part of this review. Please refer to the other detailed reviews.

6 Metabolic Network Analysis and Flux Analysis

Metabolic pathways and their fluxes are at the core of metabolic engineering. According to the development of metabolic network analysis and its applications, we classified this field into four parts, namely metabolic network topology analysis, flux balance analysis with specified applications of flux analysis, pathway analysis (extreme pathway and elementary mode analysis) and platform construction. Regarding the important part of metabolic engineering, metabolic control analysis, there are few published results. Only Sun et al. determined the flux control coefficients of the four enzymes involved in the upper pathway of biphenyl degradation in *Rhodococcus pyridinovorans* from transient metabolite concentrations [120]. This is the first experimental evidence of the control step in the pathway of biphenyl degradation using metabolic control analysis.

6.1 Metabolic Network Topology Analysis

Topology means we just consider the connection of metabolites. In this field, Ma et al. have contributed many highly influential works. They reconstructed the metabolic networks of 80 fully sequenced organisms from genome data and an extensively revised bioreaction database. Then they calculated the path length. The distribution of the connection degree of these networks is shown to follow the power law, indicating that the overall structure of all the metabolic networks has the characteristics of a small world network. However, clear differences exist in the network structure of the three domains of organisms. Eukaryotes and archea have a longer average path length than bacteria [121]. This paper had been recommended to be the "faculty 1000" best paper. They also use a reaction graph representation of a metabolic network for the identification of its global connectivity structure and for decomposition and find a bow-tie connectivity structure similar to that previously discovered for metabolite graph existing in the reaction graph [122].

6.2 Flux Balance Analysis (FBA) and Specified Applications of Flux Analysis

Flux balance analysis (FBA) is the most important approach for analyzing the metabolic network, especially from the perspective of genome scale metabolic networks. It is also a constraint-based approach, which can be used to obtain an optimal solution within the feasible range of cellular capabilities with optimizing methods, such as linear programming. Although there are many applications of FBA in different strains, it is difficult to find the real applications by Chinese researchers. The reasons should be: (1) it lacks our own genome scale model and it

is a little difficult to construct such a model based on the databases and papers (time consuming and need some special skills); (2) many of the interested strains do not even have genome information, so genome sequencing and genomics should be the first choice to target; (3) FBA can just provide some "guess" information, so researchers are more willing to use the new pathway analysis tools including extreme pathway and elementary mode analysis. In the few examples of FBA application, Wang et al. used the genome scale model of *E. coli* to estimate the optimized succinic acid production pathways [37], and Jiang et al. used simplified metabolic network of yeast, which contains 37 metabolites, 54 reactions to simulate the production of biomass [123]. Many Chinese research groups had realized the problems of this and are constructing their own genome scale metabolic network models, such as *B. subtilis* (unpublished papers).

Flux analysis is one of the most important tools of metabolic engineering. Chinese researchers also used this tool to serve the strain improvement. For example, Luo et al. analyzed the metabolic flux in *E. coli* expressing human-like collagen in fed-batch culture [124]; Gao et al. analyzed the metabolic fluxes for hyaluronic acid (HA) production by *Streptococcus zooepidemicus* [125]; Bai et al. analyzed the wild-type and a mutant *Lactobacillus lactis* strain for l(+)-lactic acid production [126, 127]. In last 5 years, there have been many published successes when using metabolic flux analysis. However, many of them just selected some simplified model and tested some metabolites to calculate the intra fluxes. There has been little breakthrough in ^{13}C labeled metabolic flux analysis in China. The ^{13}C labeled metabolic flux analysis originated from 1995 and it had been adopted by many metabolic engineering labs as one general tool. It seems that Chinese researchers should make more effort in this. There are many technical barriers and cost issues. So international cooperation should be a good choice from the beginning. Zhejiang University of China cooperates with Kyushu Institute of Technology of Japan to implement a series of research in ^{13}C labeled metabolic flux analysis in yeast [128] and *E. coli* [129]. Recently, their laboratories published a new hybridized genetic algorithm, which can simulate the flux distributions without the requirement of the information on the partial derivatives [130].

6.3 Pathway Analysis

Generally, pathway analysis deals with the discovery and analysis of meaningful metabolic networks. In contrast to FBA, it characterizes the complete space of admissible steady-state flux distributions by particular flux vectors. Currently, there are two pathway analysis tools: elementary modes analysis (EMs) and extreme pathway analysis.

In order to reduce the number of EMs used to reconstruct a real flux distribution, Wang and Yang et al. found a power function relationship between the decrease of relative error and the increase of the number of the selecting EMs, and a logarithmic relationship between the increases of the number of non-zero weighted EMs and

that of the number of the selecting EMs. Their discoveries showed that it is possible to reconstruct a given flux distribution by a selected subset of EMs from a large metabolic network and furthermore, they help us identify the 'governing modes' to represent the cellular metabolism for such a condition [131]. Jiang et al. used EM analysis to predict the relationship between the gene deletion genotype and different phenotypes of yeasts. Their result showed EM to be an effective tool for bridging the gap between metabolic network and growth phenotype [123].

Much more effort is needed in this promising research field in near future.

6.4 Platform Construction

Ruoyu Rou et al. (FluxExplorer: a general platform for modeling and analyses of metabolic net-works based on stoichiometry) introduce a new platform for modeling and analysis of metabolic networks: FluxExplorer. FluxExplorer for metabolic modeling and analysis which is based on stoichiometry is a publicly available tool for systems biology research. This platform integrates various analytic approaches, including flux balance analysis, minimization of metabolic adjustment, extreme pathways analysis, shadow prices analysis, and singular value decomposition, providing a thorough characterization of the metabolic system. The calculation modules are independent of the commercial program MATLAB, like GAMS. This platform can be used to reconstruct metabolic networks. Its function also include FBA and shadow prices analysis, minimization of metabolic adjustment (MOMA) of a mutant network, analysis of the topological properties of a metabolic network, and other application. Then this platform is used to analyze the mitochondrial metabolic network [132].

7 Outlook

In this review we have introduced part of the important industrial biotechnology contributions of Chinese researchers to modern biotechnology in the past 5–7 years. Although China is still a developing country, many of the research centers and scientists are actually world renowned. However, it is clear that, compared with the advanced research level throughout the world, we have a long way to go. Chinese researchers should take both follow-up research and independent innovation seriously. R&D priority should shift from active follow-up to dominant independent innovation and independent innovation should aim at the international frontiers of science and the national targets. Meanwhile, we need to arrange our research work more systematically.

Hopefully the Chinese government will always take a positive attitude on biotechnology. In China there are at least 2,500 enterprises with more than 50,000 employees involved in modern biotechnology. Modern biotechnology development

is one of the fundamental national strategies and will play a key role in the sustainable development of the Chinese economy. With the booming economy development, we really believe China could make manifold valuable contributions to the shaping of the emerging landscape in the biology of the new century.

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