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Biotechnology in China III: Biofuels and Bioenergy

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Biotechnology in China III: Biofuels and Bioenergy

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Preface

Fueled by rapid economic growth, China's crude oil consumption has increased drastically, from 217 million tons (Mt) in 2001 to 423 Mt in 2010, making it the second largest oil consumer in the world, only after the United States. Since the domestic oil production has only increased slightly during the same period of time, from 164 to 184 Mt, there is a continuously increasing gap which must be filled by imported oil. Taking into account the trend of economic growth, China's strong dependence on foreign oil cannot be mitigated in the future without developing self-dependent alternatives, which not only secure the sustainability of its own economy, but also affect the rest of the world.

China is also one of the main grain producers in the world, producing more than 500 Mt of grain per year, and correspondingly generating 600–700 Mt agricultural residues. Due to the lack of economically viable technologies for their utilization, most agricultural residues are burnt in the field by farmers, which pollutes environments and even presents public hazards such as the disruption of air transportation by smoke clouds in the sky. Producing biofuels, bioenergy and bio-based chemicals through the refinery of lignocellulosic biomass has been acknowledged worldwide as an alternative to the oil refinery since the oil crisis occurred in the 1970s. It has also recently been highlighted again because of high oil prices and global climate change caused by the over-consumption of petroleum-based products, particularly vehicle emissions. Without doubt, the successful development of biomass refineries in China will help it to sustain its own economy, and in the meantime contribute to the whole world.

Driven by these imperatives, governmental funding for R&D of biomass refineries has increased significantly during the 11th-Five Year Economic and Social Development of China from 2005 to 2010. This momentum is expected to be maintained for a long term to make such a pathway economically competitive. We are honored to be invited by the Series Editor Professor T. Scheper and Springer to edit the consecutive volume on "Biotechnology in China", with a focus on biofuels and bioenergy to present major progress achieved by Chinese scientists and challenges to be addressed collectively by international communities.

The sugar platform is the prerequisite for the bioconversion of lignocellulosic biomass, and high efficient and low cost cellulases are the bottleneck. This is therefore addressed first by Professors X. Fang and Y. B. Qu. The major part of this volume is on liquid transportation fuels, including bioethanol, biodiesel, biobutanol and branched-chain higher alcohols, which are presented by Professors F. W. Bai, N. Y. W. Ho, D. H. Liu, J. Xu, Y. Li and Q. H. Wang. Meanwhile, biogas, which has been well established in China, as well as emerging biohydrogen and microbial fuel cells are addressed by Professors H. Q. Yu, A. J. Wang and G. P. Sheng. Finally, fuels and chemicals from hemicellulose sugars that are less efficiently converted by native, even genetically modified microorganisms are discussed by Professors H. Huang and G. T. Tsao. We expect that this special volume to be a window for international colleagues to learn the current R&D progress in biofuels and bioenergy in China.

On behalf of my co-editors Professors H. Huang and G. T. Tsao, I express our sincere thanks to all authors and reviewers for their dedication, contributions and valuable comments, Professor T. Scheper and Dr. M. Hertel for their encouragement, Ms. K. Bartsch for her delicate coordination and Dr. C. G. Liu for his assistance in the art work. Also, I greatly appreciate the generosity of my institute Dalian University of Technology (DUT), my colleagues and graduates at DUT and my family for all their support.

Dalian, China Feng-Wu Bai

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Cellulolytic Enzyme Production and Enzymatic Hydrolysis for Second-Generation Bioethanol Production

Mingyu Wang, Zhonghai Li, Xu Fang, Lushan Wang and Yinbo Qu

Abstract Second-generation bioethanol made from lignocellulosic biomass is considered one of the most promising biofuels. However, the enzymatic hydrolysis of the cellulose component to liberate glucose for ethanol fermentation is one of the major barriers for the process to be economically competitive because of the recalcitrance of feedstock. In this chapter, the progress on the understanding of the mechanisms of lignocellulose degradation, as well as the identification and optimization of fungal cellulases, cellulolytic strains, and cellulase production is reviewed. The physiologic functions and enzymatic mechanisms of two groups of enzymes involved in lignocellulose degradation, cellulases and hemicellulases, are discussed, and the synergism of the cellulase components during lignocellulose degradation is addressed. Furthermore, the methods for screening filamentous fungal strains capable of degrading lignocellulose are evaluated and the production of cellulases by these fungal strains is discussed. Aside from traditional mutagenesis for improving the secretion level and enzymatic activities of cellulases from filamentous fungal species, genetic engineering of strains and protein engineering on cellulase molecules are also highlighted.

Keywords Bioethanol · Cellulases · Hemicellulase · hydrolysis · Lignocellulose

Contents

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1 Introduction

Bioethanol produced from lignocellulosic biomass is suggested as the most promising renewable and carbon-neutral alternative to petroleum-based transportation fuels. It has been a focus of scientific research and industrial demonstration for its potential in alleviating fossil fuel dependence as well as mitigating greenhouse gas emissions [1, 2]. Bioethanol is also receiving significant attention in China for promotion of the country's rural economy, which is one of the government's top priorities.

There are five fuel ethanol plants in China, producing 1.52 million tons of fuel ethanol annually from starch-based feedstock, mainly from corn and wheat, except the COFCO plant in the southwest Guangxi Province, which produces fuel ethanol from cassava chips. Significant government subsidies have also been granted to these ethanol producers since they started. Considering China's large population and dwindling arable land because of rapid urbanization, fuel ethanol production from starch-based feedstock is not sustainable for addressing the country's energy issue without endangering food security.

Lignocellulosic biomass is abundantly available in China, particularly agricultural residues such as corn stover, rice straw, and wheat straw. It has great potential as a feedstock for the production of fuel ethanol, also known as bioethanol or second-generation fuel ethanol. However, liberating sugars from lignocellulosic biomass at low cost to develop economically competitive bioethanol production and other bio-based chemicals is very challenging. Two major approaches have been developed: acid hydrolysis and enzymatic hydrolysis. In the 1950s, an ethanol plant with a 4,000-t annual production capacity was established in Heilongjiang Province, Northeastern China, wherein an acid was used to hydrolyze sawdust based on the technology from the former USSR; however, the plant was closed in the 1990s because of economic and environmental concerns. Despite continuous efforts, the dilute acid hydrolysis approach is still hampered by its nonselectivity, by-product formation, low ethanol yields (50–60% of theoretical values) [3], and the high cost of wastewater treatment. In contrast, enzymatic hydrolysis, which effectively overcomes these disadvantages, has received increasing attention and has been extensively developed in China [4–9].

Unlike starch, cellulose is the primary structural component of plant cell walls and is therefore resistant to biodegradation in nature. Cellulose is insoluble,

Table 1 Comparison of dosage requirement of amylase, glucoamylase, and cellulase during hydrolysis

Enzyme	Activity	Enzyme loading
Amylase[a]	192 KNU/mL	0.2 KNU/g starch
Glucoamylase[b]	652 AGU/mL	3.26 AGU/g starch
Cellulase[c]	100 FPU/mL	20 FPU/g glucan

KNU kilo Novo unit, *AGU* amyloglucosidase unit, *FPU* filter paperase unit
[a] Liquozyme SC DS was used [10]
[b] Spirizyme was used [10]
[c] Accellerase™ 1000 was used [11]

making its enzymatic hydrolysis heterogeneous, which significantly compromises the reaction rate. Therefore, higher cellulase loadings are needed for cellulose hydrolysis compared with hydrolysis of starch by amylase and glucoamylase (Table 1). Merino and Cherry [12] suggested that the required enzyme concentration for cellulose degradation is 40-fold to 100-fold higher than that of starch hydrolysis, resulting in a high enzyme cost during cellulose degradation. Therefore, the breakdown of lignocellulosic biomass to liberate sugars is much more expensive [13], which accounts for a large fraction of bioethanol production costs [14]. One strategy is reducing the cellulase production cost through improving cellulase titers during the fermentation, and another is optimizing the cellulase composition to enhance the synergetic effect of different enzymatic components during cellulose hydrolysis so that the specific activity of cellulase is improved and the enzyme dosage is minimized.

In this chapter, we review the updated progress in cellulase research and production and enzymatic cellulose hydrolysis for the production of the second-generation bioethanol.

2 Lignocellulose Biodegradation

2.1 Cellulolytic Enzymes

Most known cellulolytic enzymes are produced and excreted by filamentous fungi, among which *Trichoderma reesei* has received special attention for its hyperenzymatic production capabilities [15–17]. It has been subsequently used as a model organism for investigations of enzymatic hydrolysis mechanisms. Proteomic analysis of these enzymes with high-resolution two-dimensional gel electrophoresis revealed many glycoside hydrolases with this species [15, 17], which can be divided into two main groups: cellulases and hemicellulases.

Cellulases are enzymes that hydrolyze cellulose, a linear polysaccharide molecule composed of repeated β–(1→4) linked D-glucopyranosyl (Glc) units. Multiple types of cellulases have been discovered, including at least two exo-β-glucanases,

or cellobiohydrolases (CBHs; EC 3.2.1.91) (CBH I and CBH II), four endo-β-glucanases (EGs; EC 3.2.1.4) (EG I, EG II, EG III, EG V), and one β-glucosidase (βG; EC 3.2.1.21) [18].

Hemicellulases are enzymes that degrade hemicelluloses, a group of polysaccharides and one of major plant cell wall components. Unlike cellulose, which is composed entirely of glucosyl moieties linked by only β-1, 4-glycosidic bonds, various types of sugar moieties linked by different bonds, intramolecular architecture, and intermolecular interactions can be found within hemicelluloses. Hemicelluloses also differ from the major plant cell wall structural component cellulose in their much smaller polysaccharide chains. Hemicelluloses can serve to cross-link cellulose microfibrils by interconnecting them as well as linking cellulose molecules to other cell wall components [19]. Common hemicelluloses include β-glucan (different from cellulose), xylan, xyloglucan, arabinoxylan, mannan, galactomannan, arabinan, galactan, and polygalacturonan. Corresponding to these hemicelluloses, various types of hemicellulases exist, each specific to a kind of hemicellulose [19–23]. These enzymes can be clustered into two groups: hemicellulases that attack the polysaccharide backbone and those that attack the side chains.

Xylan, whose structure differs from plant to plant, is the second most abundant polysaccharide in herbs and hardwoods, demanding the collaboration of a group of enzymes during their degradation. Multiple enzymes including endo-β-xylanase (EC 3.2.1.8), β-xylosidase (EC 3.2.1.37), α-glucuronidase (EC 3.2.1.139), α-L-arabinofuranosidase (EC 3.2.1.55), and acetylxylan esterase (EC 3.1.1.6) act synergistically in this process (Fig. 1). Endo-1,4-xylanases, cleave internal β-1, 4-xylosidic bonds on the xylan polysaccharide backbone. Unlike EGs, whose cleavage sites are random, endo-1,4-xylanases recognize specific bonds for cleavage on the basis of polysaccharides properties such as chain length and branching levels [20]. Endoxylanases were initially classified into two groups by their ability to hydrolyze the 1, 3-α-L-arabinofuranosyl branching points of arabinoxylans: hydrolyzing and nonhydrolyzing endoxylanases, which have different pI values and molecular weights [21]. However, these patterns were shown to account for only 70% of all endoxylanases, and a classification system of all glycoside hydrolases (glycoside hydrolase families) is better recognized now [22]. Products from xylan degradation by endoxylanases are a mixture of β-D-xylopyranosyl oligomers of various lengths, which serve as substrates for β-xylosidases that subsequently hydrolyze them to xylose from the nonreducing end of these oligomers [20]. Multiple other enzymes are also involved in xylan degradation, primarily due to the complex nature of these polysaccharides: α-L-arabinofuranosidase cleaves the α-glycosidic bonds between arabinose and xylose moieties in xylan; α-glucuronidases cleave the α(1→2) bonds linking the (methyl) GlcU units in xylan [19]; acetylxylan esterase removes the *O*-acetyl groups at the 2- and 3-positions of β-D-xylopyranosyl residues; ferulic acid esterase (EC 3.1.1.73) cleaves the ester bond between the arabinose and ferulic acid side chains; and *p*-coumaric acid esterase (EC 3.1.1.73) cleaves the ester bond between arabinose and *p*-coumaric acid [20].

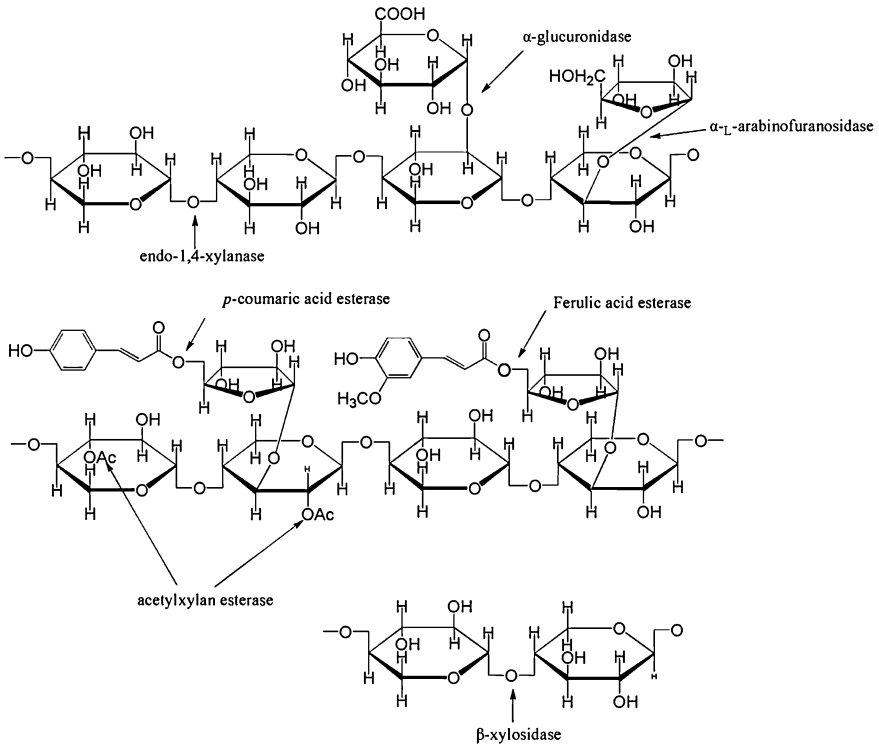

Fig. 1 Chemical structure and degradation of hemicellulose

2.2 Mechanism of Cellulose Biodegradation

The biodegradation of crystalline cellulose is slow because the substrate is insoluble and poorly accessible to enzymes. The biodegradation mechanism of cellulose has been studied over the last 50 years by scientists worldwide. EG, CBH, and βG have been shown to act synergistically in this bioprocess, during which EG first randomly cleaves the internal glycosidic bonds of the cellulose chain, and the resulting exposed reducing or nonreducing ends are subsequently attacked by CBH, releasing cellobiose, the substrate of βG, which eventually hydrolyzes cellobiose to glucose (Fig. 2) [24]. The optimized ratio of cellulolytic enzymes was investigated, and it was found that the best saccharification of crystalline cellulose is achieved with an enzyme blend (60:20:20 CBH I–CBH II–EG I) wherein a saturated level of βG was included to eliminate cellobiose inhibition [25]. In a different report, the impact of the cellulase mixture composition on cellulose conversion was modeled, and the findings suggested different optimum ratios for substrates with different characteristics, specifically degrees of polymerization (DP) and surface area [26]. These theoretical and experimental conclusions, however, demand further examination, as

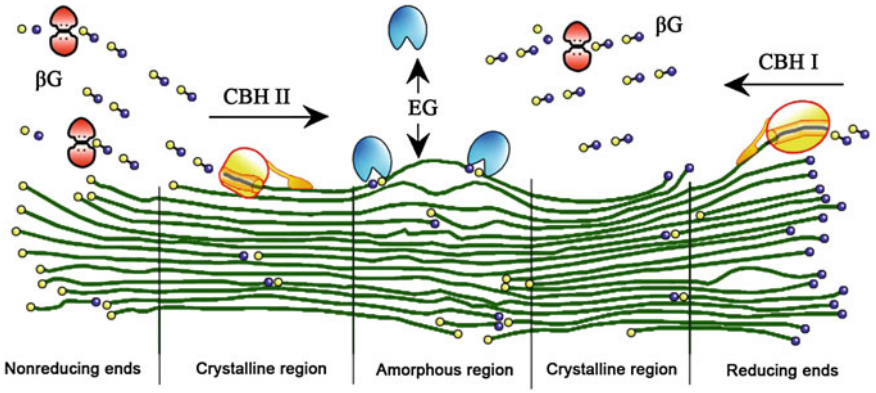

Fig. 2 Mechanism of cellulose biodegradation. *CBH* cellobiohydrolase (or exo-*β*-glucanase), *EG* endo-*β*-glucanase, *βG* *β*-glucosidase

the behavior of the complicated enzymatic lignocellulose degradation is difficult to model and prone to experimental errors.

CBHs have a higher reactivity on crystalline cellulose than EGs. Both CBHs and EGs digest long polysaccharide chains into smaller fragments, thereby reducing the DP of the substrate. CBHs have been shown to be primarily responsible for solubilization of cellulose and only decrease the DP incrementally, whereas EGs decrease DP significantly but only play a minor role in cellulose solubilization [27]. Research on the cellulolytic enzyme system in *T. reesei* suggested that CBHs are the most abundant proteins in this system, representing more than 37% of the total protein content [17]. Therefore, understanding the structures of CBHs, as well as their catalytic mechanisms, is important when attempting to improve the enzymatic hydrolysis rate of lignocellulose [24]. Using CBH I, which catalyzes the cleavage of cellulose from the reducing end of the polysaccharide chain as an example, we attempt to address the complexity of the mechanism of these enzymes and also the synergistic relationship between different domains in these enzyme molecules.

Previous studies on CBH I identified two functional domains in this enzyme: the cellulose-binding domain (CBD; also referred to as the cellulose-binding module) and the catalytic domain (CD), which are linked by a flexible linker [18]. Further structural investigations revealed a synergistic relationship between these two domains, whose collaborative efforts lead to crystalline cellulose degradation [28, 29]. On the basis of these observations, we suggested an intramolecular synergic model for CBH I during its degradation of cellulose, which is shown in Fig. 3 in detail.

In this synergic model, multiple enzymes are involved in the degradation of cellulose, of which EGs first randomly cleave the internal amorphous regions, thereby generating reducing ends in the polysaccharide chains in preparation for CBH I degradation [18, 27]. CBH I is subsequently recruited to these sites by the binding of the CBD to the hydrophobic surfaces of crystalline cellulose [30]. It was suggested that the CD assists in this recruitment process and synergistically

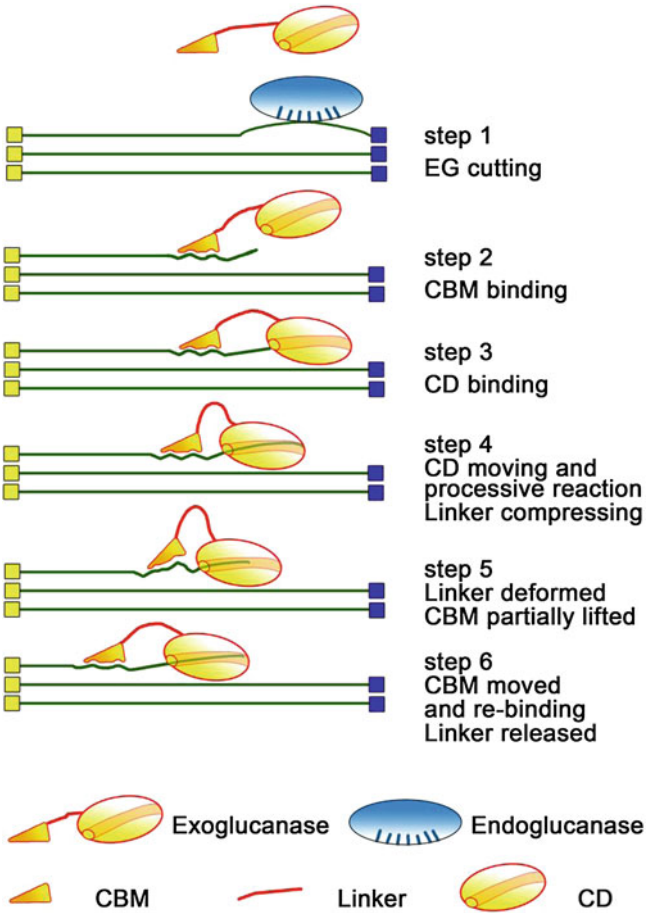

Fig. 3 Model of synergistic effect of the cellulose-binding module (*CBM*; or cellulose-binding domain) and the catalytic domain (*CD*) in CBH I

accelerates the binding of CBH I to crystalline cellulose. This results in an increase in local cellulase concentration on the cellulose surface [28, 29, 31–33].

As shown in Fig. 3, the binding of the CBD to crystalline cellulose is hypothesized to result in a change in the spatial coordination of the hydroxyl groups on the surface of the polysaccharide chain and, subsequently, the hydrogen bond network; this results in the "peeling" of the cellulose chain from the crystalline structure, the disruption of the surface of this structure, and the decrease in the regional DP [18, 29]. This interaction between the CBD and crystalline cellulose was observed using purified CBD from EG III of *T. reesei*, which is homologous to the CBD from CBH I. The addition of purified CBD to cellulose decreases the number of hydrogen bonds by 17.0% and the crystalline index by 16.5% [34]. In another report, the turbidity of cellulose CF11 increased by 128.9%

after treatment with purified CBD of CBH I from *Penicillium janthinellum*; this suggests the dissociation of small fibrils from cellulose assisted by the CBD [32], and agrees with the hypothesis that the CBD disrupts the regional hydrogen network of crystalline cellulose. The "free" polysaccharide chain released from this CBD-assisted disruption is then recognized by the CD and subsequently enters its catalytic tunnel. Four cellobiose units enter the tunnel, where cleavage of the glycosidic bond takes place [35, 36]. The energy released from this reaction is used in changing the enzymatic structure, causing the enzyme molecule to move forward along the polysaccharide chain, and compressing the linker [37]. Further cleavage results in further compression of the linker and eventually results in the dissociation of the CBD region from the polysaccharide chain despite the tight bond between the three Tyr residues at its active site and the pyranoid rings on the polysaccharide chain [38, 39]. The CBD then moves forward by four cellobiose units (the length of the linker), rebinds with the polysaccharide chain, and again disrupts the regional hydrogen-bond network for further digestion by the CD [31, 40]. This clear intramolecular synergism between the CBD, the CD, and the linker helps the CBH I molecule crawl along the polysaccharide chain for its complete digestion. A dual function is proposed for the CBD in this model: binding and, consequently, increasing the regional concentration of CBH I on the cellulose surface, and disrupting the hydrogen-bond network and, subsequently, the crystalline surface [29]. Indeed, real-time observations of the movement of CBH I from *T. reesei* and its CD along cellulose were made using a high-speed atomic force microscope; the similar movement rates and therefore the hydrolysis rates of the holoenzyme and the CD suggest that the CBD does not participate in catalysis but rather helps increase the regional enzyme concentration on the cellulose surface [41]. The role of the CBD in the disruption of the hydrogen-bond network could not be identified in these experiments, but a role cannot be ruled out either because unlike the highly crystalline and highly pure cellulose from green algae used in this investigation, the "regular" substrates for CBH are cellulose from plants in a more complicated environment. It was suggested that the product of cellulose degradation by CBH I, cellobiose, is an inhibitor of CBH I. This inhibition mechanism was investigated, and it was found that the binding of cellobiose to the active site of CBH I prevents further reactions, and that cellobiose induces conformational change in CBH I, thereby decreasing its activity [42].

This model explains a phenomenon long observed by scientists: the rate of cellulose hydrolysis by cellulases drops drastically during extended digestion. CBH I only hydrolyzes the one polysaccharide chain residing on the surface of the crystalline cellulose because binding of the CBD is required for disruption of the hydrogen-bond network. The changes in the packing and arrangement of microfibrils during later stages of hydrolysis lead to inhibition of CBH activity, as observed by Wang et al. [28].

This model for cellulose biodegradation is complicated in itself, but further evidence suggests that this may not be the full story. Ma et al. [43] showed that the CBH I from *T. reesei* is not uniformly adsorbed onto cellulose; some enzyme molecules are reversibly bound to cellulose, but the binding is irreversible for others. It was further

suggested that the irreversibly bound enzyme molecules undergo a conformational change and are inactivated. This shows that the cellulose degradation mechanism is still far from being fully understood, and many problems remain.

2.3 Factors Affecting Enzymatic Hydrolysis

The enzymatic hydrolysis of cellulose involves EG, CBH, and βG working in synergy as discussed in Sect. 2.2. The last step in this complicated process involves the degradation of cellobiose to glucose by βG. However, *T. reesei* only secretes low levels of βG, resulting in the accumulation of cellobiose during hydrolysis, which in turn contributes to the inhibition of CBH and EG activity. This inhibition effect caused by cellobiose accumulation is more than that caused by glucose accumulation [44]. Supplementing the native *T. reesei* enzyme cocktail with additional βG from other species of fungi with higher specific activities, better affinity for substrates, and lower inhibition by glucose is therefore common to mitigate cellobiose inhibition [44, 45]. Zhang et al. [9] showed that the addition of commercial *Aspergillus niger* βG preparation with 30 cellobiase units per gram of glucan was enough to eliminate cellobiose inhibition completely. Chen et al. [44] showed improvement in saccharification with the addition of purified βG from *Penicillium decumbens* to the cellulase cocktail of *T. reesei*. Ma et al. [46] introduced the βG I encoding sequence *pbgl1* from *P. decumbens* into the genome of *T. reesei* Rut C-30 and placed it under the control of the *cbh1* promoter from *T. reesei.* This recombinant *T. reesei* strain presented significantly increased βG activity, filter paper activity, and saccharification of pretreated cornstalk compared with the parent strain. Shen et al. [47] reported the heterologous expression of the βG-encoding gene *bgl1* from *Saccharomycopsis fibuligera* in the industrial yeast species *Saccharomyces cerevisiae*. This recombinant yeast strain gained cellobiose-hydrolyzing ability, and improved biomass hydrolysis efficiency during cellulose degradation when used in combination with cellulase preparations. Han and Chen [48, 49] extracted βG from postharvest corn stover, and showed that supplementation of commercial fungal cellulase with this purified βG is more effective than supplementation with βG from *A. niger*.

Lignocellulosic materials are primarily composed of three components: cellulose, hemicelluloses, and lignin. These are entangled together, forming a complicated matrix in which cellulose is well protected. Most cellulolytic microbes encode an array of hemicellulases along with cellulases to digest hemicelluloses, break down the matrix, and thus increase accessibility of their cellulases to cellulose. Kumar and Wyman [50] identified the relationship between xylanase/xylosidase activity and cellulose hydrolysis activity. Zeng and Chen [51] reported that the addition of ferulic acid esterase to steam-exploded rice straw accelerates its hydrolysis rate by breaking some of the ester bonds between lignin and hemicellulose, and improving accessibility to cellulose. Zhang et al. [9] reported that supplementation of pretreated

lignocellulose samples with xylanase and pectinase significantly increases the conversion of cellulose and hemicellulose, with pectinase being more effective.

Hemicellulose degradation, particularly xylan, has also been shown to involve multiple enzymes working in synergy. It was suggested that the acetyl groups in acetylated β-D-xylopyranosyl residues hinder the binding of endoxylanases to the xylan backbone [20], and acetylxylan esterases remove this hindrance and increase the accessibility of endoxylanases to the xylan molecule. On the other hand, β-xylosidases are believed to degrade xylooligosaccharides, the product of xylan degradation by endoxylanases and also the inhibitor of the latter enzyme. The presence of β-xylosidases is thus essential for the complete hydrolysis of xylan, as well as the full activity of endoxylanases [20, 52]. The synergistic effect of enzymes involved in xylan degradation is a complicated process that demands further investigation because of the heterogeneity and the complicated structure of the substrate. Indeed, previous research identified the complexity of the synergism during the degradation of xylan [53].

The enzymatic hydrolysis of lignocellulose is a complicated process that is affected by many factors. Liu et al. [6] suggested that different pretreatment methods result in different lignocellulosic biomass compositions and structures after pretreatment and that these changes significantly influence the ethanol yield from lignocellulosic biomass. Furthermore, the absorption and desorption of cellulase were shown to be related to the lignin content of the biomass, which has a significant effect on enzymatic hydrolysis [6]. Yu et al. [54] treated corn straw biologically with white rot fungi prior to alkaline/oxidative pretreatment and showed that this biological treatment decreases the lignin content significantly and improves cellulase desorption. Many other pretreatment methods have also been used by Chinese scientists, including microwave and alkaline pretreatment, dilute acid pretreatment, organosolv pretreatment, steam explosion, biodelignification, and hot compressed water pretreatment [4]. These pretreatment approaches generally aim to break the physical barriers of lignocellulose and remove lignin, which competitively binds to CBH I and inhibits the binding of CBH I to cellulose [4, 55]. In lignocellulosic biomass, hemicelluloses are entangled with cellulose, which protects the latter from cellulase degradation. In some pretreatment approaches, hemicelluloses are also removed, thereby exposing cellulose to cellulases and affecting the types of enzymes required for later enzymatic hydrolysis. One such example is steam explosion, in which hemicelluloses are removed from lignocellulose, thereby affecting the types of enzymes required and decreasing the necessity for hemicellulases in subsequent enzymatic hydrolysis steps [56].

Aside from investigations on different pretreatment methods, multiple reports have focused on other factors that affect cellulases and lignocellulose biodegradation. Further investigation by Chinese scientists identified a series of nonenzyme factors that improve the enzymatic hydrolysis efficiency of lignocellulosic biomass. This suggests that reducing biomass recalcitrance using microbes is a complicated process that involves multiple factors that are not fully understood. Wang et al. [57] found a low molecular weight peptide called short fiber generating factor in the supernatant of *Trichoderma pseudokoningii* S38 culture.

Short fiber generating factor was observed to enhance the enzymatic hydrolysis of cotton fiber because of its oxidation of the hydroxyl groups in cellulose, which leads to the depolymerization of cellulose, the formation of short fibers, and ultimately, the increased susceptibility of cellulose for hydrolysis [57]. Another component hypothesized to have cellulose disruption activity is swollenin, a novel plant expansin-like protein [58]. Yao et al. [59] isolated this protein (SWO2) from the cellulolytic fungus *T. pseudokoningii* S38, which shares a significant similarity with *T. reesei* swollenin as found by sequence analysis. Zhou et al. [60] found that the recombinant swollenin (SWO2) from *T. pseudokoningii* S38 has a significant synergistic effect in cellulose hydrolysis with low doses of cellulases in disrupting biomass recalcitrance. This effect was enhanced when cellulose was first pretreated with SWO2 before the enzymatic hydrolysis took place [60].

The addition of sorbitol reportedly improves the thermostability of cellulases and xylanases, which improves the conversion of cellulose and hemicelluloses [61]. A similar observation was made by Ouyang et al. [7], who reported that the addition of polyethylene glycol 4000 helps stabilize cellulolytic enzymes and improves cellulose degradation by 41.1–78.9%.

As discussed above, the biodegradation of lignocellulose is a very complicated process that involves multiple enzymes and factors. Elucidation of the detailed mechanism of enzymatic lignocellulose degradation, identification of novel factors that are involved in lignocellulose degradation, and understanding the synergic relationships between cellulases, hemicellulases, and various nonenzyme factors, as well as between the functional domains within enzymatic molecules, have been and will remain a focus of study. Understanding these important mechanisms will assist scientists in engineering cheaper cellulases and more effective enzyme preparations, optimizing lignocellulose degradation strategies, and eventually decreasing the operational cost of enzymatic hydrolysis processes.

3 Cellulase Production by Filamentous Fungus

3.1 Screening of Cellulase-Producing Microorganisms

Although a large number of microorganisms are capable of degrading cellulose, only a few of these microorganisms produce significant quantities of enzymes capable of completely hydrolyzing crystalline cellulose. Fungi have received the most attention in industry because of their higher enzyme productivity. Agar plates containing carboxymethyl cellulose or acid-swollen cellulose have been used to screen cellulase-producing filamentous fungi [62, 63]. Hypercellulolytic strains, which result in larger hydrolysis zones surrounding the colonies after 3–6 days of incubation, are subsequently selected and studied. Furthermore, fungal species that produce high cellulase and hemicellulase activity are also isolated using double-layer agar plates supplemented with holocellulose, which consists of both cellulose and hemicellulose (Fig. 4) [64, 65].

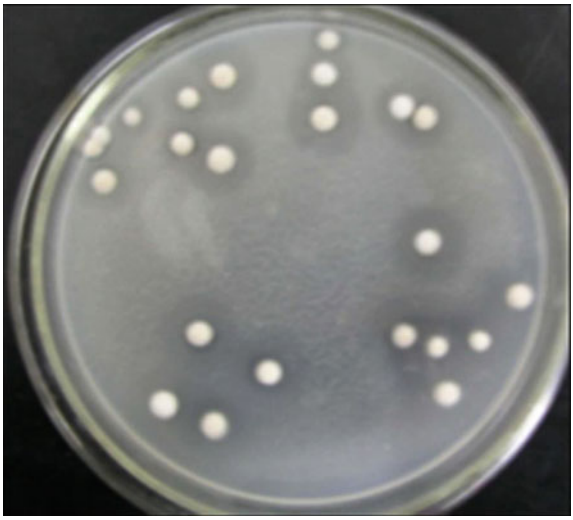

Fig. 4 Double-layer agar plate for screening cellulase-producing filamentous fungi

Another plating technique was used by Chinese scientists in selecting fungal strains secreting βG with high activity. Esculin and ammonium ferric citrate were added to the top layer of a double-layer agar plate. Esculin can be digested by βG, generating esculetin, which reacts with ferric ions and turns black. Black circles were observed around colonies secreting βGs, and the size of the circle can be used for determining the level of βG secretion. Through this approach, *P. decumbens* strain JU5-5 was obtained by Liu et al. [66], which has 100% greater βG secretion than the traditional industrial strain JU-A10.

The genera *Trichoderma* and *Aspergillus* are the most extensively studied cellulase-producing microorganisms. Enzymes produced by these fungi are commercially available for industrial production. Microorganisms of the genus *Trichoderma* produce relatively large quantities of EGs and CBHs, but only low levels of βG, whereas *Aspergillus* species produce relatively large quantities of EGs and βGs and low levels of CBHs.

To obtain higher titers and more effective cellulolytic systems, many novel cellulase-producing strains have been screened by Chinese scientists. Although *T. reesei* is the most extensively studied cellulase-producing industrial microorganism for its high cellulolytic capabilities, many other novel cellulase-producing strains have also been screened.

T. reesei produces only low levels of βG despite its high level of extracellular protein expression. Chinese scientists therefore attempted to look for *Trichoderma* strains that have higher βG activity. Two new *Trichoderma viride* strains, obtained using random mutagenesis, were isolated by Jiang et al. [67] and Xu et al. [68]. These strains were observed to produce higher βG activity than *T. reesei*. Wang et al. [69] suggested that the optimum temperature for the psychrophilic cellulases produced by oceanic bacteria is 15–25 °C, lower than that of regular cellulases (45–55 °C). Recently, psychrophilic cellulases produced by *Trichoderma* and

Table 2 *Penicillium* species producing lignocellulose-degrading enzymes

Species	References
P. decumbens	[67]
P. funiculosum	[78]
P. janthinellum	[32]
P. chrysogenum	[79]
P. canescens	[80]
P. purpurogenum	[81]
P. brasilianum	[82]
P. simplicissimum	[83]
P. citrinum	[84]
P. melinii	[85]
P. verruculosum	[86]
P. pinophilum	[87]
P. echinulatum	[88]
P. occitanis	[89]
P. minioluteum	[90]

Penicillium species were reported and their conditions for cellulase production were optimized [70, 71]. A strain producing alkaline cellulase was isolated and studied by Chinese scientists [72]. Interestingly, this strain secretes cellulases with an optimum pH of 9.5, making it potentially useful for hydrolyzing alkali-treated biomass. To obtain halophilic or salt-tolerant cellulases, a megagenomic library was constructed from environmental DNA samples and screened for potential halophilic cellulases. An EG from glycoside hydrolase family 5 was identified through this approach, and was cloned and expressed in *Escherichia coli*, and purified. This purified protein was shown to be salt-tolerant and maintains about 70% residual activity after pretreatment with 4 M NaCl or KCl for 10 days [73]. In the search for thermostable cellulases, an attempt was made by Qin et al. [74] to overexpress EG Cel5A from *T. reesei* in *S. cerevisiae*. This recombinant EG has higher glycosylation content, as well as a dramatically better themostability at 50 °C. In another study, Cel45A from *P. decumbens* was purified, the first such attempt with a glycoside hydrolase family 45 protein; the enzyme was shown to have an optimum temperature of 60 °C and significantly hydrolyzes konjac glucomannan, phosphoric acid−swollen cellulose, and sodium carboxyl methyl cellulose, with the highest activity against the first substrate [75].

Penicillium species with the ability to produce high cellulase and hemicellulase titers have been described [8, 64, 76, 77]. They usually have higher βG activity than *Trichoderma* sp. [76], making them potent for the hydrolysis of lignocellulosic materials. Interestingly, the enzyme from *Penicillium* sp. ECU0913, which has both high cellulase and high hemicellulase activity, efficiently hydrolyzes pretreated corn stover without any accessory enzymes [61]. Many *Penicillium* strains that produce cellulolytic enzymes were found (Table 2), of which *P. decumbens*, *P. janthinellum*,, and *P. funiculosum* were the most studied.

In 1979, a fast-growing cellulolytic fungus, *P. decumbens* 114–2, was isolated from soil by scientists at Shandong University [64], which they then studied in detail for almost 30 years [6–8, 44, 64, 75, 77, 91–97]. Some cellulases (cel7B and cel5A) from *P. decumbens* have been purified and characterized, establishing the strain improvement strategy of this fungus [93, 95]. The industrial production of cellulolytic enzymes from this species has been conducted since 1996.

3.2 Mechanisms of Cellulase Synthesis

Apart from the development of economically feasible systems for cellulose degradation, there has also been continuing interest in understanding the mechanisms of cellulase synthesis and production to identify feasible approaches for increasing cellulase production [94].

Sun et al. [17] investigated the proteome profiling map of the cellulases secreted by *T. reesei* Rut C-30 using two-dimensional gel electrophoresis. CBH I and CBH II were found to represent about 37% of the total extracellular proteins, and the CBH II concentration produced with nonpretreated rice straw powder was about threefold higher than that with alkali-treated straw. This interesting result suggests that the synthesis of CBH II is controlled by other factors aside from cellulose. Sun et al. [96] reported the differences in the composition and expression levels of *P. decumbens* cellulases under induced and basal conditions. The basal cellulase in *P. decumbens* was demonstrated to be composed of CBH I, CBH II, EG I, EG II, and βG, whereas two EGs were expressed only under induction conditions. Furthermore, the basal and induced EGs from *P. decumbens* were encoded by different genes.

To understand the mechanism of the two transcription repressors Cre1 and ACE I in *T. reesei*, Su et al. [98] developed a new strategy wherein a plasmid that encodes a chimeric transcription activator containing the DNA binding domains from Cre1 and ACE I and the effector domain from the activator ACE II was constructed and transformed into *T. reesei*. The recombinant strain had higher cellulase activity than its parent strain and had a different colony appearance. The results also provide an overview of the set of genes that might be regulated by Cre1 or ACE I. These results contribute to further understanding the regulatory roles of these two repressors in cellular pathways and provided a new method for strain improvement through genetic manipulation.

Liu et al. [99] studied the differences in gene sequences of CBH I gene (*cbh1*) from wild-type and mutant *P. decumbens* strains and found that the mutant strain JU-A10 is a multiple mutant of the wild-type strain in the sequences upstream of the gene. The enhanced CBH activity of the mutant may be due to a single base mutation of the upstream sequence of *cbh1*, which affects the transcription regulation of the mutant instead of the protein coding sequences. This discovery suggests the critical role of the promoter region of cellulose-encoding genes, which is helpful in constructing hyperproducing strains of *P. decumbens*. An apparent abolishment of glucose repression was also identified in strain JU-A10,

with enhanced observed cellulase and hemicellulase production in glucose-containing media. Genomic analysis of this strain revealed a single nucleotide deletion at the +1205 position in the *creA* gene, which encodes a carbon catabolite repressor protein. This frameshift mutation changes the amino acid sequence downstream from the site of the mutation (unpublished data). Numerous other mutations have also been identified from this mutant strain through genomic analysis, in addition to the changes in the *creA* gene.

Another focus for Chinese scientists is identifying inducers for cellulolytic enzyme production, which could potentially benefit the cellulase industry. Wang et al. [100] observed that the concentrations of ATP and cyclic AMP (cAMP) influence cellulase production. Cellulase synthesis is repressed by high concentrations of intracellular ATP, whereas exogenous cAMP increases cellulase synthesis. The effects of wheat bran on the hydrolysis of extracellular biomass were investigated in *P. decumbens* by Sun et al. [96]. The soluble cello-oligosaccharide composition of wheat bran was shown to be one of the most significant factors in cellulase production. This significant discovery may be critical in the cellulase industry because wheat bran, as an inducer in cellulase and xylanase production, is inexpensive.

3.3 Optimization of Cellulase-Producing Strains, Cellulases, and Their Production

Traditional random mutagenesis techniques, such as ultraviolet (UV) irradiation, *N*-methyl-*N*′-nitro-*N*-nitrosoguanidine treatment, low-energy ion beam implantation, and atmospheric pressure nonequilibrium discharge plasma, are useful and effective approaches for the development of fungal strains with increased cellulase production. Through these approaches, mutants of *T. reesei* and *P. decumbens* with highly increased cellulolytic capabilities have been obtained (Fig. 5) [16, 24, 101].

Genome shuffling is another effective approach for the rapid engineering of microbial strains with desirable industrial phenotypes, and it is thus used for the development of cellulase-producing strains. Cheng et al. [91] used this technique to enhance cellulase production by repeated protoplast fusions, and the GS2-15, GS2-21, and GS2-22 fusants obtained showed 100, 109, and 94% increased filter paperase activity compared with their parent strain. Xu et al. [68] used genome shuffling to improve the cellulase production of the wild-type strain *T. viride* TL-124. The initial mutants were generated through random mutagenesis and were then subjected to recursive protoplast fusion. The cellulase activity of the resulting strains was assayed after solid-state fermentation using wheat straw as the substrate. The shuffled strain *T. viride* F161, which was selected from among approximately 2,000 strains after two rounds of genome shuffling, exhibited a total cellulase activity of 4.17 U g^{-1} dry weight, which was 1.97-fold higher than that of wild-type strain *T. viride* TL-124 (2.12 U g^{-1} dry weight).

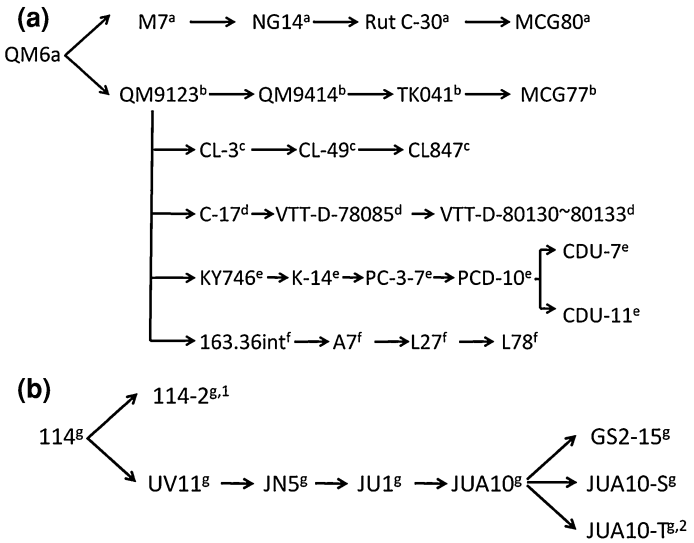

Fig. 5 The genealogy of *Trichoderma reesei* (**a**) and *Penicillium decumbens* (**b**). *a–g* strains isolated/constructed by Rutgers University, USA; the US Army Natick Laboratories; Cavla Laboratories, France; VTT Technical Research Centre of Finland; Kyowa Hakko Kirin, Japan, formerly Kyowa Hakko Kogyo, Japan; Chiron Corporation, formerly Cetus Corporation, USA; the State Key Laboratory of Microbial Technology, Shandong University, China. *1* Filter paperase unit (FPU) 0.35 IU/mL, *2* FPU 18.9 IU/mL

The genealogy of the representative strains created in our laboratory with the aforementioned approaches is presented in Fig. 5b. Large numbers of mutant strains that exhibit a diversity of characteristics were obtained through UV and *N*-methyl-*N*′-nitro-*N*-nitrosoguanidine treatment, and various other techniques. *P. decumbens* strains JU15 and JU1, which are resistant to catabolite repression, were isolated using a culture medium containing glucose [67].

Aside from these traditional mutation techniques, genetic engineering was also exploited to improve the expression levels of cellulases and hemicellulases in fungi. For example, Liu et al. [102] modified the promoter region of *cbh1* and alleviated the glucose repression effects by deleting the binding sites of the CreI protein, as well as repeatedly inserting the CCAAT box and the ACE II binding site into the modified *cbh1* promoter in *T. reesei*. Su et al. [98] obtained higher cellulase activity in *T. reesei* by reconstructing the effector domain of ACE II. Zhang et al. [103] replaced the promoter of the gene encoding *β*G with a four-copy *cbh1* promoter in *T. reesei*. The recombinant strain showed a significant increase in *β*G activity, filter paper activity, and better saccharification of corncob residues. A similar approach was utilized by Wang et al. [104], who created a *T. reesei* strain with higher CMCase activity by replacing the coding sequence of CBH I with that of EG III. The genome sequencing of *P. decumbens* was recently completed (unpublished data), and will provide insight into the role of cellulase genes and the regulatory mechanisms of cellulose degradation. To improve gene

targeting efficiency in *P. decumbens*, Li et al. [94] developed a highly efficient gene targeting system by deleting *pku70*, which is involved in the nonhomologous end-joining pathways, and succeeded in significantly improving gene targeting efficiency. The development of gene manipulation platforms in species of cellulase-producing fungi will efficiently improve the efficiency of engineering these species for better lignocellulose degradation.

Aside from the genetic engineering of cellulase-producing fungal strains, protein engineering of cellulases is also a focus of research in China. Xiao et al. [105] applied an error-prone PCR technique in random mutagenesis of EG III from *T. reesei*, and obtained a psychrophilic enzyme called w-3. Catalytic analysis of this enzyme suggested a 19% increase in specific activity and a 41% increase in K_{cat}/K_m at 30 °C. The sequence analysis suggested a 25 amino acid residue deletion at the C-terminus and a significant decrease in its hydrophobicity. The same technique was used to investigate EG III from *T. reesei* in search of an alkaline EG. An N321T mutant with an increased optimum pH was obtained. Further site-directed mutagenesis suggests the important role of this amino acid residue for the activity of EG III, and it resulted in an N321H mutant with a broader range of pH tolerance [106]. Another example of the genetic engineering of enzymes is the research on EG II from *T. reesei* during the search for an alkaline EG. Qin et al. [107, 108] subjected residue 342 to saturated mutagenesis and modified the enzyme through random mutagenesis and two rounds of DNA shuffling. A series of modified enzymes were produced during these investigations, including an N342 variant with an optimum pH of 5.8, one unit higher than the wild-type enzyme and with a 1.5-fold higher K_{cat}/K_m at pH 6.5, an N39R/L218H/W276R/N342T variant that has a pH optimum of 6.2 and a 1.4-fold higher K_{cat}/K_m at pH 6.2, as well as three variants L218H, Q139R/N342T, and Q139R/L218H/W276R/N342T that have more than 3.5-fold increased activity at pH 7.0 [107, 108].

Research on optimizing processes of cellulase production is another focus of study in China, which aims to improve the productivity and efficiency of cellulase production. Yu and Koo [109] used mixtures of Avicel and wheat bran as carbon sources and obtained higher secreted cellulase activity [11.67 filter paperase units (FPU) per milliliter] with *T. reesei* Rut C-30 in a 2.5-L fermentor. Duan et al. [110] investigated the combined effects of cellulose powder CF11 and glucose on EG production from *T. pseudokoingii* S38, and demonstrated that the use of both substrates as carbon sources increases the volumetric product efficiency, as well as the specific activity of EG production compared with the use of only one substrate.

Qu et al. [76] suggested that the high price of cellulases is partly due to the high cost of cellulose powder and inorganic salts in media. A new cellulase production process using industrial wastes was therefore developed with *P. decumbens* JU1. In this process, spent ammonium sulfite liquor and cellulosic wastes (clarifier sludge and digester fines) from a paper mill were used as the medium for fungal growth, as well as cellulase production. Yang and Yu [111] used bagasse pretreated with alkali and microwave radiation instead of cellulose powder, thereby addressing the high prices of cellulose powders. In another study, solid residues from the evaporation of acidic liquid generated during industrial

production of sodium glutamate were used to substitute for ammonium sulfate as the nitrogen source during cellulase production, and a similar cellulase production rate was observed [112].

The optimization of fermentation conditions has also been attempted to improve cellulolytic enzyme production by Chinese scientists. Dong et al. [92] reported that the CMCase production of *P. decumbens* JU-A10 was increased through response surface analysis and that the productivity with Chinese sorghum straw as a carbon source was significantly higher than that of corn stover. Yu and Koo [113] increased cellulase production with *T. reesei* from 21.25 FPU/mL to 30.6 FPU/mL through fed-batch culture and suggested that cell mass has a significant effect on cellulase activity and productivity. Qu and Gao [114] used a mixed culture of *P. decumbens* and *Aspergillus* sp. and improved the cellulolytic enzyme system during solid-state fermentation.

4 Conclusions

The enzymatic hydrolysis of lignocellulosic biomass for the production of second-generation bioethanol is considered one of the most promising approaches for abundant and reliable biofuels, and it has therefore received attention from scientists worldwide, including Chinese scientists. In this review, we have attempted to summarize current understanding of the mechanism of lignocellulose degradation, as well as the efforts to optimize the production of cellulases, the hydrolysis of lignocelluloses, and the fungal strains that produce cellulases. As can be seen, rendering of biomass recalcitrance is a complicated process that requires the synergistic effort of multiple factors, including cellulases, hemicellulases, and nonenzyme factors. Synergism was also discovered within each group of enzymes and even within specific enzyme molecules. This process is still far from being completely understood; new factors are being discovered and new findings regarding the mechanism of this process are being reported.

The oil crisis in the 1970s as well as the recent one have prompted scientific and industrial efforts to search for a realistic substitute or at least a supplement for crude oil. The rapid economic progress and improvement of living standards in China since the 1980s demand a rapid increase in energy production. This has led to a shift in the focus of the Chinese government and scientists to sustainable energy production, including the development of technologies for second-generation bioethanol. In this review, the efforts of Chinese scientists in the development of better enzymes, better strains, and better approaches for lignocellulose-based bioethanol production has been summarized. These efforts have dramatically increased the productivity and efficiency and have decreased the costs of bioethanol production. Today, numerous governments, institutions, and international corporations have initiated and succeeded in searches for viable and economic protocols in the enzymatic hydrolysis of lignocelluloses. We are optimistic that industrial-scale production of second-generation

bioethanol will soon be achieved at a reasonably low cost, which will hopefully lead to a sustainable and better tomorrow.

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References

1. Rass-Hansen J, Falsig H, Jørgensen B et al (2007) Bioethanol: fuel or feedstock? J Chem Technol Biotechnol 82(4):329–333
2. Searchinger T, Heimlich R, Houghton RA et al (2008) Use of U.S. croplands for biofuels increases greenhouse gases through emissions from land-use change. Science 319(5867): 1238–1240
3. Palmqvist E, Hahn-Hagerdal B (2000) Fermentation of lignocellulosic hydrolysates II: inhibitors and mechanisms of inhibition. Bioresour Technol 74(1):25–33
4. Fang X, Shen Y, Zhao J et al (2010) Status and prospect of lignocellulosic bioethanol production in China. Bioresour Technol 101(13):4814–4819
5. Chen H, Han Y, Xu J (2008) Simultaneous saccharification and fermentation of steam exploded wheat straw pretreated with alkaline peroxide. Process Biochem 43(12): 1462–1466
6. Liu K, Lin X, Yue J et al (2010) High concentration ethanol production from corncob residues by fed-batch strategy. Bioresour Technol 101(13):4952–4958
7. Ouyang J, Dong Z, Song X et al (2010) Improved enzymatic hydrolysis of microcrystalline cellulose (Avicel PH101) by polyethylene glycol addition. Bioresour Technol 101(17): 6685–6691
8. Qu Y, Zhu M, Liu K et al (2006) Studies on cellulosic ethanol production for sustainable supply of liquid fuel in China. Biotechnol J 1(11):1235–1240
9. Zhang M, Su R, Qi W et al (2010) Enhanced enzymatic hydrolysis of lignocellulose by optimizing enzyme complexes. Appl Biochem Biotechnol 160(5):1407–1414
10. Wu X, Zhao R, Wang D et al (2006) Effects of amylose, corn protein, and corn fiber contents on production of ethanol from starch-rich media. Cereal Chem 83(5):569–575
11. Singh R, Varma AJ, Seeta Laxman R et al (2009) Hydrolysis of cellulose derived from steam exploded bagasse by *Penicillium* cellulases: comparison with commercial cellulase. Bioresour Technol 100(24):6679–6681
12. Merino ST, Cherry J (2007) Progress and challenges in enzyme development for biomass utilization. Adv Biochem Eng Biotechnol 108:95–120
13. Wingren A (2005) Ethanol from softwood: techno-economic evaluation for development of the enzymatic process. PhD dissertation, Lund University
14. Sassner P, Galbe M, Zacchi G (2008) Techno-economic evaluation of bioethanol production from three different lignocellulosic materials. Biomass Bioenerg 32(5):422–430
15. Vinzant TB, Adney WS, Decker SR et al (2001) Fingerprinting *Trichoderma reesei* hydrolases in a commercial cellulase preparation. Appl Biochem Biotechnol 91–93:99–107
16. Esterbauer H (1991) Production of *Trichoderma* cellulase in laboratory and pilot scale. Bioresour Technol 36(1):51–65
17. Sun W, Cheng C, Lee W (2008) Protein expression and enzymatic activity of cellulases produced by *Trichoderma reesei* Rut C-30 on rice straw. Process Biochem 43(10): 1083–1087

18. Lynd LR, Weimer PJ, van Zyl WH et al (2002) Microbial cellulose utilization: fundamentals and biotechnology. Microbiol Mol Biol Rev 66(3):506–577
19. Kumar R, Singh S, Singh OV (2008) Bioconversion of lignocellulosic biomass: biochemical and molecular perspectives. J Ind Microbiol Biotechnol 35(5):377–391
20. Polizeli ML, Rizzatti AC, Monti R et al (2005) Xylanases from fungi: properties and industrial applications. Appl Microbiol Biotechnol 67(5):577–591
21. Wong KK, Tan LU, Saddler JN (1988) Multiplicity of β-1, 4-xylanase in microorganisms: functions and applications. Microbiol Rev 52(3):305–317
22. Collins T, Gerday C, Feller G (2005) Xylanases, xylanase families and extremophilic xylanases. FEMS Microbiol Rev 29(1):3–23
23. Moreira LR, Filho EX (2008) An overview of mannan structure and mannan-degrading enzyme systems. Appl Microbiol Biotechnol 79(2):165–178
24. Fang X, Qin Y, Li X et al (2010) Progress on cellulase and enzymatic hydrolysis of lignocellulosic biomass. China J Biotechnol 26(7):864–869
25. Baker JO, Ehrman CI, Adney WS (1998) Hydrolysis of cellulose using ternary mixtures of purified cellulases. Appl Biochem Biotechnol 70–72:395–403
26. Levin SE, Fox JM, Clark DS et al (2011) A mechanistic model for rational design of optimal cellulase mixtures. Biotechnol Bioeng 108(11):2561–2570
27. Zhang YH, Lynd LR (2004) Toward an aggregated understanding of enzymatic hydrolysis of cellulose: noncomplexed cellulase systems. Biotechnol Bioeng 88(7):797–824
28. Wang L, Zhang Y, Gao P et al (2006) Changes in the structural properties and rate of hydrolysis of cotton fibers during extended enzymatic hydrolysis. Biotechnol Bioeng 93(3):443–456
29. Wang L, Zhang Y, Gao P (2008) A novel function for the cellulose binding module of cellobiohydrolase I. Sci China C Life Sci 51(7):620–629
30. Lehtio J, Sugiyama J, Gustavsson M et al (2003) The binding specificity and affinity determinants of family 1 and family 3 cellulose binding modules. Proc Natl Acad Sci USA 100(2):484–489
31. Linder M, Teeri TT (1996) The cellulose-binding domain of the major cellobiohydrolase of *Trichoderma reesei* exhibits true reversibility and a high exchange rate on crystalline cellulose. Proc Natl Acad Sci USA 93(22):12251–12255
32. Wang T, Zou Y, Shi Y et al (2000) Expression and characteristic of secretary CBD_{CBH1} from *Penicillium janthinellum* in *E.coli*. Chin J Biochem Mol Biol 16(5):644–649
33. Zhong L, Matthews JF, Hansen PI et al (2009) Computational simulations of the *Trichoderma reesei* cellobiohydrolase I acting on microcrystalline cellulose Ibeta: the enzyme-substrate complex. Carbohydr Res 344(15):1984–1992
34. Xiao Z, Gao P, Qu Y et al (2001) Cellulose-binding domain of endoglucanase III from *Trichoderma reesei* disrupting the structure of cellulose. Biotechnol Lett 23(9):711–715
35. Divne C, Stahlberg J, Teeri TT et al (1998) High-resolution crystal structures reveal how a cellulose chain is bound in the 50 angstrom long tunnel of cellobiohydrolase I from *Trichoderma reesei*. J Mol Biol 275(2):309–325
36. Varrot A, Frandsen TP, von Ossowski I et al (2003) Structural basis for ligand binding and processivity in cellobiohydrolase Cel6A from *Humicola insolens*. Structure 11(7):855–864
37. Zhao X, Rignall TR, McCabe C et al (2008) Molecular simulation evidence for processive motion of *Trichoderma reesei* Cel7A during cellulose depolymerization. Chem Phys Lett 460(1–3):284–288
38. Carrard G, Linder M (1999) Widely different off rates of two closely related cellulose-binding domains from *Trichoderma reesei*. Eur J Biochem 262(3):637–643
39. Mattinen ML, Linder M, Teleman A et al (1997) Interaction between cellohexaose and cellulose binding domains from *Trichoderma reesei* cellulases. FEBS Lett 407(3):291–296
40. Receveur V, Czjzek M, Schulein M et al (2002) Dimension, shape, and conformational flexibility of a two domain fungal cellulase in solution probed by small angle X-ray scattering. J Biol Chem 277(43):40887–40892

41. Igarashi K, Koivula A, Wada M et al (2009) High speed atomic force microscopy visualizes processive movement of *Trichoderma reesei* cellobiohydrolase I on crystalline cellulose. J Biol Chem 284(52):36186–36190
42. Zhao Y, Wu B, Yan B et al (2004) Mechanism of cellobiose inhibition in cellulose hydrolysis by cellobiohydrolase. Sci China C Life Sci 47(1):18–24
43. Ma A, Hu Q, Qu Y et al (2008) The enzymatic hydrolysis rate of cellulose decreases with irreversible adsorption of cellobiohydrolase I. Enzyme Microb Technol 42(7):543–547
44. Chen M, Qin Y, Liu Z et al (2010) Isolation and characterization of a β-glucosidase from *Penicillium decumbens* and improving hydrolysis of corncob residue by using it as cellulase supplementation. Enzyme Microb Technol 46(6):444–449
45. Chauve M, Mathis H, Huc D et al (2010) Comparative kinetic analysis of two fungal β-glucosidases. Biotechnol Biofuels 3(1):3
46. Ma L, Zhang J, Zou G (2011) Improvement of cellulase activity in *Trichoderma reesei* by heterologous expression of a beta-glucosidase gene from *Penicillium decumbens*. Enzyme Microb Technol. doi: 10.1016/j.enzmictec.2011.06.013
47. Shen Y, Zhang Y, Ma T et al (2008) Simultaneous saccharification and fermentation of acid-pretreated corncobs with a recombinant *Saccharomyces cerevisiae* expressing beta-glucosidase. Bioresour Technol 99(11):5099–5103
48. Han Y, Chen H (2007) Synergism between corn stover protein and cellulase. Enzyme Microb Technol 41(5):638–645
49. Han Y, Chen H (2008) Characterization of β-glucosidase from corn stover and its application in simultaneous saccharification and fermentation. Bioresour Technol 99(14): 6081–6087
50. Kumar R, Wyman CE (2009) Effect of xylanase supplementation of cellulase on digestion of corn stover solids prepared by leading pretreatment technologies. Bioresour Technol 100(18):4203–4213
51. Zeng W, Chen H (2009) Synergistic effect of feruloyl esterase and cellulase in hydrolyzation of steam-exploded rice straw. China J Biotechnol 25(1):49–54
52. van Peij NN, Brinkmann J, Vrsanska M et al (1997) β-xylosidase activity, encoded by *xlnD*, is essential for complete hydrolysis of xylan by *Aspergillus niger* but not for induction of the xylanolytic enzyme spectrum. Eur J Biochem 245(1):164–173
53. de Vries RP, Kester HC, Poulsen CH et al (2000) Synergy between enzymes from *Aspergillus* involved in the degradation of plant cell wall polysaccharides. Carbohydr Res 327(4):401–410
54. Yu H, Zhang X, Song L et al (2010) Evaluation of white-rot fungi-assisted alkaline/ oxidative pretreatment of corn straw undergoing enzymatic hydrolysis by cellulase. J Biosci Bioeng 110(6):660–664
55. Jing X, Zhang X, Bao J (2009) Inhibition performance of lignocellulose degradation products on industrial cellulase enzymes during cellulose hydrolysis. Appl Biochem Biotechnol 159(3):696–707
56. Chen H, Liu L (2007) Unpolluted fractionation of wheat straw by steam explosion and ethanol extraction. Bioresour Technol 98(3):666–676
57. Wang W, Liu J, Chen G et al (2003) Function of a low molecular weight peptide from *Trichoderma pseudokoningii* S38 during cellulose biodegradation. Curr Microbiol 46(5): 371–379
58. Saloheimo M, Paloheimo M, Hakola S et al (2002) Swollenin, a *Trichoderma reesei* protein with sequence similarity to the plant expansins, exhibits disruption activity on cellulosic materials. Eur J Biochem 269(17):4202–4211
59. Yao Q, Sun T, Liu W et al (2008) Gene cloning and heterologous expression of a novel endoglucanase, swollenin, from *Trichoderma pseudokoningii* S38. Biosci Biotechnol Biochem 72(11):2799–2805
60. Zhou Q, Lv X, Zhang X et al (2011) Evaluation of swollenin from *Trichoderma pseudokoningii* as a potential synergistic factor in the enzymatic hydrolysis of cellulose with low cellulase loadings. World J Microbiol Biotechnol. doi:10.1007/s11274-011-0650-5

61. Shi Q, Sun J, Yu H et al (2011) Catalytic performance of corn stover hydrolysis by a new isolate *Penicillium* sp ECU0913 producing both cellulase and xylanase. Appl Biochem Biotechnol 164(6):819–830
62. Montenecourt BS, Eveleigh DE (1977) Semiquantitative plate assay for determination of cellulase production by *Trichoderma viride*. Appl Environ Microbiol 33(1):178–183
63. Saddler JN (1982) Screening of highly cellulolytic fungi and the action of their cellulase enzyme systems. Enzyme Microb Technol 4:414–418
64. Qu Y, Gao P, Wang Z (1984) Screening of catabolite repression-resistant mutants of cellulase producing *Penicillium* spp. Acta Microbiol Sin 3:238–1243
65. Ma D, Gao P, Wang Z (1990) Preliminary studies on the mechanism of cellulase formation by *Trichoderma pseudokoningii* S-38. Enzyme Microb Technol 12:631–635
66. Liu C, Cai M, Ma X (1993) Screening of a β-glucosidase product repression resistant *Penicillium decumbens* strain. Microbiol Res Appl 613:5–8
67. Jiang X, Geng A, He N et al (2011) New isolate of *Trichoderma viride* strain for enhanced cellulolytic enzyme complex production. J Biosci Bioeng 111(2):121–127
68. Xu F, Wang J, Chen S et al (2011) Strain improvement for enhanced production of cellulase in *Trichoderma viride*. Prikl Biokhim Mikrobiol 47(1):61–65
69. Wang F, Wang T, Zhang G et al (2003) Screening and characterization of the cold-adaptive cellulase-producing bacteria. Mar Sci 27(5):42–45
70. Chen L, Chi N, Zhang Q (2009) Breeding and fermentation medium optimization of cold-active cellulase strain CNY086(I). Microbiology 36:1547–1552
71. Chen L, Chi N, Zhang Q (2011a) Research of cold-active cellulase produced by SWD-28 (*Penicillium* sp.). Biotechnology 21:84–88
72. Wu Q, Yuan L, Lu F et al (2010) Screening and identification of an alkaline cellulase-producing strain. Biotechnol Bull 9:205–209
73. Liu J, Liu W, Zhao X et al (2011) Cloning and functional characterization of a novel endo-β-1, 4-glucanase gene from a soil-derived metagenomic library. Appl Microbiol Biotechnol 89(4):1083–1092
74. Qin Y, Wei X, Liu X et al (2008) Purification and characterization of recombinant endoglucanase of *Trichoderma reesei* expressed in *Saccharomyces cerevisiae* with higher glycosylation and stability. Protein Expr Purif 58(1):162–167
75. Liu G, Wei X, Qin Y et al (2010) Characterization of the endoglucanase and glucomannanase activities of a glycoside hydrolase family 45 protein from *Penicillium decumbens* 114-2. J Gen Appl Microbiol 56(3):223–229
76. Jørgensen H, Morkeberg A, Krogh KBR et al (2005) Production of cellulases and hemicellulases by three *Penicillium* species: effect of substrate and evaluation of cellulase adsorption by capillary electrophoresis. Enzyme Microb Technol 36(1):42–48
77. Qu Y, Zhao X, Gao P et al (1991) Cellulase production from spent sulfite liquor and paper-mill waste fiber. Appl Biochem Biotechnol 28–29:363–368
78. Hoffman RM, Wood TM (1985) Isolation and partial characterization of a mutant of *Penicillium funiculosum* for the saccharification of straw. Biotechnol Bioeng 27(1):81–85
79. Hou Y, Wang T, Long H et al (2007) Cloning, sequencing and expression analysis of the first cellulase gene encoding cellobiohydrolase 1 from a cold-adaptive *Penicillium chrysogenum* FS010. Acta Biochim Biophys Sin 39(2):101–107
80. Chulkin AM, Loginov DS, Vavilova EA et al (2009) Enzymological properties of endo-(1–4)-beta-glucanase Eg12p of Penicillium canescens and characteristics of structural gene egl2. Biochemistry (Mosc) 74(6):655–662
81. Kurasawa T, Yachi M, Suto M et al (1992) Induction of cellulase by gentiobiose and its sulfur-containing analog in *Penicillium purpurogenum*. Appl Environ Microbiol 58(1):106–110
82. Krogh KB, Morkeberg A, Jorgensen H et al (2004) Screening genus *Penicillium* for producers of cellulolytic and xylanolytic enzymes. Appl Biochem Biotechnol 113–116: 389–401

83. Yu H, Zeng G, Huang G et al (2005) Lignin degradation by *Penicillium simplicissimum*. Environ Sci 26(2):167–171
84. Dutta T, Sahoo R, Sengupta R et al (2008) Novel cellulases from an extremophilic filamentous fungi *Penicillium citrinum*: production and characterization. J Ind Microbiol Biotechnol 35(4):275–282
85. Rosenthal AL, Nordin JH (1975) Enzymes that hydrolyze fungal cell wall polysaccharides: The carbonhydrate constitution of mycodextranse, an endo-α (1→4)-D-glucanase from *Pencillium melinii*. J Biol Chem 250(14):5295–5303
86. Solov'eva IV, Okunev ON, Vel'kov VV et al (2005) The selection and properties of *Penicillium verruculosum* mutants with enhanced production of cellulases and xylanases. Mikrobiologiia 74(2):172–178
87. Jeya M, Joo AR, Lee KM et al (2009) Characterization of endo-β-1,4-glucanase from a novel strain of Penicillium pinophilum KMJ601. Appl Microbiol Biotechnol 85(4): 1005–1014
88. Rubini MR, Dillon AJ, Kyaw CM et al (2010) Cloning, characterization and heterologous expression of the first *Penicillium echinulatum* cellulase gene. J Appl Microbiol 108(4): 1187–1198
89. Bhiri F, Chaabouni SE, Limam F et al (2008) Purification and biochemical characterization of extracellular β-glucosidases from the hypercellulolytic Pol6 mutant of *Penicillium occitanis*. Appl Biochem Biotechnol 149(2):169–182
90. Garcia B, Castellanos A, Menendez J et al (2001) Molecular cloning of an α-glucosidase-like gene from *Penicillium minioluteum* and structure prediction of its gene product. Biochem Biophys Res Commun 281(1):151–158
91. Cheng Y, Song X, Qin Y et al (2009) Genome shuffling improves production of cellulase by *Penicillium decumbens* JU-A10. J Appl Microbiol 107(6):1837–1846
92. Dong X, Du C, Lin J (2008) Fermentation condition optimization by *Penicillium decumbens* Ju-A10 for CMCase production using response surface analysis (RSA). Ind Microbiol 38(2):20–22
93. Gao L, Wang F, Gao F et al (2011) Purification and characterization of a novel cellobiohydrolase (PdCel6A) from *Penicillium decumbens* JU-A10 for bioethanol production. Bioresour Technol 102(17):8339–8342
94. Li Z, Du C, Zhong Y et al (2010) Development of a highly efficient gene targeting system allowing rapid genetic manipulations in *Penicillium decumbens*. Appl Microbiol Biotechnol 87(3):1065–1076
95. Wei X, Qin Y, Qu Y (2010) Molecular cloning and characterization of two major endoglucanases from *Penicillium decumbens*. J Microbiol Biotechnol 20(2):265–270
96. Sun X, Liu Z, Qu Y et al (2008) The effects of wheat bran composition on the production of biomass-hydrolyzing enzymes by *Penicillium decumbens*. Appl Biochem Biotechnol 146(1–3):119–128
97. Sun X, Liu Z, Zheng K et al (2008) The composition of basal and induced cellulase systems in *Penicillium decumbens* under induction or repression conditions. Enzyme Microb Technol 42(7):560–567
98. Su X, Chu X, Dong Z (2009) Identification of elevated transcripts in a *Trichoderma reesei* strain expressing a chimeric transcription activator using suppression subtractive hybridization. World J Microbiol Biotechnol 25(6):1075–1084
99. Liu Z, Sun X, Qu Y (2008) Cloning cellobiohydrolase I from Penicillium decumbens 114-2 with TAIL-PCR and comparing with its derepressed mutant JU-A10. Acta Microbiol Sin 48(5):667–671
100. Wang D, Qu Y, Gao P (1995) Regulation of cellulase synthesis in mycelial fungi. Biotechnol Lett 17(6):593–598
101. He J, Yu B, Zhang K et al (2009) Strain improvement of *Trichoderma reesei* Rut C-30 for increased cellulase production. Indian J Microbiol 49(2):188–195

102. Liu T, Wang T, Li X et al (2008) Improved heterologous gene expression in *Trichoderma reesei* by cellobiohydrolase I gene (*cbh1*) promoter optimization. Acta Biochim Biophys Sin 40(2):158–165
103. Zhang J, Zhong Y, Zhao X et al (2010) Development of the cellulolytic fungus *Trichoderma reesei* strain with enhanced *β*-glucosidase and filter paper activity using strong artificial cellobiohydrolase 1 promoter. Bioresour Technol 101(24):9815–9818
104. Wang T, Liu T, Wu Z et al (2004) Novel cellulase profile of *Trichoderma reesei* strains constructed by *cbh1* gene replacement with *eg3* gene expression cassette. Acta Biochim Biophys Sin 36(10):667–672
105. Xiao Z, Wang P, Qu Y et al (2002) Cold adaptation of a mesophilic cellulase, EG III from *Trichoderma reesei*, by directed evolution. Sci China C Life Sci 45(4):337–343
106. Wang T, Liu X, Yu Q et al (2005) Directed evolution for engineering pH profile of endoglucanase III from *Trichoderma reesei*. Biomol Eng 22(1–3):89–94
107. Qin Y, Wei X, Song X et al (2008) Engineering endoglucanase II from *Trichoderma reesei* to improve the catalytic efficiency at a higher pH optimum. J Biotechnol 135(2):190–195
108. Qin Y, Wei X, Song X et al (2008) The role of the site 342 in catalytic efficiency and pH optima of endoglucanase II from *Trichoderma reesei* as probed by saturation mutagenesis. Biocatal Biotransfor 26(5):378–382
109. Yu X, Koo YM (1998) Cellulase production by *Trichoderma reesei* in submerged fermentation. Ferment Ind 24:20–25
110. Duan XY, Liu SY, Zhang WC et al (2004) Volumetric productivity improvement for endoglucanase of *Trichoderma pseudokoingii* S-38. J Appl Microbiol 96(4):772–776
111. Yang X, Yu X (2008) Cellulase production by *Trichoderma reesei* from bagasse pretreated by alkali and microwave. Chin J Bioprocess Eng 6:61–65
112. Yao L, Yue J, Zhao J et al (2010) Application of acidic wastewater from monosodium glutamate process in pretreatment and cellulase production for bioconversion of corn stover—feasibility evaluation. Bioresour Technol 101(22):8755–8761
113. Yu X, Koo YM (1999) Cellulase production by *Trichoderma reesei* Rut C-30 with batch and fed-batch fermentation. Food Ferment Ind 25:16–19
114. Qu Y, Gao P (1992) Improvement of cellulase composition by solid-state mixed culture of *Penicillium decumbens* and *Aspergillus* sp. Ind Microbiol 22:1–9

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Bioethanol from Lignocellulosic Biomass

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Abstract China is suffering from a sustained shortage of crude oil supply, making fuel ethanol and other biofuels alternative solutions for this issue. However, taking into account the country's large population and dwindling arable land due to rapid urbanization, it is apparent that current fuel ethanol production from grain-based feedstocks is not sustainable, and lignocellulosic biomass, particularly agricultural residues that are abundantly available in China, is the only choice for China to further expand its fuel ethanol production, provided economically viable processes can be developed. In this chapter, cutting edge progress in bioethanol is reviewed, with a focus on the understanding of the molecular structure of the feedstock, leading pretreatment technologies, enzymatic hydrolysis of the cellulose component and strategies for the co-fermentation of the C5 and C6 sugars with engineered microorganisms. Finally, process integration and optimization is addressed with a case study on the COFCO Corporation's pilot plant, and challenges and perspectives for commercial production of bioethanol are highlighted.

Keywords Co-fermentation · Enzymatic hydrolysis · Lignocellulosic biomass · Pretreatment · Process integration and optimization

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Contents

1 Introduction

Although the historical production of fermented beverages and alcohol in China dates back to 2000 years ago in the Han Dynasty, fuel ethanol production is a recent event in China that was initiated 10 years ago by the government to offset the rapidly enlarging gap between the country's crude oil consumption, driven up by its rapid economic growth, and dwindling domestic reserves and production. The first fuel ethanol plant was put into operation in August 2003 in Jilin Province, in the northeast of China, with corn as the feedstock. Currently, there are five fuel ethanol producers across the country, producing 1.52 million tons of fuel ethanol annually from starch-based feedstocks including corn, wheat and cassava. Taking into account the country's large population and dwindling arable land due to rapid urbanization, it is apparent that current fuel ethanol production is not sustainable.

On the other hand, as one of the major grains producers in the world, agricultural residues are abundantly available in China, with an estimated 600–700 million tons produced annually [1]. Since no economically viable technologies are available for their conversion, most is burned by farmers in the field, not only polluting the environment, but also causing other problems such as the disruption of air transportation by smoke clouds in the sky [2]. It has been acknowledged worldwide that agricultural residues are one of the best choices to replace grains for fuel ethanol production, without endangering food security, although many challenges still exist for their commercial conversions, due to their recalcitrance to degradation [3] as well as their unique chemical composition. In particular, pentose sugars contained in hemicelluloses cannot be fermented into ethanol and CO_2 as efficiently as hexose sugars by conventional ethanologenic species like

Saccharomyces cerevisiae, and thus recombinants engineered with the pentose pathways are needed [4]. However, relentless efforts for decades and unprecedented progress in biotechnology are paving the way to overcome these bottlenecks leading to a promising harvest [5].

In this chapter, cutting edge progress in bioethanol production from lignocellulosic biomass is reviewed, with a focus on the characteristics of the feedstock, leading pretreatment technologies, enzymatic hydrolysis of the pretreated cellulose component, co-fermentation of the pentose and hexose sugars released from the hydrolysis of cellulose and hemicelluloses, and process integration and optimization. Remaining challenges and perspectives for the commercial production of bioethanol are also highlighted.

2 Understanding Lignocellulosic Biomass

Understanding lignocellulosic biomass, particularly its chemical composition, is a prerequisite for developing effective pretreatment technologies to deconstruct its rigid structure, designing enzymes to liberate sugars, particularly cellulase to release glucose, from recalcitrant cellulose, as well as engineering microorganisms to convert sugars into ethanol and other bio-based chemicals.

Lignocellulosic biomass is mainly composed of plant cell walls, with the structural carbohydrates cellulose and hemicellulose and heterogeneous phenolic polymer lignin as its primary components. However, their contents varies substantially, depending on the species, variety, climate, soil fertility and fertilization practice, but on average, for agricultural residues such as corn stover, wheat and rice straw, the cell walls contain about 40% cellulose, 30% hemicellulose and 15% ligin on a dry weight basis [6].

The distinctive feature of plant cell walls is their two-part structure, as illustrated in Fig. 1. A primary cell wall is developed with cell division, and enlarged during cell growth to a fiberglass-like structure, with crystalline cellulose microfibrils embedded in a matrix of polysaccharides such as hemicelluloses. The primary wall of adjacent cells is held together by a sticky layer, called the middle lamella, composed of pectins, to form the conducting tissue system arranged in numerous vascular bundles. On the other hand, when cells cease to grow, a secondary cell wall is gradually deposited between the plasma membrane and the primary cell wall for better mechanical strength and structural reinforcement through the incorporation of lignin into xylem fibers, which accounts for the bulk of lignocellulosic biomass that can be converted to fuels and chemicals [7].

The development of the conducting tissue system with the rigid secondary cell wall is a critical adaptive event in the evolution of land plants, which not only facilitates the transport of water and nutrients as well as extensive upright growth, but also raises its recalcitrance to degradation due to the interaction and cross-linking of cellulose, hemocellulose and lignin [3].

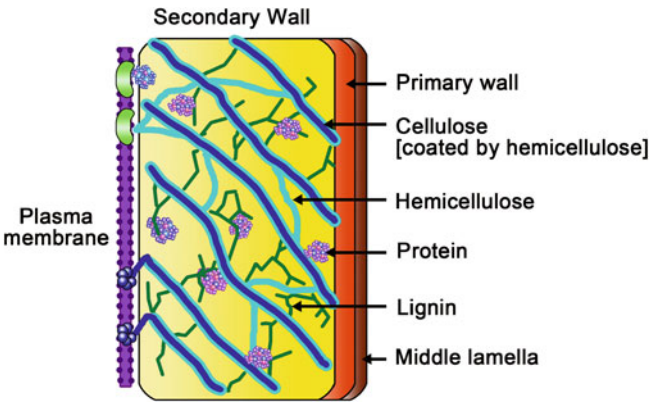

Fig. 1 Schematic diagram of plant cell walls

2.1 Cellulose

Cellulose is a polysaccharide composed of linear glucan chains that are linked together by β-1,4-glycosidic bonds with cellobiose residues as the repeating unit at different degrees of polymerization depending on resources, and packed into microfibrils which are held together by intramolecular hydrogen bonds as well as intermolecular van der Waals forces [8]. Although polymorphy has been documented for cellulose, native cellulose occurs as cellulose I, which is a mixture of two polymorphs I_α and I_β [9, 10]. Cellulose I_α is synthesized simultaneously with the extension of the microfibril network, and thus is dominant in lower plants to form the primary wall, and also in some bacteria. While, cellulose I_β is deposited within the secondary wall of higher plants for strength. The decipherment of crystalline structure indicates that cellulose I_α is characterized by the triclinic unit containing one chain, while there are two chains in the monoclinic unit of cellulose I_β providing more intramolecular hydrogen bonds, making it more stable [11]. Harsh conditions are therefore needed to transform cellulose I_β of plant biomass into amorphous polymorphs that can be attacked more efficiently by cellulases.

2.2 Hemicelluloses

Hemicelluloses are a heterogeneous group of polysaccharides with the β-(1→4)-linked backbone structure of pentose (C5) sugars, such as xylose and arabinose, and hexose (C6) sugars, including mannose, galactose and glucose as the repeating units, which have the same equatorial configuration at C1 and C4, as illustrated in Fig. 2 [12]. The structural similarity of hemicelluloses to the β-1,4-glycosidic bonds of the cellulose molecule benefits from a conformational homology, which can lead to a strong non-covalent association with cellulose microfibrils.

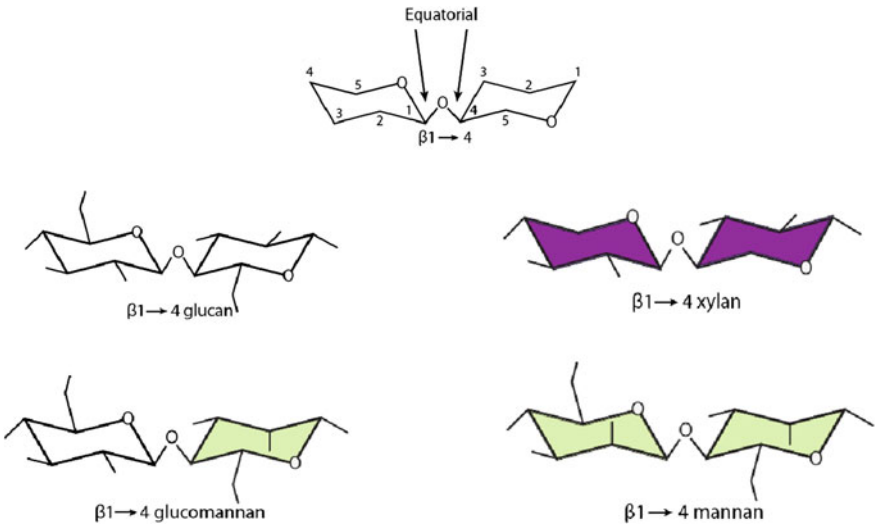

Fig. 2 Repeating units of hemicelluloses (Reprinted from [12] with permission)

Unlike cellulose which is crystalline and resistant to degradation, hemicelluloses are random and amorphous, and thus easily hydrolyzed to monomer sugars. However, hemicelluloses are embedded and interact with cellulose and lignin, which significantly increase the strength and toughness of plant cell walls.

Xyloglucan and xylans are major hemicelluloses in plant biomass. Xyloglucan is abundant in the primary walls, with the oligosaccharide composed of xylose (X) and glucose (G) with various side chains, XXXG or XXGG for vascular plants including grain crops, as the repeating unit. Xylans are polysaccharides with β-(1→4)-linked xylose residues as a backbone, which are often acetylated at the O-3 position of xylose residues and/or modified by α-(1→2)-linked glucuronosyl and 4-*O*-methyl glucuronosyl residues. Xylans, also known as glucuronoxylans, are the dominant noncellulosic polysaccharide in the secondary walls of dicots. A schematic illustration of xyloglucan and xylans is given in Fig. 3. The major sugars in the hydrolysate of hemicelluloses are therefore xylose, arabinose, glucose and galactose.

2.3 Lignin

Although lignin is a non-sugar-based polymer and cannot be used as feedstock for ethanol production via microbial fermentation, it exerts a significant impact on the economic performance of the corresponding bioconversion processes, since most inhibitors of microbial growth and fermentation come from this compound during the pretreatment that is needed to render cellulose amenable to enzymatic attack. Meanwhile, as the second most abundant component in biomass after cellulose, lignin yields

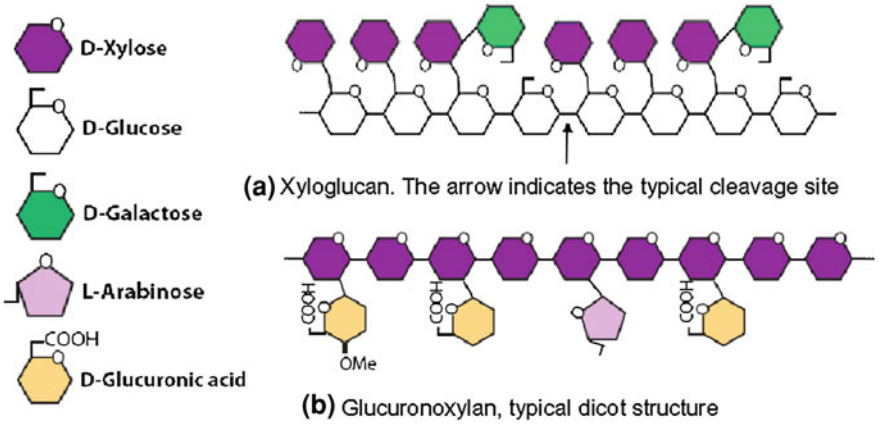

Fig. 3 Diagram of xyloglucan and xylans (Reprinted from [12] with permission)

more energy when burned, and thus is a good selection for combined heat and power (CHP) production in an eco- and environment-friendly mode of the biorefinery [13]. Moreover, lignin is an excellent starting material for various products including transportation fuels and value-added chemicals, which may add credits to bioconversion processes and make bioethanol more economically competitive.

It is apparent that understanding the fundamentals of lignin biosynthesis is the prerequisite for developing more efficient pretreatment and conditioning processes and subsequent enzymatic hydrolysis of cellulose, as well as engineering microorganisms with improved tolerance to inhibitors so that they can ferment the hydrolysate more rapidly with high yields. As illustrated in Fig. 4, lignin biosynthesis starts with the deamination of phenylalanine to cinnamic acid, followed by the modification of the aromatic ring by hydroxylation and *O*-methylation and reduction of the side chain to an alcohol moiety, resulting in the three major monolignols: *p*-coumaryl, coniferyl and sinapyl alcohols, which are exported across the plasma membrane into the apoplast.

The proportion of these monolignols varies substantially among plant species and tissues in the same plant as well as subcellular locations, and is also affected by the developmental stage and environmental stimuli. In addition to the three canonical monolignols, many other compounds are also involved in the biosynthesis of lignin, particularly ferulates, coniferaldehyde and acylated monolignols [13], which will be liberated during the pretreatment of lignocellulosic biomass.

2.4 Other Components

In addition to the three major components, cellulose and hemicelluloses that can be hydrolyzed to sugars for ethanol fermentation, and lignin left after fermentation for CHP production to drive the production facilities, other components like proteins and ashes also affect the process economics, and have not been addressed

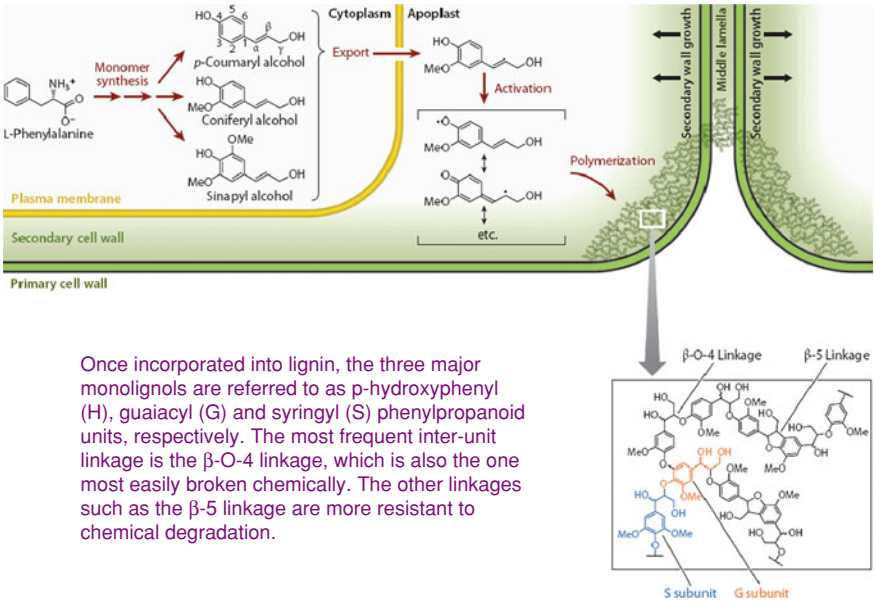

Fig. 4 Schematic diagram of lignin biosynthesis (Adapted from [13] with modifications)

adequately elsewhere. For example, fermentation nutrients are usually needed to nourish ethanologenic microorganisms, either *S. ceresive* or *Zymomonas mobilis* that can be engineered for ethanol production from lignocellulosic biomass, due to insufficient nutrition in the feedstock, which raises a concern about the supplementation of nutritional components to satisfy the basic requirements for cell growth and ethanol fermentation.

Like cellulose, hemicelluloses and lignin, nutritional components in lignocellulosic biomass also vary with species, variety, climate, soil fertility and fertilization practice. For major agricultural residues including corn stover, wheat and rice straw that are abundantly available in China, the protein content is approximately 5% [6], much lower than that in starch-based feedstocks like corn. Meanwhile, ethanologenic microorganisms cannot break down these proteins into assimilable amino acids, and thus protease treatment, which has been practiced in ethanol production from starch-based feedstocks, particularly cassava chips, may be supplemented to hydrolyze proteins, providing nitrogen sources to support microbial growth and ethanol fermentation. Otherwise, a supplementary nitrogen source from ammonia or urea needs to be provided. Corn steep liquor (CSL) is a cost-effective nutrient for providing not only assimilable nitrogen sources but also trace minerals as well as vitamins, particular for ethanol production from corn stover, since CSL is a by-product of the corn wet-milling process and its reliable supply is guaranteed. As for macronutrients such as phosphor, potassium, calcium and other minerals, they are normally sufficient due to the high ash content, up to 10% in lignocellulosic biomass [6].

3 Pretreatment

The self-assembly architecture of plant cell walls, with crystalline cellulose microfibrils interacting and entangling with hemicelluloses and lignin, creates ligin carbohydrate complexes (LCCs) [14], which are inaccessible for cellulases to bind onto surfaces of cellulose molecules. Therefore, after a preliminary size reduction to 10–30 mm through mechanical methods such as chopping, pretreatment is needed to deconstruct LCCs for efficient enzymatic hydrolysis of cellulose [15]. The smaller the size, the more efficient the mass and heat transfer will be for subsequent pretreatment and enzymatic hydrolysis. However, power requirement increases significantly with reduction in size. Therefore, a compromise between size reduction and energy consumption is needed from the economic point of view. Pretreatment technologies can be classified in general into four categories: physical pretreatment, chemical pretreatment, solvent fractionation and biological decomposition [16]. An ideal pretreatment process should maximize sugar yield from cellulose and hemicelluloses, and in the meantime minimize energy consumption and environmental impact. Unfortunately, none of them alone can satisfy all of these criteria.

3.1 Physical Pretreatment

Physical pretreatments do not use any chemicals. Size reduction by mechanical methods such as grinding or milling is one of them, through which the surface area of biomass is increased, and the degree of polymerization (DP) and crystallinity of cellulose is decreased to some extent, but the power requirement for reducing the feedstock from millimeter size to fine particles of micrometers is extremely high, which is unacceptable from the engineering point of view. Radiation such as microwaves that can penetrate and heat the feedstock instantly has also been studied [17]. However, it is problematic to process the feedstock in large quantities, not to mention the power requirement to generate the radiation. Therefore, more attention regarding physical pretreatment has been focused on the hydrothermal processes of steam explosion (SE) and liquid hot water (LHW) treatment.

SE involves heating the feedstock at elevated temperature and pressure for a short duration, followed by depressurizing the system to disrupt the structure of LCCs. Due to lower capital investment, less impact on the environment, and simple process design and operation, the SE process has been tested at pilot scales worldwide. The mechanism underlying the pretreatment is assumed to be the partial degradation of LCCs catalyzed by acetic acid released from acetylated hemicelluloses and other organic acids such as formic and levulinic acids, making the process autohydrolytic in nature [18]. The major parameters of the SE process are temperature or pressure and holding time, which should be optimized based on the characteristics of feedstocks. In general, a temperature from 160 to 260°C (corresponding pressure of 0.69–4.83 MPa) is applied, with a holding time of a few minutes [15].

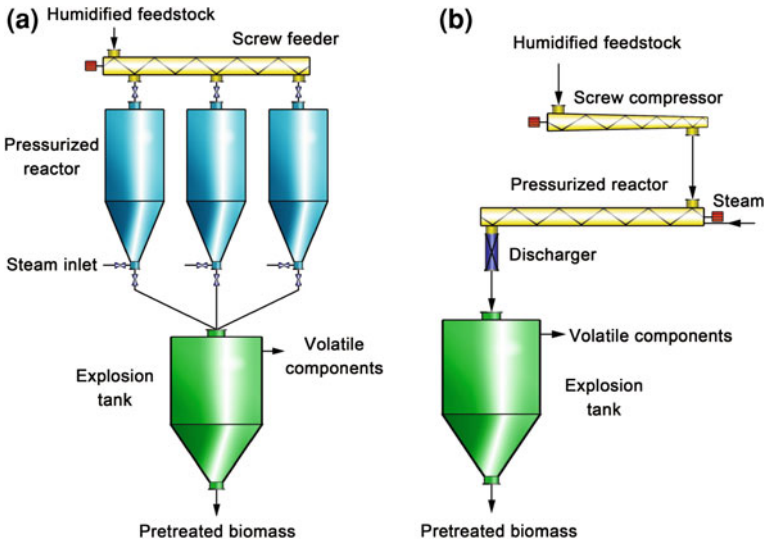

Fig. 5 Process diagram of steam explosion. Batch process (**a**), and continuous mode (**b**)

However, the sugar yield of the pretreatment is improved at low temperature and decreased holding time, whereas the development of cellulose more accessible for cellulase attack requires more severe conditions, in which sugar loss is inevitable. Therefore, a trade-off between these two contradictory factors is needed. The comprehensive impact of temperature and holding time can be evaluated quantitatively by the severity factor R_0, which is calculated using the correlation $R_0 = log[te^{(T-100)/14.75}]$, where T and t represent temperature (°C) and holding time (min), respectively, and 14.75 is the active energy of the reaction [19].

Both batch and continuous processes have been developed for the SE process, as illustrated in Fig. 5. The batch process is simple. Humidified feedstock is fed through a screw feeder into the reactor, which is then pressurized by saturated steam and maintained for a period of time. After the reaction, the material is discharged into the explosion tank at atmospheric pressure, in which volatile components are separated, and pretreated biomass is left for washing to collect sugars released by the hydrolysis of hemicelluloses. To overcome the disadvantage of the discontinuity, multiple reactors can be operated alternately. By contrast, the continuous system is more productive and effective, but the design of the reactor and discharger is more complicated due to the high solid content of the feedstock as well as the high pressure required by the pretreatment.

LHW is another hydrothermal pretreatment which can enhance sugar extraction [20]. As illustrated in Fig. 6, slurry is pre-heated via a heat exchanger, which not only saves steam consumption for heating the slurry, but also cooling water to cool down the pretreated material. The pre-heated slurry is further heated by steam via another heat exchanger, and passes through the reactor for pretreatment. Theoretically, the reactor should be operated at plug flow. Therefore, tubular

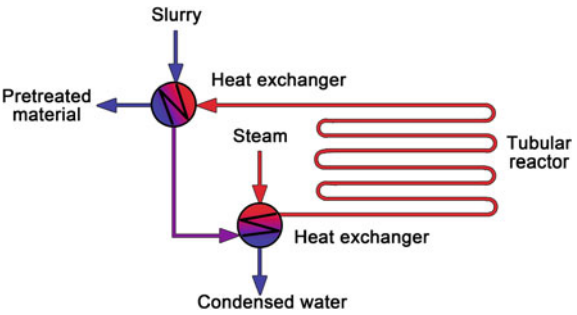

Fig. 6 Schematic diagram of the LHW process

reactors are preferred, and residence time and temperatures can be optimized for different types of feedstocks. Compared to SE with high solid uploading, the solid concentration in the slurry for the LHW process is much lower.

During the LHW pretreatment, the pH of the biomass can drop below 4, which results in the formation of inhibitors due to the degradation of sugars under acidic conditions [21]. Thus, a pH control strategy can be applied to the system to maintain the pH value above 4, preferably between 5 and 7, by adding a base as needed [22, 23]. Since the alkali is not a catalyst as in alkaline pretreatment to be addressed below, this process is termed as pH-controlled hot water pretreatment.

3.2 Chemical Pretreatment

High temperatures applied during the hydrothermal pretreatments under SE and LHW conditions dehydrate sugars and produce inhibitors such as furfural from xylose and hydroxymethyfurfural from glucose. To address this problem, acids can be supplemented to facilitate the deconstruction of LCCs under less severe conditions, either lower temperature or shorter reaction time. Among various acids, sulfuric acid is most commonly used. Although the temperatures in concentrated acid pretreatment are much lower, acid recovery presents a big challenge for the economic viability of the process. Therefore, dilute acid with concentrations less than 2% is preferred, which can be conveniently neutralized by lime or ammonium during the conditioning process [24]. Dilute acid pretreatments have been intensively studied over the years with various feedstocks and reactors at different scales [25–28]. Recently, the National Renewable Energy Laboratory (NREL) updated its technical report on Process Design and Economics for Biochemical Conversion of Lignocellulosic Biomass to Ethanol, in which the dilute-acid pretreatment was described in details (Fig. 7).

Milled corn stover is conveyed into a screw feeder and dilute acid is injected at the discharge point. The feedstock is then fed into a mixing and heating screw, and further conveyed into the vertical presteamer. Hot water is added to bring the effluent to 30% total solids. The presteamer is operated at 100°C, with a retention time of 10 min. The feedstock is then discharged through the screw feeder, and

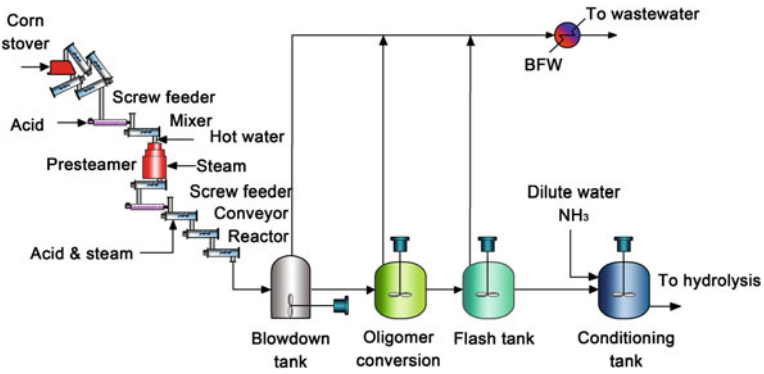

Fig. 7 Process diagram of the NREL projected dilute-acid pretreatment of corn stover [28]

acid is added at the discharge point at a concentration of 18 mg/g dry biomass before feeding into the horizontal reactor, which is operated at 158°C (0.55 MPa), with a residence time of 5 min. The feedstock from the horizontal reactor is discharged into a blowdown tank operated at 130°C (0.28 MPa). The slurry from the blowdown tank goes into the oligomer conversion tank, where an additional 4.1 mg acid/g feedstock is added, making the total acid loading 22.1 mg/g dry biomass. The oligomer conversion tank is also maintained at 130°C, with a residence time of 20–30 min. Subsequently, the feedstock is discharged into a flash tank operated at atmospheric pressure. At this stage, the hydrolysate containing 30% total solids and 16.6% insoluble solids is pumped into the conditioning tank, in which the slurry is diluted to slightly higher than 20% total solids for enzymatic hydrolysis and cooled to 75°C. Ammonia is sparged into the dilution water to adjust the hydrolysate pH to 5 as well as to provide a nitrogen source for subsequent microbial growth and ethanol fermentation. All volatile components from the blowdown tank, oligomer conversion tank and flash tank are condensed and collected [29].

Although dilute acid pretreatment seems more economically competitive, some disadvantages like corrosion, which requires expensive acid-resistant stainless steel or coatings, and inhibitors produced during the pretreatment under high temperatures, have led to the exploration of alternatives, one of them being alkaline pretreatment. Various alkalis including sodium hydroxide, lime and aqueous ammonia have been studied [30–32]. Basically, alkaline pretreatment is a delignification process, and the underlying mechanism is the saponification of intermolecular ester bonds crosslinking xylan hemicelluloses and lignin [33]. In addition, alkaline pretreatment also removes acetyl and other acidic substitutions on hemicelluloses that protect cellulose from attack by cellulase [34]. Moreover, alkaline pretreatment causes swelling of the lignocellulosic biomass, leading to the decrease of DP and crystallinity of cellulose and increase of the surface area to facilitate the enzymatic hydrolysis of cellulose. The effectiveness of alkaline pretreatment depends on the characteristics of lignocellulosic biomass and reaction conditions. In general, alkaline pretreatment is more efficient with herbaceous crops and agricultural residues with relatively low lignin content.

In comparison with the pretreatment technologies discussed previously, low temperature and pressure, even ambient conditions, can be applied under alkaline pretreatment conditions. However, the time required by the alkaline pretreatment process is hours, days or weeks rather than minutes, making it difficult to achieve the feedstock processing capacity required by commercial production of bulk commodities like ethanol. Moreover, a significant amount of salt produced during the pretreatment is a big problem, which not only affects microbial growth and fermentation, but also raises an environmental concern. Although an alternative strategy using ammonia—for example, the ammonia recycling percolation (ARP) process in which aqueous ammonia is recycled through a column containing lignocellulosic biomass such as corn stover [35]—can overcome these disadvantages, it is not cost-effective due to the high cost of the recovery of ammonia. A modified ARP process operated with low liquid ammonia throughput can address this issue to some extent [36], but is still not practical for commercial application.

Ammonia fiber explosion (AFEX) is a hybrid of the SE and ARP processes, in which biomass is pretreated with liquid anhydrous ammonia at mild temperatures (60–100°C) and high pressure [37]. When the pressure is released, the rapid expansion of ammonia gas causes swelling of the biomass, which correspondingly disrupts LCCs and creates more accessible surfaces for enzymatic hydrolysis. Since temperatures in the AFEX process are much lower than those applied to the SE process, not only can energy consumption be reduced, but also the formation of inhibitory by-products prevented. In addition, washing is not necessary for the process, which benefits for high solid loading hydrolysis. Meanwhile, ammonia remaining in the pretreated biomass facilitates microbial growth and fermentation. However, ammonia recovery by evaporation is needed, which complicates the system design and requires more capital investment and energy consumption [38].

3.3 Solvent Pretreatment

Solvent pretreatment is a fractionating process, in which an organic or aqueous organic solvent is used with or without catalysts to deconstruct LCCs [39, 40]. Methanol, ethanol, ethylene glycol, triethylene glycol, tetrahydrofurfuryl alcohol, glycerol, n-butanol, acetone, phenol etc. have been explored to extract lignin as well as hydrolyze hemicelluloses to render cellulose for enzymatic hydrolysis. The advantage of organic solvents over other chemical pretreatments is that relatively pure and low-molecular-weight lignin can be recovered as a by-product. However, the high cost of organic solvents and the intensive energy consumption associated with solvent recovery make this strategy economically uncompetitive, not to mention the difficulty in the treatment of concentrated black liquors left after solvents are evaporated and the loss of sugars in the liquors.

Ionic liquids (ILs) are salts composed of a small anion and a large organic cation, existing as liquids at room temperature with low vapor pressure [41]. Based on the understanding of the chemistry of the anion and cation, a wide variety of ILs can be

designed to dissolve cellulose or lignin from lignocellulosic biomass and deconstruct the crystalline structure of cellulose molecules for enzymatic hydrolysis. Thus, IL pretreatment has been extensively investigated recently. Meanwhile, almost all ILs can be recovered, which not only reduces their usage, but also makes them more environmentally friendly [42]. However, there are still many challenges for ILs to be practical in the pretreatment of lignocellulosic biomass for the production of bulk commodities like ethanol, and regeneration of ILs is one of them.

3.4 Biological Pretreatment

Compared with physical and chemical pretreatments in which expensive equipment, chemicals and intensive energy consumption are needed, biological pretreatment by solid fermentation employs microorganisms that degrade lignocellulosic biomass at mild conditions without special requirements for equipment [43]. Both bacteria and fungi have been explored, but rot fungi associated with wood decay are the predominant species in lignocellulose degradation for the purpose of biofuel production, particularly white-rot fungi due to their abundant ligninolytic enzymes, including lignin peroxidase, manganese peroxidase, laccases and other enzymes, and better selectivity in lignin degradation [44].

Although biological pretreatment is energy-saving and environmentally friendly, its disadvantages are apparent. Firstly, the extremely low degradation rate requires times as long as weeks for a significant change in the structure of the lignocellulosic biomass, making the process mismatched with the subsequent hydrolysis of cellulose and fermentation of sugars. Secondly, significant biomass is lost during the process, not only the lignin which is mineralized into low-molecular-weight fragments that might be further catabolized into the useless final product CO_2 [45], but also sugars released from hemicelluloses and even cellulose by the hydrolytic enzymes (simultaneous decay with lignin degradation) as a carbon source to support the growth of the microorganisms [46]. Finally, the control of microbial growth and metabolism under open and solid fermentation conditions with mixture species is unreliable, which inevitably affects the subsequent processes such as cellulose hydrolysis and ethanol fermentation. Therefore, biological pretreatment is less attractive from the viewpoint of commercial application.

4 Enzymatic Hydrolysis of Cellulose and Co-Fermentation of C5 and C6 Sugars

Following pretreatment, enzymatic hydrolysis is needed to further depolymerize the cellulose component to glucose, which can be used for ethanol fermentation together with sugars released from the hydrolysis of hemicelluloses during

the pretreatment. Despite intensive R & D worldwide for decades, two barriers still remains to be overcome for developing viable processes to make bioethanol economically competitive.

Unlike amylases and glucoamylases that are available at low prices for commercial production of various bulk products including ethanol from starch-based feedstocks, cellulases to liberate glucose from cellulose for bioethanol production are more expensive due to the difficulty of their fermentation production as well as the heterogeneous characteristic of the enzymatic hydrolysis which significantly compromises the reaction rate and increases the enzyme dosage [47]. See "Cellulolytic Enzyme Production and Enzymatic Hydrolysis for Second-generation Bioethanol Production" for details. On the other hand, the ethanologenic species, either *S. cerevisiae* which has been used for ethanol production from sugar- and starch-based feedstocks since the establishment of the industry, or *Z. mobilis* which has been intensively studied over the years due to its unique Entner–Doudoroff (ED) pathway for ethanol production with less biomass accumulation [48], cannot ferment pentose sugars in the hydrolysates into ethanol at rates and yields that are acceptable from the viewpoint of industrial production. Although the pentose sugars can be converted into other products like furfural through intramolecular dehydration of xylose by chemical catalysis [49], and xylitol, lactic acid and 2,3-butanediol by fermentations [50], all these processes seem not to be economically competitive at present, and most effort is still focused on the co-fermentation of the pentose and hexose sugars for bioethanol production by engineered strains.

4.1 Strategies for Hydrolysis and Fermentation

Based on the considerations of cellulase production and the process configurations of cellulose hydrolysis and ethanol fermentation, separate hydrolysis and co-fermentation, simultaneous saccharification and co-fermentation and consolidated bioprocessing have been developed, and are illustrated schematically in Fig. 8.

4.1.1 Separate Hydrolysis and Co-Fermentation

For the separate hydrolysis and co-fermentation (SHCF) process, cellulose is completely hydrolyzed to glucose by cellulases under optimum conditions, particularly temperatures around 50°C that facilitate the enzymatic hydrolysis, and correspondingly reduce the enzyme dosage, but cannot be tolerated by microorganisms performing ethanol fermentation at temperatures around 35°C. After complete hydrolysis of cellulose, lignin is left, which can be recovered by a filter and processed as value-added by-products. In the meantime, the viscosity of the hydrolysate is very low, which is suitable for high gravity (HG) fermentation to

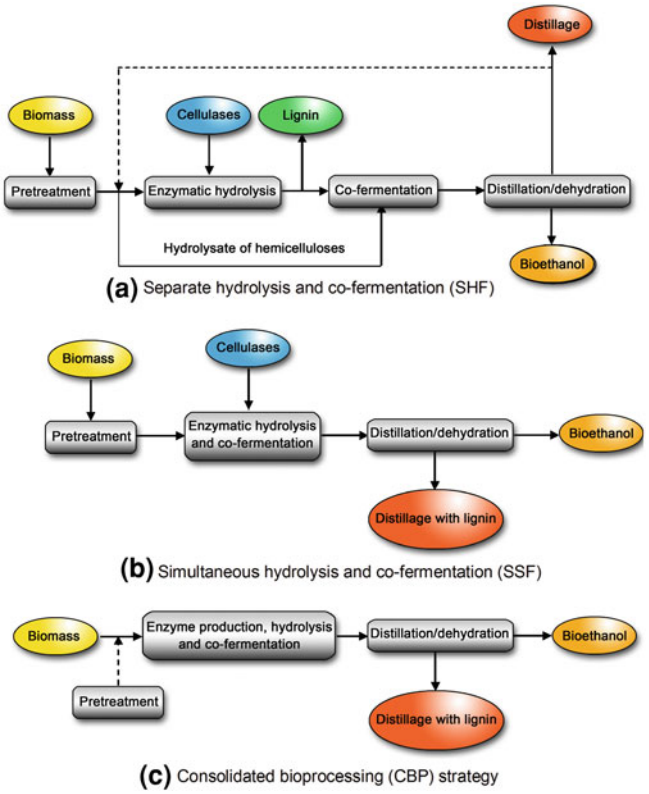

Fig. 8 Process engineering strategies for bioethanol production

reduce energy consumption for ethanol distillation as well as distillage treatment, due to the significant reduction in the distillage discharged from the distillation system. Such an idea was tested in the Iogen process, the first demonstration plant established in the world for bioethanol production through the biochemical conversion pathway [51].

However, the accumulation of glucose during the hydrolysis significantly inhibits β-glucosidase, which sequentially results in the accumulation of cellobiose that inhibits the activities of exo-β-glucanase or cellobiohydrolase (CBH) and endo-β-glucanase (EG). Supplementation of β-glucosidase may be one of the solutions to this problem if the cost of the enzyme is not too high—for example, β-glucosidase from *Aspergillus niger* [52]. On the other hand, another concern with the SHCF process is microbial contamination during the hydrolysis of cellulose and the transport of the hydrolysate through pipelines, which can deteriorate during ethanol fermentation and compromise ethanol yield, since the bulk amount of medium for ethanol fermentation is never sterilized in the

industry due to the energy consumption and sugar loss associated with the operation.

4.1.2 Simultaneous Saccharification and Co-Fermentation

For ethanol fermentation from starch-based feedstocks, the mash is liquefied at elevated temperatures of 90–110°C by thermo-tolerant amylase, the endo-enzyme hydrolyzing starch randomly into dextrins, and further hydrolyzed by glucoamylase, the exoenzyme hydrolyzing the dextrins from the non-reducing end to release glucose at 60–62°C for 20–30 min to achieve the dextrose equivalent of 15–20 only, which is then cooled down to 30–32°C and pumped into fermentors to initiate ethanol fermentation. Since most dextrins are hydrolyzed into sugars during the fermentation, the process is termed simultaneous saccharification and fermentation (SSF), and has been widely practiced in the industry. When a similar strategy is applied to ethanol production from ligonocellulosic biomass, the term simultaneous saccharification and co-fermentation (SSCF) is used, taking into account the unique characteristics of the hydrolysate that includes both C5 and C6 sugars. However, the saccharification of the dextrins/pretreated cellulose and the fermentation/co-fermentation of glucose/C5 and C6 sugars are by no means simultaneous, but sequential in nature.

The SSCF process is simple in design and easy to operate. Most importantly, higher ethanol yields can be achieved due to the alleviation of product inhibition in cellulases, which results in more complete hydrolysis of the cellulose component [53]. However, temperatures for the enzymatic hydrolysis and ethanol fermentation are significantly different, making the simultaneous optimization of the two unit operations impossible, and the SSCF process must be operated at lower temperatures to accommodate microbial growth and ethanol fermentation, normally at 30–35°C. Thus, the rate of the enzymatic hydrolysis is inevitably compromised, and a much longer time is needed to complete the hydrolysis. Moreover, lignin cannot be separated from cellulose prior to fermentation, which makes the fermentation broth extremely viscous, and the mixing and heat and mass transfer performance is correspondingly affected. Therefore, the SSCF process cannot operate under HG conditions, and energy consumption is high for the distillation of the fermentation broth with low ethanol concentrations as well as for the treatment of distillage since the amount of the discharge is much larger. For example, a time as long as 96 h was reported for the fed-batch SSCF system to convert pretreated wheat straw with 11% water insoluble solids and produce only 3.3% (w/v) ethanol [54].

A hybrid process like the SSF process practiced in ethanol fermentation from starch-based feedstocks can be developed, in which a pre-hydrolysis under optimum temperature conditions is applied to the enzymatic hydrolysis of cellulose, followed by the SSCF process to shorten the time required by the hydrolysis and

fermentation and improve the productivity of the system, but the impact of lignin on the fermentation cannot be overcome.

4.1.3 Consolidated Bioprocessing

Cellulases are produced separately and added to hydrolyze the cellulose component of pretreated biomass for the SHCF and SSCF processes, which is one of the major barriers for cost reduction of bioethanol due to the high cost of the enzyme as well as the high enzyme dosage required by the processes. In nature, many organisms, particularly microorganisms, can utilize native cellulose as a carbon source and energy to support their growth and metabolism, through synthesis and secretion of unique cellulases and subsequent hydrolysis of cellulose by the synergic functions of these enzymes [55]. Such a natural phenomenon has inspired scientists to develop mimic systems, either an individual microorganism or a microbial community, to produce ethanol and other chemicals directly from lignocellulosic biomass, even without pretreatment. All problems found with the biochemical conversion of lignocellulosic biomass seem to be solvable by this so-called consolidated bioprocessing (CBP) strategy, which was evolved from the concept of direct microbial conversion [56].

However, no natural microorganism is available for commercial production of bioethanol with the CBP strategy. Thus, the development of CBP strains is the core of the CBP process. Currently, both bacterial and yeast species have been explored for this purpose with the following strategies: (1) engineering a cellulase producer to be ethanologenic, and (2) engineering an ethanologen to be cellulolytic [57]. For the first strategy, anaerobic cellulolytic bacteria from the genus *Clostridium* are good candidates [58], and the targets for the metabolic engineering of this species include increasing ethanol titer by improving ethanol tolerance through rational designs based on the understanding of the mechanisms underlying its response to ethanol inhibition and random approaches such as the selection of mutants through an evolutionary adaptation procedure, and on the other hand improving ethanol yield by blocking the synthesis of major by-products, as illustrated by the progress with the thermophilic bacterium *Thermoanaerobacterium saccharolyticum* [59]. As for the second strategy, the primary concerns are expression and secretion of functional cellulases in ethanologenic species, particularly *S. cerevisiae*, which has been engineered with genes encoding glycoside hydrolases including cellulases and hemicellulases through cell surface display techniques [60, 61]. Unfortunately, expression of the cellobiohydrolases (CBH I and CBH II) from *Trichoderma reesei* is generally poor, not to mention the challenges of engineering the species with more other enzymes or pathways required by the efficient production of bioethanol.

Theoretically, the CBP strategy can completely eliminate cellulase production and integrate all three major steps of the bioconversion into a single cell. However, there are many unknowns to be elucidated in order to make it significant in the production of bioethanol and other biofuels. For example, the production of cellulolytic enzymes, hydrolysis of cellulose and hemicelluloses and fermentation

of released sugars need to be well coordinated within the single cell and between cells and their surroundings at different scales, from molecular levels involving gene expression and regulation to the intracellular metabolic network, as well as the kinetics of heterogeneous hydrolysis.

4.2 Strain Development

Unlike conventional sugar- and starch-based feedstocks, hydrolysates of lignocellulosic biomass contain significant amount of pentose sugars such as xylose and arabinose, in addition to hexose sugars of glucose, mannose and galactose. Unfortunately, the ethanologenic species, either *S. cerevisiae* or *Z. mobilis*, cannot ferment the pentose sugars into ethanol efficiently. If only hexose sugars from lignocellulosic biomass are fermented, with pentose sugars left behind, feedstock consumption for bioethanol production will be significantly high, and in the meantime the unfermented pentoses will remain with the distillage and increase the capital investment and energy consumption in the treatment of the distillage.

Two strategies, engineering pentose-utilizing microorganisms with ethanol production pathways or ethanol producers with pentose-metabolizing pathways, can be developed for developing recombinants to ferment both pentose and hexose sugars in the hydrolysate into ethanol [62]. Although pentose-utilizing bacteria like *Escherichia coli* and *Klebsiella oxytoca* can be engineered for ethanol production [63], their poor ethanol tolerance significantly compromises ethanol titers, making ethanol purification by distillation highly energy-intensive, and in the meantime the neutral pH values required for their growth and ethanol fermentation increase the contamination risk of the fermentation system, not to mention the problems associated with their biomass treatment. Therefore, engineering the ethanologenic species *Z. mobilis* and *S. cerevisiae* with pentose-metabolizing pathways is preferred.

In nature, bacteria employ the isomerase pathway to direct xylose to their central metabolism, whereas fungi use the reductase and dehydrogenase pathways to convert xylose to xylulose via the intermediate xylitol. Thus, an overall strategy for engineering *Z. mobilis* and *S. cerevisiae* with xylose-metabolizing pathways is illustrated in Fig. 9 [64].

4.2.1 *Z. mobilis*

Z. mobilis, a facultative anaerobic Gram-negative bacterium, can ferment glucose into ethanol and CO_2 through the ED pathway, which generates more ethanol due to less biomass production compared with the Embden–Meyerhof pathway in *S. cerevisiae* [48]. In addition, *Z. mobilis* can tolerate concentrations as high as 120 g/L ethanol [63], much higher than that tolerated by other bacteria, and its biomass is generally recognized as safe (GRAS) for animal feed, making this species suitable for metabolic engineering with pentose-fermenting ability.

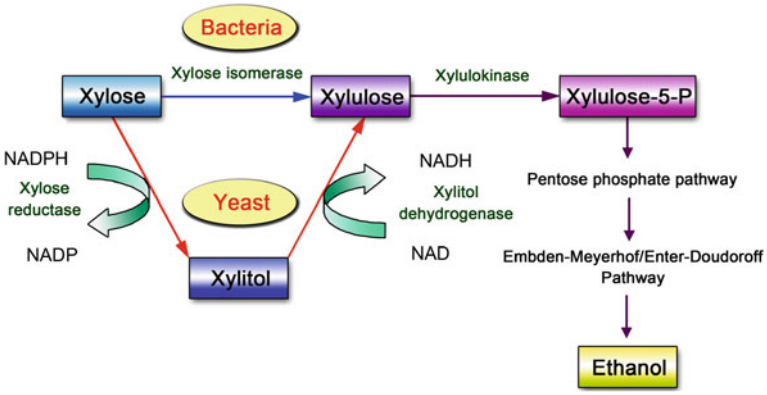

Fig. 9 Simplified xylose-metabolizing pathways in bacteria and yeast (Adapted from [60])

Zhang et al. transferred four genes responsible for xylose assimilation and pentose phosphate pathways—xylose isomerase (*xylA*), xylulose kinase (*xylB*), transketolase (*tktA*) and transaldolase (*talB*)—into *Z. mobilis*, enabling the recombinant to use xylose for growth and fermentation [65]. Shortly afterwards, Deanda et al. engineered this species with arabinose utilization by expressing five genes from *E. coli* encoding L-arabinose isomerase (*araA*), L-ribulokinase (*araB*), L-ribulose-5-phosphate-4-epimerase (*araD*), transaldolase (*talB*) and transketolase (*tktA*) [66]. To overcome the disadvantage of genetic instability of the plasmid-bearing recombinants, genomic integration of these heterologous genes was further developed [67].

Although significant progress has been made in engineering *Z. mobilis* to co-ferment pentose and hexose sugars for ethanol production, no commercial applications have been reported to date, due to the incomplete understanding of the species as well as the complexity of industrial substrates, particularly the inhibition of various toxic by-products released during the pretreatment of lignocellulosic biomass and the molecular mechanisms underlying the responses of the species to environmental stresses. With the sequencing of the *Z. mobilis* genome and elucidation of more functional genes, together with the applications of synthetic and systems biology methodologies [68–71], more efficient strains are expected to be engineered. Under the support of the DOE project, the Integrated Corn-Based Bio-Refinery (ICBR), DuPont and Broin Companies have established a partnership to produce cellulosic ethanol from corn stover by genetically modified *Z. mobilis*, which might be a milestone for commercial application of this species [72].

4.2.2 *S. cerevisiae*

Currently, ethanol production from starch- and sugar-based feedstocks is solely using strains from *S. cerevisiae*, which exhibits significant advantages over other

species. For example, ethanol tolerance of *S. cerevisiae* is the highest, and more than 20% ethanol can be tolerated by the species [73], which not only saves energy consumption for ethanol distillation, but also for the treatment of distillage due to the significant reduction in distillage discharged from the distillation system [48]. Moreover, *S. cerevisiae* prefers an acidic environment with a pH value below 4.5, which can effectively prevent ethanol fermentation from microbial contamination, since fermentors used by the industry for ethanol fermentation are too large to be sterilized by vapor. In addition, although the natural *Saccharomyces* yeast is unable to ferment xylose, there are other yeast species such as *Pichia stipitis* able to ferment xylose.

Since the 1980s, substantial research efforts have been focused on the development of genetically engineered *Saccharomyces* yeast to effectively ferment xylose, the most abundant pentose in the hydrolysate of lignocellulosic biomass. This was due in part to the failed attempts to discover new yeast species or strains that could effectively co-ferment glucose and xylose to ethanol. Fortunately, the remarkable advances in recombinant DNA techniques have provided the necessary tools to genetically modify the yeast and made it able to co-ferment both glucose and xylose to ethanol as described below.

Early studies had shown that *S. cerevisiae* can ferment xylulose to ethanol, albeit not efficiently. Therefore, theoretically the yeast is only missing the enzyme(s) to convert xylose to xylulose in order to be able to ferment xylose. It was known that bacteria could convert xylose to xylulose with a single enzyme that does not require co-factors. In contrast, the xylose-to-xylulose system from xylose-fermenting yeasts such as *P. stipitis* required two enzymes, as illustrated in Fig. 9, which not only were very difficult to clone at that time, but also not an ideal system as stated above.

Initially, there were nearly ten laboratories worldwide attempting to clone a bacterial xylose isomerase gene into the yeast. Ho and co-workers at Purdue University were the first group to accomplish the cloning of the xylose isomerase gene from *E. coli* into the yeast (unpublished). However, the protein molecules synthesized in *S. cerevisiae* by the cloned gene had no xylose isomerase activity. Subsequently, other isomerase genes from different bacteria were cloned and similar negative results were obtained. Failing to produce an active xylose isomerase in *S. cerevisiae* by cloning the xylose isomerase genes, there was only one potential approach remaining to make the yeast ferment xylose into ethanol: cloning the xylose reductase (XR) and xylitol dehydrogenase (XD) genes from *P. stipitis*. However, scientists predicted that any recombinant yeast containing these cloned genes encoding the imperfect enzyme system would not be able to sustain the fermentation of xylose to ethanol, and the result would only be the production of xylitol!

In the early 1990s, three groups reported the successful cloning of the XR and XD genes into *S. cerevisiae* to make the yeast ferment xylose [74, 75]. However, the recombinant yeast fermented xylose extremely slowly and produced little ethanol and the main product was xylitol as predicted. In 1993, Ho's group reported the successful development of the recombinant *Saccharomyces* yeast

1400 (pLNH32) that could ferment high concentrations of xylose almost completely to ethanol with little xylitol accumulated. In addition, the yeast could co-ferment glucose and xylose without a significant lag period between the fermentation of these two sugars [76].

The Purdue strain was developed by transforming an industrial strain, 1400, with a high copy number 2μ plasmid pLNH 32, which contains the cloned and overexpressed *XR*, *XD* and xylulokinase (*XK*) genes [76]. The 2μ plasmid is a broad host plasmid, designed to be able to transform any *S. cerevisiae*, including industrial wild-type strains. Such a plasmid can be used to screen better hosts for cellulosic ethanol production. Furthermore, Ho's group developed a unique new gene integration technique, facilitating effective integration of multiple genes into the yeast chromosome in multiple copies [77, 78], which is easy to perform and guarantees that the genes cloned on the integration plasmid are transferred into the host strains and integrated into their genome in as many copies as desired to provide the highest activity of the cloned enzymes. This technique allows the integration of the XR-XD-XK genes together as a cassette into the yeast chromosome in sufficient copies for the resulting yeast to ferment xylose efficiently.

The best strain developed by Ho's group prior to 2007 is 424A (LNH-ST), which was screened from 10 different strains of *S. cerevisiae* by first transforming each of them with the 2μ plasmid pLNH32, to make sure that these strains were able to ferment xylose as well as co-ferment glucose/xylose effectively in the presence of the plasmid, followed by integrating genes into the chromosomes of the selected yeast strains to develop the "stable yeast". The co-fermentation of glucose/xylose by 424A(LNH-ST) is shown in Fig. 10. This strain is currently available for industry to produce cellulosic ethanol. 424A(LNH-ST) as well as other strains developed by the integration technique have all been validated by ethanol producers to be able to co-ferment glucose and xylose to ethanol and also able to ferment glucose and xylose present in actual hydrolysates from different feedstocks [78]. 424A(LNH-ST) has also been used by companies for the production of cellulosic ethanol from wheat straw and other feedstocks in demonstration plants as early as 2004. Dr. Ho and her coworkers have continued to improve the strain by making it co-ferment other sugars like arabinose, together with glucose, xylose, mannose and galactose [79], and by making it more resistant to ethanol and acetic acid inhibition [80, 81]. A new and improved derivative of 424A(LNH-ST) has been developed that can ferment all sugars present in hydrolysates produced from any cellulosic biomass and produce more than 10% ethanol without requiring special detoxification to remove inhibitors in the hydrolysates [82]. It will be available for industrial production of cellulosic ethanol in the near future.

4.3 Process Integration and Optimization

Various technologies for pretreatment, enzymatic hydrolysis and fermentation strains have been developed in recent decades for bioethanol production from

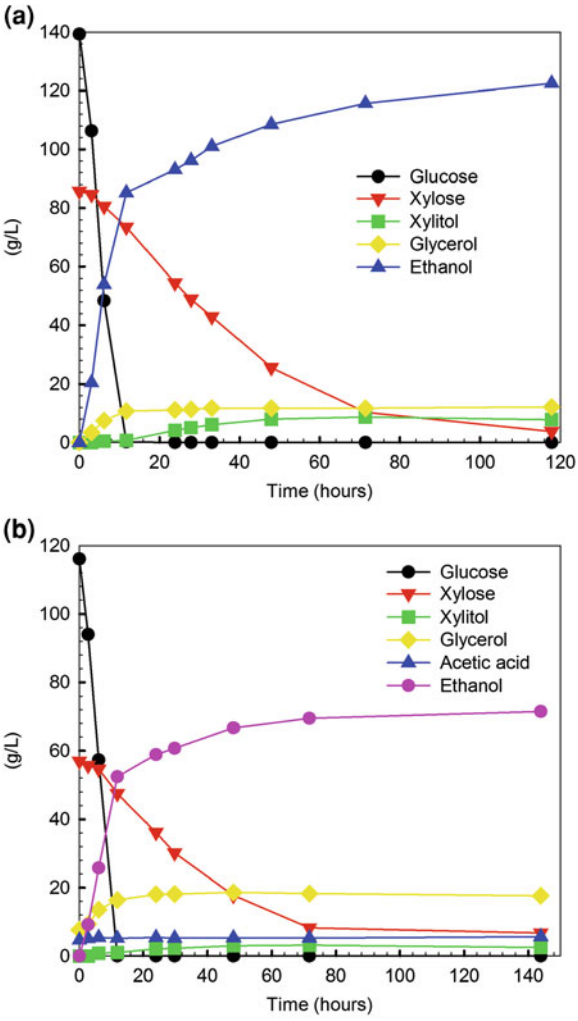

Fig. 10 Co-fermentations of glucose and xylose in simulated medium (**a**) and wheat straw hydrolysate (**b**) by the recombinant *S. cerevisiae* 424A(LNH-ST)

lignocellulosic biomass. Process integration aims to optimize these units on the system level, and thus improve the techno-economic performance of the system, making bioethanol economically competitive with petroleum-based fuels.

Unlike ethanol production from sugar- and starch-based feedstocks that can be carried out at HG conditions with more than 10% ethanol achieved, ethanol concentration that can be achieved with lignocellulosic biomass is much lower due to the problematic characteristics of the feedstock, and a larger quantity of water needs to be introduced into the system via feedstock, steam and addition of chemicals, which inevitably reduces the efficiency of the facility and enhances the energy consumption of ethanol distillation and distillage treatment. Therefore, the

most vital consideration for technology integration and process optimization is to minimize the water usage of the process without significantly compromising the performance of the enzymes and microorganisms. Taking the COFCO–SINOPEC–Novozyme second generation fuel ethanol project with an annual production capacity of 62 million liters as an example, the overall process involves feedstock handling, size reduction, pretreatment, substrate conditioning, enzymatic hydrolysis, fermentation, ethanol distillation, residue dewatering and biogas production.

The prior feedstock for the project is corn stover, which contains 10–15% moisture under field-dried conditions and detectable impurities. After a primary size reduction by a shredder, the feedstock is screened to remove dirt and grit and passed through a magnetic separator to remove tramp metals. It is then further reduced by the secondary shredder to 20–50 mm. Steam explosion is used for the biomass pretreatment, and solid contents are controlled at 30–40% during the pretreatment process. The feedstock is pre-heated by the flash vapor, which not only saves energy consumption, but also reduces condensed water to ensure the high solid content. The temperature and residence time can be controlled in the ranges of 130–220°C and 5–120 min, depending on the feedstocks and the size reduction. A small amount of acid is supplemented to accelerate the hydrolysis of hemicelluloses to deconstruct the LCCs more efficiently and enhance the accessibility of cellulases to the surface of cellulose. In addition, the addition of acid can also lessen the severity of the pretreatment conditions, reduce degradation of sugars and enhance pentose recovery.

The pretreated substrate is transferred into the hydrolysis reactor with initial dry matter content of 20–25% after neutralization by alkalis such as lime, sodium hydroxide and ammonia. The mixing of the substrate with enzymes presents challenges due to the high viscosity and poor fluidity of the slurry at the early stage of the hydrolysis. Laboratory trials and scaling-up practice indicated that feeding substrate and enzymes in a fed-batch mode can improve the mixing performance and facilitate the enzymatic hydrolysis. The temperature and pH are set at 50°C and 5.0 respectively under the optimal conditions for the cellulases (Cellic CTec2) developed by Novozyme for the pre-hydrolysis of the pretreated feedstock, followed by the co-fermentation of the hexose and pentose sugars by the genetically engineered *S. cerevisiae* developed by Dr. Nancy Ho at Purdue University and licenced to COFCO. The yeast seed is cultivated with the hydrolysate supplemented with CSL. Due to the high concentrations of inhibitors and low content of nutrients in the hydrolysate, an extended time is required for the seed culture, and much higher inoculation is needed to initiate the fermentation, which is completed within 96–120 h.

The broth containing 5–7% (v/v) ethanol is then distilled for ethanol recovery. It is worth noting that this unit operation is more energy-intensive than that for ethanol production from sugar- and starch-based feedstocks. The distillage discharged from the distillation system is filtered to separate lignin residues remaining after the fermentation, and the filtrate is digested anaerobically for biogas production, while the cake is dewatered. Both biogas and lignin residues can be co-fired to generate steam.

5 Conclusions

It has been acknowledged that bioethanol is one sustainable solution to the current energy issue, particularly for countries like China which are suffering from a shortage of crude oil supply and strongly depend on imported oil for their economic and social development. However, although significant progress has been achieved in biomass pretreatment, cellulase production and co-fermentation of the pentose and hexose sugars in recent decades, bioethanol is still not economically competitive compared with petroleum-based fuels, making cost reduction the biggest challenge. Taking into account the multi-disciplinary nature of the whole process, the portfolio that incorporates a deep understanding of the characteristics of lignocellulosic biomass, innovations for developing more efficient cellulases and microbial strains for enhanced rates and yields and process integration and optimization for reducing energy consumption requires relentless effort. Moreover, the development of a biomass-based bio-refinery to utilize the feedstock more comprehensively, and in the meantime add more value-added co-products like bio-based materials from the lignin component into the production train, would offset the cost of bioethanol and make it more economically competitive.

References

1. Xie GH, Wang XY, Ren LT (2010) China's crop residues resources evaluation. Chin J Biotechnol 26:855–863
2. Li LJ, Wang Y, Zhang Q et al (2008) Wheat straw burning and its associated impacts on Beijing air quality. Sci China Ser D: Earth Sci 51:403–414
3. Himmel ME, Ding SY, Johnson DK et al (2007) Biomass recalcitrance: Engineering plants and enzymes for biofuels production. Science 315:804–807
4. Vleet JHV, Jeffries TW (2009) Yeast metabolic engineering for hemicellulosic ethanol production. Curr Opin Biotechnol 20:300–306
5. Service RF (2007) Biofuel researchers prepare to reap a new harvest. Science 315:1488–1491
6. Pauly M, Keegstra K (2008) Cell wall carbohydrates and their modifications as a resource for biofuels. Plant J 54:559–568
7. Sticklen MB (2008) Plant genetic engineering for biofuel production: towards affordable cellulosic ethanol. Nat Rev Genet 9:433–443
8. Klemm D, Heublein B, Fink HP et al (2005) Cellulose: fascinating biopolymer and sustainable raw material. Angew Chem Int 44:3358–3393
9. Atalla RH, Vanderhart DL (1984) Native cellulose: a composite of two distinct crystalline forms. Science 223:283–285
10. O'sullivan AC (1997) Cellulose: the structure slowly unravels. Cellulose 4:173–207
11. Heiner AP, Sugiyama J, Teleman O (1997) Crystalline cellulose I_{α} and I_{β} studied by molecular dynamics simulation. Carbohyd Res 273:207–223
12. Scheller HV, Ulvskov P (2010) Hemicelluloses. Annu Rev Plant Biol 61:263–289
13. Bonawitz ND, Chapple C (2010) The genetics of lignin biosynthesis: Connecting genotype to phenotype. Annu Rev Genet 44:337–363
14. Chundawat SPS, Beckham GT, Himmel ME et al (2011) Deconstruction of lignocellulosic biomass to fuels and chemicals. Annu Rev Chem Biomol Eng 2:6.1–6.25

15. Kumar P, Barrett DM, Delwiche MJ et al (2009) Methods for pretreatment of lignocellulosic biomass for efficient hydrolysis and biofuel production. Ind Eng Chem Res 48:3713–3729
16. da Costa Sousa L, Chundawat SPS, Balan V et al (2009) 'Cradle-to-grave' assessment of existing lignocellulose pretreatment technologies. Curr Opin Biotechnol 20:339–347
17. Binod P, Satyanagalakshmi K, Sindhu R et al (2011) Short duration microwave assisted pretreatment enhances the enzymatic saccharification and fermentable sugar yield from sugarcane bagasse. Renewable Energy (In press)
18. Ramos LP (2003) The chemistry involved in the steam treatment of lignocellulosic materials. Quim Nova 26:863–871
19. Abatzoglou N, Chornet E, Belkacemi K (1992) Phenomenological kinetics of complex systems: the development of a generalized severity parameter and its application to lignocellulosic fraction. Chem Eng Sci 47:1109–1122
20. Liu C, Wyman CE (2005) Partial flow of compressed-hot water through corn stover to enhance hemicellulose sugar recovery and enzymatic digestibility of cellulose. Bioresour Technol 96:1978–1985
21. Pedersen M, Johansen KS, Meyer AS (2011) Low temperature lignocellulose pretreatment: effects and interactions of pretreatment pH are critical for maximizing enzymatic monosaccharide yields from wheat straw. Biotechnol Biofuels 4:11
22. Mosier N, Hendrickson R, Ho N et al (2005) Optimization of pH controlled liquid hot water pretreatment of corn stover. Bioresour Technol 96:1986–1993
23. Kim Y, Hendrickson R, Mosier NS et al (2009) Liquid hot water pretreatment of cellulosic biomass. In: Mielenz JR (ed) Biofuels: Methods and Protocols. Methods in Molecular Biology Series. Springer
24. Jennings EW, Schell DJ (2011) Conditioning of dilute-acid pretreated corn stover hydrolysate liquors by treatment with lime or ammonium hydroxide to improve conversion of sugars to ethanol. Bioresour Technol 102:1240–1245
25. Lloyd TA, Wyman CE (2005) Combined sugar yields for dilute sulfuric acid pretreatment of corn stover followed by enzymatic hydrolysis of the remaining solids. Bioresour Technol 96:1967–1977
26. Saha BC, Iten LB, Cotta MA et al (2005) Dilute acid pretreatment, enzymatic saccharification and fermentation of wheat straw to ethanol. Process Biochem 40:3693–3700
27. Zhu Y, Lee YY, Elander RT (2004) Dilute-acid pretreatment of corn stover using a high-solids percolation reactor. Appl Biochem Biotechnol 117:103–114
28. Schell DJ, Farmer J, Newman M et al (2003) Dilute–sulfuric acid pretreatment of corn stover in pilot-scale reactor. Appl Biochem Biotechnol 105–108:69–85
29. Humbird D, Davis R, Tao L et al (2011) Process design and economics for biochemical conversion of lignocellulosic biomass to ethanol: Dilute-acid pretreatment and enzymatic hydrolysis of corn stover. Technical Report, NREL/TP-5100-47764
30. Gupta R, Lee YY (2010) Pretreatment of corn stover and hybrid poplar by sodium hydroxide and hydrogen peroxide. Biotechnol Prog 26:1180–1186
31. Kim S, Holtzapple MT (2005) Lime pretreatment and enzymatic hydrolysis of corn stover. Bioresour Technol 96:1994–2006
32. Kim TH, Kim JS, Sunwoo C et al (2003) Pretreatment of corn stover by aqueous ammonia. Bioresour Technol 90:39–47
33. Sun Y, Cheng J (2002) Hydrolysis of lignocellulosic materials for ethanol production: a review. Bioresour Technol 83:1–11
34. Chang VS, Holtzapple MT (2000) Fundamental factors affecting biomass enzymatic reactivity. Appl Biochem Biotechnol 84–86:5–37
35. Kim TH, Lee YY (2005) Pretreatment and fractionation of corn stover by ammonia recycle percolation process. Bioresour Technol 96:2007–2013
36. Kim TH, Lee YY (2006) Pretreatment of corn stover by low-liquid ammonia recycle percolation process. Appl Biochem Biotechnol 133:41–57
37. Teymouri F, Laureano-Perez L, Alizadeh H et al (2005) Optimization of the ammonia fiber explosion (AFEX) treatment parameters for enzymatic hydrolysis of corn stover. Bioresour Technol 96:2014–2018

38. Balan V, Bals B, Chundawat SPS et al (2010) Lignocellulosic biomass pretreatment using AFEX. In: Mielenz JR (ed) Biofuels: Methods and Protocols. Methods in Molecular Biology Series. Springer
39. Zhao XB, Cheng KK, Liu DH (2009) Organosolv pretreatment of lignocellulosic biomass for enzymatic hydrolysis. Appl Microbiol Biotechnol 82:815–827
40. Park N, Kim HY, Koo BW et al (2010) Organosolv pretreatment with various catalysts for enhancing enzymatic hydrolysis of pitch pine. Bioresour Technol 101:7046–7053
41. Holm J, Lassi U (2011) Ionic Liquids in the pretreatment of lignocellulosic biomass. In: Kokorin A (ed) Ionic liquids: applications and perspectives. InTech, Rijeka
42. Mora-Pale M, Meli L, Doherty TV et al (2011) Room temperature ionic liquids as emerging solvents for the pretreatment of lignocellulosic biomass. Biotechnol Bioeng 108:1229–1245
43. Keller FA, Hamilton JE, Nguyen QA (2003) Microbial pretreatment of biomass: potential for reducing severity of thermochemical biomass pretreatment. Appl Biochem Biotechnol 105–108: 27–41
44. Dashtban M, Schraft H, Syed TA et al (2010) Fungal biodegradation and enzymatic modification of lignin. Int J Biochem Mol Biol 1:36–50
45. Steffen KT, Hofrichter M, Hatakka A (2000) Mineralisation of ^{14}C-labelled synthetic lignin and ligninolytic enzyme activities of litter-decomposing basidiomycetous fungi. Appl Microbiol Biotechnol 54:819–825
46. Hammel KE (1997) Fungal degradation of lignin. In: Cadisch G, Giller KE (eds) Plant litter quality and decomposition. CABI
47. Wen F, Nair NU, Zhao H (2009) Protein engineering in designing tailored enzymes and microorganisms for biofuels production. Curr Opin Biotechnol 20:412–419
48. Bai FW, Anderson WA, Moo-Young M (2008) Ethanol fermentation technologies form sugar and starch feedstocks. Biotechnol Adv 26:89–105
49. Mamman AS, Lee JM, Kim YC et al (2008) Furfural: Hemicellulose/xylose derived biochemical. Biofuel Bioprod Biorefin 2:438–454
50. Saha BC (2003) Hemicellulose bioconversion. J Ind Microbiol Biotechnol 30:279–291
51. Tolan JS (1999) Alcohol production from cellulosic biomass: the Iogen process, a model system in operation. In: Jacques K, Lyons TP, Kelsall DR (eds) The alcohol textbook, 3rd edn. Nottingham University Press, Nottingham
52. Wang C, Wu G, Chen C et al (2011) High production of β-glucosidase by *Aspergillus niger* on corncob. Appl Biochem Biotechnol. (In press)
53. Öhgren K, Bura R, Lesnicki G et al (2007) A comparison between simultaneous saccharification and fermentation and separate hydrolysis and fermentation using steam-pretreated corn stover. Process Biochem 42:834–839
54. Olofsson K, Palmqvist B, Lidén G (2010) Improving simultaneous saccharification and co-fermentation of pretreated wheat straw using both enzyme and substrate feeding. Biotechnol Biofuels 3:17
55. Lynd LR, Weimer PJ, van Zyl WH et al (2002) Microbial cellulose utilization: fundamentals and biotechnology. Microbiol Mol Biol Rev 66:506–577
56. Lynd LR, Elander RT, Wyman CE (1996) Likely features and costs of mature biomass ethanol technology. Appl Biochem Biotechnol 57–58:741–761
57. Xu Q, Singh A, Himmel ME (2009) Perspectives and new directions for the production of bioethanol using consolidated bioprocessing of lignocellulose. Curr Opin Biotechnol 20: 364–371
58. Jin M, Balan V, Gunawan C et al (2011) Consolidated bioprocessing (CBP) performance of *Clostridium phytofermentans* on AFEX-treated corn stover for ethanol production. Biotechnol Bioeng 108:1290–1297
59. Shaw AJ, Podkaminer KK, Desai SG et al (2008) Metabolic engineering of a thermophilic bacterium to produce ethanol at high yield. Proc Natl Acad Sci USA 105:13769–13774
60. Fujita Y, Ito J, Ueda M et al (2004) Synergistic saccharification and direct fermentation to ethanol of amorphous cellulose by use of an engineered yeast strain codisplaying three types of cellulolytic enzyme. Appl Environ Microbiol 70:1207–1212

61. Katahira S, Fujita Y, Mizuike A et al (2004) Construction of a xylan-fermenting yeast strain through codisplay of xylanolytic enzymes on the surface of xylose-utilizing *Saccharomyces cerevisiae* cells. Appl Environ Microbiol 70:5407–5414
62. Stephanopoulos G (2007) Challenges in engineering microbes for biofuels production. Science 315:801–804
63. Dien BS, Cotta MA, Jeffries TW (2003) Bacteria engineered for fuel ethanol production: current status. Appl Microbiol Biotechnol 63:258–266
64. Aristidou A, Penttilä M (2000) Metabolic engineering applications to renewable resource utilization. Curr Opin Microbiol 11:187–198
65. Zhang M, Eddy C, Deanda K et al (1995) Metabolic engineering of a pentose metabolism pathway in ethanologenic *Zymomonas mobilis*. Science 267:240–243
66. Deanda K, Zhang M, Eddy C et al (1996) Development of an arabinose-Fermenting *Zymomonas mobilis* strain by metabolic pathway engineering. Appl Env Microbiol 62:4465–4470
67. Mohagheghi A, Evans K, Chou YC et al (2002) Cofermentation of glucose, xylose, and arabinose by genomic DNA-integrated xylose/arabinose fermenting strain of *Zymomonas mobilis* AX101. Appl Biochem Biotechnol 98–100:885–898
68. Seo JS, Chong H, Park HS et al (2005) The genome sequence of the ethanologenic bacterium *Zymomonas mobilis* ZM4. Nat Biotechnol 23:63–68
69. Lee KY, Park JM, Kim TY et al (2010) The genome-scale metabolic network analysis of *Zymomonas mobilis* ZM4 explains physiological features and suggests ethanol and succinic acid production strategies. Microb Cell Fact 9:94
70. Picataggio S (2009) Potential impact of synthetic biology on the development of microbial systems for the production of renewable fuels and chemicals. Curr Opin Biotechnol 20:325–329
71. Mukhopadhyay A, Redding AM, Rutherford BJ (2008) Importance of systems biology in engineering microbes for biofuel production. Curr Opin Biotechnol 19:228–234
72. Reisch M (2006) Fuels of the future: Chemistry and agriculture join to make a new generation of renewable fuels. Chem Eng News 84(47):30–32
73. Thomas KC, Hynes SH, Ingledew WM (1996) Practical and theoretical considerations in the production of high concentration of alcohol by fermentation. Process Biochem 31:321–331
74. Kotter P, Amore R, Hollenberg CP, Ciriacy M (1990) Isolation and characterization of the *P. stipitis* xylitol dehydrogenase gene XYL2, and construction of a xylose-utilizing *Saccharomyces cerevisiae* transformant. Curr Genet 18:493–500
75. Tantirungkij M, Nakashima N, Seki T, Yoshida T (1993) Construction of xylose-assimilating *Saccharomyces cerevisiae*. J Ferment Bioeng 75:83–88
76. Ho NWY, Chen Z, Brainard A (1998) Genetically engineered *Saccharomyces* yeast capable of effective cofermentation of glucose and xylose. Appl Environ Microbiol 64:1852–1859
77. Ho NWY, Chen Z, Brainard A (1997) Genetically engineered yeast capable of effective fermentation of xylose to ethanol. Proceedings of Tenth International Symposium on Alcohol Fuels, Colorado Springs, CO, USA, 7–10 Nov P738.
78. Toon ST, Philippidis GP, Ho NYW et al (1997) Enhanced cofermentation of glucose and xylose by recombinant *Saccharomyces* yeast strains in batch and continuous operating modes. Appl Biochem Biotech 63–65:243–255
79. Bera AK, Sedlak M, Khan A et al (2010) Establishment of L-arabinose fermentation in *Saccharomyces cerevisiae* 424A(LNH-ST) by genetic engineering. Appl Microbiol Biotech 87:1803–1811
80. Casey E, Sedlak M, Ho NWY et al (2010) Effect of acetic acid and pH on the co-fermentation of glucose and xylose to ethanol by recombinant *S. cerevisiae*. FEMS Yeast Res 10:385–393
81. Athmanathan A, Sedlak M, Ho NYW et al (2011) Effect of product inhibition on xylose fermentation to ethanol in glucose-xylose co-fermenting *S. cerevisiae* 424A (LNH-ST). Biol Eng 3:111–124
82. Bera AK, Ho NYW, Khan A et al (2011) A genetic overhaul of *Saccharomyces cerevisiae* 424A(LNH-ST) to improve xylose fermentation. J Ind Microbiol Biotechnol 38:617–626

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Biodiesel From Conventional Feedstocks

Wei Du and De-Hua Liu

Abstract At present, traditional fossil fuels are used predominantly in China, presenting the country with challenges that include sustainable energy supply, energy efficiency improvement, and reduction of greenhouse gas emissions. In 2007, China issued *The Strategic Plan of the Mid-and-Long Term Development of Renewable Energy*, which aims to increase the share of clean energy in the country's energy consumption to 15% by 2020 from only 7.5% in 2005. Biodiesel, an important renewable fuel with significant advantages over fossil diesel, has attracted great attention in the USA and European countries. However, biodiesel is still in its infancy in China, although its future is promising. This chapter reviews biodiesel production from conventional feedstocks in the country, including feedstock supply and state of the art technologies for the transesterification reaction through which biodiesel is made, particularly the enzymatic catalytic process developed by Chinese scientists. Finally, the constraints and perspectives for China's biodiesel development are highlighted.

Keywords Biodiesel · Conventional feedstocks · Greenhouse gas emission · Renewable energy

Contents

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1 Introduction

Compared with mineral diesel, biodiesel that is produced from plant oils or animal fats through the transesterification reaction with alcohols is more renewable, and thus benefits not only energy substitution and reduction of the dependency on imported oil, but also the rural economy through the engagement of the feedstock production, two priorities in the agenda of the Chinese government, since the country has experienced a drastic increase in oil imports within the past decade, and in the meantime has the largest agricultural population in the world. On the other hand, biodiesel consumption generates less environmental pollutants such as SO_x and NO_x, and mitigates CO_2 emission. Biodiesel is also biodegradable, making it especially suitable for farm and marine applications where the risk of fuel leaking to the environment is high [1–3].

Execution of B5, B10 or B20 Standards, with 5, 10 or 20% biodiesel supplemented into petroleum-based diesel, respectively, has been enforced in many countries including the USA and European countries. Although biodiesel development in China lags behind these countries, taking into account its output and R&D investment, more effort has been made within the last few years, due to high prices of crude oils and petroleum-based transportation fuels such as gasoline and diesel, which are making biodiesel more economically competitive. In 2007, China enacted the Biodiesel for Blending with Diesel Fuel (B100) National Standard, and in 2008, the National Development and Reform Committee approved three demonstration projects for biodiesel in Sichuan, Guizhou, and Hannan, which are owned by the China National Petroleum Company (PetroChina), China Petrochemical Corporation (Sinopec), and China National Offshore Oil Corporation (CNOOC), respectively, with a focus on feedstock development and innovative technologies for cost-effective biodiesel production. In 2011, the China Standardization Committee enacted the B5 Standard.

At present, the annual production capacity of biodiesel in China exceeds one million tons, with private companies as the major players, producing biodiesel from junk oil, acidified oil, and woody plant oil, mostly by chemical catalysis. Meanwhile, research institutes and universities such as the China Research Institute of Petroleum Processing, Tsinghua University, Beijing University of Chemical Technology, South China University of Technology, Zhejiang University of Technology, and Sichuan University are devoted to developing innovative technologies for biodiesel production and collaborating with industry [4–6]. For example, Hunan Biorivers Co. Ltd. established a 20, 000 ton/year biodiesel

production facility in 2006 using enzymatic catalysis of transesterification developed by Tsinghua University, which was expanded to 40, 000 ton/year in 2008.

Despite the aforementioned advantages of biodiesel, its development in China is challenged by the reliable supply of feedstocks at large scale and low cost, innovative processing technologies for cost-effective production, and preferential policies for increasing market share, which are discussed in this chapter.

2 Conventional Oil Feedstocks Used for Biodiesel Production

In the USA and European countries, biodiesel production mainly uses rapeseed, soybean, and other oil crops as the feedstocks, while in China biodiesel production cannot rely on these crops, which are sources for edible oils. The Chinese government has issued regulations and prohibited projects transforming edible oils, such as rapeseed oil, into biodiesel.

Currently, the main feedstocks for biodiesel production in China are waste oils. However, with the rapid development of the biodiesel industry in recent years, the price of waste oils has increased, making many small and medium biodiesel enterprises struggle to survive. Although biodiesel production from algae exhibits good potential, this strategy is not economically competitive. In the long term, oil plants such as castor and Jatropha will be sustainable feedstocks for China's biodiesel production.

2.1 Low-Grade Oils and Waste Oils/Greases

As virgin oil feedstock is not feasible for biodiesel production in China, low-grade oils and waste oils/greases have been explored for this purpose. Low-grade oils include acidified oil recovered from vegetable oil refining process, while waste oils/greases are recovered with grease traps from restaurant kitchen sewage systems, waste cooking oil from fried oil in restaurants and food processing plants, and animal fat and oil from the meat processing industry. In 2010, China consumed about 24 million tons of edible oil and generated 4–5 million tons of waste oils, of which about 2 million tons were collectable [7].

2.2 Potentials of Oil Plants for Large-Scale Biodiesel Production

There are more than 150 oil-bearing plant families in China and the oil content of some species can reach as high as 40% [8]. Although accurate data about the production and utilization of energy plants are not yet available, China's agriculture and forestry have a broad variety of energy plant resources as listed in Table 1.

Table 1 Oleaginous crops—overview of production potentials

Variety	Distribution	Planting area	Average yield (ton/ha/year)	Possible areas	Availability of land
Jatropha curcas L.	Guangdong, Guangxi, Yunnan, Sichuan, Guizhou, Taiwan, Fujian, Hainan	More than 16,000 ha in Sichuan (primary area)	Kernel: 9.75	Mainly in tropical very dry to moist through subtropical thorn to wet forest life zones	At least 2,000,000 ha
Pistacia chinensis Bunge	Hebei, Henan, Anhui, Shanxi,	About 66,700 ha	Kernel: 7.5	Centralized in Taihangshan Mountains, covering middle and south of Hebei province and the north of Henan province	At least 300,000 ha
Cornus wilsoniana	Hunan, Hubei, Jiangxi, Guizhou, Sichuan, Guangdong, Guangxi	About 10,000 ha	Kernel: 4.5–9.0	Mainly in southern provinces	At least 2,000,000 ha
Xanthoceras sorbifolia Bunge	Shanxi, Shaanxi, Hubei, Neimenggu, Ningxia, Gansu, Henan	About 25,000 ha	Fruit: 45 (but only kernel for biodiesel production)	Mainly in northwest and north China	At least 4,000,000 ha

Taking *Jatropha curcas* L. as an example, it is widely distributed from dry subtropical regions to tropical rain forests. Its current production is mainly in Guangdong, Guangxi, Yunnan, Sichuan, Guizhou, Taiwan, Fujian, and Hainan Provinces. Other tropical and subtropical regions are also potentially suitable for the plant to grow. Scientists from Sichuan University and Southwest Forestry Institute are the main players in *J. curcas* research and planting, investigating how to increase the output of the plant seed as well as process the seed oil for biodiesel production. Since 2003, Guizhou Province has been collaborating with Germany's Daimler Chrysler in the development of large-scale liquid biofuel production from *J. curcas* seed, which aims at improving the barren area by building up ecological protective systems and promoting rural economic development through sustainable agricultural production, which can improve farmers' income and reduce their migration.

The seed yield of *Jatropha curcas* can approach to 9.75 ton/ha with 40% oil based on dry mass. Economic benefits can be further enhanced by the refining of the feedstock with by-products such as value-added insecticide from the dregs and fertilizer from the remaining residues [8]. Currently, the seed of *J. curcas* is mainly imported from Thailand and India, but China is developing the cultivars from local *Jatropha* varieties. Although *J. curcas*. is promising as a feedstock for large-scale biodiesel production, other factors such as the high labor cost of harvesting the plant seed need to be taken into consideration.

3 Current Technologies for Biodiesel Production

The transesterification reaction is the core of biodiesel production, and refers to the formation of fatty acid alkyl-ester (mainly methyl ester or ethyl ester) by the reaction between triglycerides and methanol or ethanol with or without a catalyst. Various vegetable oils and animal fats as well as waste oils can be used as the raw materials for the transesterification reaction to produce biodiesel. After the transesterification reaction, the molecular weight of natural grease falls to one-third of the original. Although short chain alcohols, such as methanol or ethanol, can be used for the transesterification reaction, methanol is most widely used because of its low price, short carbon chain, strong polarity, and fast reaction rate. The transesterification reaction can occur with the use of acid, alkali, or enzyme as catalysts, or in a supercritical fluid system without catalyst.

3.1 Homogeneous Catalyst-Mediated Process

3.1.1 Homogeneous Alkali-Catalyzed Reaction

Homogeneous alkali-mediated biodiesel production is a mature technology with wide applications in the USA and Europe. Commonly used alkali catalysts include sodium hydroxide, potassium hydroxide, sodium carbonate, and potassium

carbonate. The traditional production process is the use of alkali metal hydroxide as the homogeneous catalyst, which has a higher solubility in methanol.

Generally speaking, with homogeneous alkali as the catalyst for biodiesel production, control for water content and free fatty acids (FFA) contained in raw materials is required. It has been demonstrated extensively that when the moisture content of raw materials is more than 0.06% and acid content is more than 1 mg/g oil, the saponification reaction occurs, resulting in emulsification, which weakens catalyst activity and reduces biodiesel yield [9–12].

Many Chinese scholars have studied soybean oil, cottonseed oil, rapeseed oil, rice bran oil, sunflower oil, fried oil, and a variety of waste oil and other fats and oils as raw materials with alkali as the catalyst for biodiesel production. Their studies have also shown that, when the raw material contains water and FFA, saponification occurs, not only consuming the alkali catalyst, but also reducing the reaction rate and leading to difficulties in separating the by-product glycerol [9–12]. Therefore, the current homogeneous alkali-mediated biodiesel preparation is mainly limited to academic research in China. In fact, since only low-quality oil feedstocks are currently available for biodiesel production, the use of the homogeneous alkali-mediated process for preparing biodiesel on a large scale is not realistic in China.

3.1.2 Homogeneous Acid-Catalyzed Reaction

The homogeneous acids used for biodiesel production are mainly concentrated sulfuric acid, phosphoric acid, and other inorganic acids. Due to the fact that concentrated sulfuric acid is cheap and abundant, it is the most commonly used homogeneous acid catalyst at present in China. However, this inorganic acid leads to serious issues such as severe corrosion of the equipment and environmental concerns. Since low-quality oils, which usually contains large amount of water and FFA, are the main oil feedstocks in China, alkali-based technology cannot be adopted for biodiesel production [13–15]. Therefore, nowadays, vast numbers of small and medium enterprises adopt the acid-catalyzed technology to produce biodiesel, leading to more waste, a more complicated production process, and other issues. These consequences cast uncertainty over the future of the acid-catalyed process for biodiesel production.

3.1.3 Two-Step Catalytic Process With Acid and Alkali

Some enterprises in China use the acid-alkali two-step catalytic process for biodiesel production, in which pre-esterification with an acid catalyst is conducted prior to alkali catalyst-mediated transesterification [16, 17]. Although this kind of acid- and alkali-catalyzed two-step catalytic process can be used with different oil feedstocks for biodiesel production, the complexity of the procedures and operations diminishes its economic and environmental benefits.

3.2 Heterogeneous Catalyst-Mediated Process

3.2.1 Heterogeneous Solid Alkali-Catalyzed Reaction

Some disadvantages associated with homogeneous alkali catalysts, such as costly separation of the catalyst from the reaction mixture, have inspired the development of heterogeneous solid catalysts for biodiesel production, and solid alkali catalysts including mainly alkali metal oxides, alkaline-earth metal oxides, hydrotalcite and hydrotalcite-like solid alkali have thus been developed [18, 19]. In general, alkali metal oxides and alkaline-earth metal oxides are hygroscopic, which leads to costly separation. In addition, their preparation requires relatively elevated temperatures and pressures. Anion exchange resin is another solid alkali catalyst for biodiesel production. Chinese researchers have reported that different anion exchange resins pretreated with NaOH solution could be used as solid alkali catalysts for biodiesel production. Some researchers used hydrotalcite and hydrotalcite-like solid alkali as the catalysts for biodiesel production. In these systems, strong and medium basicity can be achieved by the presence of $O^{2-}$and OH^{-}species. However, these types of solid alkali catalysts are easily poisoned by H_2O and CO_2. Researchers have also investigated activated carbon, MgO, CaO, ZrO_2 and TiO_2 as the solid alkali catalysts for biodiesel production [18–20].

Although heterogeneous catalysts have the advantages of being reusable and easy to separate from the reaction products, their performance is generally poorer than that of the commonly used homogeneous catalysts. Though many researchers have investigated heterogeneous catalyst-mediated biodiesel production, few practical applications have been reported.

3.2.2 Heterogeneous Solid Acid-Catalyzed Reaction

Many scholars are engaged in the study of solid acid-catalyzed biodiesel production in China. The solid acid catalysts include zeolites, heteroplyacids, and ion-exchange resins. [21, 22]. In general, these solid acid catalysts adapt well to feedstocks with high FFA content, and have the advantage of easy separation. However, solid acid catalysts have the disadvantages of low activity, slow reaction rate, high temperature, and poor yields. Even though some companies have claimed that solid acid catalyst-mediated biodiesel production technology is mature, the separation issue as well as high cost hinders its practical application.

Some scholars are also working on acid ionic liquids for biodiesel production [21, 22]. Ionic liquid, as a type of new environmentally friendly solvent and liquid acid catalyst, shows the high-density reactivity sites of liquid acid and the non-volatile property of solid acids. It is reported that ionic liquids can be used as a solvent when it is present as catalysts. Although using ionic liquid as catalysts for biodiesel production has been reported in China, the cost, adaptation of materials, and other issues associated with ionic liquid-mediated biodiesel preparation need to be further studied.

3.3 Lipase-Mediated Process

In recent years, enzymatic approaches for biodiesel production have received much attention, since they have many advantages over chemical methods: moderate reaction conditions, low alcohol to oil ratio, easy product recovery, and environmentally friendly. In addition, FFAs contained in waste oils and fats can be simultaneously completely converted to alkyl esters. In terms of the forms of biocatalyst, enzyme-mediated biodiesel production can be classified into immobilized lipase, whole cell catalyst, and liquid lipase-mediated alcoholysis, respectively. The related R&D as well as the progress of industrialization is discussed here.

3.3.1 Immobilized Lipase-Mediated Transformation

A solvent-free system was first proposed for lipase-mediated methanolysis for biodiesel production. In such a system, it was demonstrated that methanol has poor solubility in oil feedstocks and too much methanol existing as drops in the system has an inhibitory effect on lipase activity [1, 2]. Stepwise methanol addition was therefore recommended for enzyme-mediated biodiesel production in the solvent-free system. In addition, glycerol, a by-product of the process, is very hydrophilic and insoluble in the oils, and is easily adsorbed onto the surface of the immobilized lipase, leading to a negative effect on lipase activity and operational stability. Several methods have been proposed to eliminate this phenomenon, such as adding silica gel to the reaction system to absorb glycerol or periodically washing the lipase with some organic solvents to remove glycerol. In practice, these methods do reduce the negative effect caused by glycerol to some extent, but make the system more complicated, especially for large-scale continuous biodiesel production [23, 24].

With the aim of reducing the aforementioned negative effects caused by methanol and glycerol, some researchers tried carrying out the enzymatic reaction for biodiesel production in organic solvent systems. According to the traditional principle of non-aqueous enzymology, enzymes show higher activity in relatively hydrophobic organic solvents with higher logP (>3), such as n-hexane and petroleum ether. Based on this knowledge, hydrophobic organic solvents have been extensively explored as the reaction medium for immobilized lipase-catalyzed biodiesel production. In reality, with such relatively hydrophobic organic solvents as the reaction media, methanol and glycerol have poor solubility in these systems. Therefore the negative effects caused by methanol and glycerol cannot be completely eliminated, and lipase still exhibits poor operational stability.

Tsinghua University developed a novel process with a relatively hydrophilic organic solvent, tert-butanol, as the reaction medium for lipase-mediated alcoholysis for biodiesel production [2]. Both methanol and the by-product glycerol are soluble in tert-butanol medium and the whole system is homogeneous, and the

Fig. 1 The first industrial plant for biodiesel production using lipase catalysis

negative effects caused by methanol and glycerol on lipase performance can be eliminated totally. It was further reported that with combined use of Lipozyme TL IM and Novozym 435 in the tert-butanol system, the highest biodiesel yield obtained was 95% and there was no obvious loss in lipase activity even after being reused for 200 cycles. This technology was validated to be suitable for the conversion of various feedstocks into biodiesel, including low-grade waste oils.

The first industrial facility in the world for lipase-mediated biodiesel production with this new technology was built on December 8, 2006, with a biodiesel production capacity of 20,000 ton/year, which was extended to 40,000 ton/year in 2008 (Fig. 1). The success of the facility has drawn attention from industries in China and abroad. Enterprises from America, Europe and Brazil are negotiating with Tsinghua University for licencing this technology for biodiesel production. A pilot-scale plant in Brazil is scheduled in 2012 with the support of CCBCE (Center for China–Brasil Climate and Energy Innovation Technology).

3.3.2 Whole-Cell-Mediated Alcoholysis

Utilizing whole cells instead of conventional immobilized lipase as the catalyst for biodiesel production is a potential way of reducing the cost of the biocatalyst, since they can avoid the complex procedures of lipase fermentation, purification and immobilization.

Rhizopus oryzae, a species producing intercellular lipase, has been studied extensively for biodiesel production, and it has been demonstrated that the

immobilization of such whole cells can be realized spontaneously during the process of cell cultivation [25]. To further stabilize the whole-cell catalysts, cross-linking treatment with glutaraldehyde solution was suggested and in this way the immobilized whole cells can be reused for many batches. Further study has revealed that the activity of such cross-linking immobilized cells was even competitive to the commercial lipase Novozym 435. Acyl migration was also observed during the whole-cell-mediated methanolysis for biodiesel production, which was promoted with the increase in water content in the reaction mixture [26–28].

Although whole-cell biocatalysts overproducing intracellular lipase can be expected to reduce the cost of lipase preparation considerably and offer a promising prospect for industrial biodiesel production, many challenges such as scaling up and process optimization need to be investigated further.

3.3.3 Liquid Lipase-Mediated Alcoholysis

Liquid lipase offers an alternative approach to enzyme-catalyzed biodiesel production, though few reports are available. Compared with immobilized lipase, soluble lipase possesses the advantages of faster reaction rate and lower cost; hence, much attention has been paid to soluble lipase-mediated methanolysis for biodiesel preparation in recent years [29–32]. Research has already showed that a soluble lipase NS81006 could be capable of catalyzing the methanolysis of triglycerides to produce biodiesel in an oil/water biphasic system and a biodiesel yield of over 90% could be obtained after 8 h reaction. Although the recovery of lipase and the enzyme's performance during continuous running need to be evaluated further, the liquid lipase-mediated reaction provides an alternative method of enzyme-mediated alcoholysis for biodiesel production.

3.4 Biodiesel Production in a Supercritical Fluid System

Supercritical fluid has properties different from gas or fluid, with its density similar to fluid, its viscosity similar to gas, and its thermal conductivity and diffusion coefficient between gas and fluid. Methanol is hydrophobic in supercritical conditions, and triglyceride dissolves well in supercritical methanol. As a result, biodiesel production in a supercritical system has advantages of fast reaction rate and high conversion yield [33, 34]. Since the supercritical fluid method is sensitive to operating temperature and pressure variations, it is feasible to change the operating conditions of the reaction to adjust the physical properties of supercritical fluid, and thus affect the mass transfer, solubility, and reaction dynamics performance correspondingly. However, the shortcomings associated with this method are high operating temperature and pressure of about 350–400 C and 45–65 MPa, respectively, and the economics of its industrial application needs further systematic evaluation [33–35].

The super critical fluid method for biodiesel production was developed by Sinopec and tested in a 200 ton/year pilot plant. Its characteristics include simple pretreatment of raw material, adaptability to various raw materials, simplified post-processing, and less wastewater discharge. In 2010, CNOOC applied this technology to build an industrialization demonstration plant with a biodiesel production capacity of 60,000 ton/year, but an update on its economic performance was not available when this chapter was prepared.

3.5 Processing of By-Product Glycerol

In the preparative process using the above-mentioned technologies, whether traditional acid or alkali catalysis, lipase catalysis or in a supercritical fluid system, glycerol is produced as a by-product up to 10%, depending on the ratio of triglyceride and fatty acid contained in the feedstock [3]. Taking into account biodiesel as a bulk commodity, the amount of glycerol produced is very significant, and must be processed properly to validate biodiesel production.

Researchers in the Department of Chemical Engineering, Tsinghua University have successfully converted the by-product glycerol into a value-added product 1,3-propanediol (PDO) by biological transformation [36, 37]. PDO is an important block chemical with a potential market for the production of the excellent polymer polytrimethylene terephthalate (PTT) through the polymerization reaction with terephthalic acid. This technology has now completed industrial demonstration.

4 Constraints and Perspectives of Biodiesel Development

4.1 Feedstocks

Currently, illegal collection, purification, and re-sale of waste oils as components for edible oils are making waste oils scarce for biodiesel producers. The establishment and enforcement of strict regulations on waste oil management in China is urgently needed to support their use in biodiesel production.

To avoid competition for feedstocks with food (edible oils) production, the biodiesel industry will rely more and more on energy crops for sustainable feedstocks. At present, breeding of high quality energy crops such as *J. curcas* and *Pistacia chinensis* Bunge has advanced. Further research will focus on their low cost and large-scale production. Meanwhile, genetically modified energy crops may increase oil productivity significantly, resulting in large cost reductions. Since most forestlands in China are located in mountainous areas, collection and transportation of energy forestry crops is labour-intensive and costly, and the corresponding infrastructure must be established.

4.2 Processing Technologies

At present, processing technologies for producing biodiesel from widely available energy crops such as *J. curcas* and *P. chinensis* have been extensively studied, but there is still more space for improvement in order to increase biodiesel yield. To date, investment in R&D is still inadequate considering the rapid increase of biodiesel demand.

Currently, many technologies are under development for biodiesel production, but industrialization or pre-industrialized progress has not been reported. Whereas CNOOC is using the supercritical fluid system developed by Sinopec to produce biodiesel in Hainan, and Hunan Biorivers Co. Ltd. is cooperating with Tsinghua University to use the enzymatic process, most small and medium-sized enterprises in China still use acid techniques to produce biodiesel, and are facing concerns about environmental pollution and low product quality not suitable for transportation fuels, but only for power generation, heating or some farm-oriented uses. It is believed that with the modification of relevant laws and regulations, and close cooperation between scientific research institutes and enterprise, advanced technologies are expected to be put into large-scale applications in the near future.

4.3 Marketing of Biodiesel

The market for biodiesel in China is still limited to local distribution, direct sale by producers, and purchase by customers, due to the low output and obstacles in the main distribution network of the three leading petrol companies Sinopec, Petro China and CNOOC. Direct sale of biodiesel to transport companies, the agricultural production sector, and public services is done in Sichuan and Fujian provinces, since fossil diesel is in short supply in some areas. However, it is difficult for consumers to trust the biodiesel quality even though the B5 standard comes into effect in 2011.

Since the price of fossil diesel keeps rising, biodiesel is now becoming more competitive. In general, the main challenges for biodiesel production include competing resources of land diversion and water usage, greenhouse gas reduction potential, and crop collection and processing cost. Given an improved economic feasibility and increased production capacity in the future, biodiesel need to be incorporated into the distribution network of the leading petrol companies Sinopec, Petro China and CNOOC.

5 Major Players of Biodiesel R&D and Industrial Production

R&D on biodiesel in China has advanced rapidly. Research sectors include selection, genetic modification and cultivation of oil plants, processing technologies, and equipment. Researchers from various universities and research institutes

Table 2 Major producers of biodiesel in China

Name and location	Designed capacity (million tons/year	Feedstock
Fujian Longyan New Energy Zhuoyue Co. Ltd, Longyan, Fujian province	20,000	Grease trap waste
Sichuan Gushan Oil & Fat Chemical Ltd, Gushan, Sichuan province	30,000	Grease trap waste, rapeseed oil
Zhenghe Bio-Energy Ltd, Wuan, Hebei province	20,000	Acidified oil, fatty acid distillates
D1Energy PLC, UK, Sichuan province	500,000	*Jatropha curcas L.*
Leo Ltd., England, & Hunan Tianyuan Clean BioEnergy Ltd., Hunan province	200,000	Nd
Daimler Chrysler, Germany, and Guizhou province	Not available	Jatropha seeds
Henan Xinyang Hongchang Group, Henan province	30,000	Local wood plant oil and grease trap waste from different provinces
Hunan Biorivers Co. Ltd.	40,000	Grease trap waste, rapeseed
Dinuo Chemical Ltd., Guiyang, Guizhou	30,000	Grease trap waste
Fujian Yuanhua Energy Science Co. Ltd., Fujian province	30,000	Waste grease and oils
Shangqiu Grease Chemical Co. and Shangqiu Administration of Road Management, Shangqiu, Henan	110,000	Waste oil
Fuzhou Gushan Oleo Chemical Co. Ltd.	100,000	Waste grease and oils
Yuanhua Energy Technology, Hangzhou Xiaoshan	50,000	Waste grease and oils
Shanghai Biodiesel Plant, Jinshan	50,000	Grease trap waste, rapeseed
NCOOC	60,000	Jatropha seeds

including Tsinghua University, Beijing University of Chemical Technology, Sichuan University, China Agriculture University, Chinese Academy of Forestry, Guangzhou Institute of Energy Conversion and Harbin Forest University are actively engaged in biodiesel R&D [3–7].

However, China's biodiesel industry is in its infancy. The total output nationwide was only tens of thousands of tons in 2010, although the production capacity was more than 1 million tons. The major industrial players involved in biodiesel production are given in Table 2.

6 Summary

Biodiesel is an emerging industry in China, and presents increasing market potential. Inspired by the business opportunities evident in the "Dual Concepts" of green energy and support for the agriculture industry, many enterprises enter this industry. Through an overview on biodiesel development in developed countries such as America, Germany, and Japan, and less developed countries such as South Africa, Brazil, Korea, India, and Thailand, we found that they provide China with good examples of the development of this alternative fuel industry from the aspects of policies, technologies, facilities, vehicle manufacturing, etc.

In 2020, the number of motor vehicles in China will reach 130–150 million, and the demand of fuels will correspondingly increase to about 256 million tons (Mt), about 85 Mt of gasoline and 171 Mt of diesel from petroleum or biodiesel. The available amount of biodiesel by that time will depend on land resources for woody oil plants as sustainable feedstocks for biodiesel production. Even if conflicts between energy forestry and traditional forestry, which focuses on timber production, could arise due to China's long-term forestry formulation policy, research on biodiesel production and investigations on land availability for energy forestry have been carried out.

When people know more about its advantages, biodiesel will be accepted more widely. The gap between market demand for biodesel and its limited output will make the product be in short supply, and thus increase its economic competitiveness. Meanwhile, tight product quality control and waste treatment will be applied to biodiesel production, and producers should increase their investment in technology innovation and improve their product quality.

References

1. Tan TW, Shang F, Zhang X (2010) Current development of biorefinery in China. Biotechnol Adv 28(5):543
2. Du W, Li W, Sun T, Chen X, Liu DH (2008) Perspectives for biotechnological production of biodiesel and impacts. Appl Microbiol Biotechnol 79(3):331
3. Min EZ, Du ZX (2010) Perspectives of biodiesel industry in China. Eng Sci 12(2):11 (Chinese)

4. Pei P, Zheng FT, Cui HX (2009) Analysis on status, potential and barrier of the biodiesel development in China. Forest Econ 3:65 (Chinese)
5. Wang DJ, Yang CL, Qiao MK (2009) Progress on biodiesel development and utilization. Chem Res 20(3):108 (Chinese)
6. Sun C, Liang W (2008) The status of biodiesel development and production in China. Nat Gas Ind 28(9):123 (Chinese)
7. Li JP, Zhang XB, Li XQ, Li Z (2008) Research on biodiesel technique and its application progress in China. Appl Chem Ind 37(3):327 (Chinese)
8. Li CZ, Jiang LJ, Cheng S (2005) Biodiesel-green energy. Chemical Industry Press, Beijing
9. Wang YY, Gu BJ (2010) Mechanism and application of biodiesel preparation by transesterification. Adv Fine Pet 11(5):21 (Chinese)
10. Lai HX, Wan X, Jiang ML (2010) Advance in production technology of biodiesel. Chem Bioeng 27(5):11 (Chinese)
11. Zhang Z, Wei HG (2009) Progress in biodiesel production technologies. Pet Plan Eng 20(1):23 (Chinese)
12. Zhang J, Wang XJ, Guo HJ (2010) Research progress on transesterification for preparation of biodiesel. Appl Chem Ind 39(6):916 (Chinese)
13. Peng BX, Shu Q, Wang GR, Wang JF (2009) Kinetics of esterification for acid-catalyzed preparation of biodiesel chemical reaction. Eng Technol 25(3):250 (Chinese)
14. Zhu LP, Lv ZF, Zhan FT, Ge YJ (2007) Study of preparation of biodiesel by acid catalysts. Appl Chem Ind 36(10):1014 (Chinese)
15. Yang ZQ, Xie WL, Li HT (2006) Research in transesterification of oils and fats via acid catalysts. Cereal Oil (3):13 (Chinese)
16. Chen Y, Wang DH, Wang BH (2008) Study on preparation process of biodiesel from waste cooking oil by homogeneous catalysis method. Pet Technol Appl 26(5):415 (Chinese)
17. Huang SJ, Chen Y, Xie Y, Chen D, Ma YG (2010) Development of calcium-based solid base catalysts for biodiesel production by transesterification. China Oil Fat 35(3):46 (Chinese)
18. Ren TQ, Li PF, Sun Y, Qiao QD (2010) Research on production of biodiesel catalyzed by solid catalysts. Ind Catal 18(11):19 (Chinese)
19. Yan SL, Lu HF, Jiang LH, Liang B (2007) Solid base catalysts for transesterification of oil with methanol to produce biodiesel. J Chem Ind Eng 58(10):2506 (Chinese)
20. Hou XG, Qiao XG, Qi YQ, Wang GF, Qin ZF, Wang JG (2006) Preparation of biodiesel from soybean oil catalyzed by solid alkali in subcritical methanol. China Oil Fat 31(10):37 (Chinese)
21. Wang LB, Huang FH, Li WL (2009) Research progress of biodiesel preparation catalyzed by solid acids. Chem Bioeng 26(4):12 (Chinese)
22. Li H, Xie WL, Shao L, Chun H (2007) Progress in biodiesel preparation from transesterification of oils and fats catalyzed by solid acids. Cereal Oil (12):8 (Chinese)
23. Wang WJ, Yang YQ, Wu SZ (2010) Research progress of biodiesel production catalyzed by lipase. Biotechnol Bull (3):54 (Chinese)
24. Yu XY, Huang ZX, Lin H (2009) Research progress on biodiesel production by bio-enzymatic catalysis. China Oil Fat 34(6):48 (Chinese)
25. Sun T, Du W, Liu DH (2009) Prospective and impacts of whole cell-mediated alcoholysis of renewable oils for biodiesel production. Biofuel Bioprod Biorefin 3(6):633
26. Li W, Du W, Liu DH, Yao Y (2008) Study on factors influencing stability of whole cell during biodiesel production in solvent-free and tert-butanol system. Biochem Eng J 41(2):111
27. Sun T, Du W, Liu DH (2010) Exploring the effects of oil inducer on whole cell -mediated methanolysis for biodiesel production. Process Biochem 45(1):514
28. Li W, Du W, Liu DH (2008) *Rhizopus oryzae* whole-cell-catalyzed biodiesel production from oleic acid in terf-butanol medium. Energy Fuel 22(1):155
29. Lv D, Du W, Zhang GL, Liu DH (2010) Mechanism study on NS81006-mediated methanolysis of triglyceride in oil/water biphasic system for biodiesel production. Process Biochem 45(4):446
30. Xin C, Du W, Liu D (2008) Response surface optimization of biocatalytic biodiesel production with acid oil. Biochem Eng J 40(3):423

31. Chen X, Du W, Liu DH (2008) Effect of several factors on soluble lipase-mediated biodiesel preparation in the biphasic aqueous-oil systems. World J Microbiol Biotechnol 24(10):2097
32. Ren HJ, Du W, Lv LL, Liu DH (2011) Study on free lipase-catalyzed ethanolysis for biodiesel preparation in an oil/water biphasic system. J Am Oil Chem Soc 88(10):1551
33. Gao LA, Song BA, Yang S (2010) Advance in production technology of biodiesel. Chem Bioeng 37(5):17 (Chinese)
34. Hu DD, Wang WQ (2006) Development in production of biodiesel fuel using supercritical esterification method. Adv Fine Pet 7(11):23
35. Yang JB, Chen MK, Tang SH, He DP, Chen T (2008) Synthesis of biodiesel in supercritical fluid. China Oil Fat 33(1):40 (Chinese)
36. Xu YZ, Liu HJ, Du W, Sun Y, Liu DH (2009) Integrated production for biodiesel and 1, 3-propanediol with lipase-catalyzed transesterification and fermentation. Biotechnol Lett 31(9):1335
37. Liu HJ, Du W, Liu DH (2007) Progress of the biodiesel and 1, 3-propanediol integrated production. Prog Chem 19(7):1185 (Chinese)

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Establishing Oleaginous Microalgae Research Models for Consolidated Bioprocessing of Solar Energy

Dongmei Wang, Yandu Lu, He Huang and Jian Xu

Abstract Algal feedstock is the foundation of the emerging algal biofuel industry. However, few algae found in nature have demonstrated the combination of high biomass accumulation rate, robust oil yield and tolerance to environmental stresses, all complex traits that a large-scale, economically competitive production scheme demands. Therefore, untangling the intricate sub-cellular networks underlying these complex traits, in one or a series of carefully selected algal research models, has become an urgent research mission, which can take advantage of the emerging model oleaginous microalgae that have already demonstrated small, simple and tackleable genomes and the potential for large-scale open-pond cultivation. The revolutions in whole-genome-based technologies, coupled with systems biology, metabolic engineering and synthetic biology approaches, would enable the rational design and engineering of algal feedstock and help to fill the gaps between the technical and economical reality and the enormous potential of algal biofuels.

Keywords Biofuels · Functional genomics · Oil-producing algae · Research models · Synthetic biology · Systems biology

Dongmei Wang and Yandu Lu have contributed equally to this work.

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Contents

1 Introduction

Energy demand and environmental concerns have significantly constrained the sustainable development of the Chinese economy, which has been experiencing record levels of energy consumption. In 2009, China's energy consumption was equivalent to over 3.1 billion tons of standard coal [1]. From 2007 to 2010, its imported crude oil increased from 46% to a record level of 56% of total consumed oil [2]. On the other hand, total CO_2 emission in China reached 67.2 billion tons in 2007, accounting for 24% of the total emission in the world (CO_2 Emissions from Fuel Combustion, 2009 ed., IEA; http://www.iea.org). At the Copenhagen Climate Summit (December 2009), China committed to a 40–45% reduction of CO_2 emission per GDP unit production by 2020 compared to the 2005 level. Renewable and environmentally friendly energy sources have therefore become a crucial factor defining the economical and social sustainability of the country.

Solar energy is the most abundant clean energy. Photosynthesis is the only known biological approach in capturing solar energy and fixing CO_2. The photosynthate derived, largely in the forms of plant biomass, has long been exploited for energy via direct biomass burning or conversion to biogas, albeit at an efficiency and scale unable to compete with fossil fuels. In a "Consolidated Bioprocessing of Solar Energy" (CBP-SE) scheme (Fig. 1), a number of traditionally discrete processing steps, including photosynthesis, accumulation of energy storage compounds, and production of ethanol, biodiesel (triacylglycerol), advanced biofuel (e.g. terpenoid and long-chain hydrocarbons), high-value chemicals and food additives (pigments, proteins and polysaccharides), are consolidated into a single processing step, typically in a single cell or cellular system. Such an ultimate reduction of processing steps from solar energy and inorganic carbon to biofuels maximizes energy and cost efficiency, and thus represents one of the most competitive strategies for producing biofuels on a large scale.

Microalgae are one promising feedstock for biofuel production in the CBP-SE scheme. In addition, they can utilize marginal land and brackish and waste water, and thus do not compete with agriculture for land and water resources [3, 4]. Thus, large-scale cultivation of oleaginous algae holds the potential to simultaneously alleviate

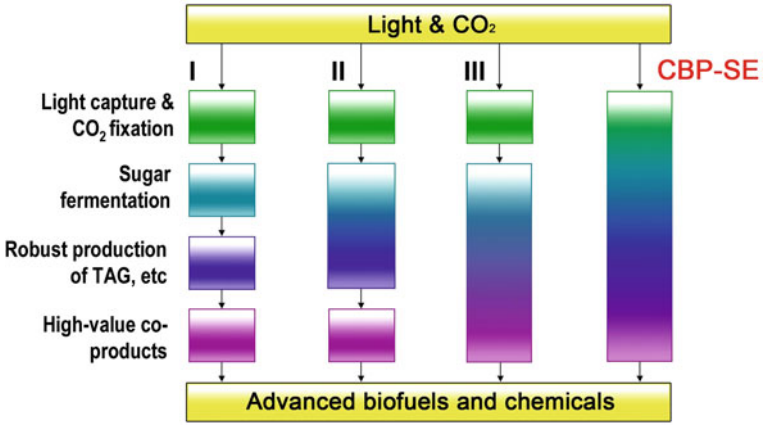

Fig. 1 Consolidated Bioprocessing of Solar Energy (CBP-SE)

energy crisis, CO_2 emission and environmental concerns [3]. The current pilot-scale algal oil production systems typically consist of identification and preservation of microalgae feedstock, large-scale cultivation to produce algal biomass, and biomass processing that typically includes harvesting, oil extraction and transesterification to produce biodiesel. However, there are presently a number of major technological bottlenecks, including low oil content of wild microalgae (usually less than 25%), low productivity (usually less than 15 g/m^2/day on average), difficulty in oil extraction (high energy consumption when dehydrating biomass over 99%), low efficiency in converting biomass energy to biodiesel (large amounts of biomass residue are wasted), low quality of mature liquid fuel produced (of high oxygen content or containing large amounts of long carbon-chain unsaturated fatty acids) [5].

Most of these challenges can be traced to the microalgal feedstock. It was estimated that if the algal biomass productivity increased from 20 to 60 g/m^2/day and lipid content from 25 to 60%, algal fuel cost per gallon would fall from nearly $10 to $2, a price level competitive with fossil fuels [5]. However, at present, autotrophic growth rates of microalgae are generally less than 15 g/m^2/day and neutral lipid content less than 25%. In addition to oil productivity, stress tolerance of the microalgal feedstock is of particular importance, which is reflected as the ability to establish and maintain dominant culture at large-scale, typically outdoor cultivation under abiotic and biotic environmental perturbation such as seasonal and diurnal climate variation, water source fluctuation, and invasion by competitors and predators. Furthermore, chemical parameters of the cellular products synthesized by the microalgal feedstock, such as length of the carbon chain, number of double bonds and content of unsaturated fatty acids, directly influence the quality of biofuel products. For example, fatty acids with more than four double bonds are vulnerable to oxidation and therefore represent an challenge for storage [5]. The viability of algal biofuels industry is therefore dependent on the availability of algal feedstock with much higher biomass productivity and lipid

content, desirable environmental stress tolerance and a product profile optimized for biofuel conversion. However, hunting in nature for such microalgae strains that are simultaneously endowed with such talents has proved to be an uphill struggle.

2 Progress and Challenges in Algal Feedstock Development

Nearly two decades ago, the "Aquatic Species Program" in the USA amassed a microalgal collection of over 3,000 strains which eventually was winnowed down to around 300 species with potential as biofuel producers. Some of the strains were put into open ponds for outdoor cultivation testing; however, none of them demonstrated a combination of the superior traits demanded [6]. For example, although the growth rate could reach 50 $g/m^2/day$ in mass culture, the yearly average was only 10 $g/m^2/day$, a level far below the industrial requirements. Most importantly, some of the key traits were mutually exclusive for a given microalgal isolate, such as high growth rate and high oil content [6]. These roadblocks were also encountered by Japanese researchers in the "Biological Fixation and Utilization of CO_2" project, where some strains were isolated that could tolerate and grow rapidly under high concentrations of CO_2. These pioneering endeavors demonstrated that only a small fraction of microalgal species and strains in nature harbors the potential for large-scale biodiesel production, and few of them are simultaneously endowed with high biomass productivity and high lipid content, strong environmental tolerance and a product profile optimized for biofuels [4].

Genetic engineering of microalgae has also been attempted to improve lipid productivity. In 1995, one of the key enzymes for lipid biosynthesis, encoded by the acetyl-CoA carboxylase (ACCase) gene, was identified. Targetting the gene, transgenic *Cyclotella cryptica* and *Navicula saprophila* were developed, but neither showed increased lipid content [7–11]. More recently, in a *Chlamydomonas reinhardtii* mutant with inactivated ADP-glucose pyrophosphorylase, an enzyme involved in starch synthesis, the triacylglycerol content exhibited a 10-fold increase, from 2 to 20.5%, under nitrogen starvation [11]. However, its growth slowed down, resulting in lower overall lipid yield than that of the wild type. So far, there have been few success stories in improving oil productivity by engineering individual genes or pathways in oleaginous microalgae. As a result, transgenic microalgae at product costs competitive with petroleum-derived fuels have been rare [12].

Such humbling outcomes resulted from a number of realities, some of which were recognized only recently. *First*, the key traits of oleaginous microalgae are inherently correlated and interact with each other, thus selection and engineering strategies that treat each trait independently would usually be futile. Many such phenotypes were observed, such as the link between photosynthesis and cellular carbon flux, the apparent conflicts between algal growth rate and oil content, the interactions between biosynthesis pathways of neutral lipids (e.g. triacylglycerol) and polar lipids (phospholipids etc.), the relationship between neutral lipid production and pigment synthesis, and the links between the various oil-induction programs induced by light

and nutrients. However, the molecular and genomic foundations for these inter-trait crosstalks remain largely unknown. *Second*, each key microalgal trait itself is determined or controlled by a complex set of genes or pathways. For example, at least four known pathways interact with each other in lipid synthesis. Such complexity in the cellular metabolic and regulatory network further confounds efforts to engineer microalgal feedstock. *Third*, for oleaginous microalgae, our understanding of the genes, pathways and genomes is quite partial and superficial. So far, there have been relatively few genome sequences available for oleaginous microalgae. Even for the laboratory model microalgae *C. reinhardtii*, a surprising large percentage of genes (nearly 60%) are still of unknown function. *Finally*, the genetic engineering toolboxes for oleaginous microalgae have typically been preliminary or limited [4], at least partially resulting from the plethora of potential oleaginous microalgal strains and the relatively tiny size of the research community focusing on a particular strain. Therefore, dissecting and engineering the genome-wide metabolic and regulatory networks underpinning the complex interactions among traits and those among genes/pathways for each trait has become an urgent mission.

3 Research Models

However, well-established research models for untangling the genome-wide sub-cellular networks in oleaginous microalgae are still largely absent. The prerequisite for formulating such research models is the choice of model algal strains. First and foremost, an ideal strain for such a purpose should possess those phenotypes that are crucial and representative in large-scale cultivation for oil production. (1) Its potential for large-scale cultivation should have been demonstrated in closed or open pond culture system. (2) It should harbor exceptional phenotypes in several, if not all, of those key traits determining the technical and economic feasibility of microalgal biofuels, such as growth rate, lipid content, biomass productivity, environmental tolerance, etc. (3) It should be suitable for a wide range of cultural and ecological conditions, such as wastewater, freshwater, brackish (<3.5% salt), marine (3.5% salt) and hypersaline (>3.5% salt) environments. (4) Despite the likely enormous collective metabolic capability related to lipid and biomass production in microalgae, a model strain should harbor a certain degree of versatility in synthesis of high-value co-products in addition to triacylglycerol, which could reduce the overall cost of microalgal biofuel. (5) Although the metabolic and regulatory diversity of oleaginous microalgae remains an uncharted territory, ideally a model strain should be representative of certain metabolic and regulatory modes of energy conversion, storage and partitioning, so that the knowledge gained on the model strain can be readily extended to many additional strains. On the other hand, as a research model for the dissection and engineering of genome-wide networks, it is crucial, in terms of microalgal genotypes, that the strain features a relatively small genome, simple gene structure, clear genetic background, abundant genomics resources, and widely accessible methods and tools for genetic manipulation.

Table 1 Biomass productivity, lipid content and lipid productivity of 30 microalgal strains cultivated in 250-mL flasks (adapted from [14])

Microalgae	Biomass productivity (mg $L^{-1}d^{-1}$)	Lipid content (%)	Lipid productivity (mg $L^{-1}d^{-1}$)
Nannochloropsis sp. RM	278.8 ± 0.0	31.0 ± 0.5	86.3 ± 0.0
Nannochloropsis sp. RP	232.7 ± 25.7	37.0 ± 0.5	86.1 ± 9.5
Nannochloropsis sp. ZM	241.8 ± 7.7	33.1 ± 1.7	79.9 ± 2.6
Pavlova lutheri	212.5 ± 10.6	37.1 ± 0.5	78.9 ± 3.9
Scenedesmus sp. DM	348.2 ± 2.6	21.8 ± 0.6	75.8 ± 0.6
Pavlova salina	240.0 ± 7.1	31.1 ± 1.4	74.6 ± 2.2
Chlorocomccum sp. UMACC 112	380.0 ± 2.6	19.5 ± 0.7	74.2 ± 0.5
Nannochloropsis sp. CS 246	231.8 ± 1.3	30.4 ± 0.3	70.4 ± 0.4
Nannochloropsis sp. MRS	270.0 ± 2.6	24.9 ± 0.7	67.2 ± 0.6
Ellipsoidium sp. LW 70/01	235.5 ± 1.3	28.4 ± 0.4	67.0 ± 0.4

Based on these criteria, one strategy for choosing and establishing model oleaginous microalgae starts with the present laboratory microalgae. It takes advantage of the abundant genomics resources and widely accessible genetic systems that are typically found in these microalgae. However, this approach suffers from several drawbacks, in that existing laboratory organisms are of low oil productivity; for example, *C. reinhardtii* [13] accumulate only 2% of triacylglycerol under nutrient starvation conditions. A potentially even bigger challenge is that after numerous generations of laboratory propagations, these laboratory strains could have largely lost the environmental tolerance that allows robust growth under large-scale (typically outdoor and open-pond) cultivation. The other strategy, instead, starts with wild microalgal strains that are able to synthesize and accumulate large amounts of lipids under large-scale cultivation, such as certain *Nannochloropsis* and *Chlorella* strains.

4 Advantages of Nannochloropsis

Nannochloropsis (Eustigmatophyceae) is a genus of unicellular photosynthetic microalgae, ranging in size from 2 to 5 μm and widely distributed in marine, fresh and brackish waters. In several pioneering large-scale outdoor cultivation facilities, *Nannochloropsis* strains such as *N. salina* and *N. oceanica* have demonstrated (Table 1) (1) rapid and robust growth, including when supplied with flue gases, (2) ability to accumulate high levels of triacylglycerols naturally or upon induction at a demonstration scale [14], and (3) exceptional tolerance to environmental stresses. Moreover, they are able to abundantly build up pigments such as astaxanthin, zeaxanthin and canthaxanthin and to accumulate high levels of polyunsaturated fatty acids (e.g. eicosapentaenoic acid, EPA) [15–17]. On the other hand, emerging genomics data on eight *Nannochloropsis* strains that represent every known species in the genus suggest that *Nannochloropsis* strains feature small genome sizes,

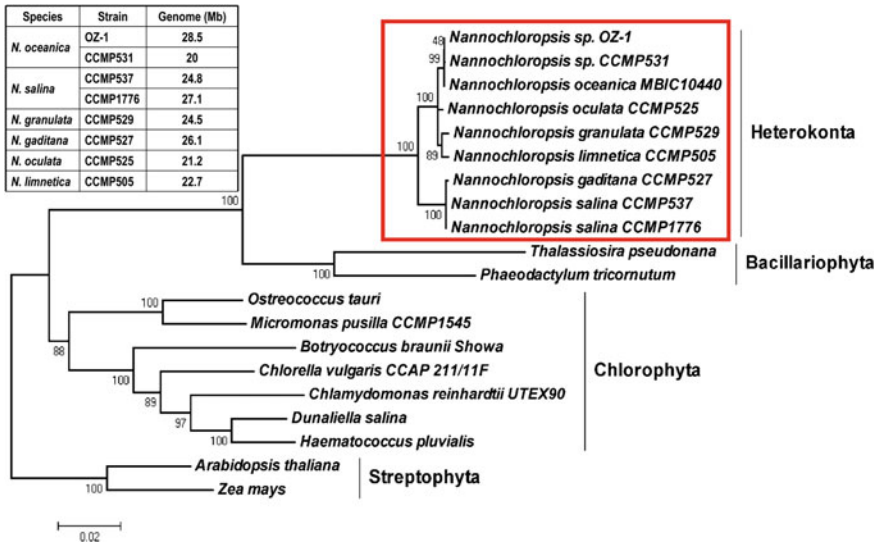

Fig. 2 The phylogenetic and genomic features of *Nannochloropsis* genus

relative compact genome structure and simple gene models with averagely fewer introns per gene (Fig. 2; unpublished data from Qingdao Institute of BioEnergy and Bioprocess Technology, Chinese Academy of Sciences). Moreover, genetic engineering has been demonstrated recently [18].

Several features distinguished *Nannochloropsis* from the existing laboratory research models of single-cell photosynthetic organisms (Table 2). The demonstrated large-scale cultivation, natural high oil productivity, eukaryotic protein-modification systems and rich metabolic capability are among the key advantages of *Nannochloropsis* when compared with most cyanobacteria. The high environmental versatility, as well as small and compact genomes, compare favorably with many other eukaryotic microalgae such as *C. reinhardtii* (genome size 120 Mb [13]) and *Dunaliella salina* (genome size over 600 Mb; unpublished data of DOE Joint Genome Institute). Furthermore, these key advantages suggest the need and the feasibility to develop *Nannochloropsis* into an platform organism for CBP-SE, where the natural or engineered genome and cell can serve as a chassis for plugging in additional functional modules, such as those product-oriented biosynthetic pathways, to enable customized production of the plethora of biofuel molecules and biochemicals directly from sunlight and CO_2 under large-scale cultivation. Under such a concept of "Synthetic *Nannochloropsis*" (Syn-Nanno), *Nannochloropsis* can potentially fill the vacancy as the "photosynthetic yeast" that supports a much wider scope of CBP-SE applications, similar to the role of *Saccharomyces cerevisia* in the sugar-based traditional fermentation industry (Fig. 3).

Table 2 Characteristics and advantages of *Nannochloropsis* as a green cell factory for CBP-SE

	Nannochloropsis spp.	*Chlamydomonas reinhardtii*	*Synechocystis* sp. PCC 6803
Genetic background	Clear Tiny genome size (20 ~ 30 Mb) Simple genome structure and organization Transformation possible [61–63]	Clear Large genome size (~ 120 Mb) [13] Complex genome structure and organization Routine genetic manipulation [64–66]	Clear Tiny genome size, (3.6 Mb) [67] Simple genome structure and organization Routine genetic manipulation [68, 69]
Growth rate	Rapid (outdoor and lab conditions)	Rapid (lab condition)	Rapid (lab condition)
Stress tolerance	Tolerates high CO_2 content [70] when fed flue gases Tolerance to variable outdoor environments	Sensitive to high CO_2 content Sensitive to variable outdoor environments	Sensitive to high CO_2 content Sensitive to variable outdoor environments
Water source	Low water-quality requirement Freshwater, seawater, and wastewater	High water-quality requirement Freshwater	Low water-quality requirement Freshwater
Biomass productivity	High [70]	Low	High
Metabolites	Enormous diversity of biosynthetic pathways [15, 17] High oil productivity Produce of high-value co-products [16]	Simple biosynthetic pathways Low oil productivity No co-products such as DHA, astaxanthin etc.	Relatively simple biosynthetic pathway Low oil productivity No co-products such as DHA, astaxanthin etc.
Post-translational modification	Yes	Yes	No
Cultivation systems	Large-scale cultivation demonstrated in variable climate and areas of China, USA, Israel	No outdoor cultivation system	Outdoor cultivation system remains to be demonstrated

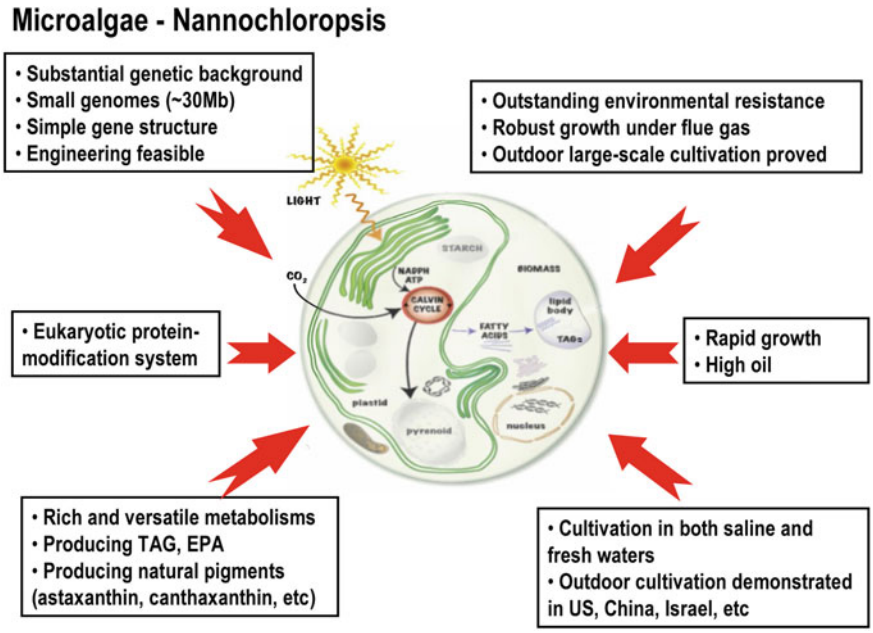

Fig. 3 The concept of Syn-Nanno for CBP-SE

5 Promises of Functional Genomics

Systems biology approaches based on functional dissection and engineering of whole cellular components, e.g. genomics, transcriptomes, proteomes and metabolomes, have ushered in the era of cellular network manipulation. For example, the second generation sequencing technologies, including 454, Solexa and SOLiD, have dramatically reduced sequencing cost while increased the data yield, both by 2–3 orders of magnitude, so that not only genomes and transcriptomes but also metagenomes can now be sampled with an unprecedented depth and accuracy. Such high throughput, genome-wide technologies, when applied to wild oleaginous microalgae and its associated ecosystems, could help understand, at single-cell, population and community levels, the integrated blueprint of genomes in structure, function and evolution, and further elucidate the cellular coordination of gene expression and the network of multiple signaling pathways, and finally identify the engineering targets for trait improvement. Experiences in addressing these fundamental questions could be gained from the other unicellular eukaryotic algae whose genomes have been unveiled, such as the green algae *C. reinhardtii* [13], *Chlorella variabilis* [19], *Micromonas* [20], *Ostreococcus tauri* and *Ostrecoccus lucimarinus* [21], the red algae *Cyanidioschyzon merola* [22], and the diatoms *Thalassiosira pseudonana* [23] and *Phaeodactylum tricornutum* [24].

(1) Functional diversity of the genes, pathways and networks underlying lipid production and related traits. In diatoms, the cells accumulate silicic acid and build

the extracellular frustule via obligate dependence upon silicon in the environment [23]. The *P. tricornutum* and *T. pseudonana* genomes allowed the identification of genes involved in silica nanofabrication in both diatoms. Moreover, variation and dynamics of gene expression in *P. tricornutum* under different Si levels unraveled the transportation and metabolism pathways of silicon as well as the associated transcriptional and post-transcriptional regulatory mechanism [25]. In oleaginous algae such as *Nannochloropsis*, the genomes are expected to reveal the diversity of the structural and regulatory genes and pathways that underlie robust lipid production. Such sequences can serve as novel phylogenetic markers or functional markers for strain-typing oleaginous microalgae, for which reliable and sensitive identification and tracking of strains have been non-trivial due to the absence of easily distinguishable morphological features in these single-cell organisms. These markers, once validated and tested over a broader range of isolates, might enable a genotyping-based strategy for strain characterization, which can be of much higher throughput than the traditional phenotyping-based selection strategies, and thus might change the paradigm of microalgal feedstock screening and selection.

(2) Evolution of functional elements underlying the key traits of oleaginous algae. In *O. tauri* and *O. lucimarinus*, chromosomes 19 and 18, respectively, were hypothesized to be exogenously acquired after the divergence of the two species because they exhibited different GC contents and distinct codon usage from the core genomes and harbored genes that were either unknown or more similar to bacteria than green algae (such as those altering the cell-surface glycosylation as a defense mechanism to the marine environment [21]). The *P. tricornutum* genome also comprised hundreds of genes horizontally transferred from bacteria [24]. These findings suggested that horizontal gene transfer contributed to the origin of microalgal genetic variation, leading to the creation of new genes and new gene functions [26]. In oleaginous microalgae, a number of important hypotheses regarding the origin and evolution of functional elements, particularly those related to the key traits, can be tested via their genome sequences, such as pan-genome or phylo-genome. For example, what are the genomic definitions of genus, species and strains? Is there a core genome shared by a group of phylogenetically closely related strains? How large are the cores? Do the cores and the accessories encode a similar set of functions or evolve in a similar manner? Are the key traits important to robust lipid production found at the core or at the accessories? Are different functions evolving at distinguishable rates or of different origin? Are the single-cell organisms such as microalgae evolving at a different rate from their multicellular relatives? Moreover, as microalgae inevitably encounter a plethora of environmental “shocks” or “stresses” during an outdoor cultivation process, it would be essential to understand the significance and nature of microevolution in genome structure and function. Key questions include the links between genotype evolution and phenotype alteration, the potential difference between coding sequence evolution versus non-coding sequence evolution, and the distinction between structural gene evolution versus regulatory gene evolution. These questions will shed light on rational strategies and practices for engineering favorable

traits or slowing down trait degeneration in microalgal feedstock development and preservation.

(3) Strategies and tools for validation of gene function. Even for the widely studied *C. reinhardtii*, nearly 40% of the protein-coding sequences on its genome remain functionally unknown. One functional characterization strategy is via the integration of the genome, transcriptome, proteome and metabolome tools. For example, almost immediately upon completion of the *O. tauri* genome, proteins were identified that play roles in key biological processes such as photosynthesis, photoperiod, flagella assembly, cellular lipid accumulation under nitrogen starvation etc. [27], and were functionally validated by gene manipulations [28]. Another approach in testing gene function builds upon small RNA profiling. In *Chlamydomonas*, small RNA interference was used to specifically knock-down the expression of major lipid droplet protein (MLDP) whose down-regulation enhanced the volume of cellular lipid-droplet under nitrogen starvation. This demonstrates the potential of genetic engineering, e.g. small RNA interference, on breeding/selection of oleaginous microalgae [27, 29, 30], even though the total lipid content of the engineered strain is still far lower than that required by large-scale biofuel production. The genetic apparatuses for small RNAs functioning are present in *Nannochloropsis*, indicating the possibility of a microRNA-based gene-specific knock-down tool to further improve key traits for biofuel production.

(4) Targets and approaches of genetic engineering. For example, in *O. tauri*, 26 selenocysteine-containing proteins were identified while only 10 were found in *Chlamydomonas*, whose genome size is almost ten times larger, suggesting the importance of micronutrients in their ecological strategies and speciation [21]. Over-represented genes involved in polyamine metabolism in *T. pseudonana* indicated significant roles of long-chain polyamines in silica nanofabrication. In oleaginous microalgae such as *Nannochloropsis*, lipid accumulation is induced by a number of stress conditions that include nitrogen depletion and exposure to high light intensity. Reconstructed gene co-expression networks and other molecular phenotype data in response to each of these environmental stimuli and their combinations should allow the intricate interactions among the different functional modules to be delineated, thus narrowing down the potential targets of genetic engineering to a prioritized list of key metabolic or regulatory junctions. Furthermore, the integrated network should allow probing the nature of regulation; for example, does the regulation occur at genome, transcriptional, post-transcriptional or post-translational levels?

6 Coupling Feedstock Development with Downstream Processing

In addition to feedstock development, several crucial research gaps must be overcome in order to achieve a full-scale operation. A comprehensive research portfolio should include: (1) a high throughput algal strain selection method and

evaluation system; (2) investigations into the mechanism and regulation of photosynthesis, CO_2 capture, and lipid synthesis; (3) design of advanced photobioreactors; (4) process optimization and mass scale cultivation system; (5) selection of cost-effective technologies for biomass harvesting and drying; (6) integrative exploitation of the components after lipid extraction; (7) an economic and environmental evaluation; and (8) a technical route and research platform for highly efficient and cost-effective commercialization of microalgal biofuel production [2].

Targeting these crucial bottlenecks, the Chinese government has provided significant funding support to encourage multi-disciplinary approaches for research assessment of the complete algal biofuel production chain. In 2009, the National High-Technology Development Program of China ("863" Program) sponsored the "CO_2-Microalgae-Fuels" project, in which existing and new technologies are being tested to assess the potential of using lipid-producing microalgae for industrial-scale fixation of CO_2. In late 2010, the National Science and Technology Support Program of China supported the "Research and demonstration of key technologies in feedstock development and low-cost cultivation of energy algae". In 2011, the National Basic Research Development Program of China ("973" Program) funded the "Scientific foundation for mass production of microalgal energy", a project in which a large research consortium consisting of over a dozen universities, research institutes and commercial entities was founded for collaboration in examining the scientific basis of large-scale energy production from microalgae. Meanwhile, the "Solar Energy Action Plan", from the Chinese Academy of Sciences (CAS), has funded the development of production systems for microalgal biodiesel and microalgal alkenes. In addition, China Petrochemical Corporation (Sinopec) and CAS have formed a joint venture to develop the key enabling technologies for large-scale production of microalgal biodiesel.

These past public and industrial funding programs in China have reported some initial successes, such as promising lipid production strains [31, 32], microalgal autotrophic culture optimization [33–45], open production systems (raceway ponds; [46]), fermentation-based microalgal biodiesel production [47–53] and new photobioreactor designs [54–59]. Moreover, the use of waste CO_2 from power plants to enhance production has been shown to be technically feasible, and hence may be deployed to reduce production costs and for greenhouse gas (GHG) emission control [60]. Furthermore, concurrent extraction of valuable co-products (e.g., β-carotene, PUFA, biofertilizers, among others) with biofuel production has significant potential to narrow the gap between microalgal oil cost and fossil fuel price [2].

7 Conclusions and Perspectives

In summary, the prerequisite of productive feedstock development for microalgal biofuels is to select and establish the research model system on functional genomics, so as to better understand the regulatory network under a series of

complex traits such as energy capture and conversion, environmental stress adaptation and product profiles. Those systems that demonstrate not only promising phenotypes but superior features as a genomics research model, such as certain *Nannochloropsis*, *Chlorella* and *Phaeodactylum* strains, are emerging as promising research models for the functional genomics of oleaginous microalgae. Due to their rich genomic resources, compact genomes, relatively simple gene structures, wide ecological adaptation, and rich collections of natural strains, together with their demonstrated capability for outdoor open-pond photosynthetic cultivation, organisms such as *Nannochloropsis* spp. and *Chlorella* spp. can serve as research models and production strains for robust, scalable and economical production of fuels and chemicals. Integrated technology development that supports the complete chain of microalgal production systems, including microalgae phylogeny, physiology, genetics, systems biology, metabolic engineering, pathogenesis, field cultivation, bioprocess engineering, biorefinery, and techno-economical analysis, should enable concrete progresses towards the era of photosynthetic biofuels.

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References

1. NBSC (2010) Statistic yearbook of China (National Bureau of Statistics of China). http://wwwstatsgovcn/tjsj/ndsj/2010/indexchhtmBeijing China
2. Li YG, Xu L, Huang YM, Wang F, Guo C, et al (2011) Microalgal biodiesel in China: opportunities and challenges. Appl Ener
3. Wijffels RH, Barbosa MJ (2010) An outlook on microalgal biofuels. Science 329:796
4. Hu Q, Sommerfeld M, Jarvis E, Ghirardi M, Posewitz M et al (2008) Microalgal triacylglycerols as feedstocks for biofuel production: perspectives and advances. Plant J 54:621–639
5. Chisti Y (2007) Biodiesel from microalgae. Biotechnol Adv 25:294–306
6. Sheehan J (1998) A look back at the US Department of Energy's aquatic species program: biodiesel from algae: National Renewable Energy Laboratory Golden, CO
7. Dunahay TG, Jarvis EE, Dais SS, Roessler PG (1996) Manipulation of microalgal lipid production using genetic engineering. Appl Biochem Biotechnol 57:223–231
8. Dunahay TG, Jarvis EE, Roessler PG (1995) Genetic transformation of the diatoms *Cyclotella cryptica* and *Navicula saprophila*. J Phycol 31:1004–1012
9. Roessler PG, Bleibaum JL, Thompson GA, Ohlrogge JB (1994) Characteristics of the gene that encodes acetyl CoA carboxylase in the diatom *Cyclotella crypticaa*. Ann NY Acad Sci 721:250–256
10. Roessler P, Ohlrogge J (1993) Cloning and characterization of the gene that encodes acetyl-coenzyme A carboxylase in the alga *Cyclotella cryptica*. J Biol Chem 268:19254
11. Li Y, Han D, Hu G, Dauvillee D, Sommerfeld M et al (2010) *Chlamydomonas* starchless mutant defective in ADP-glucose pyrophosphorylase hyper-accumulates triacylglycerol. Metab Eng 12:387–391
12. Leon-Banares R, Gonzalez-Ballester D, Galvan A, Fernandez E (2004) Transgenic microalgae as green cell-factories. Trends Biotechnol 22:45–52

13. Merchant SS, Prochnik SE, Vallon O, Harris EH, Karpowicz SJ et al (2007) The *Chlamydomonas* genome reveals the evolution of key animal and plant functions. Science 318:245
14. Rodolfi L, Chini Zittelli G, Bassi N, Padovani G, Biondi N et al (2009) Microalgae for oil: strain selection, induction of lipid synthesis and outdoor mass cultivation in a low-cost photobioreactor. Biotechnol Bioeng 102:100–112
15. Rebolloso-Fuentes M, Navarro-Perez A, Garcia-Camacho F, Ramos-Miras J, Guil-Guerrero J (2001) Biomass nutrient profiles of the microalga *Nannochloropsis*. J Agric Food Chem 49:2966–2972
16. Lubián LM, Montero O, Moreno-Garrido I, Huertas IE, Sobrino C et al (2000) *Nannochloropsis* (Eustigmatophyceae) as source of commercially valuable pigments. J Appl Phycol 12:249–255
17. Karlson B, Potter D, Kuylenstierna M, Andersen R (1996) Ultrastructure, pigment composition, and 18S rRNA gene sequence for *Nannochloropsis granulata* sp nov. (Monodopsidaceae, Eustigmatophyceae), a marine ultraplankter isolated from the Skagerrak, northeast Atlantic Ocean. Phycologia 35:253–260
18. Li SS, Tsai HJ (2009) Transgenic microalgae as a non-antibiotic bactericide producer to defend against bacterial pathogen infection in the fish digestive tract. Fish Shellfish Immunol 26:316–325
19. Blanc G, Duncan G, Agarkova I, Borodovsky M, Gurnon J et al (2010) The *Chlorella variabilis* NC64A genome reveals adaptation to photosymbiosis, coevolution with viruses, and cryptic sex. Plant Cell Online 22:2943
20. Worden AZ, Lee JH, Mock T, Rouzé P, Simmons MP et al (2009) Green evolution and dynamic adaptations revealed by genomes of the marine picoeukaryotes *Micromonas*. Science 324:268
21. Palenik B, Grimwood J, Aerts A, Rouzé P, Salamov A et al (2007) The tiny eukaryote *Ostreococcus* provides genomic insights into the paradox of plankton speciation. Proc Natl Acad Sci 104:7705
22. Nozaki H, Takano H, Misumi O, Terasawa K, Matsuzaki M et al (2007) A 100%-complete sequence reveals unusually simple genomic features in the hot-spring red alga *Cyanidioschyzon merolae*. BMC Biol 5:28
23. Armbrust E, Berges JA, Bowler C, Green BR, Martinez D et al (2004) The genome of the diatom *Thalassiosira pseudonana*: ecology, evolution, and metabolism. Science 306:79
24. Bowler C, Allen AE, Badger JH, Grimwood J, Jabbari K et al (2008) The *Phaeodactylum* genome reveals the evolutionary history of diatom genomes. Nature 456:23–244
25. Sapriel G, Quinet M, Heijde M, Jourdren L, Tanty V et al (2009) Genome-wide transcriptome analyses of silicon metabolism in *Phaeodactylum tricornutum* reveal the multilevel regulation of silicic acid transporters. PLoS One 4:e7458
26. Xiong AS, Peng RH, Zhuang J, Gao F, Zhu B et al (2009) Gene duplication, transfer, and evolution in the chloroplast genome. Biotechnol Adv 27:340–347
27. Moellering ER, Benning C (2010) RNA interference silencing of a major lipid droplet protein affects lipid droplet size in *Chlamydomonas reinhardtii*. Eukaryotic cell 9:97
28. Corellou F, Schwartz C, Motta JP, Djouani-Tahri EB, Sanchez F et al (2009) Clocks in the green lineage: comparative functional analysis of the circadian architecture of the picoeukaryote *Ostreococcus*. Plant Cell Online 21:3436
29. Molnár A, Schwach F, Studholme DJ, Thuenemann EC, Baulcombe DC (2007) miRNAs control gene expression in the single-cell alga *Chlamydomonas reinhardtii*. Nat 447:1126–1129
30. Zhao T, Li G, Mi S, Li S, Hannon GJ et al (2007) A complex system of small RNAs in the unicellular green alga *Chlamydomonas reinhardtii*. Genes Dev 21:1190
31. Yang BJ, Zheng L, Chen JH, Zang JY, Wang XR et al (2009) Cluster analysis on fatty acid composition of *Enteromorpha prolifera* off northern China coast. Oceanol Limnol Sin 40:627–632
32. Hu Z, An M, Duan S, Xu N, Sun K et al (2009) Effects of nitrogen sources on the growth, contents of total lipids and total hydrocarbons of *Botryococcus braunii*. Acta Ecol Sin 29:3288–3294

33. Li Y, Huang J, Sandmann G, Chen F (2008) Glucose sensing and the mitochondrial alternative pathway are involved in the regulation of astaxanthin biosynthesis in the dark-grown *Chlorella zofingiensis* (Chlorophyceae). Planta 228:735–743
34. Huang J, Liu J, Li Y, Chen F (2008) Isolation and characterization of the phytoene desaturase gene as a potential selective marker for genetic engineering of the astaxanthin producing green alga *Chlorella zofingiensis* (Chlorophyta). J Phycol 44:684–690
35. Huang JC, Chen F, Sandmann G (2006) Stress-related differential expression of multiple [beta]-carotene ketolase genes in the unicellular green alga *Haematococcus pluvialis*. J Biotechnol 122:176–185
36. Huang JC, Wang Y, Sandmann G, Chen F (2006) Isolation and characterization of a carotenoid oxygenase gene from *Chlorella zofingiensis* (Chlorophyta). Appl Microbiol Biotechnol 71:473–479
37. Liu J, Zhong Y, Sun Z, Huang J, Sandmann G et al (2010) One amino acid substitution in phytoene desaturase makes *Chlorella zofingiensis* resistant to norflurazon and enhances the biosynthesis of astaxanthin. Planta 232:61–67
38. Li Y, Huang J, Sandmann G, Chen F (2009) High light and sodium chloride stress differentially regulate the biosynthesis of astaxanthin in *Chlorella zofingiensis* (Chlorophyceae). J Phycol 45:635–641
39. Chen T, Wei D, Chen G, Wang Y, Chen F (2009) Employment of organic acids to enhance astaxanthin formation in heterotrophic *Chlorella zofingiensis*. J Food Process Preserv 33:271–284
40. Wu Z, Chen G, Chong S, Mak N, Chen F, et al. (2010) Ultraviolet-B radiation improves astaxanthin accumulation in green microalga *Haematococcus pluvialis*. Biotechnol Lett: 1–4
41. Liu J, Huang J, Sun Z, Zhong Y, Jiang Y et al (2011) Differential lipid and fatty acid profiles of photoautotrophic and heterotrophic *Chlorella zofingiensis*: assessment of algal oils for biodiesel production. Bioresour Technol 102:106–110
42. Zhang X, Pan L, Wei X, Gao H, Liu J (2007) Impact of astaxanthin-enriched algal powder of *Haematococcus pluvialis* on memory improvement in BALB/c mice. Environ Geochem Health 29:483–489
43. Liu J, Zhang X, Sun Y, Lin W (2010) Antioxidative capacity and enzyme activity in *Haematococcus pluvialis* cells exposed to superoxide free radicals. Chin J Oceanol Limnol 28:1–9
44. Qin S, Liu GX, Hu ZY (2008) The accumulation and metabolism of astaxanthin in *Scenedesmus obliquus* (Chlorophyceae). Process Biochem 43:795–802
45. Lu Y, Jiang P, Liu S, Gan Q, Cui H et al (2010) Methyl jasmonate-or gibberellins A3-induced astaxanthin accumulation is associated with up-regulation of transcription of [beta]-carotene ketolase genes (bkts) in microalga *Haematococcus pluvialis*. Bioresour Technol 101: 6468–6474
46. Deng Z, Yan C, Lu F, Hu Q, Hu Z (2008) Growth kinetics of 1–2 mm and 3–4 mm colonies of Nostoc spheroides (Cyanophyta) in outdoor culture. Biotechnol Lett 30:1741–1746
47. Xu H, Miao X, Wu Q (2006) High quality biodiesel production from a microalga *Chlorella protothecoides* by heterotrophic growth in fermenters. J Biotechnol 126:499–507
48. Miao X, Wu Q (2006) Biodiesel production from heterotrophic microalgal oil. Bioresour Technol 97:841–846
49. Xiong W, Li X, Xiang J, Wu Q (2008) High-density fermentation of microalga *Chlorella protothecoides* in bioreactor for microbio-diesel production. Appl Microbiol Biotechnol 78:29–36
50. Li X, Xu H, Wu Q (2007) Large scale biodiesel production from microalga *Chlorella protothecoides* through heterotrophic cultivation in bioreactors. Biotechnol Bioeng 98: 764–771
51. Gao C, Zhai Y, Ding Y, Wu Q (2010) Application of sweet sorghum for biodiesel production by heterotrophic microalga *Chlorella protothecoides*. Appl Energy 87:756–761
52. Wen ZY, Chen F (2003) Heterotrophic production of eicosapentaenoic acid by microalgae. Biotechnol Adv 21:273–294

53. Wei D, Liu L (2008) Optimization of culture medium for heterotrophic *Chlorella protothecoides* producing total fatty acids. Chem Bioeng 3 25(3):35–40
54. Xie J, Zhang Y, Li Y, Wang Y (2001) Mixotrophic cultivation of Platymonas subcordiformis. J Appl Phycol 13:343–347
55. Wang Y, Li Y, Shi D, Shen G, Ru B et al (2002) Characteristics of mixotrophic growth of *Synechocystis* sp in an enclosed photobioreactor. Biotechnol Lett 24:1593–1597
56. Yu G, Li Y, Shen G, Wang W, Lin C et al (2009) A novel method using CFD to optimize the inner structure parameters of flat photobioreactors. J Appl Phycol 21:719–727
57. Xu L, Weathers PJ, Xiong XR, Liu CZ (2009) Microalgal bioreactors: challenges and opportunities. Eng Life Sci 9:178–189
58. Su Z, Kang R, Shi S, Cong W, Cai Z (2008) An economical device for carbon supplement in large-scale micro-algae production. Bioprocess Biosyst Eng 31:641–645
59. Su Z, Kang R, Shi S, Cong W, Cai Z (2010) An effective gas-liquid transmission device in the enclosed microalgae cultivation. Appl Biochem Biotechnol 160:428–437
60. Wang B, Li Y, Wu N, Lan CQ (2008) CO_2 bio-mitigation using microalgae. Appl Microbiol Biotechnol 79:707–718
61. Vick B, Kilian O (2009) VCP-based vectors for algal cell transformation. US Patent App. 20,090/317,904
62. Cha TS, Chen CF, Yee W, Aziz A, Loh SH (2011) Cinnamic acid, coumarin and vanillin: Alternative phenolic compounds for efficient Agrobacterium-mediated transformation of the unicellular green alga, *Nannochloropsis* sp. J Microbiol Methods 84:430–434
63. Chen HL, Li SS, Huang R, Tsai HJ (2008) Conditional production of a functional fish growth hormone in the transgenic line of *Nannochloropsis oculata* (Eustigmatophyceae). J Phycol 44:768–776
64. Boynton JE, Gillham NW, Harris EH, Hosler JP, Johnson AM et al (1988) Chloroplast transformation in *Chlamydomonas* with high velocity microprojectiles. Science 240:1534
65. Blowers AD, Bogorad L, Shark KB, Sanford JC (1989) Studies on *Chlamydomonas* chloroplast transformation: foreign DNA can be stably maintained in the chromosome. Plant Cell Online 1:123
66. Shimogawara K, Fujiwara S, Grossman A, Usuda H (1998) High-efficiency transformation of *Chlamydomonas reinhardtii* by electroporation. Genetics 148:1821
67. Nakao M, Okamoto S, Kohara M, Fujishiro T, Fujisawa T et al (2010) CyanoBase: the cyanobacteria genome database update. Nucleic Acids Res 38:D379
68. Porter RD (1986) Transformation in cyanobacteria. Crit Rev Microbiol 13:111
69. Vioque A (2007) Transformation of cyanobacteria. In: León R, Gaván A, Fernández E, (eds) Transgenic Microalgae as Green Cell Factories. Springer pp 12–22
70. Tredici MR (2008) Microalgae biofuels: Potential and limitations. Microalgae Biomass Summit Seattle, Wash: Algal Biomass Organization 815–820

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Biobutanol

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Abstract China initiated its acetone–butanol–ethanol (ABE) industry in the 1950s; it peaked in the 1980s, and ended at the end of the last century owing to the development of more competitive petrochemical pathways. However, driven by the high price of crude oil and environmental concerns raised by the over-consumption of petrochemical products, biofuels and bio-based chemicals including butanol have garnered global attention again. Currently, butanol produced from ABE fermentation is mainly used as an industrial solvent or a platform chemical for several bulk derivatives, and is also believed to be a potential biofuel. A number of plants have been built or rebuilt in recent years in China for butanol production with the ABE process. Chinese researchers also show great interest in the improvement of the production strains and corresponding processes. They have applied conventional mutagenesis methods to improve butanol-producing strains such as the *Clostridium acetobutylicum* mutant strains EA2018 (butanol ratio of 70%) and Rh8 (butanol tolerance of 19 g/L). The omics technologies, such as genome sequencing, proteomic and transcriptomic analysis, have been adapted to elucidate the characteristics of different butanol-producing bacteria. Based on the group II intron method, the genetic manipulation system of *C. acetobutylicum* was greatly improved, and some successful engineering strains were developed. In addition, research in China also covers the downstream processes. This article reviews up-to-date progress on biobutanol production in China.

Keywords Acetone–butanol–ethanol industry · Biobutanol · China · *Clostridium acetobutylicum* · Genomics · Strain engineering

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Contents

1 Introduction

ABE fermentation means the production of acetone, butanol, and ethanol via fermentation, usually using the clostridial strains. It was initiated in the early part of the twentieth century in the UK, and achieved its peak in the 1950s. The driving forces for ABE fermentation development were different in different historical periods, including the artificial synthesis of rubber at the beginning of the twentieth century, the manufacture of cordite in World Wars I and II, and the development of nitrocellulose lacquer for the automobile industry after World War I. Along with the rapid development of the petroleum industry, ABE fermentation lost its economic competitiveness for producing acetone or butanol, which resulted in a dramatic decline in ABE fermentation in the last half of the twentieth century [26]. In China, the ABE industry was initiated in 1950s and reached its peak in the 1980s. All ABE plants in China were closed at the end of the last century owing to the severe competitive impact of petro-based butanol [38].

The oil crisis in the 1970s warned that humanity's dependence on oil was not sustainable and that industrial processes based on renewable resources must be developed. The current serious shortage of oil re-stimulates governments and scientists to find substitutes for petrochemical products from renewable biomass. Butanol (butyl alcohol or 1-butanol, C_4H_9OH, MW 74.12), is an important chemical and solvent. It is mainly used to synthesize butyl acrylate and methacrylate esters for latex surface coating, enamels and lacquers, and butyl glycol ether, butyl acetate and plasticizers. Additionally, butanol can be used directly as the diluent for brake fluid formulations, and as solvent for the production of hormones, vitamins, and antibiotics. Although ethanol has been extensively recognized as the typical biofuel, butanol, as an alternative biofuel, has several significant advantages over ethanol, such as higher energy content, lower water absorption,

better blending ability with gasoline, and direct use in conventional combustion engines without modification [29].

Recently, a number of plants have been built or rebuilt in China to restart ABE production with an expected production capacity of 1,000,000 tons of solvents per year, and were summarized in Ni's review [38]. Meanwhile, biobutanol research was restarted using clostridial strains from public collection centers or industrial strains. ABE research in China has been supported by the National High Technology Research and Development Program of China (863 Project, No.2006AA02Z237, 4.8 million RMB) and the National Natural Science Foundation of China (NSFC, over 2.1 million RMB). In China's Twelfth Five Year Plan (2011–2015), over 22 million RMB will be used to support biobutanol research. Much progress on biobutanol R&D involved in genomics, proteomics, metabolic engineering, and genetic manipulation tools has been published. The main results of biobutanol research in China from published articles will be reviewed in this chapter.

2 Advances in Butanol-Producing Microorganisms

2.1 Strains Used for Biobutanol Industry and Research

The strains for industrial ABE fermentation are all clostridial bacteria, including *Clostridium acetobutylicum*, *C. beijerinckii*, *C. saccharobutylicum*, and *C. saccharoperbutylacetonicum* [25]. The *Clostridium* strains used in Chinese solvent plants were isolated and improved locally by individual plant and research institutes, such as *C. acetobutylicum* No. 2 with excellent phage-resistant properties from the Shanghai Solvent Plant [18]. The main Chinese research groups and their operating strains are summarized in Fig. 1.

The Shanghai Cooperative Bio-butanol Group (SCBG) (Shanghai Institute of Plant Physiology and Ecology, Chinese Academy of Sciences) mainly focus on *C. acetobutylicum* EA2018 [53, 54], which was developed by chemical mutagenesis breeding of a soil-screened wild-type *C. acetobutylicum.* This strain could produce a higher ratio of butanol in solvents (70%), and has been licensed to several commercial producers in China. Li Yin's group (Institute of Microbiology, Chinese Academy of Sciences) takes *C. acetobutylicum* DSM1731 [3] as the main research material, and focuses on strain improvement by metabolic engineering to enhance solvent production, improve butanol yield, and increase the microbial tolerance against solvents. Another research group led by Prof. Li Fuli (Qingdao Institute of Bioenergy and Bioprocess Technology, Chinese Academy of Sciences) uses *C. beijerinckii* ATCC55025 as the research strain, which is an asporogenic mutant of *C. beijerinckii* ATCC4259 and can use wheat bran for ABE fermentation [43]. There are also some other groups focused on biobutanol production from various carbon resources.

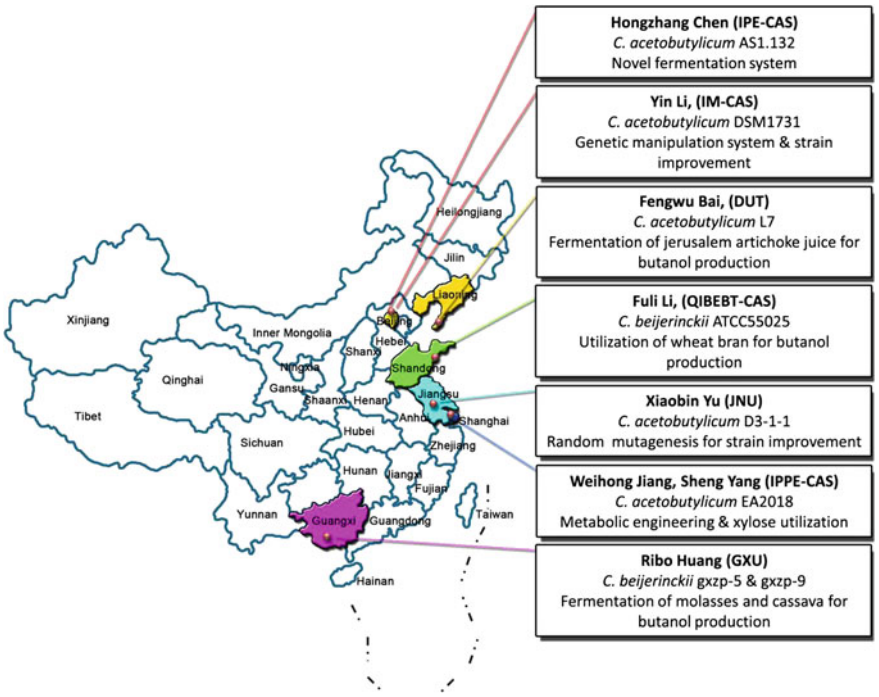

Fig. 1 The main research activities on biobutanol in China. This figure was summarized from published articles. IPE-CAS: Institute of Process Engineering, Chinese Academy of Sciences, Beijing. IM-CAS: Institute of Microbiology, Chinese Academy of Sciences, Beijing. DUT: Dalian University of Technology, Dalian, Liaoning Province. QIBEBT-CAS: Qingdao Institute of Bioenergy and Bioprocess Technology, Chinese Academy of Sciences, Qingdao, Shandong Province. JNU: Jiangnan University, Wuxi, Jiangsu Province. IPPE-CAS: Shanghai Institute of Plant Physiology and Ecology, Chinese Academy of Sciences, Shanghai. GXU: Guangxi University, Nanning, Guangxi Province

2.2 Genetic Manipulation Methods

For genetic manipulation of *C. acetobutylicum*, gene knockout/down methods have been well developed, such as the homologous recombination method based on replicable/non-replicable vectors [14, 17, 18, 46], and antisense RNA (asRNA) strategies [8, 48]. From 2007, Chinese researchers started to contribute to this field, based on the Group II intron system.

2.2.1 Gene Disruption Using Group II Intron

In 2001, a novel method of gene inactivation based on a group II intron was adapted for some bacteria [27]. The system was derived from *Lactococcus lactis* Ll.LtrB group II intron. The principle of retrohoming is that the group II intron,

which self-excised from the RNA, can form a ribonucleoprotein complex with a conserved IEP (intron-encoded protein), which can recognize the DNA target site and make an invasion event [36]. Further research revealed that the target-site recognition rules were determined by two sites located on the intron RNA (EBS1d and EBS2). Therefore, the modification of the two sites according to a statistical mathematical model will lead to artificial insertion of the intron into target sites. A commercial gene-knockout system, Targetron (Sigma-Aldrich, St. Louis, MO, USA), has been developed based on these rules. Minton's group in the University of Nottingham, UK [19] and Jiang's group (Shanghai Institute of Plant Physiology and Ecology, Chinese Academy of Sciences) [45] have almost simultaneously reported the application of such a gene-knockout system in *C. acetobutylicum*. Following the establishment of this method, the genetic manipulation of *C. acetobutylicum* has become feasible in many laboratories. Green [13] believes that this method based on the group II intron system for gene knockout of *C. acetobutylicum* has made significant advances and that it is now possible to construct multi-step biosynthetic pathways paving the way for new synthetic clostridia.

2.2.2 Intron-Anchored Targeted Gene Deletion in *C. acetobutylicum*

In 2011, Li's group in the Institute of Microbiology, CAS, proposed an intron-anchored gene deletion approach for *C. acetobutylicum*, which combines the advantage of the group II intron "ClosTron" system and homologous recombination [23]. In this approach, an allele homologous to the upstream or downstream of the intron target site was constructed into the intron. Upon introducing this construct into the target microorganism, an intron retrotransposition might occur initially. The deletion of the target genes could then be achieved by homologous recombination. Using this method, the *ctfA/B* and CAC1493/1494 operons were successfully deleted. To date, the group II intron system has been used to inactivate genes in at least ten different bacterial species [44], for most of which targeted gene deletion is impossible. Therefore, the approach developed in this study has the potential to be applied for gene deletion in those species where first-step insertion via intron retrotransposition has been established.

2.2.3 Engineering *C. acetobutylicum* to Accept Unmethylated DNA

It is difficult to genetically manipulate the important genus *Clostridium*, due to the existence of the restriction and modification (RM) systems. Most RM systems comprise a DNA methyltransferase (MTase) and a restriction endonuclease (REase). The MTase enables recognition of 'self' DNA by methylation of specific nucleotides within particular DNA sequences, whereas the REase enzymatically cleaves the 'foreign' unmodified DNA. Li's Group in the Institute of Microbiology, CAS, reported the construction of a CAC1502 disrupted mutant

C. acetobutylicum SMB009 [10], using the ClosTron system based on group II intron insertion. The resulting strain SMB009 lost the type II restriction endonuclease activity, and can be transformed with unmethylated DNA as efficiently as with methylated DNA.

In previous studies, the methylation step is inevitable for transforming foreign DNA into *C. acetobutylicum* because of the presence of a type II restriction endonuclease Cac824I [35]. The strategy developed by Dong et al. [10] makes it easier to genetically modify the clostridial species using unmethylated DNA, and improves the manipulation efficiency significantly. This will help to advance the understanding of the clostridial physiology from the molecular level, and to expand the application of *Clostridium* strains for medical and biotechnological purposes.

2.2.4 Electrotransformation of *C. acetobutylicum* in Air

Clostridia are typically strict anaerobes. The manipulation of *C. acetobutylicum* is usually performed in an anaerobic chamber, and is thus laborious and time-consuming. Dong et al. reported work to demonstrate that electrotransformation of *C. acetobutylicum* in air is feasible [9]. The CAC2634 gene encoding PerR is a known peroxide regulon repressor. They disrupted CAC2634 in their previously constructed RM system-deficient *C. acetobutylicum* mutant SMB009 using the group II method. The resulted mutant SMB012 was proved to be electrotransformable in air with an efficiency of 1.2–3.1 $\times$ 10^3 transformants/μg DNA. The electrotransformation process of *C. acetobutylicum* could be significantly simplified, especially when operating multiple electrotransformations.

2.3 Strain Improvement

2.3.1 Metabolic Engineering

Although some microorganisms such as *Escherichia coli*, yeast, *Lactobacillus brevis*, and *Cyanobacteria* have been engineered to produce biobutanol, clostridial strains are still the main butanol-forming strains, due to their excellent fermentation characteristics. Butanol constitutes 60–70% (w/w) of the total ABE solvents [26, 53, 54], and eliminating the production of acetone or ethanol was expected to increase butanol ratio. Jiang et al. reported their work on weakening the acetone pathway to increase the butanol ratio [24]. Acetoacetate decarboxylase (AADC) encoded by the *adc* gene is one of the key enzymes to form acetone. They disrupted the *adc* gene using TargeTron technology in *C. acetobutylicum* EA2018 [45]. The *adc*-disrupted mutant strain 2018adc showed an increased butanol ratio from 70 to 80.05%, with an decreased acetone titer of 0.21 g/L. However, the growth of 2018adc is inhibited and the titers of ethanol and butanol are reduced to

half those of its control strain 2018p (EA2018 bearing pIMP1-Pptb vector) in P2 medium. In addition, the authors found that regulating the culture pH by adding calcium carbonate can increase the butanol titer of strain 2018adc. Regulating the in-vivo electron flow in this strain by adding methyl viologen could also increase the yield of butanol from 57 to 70.8%. This work is believed to the first example of a TargeTron-based knockout mutant of *C. acetobutylicum* defective in central fermentative metabolism [28].

2.3.2 Engineering the Robustness of *C. acetobutylicum*

To improve the aero- and solvent tolerance of *C. acetobutylicum*, Zhu et al. cloned and over-expressed the *gshAB* genes from *E. coli* into *C. acetobutylicum* DSM1731 to biosynthesize glutathione (GSH), and thus increased the robustness of *C. acetobutylicum* and achieved better solvent production [22]. The *gshA* gene encodes γ-glutamylcysteine synthetase (γ-GCS) and the *gshB* gene encodes glutathione synthetase (GS) in *E. coli*, via which glutathione is synthesized. Zhu et al. constructed plasmids pITA, pITB, and pITAB on the base of parent plasmid pITF [10] so as to introduce *gshA*, *gshB*, and *gshAB* into *C. acetobutylicum* DSM1731, respectively. Biosynthesis of GSH in *C. acetobutylicum* DSM1731 increased the aerotolerance and butanol-producing capability, and decreased the growth inhibition of butanol. Strain DSM1731(pITAB) produced 19.7 g/L total solvents, including 14.8 g/L butanol, 3.7 g/L acetone, and 1.2 g/L ethanol in 64 h of fermentation. Production of butanol by DSM1731_pITAB increased by 66 and 37% when compared to the control strain DSM1731_pITB and wild-type strain (DSM1731), respectively. It was postulated that GSH might be involved in scavenging reactive oxygen species (ROS) and maintenance of redox balance in cells with concomitant improvement in aerotolerance. Furthermore, scavenging ROS helps cells resist solvent, acid and osmotic pressure, thus increase the butanol tolerance, which resulted in increased butanol production. This study showed for the first time that GSH biosynthesis in *C. acetobutylicum* could increase the production of butanol and improve the butanol tolerance. The results demonstrated that introducing this metabolic redundancy could improve the robustness of the host to achieve better solvent production, which is one of the most important physiological functionalities for efficient production of fuels and chemicals [55]. Therefore, strategies of exploiting and engineering functional metabolic redundancy may also help microbes to better adapt themselves to the industrial environments.

2.3.3 Improvement of Xylose Utilization

For converting lignocellulosic hydrolysate into biofuels, it is important for *C. acetobutylicum* to utilize a variety of carbohydrates, including pentose and hexose. Lignocellulose is the most abundant renewable resource, and D-xylose is a significant component of the hydrolysate [20, 58]. Making good use of

lignocelluloses can reduce the costs of substrates. WhenD-glucose and D-xylose are simultaneously present in the medium, utilization of D-xylose is severely inhibited byD-glucose [40], a phenomenon called "carbon catabolite repression" (CCR) [37]. To enhance efficient utilization of lignocellulosic hydrolysates for ABE production, Ren et al. [43] attempted to reduce or eliminate CCR in *C. acetobutylicum*. The gene CAC3037 in *C. acetobutylicum* ATCC824 was firstly confirmed as the *ccpA* gene encoding CcpA by the complementary investigation in a mutant of *B. subtilis*. Then CAC3037 was disrupted using the group II intron method [45], generating mutant strain 824ccpA. Under pH-controlled conditions, 824ccpA can use a mixture of D-xylose andD-glucose simultaneously without CCR. When the ratio ofD-glucose to D-xylose was 1:1, the strain of 824ccpA showed the maximal solvent titers (acetone, butanol and ethanol are 4.94, 12.05 and 1.04 g/L, respectively), which was almost the same solvent levels as with maize- or molasses-based fermentation by wild-type. Disruption of *ccpA* offers a successful genetic modification strategy for simultaneously utilizing the sugars in lignocellulosic materials by *Clostridium*, which is essential for further exploitation of lignocellulose for the production of solvents and biofuels. In addition, the gene *talA*, which encodes transaldolase in *E. coli* K-12, was cloned and overexpressed in *C. acetobutylicum* ATCC824 [16]. The resulting strain 824-TAL showed improved ability for xylose utilization and solvent production using xylose as the sole carbon source compared with *C. acetobutylicum* ATCC824. It suggested that transaldolase is weak in the pentose phosphate pathway (PPP) of *C. acetobutylicum*. The xylose utilization pathway and regulons in *C. acetobutylicum* have been well studied by Gu et al. via a comparative genomic approach, providing comprehensive insights into xylose catabolism and its regulation [15].

Besides the engineering work by Chinese researchers, scientists in other countries have also reported the metabolic engineering of other key metabolic pathway genes involved in solvent production, such as *adc*, *ctfAB*, *adhE*, *pta*, *buk*, and *ctfB*, transcriptional regulator *solR* and *spo0A*, genes for molecular pumps, and chaperones such as *groES*, *dnaKJ*, *hsp18*, and *hsp90* [29]. These studies help understanding of the physiology and metabolism of *C. acetobutylicum*, based on which Chinese scientists are attempting further metabolic engineering. Chinese researchers are also trying to solve some key problems in butanol production, including (1) improving the capability to utilize a variety of low-price and non-cereal substrates by genetic modification and adaptation; (2) eliminating byproducts so as to obtain the sole butanol-producing strain by metabolic engineering; (3) improving butanol tolerance by genome-scale evolution, and (4) increasing productivity by physiological functionality engineering.

2.4 *New Insights into Genome-Scale Analysis of Physiology of* C. acetobutylicum *and Solvent Production Using Omics Technologies*

Omics technologies acting as system biotechnologies have been widely developed in recent years. Omics study can help understand organisms at the global level. Many articles have been published on genomics, transcriptomics, proteomics and metabolomics in *C. acetobutylicum* [1, 2, 31, 39, 47]. After the announcement of the genome of the typical strain *C. acetobutylicum* ATCC824 in 2001 by American researchers, the genome sequences of two other *C. acetobutylicum* strains (EA2018 and DSM1731) were also published recently.

C. acetobutylicum EA2018 is a high-butanol-producing, non-spore-forming strain, generated by N-methyl-N-nitro-N-nitrosoguanidine (NTG) treatment [53, 54]. The butanol ratio and starch conversion rate of the EA2018 strain were 10 and 5% higher, respectively, than the ATCC824 strain [7]. The size of the EA2018 chromosome is 650 bp smaller than that of the typical strain ATCC824, and the size of the EA2018 megaplasmid is 4 bp smaller than that of ATCC824; in addition, a total of 46 deletion sites and 26 insertion sites were found across the EA2018 genome, including one deletion site in the megaplasmid [22]. EA2018 was found to be 99.8% identical to ATCC824, with 72 insertion/deletionss and 451 single nucleotide variations (SNVs), some of which may be related to the hyper-butanol-producing characteristics of EA2018. Further comparative analysis on transcriptomic profiling of gene expression in EA2018 and ATCC824 revealed increased expression levels of several key genes, such as *spo0A* and *adhEII*. In addition, the variation in CEA_G2622 (CAC2613 in ATCC824), a putative transcriptional regulator involved in xylose utilization, may accelerate the utilization of substrate xylose. This information should be valuable for further genetic modification of *C. acetobutylicum* to improve butanol production.

Butanol toxicity is one of the most critical factors affecting ABE fermentation, and can disrupt the phospholipid components of the cell membrane causing an increase in membrane fluidity [49]. It was also found that the cell growth, nutrient transport and rate of sugar uptake were inhibited and the membrane-bound ATPase activity was negatively affected by butanol [4, 49]. Fermentations of *C. acetobutylicum* therefore rarely produce butanol higher than 13 g/L. Mao et al. used 1% (v/v) diethyl sulfate (DES) mutagenesis and genome shuffling strategies to generate a mutant strain Rh8 from *C. acetobutylicum* DSM1731. The mutant strain Rh8 exhibits enhanced butanol tolerance of 19 g/L, and could grow in the presence of 18 g/L butanol, compared with the severe inhibition of strain DSM1731 by 12 g/L butanol. In pH control batch fermentation, the production of acetone and butanol in Rh8 increased by 18 and 23% respectively compared to DSM1731. Compared with the genome sequence of *C. acetobutylicum strain* ATCC824 and EA2018, the strain DSM1731 has 11.1 kb plasmid, pSMBb, which has not been previously reported in this species. 345 SNVs were identified between

the chromosomes of the strains, and the size of the DSM1731 megaplasmid is 4 bp smaller than that of ATCC824 [3]. The proteomic analysis of *C. acetobutylicum* DSM1731 and its mutant Rh8 showed that 102 expressed proteins are significantly down/upregulated, involved in protein folding, solvent formation, amino acid metabolism, protein synthesis, nucleotide metabolism, transport, and others. More than 70% of the 102 differentially expressed proteins were either upregulated or downregulated in both the acidogenic and solventogenic phases in Rh8, whereas they were upregulated or downregulated only in the solventogenic phase in DSM1731. Genes encoding for 52 proteins of the 102 proteins are involved with response to butanol stress or the transition from acidogenesis phase to solventogenesis. These results showed that the mutant strain Rh8 has developed a mechanism to prepare itself for butanol challenge before butanol is produced, which gives rise to the increased production of butanol. Mao et al. also performed comparative membrane proteome analysis between the wild-type and the butanol-tolerant mutant strain Rh8 [34]. A total of 73 significantly differently expressed proteins were identified, of which 92% were involved in ion and metabolite transport, components of the cellular membrane or wall machinery, involved in protein processing and stability, associated with surface coat and flagellar formation, and involved in respiratory chain and energy metabolism. The increased protein expression levels in membrane structure and surface stabilization, sporulation, and lipid metabolism, and also the increased expression of ATPase and reduced mobilization and peptide transport, might save energy to cope with butanol challenge, showing that the butanol-tolerant mutant Rh8 might have evolved a more stabilized membrane structure and a cost-efficient energy metabolism strategy to cope with the butanol challenge. Further characterizations of the differentially expressed membrane proteins identified in this study can help to understand and elucidate the molecular mechanisms underlying the complex phenotype of butanol tolerance and butanol production. The study, for the first time, reports the systematic analysis of the membrane proteome of *C. acetybutylicum*. The comparative cytoplasmic proteomics and comparative membrane proteomics gave new sights into the effect of butanol on cellular physiology and the molecular basis of butanol tolerance in *C. acetobutylicum*.

For ABE fermentation, the process of solvent production often collapses when cells are grown in pH-uncontrolled glucose medium ("acid crash" phenomenon). Acetic acid and butyric acid was previously believed to be the reason for this phenomenon. Wang et al. found that formic acid plays an important role in the "acid crash" of ABE fermentation [50]. In pH-uncontrolled glucose medium or glucose-rich medium, *C. acetobutylicum* failed to produce solvents and could accumulate 0.5–1.24 mM formic acid in cells. By expressing formate dehydrogenase from *Candida boidinii* in *C. acetobutylicum*, intracellular formic acid concentration decreased to below detection level, and the engineered strain could restore solvent production. Hence, it was suggested that formic acid triggers the "acid crash" of ABE fermentation in *C. acetobutylicum,* rather than acetic acid and butyric acid.

3 Progress in ABE Fermentation Process

3.1 Substrates

The main substrates for ABE fermentation in China include three kinds: starchy feedstock (corn, cassava and potato), molasses and hydrolysate. In 2010, the total yield of corn was 1.77 billion tons, according to the National Bureau of Statistics of China. Currently, corn is the major substrate used in most of the ABE fermentation plants in China. In addition to corn, corn starch (after removal of corn oil and protein) is also used as the substrate in some plants [38]. The main strains used for ABE fermentation in China are *C. acetobutylicum*-derived mutants, such as *C. acetobutylicum* No. 2, *C. acetobutylicum* AS1.70 and *C. acetobutylicum* EA2018. This species is preferabe for utilizing starchy feedstock as substrate for ABE fermentation [25]. Besides starchy feedstock, molasses can also be used as low-cost substrate in solvent fermentation. Molasses is a viscous by-product of the processing of sugar cane or sugar beets into sugar. There are about 6 million acres of land under cassava cultivation in Guangxi Province, with 8 million tons yield per year. The yield of sugar cane and sugar beet in China is now more than 57 million tons and 6 million tons, respectively. Fan et al. used sugar beet molasses for butanol production by fed batch fermentation [12]. The concentrations of butanol and total solvents reached 14.15 and 19.65 g/L, respectively, with an over 70% ratio of butanol.

In contrast to the limited supply of starch and molassess, the abundant agricultural residues can be a potential feedstock for ABE fermentation in China. It provides enough feedstock for biobutanol production and avoids competition for food with an increasing population. There is now great interest in exploring the hydrolysate of different lignocellulosic substrates for ABE production all over the world. Nasib Qureshi [41] used acid- and enzyme-hydrolyzed corn fiber as substrate for butanol production by *C. beijerinckii* BA101. Wheat straw hydrolysate could also be utilized for ABE fermentation [42]. In China, Li et al. [30] used enzymatic hydrolysis of steam-exploded cornstalk stover for ABE production; the maximal butanol productivity reached 0.31 g/(L h). The hydrolysate of wheat bran was also used for ABE fermentation. The *C. beijerinckii* ATCC 55025 used by Xu Ping's lab could produce a total of 11.8 g/L solvent after 72 h fermentation of the hydrolysate of wheat bran [32]. Another new hydrolysate is the acid hydrolysate of Jerusalem artichoke juice. Bai Fengwu's team used Jerusalem artichoke juice as substrate; the final butanol titer reached 11.21 g/l [5]. Although lignocellulosic hydrolysates have been proved to be a potential substrate for ABE fermentation, industrialization still needs more efforts from other fields, such as the treatment of lignocellulosic materials, and an economic separation process.

3.2 Fermentation Process

The fermentation process mainly comprises batch fermentation, fed-batch fermentation, continuous fermentation, cell immobilization, and fermentation coupled with

separation. In Chinese plants, the continuous fermentation process for butanol has been well established to improve bioreactor productivity by keeping the culture at a steady state in fermentation. The steady state is controlled by continuous addition of fresh medium, which is exactly balanced by the removal of cell suspension from the bioreactor. Compared with batch fermentation and fed-batch fermentation, this process could reduce the fermentation period, increase the rate of equipment utilization, and decrease the production cost. The use of continuous fermentation for ABE production in China dates back about 50 years. However, general *Clostridium* strains seem not to be suitable for continuous fermentation, because solventogenic clostridia could form spores in the process and lose their solvent production activity. In addition, the strains would exhibit physiological decay, referred to as degeneration, after a serious of inoculations in culture medium [11]. In the 1950s, Chinese researchers began to study continuous ABE fermentation and its industrial application. Chiao published the first academic article on this subject in China [6]. In the review by Ni et al. [38], there was a detailed description of Chinese ABE continuous fermentation. In brief, the whole system included six to eight 200–500 m^3 fermenters. The first two tanks were activation fermenters with periodic addition of seed culture from smaller seed tanks. The hallmark of this system was that the cells could maintain the active phase using this configuration and inoculation procedure. From the first fermenter to the last, there is a pressure gradient to keep the whole system continuously operating in an overflow manner. In this way, the ABE process could be continuously operated for about 170–480 h without sterilization and loading. This continuous fermentation has higher productivity (20–50%) than batch fermentation.

3.3 Separation

In the ABE fermentation process, when the solvents accumulate to a specific concentration (usually less 30 g/L), the cells show significant growth inhibition and limited production activity. Butanol is recognized as the most toxic product among the solvents. Therefore, developing a series of butanol recovery methods to remove the toxicity of butanol to cells is an alternative way to improve productivity. Conventionally, the solvents were separated from the fermentation broth by distillation. For in-situ recovery, several other processes have been established, including absorption, liquid–liquid extraction, perstraction, reverse osmosis, pervaporation and gas stripping.

(a) Absorption. Absorption is an efficient technique of removing butanol from the fermentation broth. The principle is that butanol is absorbed by absorbents and then desorbed by heat or displacers to obtain pure butanol. This method can be used for in-situ recovery. Polyvinyl pyridine (PVP) and silicalite are the common absorbents. Compared to conventional batch fermentation, product concentration and productivity increased by 54 and 130%, respectively, and sugar utilization increased to 73.3 g/L in absorption-coupling fermentation [51, 52]. The results proved that adsorption coupled with the fermentation process was an effective method for butanol recovery in improving total product concentration and sugar

utilization rate. However, the capacity and selectivity for butanol are currently still quite low, which, together with the high price of absorbents, form great obstacles to the promotion of this technology.

(b) Liquid–liquid extraction. Liquid–liquid extraction is another efficient recovery technology that can remove butanol from the fermentation broth in situ. This method is built on the principle that solubilities of chemicals vary in different solutions and distribution coefficients of the chemicals vary between two immiscible phases. In this case, when a water-insoluble organic extractant is mixed with the liquid fermentation broth, butanol will be selectively concentrated in the organic phase, because it is more soluble than in the aqueous phase. Hu et al. [21] compared four different biodiesels (waste oil, rapeseed oil, palm oil, and KFC waste oil) for solvent extraction, finding that when palm oil was added to the fermentation (24 h, oil:water volume ratio is 0.4:1), the fermentation intensity increased by 10.9% compared to the traditional fermentation, and the concentration of butanol in the organic phase reached to 6.44 g/L. Liquid–liquid extraction has high capacity and selectivity. However, it also has some problems, such as the toxicity of the extractant to the cell and emulsion fermentation.

(c) Pervaporation. Pervaporation is another recovery technique that relies on membranes. Membrane selectivity is mainly used to remove liquid from the volatile compounds of fermentation. The volatile components of fermentation broth or organic compounds undergo selective vaporization through the membrane, and nutrients, sugar, and microbial cells remain below. Currently, ceramic membranes, polymer membranes, and liquid membranes have been widely used in pervaporation separation of organic compounds (such as butanol, ethanol, etc.). Zhou et al. used homemade silicalite-1/silicone rubber hybrid pervaporation membranes to separate butanol, acetone, and ethanol from water [56]. The effects of acetone and ethanol on pervaporation separation of butanol were studied at different concentrations and temperatures, and the conclusion drawn that membrane permeability of butanol would increase in the presence of acetone and ethanol. Luo et al. tried to separate and concentrate butanol by pervaporation [33]. The butanol concentration increases with the rise of temperature, permeation pressure, and cross-flow rate. The selectivity of pervaporation for butanol is the highest at 50°C. The advantages of pervaporation are high efficiency, low energy consumption, and less investment. However, the membrane needs to be regenerated, because a swelling effect makes the membrane more permeable, resulting in less selectivity of solvents.

There are no reports by Chinese researchers involved in gas stripping, perstraction, and reverse osmosis.

4 Conclusions

In China, research on ABE fermentation covers almost every aspect of this field. The excellent solvent-producing strains, such as EA2018 and Rh8, have been improved by native researchers using conventional mutagenic methods. Using the

modern omics technologies, the advanced characteristics of these strains were elucidated in systematic levels. Chinese researchers also contributed to developing the currently most efficient gene knockout tool based on group II intron in *C. acetobutylicum*, and some improvements in genetic manipulation on *C. acetobutylicum* were achieved by Chinese researchers,viz., intron-anchored targeted gene deletion, acceptance of unmethylated DNA, and electrotransformation in air. Some efforts are also being made to developing more efficient genetic tools focusing on genome engineering. Currently, several engineered strains have been reported by Chinese researchers, aiming to improve the strain's fermentation performance. In addition, fermentation and separation technologies have also been widely studied in China. Chinese researchers are also trying to develop the ABE process using lignocellulosic feedstocks, for an sustainable and economical process. We believe that more achievements in biobutanol production will be published by Chinese researches in the future. Meanwhile, Chinese researchers should pay more effort to contribute to the academic field with creative ideas and fundamental research.

References

1. Alsaker KV, Papoutsakis ET (2005) Transcriptional program of early sporulation and stationary-phase events in *Clostridium acetobutylicum*. J Bacteriol 187:7103–7118
2. Alsaker KV, Spitzer TR, Papoutsakis ET (2004) Transcriptional analysis of *spo0A* overexpression in *Clostridium acetobutylicum* and its effect on the cell's response to butanol stress. J Bacteriol 186:1959–1971
3. Bao G, Wang R, Zhu Y et al (2011) Complete genome sequence of *Clostridium acetobutylicum* DSM 1731, a solvent producing strain with multi-replicon genome architecture. J Bacteriol: doi:10.1128/JB.05596-11
4. Bowles LK, Ellefson WL (1985) Effects of butanol on *Clostridium acetobutylicum*. Appl Environ Microbiol 50(5):1165–1170
5. Chen L, Xin C, Deng P et al (2010) Butanol production from hydrolysate of Jerusalem artichoke juice by *Clostridium acetobutylicum* L7. Chin J Biotechnol 26(7):991–996
6. Chiao J, Cheng Y, Shen Y et al (1960) Studies on the continuous acetone-butanol fermentation. Acta Microbiol Sin 10:137–148
7. Chiao J, Sun Z (2007) History of the acetone-butanol-ethanol fermentation industry in China: development of continuous production technology. J Mol Microbiol Biotechnol 13:12–14
8. Desai R, Papoutsakis E (1999) Antisense RNA strategies for metabolic engineering of *Clostridium acetobutylicum*. Appl Environ Microbiol 65:936–945
9. Dong H, Tao W, Zhu L et al (2011) CAC2634-disrupted mutant of *Clostridium acetobutylicum* can be electrotransformed in air. Lett Appl Microbiol 53(3):379-82. doi:10.1111/j.1472-765X.2011.03111.x
10. Dong H, Zhang Y, Dai Z et al (2010) Engineering *Clostridium* strain to accept unmethylated DNA. PLoS One 5(2):e9038
11. Dyr J, Munk V (1954) Biosynthesis of riboflavin by *Clostridium acetobutylicum*. Chekhoslovatskaia Biol 3(1):23–29
12. Fan J, Feng W, Di S et al (2010) Production of butanol from sugar beet molasses by fed-batch fermentation. Chin J Bioprocess Eng 8:6–9
13. Green EM (2011) Fermentative production of butanol–the industrial perspective. Curr Opin Biotech 22:337–343

14. Green EM, Boynton ZL, Harris LM et al (1996) Genetic manipulation of acid formation pathways by gene inactivation in *Clostridium acetobutylicum* ATCC 824. Microbiology 142:2079–2086
15. Gu Y, Ding Y, Ren C et al (2010) Reconstruction of xylose utilization pathway and regulons in Firmicutes. BMC Genomics 11(1):255
16. Gu Y, Li J, Zhang L et al (2009) Improvement of xylose utilization in *Clostridium acetobutylicum* via expression of the *talA* gene encoding transaldolase from *Escherichia coli*. J Biotechnol 143(4):284–287
17. Harris LM, Welker NE, Papoutsakis ET (2002) Northern, morphological, and fermentation analysis of *spo0A* inactivation and overexpression in *Clostridium acetobutylicum* ATCC 824. J Bacteriol 184(13):3586–3597
18. Heap JT and Minton NP (2009) Methods. PCT/GB2009/000380
19. Heap JT, Pennington OJ, Cartman ST et al (2007) The ClosTron: a universal gene knock-out system for the genus *Clostridium*. J Microbiol Methods 70(3):452–464
20. Ho NW, Chen Z, Brainard AP (1998) Genetically engineered *Saccharomyces* yeast capable of effective cofermentation of glucose and xylose. Appl Environ Microbiol 64:1852–1859
21. Hu C, Du Y, Yang Y (2007) Preliminary study on coupling between biodiesels and acetone-butanol fermentation. Chin J Process Eng 5(1):27–33
22. Hu S, Zheng H, Gu Y et al (2011) Comparative genomic and transcriptomic analysis revealed genetic characteristics related to solvent formation and xylose utilization in *Clostridium acetobutylicum* EA 2018. BMC Genomics 12:1471–2164
23. Jia K, Zhu Y, Zhang Y et al (2011) Group II intron-anchored gene deletion in *Clostridium*. PLoS One 6(1):e16693
24. Jiang Y, Xu C, Dong F et al (2009) Disruption of the acetoacetate decarboxylase gene in solvent-producing *Clostridium acetobutylicum* increases the butanol ratio. Metab Eng 11: 284–291
25. Jones DT, Keis S (1995) Origins and relationships of industrial solvent-producing clostridial strains. FEMS Microbiol Rev 17(3):223–232
26. Jones DT, Woods DR (1986) Acetone-butanol fermentation revisited. Microbiol Rev 50(4):484–524
27. Karberg M, Guo H, Zhong J et al (2001) Group II introns as controllable gene targeting vectors for genetic manipulation of bacteria. Nat Biotechnol 19(12):1162–1167
28. Lütke-Eversloh T, Bahl H (2011) Metabolic engineering of *Clostridium acetobutylicum*: recent advances to improve butanol production. Curr Opin Biotechnol 22:634–647
29. Lee SY, Park JH, Jang SH et al (2008) Fermentative butanol production by clostridia. Biotechnol Bioeng 101(2):209–228
30. Li D, Chen H (2007) Fermentation of acetone and butanol coupled with enzymatic hydrolysis of steam exploded cornstalk stover in a membrane reactor. Chin J Process Eng 7(6):1212–1216
31. Liu S, Qureshi N (2009) Proteome analysis and comparison of *Clostridium acetobutylicum* ATCC 824 and Spo0A strain variants. New Biotechnol 26:117–121
32. Liu Z, Ying Y, Li F et al (2010) Butanol production by *Clostridium beijerinckii* ATCC 55025 from wheat bran. J Ind Microbiol Biotechnol 37(5):495–501
33. Luo J, Yi S, Su Y et al (2010) Separation and concentration of butanol from acetone-butanol-ethanol mixed solution by pervaporation. Chem Eng 38(2):43–46
34. Mao S, Luo Y, Bao G et al (2011) Comparative analysis on the membrane proteome of *Clostridium acetobutylicum* wild type strain and its butanol-tolerant mutant. Mol BioSyst 7:1660–1677
35. Mermelstein LD, Welker NE, Bennett GN et al (1992) Expression of cloned homologous fermentative genes in *Clostridium acetobutylicum* ATCC 824. Biotechnology (NY) 10(2): 190–195
36. Mills DA, Manias DA, McKay LL et al (1997) Homing of a group II intron from *Lactococcus lactis* subsp. *lactis* ML3. J Bacteriol 179(19):6107
37. Mitchell WJ (1998) Physiology of carbohydrate to solvent conversion by clostridia. Adv Microb Physiol 39:31–130

38. Ni Y, Sun Z (2009) Recent progress on industrial fermentative production of acetone-butanol-ethanol by *Clostridium acetobutylicum* in China. Appl Microbiol Biotechnol 83(3): 415–423
39. Nolling J, Breton G, Omelchenko M et al (2001) Genome sequence and comparative analysis of the solvent-producing bacterium *Clostridium acetobutylicum*. J Bacteriol 183:4823–4838
40. Ounine K, Petitdemange H, Raval G et al (1985) Regulation and butanol inhibition of D-xylose and D-glucose uptake in *Clostridium acetobutylicum*. Appl Environ Microbiol 49:874–878
41. Qureshi N, Ezeji TC, Ebener J et al (2008) Butanol production by *Clostridium beijerinckii*. Part I: Use of acid and enzyme hydrolyzed corn fiber. Bioresour Technol 99(13):5915–5922
42. Qureshi N, Saha BC, Cotta MA (2007) Butanol production from wheat straw hydrolysate using *Clostridium beijerinckii*. Bioprocess Biosyst Eng 30(6):419–427
43. Ren C, Gu Y, Hu S et al (2010) Identification and inactivation of pleiotropic regulator CcpA to eliminate glucose repression of xylose utilization in *Clostridium acetobutylicum*. Metab Eng 12:446–454
44. Rodriguez SA, Davis G, Klose KE (2009) Targeted gene disruption in *Francisella tularensis* by group II introns. Methods 49(3):270–274
45. Shao L, Hu S, Yang Y et al (2007) Targeted gene disruption by use of a group II intron (targetron) vector in *Clostridium acetobutylicum*. Cell Res 17:963–965
46. Soucaille P, Figge R, Croux C (2008) Process for chromosomal integration and DNA sequence replacement in clostridia. PCT/EP2006/066997
47. Tomas CA, Beamish J, Papoutsakis ET (2004) Transcriptional analysis of butanol stress and tolerance in *Clostridium acetobutylicum*. J Bacteriol 186(7):2006–2018
48. Tummala SB, Welker NE, Papoutsakis ET (2003) Design of antisense RNA constructs for downregulation of the acetone formation pathway of *Clostridium acetobutylicum*. J Bacteriol 185(6):1923–1934
49. Vollherbst-Schneck K, Sands J, Montenecourt B (1984) Effect of butanol on lipid composition and fluidity of *Clostridium acetobutylicum* ATCC 824. Appl Environ Microbiol 47(1):193–194
50. Wang S, Zhang Y, Dong H et al (2011) Formic acid triggers the "acid crash" of acetone-butanol-ethanol fermentation by *Clostridium acetobutylicum*. Appl Environ Microbiol 77(5): 1674–1680
51. Yang X, Tsai GJ, Tsao GT (1994) Enhancement of in situ adsorption on the acetone-butanol fermentation by *Clostridium acetobutylicum*. Sep Tectmol 4(2):81–92
52. Yang X, Tsao GT (1995) Enhanced acetone-butanol fermentation using repeated fed-batch operation coupled with cell recycle by membrane and simultaneous removal of inhibitory products by adsorption. Biotechnol Bioeng 47:444–450
53. Zhang Y, Chen J, Yang Y et al (1996) Breeding high-ratio butanol strains of *Clostridium acetobutylicum* and application to industrial production. Indust Microbiol 26:1–6
54. Zhang Y, Chen J, Yang Y et al (1996) Breeding of high-ratio butanol strains of *Clostridicum acetobutylicum* and application to industrial production. Ind Microbiol 26(4):1–6
55. Zhang Y, Zhu Y, Li Y (2009) The importance of engineering physiological functionality into microbes. Trends Biotechnol 27(12):664–672
56. Zhou H, Su Y, Yi S et al (2010) Effect of acetone and ethanol on pervaporation separation of butanol. CIESC J 61(5):1143–1150
57. Zhu L, Dong H, Zhang Y et al (2011) Engineering the robustness of *Clostridium acetobutylicum* by introducing glutathione biosynthetic capability. Metab Eng 13:426–434
58. Zverlov VV, Berezina O, Velikodvorskaya GA et al (2006) Bacterial acetone and butanol production by industrial fermentation in the Soviet Union: use of hydrolyzed agricultural waste for biorefinery. Appl Microbiol Biotechnol Bioeng 71:587–597

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Branched-Chain Higher Alcohols

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Abstract China's energy requirements and environmental concerns have stimulated efforts toward developing alternative liquid fuels. Compared with fuel ethanol, branched-chain higher alcohols (BCHAs), including isopropanol, isobutanol, 2-methyl-1-butanol, and 3-methyl-1-butanol, exhibit significant advantages, such as higher energy density, lower hygroscopicity, lower vapor pressure, and compatibility with existing transportation infrastructures. However, BCHAs have not been synthesized economically using native organisms, and thus their microbial production based on metabolic engineering and synthetic biology offers an alternative approach, which presents great potential for improving production efficiency. We review the current status of production and consumption of BCHAs and research progress regarding their microbial production in China, especially with the combination of metabolic engineering and synthetic biology.

Keywords Alcohol tolerance · Biofuels · Branched-chain higher alcohols · Metabolic engineering · Synthetic biology

Contents

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1 Introduction

One of the great challenges in the twenty-first century is to meet the growing demand for fuels for transportation, agricultural use, industrial processes, and household use, as well as to provide feedstocks for their sustainable production because of the inevitable depletion of global fossil fuel resources [1, 2], which has been highlighted by high oil prices. More importantly, the future fuel supply must be met with substantial reduction of greenhouse gas emissions. Presently, the increasing concerns are being met with biofuels from biomass, the largest and most renewable energy source in the world [3].

Currently, ethanol is the major biofuel worldwide, and is being produced commercially from sugar- and starch-based feedstocks such as sugarcane, corn, and cassava. However, ethanol has significant limitations. For example, its energy density is less than that of gasoline, and it tends to absorb water from the surroundings, making it more corrosive when stored or distributed with existing infrastructures. Compared with ethanol, higher alcohols offer advantages owing to their higher energy density and lower hygroscopicity (Table 1).

Moreover, higher alcohols are flexible fuel oxygenates with properties that allow blending with both gasoline and diesel fuels [4, 5]. When used as a blended product, higher alcohols can reduce harmful emissions such as CO, NO_x, SO_x, and particulates that cause smog, making them attractive alternatives to methyl tertiary butyl ether (MTBE). In addition, branched-chain higher alcohols (BCHAs) have higher octane numbers than their straight-chain counterparts, resulting in less knocking in engines [6]. Moreover, as superior organic solvents and essential chemical materials, BCHAs are also widely used in the plastics, cosmetic, perfume, flavoring, ink, paint, and resin industries as well as in organic synthesis. For example, isobutanol is converted into acetate ester, which is used extensively as a lacquer solvent, and isopropanol is used heavily in herbicide syntheses and in solvents for coatings and inks.

Higher alcohols, including BCHAs, are now produced commercially from olefins in a multistep process that includes the oxo (hydrocarbonylation) reaction and subsequent hydrogenation and separation steps. The market for several higher alcohols is currently large and highly dependent on oil prices. Expansion into the fuel additive market would likely be accelerated if superior performance and low-cost production could be demonstrated [7].

Table 1 The characteristics of some liquid fuels

Physical property	Gasoline	Ethanol	1-Propanol	1-Butanol	1-Pentanol	Isopropanol	Isobutanol	2-Methyl-1-butanol	3-Methyl-1-butanol
Molecular weight	72–170	46	60	74	88	60	74	88	88
Density at 20 °C (g/cm^3)	0.700–0.790	0.794	0.800	0.810	0.810	0.785	0.802	0.805	0.809
Boiling point at 1 atm (°C)	32–210	78	97	118	138	62.5	108	132	132
Solubility[a]	Immiscible	Miscible	Miscible	7.7	Miscible	Miscible	8.0	Immiscible	Immiscible
Energy density (MJ/kg)	44.5	29.7	33.6	36.1	37.7	33.6	36.1	37.7	37.7
Net heat of combustion (BTU/gal)	124,000	80,000	86,000	93,000	94,000	NA	95,000	NA	NA
$(R + M)/2$[b]	87	112	NA	87	NA	106	103.5	147	147
Blend RVP (psi at 100 °F)	14.7	18–22	1.3	4.3	NA	14	5.0	NA	NA
Hygroscopicity	Low	High	Low	Low	Low	Low	Low	Low	Low
Compatible with current infrastructure	Yes	No	Yes	Yes	Yes	Yes	Yes	Yes	Yes

NA not available, *RVP* Reid vapor pressure

[a] Water solubility at 20 °C (g/100 mL)

[b] Average of research octane number and motor octane number (US/Canadian formula for determining octane level in gasoline)

In China, biofuels developed from biomass are playing more and more essential roles in the national energy structure [8]. In 2010, the total biofuel production in China was 1.58 million tons, including 1.52 million tons of fuel ethanol and 0.3 million tons of biodiesel, which were produced mainly from grains and recycling cooking oils [9, 10]. With limited arable land per capita, this path to biofuel development risks the security of grain supply in China. Despite these obstacles, the Chinese government still sees potential benefits from biofuels, provided that they are produced commercially from nonfood feedstocks, and put in place a national goal of increasing their share in fuel consumption to 15% by 2020 from only 2% presently. In addition, the government has also pledged as part of the Copenhagen agreements to cut carbon emissions by 40–45% per unit GDP production compared with the 2005 level. This commitment translates into an investment of at least 1% of GDP (CNY 240 billion) in China's clean energy sector in the next 5 years. Except bioethanol and biodesiel, developing higher alcohols as biofuels is one of most important parts of the long-term plan of China's clean energy [11, 12].

China has the second largest market for chemicals in the world after the USA [13]. The robust Chinese economy is stimulating the growth of the chemical industry, even though the world is still suffering from an economic recession. In 2007, China recorded a GDP growth rate of 11.4%, but the chemical industry grew at a rate of 26% during the same year. It is projected that the consumption of power parity of China will surpass that of the USA within the next 10 years, and this will emerge as a driving force for domestic producers, and is encouraged by the government as a solution to alleviate its dependence on exports for trade balances with the West. This also indicates opportunities for the use of chemicals such as higher alcohols.

Presently, great efforts are being made for bioethanol production in China [14]. However, unfortunately, BCHAs, such as isopropanol, isobutanol, 3-methyl-1-butanol, and 2-methyl-1-butanol, have been produced from a renewable source with lower yields, not high enough to make them viable as a gasoline substitute, not only in China but also worldwide. Developing BCHAs is still an extremely challenging path to be explored. We summarize here the current status of production and consumption of the main BCHAs and some preliminary progress and the possibility of developing microbial production of BCHAs in China, especially with the combination of metabolic engineering and synthetic biology.

2 Market Consumption and Supply of BCHAs in China

2.1 Fuel Market Analysis

As in the international market, there are two primary uses for BCHAs in China, either as base chemicals or as high-energy, low-emission fuel oxygenates [15]. For base chemicals, BCHAs are used as feedstocks for manufacturing many

product categories, including cosmetics, perfumes, flavorings, inks, solvents, and resins. For fuel oxygenates, BCHAs are flexible chemicals with properties that allow blending with both gasoline and diesel fuels.

Based on an average selling price of $781 per ton, the fuel additive market in China exceeded $1.5 billion in 2007, with an expected growth rate of over 17% annually, which will drive the market to over $17.6 billion by 2022 [16, 17]. Currently, China is the world's largest MTBE market, with 87% being used as a gasoline additive in 2007 [18, 19]. The fuel additive market in China is driven by the demand for gasoline. China's demand for gasoline has grown substantially and is expected to continue to grow in double digits owing to the rapid increase in the number of automobiles. In any event, there is little doubt that China will continue to increase its reliance on oil, and gasoline usage will continue to grow dramatically. Market demand for MTBE or alternative fuel additives will be significant. Therefore, there is reason to assume that BCHAs might replace MTBE in the fuel additive market.

2.2 Isopropanol

Isopropanol can be used directly as a fuel supplement added to gasoline or as a feedstock for the transesterification of fats into biodiesel. The use of diisopropyl ether as a fuel oxygenate may become a significant outlet for isopropanol [20]. It is estimated that the demand for isopropanol in China will increase at a rate of 8–10% in the next 5 years and the production capacity is expected to be 230,000 t in 2015, indicating a high market potential [21].

There are two commercial routes for manufacturing isopropanol by combining water and propylene to produce it. The indirect method is based on the hydration of refinery-grade propylene using sulfuric acid to form isopropyl sulfate, which is then hydrolyzed with steam to form sulfuric acid and isopropanol. The crude isopropanol is distilled to the desired purity. The indirect process can use refinery-grade propylene streams and lower operating pressures than the direct process, but suffers from higher corrosion and maintenance costs. Owing to the complicated process and the problem of a high level of pollution, this method was abandoned in the 1980s. Instead, the direct hydration of chemical-grade (90–99%) propylene without sulfuric acid consumption was developed. Propylene and water are heated and the liquid–vapor mixture under pressure passes into a trickle-flow reactor containing sulfonated polystyrene cation ion-exchange resins. Alternatively, the reaction can be performed in the gas phase over a phosphoric acid based fixed-bed catalyst. The main limitation of the model route is the high energy consumption [21]. Besides, isopropanol can also be produced by the hydrogenation of acetone in the liquid phase. This process is only suitable when excess acetone is available.

China started its isopropanol industry rather early. Jinzhou Petrochemical Company, China National Petroleum Corporation (CNPC), constructed a 20,000 t/year isopropanol unit with the propylene vapor-phase hydration process in 1977.

After two expansions, in 1992 and 1999, the capacity reached 100,000 t/year. Shandong Dongying Hi-Tech Spring Chemical Industrial Company constructed a 30,000 t/year isopropanol unit with the propylene direct hydration process developed by Dalian Institute of Chemical Physics, Chinese Academy of Sciences, which was put into operation in November 2005. Considering the shortage of supply of isopropanol in the domestic market, CNPC Jinzhou Petrochemical Company invested CNY 418 million and constructed a 150,000 t/year isopropanol unit during the 11th Five-Year Program (2006–2010), and this was put into operation in 2007. Although isopropanol production in China has increased constantly in recent years, its market demand still outpaces domestic production and the shortfall has to be supplied by imports. In 2000, 55,000 t of isopropanol was imported, but imports increased to 112,000 t in 2005.

The most important application of isopropanol in China is as a solvent, which accounts for about 70% of the total amount used, mainly in ink production and the pharmaceutical and pesticide sectors. China has become the second largest producer of coatings in the world. The distribution readjustment to the coatings sector in China has been greatly accelerated in recent years and products are developing toward high-grade varieties. Many well-known foreign producers have come to China and invested in the construction of high-grade coatings units. The demand for isopropanol in the coatings sector has therefore presented a trend of rapid growth.

In contract to the situation in advanced countries, applications of isopropanol in many sectors have not yet been explored in China. For example, isopropanol has very strong sterilizing ability (more efficient than ethanol), and can be used in the sterilization of skin, hands, and medical apparatuses as well as artificial limbs. In some countries, isopropanol is the most popular antiseptic in public places such as beauty parlors, but it is seldom used in China, even during the severe acute respiratory syndrome epidemic in 2003. Domestic firms should highlight research into applications of isopropanol, especially its applications in the production of solvents for surface coatings, dewaxing solvents for lube oils, cleaning agents for electronics, antiseptics for public places, chemicals in daily use, and resins so as to promote growth in the consumption of isopropanol.

2.3 Isobutanol

Isobutanol is a bulk chemical and has many industrial applications, such as solvents, paint additives, ink ingredients, and extractants for organic compounds. Many isobutanol derivatives are used in the chemical industry. Isobutyl acetate is one of them, and is used for production of lacquers and coatings. Isobutyl esters such as diisobutyl phthalate are another category, and are used as plasticizer agents in plastics, rubbers, and other dispersions. Isobutanol can also be dehydrated to produce butenes, which are building blocks for the production of materials such as lubricants, synthetic rubber, xylene, and polyester terephthalate [22].

The current market for isobutanol is about 500,000 t/year. The traditional strategy for isobutanol production is carbonylation of propylene. Two processes are commonly used in the chemical industry. The major process is hydroformylation, which generates a mixture of isobutanol and isobutyraldehyde. Another process is Reppe carbonylation. However, these petrochemical pathways are not sustainable, and great concerns have been raised regarding environmental pollution.

In recent years, with the rapid growth of the construction of new carbonyl alcohol production facilities [23], the production capacity of isobutanol (one of the main by-products of carbonyl alcohol production) in China has been increased. In 2006, the total domestic production capacity of isobutanol was 81,500 t/y. In 2010, China National Offshore Oil Corporation (CNOOC) initiated a 1.5 billion tons per year oil refinery project to use heavy crude oil from the Bohai Sea Oil Field, and Bohai Chemical Group will construct a 230,000 t/year butanol/octanol production facility as the CNOOC project's downstream support unit, which will significantly increase China's isobutanol production capacity. However, with the rapid development of the country's chemical industry, it will be difficult to be self-sufficient in raw materials for the domestic market, and the dependence on imports of isobutanol will persist for quite a long time. In 2008, China's isobutanol consumption was 260,000 t, and more than half of the isobutanol was imported. Therefore, the development of alternative isobutanol production processes has great potential.

2.4 2-Methyl-1-butanol, and 3-Methyl-1-butanol

3-Methyl-1-butanol and 2-methyl-1-butanol are normally used as apple or banana flavoring agents for wine. They can also be used as chemical intermediates and solvents in pharmaceutical products. The market consumption of 3-methyl -1-butanol and 2-methyl-1-butanol is increasing continuously: however, the production capacity is still low. The domestic market for 3-methyl-1-butanol and 2-methyl-1-butanol is more than 100,000 t/year, but China's production capacity is only around 30,000 t [24].

3-Methyl-1-butanol and 2-methyl-1-butanol were first isolated from fusel oils, by-products of ethanol fermentation by yeast [25]. These compounds can also be derived from the chlorination of pentane followed by hydrolysis [26]. Another alternative process is the oxo process, a general strategy for the manufacture of C_4 and higher alcohols [27]. Both the chlorination process and the oxo process are current commercial processes for the production of 3-methyl-1-butanol and 2-methyl-1-butanol, but the oxo process via the hydroformylation reaction is the more popular. Two main technologies are used for the process. The first was brought on stream by Ruhrchemie in Germany and Exxon in USA in the 1940s and is generally referred to as "high-pressure cobalt catalyst technology." The active catalyst species is cobalt hydrocarbonyl, and a pressure of 200–300 atm is required to maintain the stability of the catalyst. In the early 1960s, Shell commercialized a

modern version of the cobalt catalyst process. This technology uses organophosphine ligands, which allows a lower operating pressure of 30–100 atm but at the expense of the catalyst activity. The Shell technology is employed primarily in the production of linear primary alcohols, whereas the high-pressure cobalt technology is frequently used in the production of branched alcohols.

3 Background and R & D Platforms for Microbial Production of BCHAs

3.1 Background

With the continuous increase in market consumption and serious environmental concerns raised by the production of BCHAs by traditional petrochemical pathways, microbial production has been considered the most promising alternative [6, 28]. High efficiency, low energy consumption, and environmental friendliness, all these characters of microbial production seem to meet perfectly the requirements of our society. However, despite microbial production having already achieved great success for many products, such as amino acids, organic acids, vitamins, and nucleotides, it is still not economically competitive for the production of BCHAs [29], which have inspired intensive research in the USA, Europe, and the Asia–Pacific region, including Japan, Korea, and China. Moreover, some energy and chemical companies have taken a further step to bring the progress made into pilot plants and commercial production [30]. There are also many small research-oriented companies joining this competitive emerging market [31].

3.2 R & D Platforms

In China, the government has put great efforts into energy- and resource-saving and pollutant reduction for sustainability, and advanced manufacturing processes such as industrial biotechnology have been given priorities in government-sponsored research, which involves three platforms [9]. The first is the National Mid- and Long-Term (2006–2020) Program for Science and Technology Development, which is the R & D road map for the social and economic development of China. The next-generation industrial biotechnology toward the development of biofuels, bio-based chemicals, and biomaterials is highlighted. The second is the Five-Year Plan for Biotechnology Development, in which specific goals are proposed for bio-related areas, including industrial biotechnology, and the progress is evaluated at the end of the program. The third is projects funded by the Ministry of Science and Technology as well as local governments. Notably, the National Basic

Research Program of China (973 Program) and the National High Technology Research and Development Program of China (863 Program) are among them. In the 863 Program of the 12th Five-Year Plan from 2011 to 2015, 11 bio-related projects have been funded, and the higher alcohol project is one of them, with a focus on microbial production of isobutanol from nongrain feedstocks and systematic technology integration for improved yield and productivity. As the national innovation system, the Chinese Academy of Sciences is also actively involved in the projects for microbial production of BCHAs [32, 33].

4 Update on Progress in Microbial Production of BCHAs

In the quest to find substitutes for petroleum-based fuels, several low-energy molecules such as ethanol have been employed because of their easy production. However, biofuels with energy density similar to that of current fuels would be preferred, and BCHAs are among them. Other properties of these alcohols also display more desirable features. For example, a lower miscibility with water and lower vapor pressure are benefits for transportation fuels. For microbial production of BCHAs, a unique approach is to employ synthetic biology and metabolic engineering to engineer and assemble the natural or chimeric synthetic pathways in cell factories for the production of the target compounds since a natural microorganism only accumulates a very tiny amount of BCHAs [34–36]. Since James Liao's group at the University of California, Los Angles [28, 37] and other groups worldwide [38, 39] developed new strategies for producing BCHAs by genetically modified microorganisms, microbial production of BCHAs has also made progress in China.

4.1 Product Toxicity

Unlike ethanol, BCHAs are more toxic to microbes, although different microorganisms respond differently to the toxicity owing to the differences in their tolerance and the length of the carbon chain, and this has been investigated with isobutanol [40, 41]. However, the underlying mechanisms are less well known. The inhibitory effect of 3-methyl-1-butanol on Gram-negative *Escherichia coli* and Gram-positive *Corynebacterium glutamicum* has been studied in the authors' laboratory. *E. coli* was more sensitive than *C. glutamicum* to 3-methyl-1-butanol (Fig. 1). Another study reported that *Bacillus subtilis* is more tolerant to higher alcohols than *E. coli* [42], indicating *B. subtilis* might be a more suitable host for microbial production of higher alcohols.

For the host, the toxicity of BCHAs has been proved to be one of the barriers limiting the final product titer in the fermentation broth. For example, no more than 2% (w/v) BCHAs can be achieved presently, making the downstream product

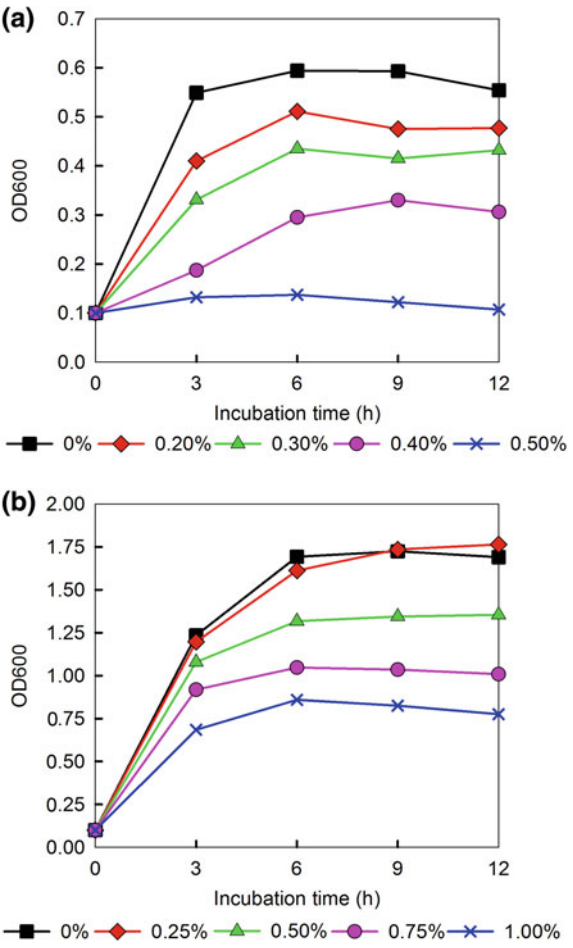

Fig. 1 Toxicity of 3-methyl-1-butanol for *Escherichia coli* (**a**) and *Corynebacterium glutamicum* (**b**) *OD600* optical density at 600 nm

purification extremely energy intensive and resulting in a significant amount of waste being discharged. Engineering host strains with improved tolerance is the primary solution [43], and has been well demonstrated in butanol production with *Clostridium acetobubutylicum* [44].

4.2 Improving Alcohol Tolerance of Microorganisms

Methods of improving the alcohol tolerance of microorganisms can be grouped into two strategies: random approach and rational design [44]. Improving alcohol tolerance by the random approach includes random mutation via evolutionary engineering. This strategy was used in the authors' laboratory to improve the 3-methyl-1-butanol tolerance of *E. coli*. A twofold increase in tolerance was

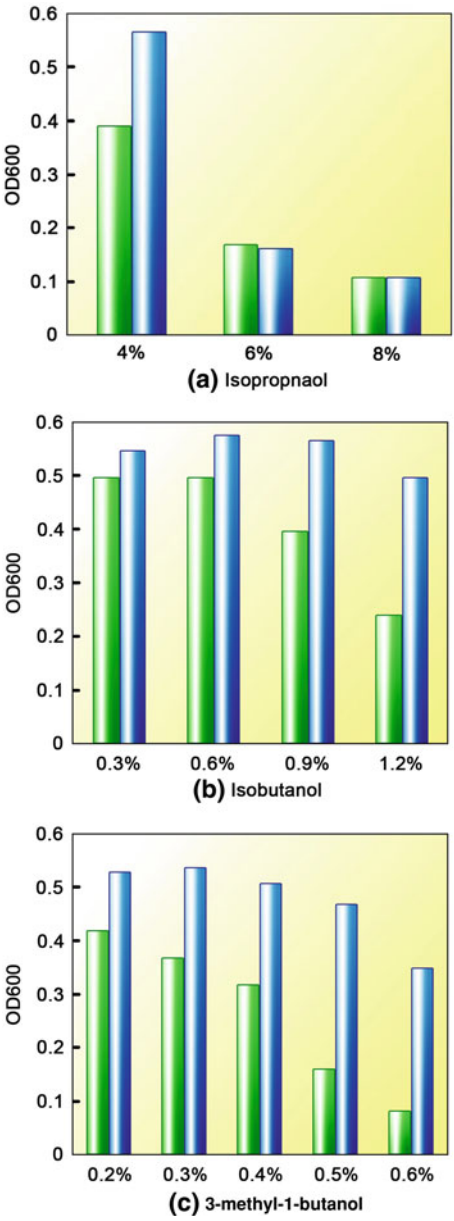

Fig. 2 Tolerance to branched-chain higher alcohols of the wild-type (*green*) and mutated (*blue*) *Escherichia coli* strains

observed with the mutated strain after 120 subcultures with increasing 3-methyl-1-butanol concentration (Fig. 2). The strain with improved 3-methyl-1-butanol tolerance has also shown a similar effect for isobutanol and isopropanol. Through

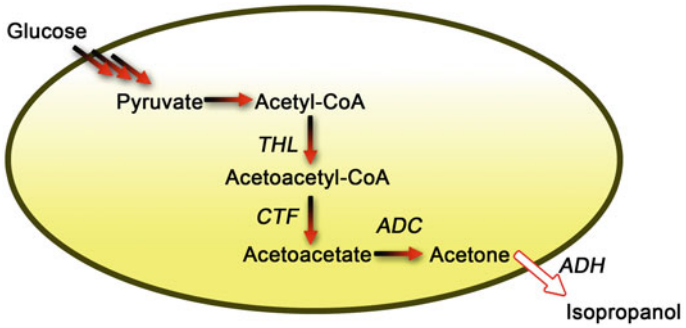

Fig. 3 Synthetic pathway and key enzymes for isopropanol production. The pathway from glucose to acetone is a natural one. When primary–secondary alcohol dehydrogenase (*ADH*) was introduced, the synthetic pathway for isopropanol production was established. *THL* thiolase, *CTF* coenzyme A transferase, *ADC* acetoacetate decarboxylase, *CoA* coenzyme A

this experiment, evolutionary engineering has been proven to be effective for improving branched-chain-alcohol tolerance of host strains.

The second strategy for improving alcohol tolerance of microorganisms is by rational design and metabolic engineering. Liao's group [41] used information from genomic analysis of an evolutionary strain with improved tolerance to isobutanol and reconstructed one strain with improved tolerance to isobutanol. Improving alcohol tolerance of microorganisms by rational design is based on sufficient information provided by omics analysis, and this is going to be one routine approach for improving industrial strains with the progress in sequencing technology.

4.3 Microbial Production of BCHAs

Many species of *Clostridium* have been investigated for isopropanol production, but the maximum concentration of isopropanol produced is no more than 30 mM [45]. Limited knowledge about metabolic regulation of the strains and the difficulty of gene manipulation have hindered further improvements in isopropanol production. However, many clostridia could produce a high amount of acetone, and Bermejo et al. [46] produced acetone in *E. coli* by introducing three genes from *C. acetobutylicum* ATCC 824 [*THL*, *CTF*, and *ADC* encoding acetyl coenzyme A (CoA) acetyltransferase, acetoacetyl-CoA transferase, and acetoacetate decarboxylase, respectively]. This engineered *E. coli* strain produced almost the same level of acetone as *C. acetobutylicum* ATCC 824 does.

Therefore, biosynthesis of isopropanol (Fig. 3) can be engineered into host strains by introducing the pathway from *Clostridium beijerinckii* or other clostridia, which produces isopropanol from acetyl-CoA via acetone [37]. First, an acetyl-CoA acetyltransferase condenses two molecules of acetyl-CoA, which is a

regular metabolite in almost all organisms, to one molecule of acetoacetyl-CoA. Next, an acetoacetyl-CoA transferase transfers CoA from acetoacetyl-CoA to acetate or to butyrate, forming acetoacetate, which is then converted to acetone and carbon dioxide by an acetoacetate decarboxylase. Finally, a primary–secondary alcohol dehydrogenase (such as the secondary alcohol dehydrogenase) converts acetone to isopropanol in an NADPH-dependent reaction.

With renewed interest in alternative fuels, the production of isopropanol, especially by a renewable route (fermentation or synthetic biosynthesis), has become an important topic to study. In the traditional fermentative process, isopropanol can be produced naturally by various strains of *Clostridium*, with maximum production levels reaching 2 g/L [45]. In the synthetic biosynthesis pathway, the production of isopropanol has been investigated in tractable heterologous organisms. The first work using a synthetic pathway for isopropanol production in *E. coli* was reported by Hanai et al. [37]. Their strategy utilized the pathway models of *Clostridium*. Various combinations of genes from *Clostridium* species were cloned and expressed in *E. coli*, and the engineered *E. coli* was able to produce 81.6 mM isopropanol after 30.5 h in shake flasks with a mole yield of 43.5% in the production phase. Afterward, Jojima et al. [38] engineered *E. coli* harboring the isopropanol-producing pathway consisting of thiolase, CoA transferase, actoacetate decarboxylase, and alcohol dehydrogenase from *Clostridium*, and produced up to 227 mM isopropanol with a mole yield of 51% from glucose under aerobic fed-batch conditions. Recently, Inokuma et al. [47] further improved isopropanol production by optimization of the fermentation conditions and removal of isopropanol using metabolically engineered *E. coli* strain TA76, producing about 2,378 mM (143 g/L) isopropanol after 240 h with a mole yield of 67.4%, which indicates great potential for commercial fermatative isopropanol production. However, the low production rate is still a significant hurdle for the low-cost fermentative production of isopropanol, and improvement is still necessary, which would be likely approached by additional metabolic engineering and optimization of all aspects of the fermentation system. Unfortunately, no engineering microorganisms for isopropanol production have been reported in China.

Isobutanol, 2-methyl-1-butanol, and 3-methyl-1-butanol are major organoleptic compounds in fermented foods, such as alcoholic beverages, soy sauce, and bread. The synthetic pathway for the production of these BCHAs was proposed by Ehrlich [48]. The Ehrlich pathway involves three enzymatic activities (Fig. 4). Firstly, branched-chain amino acids are transaminated to the corresponding oxoacids by branched-chain amino acid transaminases. The ammonia binds to 2-oxoglutarate and the glutamate so formed can subsequently serve as a nitrogen donor for all other nitrogen-containing compounds in the cell [49, 50]. Secondly, branched-chain oxoacids are converted to branched-chain aldehydes via a decarboxylation reaction. Finally, the branched-chain aldehyde is reduced to the corresponding BCHA by an alcohol dehydrogenase. BCHAs can also be synthesized from the corresponding intermediate α-keto acids in the branched-chain amino acid (L-valine, L-leucine, and L-isoleucine) metabolic pathway by decarboxylation and reduction. Therefore, these α-keto acids are formed via two major pathways:

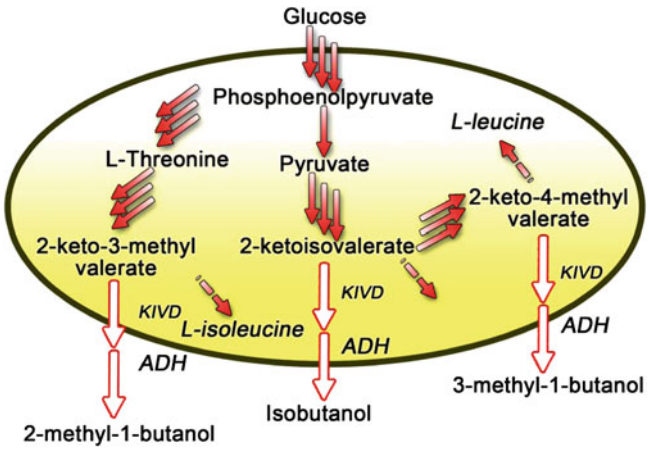

Fig. 4 Synthetic pathway and key enzymes for production of isobutanol, 3-methyl-1-butanol (isopentanol), and 2-methyl-1-butanol. When 2-keto acid decarboxylase (*kivd*) and alcohol dehydrogenase (*ADH*) are introduced, the 2-keto acid intermediates of L-valine, L-leucine, and L-isoleucine biosynthesis are directed for the production of isobutanol, 3-methyl-1-butanol, and 2-methyl-1-butanol

catabolic and anabolic pathways involved in de novo synthesis of branched-chain amino acids through their biosynthetic pathways from glucose.

As an alternative to the petrochemical production, isobutanol can also be produced from renewable bio-based feedstocks by metabolically engineered microorganisms. The team led by Liao [28] made great progress in constructing the synthetic pathway for isobutanol production in *E. coli* as well as *C. glutamicum*. The engineered strain produced 22 g/L isobutanol after 112 h [28]. This team also engineered a *C. glutamicum* strain which can produce 4.9 g/L isobutanol [51]. To use lignocellulose as the feedstock for isobutanol production, Liao and his colleagues [52] successfully engineered *Clostridium cellulolyticum* for direct conversion of cellulose to isobutanol through the process of consolidated bioprocessing. The US Department of Energy's BioEnergy Science Center (BESC) at Oak Ridge National Laboratory also developed the technology for isobutanol production directly from cellulose.

Some researchers have used *Cyanobacteria* for isobutanol production, since these organisms can use carbon dioxide as the feedstock. Heterologous expression of valine synthesis enzymes has been used to convert pyruvate to ketoisovalerate within *Synechococcus elongatus* PCC 7942. Intracellular ketoisovalerate is then converted to isobutyraldehyde at a rate of 6.23 mg/L/h by coexpression of the keto acid decarboxylase gene. Isobutyraldehyde is further converted to isobutanol by coexpression of alcohol dehydrogenase from *E. coli*. Isobutanol was recovered from the liquid phase in about 50% yield [53].

Two research groups in China have also been engaged in engineering microorganisms for isobutanol biosynthesis. The team led by Jianping Wen of Tianjin University [42, 54] engineered *B. subtilis* 168 for isobutanol production through

overexpression of the ketoisovalerate decarboxylase and alcohol dehydrogenase genes and optimizing the fermentation conditions. The engineered strain produced 0.6 g/L isobutanol after 36 h. The team led by Xue-Li Zhang of Tianjin Institute of Industrial Biotechnology engineered *E. coli* for isobutanol production (Two patents, CN201110155176.0 and CN201120096157.0, have been applied). The native fermentation pathways of *E. coli* competing for pyruvate (lactate dehydrogenase, pyruvate-formate lyase, alcohol dehydrogenase, fumarate reductase) were firstly deleted, and the essential genes of the isobutanol synthetic pathway were then integrated into the chromosome of the engineered strain. Expression of these essential genes was further fine-tuned to obtain the optimal strength for isobutanol production. Since NADPH is required for isobutanol production and NADH is produced during glycolysis, cofactor engineering was utilized to obtain the maximum yield for anaerobic isobutanol production. After an engineered strain coupling isobutanol production with cell growth had been obtained, metabolic evolution was utilized to improve both the anaerobic cell growth and isobutanol production.

A previous report showed that 3-methyl-1-butanol was identified in a small quantity in the fusel alcohols produced by *Saccharomyces cerevisiae* during ethanol fermentation [55]. Compared with the wild-type cells producing 3-methyl -1-butanol at 92.3 mg/L at 24 h, the mutant showed a 1.8–2.3-fold increase in production. In 2008, Liao's group [28] demonstrated the production of 3-methyl -1-butanol in *E. coli*. They engineered an *E. coli* strain to produce 3-methyl -1-butanol from glucose via the host's amino acid biosynthetic pathways [28]. Recently, they further improved the 3-methyl-1-butanol production by random mutagenesis of L-leucine biosynthesis and two-phase fermentation and obtained 9.5 g/L 3-methyl-1-butanol after 60 h with a yield of 0.11 g/g glucose, showing promise in using *E. coli* as a host for 3-methyl-1-butanol biofuel production [56]. Microbial production of 3-methyl-1-butanol and 2-methyl-1-butanol in China has not been reported yet. A preliminary study is currently being conducted in the authors' laboratory.

5 Concluding Remarks

Although R & D into microbial production of BCHAs in China is relatively weak, the lag is not significant, since the worldwide research in this area is still in its infancy. Driven by the energy requirements and environmental concerns, more efforts devoted to fundamentals and technological innovations will enable our next-generation biofuels to be more competitive in the market, and contribute to the sustainability of the country's economic and social development.

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References

1. An H, Wilhelm WE, Searcy SW (2011) Biofuel and petroleum-based fuel supply chain research: a literature review. Biomass Bioeng 35:3763–3774
2. Cherubini F, Strømman AH (2011) Life cycle assessment of bioenergy systems: state of the art and future challenges. Bioresour Technol 102:437–451
3. Zinoviev S, Müller-Langer F, Das P, Bertero N, Fornasiero P, Kaltschmitt M, Centi G, Miertus S (2010) Next-generation biofuels: survey of emerging technologies and sustainability issues. ChemSusChem 3:1106–1133
4. Peralta-Yahya PP, Keasling JD (2010) Advanced biofuel production in microbes. Biotechnol J 5:147–162
5. Connor MR, Liao JC (2009) Microbial production of advanced transportation fuels in non-natural hosts. Curr Opin Biotechnol 20:307–315
6. Mainguet SE, Liao JC (2010) Bioengineering of microorganisms for C_3 to C_5 alcohols production. Biotechnol J 5:1297–1308
7. Olson ES, Sharma RK, Aulich TR (2010) Higher-alcohols biorefinery: improvement of catalyst for ethanol conversion. Appl Biochem Biotechnol 113–116:913–932
8. Zhuang D, Jiang D, Liu L, Huang Y (2011) Assessment of bioenergy potential on marginal land in China. Renew Sust Energ Rev 15:1050–1056
9. Zhang XQ (2011) Annual report on bioindustry in China: 2010. Chemical Industry Press, Beijing
10. Wu ZJ (2011) The problems and suggestions for the development of biodiesel industry in China. China Biodiesel 2(2):5–6
11. Ni Y, Sun Z (2009) Recent progress on industrial fermentative production of acetone-butanol-ethanol by *Clostridium acetobutylicum* in China. Appl Microbiol Biotechol 83(3):415–423
12. Wang Q (2011) Time for commercializing non-food biofuel in China. Renew Sust Energ Rev 15:621–629
13. Liu J, Diamond J (2005) China's environment in a globalizing world. Nature 435:1179–1186
14. Fang X, Shen Y, Zhao J, Bao X, Qu Y (2010) Status and prospect of lignocellulosic bioethanol production in China. Bioresour Technol 101:4814–4819
15. Xiang M, Li D, Xiao H, Zhang J, Qi H, Li W, Zhong B, Sun Y (2008) Synthesis of higher alcohols from syngas over Fischer–Tropsch elements modified K/β-Mo2C catalysts. Fuel 87:599–603
16. Ou X, Zhang X, Chang S (2010) Alternative fuel buses currently in use in China: life-cycle fossil energy use, GHG emissions and policy recommendations. Energy Policy 38:406–418
17. Hu Z, Tan P, Yan X, Lou D (2008) Life cycle energy, environment and economic assessment of soybean-based biodiesel as an alternative automotive fuel in China. Energy 33:1654–1658
18. Sun X, Wang X (2008) Status quo of restricted MTBE market worldwide. Chem Ind 26(6): 16–22
19. Lin CW, Chiang SB, Lu SJ (2005) Investigation of MTBE and aromatic compound concentrations at a gas service station. Environ Monit Assess 105:327–339
20. Lee I, Johnson LA, Hannond EG (1995) Use of branched chain esters to reduce the crystallization temperature of biodiesel. J Am Oil Chem Soc 72:1155–1160
21. Cui XM (2008) Production technologies and market analysis of isopropanol. Shanghai Chem Ind 33:31–34
22. Cheng J, Jiang C (2007) Analysis on process technology and market situation of isobutyl alcohol worldwide. Chem Ind 25(10):28–31
23. Liu Y, Xue HF (2010) Domestic and international market analysis and suggestions for carbonyl alcohol technology. Chem Ind Eng Prog 29:970–975
24. Wutai Consulting (2011) Marketing research report of 3-Methy-1-butanol. http://www.timesprc.com.cn/jx/2120.htm
25. Hazelwood LA, Daran JM, van Maris AJ, Pronk JT, Dickinson JR (2008) The Ehrlich pathway for fusel alcohol production: a century of research on *Saccharomyces cerevisiae* metabolism. Appl Environ Microbiol 74:2259–2266

26. Ayres EE (1929) Amyl alcohols from the pentanes. Ind Eng Chem 21:899–904
27. Faith ML, Clark RL, Donald B (1965) Keyes "industrial chemicals". Wiley, New York
28. Atsumi S, Hanai T, Liao JC (2008) Non-fermentative pathways for synthesis of branched-chain higher alcohols as biofuels. Nature 451:86–89
29. Dellomonaco C, Fava F, Gonzalez R (2010) The path to next generation biofuels: successes and challenges in the era of synthetic biology. Microb Cell Fact 9:3
30. Manzer LE (2010) Recent developments in the conversion of biomass to renewable fuels and chemicals. Top Catal 53:1193–1196
31. Yan Y, Liao JC (2009) Engineering metabolic systems for production of advanced fuels. J Ind Microbiol Biotechnol 36:471–479
32. Ma YH (2010) Development report of industrial biotechnology in China. Science Press, Beijing
33. Pei L, Schmidt M, Wei W (2011) Synthetic biology: an emerging research field in China. Biotechnol Adv. doi:10.1016/j.biotechadv
34. Nielsen J (2011) Biofuels: chimeric synthetic pathways. Nat Chem Biol 7:195–196
35. Clomburg JM, Gonzalez R (2010) Biofuel production in *Escherichia coli*: the role of metabolic engineering and synthetic biology. Appl Microbiol Biotechnol. 86:419–434
36. Derrick S, Large PJ (1993) Activities of the enzymes of the Ehrlich pathway and formation of branched-chain alcohols in *Saccharomyces cerevisiae* and *Candida utilis* grown in continuous culture on valine or ammonium as sole nitrogen source. J Gen Microbiol 139: 2783–2792
37. Hanai T, Atsumi S, Liao JC (2007) Engineered synthetic pathway for isopropanol production in *Escherichia coli*. Appl Environ Microbiol 73:7814–7818
38. Jojima T, Inui M, Yukawa H (2008) Production of isopropanol by metabolically engineered *Escherichia coli*. Appl Microbiol Biotechnol 77:1219–1224
39. Chen X, Nielsen KF, Borodina I, Kielland-Brandt MC, Karhumaa K (2011) Increased isobutanol production in *Saccharomyces cerevisiae* by overexpression of genes in valine metabolism. Biotechnol Biofuels 4:21
40. Minty JJ, Lesnefsky AA, Lin F, Chen Y, Zaroff TA, Veloso AB, Xie B, McConnell CA, Ward RJ, Schwartz DR, Rouillard JM, Gao Y, Gulari E, Lin XN (2011) Evolution combined with genomic study elucidates genetic bases of isobutanol tolerance in *Escherichia coli*. Microb Cell Fact 10:18
41. Atsumi S, Wu TY, Machado IM, Huang WC, Chen PY, Pellegrini M, Liao JC (2011) Evolution, genomic analysis, and reconstruction of isobutanol tolerance in *Escherichia coli*. Mol Syst Biol 6:449
42. Li S, Wen J, Jia X (2011) Engineering *Bacillus subtilis* for isobutanol production by heterologous Ehrlich pathway construction and the biosynthetic 2-ketoisovalerate precursor pathway overexpression. Appl Microbiol Biotechnol 91:577–589
43. Dunlop MJ, Dossani ZY, Szmidt HL, Chu HC, Lee TS, Keasling JD, Hadi MZ, Mukhopadhyay A (2011) Engineering microbial biofuel tolerance and export using efflux pumps. Mol Syst Biol 7:487
44. Jia K, Zhang Y, Li Y (2010) Systematic engineering of microorganisms to improve alcohol tolerance. Eng Life Sci 10:422–429
45. Chen J-S, Hiu SF (1986) Acetone-butanol-isopropanol production by *Clostridium beijerinckii* (synonym, *Clostridium butylicum*). Biotechnol Lett 8:371–376
46. Bermejo LL, Welker NE, Papoutsakis ET (1998) Expression of *Clostridium acetobutylicum* ATCC 824 genes in *Escherichia coli* for acetone production and acetate detoxification. Appl Environ Microbiol 64:1079–1085
47. Inokuma K, Liao JC, Okamoto M, Hanai T (2010) Improvement of isopropanol production by metabolically engineered *Escherichia coli* using gas stripping. J Biosci Bioeng 110:696–701
48. ter Schure EG, Flikweert MT, van Dijken JP, Pronk JT, Verrips CT (1998) Pyruvate decarboxylase catalyzes decarboxylation of branched-chain 2-oxo acids but is not essential for fusel alcohol production by *Saccharomyces cerevisiae*. Appl Environ Microbiol 64: 1303–1307

49. Dickinson JR, Norte V (1993) A study of branched-chain amino acid aminotransferase and isolation of mutations affecting the catabolism of branched-chain amino acids in *Saccharomyces cerevisiae*. FEBS Lett 326:29–32
50. Sentheshanmuganathan S (1960) The mechanism of the formation of higher alcohols from amino acids by *Saccharomyces cerevisiae*. Biochem J 74:568–576
51. Smith KM, Cho K-M, Liao JC (2010) Engineering *Corynebacterium glutamicum* for isobutanol production. Appl Microbiol Biotechnol 87:1045–1055
52. Higashide W, Li Y, Yang Y, Liao JC (2011) Metabolic engineering of *Clostridium cellulolyticum* for production of isobutanol from cellulose. Appl Environ Microbiol 77: 2727–2733
53. Atsumi S, Higashide W, Liao JC (2009) Direct photosynthetic recycling of carbon dioxide to isobutyraldehyde. Nat Biotechnol 27:1177–1180
54. Jia X, Li S, Xie S, Wen J (2011) Engineering a metabolic pathway for isobutanol biosynthesis in *Bacillus subtilis*. Appl Biochem Biotechnol. doi: 10.1007/s12010-011-9268-1
55. Abe F, Horikoshi K (2005) Enhanced production of isoamyl alcohol and isoamyl acetate by ubiquitination-deficient *Saccharomyces cerevisiae* mutants. Cell Mol Biol Lett 10:383–388
56. Connor MR, Cann AF, Liao JC (2010) 3-Methyl-1-butanol production in *Escherichia coli*: random mutagenesis and two-phase fermentation. Appl Microbiol Biotechnol 86:1155–1164

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Advances in Biogas Technology

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Abstract Biogas technology has been practiced for over a century and is widely used in full-scale facilities in China. However, there are still many technological and economic barriers to be overcomed in its applications. Recent advances and multi-disciplinary cooperations in microbiology, biochemistry, and engineering science are bringing new promises of a better understanding and control of the anaerobic digestion processes, and thus a renaissance of this technology. In particular, great progress in biogas technology has been achieved in China in the approach to larger-scale and more widespread applications. This chapter overviews the recent advances in biogas technology in China, evaluates the current challenges, and discusses the emerging technologies and future perspectives.

Keywords Anaerobic digestion · Lignocellulosic waste · Methanogen · Municipal solid waste · Process engineering

Contents

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1 Methanogens Involved in Biogas Fermentation

1.1 Methanogens

Methanogens involved in biogas fermentation are very diverse. Taxonomically, methanogens belong to the Euryarchaeota, and have been identified as Genera 25 of Class 3. The latest taxonomic system is shown in Figure 1 [1].

In recent years, psychrophilic methanogens and acidophilic methanogens with specific environmental adaptability have also been discovered. Psychrophilic methanogens that grow below 10 degrees celsius were not isolated until the 1990s [2]; by the 2000s, 84 species of methanogens had been isolated and named [3]. Most acidophilic methanogens grow in specific acidic condition. These acidic environments are mainly geothermal environments and mining areas that generally contain contaminants. The main species of methanogen are *Thiobacillus thiooxidans*, *Thiobacillus ferrooxidans* etc. [4].

In biogas fermentation systems, the non-methanogens and methanogens usually coexist. These bacterias may benefit or inhibit each other, and thus a balanced community structure can be maintained. Non-methanogens can (1) provide substrates for the growth and methane production of methanogens, (2) maintain appropriate oxidation–reduction potential (ORP) for methanogens, (3) remove toxic substances that may inhibit methanogens, and (4) eliminate feedback inhibition for methanogens. Meanwhile, non-methanogens and methanogens together maintain an appropriate pH during biogas fermentation.

1.2 Conditions for Predominance of Methanogens

Temperature: The optimal temperature range for methanogen growth and methane production is 25–30 C [5]. *Trace metal elements*: Addition of trace metal elements can change the dominant species of methanogen, and facilitate increased acid utilization and toxic resistance [6, 7]. *Salts*: Sulfate-reducing bacterias (SRB) compete with methanogens for substrate. Additionally, the sulfide produced has a significant toxic effect on methanogens [8]. *pH*: The optimal pH range for methanogen growth is 6.8–7.5. The medium pH affects the cell surface charge, the ionization of organic compounds, and the microbial resistance to high temperature. In addition, pH also significantly influences enzyme activity [9]. *ORP*: ORP is a more accurate indicator of the system's anaerobic status than DO [10]. The optimal ORP range for methanogens is below −350 mV. Anaerobic environments are characterized by low ORP.

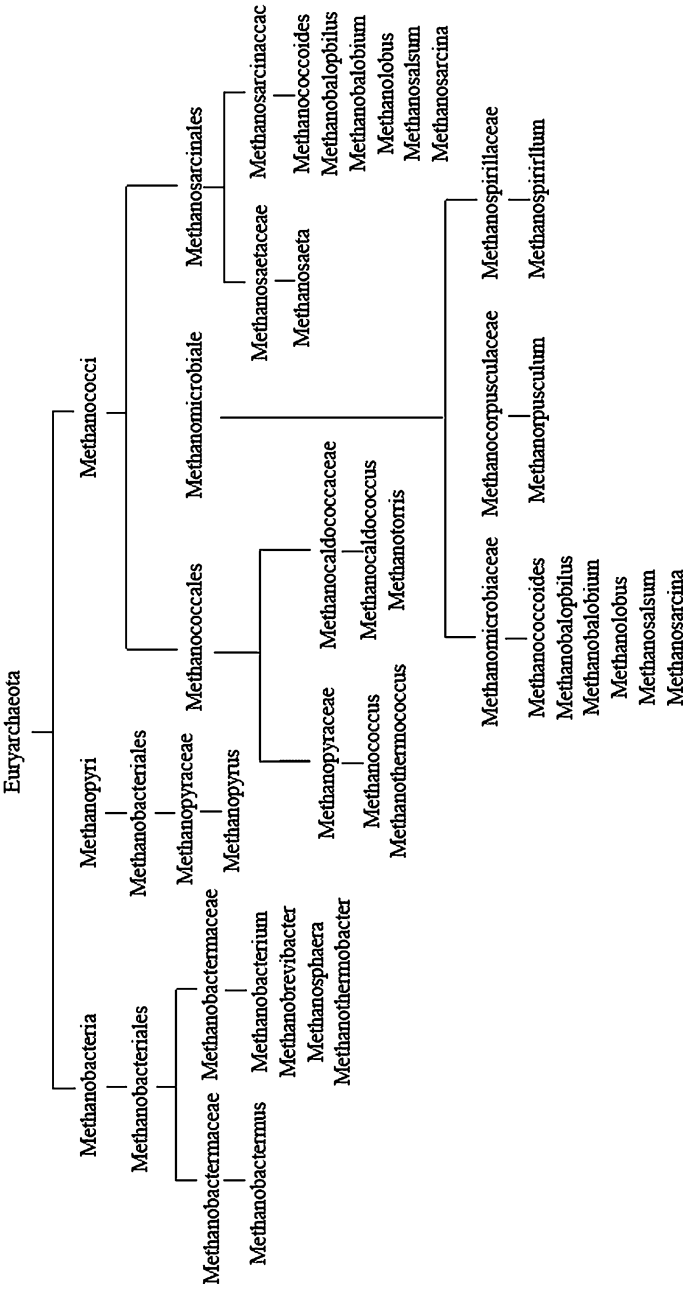

Fig. 1 Taxonomic system of methanogens

2 Biogas Fermentation Processes

2.1 Anaerobic Digesters

An anaerobic digester (AD), also known as a methane fermentation reactor, is a central device in a biogas system. Relatively mature reactor configurations, such as anaerobic filter (AF), up-flow anaerobic sludge bed (UASB), continuous stirred-tank reactor (CSTR), and up-flow blanket filter (UBF), have been widely used to treat urban sewage and wastewaters from alcohol, starch, and organic acid fermentation industries and at the same time to recover methane. Most biogas facilities for combined treatment of agricultural solid waste and livestock manure are still on household scales. Many obstacles remain in the pretreatment of biomass, further treatment of residue and slurry, and promotion of their effective use. In addition, challenges in scale-up, economy, environmental protection, and recycling issues are yet to be addressed [11].

Statistics for 2002 showed that the major reactor types adopted for biogas projects in China were UASB, CSTR, internal circulation (IC), and expanded granular sludge bed (EGSB) [12]. Among the existing large- and medium-sized methane projects, there were 200 sites of UASB and 128 sites of CSTR, which in all accounted for more than 80% of the total market in China.

Due to numerous advantages, such as needing no mechanical stirring, being applicable to wastewater of various organic loads, easy operation, small footprint, and high performance in granular sludge cultivation, UASB reactors have been widely used in China, accounting for 49.26% of the total market. UASBs enable reduced biomass washout, and thus usually have high fermentation efficiency. However, they are not suitable for wastewaters that contain high suspended solid (SS) content of more than 3,500 mg/L. In such cases, CSTRs could be a more desirable choice, as they demonstrate superior capability for treatment of high SS-containing and high temperature wastewaters. For this reason, CSTRs are more frequently adopted in alcohol wastewater treatment processes.

In China, the largest anaerobic fermentation tank group of CSTRs is located in Taichang Xintai Alcohol Company, Jiangsu Province. This facility was first built and put into operation in 1999, with a total volume of 13,200 m^3. It is capable of processing 2,000 m^3/d of cassava wastewater and producing 40,000 $m^3$of biogas. The largest single-tank anaerobic fermenter is in Nanyang alcohol plant of Henan province, which has a volume of 5,000 m^3. The largest UASB anaerobic project is in Xuzhou Fangting winery, with a tank group total volume of 10,000 m^3. In China, the maximum single-tank volume of UASB has reached 4,000 m^3.

In recent years, ICs and EGSBs are gaining increasing popularity in China due to their high efficiency and small footprint. These reactors are especially suitable for wastewater with low SS and low organic concentration, such as paper mill wastewater, beer wastewater, and starch wastewater.

The development of anaerobic reactors has evolved from the traditional civil engineering model to the equipment model in China. The content of engineering

Table 1 Anaerobic biogas projects of different industries [15]

Industry	Number	Proportion (%)
Fermentation alcohol	156	38.42
Sugar alcohol	15	3.69
Beer	47	11.58
Beverage	16	3.94
Starch, sugar, food	73	17.98
Citrate acid	21	5.17
Pharmaceutical	20	4.93
Monosodium glutamate	24	3.44
Meat	23	5.67
Petrochemical	12	2.96
Paper	9	2.21

equipment construction technology and the corresponding investment have significantly increased. At present, although some large-scale anaerobic engineering structures have started to use reinforced concrete, most anaerobic reactors still use steel structure. There is still a long way to go for the standardization, automation and complete set of the equipment in this area.

In the past decade, China has been putting great efforts into standardization, serialization, and industrialization of CSTR and UASB reactors for anaerobic fermentation. A series of full-mixing anaerobic fermentation tanks, eject pump stir, and gas stir have been developed. For UASBs, there are now two series of standardized configurations, rectangle and round types. Recently, some new material and new technology developed by other countries have been introduced into China and incorporated into the reactor design, such as the two-fold bite 1:3 assemble technology from Lipp GmbH, Germany, and the canning technology from Farmatic, Germany. Such improvements in construction methods have brought distinct economic benefits. For example, with the employment of assembling or rolling techniques, the construction period has been considerably shortened and 50% of the material cost can be saved compared to conventional steel structures. In addition, some confer additional advantages of high corrosion-resistance and strength. Such modern manufacturing technologies have been widely accepted in China, and currently there are already dozens of devices in China constructed using these technologies [12].

2.2 Substrates

2.2.1 High-Strength Wastewater

The data from the National Statistical Yearbook show that, by 2002, 8.5 billion tons of organic wastewater and 24.4 million tons of waste were produced each year from over 10 light industries, including the alcohol, sugar, beer, wine, liquor,

Table 2 Selected anaerobic biogas projects in China [15]

Province/city	Number	Proportion (%)
Shandong	105	29.09
Sichuan	37	10.25
Jiangsu	36	9.97
Henan	20	5.54
Anhui	17	4.71
Guangdong	17	4.71
Zhejiang	17	4.71
Liaoning	16	4.43
Beijing	16	4.43
Hebei	15	4.16
Jilin	14	3.88
Guangxi	11	3.05
Hunan	11	3.05
Hubei	11	3.05
Tianjin	9	2.49
Heilongjiang	9	2.49

starch, monosodium glutamate, beverage, and paper industries. If the organic matter contained in these waste streams can be converted into biogas, then 63.5 million $m^3$of biogas would be produced per year [12].

By October 2003, 406 sites of industrial-scale biogas projects had been built. The total volume of anaerobic engineering facilities reached 1.5 million m^3, with an average of 2,500 m^3per block. The annual capacities for wastewater treatment and biogas production were 150 million $m^3$and 10 billion m^3, respectively. Tables 1 and 2 list the biogas plants classified by waste type and regional location.

As can be seen from Table 1, China's biogas projects have been widely applied for treatment of wastewater from about 20 industries, of which the wastewater from the alcohol, starch, beer, fermentation, slaughtering and pharmaceutical industries accounted for about 90%.

Taicang Xintai Alcohol Co., Ltd. produces 56,000 tons of alcohol each year. Cassava is used as the raw material, and the wastewater discharged was 1,500–2,000 ton/year [13]. This is a type of acidic organic wastewater that has high density and high SS concentration. The chemical oxygen demand (COD) concentration is 50,000–60,000 mg/L with an average of 57,500 mg/L. The SS is about 20,000–30,000 mg/L and pH is 3–4.

In 1998, the company invested 20 million RMB Yuan to build a biogas technology-based sewage treatment plant, which covers an area of 1,600 m^2. The entire treatment process includes full-mixing anaerobic digestion (CSTR 2,200 $m^3 \times 6$) + UASB anaerobic treatment (1,000 m^3) + SBR aerobic treatment (1,884 $m^3 \times 2$).

To further recover electricity from the produced biogas, in 2002 and 2003 the company added 8.8 million Yuan investment to upgrade the process based on the existing gas production plant and captive power plant. A new 600 kW generator that burns biogas and a set of gas purification equipment for the existing stream

Table 3 Development of straw biogas fermentation technology in China

Feedstock	Pretreatment method	Fermentation conditions	Efficiency	Reference
Corn stover	8% NaOH, 15 C, 20 d	35 C, 150 rpm	Improving 207.1%	[19]
	5% ammonia, 15 C, 20 d		Improving 105.8%	
	4% carbamide, 15 C, 20 d		Improving 15.9%	
	P. florida		Improving 165.1%	
	Compound bacteria	25 C, 30 d	Improving 57.4%	[20]
	Coriolus versicolor and white-rot fungi	37 C	703.1 mL/d	[21]
	Lv jieling, 55 C, 5 d	37 C, 11 d	422.4 mL/g TS	[22]
	Enzymatic microorganism, 55 C, 5 d		455.7 mL/g TS	
	Speed decay fungi, 55 C, 5 d		418.8 mL/g TS	
	Lime water, 55 C 5 d		401.4 mL/gT S	
	Straw : pig manure = 7:3	35 C	0.8−1.0 $m^3/(m^3$ d)	[23]
	Steam explosion	50 C	7.54 mL/ (g VSS d)	[24]
	4% NaOH, 100 C, 3 h	35 C	Improving 117.9%	[25]
	6% NaOH, ultrasonic treatment at 360 kHz for 60 min		Improving 149.8%	
Rice straw	5 wt%*white-rot fungi*	55 C, 59 d	282.1 L/kg VS	[26]
		35 C, 59 d	243.3 L/kg VS	
		25 C, 59 d	184.5 L/kg VS	
	5 wt%*white-rot fungi*, 7 d	25 C, 45 d	0.457 m^3/kg TS	[27]
	1%NaOH, 90 C, 2.5 h 100 μmol/L Mg^{2+}, 0.125%, Twain 80	37 C, 26 d	Improving 569.8%	[28]
	Trichoderma pseudokoningii and *white-rot fungi*	35 C, 110 r/min	Improving 55.6%	[29]
	Trichoderma pseudokoningii		Improving 30.5%	[29]
Wheat straw	*Lv jieling*, 50 C, 3 d	25 C, 35 d	31.14 m^3	[30]
	Speed decay fungi, 50 C, 5 d		32.23 m^3	
Calla stalk	–	23 C, 22 d	1006.36 mL/g VS	[31]
Reed straw	5% NaOH, 25 C, 15 d	35 C, 100 r/min	1,269 mL/d	[32]
Rape stalk	Compound bacteria	25 C	Improving 37.6%	[33]

generator were installed. With these improvements, the annual biogas output of the project reached 12 million m^3, and the annual power generating capacity reached 11 million kWh, including 1.5 million kWh from biogas burning and 9.5 million kWh from the stream generator.

In China, the main challenges for biogas fermentation from organic wastewater include: (1) Lack of policy support. Large and medium-sized biogas projects involve both environmental pollution control and energy recycling. To promote the application of such projects, the government needs to incorporate it into the state construction plan, offer more financial support, and give more priority for its development. (2) Lack of economic incentives. Biogas projects usually need high initial investment. Thus preferential policies should be given, such as investment incentives, tax exemption/release and tax repayment during the project construction and implementation. (3) Lack of technology standards and specifications. Design and construction of biogas plants still lack the appropriate technical standards and specifications. The defective technical service network brings difficulties in project operation, management and maintenance. The losses and adverse effects from unreasonable design as well as the lax construction standards also affected the development of industrial biogas projects. (4) Lack of information sources and channels in development of biogas projects. The main organic wastewater discharge in China is from small and medium enterprises, which are numerous and are widely spread all over the country. However, many of these companies lack information sources and channels for developing biogas projects. In recent years, the loss resulting from fraud presents another obstacle to the development of industrial-based biogas projects.

2.2.2 Lignocellulosic Waste

China is a large agricultural country with 80% of its population living in rural areas. About 700 million tons of crop straw are produced each year, of which, about 370 million tons of straw can potentially be used as an energy source [14]. Anaerobic methane fermentation is an important avenue for recovering energy from straw and other biomass resources. Using this technology, the large amount of straw can be efficiently used for energy production at relatively small scale with low investment [15].

Intensive investigations on improving biogas production from lignocellulosic wastes have been carried out in China. Table 3 shows the recent development of straw biogas fermentation technology in China [16–30].

By the end of 2009, about 10 large-scale straw biogas projects had been built and put into operation in China, and 16 pilot-scale projects were operated in 13 provinces (cities, districts) including Heilongjiang, Inner Mongolia, Tianjin, Hebei, Henan, Shandong, Shanxi and Shaanxi. Recently, Jiangsu, Guangxi and several other provinces also started to play an active role in exploring regional straw biogas technology and improving its management [31].

One established large-scale straw biogas demonstration project is located in Tangshan City, Hebei Province. This project adopts special double-membrane technology, and has a total reactor volume of 600 $m^3$and annual biogas production of 300,000 m^3. The biogas production can meet the needs of household use for fuel and lighting. Thus, it holds great promise in resolving the contradictions between fuel, feed and fertilizer.

Zibo, in Shandong Province of China, has successfully started two large-scale straw biogas projects and found a new way to make better use of corn stalks from more than 600 hectares of land. 20 million Yuan has been invested to build a 1-km^2 large-scale straw biogas project, including a 1,000-m^3 methane fermentation tank and a 600-m^3 air tank. Now the main part of this project has been finished. Upon the completion of project construction, it will annually consume 200 hm^2 of corn stover, produce 1,000 m^3 Of biogas per day, and will be able to meet the daily energy needs of 700–1,000 residents. In addition, it will produce 1,200 tons of high-quality solid organic fertilizer and 30,000 tons of liquid organic fertilizer, bringing an annual direct economic benefit of 40 million Yuan.

Another straw biogas project is also under construction in Zibo City. It has a total investment of 500 million Yuan and total fermentation tank volume of 3,000 m^3. The first-stage construction is under way, with an initial investment of 180 million Yuan. A 1,000-m^3 large-scale biogas fermentation tank will be built in this plant, covering an area of 1 hm^2. So far, more than half the project has been finished. It is expected to provide a direct economic benefit of more than 80 million Yuan after completion.

In addition, a centralized straw biogas supply system is planned in Jintan City, Jiangsu Province. This system will adopt medium-temperature fermentation technology. The main works consist of four 75-m^3 red-mud plastic anaerobic tanks, one 21-m^3 regulating reservoir, one 100-m^3 reservoir bag, and one 64-m^3 liquid storage tank. When finished, the project will handle annually about 80 tons of crop stalks and produce biogas to meet the need of 100 households [15].

The main challenges for lignocellulosic waste biogas technology facing China are as follows:

1. Efficient pretreatment technology is still lacking. It is difficult to handle straw in a fermentation system without pretreatment, because the straw easily floats and crusts in the fermentation liquor. The solid-state fermentation technology also faces problems such as poor mass transfer and gas release, high mixing resistance and easy acidification. Thus, straw pretreatment is necessary to overcome these restrictions. However, pretreatment technology is currently still in its early stages of development in China. No pretreatment methods are mature enough to be ready for large-scale biogas production application.
2. High-rate anaerobic digestion reactors specially designed for straw treatment are needed. Most of the existing anaerobic digestion reactors for biogas production have been designed for digestion of easily degradable substrates, such as manure and food-processing wastewater. These anaerobic digestion reactors have a very wide scope of applications, but they are not suitable for anaerobic

digestion of straw. Thus, crop straws are often used as "auxiliary material" instead of "main material" in anaerobic digestion process.

3. Straw biogas technology is in its infancy. At present, research on straw biogas technology in China has just begun, and no straw biogas project has run for more than 3 years so far. None of these processes, except two-phase anaerobic digestion technology, has obtained identification and approval from the provincial science and technology departments. Moreover, there are some technical, economic barriers and management problems. The total operating performance of biogas systems is not satisfactory and these processes are still to be practically certified.
4. Compared to the animal manure biogas projects, straw-based biogas projects need more work on material collection, storage and pretreatment, which all increase the cost; the quality and availability of straw varies significantly with the season, while purchasing straw directly from farmers may lead to poor price controllability and further raise the cost. The existing straw biogas projects in China mostly adopt a concentrated biogas supply mode, and their products are biogas and fermentation residue. As a possible benefit, the biogas can be sold at a low price to farmers as an energy source for cooking, while the fermentation residues can be made into organic fertilizer. But the idea of using fermentation residues as fertilizer has not been completely accepted by farmers, and thus the residues are basically provided to them for free at the present stage. Besides, clean production mechanisms have not been introduced, and the project benefit is still not straightforward. In summary, high investment and running cost, and low benefit from products, present significant barriers to the commercial application of straw-based biogas technologies.
5. Straw biogas projects require more support at state policy level. In recent years, especially since 2008, the Chinese government has put great emphasis on the construction of biogas projects in rural areas. The investment funds from central government, however, are mainly used to build straw biogas projects, including construction and equipment investment, while less has spent on improving the operational performance and the utilization of the final products. The poor market profitability has severely weakened the operational performance, so some projects are running below capacity or have even stopped completely.

2.2.3 Livestock Waste

Since the mid-1990s, large-scale livestock farm pollution has gained increasing attention. Especially in recent years, with rapid economic growth and improvement in living standards, China's livestock industry has also seen a rapid development. The number of cattle, pigs, sheep and poultry has shown an upward trend. According to national statistics, in 2007 the total amount of livestock manure was 2.6 billion tons, and it is expected to reach 41 million tons by 2020, The manure,

Table 4 Biogas projects for livestock waste treatment in China

Project	Raw materials	Digester scale temperature	Treatment capacity tons/d	Biogas production m^3/d
Inner Mongolia Hongwu high-tech agriculture and animal husbandry Co. Ltd.	Pig manure	CSTR 350 m^3 28 C	28	375
Hangzhou Zhengxing animal husbandry Co. Ltd	Chicken manure and cattle sewage	UASB 600 m^3 ambient temperature	150	160
Jiaxing Xiucheng district Lvjiayuan animal husbandry Co. Ltd	Pig manure sewage	Anaerobic AF 800 m^3 ambient temperature	100	220
Shandong Luozhuang intensive pig breeding farm	Pig manure	UASB 2,000 m^3 ambient temperature	100	1,450
Shandong Laizhou Nanguan village farm	Pig manure	USR 1,000 m^3	100	6,000
Inner Mongolia Mengniu Aoya biogas demonstration project	Cattle manure	CSTR 2,500 m^3 × 4	280	10,000
Engniu Wenshang modern pasture biogas power generation project	Cattle manure	CSTR 2,700 m^3	800	6,000–8,000
Shandong Minhe animal husbandry 3 MW biogas plant combined with heat, power and fertilizer production	Chicken manure	CSTR 3,200 m^3 × 8	500	30,000

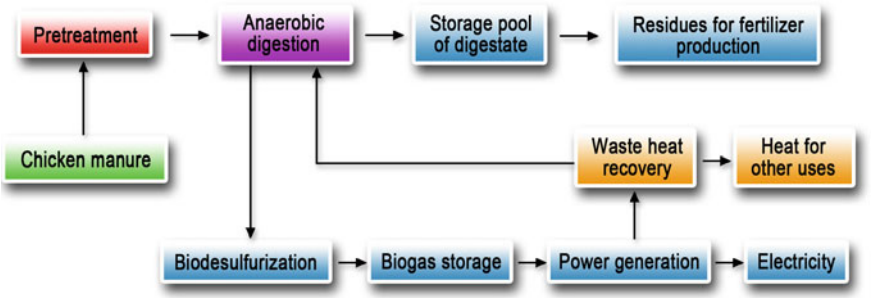

Fig. 2 Schematic diagram of the Deqingyuan biogas power project

if not properly handled, will degrade the ecological environment of farmland, cause water and air pollution, spread diseases and bring a range of environmental and health problems to the surrounding residents. On the other hand, these manures have good biodegradability and have a high potential to be utilized as a low-cost feedstock for biogas fermentation. With ever-intensifying environmental pollution and energy shortages, bioenergy production from livestock manure is becoming more and more attractive.

Farm biogas projects now mostly use livestock manure as the raw material. Carbon material is broken down into combustible gas (methane) through microbial metabolism under anaerobic conditions. Anaerobic fermentation of livestock manure and sewage can not only obtain high-quality energy (biogas) but also clean up the environment, thus realizing multi-level utilization of the biomass resources. In this respect, China now possesses mature technology and the ancillary equipment for large-scale biogas production, which is approaching the international level. Regarding biogas project packaged technology, the whole design, including pretreatment, anaerobic fermentation, biogas transport and distribution, fertilizer production and post-treatment of fermentation liquor can be performed based on the classification of pig manure, chicken manure, cattle manure and other raw material; regarding the fermentation process, the mechanism of anaerobic fermentation has been studied and highly efficient fermentation reactors, such as UASB and EGSB, have been employed for manure treatment; regarding ancillary equipment, China has successfully developed a series of devices, including biogas combustion generators, industrial canning, automatic control devices, desulfurization and dehydration equipment and solid–liquid separation equipment [32]. The major biogas projects for livestock waste treatment in China are summarized in Table 4.

The ecologically friendly park of Beijing Deqingyuan Agricultural Scientific Co. Ltd. is the largest egg chicken-raising base in Asia. It raises 2.6 million egg chickens each year, and produces 212 tons/d (TS 30%) of chicken manure. To treat the manure, a large-scale thermoelectric biogas power plant was constructed. The total installed electrical generation capacity of this project is 1 MW. In April 2009, the Deqingyuan biogas power project started operation achieving net electricity

Table 5 Major engineering facilities of the Deqingyuan biogas power project

Treatment unit	Engineering facility	Dimension	Number	Dolid retention time, d
Pretreatment	Collecting pool	1,000 m^3	1	2
	Hydrolysis pool	1,000 m^3	1	1.5
Anaerobic digestion	Anaerobic digester	3,000 m^3	4	20
	Second fermenter	4,000 m^3	1	7
Biogas slurry	Storage pool	50,000 m^3	1	90
Treatment and utilization of biogas	First biodesulfurizer	20 m^3	4	
	Second biodesulfurizer	120 m^3	1	
	Two-film dry biogas storage	2,150 m^3	1	
	Generator	1,064 kW	2	

generation, which marked a new stage of Deqingyuan's recycling economy and sustainable development. Currently, the biogas yield of this plant is 7 million $m^3$and the electricity yield is 14 million kWh. The Deqingyuan biogas power plant is established under joint support from the United Nations Development Program (UNDP) and Global Environment Facility (GEF): The demonstration project of biogas power technology in the world. It is an important demonstration of energy conservation, emission reduction and low carbon economy development in China. The schematic diagram of the Deqingyuan biogas power project is shown in Fig. 2. Major engineering facilities are given in Table 5.

The major operational parameters of the Deqingyuan biogas power project are described below:

1. Anaerobic fermentation is performed at 38 C in the fermenter kettle with compound CSTR technique (10% TS). The biogas production rate and yield was 1.9 $m^3/(m^3$ d) and 7 million m^3, respectively.
2. This project adopts the combined heat, power and fertilizer production process. The biogas produced is utilized for electricity production by two 1,064 kW electric generators (Jenbacher). The annual average electricity and heat generation are 14 million kWh and 56 million MJ, respectively. The electrical efficiency of the generators is 38%, while the total heat and electrical efficiency of the generators is 80%. The biogas slurry can be used as fertilizer in the surrounding apple/vineyard (1 million acres) and the feed base (1 million acres). Thus, zero discharge of farm wastes can be achieved.
3. This project uses hydrolytic sand removing techniques for the pretreatment. The egg-chicken manure is characterized by high sand content. Pretreatment by hydrolysis can remove 80–90% of the sand, ensuring the normal operation of subsequent processes.
4. Biodesulfurization technology is used to remove H_2S from the produced biogas. The content of H_2S in the biogas was 3,000–4,000 ppm. After the two-stage biodesulfurization process, the content of H_2S can be reduced to less than 200 ppm, which can meet the requirements of the electricity generator operation.

Table 6 Municipal solid waste biogas plants running/under construction in China

Location		Start	Feedstock	Technology developer	Capacity, million tons/year	Cost, Yuan
Beijing	Dong Cun Taihu Coun.	2007	Restaurant- & MSW, manure	Linde Valorga Biomax	0.2	Inv.18 m, Fee13.5/t
Shanghai	Jinshan	2008	MSW, BMW		0.22	Inv. 32 m
	Putuo	2007	Municipal wet waste	Valorga Biomax	0.18– 0.29	Inv.30 m, Fee17/t
Guangzhou	Likeng (Guandong)	2007	Municipal wet waste	Valorga Biomax	0.36	Inv.32 m
Changsha	Huiming (Hunan)	2005	MSW		0.73	Inv.11 m
Mianyang	Sichuan	2002	MSW	Tunnel type	0.25 AD: 3,600 t/a	
Yingkou	Liaoning	2007	MSW, SS	Tsinghua Tongfang	0.27	Inv. 20 m
Shenyang	Liaoning	2010	BMW (source separation)	Wet AD recommended	0.12–0.20	Inv.12 m, Fee > 6/t

5. This project also installed two film dry biogas storage and biogas boilers (2 tons/h), which ensures zero emission of biogas in the generator maintenance period.

Currently, challenges for livestock waste-based biogas fermentation technology in China mainly lie in two aspects: (1) The industry chains of the biogas projects for livestock and poultry farms are not complete, which lowers the benefit of biogas projects. Even worse, secondary pollution may occur. (2) The level of industrialization is limited, because the key parts and matching equipment are mostly manufactured in small batches. The level of standardization is low. Specialized complete equipment and standardized biogas projects have not yet been developed.

2.2.4 Municipal Solid Waste

Along with the change of city garbage status and mode management, China's urban domestic waste management mode is evolving from pure terminal treatment to source control and comprehensive management. Many large landfill plants have been built in recent years. For the landfill disposal process, methane release is one critical issue needs to be addressed, and the problem is becoming more and more prominent [33]. To address this, several departments in China, including the state planning commission, state environmental protection administration, state economic and trade commission, and ministries of finance, construction and science & technology, jointly compiled and released the "National action plan on China city waste landfill gas collection and use" in 2002. It is estimated that, by 2015, the harmless handling of urban waste landfill in China will reach 60–70%, and the annual garbage treatment capacity will reach 179 million tons, 60% of which (about 110 million tons) will be subjected to sanitary landfill disposal. If each kilogram of waste produces 0.06–0.44 m^3 Biogas, and on the assumption that we recycle all the biogas generated from the landfill, an equivalent of 10–70 billion m^3 of biogas can then be obtained. The minimum value of 10 billion m^3 is equal to the present coal-bed methane production in China, and the maximum value of 70 billion m^3 is equal to one fifth of the natural gas production of the whole country. As can be seen, the amount of biogas generated from the landfill can be amazing. Meanwhile, reducing the methane emissions is also important for environmental protection [34].

In China, the technology of landfill methane production from city garbage started late, and the relevant research began only about two years ago. In 1997, the environmental protection administration initiated the program of "Promoting China's Urban Waste Landfill Methane Gas Collection and Use" in Nanjing, Anshan and Ma'anshan. At present, Nanjing has already successfully reached the goal of power generation from collected landfill gas. The project "Collect Methane Used in Automobile Fuel" in Anshan and project "Collect Methane Used in Hospital Poisonous and Harmful Waste Incineration" in Ma'anshan have also been

completed. Table 6 summarizes the large and medium-sized municipal solid waste biogas plants running or under construction in China [35].

Despite much progress achieved in recent years, there are still many challenges in the practical application and promotion of landfill biogas technologies.

1. The high cost of power output is a big problem. Usually landfills do not have a power transmission system. Therefore, selling electricity generated from the landfill gas power system requires permission from the power company. This procedure is necessary to make landfill electricity output legal and standard in internet protocols, but practically it can be very complicated. Thus, the price of landfill-derived biogas cannot compare with that from other conventional sources.
2. Lack of operational economic incentives. Although the Chinese government has introduced some economic incentive policies to promote the development of renewable energy, including landfill gas power generation, there still exist many problems in policy implementation, such as lack of grid access permission, standard power purchase agreement and electricity price calculation method, etc.
3. Lack of Chinese-made special equipment for power generation from methane. For example, the landfill gas power generation equipment in Tianziling, Hangzhou was purchased from an American company.
4. Difficulty in popularizing the technology. Unstable landfill biogas production increases the difficulty in estimating the power-generation capability and controlling the practical power generation operation.

3 Emerging Technologies

3.1 Anaerobic Membrane Reactor

The anaerobic membrane bioreactor (AnMBR) was initiated in 1978. COD removal of 90% and NH_3-N removal of 75% were achieved in an early system that integrated an anaerobic nitration pool and a membrane system for sewage treatment. Incorporating the membrane separation technology into the anaerobic process brings many advantages. In such a system, the hydraulic retention time (HRT) is uncoupled from the solid retention time (SRT), and thus high biomass concentration can be maintained under high hydraulic load and sufficient mixing, which ensures high treatment efficiency. Due to the high biomass concentration and sufficient retention of some slow-growing microorganisms, the AnMBR generally has good toxic resistance, and is especially suitable for the treatment of high organic-load and biorefractory wastewater, such as industrial wastewater and landfill leachate.

The biological treatment unit of the AnMBR usually adopts UASB, fixed-bed, fluidized-bed and two-phase-type reactors. Compared to aerobic MBR, the

AnMBR is highly cost-effective, because it needs no aeration; instead methane and biological resources can be recovered in this process. However, AnMBRs have much stricter requirements on their operating conditions. The main operational parameters include: volume organic load of 2.9–50 kg COD/(m^3 d), HRT of 12 h, SRT of 6–300 day, MLSS of 8–50 g/L, transmembrane pressure of 100–500 kPa, membrane flux of 18–150 L/(m^2 h), and COD removal of 85–97%. The side-stream-type AnMBR has been primarily adopted in practice.

Wang et al. [36] designed an AnMBR to treat the actual sewage from Tsinghua University. In this system, additional fine fiber filling was packed at the top of the anaerobic sludge bed to prevent sludge washout, and hollow fiber membranes were set in the lower part of reactor. The membrane uses polyethylene material, and has an aperture size of 0.03 μm and filtering area of 0.3 m^2. The results showed that the system could withstand severe organic load fluctuation. When the organic load changed dramatically from 0.5 to 12.5 kg/(m^3 d), the average effluent COD remained less than 20 mg/L, and the total COD removal was about 97% with no SS detected in the effluent. Guan et al. [37] used a two-phase AnMBR to treat organic wastewater polluted by industrial starch and grape bran. The COD and SS concentrations were 1,500–1,700 mg/L and 1,000–4,000 mg/L, respectively. The acidogenic reactor and methane-producing reactor were placed in a 35 C incubator, while the membrane separation unit was set between the two-phase reactors and operated under ambient temperature. A polypropylene hollow fiber membrane with an aperture size of 0.1–0.49 μm was adopted. A high COD removal of above 95% and SS removal of over 92% were achieved in this system. In addition, the introduction of a membrane unit significantly enhanced operational stability of the two-phase anaerobic nitrification system. He et al. [38] treated high organic-concentration food wastewater using an AnMBR, which consists of a complete-mixing anaerobic biological reactor and a plate-frame ultrafiltration membrane module. Polyether sulphone was used as the membrane material. This system removed 80–90% of the COD even at a high COD load of 2.3 kg/(m^3 d). The removals of SS, chroma and bacteria reached as high as 100, 98 and 99%, respectively.

3.2 Dry Digestion Technology

Dry digestion is an innovative technology that allows direct biogas production from solid-state organic wastes. The content of solid organic wastes can reach 35%, which is considerably higher than that in traditional anaerobic digesters (8%). Normally, a solid organic waste content of 20% is more suitable. Dry digestion can not only improve gas production rate and efficiency per tank volume, but also simplify the operation and reduce the cost. The digested products can be directly utilized as fertilizers or soil conditioners without dewatering. With all these advantages, the dry digestion technology is attracting increasing interest for the treatment of municipal solid waste and lignocellulosic waste [39].

Table 7 Overview of biomass gasification projects in China

Reactor	Intensity, kg/(m^2 h)	Power, kJ/h	Use
Updraft fixed-bed biomass gasifier	240	2,900	Heat production
	180	1,600	Heating boiler
Downdraft fixed-bed biomass gasifier	200	300	Tea drying
	200	660	Wood drying
	200	1,490	Gas boiler
	200	1,000	Gas supply
	200	2,000	Gas supply
	637	900	Electricity production (30 kW)
	510	920	Electricity production
Stratified downdraft fixed-bed biomass gasifier	150	160 kW	Electricity production
	150	60 kW	Electricity production
	398	2–5 kW	Electricity production
Circulating fluidized bed	1,900	4,200	Gas boiler
	2,000	67	Gas supply
	1,000	160 kW	Electricity production
Internally circulating fluidized bed		167	Gas supply

In China, studies on household dry anaerobic digesters started in the 1980s and great progress has been made since then. Wu et al. [40] proposed a wet-dry integrated digester and successfully applied it in the Huanghuaihai region to achieve high gas production at low construction costs. Sun et al. [41] designed a full-scale dry anaerobic digestion system, which consists of a sealed cylindrical digester and cylindrical red mud plastic top cover digester. These digesters have simple designs and need no daily feeding and discharge, thus they hold great potential to be promoted in rural areas. Investigation of automatic discharge dry digestion technology and related semi-continuous dry digestion technology was initiated early in 1986 [42]. The automatic discharge technology was successfully demonstrated at laboratory and pilot scales. This technology was approved by the Ministry of Agriculture of China (MAC) in August 1988, and has been commercially available since then. Ma et al. [43] designed a constant-pressure gas separation tank dry anaerobic digester and successfully demonstrated it in 30 household projects. The gas production from such a system can basically meet the energy needs for cooking and lighting of a family of four with two meals a day. In addition, Kang et al. [44] also invented an efficient small-scale voltage-stabilized automatic filtration dry anaerobic digester.

Studies of dry anaerobic digestion technology for large-scale application in China began in the 21st century. Gan et al. [45] designed a horizontal stirred digester equipped with a helical ribbon to treat livestock wastes and investigated the operational parameters. Yan et al. [46] designed a heating and preserving equipment for a livestock dry anaerobic treatment system, which can be used for medium and large intensive farms. The optimum operational parameters

(the temperature of heat medium jacket, flux, heat time and so on) for heat transfer needed by each phase were devised theoretically in the normal operation of dry anaerobic digestion process. Meanwhile, the feasibility of such a technology was experimentally demonstrated, which lays the foundation for the engineering application of the dry anaerobic digestion process.

3.3 Biomass Gasification

A broad range of wood, grass and agricultural residues can be converted into bio-oil, charcoal and producer gas or syngas through biomass gasification. In contrast to anaerobic digestion, biomass gasification is a thermochemical process. Under high temperature and pressure, the compounds in biomass are converted into a combustible gas similar to the biogas from anaerobic digestion. Like conventional biogas, the biogas created from biomass gasification can be used for power generation, heating, co-generation, etc. [47–49].

In China, the study and application of biomass gasification started early in the 1940s, in which period biogas produced from wood gasification was used to drive cars in some cities. In the 1950s, a series of wood gasifiers were designed and operated to power drainage and irrigation machinery in agricultural production. Fixed-bed gasification technology was also used for the treatment of rice husks in the food processing industry in China. In the same period, a complete wood dry distillation production line was introduced from Poland. However, the in-depth investigation of biomass gasification technology was not carried out till the late 1970s and early 1980s.

At present, the most widely used biomass gasification technologies in China are updraft fixed-bed biomass gasifier, ND series biomass gasifier, XFL series straw gasifier, wood dry distillation technology, and rice husk gasified electricity production technology. A brief comparison of these typical technologies is given in Table 7 [40, 47, 50–56].

4 Perspectives of Biogas Technologies in China

4.1 Industrialization and Standardization

The development of medium and large biogas projects in China started in the late 1970s, but progress was slow until the 2000s. At the national planning level, MAC issued the “Energy and Environmental Engineering Construction Plan of Medium and Large Livestock Farms” in 2000, the Ministry of Environmental Protection of China (MEPC) announced “Regulation on Contamination Control and Management of Livestock Farms” and “Discharge Standard of Pollutants for Livestock

and Poultry Raising Industry" (GBl8596-2001) in 2001. In 2005, MAC issued "Biogas Construction Plan of the Whole Country for 2006–2010", in which the construction of 5,000 biogas projects in key areas was planned. In addition, "Well-off Environmental Protection Action Plan" was announced by MEPC in 2006, which planned to complete 500 demonstration projects of mass livestock and poultry treatment to improve the contamination control. The implementation of all these plans has been partially completed and some are still under way.

Laws and regulations play an important role in the development of biogas engineering. After the issuing of "Renewable Resources Law of the People's Republic of China" in 2005, a series of relevant regulations have been released, such as "Tentative Management Measures for Allocation of Prices and Expenses for Generating Electricity by Renewable Energy", "Renewable Energy Relevant Management Regulations" and "Renewable Energy Development Interim Measures for the Administration of the Special Fund". In addition, "Agricultural Ecological Environment Management Regulations" has been implemented in 20 provinces/cities in China.

4.2 Matching Facilities

China has formed certain production capabilities in manufacturing matching facilities for biogas projects. Currently, a series of residential facilities have already been successfully manufactured and marketed, which have seen widespread application in practical biogas projects. The major matching facilities include: (1) Dregs and liquid separator: vertical centrifugal machine, horizontal decanter centrifuge, frame vertical pressure filter. (2) Biogas silo: wet and dry biogas silo. (3) Biogas desulfurization devices: desulfurization using dry desulfurizers, desulfurization using liquid sodium carbonate, desulfurization by air introduction. Desulfurizers have different sizes and styles. (4) Sludge dewatering: belt-type, frame-type and horizontal screw-style dewatering machines. (5) Biogas electricity generators: oil and gas mixing combustion generators of different types. The manufacturing technology of pure biogas electricity generators is still under investigation.

4.3 Future Trends in Biogas Engineering

Currently, research interest in biogas engineering in China has already transferred from biogas production alone to integrated consideration of the environmental and ecological factors and comprehensive utilization of biogas. Meanwhile, with the increasing public awareness and government support, biogas engineering in China is moving rapidly toward scaling-up and standardization. This development trend can be seen from the following aspects:

1. The feedstock of biogas engineering is expanding from single feedstock (mainly livestock) to diversified source utilization. Adding even small amounts of other biomass (such as lignocellulosic waste and municipal solid waste) to a manure digester can further increase biogas production and provide a beneficial disposal option for other wastes.
2. The location of construction of biogas engineering is expanding from rural to small towns. Mass production and utilization of biogas is the development trend of current international biogas technology. This is an efficient way to reduce the cost and improve the economic benefit of biogas projects. Small towns could thus be a highly desirable area to achieve efficient organic waste utilization and biogas production.
3. More emphasis is being put on the end uses of biogas. Currently, the most common use of biogas in China is for power generation and heating, but new technologies may broaden the use, for example producing renewable natural gas and compressed renewable natural gas vehicle fuels.
4. The operation and management of biogas engineering are gradually approaching international standard. Operational management plays a key role in biogas process engineering. The developing direction of biogas engineering management in the future will be ensuring professional standardization and introducing orderly development mechanisms.

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References

1. Yu FB, Luo XP, Guang LB et al (2008) Research advances in biogas fermentation microorganism. J Anhui Agric Sci 36(35):15658–15660 (in Chinese)
2. Simankova MV, Kotsyurbenko OR, Lueders T et al (2003) Isolation and characterization of new strains of methanogens from cold terrestrial habitats. Syst Appl Microbiol 26:312–318
3. Min H (1985) Energy metabolism in methanogenic bacteria. China Biogas 1:7–11 (in Chinese)
4. Robbins EI (2000) Bacteria and archaea in acidic environments and a key to morphological identification. Hydrobiologia 433:61–89
5. Pei ZJ, Wang DW, Zhang N et al (2009) Effect of temperature on the efficiency of methanogenic bacteria. Heilongjiang Agric Sci 5:128–129 (in Chinese)
6. Streicher C (1991) Improvement of the anaerobic digestion of diluted whey in a fluidized bed by nutrient additions. Environ Technol 12:333–341
7. Murray WD, Vanden BL (1981) Effect of nickel, cobalt and molyb-denum on perfomance of methanogenic fixed–film reactors. Appl Environ Microbiol 42:502–505
8. Abram JW, Nedwell DB (1987) Inhibition of methanogenesis by sulphate reducing bacteria competing for transferred hydrogen. Arch Microbiol 117:89–92
9. Li G, Yang LZ, Ou YF (2001) Control factors of anaerobic digestion and effect of pH and Eh. J Southwest Jiaotong University 36(5):518–521 (in Chinese)
10. Hu JJ, Zhou QY (1988) Environmental engineering microbiology. Higher Education Press, Beijing (in Chinese)

11. Ma DJ, Kong XD, Tang GS (2010) The technical parameters analysis of biomass biogas. Public Commun Sci Technol 15:135–136 (in Chinese)
12. Shen ZH (2005) Medium-sized biogas project status, problems and measures on industrial organic wastewater in China. China Biogas 23:161–166 (in Chinese)
13. Yan L, Zeng YW (2005) The feasibility analysis of power generation industry from biogas in China. China Biogas 23:233–237 (in Chinese)
14. Bian BX, Zhao YC (2005) Treatment and application of agriculture solid waste. Chem Ind Press, Beijing
15. Wang J (2009) The situation of straw resources and benefit analysis of straw biogas. Environ Prot Circul Econ 29(12):39–41 (in Chinese)
16. Zhong WZ, Zhang ZZ, Qiao W, Fu PC, Liu M (2011) Comparison of chemical and biological pretreatment of corn straw for biogas production by anaerobic digestion. Renew Energy 36(6):1875–1879
17. Yan ZY, Yuan YX, Liu XF et al. (2006) A study on biogas fermentation with straw. 2006 China biomass science and technology forum 2006:104–105 (in Chinese)
18. Liu QY, Bian Y, Li JY (2008) Experimental study on the effects of white-rot fungus on the production ablity of methane with corn straw stalk. J Anhui Agric Sci 36(29):12841–12842
19. Bian Y, Liu QY, Li JY (2007) Experimental investigation of making biogas from dry fermented corn stalk. J Shenyang Agric Univ 38(3):440–442 (in Chinese)
20. Li SM, Wei YJ, Zhang XJ et al (2008) Research on the biogas production from lignocellulosic material. Renew Energy Resour 26(1):50–54 (in Chinese)
21. Song YM, Chen HZ (2008) Study on biogas production by thermophilic solid-state fermentation from steam exploded corn stalk. Chin J Environ Eng 2(11):1564–1570 (in Chinese)
22. Zhang T (2009) The impact of ultrasound combined with dilute soda pretreatment on straw anaerobic pretreatment process. Hubei University of Technology master's degree thesis (in Chinese)
23. Li LH, Li D, Sun YM, Ma LL et al (2010) Effect of temperature and solid concentration on anaerobic digestion of rice straw in South China. Int J Hydrogen Energy 35(13):7261–7266
24. Liu WW, Ma H, Wang JX et al (2007) Study on dry biogas anaerobic fermentation of rice straw. Bi-annual conference of chinese society of agricultural engineering 2007:1–5 (in Chinese)
25. Wang YZ, Yang L, Zhang T et al (2010) Effect of metal ions and surfactant addition on biogas production. Renew Energy Resour 28(2):30–32 (in Chinese)
26. Wang SY (2009) Effect of biogas solid-state fermentation of straw. Hubei University of Technology master's degree thesis (in Chinese)
27. Huang RY, He WN, Tang HJ et al (2008) Straws biogas fermentation with bacterium additive pretreatment. China Biogas 26(4):24–26 (in Chinese)
28. Su YY, Zhang WD (2003) Potential of the biogas fermentation with calla straw. Agric Technol 23(6):53–57 (in Chinese)
29. Sun C, Liu RH, Qin GD (2010) Experiments on pretreatment and anaerobic digestion of asparagus stalk for biogas production. Agric Eng 41(8):94–99 (in Chinese)
30. Wan CY, Huang FH, Liu R et al (2010) Effect on increasing biogas production using rape straw by microbiological pretreatment. Trans Chin Soc Agric Eng 26(6):267–271 (in Chinese)
31. Chen L, Zhao LX, Dong BC et al (2010) The status and trends of the development of biogas plants for crop straws in China. Renew Energy Resour 28(3):145–148 (in Chinese)
32. Zhao LX (2007) Medium and large scale biogas project. Chem Ind Press, Beijing (in Chinese)
33. An J, Chang J, Zhu ZQ (2008) The situation and development of city garbage stuffing and burying and biogas technology. Guangxi Agric Sci 23(5):60–63 (in Chinese)
34. Shi JL, Li JF, Hu RQ, Song YQ (2002) Some problems in the utilization of urban LFG. China Energy 10(9):24–26

35. Bernhard R, Zhao YC, Ji R et al (2007) Biogas from municipal and agricultural bioorganic waste: renewable energy for China. International symposium MBT
36. Cheng W, Xia H, Yi Q (1999) Domestic wastewater treatment using an anaerobic bioreactor coupled with membrane filtration. Process Biochem 35:335–340
37. Guan YT, Jiang ZP, Zhu WP et al (1998) Study on two-phase anaerobic technology for organic wastewater treatment. Environ Sci 19(6):56–59 (in Chinese)
38. He YL, Wu ZC, Li CJ (1999) Application of the anaerobic MBR for treatment of high concentration food wastewater. Environ Sci 20(6):53–55 (in Chinese)
39. Wu J, Xu LJ, Xie JL (2006) The effect of alkali-pretreatment on anaerobic digestion of solid organic waste. Acta Sci Circumst 26(2):252–255
40. Wu XQ, Liu KX, Li Y (1985) The application of wet-dry anaerobic fermentation process in the rural of Huanghuaihai region. Res Agric Mod 8:40–42 (in Chinese)
41. Sun GC, Sha TL, Guo XM (1986) The productive experiment of dry anaerobic fermentation. J Sol Energy 7(1):10–15 (in Chinese)
42. Ye S, Wei JS, Zhao HL (1989) Automatic discharging dry anaerobic fermentation device. China Biogas 4:17–19 (in Chinese)
43. Ma YR, Hui KJ, Rong LH (1990) The benefit of gas separation storage-constant voltage dry anaerobic biogas digestion technology in the dry and cold region of Ningxia province. China Biogas 8(2):25–26 (in Chinese)
44. Kang HD (2005) small-scale efficient voltage stabilized auto-aimless filtration dry anaerobic digester. [EB/OL] http://www/patent.com.cn
45. Gan RH (2004) Study on the stirring reactor for the poultry and livestock manure digestion. Huazhong Agricultural University master's degree thesis (in Chinese)
46. Yan SP (2004) Studies on fermentation tin's heating and heat preservation in the poultry and domestic dejects digestion with high temperature anaerobic dry fermentation. Huazhong Agricultural University master's degree thesis (in Chinese)
47. Liu RH, Niu WS, Zhang DL (2005) Biomass thermal and chemical conversion technology. Chem Ind Press, Beijing (in Chinese)
48. Peter T (2010) Biogas: Rethinking the midwest's potential. Clean Wisconsin
49. The report of European biomass energy development and utilization (2006). 8http://www.newenergy.com.cn
50. Chen GY, Gao WX, Yan BB, Jia JN (2006) Present research status and development of biomass gasification technologies. Gas Heat 26(7):2–8 (in Chinese)
51. Deng XL, Gao YW, Xu Y et al (2007) Progress in biomass gasification and its requipments. Biomass Chem Eng 41(6):37–41 (in Chinese)
52. Fan JC, Wang YG, Gao H (2004) Economic benefit evaluation of biomass gasification and subsequent electricity generation. Energy Eng 1:20–23 (in Chinese)
53. Sheng JJ (2007) Progress on biomass gasification and power generation. Energy Conservation Technol 25(1):76–80 (in Chinese)
54. Wu CZ (2003) The electricity production by biomass gasification technology: (1) The working principle and technological process of electricity production by biomass gasification. Renew Energy 1:41–43 (in Chinese)
55. Ying H, Jiang JC (2007) Conversion technology and utilization of biomass energy(IV)-technology and application of biomass gasifiacation. Biomass Chem Eng 41(6):47–55 (in Chinese)
56. Zhao LC, Li XW, Wang GL et al (2008) The application and investigation of biomass gasification. Renew Energy 26(6):55–58 (in Chinese)

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Biohydrogen Production from Anaerobic Fermentation

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Abstract Significant progress has been achieved in China for biohydrogen production from organic wastes, particularly wastewater and agricultural residues, which are abundantly available in China. This progress is reviewed with a focus on hydrogen-producing bacteria, fermentation processes, and bioreactor configurations. Although dark fermentation is more efficient for hydrogen production, by-products generated during the fermentation not only compromise hydrogen production yield but also inhibit the bacteria. Two strategies, combination of dark fermentation and photofermentation and coupling of dark fermentation with a microbial electrolysis cell, are expected to address this issue and improve hydrogen production as well as substrate utilization, which are also discussed. Finally, challenges and perspectives for biohydrogen production are highlighted.

Keywords Biohydrogen · Dark fermentation · Microbial electrolysis cell · Microbial fuel cell · Organic wastes · Photofermentation

Contents

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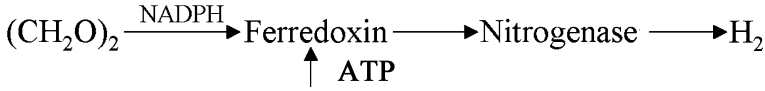
1 Fundamentals of Anaerobic Hydrogen Fermentations

Biological hydrogen production under anaerobic conditions can be classified into two categories according to the types of microorganisms employed: photofermentative hydrogen production and dark-fermentative hydrogen production.

1.1 Photofermentation

Photofermentative hydrogen production in purple non-sulfur photosynthetic bacteria is catalyzed by nitrogenase under nitrogen-deficient conditions using light energy and reduced compounds [1]. The overall biochemical pathway of the photofermentation process can be expressed as follows:



Hydrogen production by photofermentative bacteria has been widely studied for a long time. Purple non-sulfur photosynthetic bacteria have photosystem I, but not photosystem II, which makes these anoxygenic bacteria suitable for hydrogen production since photosystem II generates oxygen, which inactivates nitrogenases irreversibly. Batch and continuous processes have been developed [2–4], and cells immobilized by supporting matrices such as poly(vinyl alcohol), agar gel, and porous glass have also been explored to enhance hydrogen production [5–8]. However, there are significant disadvantages in the photofermentative hydrogen production, such as high energy demand by the nitrogenases and low light energy harvest efficiency and hydrogen productivity. Therefore, photofermentation alone is not economically competitive for hydrogen production, but it can be integrated with dark fermentation to improve substrate conversion as well as hydrogen production [9].

1.2 Dark Fermentation

Dark-fermentative microorganisms are mainly anaerobic bacteria, which can generate molecular hydrogen during the decomposition of carbohydrate substrates. The main soluble products are organic acids, including acetic, propionic, and butyric acids, and ethanol [10]. The dark fermentation is exergonic. However,

depending on the species and the operating environments, prevalent end products can be significantly different. Generally, there are two widely known fermentation types, butyric-type fermentation and propionic-type fermentation, according to the main end products [11]. The butyric-type fermentation results in the formation of H_2, CO_2, butyrate, and acetate as the dominant products, whereas the propionic-type fermentation produces mainly propionate, acetate, and some valerate, with little hydrogen being produced, and this should be controlled from the viewpoint of hydrogen production. In 1994, a third type, ethanol-type fermentation, was discovered by Ren et al. [12], with ethanol, acetic acid, H_2, and CO_2 as major end products. The maximum theoretical yields of hydrogen production are 2–3 mol/mol glucose in butyric-type fermentation and ethanol-type fermentation.

For practical applications, dark-fermentative hydrogen production has more advantages than photofermentation, such as significantly high hydrogen production rate, energy savings, broad spectrum of feedstocks, simple process control, and lower maintenance costs.

2 Biohydrogen Production from Wastewater

In China, Ren et al. [12] initiated hydrogen production with dark fermentation from wastewater rich in carbohydrates in the 1990s, and this has attracted great interest since then.

2.1 Hydrogen-Producing Bacteria

The success of hydrogen production depends on the choice of the host microorganisms. During recent decades, intensive research has been conducted to isolate high-performance hydrogen-producing microorganisms [13–23], which are summarized in Table 1.

Among them, *Ethanoligenens harbinense* B49 isolated from a continuous stirred tank bioreactor (CSTR) is worthy of note, because the "ethanol-type" fermentation was identified with this bacterium for the first time on the basis of its main soluble end products acetate and ethanol [17]. Afterward, another strain, *E. harbinense* YUAN-3, was isolated and nominated as the model strain of the genus *Ethanoligenens* [18]. The maximum hydrogen yield and production of *E. harbinense* YUAN-3^T are 2.81 mol H_2/mol glucose and 27.6 mmol H_2/g dry cell weight (DCW)/h, respectively. Additionally, the strain exhibits self-aggregation ability during shake cultivation, which is an advantage for its future application.

To improve hydrogen production, genetic engineering approaches have been applied for elucidating functional genes and their structures and regulation mechanisms. The iron hydrogenase gene (*hydA*), which is the principal gene

Table 1 Pure and mixed cultures for hydrogen production

Microorganism	Conditions	pH	Substrate	H_2 yield	References
Pure cultures					
Dark-fermentative bacteria					
Clostridium beijerinckii AM21B	Batch	6.5	Glucose	2.0 mol H_2/mol glucose	[13]
Clostridium beijerinckii Fanp3	Batch	6.47–6.98	Glucose	2.52 mol H_2/mol glucose	[14]
Pantoea agglomerans	Batch	6.0	Glucose	2,246 mL H_2/L culture	[15]
Clostridium beijerinckii RZF-1108	Batch	7.0	Glucose	1.97 mol H_2/mol glucose	[16]
Ethanoligenens harbinense B49	Batch	3.9–4.2	Glucose	1.6–1.8 mol H_2/mol glucose	[17]
Ethanoligenens harbinense YUAN-3	Batch		Glucose	2.81 mol H_2/mol glucose	[18]
Klebsiella pneumoniae ECU-15	Batch	6.6	Glucose	2.07 mol H_2/mol glucose	[19]
Photofermentative bacteria					
Rhodopseudomonas faecalis strain RLD-53	Batch	7.0		2.84 mol H_2/mol acetate	[20]
Rhodopseudomonas palustris CQK 01	Batch		Glucose	12.04 mmol H_2/300 mL	[21]
Mixed cultures					
Sewage sludge	Continuous	6.7	Sucrose	1.7 mol H_2/mol glucose	[13]
Mixed culture	Continuous	No control	Sucrose	3.47 mol H_2/mol sucrose	[22]
Boiling treated anaerobic sludge	Batch		Municipal solid waste	134 mL H_2/g volatile solids	[23]

involved in hydrogen production, was isolated from *E. harbinense* YUAN-3 and overexpressed in *Escherichia coli* BL-21 [24]. The *hydA* gene consists of an open reading frame of 1,743 bp, encoding 580 amino acids for the hydrogenase with an estimated molecular mass of 63.18 kDa, and the purified enzyme from the recombinant exhibits catalytic activity in vitro as well as in vivo. The acetate kinase (*ack*) gene, which is responsible for the dephosphorylation of acetyl phosphate with the concomitant production of acetate and ATP, was isolated from *E. harbinense* B49 [25]. This gene contains an open reading frame of 1,200 bp, encoding 399 amino acids for the enzyme with an estimated molecular mass of 43.22 kDa and an isoionic point of 5.93. The *ack* gene was heterologously expressed in *E. coli* BL21 (DE3) as an inclusion body, and the specific activity of the refolded enzyme was 42.12 U at 25 °C, with 14.36% recombinant protein renatured. The activity of *ack* is closely related to acetate excretion in dark fermentation, which then regulates metabolism in hydrogen-producing bacteria.

2.2 Key Factors Affecting Hydrogen Fermentation

Hydrogen production is influenced by various parameters, including products accumulated such as acetate, lactate, and butyrate, temperature, pH, and metal ions [26].

In fermentative hydrogen production, ethanol, acetate, propionate, and butyrate are produced as the soluble metabolites, which can cause a sharp drop in the pH value and affect the corresponding metabolic balance, subsequently inhibiting the bacterial hydrogen production [11, 12]. Several studies have reported the inhibitory effects of added ethanol, butyrate, and acetate on fermentative hydrogen production. Zheng and Yu [27] reported that the specific hydrogen production rate and the substrate degradation efficiency both decreased on increasing the added sodium butyrate concentration from 0 to 228 mmol/L. Wang et al. [28] illustrated that added acetate had a significant influence on both substrate degradation and hydrogen production as well as the profile of soluble products, and the $C_{I,50}$ values for the specific hydrogen production rate and hydrogen yield were 11.05 and 31.90 g/L. Wang et al. [29] found that substrate degradation efficiency, hydrogen yield, and hydrogen production rate decreased as ethanol, acetate, propionate, and butyrate addition increased from 0 to 300 mmol/L, but ethanol inhibition was not significant compared with that for organic acids. Tang et al. [30] also found that acetate exhibited severer inhibition than ethanol in *E. harbinese* B49.

Temperature is a very important parameter because it affects the activity of essential enzymes (e.g., hydrogenases), which consequently affects hydrogen production by fermentative bacteria [31, 32]. In most cases, hydrogen production was tested under mesophilic conditions. Although, mesophilic hydrogen production is preferred to prevent the need for external heating, hydrogen fermentation at higher temperatures has a higher hydrogen yield, owing to the suppression of hydrogen-consuming bacteria and the capability to utilize various substrates. Luo et al. [33] studied fermentative hydrogen production from cassava at initial pH 7 and 37 and 60 °C, representing mesophilic and thermophilic conditions, correspondingly, and found a maximum hydrogen yield of 53.8 mL H_2/g volatile solids under the thermophilic condition, 53.5% higher than that achieved under the mesophilic condition.

Metal ions as cofactors of many enzymes are essential for cell growth and function during hydrogen production. Several studies have been conducted on how the external concentrations of various ions affect the fermentative hydrogen production using different kinds of inocula [17, 34–38]. However, the results obtained are quite different from each other, confirming that biohydrogen production and the optimal dose (as well as its inhibiting or stimulating effect) of Cu^{2+}, Mg^{2+}, Fe^{2+}, and Ni^{2+} are strictly correlated to the inoculum and the operating condition applied. Thus, to improve the fermentative hydrogen production process for each specific inoculum, dedicated research for addition of each metal iron is needed.

2.3 Hydrogen Fermentation Processes

Biohydrogen fermentation can be conducted in either batch or continuous mode. Batch fermentation has been shown to be suitable for initial optimization studies, but large-scale operation would likely have to be performed on a continuous or at least semicontinuous (fed or sequencing batch) basis. In China, many studies have employed CSTRs, upflow anaerobic sludge blanket (UASB) reactors, expanded granular sludge bed (EGSB) reactors, and anaerobic baffled reactors (ABRs) for biohydrogen production.

CSTRs have been the most widely applied bioreactor systems by far. They are simple to construct, easy to operate, and offer effective homogenous mixing. In 1992, Ren et al. patented a CSTR design (patent number ZL92114474.1) for continuous biohydrogen production from wastewater. The bioreactor can be operated at high concentrations of volatile suspended solids (VSS) ranging from 10 to 30 g/L, with a maximum hydrogen yield and a maximum production rate of 10.4–11.4 m^3/m^3/day and 36–40 mL/g VSS/h, respectively. Compared with CSTRs, UASB reactors retain large amounts of granular sludge, and are operated for shorter hydraulic retention times (HRTs) and thus higher organic loading rates (OLRs). Mu and Yu [39] treated sucrose-rich synthetic wastewater in a UASB reactor using hydrogen-producing granular sludge with a diameter of 1.0–3.5 mm, an average density of 1.036 g/mL, and a settling velocity ranging from 32 to 75 m/h. Guo et al. [40] employed an EGSB reactor with granular activated carbon as the carrier of active sludge for hydrogen production from molasses-containing wastewater. Without pH control, the reactor operated at 35 °C, exhibited good hydrogen production performance with a yield of 3.47 mol H_2/mol sucrose, and a specific production rate of 3.16 mmol H_2/g VSS/h. A similar EGSB reactor was also used to treat starch-containing wastewater for hydrogen production, with a maximum hydrogen production rate of 1.64 L/L/day at an OLR of 1.0 g starch/L/day, pH 4.42, and HRT of 4 h, and the average chemical oxygen demand (COD) removal rate was 31.1% at an OLR of 0.125 g starch/L/day and HRT of 24 h [41]. Li et al. [42] investigated hydrogen production from molasses-containing wastewater using a three-compartment ABR with an effective volume of 27.48 L. At the stable operational conditions, the ABR exhibited a hydrogen yield of 32.51 L/day, a specific hydrogen production rate of 0.13 L/g molasses VSS/day, and a substrate conversion rate of 0.13 L/g COD.

Apparently, a short HRT is preferred to reduce capital investment in bioreactors [43]. Chu et al. [44] found that the population of hydrogen-producing bacteria decreased gradually with the increase of the HRT: 9.2×10^8, 8.2×10^8, 2.8×10^8, and 6.2×10^7 cells per milliliter at HRTs of 6, 8, 12, and 14 h, respectively. At the optimal HRT of 6–8 h, the hydrogen yield was 1.4–1.5 mol/mol glucose. On the other hand, a high OLR can reduce energy consumption for hydrogen production. Yu et al. [45] investigated hydrogen production from high-concentration winery wastewater with COD concentrations of 14–36 g/L, and found the specific hydrogen production rate increased with the increase of the COD concentration, and the maximum hydrogen production rate of 9.33 L H_2/g

VSS/day and maximum hydrogen yield of 1.37–2.14 mol/mol hexose were obtained at a COD concentration of 34 g/L. Zhao et al. [46] optimized the hydrogen production from sucrose in a UASB reactor using response surface methodology, finding that the maximum hydrogen yield of 1.62 mol/mol hexose was achieved when the wastewater contained 14.5 g/L sucrose and the bioreactor was operated at an HRT of 16.4 h.

The suitability of any process lies in its capacity to be scaled up to the industrial level. In 1999, a pilot-scale study for biohydrogen production was performed in a continuous fermentor with a working volume of 1.48 m^3 for over 200 days. The system was fed with molasses-containing wastewater and was operated at an OLR from 3.1 to 85.6 kg COD/m^3/day. The hydrogen yield first increased with the increase of the OLR, but decreased when the OLR exceeded 68.2 kg COD/m^3/day. The maximum hydrogen production rate was 5.57 m^3 H_2/m^3/day, with a specific production rate of 0.75 m^3 H_2/kg VSS/day. At an OLR of 35–55 kg COD/m^3 reactor/day, the hydrogen yield reached 26.13 mol/kg $COD_{removed}$ [47]. This achievement was selected as one of the ten top science and technology breakthroughs in China in 2000.

In 2005, the first full-scale demonstration project of fermentative hydrogen production was conducted for 1 year, with a capital investment of CNY 21 million. The system consisted of seed sludge acclimation and enrichment, fermentative hydrogen production, hydrogen purification, fermentative methane production from residues of hydrogen production, and wastewater treatment. A 100-m^3 CSTR (working volume of 64.5 m^3) was designed for the fermentative hydrogen production from molasses-containing wastewater. Under the operation parameters of an OLR of 14–45 kg COD/m^3/day, pH 4.4–5.0, and alkalinity over 500 mg/L ($CaCO_3$), the reactor performed the ethanol-type fermentation with an average hydrogen productivity of 285 m^3/day and a maximum productivity of 588 m^3/day. Through bioaugmentation by supplying efficient hydrogen-producing microorganisms, the average hydrogen productivity was enhanced to 334 m^3/day, corresponding to a volumetric hydrogen production rate of 5.26 m^3/m^3/day and a specific rate of 0.83 m^3 H_2/kg VSS/day. Over 95% of the total sugars in the feedstock were degraded. The hydrogen purification system, which consisted of a desulfurization tower and a pressure swing adsorption tower for CO_2 removal, improved the hydrogen content in the biogas from 40–54% to 99.99%. The estimated cost of producing 99.9% H_2 was CNY 1.37 per cubic meter. The hydrogen produced was used to generate electricity via fuel cells. The effluent from the process was used for fermentative methane production followed by a wastewater treatment process. The final effluent met the national discharge standards.

3 Biohydrogen Production from Lignocellulosic Biomass

China is a large agriculture country, with 80% of its population living in rural areas. About 700 million tons of lignocellulosic wastes such as corn stover and wheat straw are produced annually. These kinds of biomass usually contain

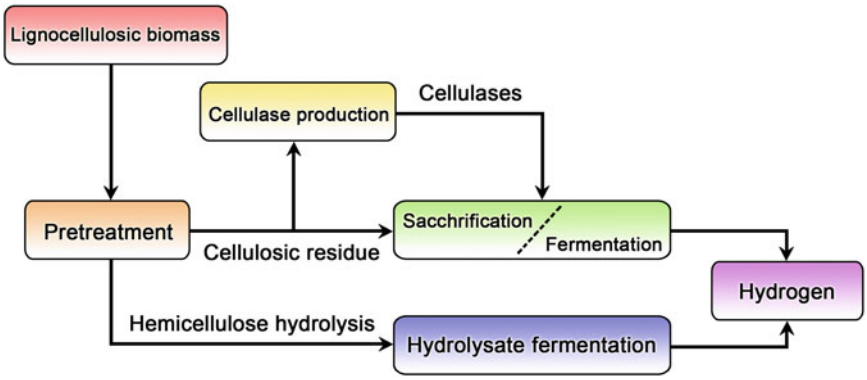


Fig. 1 General scheme for converting lignocellulosic wastes into hydrogen

38–50% cellulose, 20–35% hemicellulose, and 15–25% lignin [48], which potentially can be used as energy sources [49]. However, because of the complex structure of lignocellulose, additional processes such as pretreatment, detoxification, and hydrolysis are required [50–52]. Figure 1 shows the general scheme for converting lignocellulosic wastes to biohydrogen.

3.1 Pretreatment

The goal of the pretreatment is to improve the hydrolysis yield and hydrogen production. Pretreatment methods can be divided into different categories: physical (milling and grinding), physicochemical (steam explosion, hydrothermolysis, and wet oxidation, etc.), chemical (alkali, dilute acid, and oxidizing agents), biological, or a combination of them [51, 53, 54]. Among these methods, physicochemical and chemical pretreatments are frequently applied for enhancing hydrogen fermentation [55–59]. For example, Fan et al. [55] investigated hydrogen production from wheat straw pretreated with HCl, and the maximum cumulative hydrogen yield of 68.1 ml H_2/g total volatile solids (TVS) was observed at 126.5 h, which is about a 136-fold increase compared with that from raw wheat straw. Similar results were also achieved with beer lees waste, and a hydrogen yield of 68.6 mL H_2/g TVS was obtained from the biomass pretreated with HCl, which is an approximately tenfold increase compared with that from the raw feedstock [56]. From a practical viewpoint, the ideal pretreatment process should achieve high yields of fermentable reducing sugars, avoid degradation of the sugars produced, and avoid the formation of inhibitors of the subsequent fermentation with respect to minimal use of energy, chemicals, and capital equipment.

3.2 Functional Microorganisms

Lignocellulosic hydrolysates contain a mixture of hexose and pentose sugars. In recent years, researchers have explored a number of pentose fermentation strains and mixed cultures [60–74], which are shown in Table 2. For example, Xu et al. [61] isolated the novel mesophilic hydrogen-producing bacterium *Clostridium* sp. HR-1 from cow dung compost, which can efficiently utilize xylose as well as glucose for hydrogen production. Under optimal conditions of pH 6.5, 37 °C, and yeast extract as a nitrogen source, the average hydrogen yields and specific hydrogen production rates were 1.63 and 2.02 mol H_2/mol sugar and 11.14 and 9.37 mmol H_2/h/g DCW for xylose and glucose. Ren et al. [63] reported a thermophilic xylose–glucose fermenting strain, *Thermoanaerobacterium thermosaccharolyticum* W16, and its maximum cumulative hydrogen yield and production rate from pure glucose and xylose were 2.42 mol H_2/mol glucose and 12.9 mmol H_2/L/h, and 2.19 mol H_2/mol xylose and 10.7 mmol H_2/L/h, respectively. Besides, this strain metabolizes the reducing sugars in the hydrolysate of corn stover efficiently, and also exhibits good tolerance to inhibitors in the hydrolysate such as acetate and furfural. However, compared with hexose-fermenting microorganisms, only a few pentose-fermenting microorganisms have been explored. Further isolation of pentose-fermenting microorganisms is needed to further the utilization of lignocellulosic hydrolysates.

Direct microbial conversion, which integrates cellulolytic enzyme production, cellulose hydrolysis, and fermentation together, is considered the most attractive strategy for converting cellulosic biomass to biofuels. It has been reported that some pure cultures can degrade cellulose to produce hydrogen directly. Ren et al. [65] reported that *Clostridium acetobutylicum* X9 fermented microcrystalline cellulose with a hydrogen production rate of 6.4 mmol H_2/g biomass (DCW)/h, with a substrate degradation of 68.3%. Further research showed that the strain [66] can ferment stream-exploded corn stover to hydrogen with a specific production rate of 3.4 mmol/g substrate. Compared with pure cultures, mixed cultures were more effective in degrading cellulose owing to the synergy of different microorganisms. For example, Liu et al. [68] demonstrated a mixed culture of thermophilic anaerobic bacteria *Clostridium thermocellum* JN4 and *T. thermosaccharolyticum* GD17 could effectively decompose cellulose and produce hydrogen with a yield of 1.8 mol H_2/mol glucose, and could utilize several kinds of natural substrates, such as corn cob and cornstalk as feedstock for production of hydrogen. However, the yields of hydrogen production from direct fermentation of cellulose by cellulolytic bacteria are very low. Successful production of biohydrogen via direct cellulose fermentation will depend upon our ability to increase the yields of hydrogen during the fermentation process. This will require a more comprehensive understanding of the interactions between gene product expression, end product synthesis patterns, and the factors that regulate carbon and electron flow in cellulolytic bacteria.

Table 2 Hydrogen yields reported in xylose and cellulose fermentations

Microorganism	Substrate	T (°C)	H_2 yield	References
Clostridium tyrobutyricum ATCC 25755	Xylose	37	0.77 mol H_2/mol xylose	[60]
Clostridium sp. HR-1	Xylose	37	1.63 mol H_2/mol xylose	[61]
	Glucose		2.02 mol H_2/mol glucose	
Enterobacter sp. CN1	Xylose	37	2.0 mol H_2/mol xylose	[62]
	Glucose		0.64 mol H_2/mol glucose	
Thermoanaerobacterium thermosaccharolyticum W16	Xylose	60	2.19 mol H_2/mol xylose	[63]
	Mix		2.23–2.37 mol H_2/mol sugars	
	Glucose		2.42 mol H_2/mol xylose	
Thermoanaerobacterium aotearoense (*Δldh* mutant)	Xylose	55	1.47 mol H_2/mol xylose	[64]
	Glucose		2.71 mol H_2/mol glucose	
	Mix		2.28 mol H_2/mol sugars	
Clostridium acetobutylicum X9	Microcrystalline cellulose	37	33.7 mmol H_2/L culture	[65]
	Steam-exploded corn stover		8.4 mmol H_2/L culture	[66]
Clostridium butyricum AS1.209	Steam-exploded corn stover	35	3.0 mmol H_2/g steam-exploded corn stover	[67]
Clostridium thermocellum JN4+ *Thermoanaerobacterium thermosaccharolyticum* GD17	Microcrystalline cellulose	60	11 mmol H_2/g cellulose	[68]
	Cornstalk powder		16.1 mmol H_2/L	
	Corn cob powder		20.4 mmol H_2/L	
Microflora	Cellulose	55	4.55 mmol H_2/g cellulose	[69]
Cow dung compost	Acid-pretreated cornstalks	36	6.7 mmol H_2/g TVS	[70]
	HCl-pretreated wheat straw		3.0 mmol H_2/g TVS	[71]
	HCl-pretreated beer lees		3.1 mmol H_2/g TVS	[72]
Cow dung compost	Cellulose	37	12.1 mmol/g cellulose	[73]
Microflora	Lime-pretreated cornstalk	60	155.4 mL/g TVS	[74]

TVS total volatile solid

3.3 Strategies for Increasing Hydrogen Yield

Apart from sugars, a variety of toxic compounds such as acetic acid, furfural, 5-hydroxymethyl furfural, and aromatic and polyaromatic compounds are usually generated during the pretreatment of lignocellulosic wastes. Because of their inhibitory effect on the fermentation, these by-products may be a limiting factor for hydrogen fermentation. Cao et al. [75] used $Ca(OH)_2$ to detoxicify HCl-pretreated corn stover hydrolysate, and the hydrogen production from the supernatant was improved after the precipitate had been removed. The choice of detoxification methods depends on both the hydrolysates to be treated (regarding the formation of harmful by-products) and the fermentation microorganisms employed (regarding their stress tolerance).

On the other hand, as lignocellulosic hydrolysates contain various monosaccharides, a single type of microorganism may not produce rapid and efficient fermentation for hydrogen production. In this sense co-culture or mixed cultures might be a choice for efficient fermentation of lignocellulosic wastes to expand the utilization of carbon sources and improve substrate conversion rates. In addition, the metabolic engineering technique may be an alternative approach to improve hydrogen production from lignocellulosic wastes. Li et al. [64] knocked out the gene encoding L-lactate dehydrogenase from *Thermoanaerobacterium aotearoense* to redirect the electron flow, which resulted in twofold and 2.5-fold increases in the hydrogen yield and production rate; the maximum hydrogen yields of the *Δldh* mutant were 2.71, 1.45, and 2.28 mol H_2/mol on glucose, xylose and a glucose–xylose mixture, respectively.

Although biohydrogen production from lignocellulosic wastes is promising, it is still not cost-effective, and more work is required to improving hydrogen production, such as searching for highly efficient hydrogen-producing bacteria, developing cost-efficient pretreatment technology, and optimizing the fermentation process.

4 Emerging Technologies for Biohydrogen Production

4.1 Limitation of Dark Fermentation

Theoretically, 4 mol H_2 can be generated per mole of glucose consumed in dark hydrogen fermentation with 2 mol acetate as a by-product. However, in addition to acetate, small-molecule organic compounds such as butyrate and ethanol are also produced, and are termed a “dead-end” or “fermentation barrier,” and limit the hydrogen yield to a maximum of 2–3 mol H_2/mol glucose. Usually, no more than one third of the total potential electrons in complex biomass can be transferred to hydrogen, and the remaining two thirds end up in the forms of these fermentation by-products. On the basis of the Gibbs free energy, additional energy is required for hydrogen production from these “dead-end” compounds.

Recently, significant progress has been made on efficient conversion of various volatile fatty acids (especially acetic acid) to hydrogen via photofermentation [76] or with a microbial electrolysis cell (MEC) using exoelectrogens [77]. Thus, combination of dark fermentation and photofermentation or coupling of dark fermentation and an MEC could convert sugars more efficiently into hydrogen.

4.2 Combination of Dark Fermentation and Photofermentation

Two schemes for the combined process of dark fermentation and photofermentation have been studied: an integrated process and a sequential process. The integrated process allows dark-fermentative and photofermentative bacteria to be co-cultured in one system, saving space and operational costs. However, the first concern with the co-culture system is the ratio of the two groups of bacteria, because dark-fermentative bacteria always grow faster than photofermentative bacteria. In 2006, Fang et al. [78] co-cultured *Clostridium butyricum* and *Rhodobacter sphaeroides*, and observed a lag phase of 10 h before hydrogen was produced. They further studied the effect of different ratios of the two bacteria, and found that the maximum hydrogen yield was 0.6 mL H_2/mL medium when the ratio of *C. butyricum* and *R. sphaeroides* was 1:5.9. In 2009, Liu et al. [79] studied a coupling system containing *C. butyricum* and *Rhodopseudomonas faecalis* RLD-53, and found that the maximum hydrogen yield reached 2.45 mL H_2/mL culture at a ratio of 1:500. They also studied the combination of suspended *E. harbinense* B49 and immobilized *R. faecalis* RLD-53, in which phosphate buffer solution (PBS) was used to reduce the effect of pH variation caused by the difference in the growth rates of the two bacteria. The results indicated that higher PBS concentrations enhanced the ratio of acetate to ethanol, and the hydrogen yield was increased by two to three times compared with dark fermentation, reaching 6.32 mol H_2/mol glucose [80]. However, owing to the great difference in the growth rate and acid-resistant capacity between photofermentative and dark-fermentative bacteria, they found that the system was very difficult to operate.

Compared with the integrated process, the sequential process is easier to operate and control because the two groups of bacteria are in individual reactors which can be operated independently [81–84]. With use of two-step coupling systems, the fermentation products from the dark fermentation are converted into hydrogen during the photofermentation as illustrated in Table 3. Tao et al. [81] adopted such a sequential process for hydrogen production. They used phototrophic *R. sphaeroides* SH_2C to produce hydrogen from the effluent of dark fermentation, achieving a total hydrogen yield of 6.63 mol H_2/mol sucrose. The effect of initial pH and C/N ratio on the dark-fermentative hydrogen production was also determined. Su et al. [83] applied anaerobic sludge as seed sludge for dark-fermentative hydrogen production from cassava starch, followed by photofermentation by *Rhodopseudomonas palustris*. The cumulative hydrogen production was 402 mL H_2/g starch, which was substantially higher than the hydrogen

yield from a single dark fermentation process. A much higher hydrogen yield of 14.2 mol H_2/mol sucrose was obtained by Chen et al. [84]. They used phototrophic *R. palustris* WP3–5 to generate hydrogen from the effluent of the dark-fermentative strain *Clostridium pasteurianum* CH4. In addition, optical fibers were introduced into their phototrophic system.

4.3 Coupling of Dark Fermentation with an MEC

An MEC is an emerging technology for hydrogen production. It was developed from a microbial fuel cell (MFC). In 2005, Liu et al. [85] reported a bioelectrochemically assisted microbial system for the first time; this produced hydrogen from the fermentation end product acetate when a low external voltage (less than 110 mV) was applied. The study showed the coulombic efficiency, electronic conversion of the substrate to hydrogen, was up to 60%, much higher than that of the MFC alone (less than 40%). More than 90% of the electrons and protons generated by anodic bacteria from acetate oxidation can be recovered as hydrogen, and the hydrogen production yield reaches 3 mol H_2/mol acetate when the coulombic efficiency is 80% and the electron recovery is 90%. Whereas only 2–3 mol H_2/mol glucose can be obtained from dark fermentation, as much as 8–9 mol H_2/mol glucose can be harvested by coupling anaerobic fermentation and an MEC, and the energy input of the system is estimated to be about 1.2 mol H_2/mol glucose.

Studies on hydrogen production from MECs have been conducted in China since 2006 [86–92], with a focus on increasing the hydrogen production yield and substrate conversion through developing new bioreactor configurations, electrode materials, and microbial community analysis, which is summarized in Table 4.

Sun et al. [93] produced an MFC–MEC combined system, in which the MEC was powered by the voltage of the MFC. The hydrogen production rate and yield were 2.2 mL/L/day and 1.2 mol H_2/mol acetate, with an anodic coulombic efficiency of 28–33% and cathodic hydrogen recovery of 88–96% using 10 mM PBS. The hydrogen production rate and yield increased to 14.9 ml/L/day and 1.6 mol H_2/mol acetate, correspondingly, with 100 mM PBS. Using the same system, Sun et al. [94] produced hydrogen from propionate, and examined the effect of external resistance. It was found that propionate removal was increased when the external resistance was lower; a current density of 343 mA/m^2 was achieved with an external resistance of 10 Ω, and the hydrogen production rate was 11.9 ml/L/day. The hydrogen yields of the MEC and the whole system were 4.8 and 2.5 mol H_2/mol propionate, respectively.

Lu et al. [95] studied hydrogen production with an MEC from the effluent of the ethanol-type hydrogen fermentation. A high organic loading of molasses wastewater (COD 22.3 g/L) was firstly treated in a CSTR. The maximum rate of hydrogen production reached 0.70 m^3 H_2/m^3/day and the hydrogen yield was 0.017 g H_2/g COD (0.27 mol H_2/mol as hexose COD). The effluent COD was 6,500 mg/L, with the following composition (mg/L as COD): residual reducing

Table 3 Hydrogen production capability of the two-step dark fermentation and photofermentation

Bacteria for dark fermentation	Bacteria for photofermentation	Feedstock/light source	H_2 production (mol H_2/mol substrate)	References
Microflora	*Rhodobacter sphaeroides* SH2C	Sucrose/tungsten	6.63	[81]
Caldimonas taiwanensis On1	*Rhodopseudomonas palustris* WP3-5	Starch/tungsten	3.09	[82]
Activated sludge	*Rhodopseudomonas palustris*	Starch/tungsten	402 ml/g starch	[83]
Clostridium pasteurianum CH4	*Rhodopseudomonas palustris* WP3-5	Sucrose/tungsten	10.0	[84]
Clostridium pasteurianum CH4	*Rhodopseudomonas palustris* WP3-5	Sucrose/optical fibers	14.2	[84]

Table 4 Performances in different types of microbial electrolysis cells

Architecture and material

Reactor	Anode and surface area	Applied voltage (V)	Conversion (%)	Hydrogen yield (substrate)	References
2-chamber	Carbon cloth, 68 cm^2	0.5	11.6	0.5 mol H_2/mol (acetate)	[86]
2-chamber	Carbon cloth, 36 cm^2	0.5	25	1.0 mol H_2/mol (acetate)	[87]
2-chamber	Activated carbon granular, 94,500 m^2	0.5	35.5	1.4 mol H_2/mol (acetate)	[87]
Single-chamber	Brush, 0.22 m^2	0.7	78 ± 4	3.0 mol H_2/mol (acetate)	[88]
Single-chamber	Brush, 0.22 m^2	0.6	—	21.0 mmol H_2/g COD (protein)	[89]

Methane control

Reactor	Strategies	Performance	References
Single-chamber	Increased applied voltage from 0.3 to 0.7 V	CH_4 reduced from 10 to 4%	[90]
2-chamber	Exposed anode biofilm in air	CH_4 reduced	[91]
Single-chamber	Low temperature	No CH_4 production at 4 and 9 °C	[92]

COD chemical oxygen demand

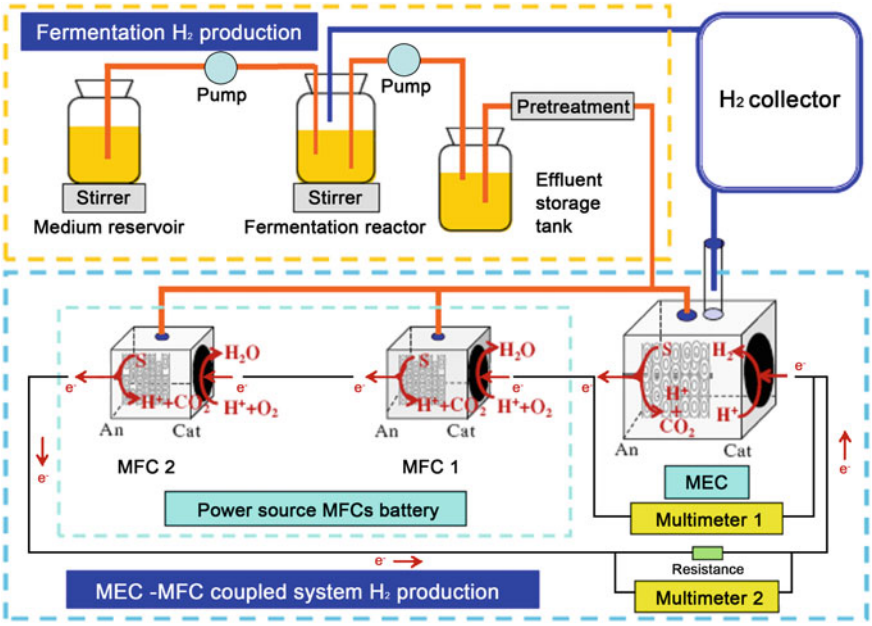


Fig. 2 Process diagram of the integrated hydrogen production, microbial electrolysis cell (*MEC*), and microbial fuel cells (*MFC*s)

sugars, 215 (based on glucose); ethanol, 2,379; acetate, 740; propionate, 351; butyrate, 204; valerate, 20. Before the effluent was fed into the MEC reactor, it was centrifuged to remove biomass and the pH was adjusted from 4.5–4.6 to 6.7–7.0 using PBS. With use of a single-chamber MEC at 0.6 V, the hydrogen production rate was 1.41 m^3 H_2/m^3/day and the overall hydrogen recovery was 83% based on both electrical energy and substrate utilization. For the whole coupling system of the fermentation and the MEC, the hydrogen production rate was 2.11 m^3 H_2/m^3/day and the overall hydrogen recovery was 96%. The energy efficiency was up to 287% based on the electrical energy input. However, the study also showed that little hydrogen was produced when the effluent was directly fed from the ethanol-type fermentation to the MEC system because of the low pH.

Wang et al. [96] developed a multiple coupling system integrating dark fermentation, MFCs, and an MEC together (Fig. 2). Hydrogen was firstly produced in continuous dark fermentation at a yield of 10 mmol H_2/g cellulose, which consumed about 71% cellulose. The COD of the fermentative effluent was 7,005 mg/L, of which 69% was volatile fatty acids, including acetic, propionic, butyric, and valeric acids, and ethanol. The pH of the effluent was 5.3, which was adjusted to 7.0 before the effluent was fed into the MFCs and the MEC. The effluent feeding into the MFCs generated a voltage of 0.43 V, which powered the MEC for hydrogen production at a yield of 33.2 mmol H_2/g COD. The overall hydrogen production for the integrated system was increased by 41% compared

with the dark fermentation alone. The total hydrogen production rate was 0.24 m^3 H_2/m^3/day, and the overall energy recovery efficiency was 23% (based on cellulose removed). No external electrical energy input was need for the MEC.

Although the combined process of dark fermentation and photofermentation or coupling of dark fermentation and an MEC could significantly enhance the hydrogen yield, how to achieve the optimal design and match the different functional processes is a key barrier that we have to overcome.

5 Perspectives

Biohydrogen is a renewable and clean energy source, making it an alternative for sustainable production, particularly when it is produced from organic wastes. However, although significant progress has been achieved in recent years in China and worldwide, developing a cost-effective system for biohydrogen production is still very challengeable, and the following bottlenecks should be addressed:

- *Limitation of dark hydrogen fermentation*. Although 4 mol H_2/mol glucose can be produced theoretically by clostridia, the yield achieved in reality is much lower since significant reducing equivalents are diverted toward by-products during the fermentation. Therefore, reducing by-product accumulation can significantly improve the hydrogen yield, which can be realized by engineering hydrogen-producing bacteria, taking advantage of understanding biological fundamentals and advances in modern biotechnology.
- *Coupling system for hydrogen production*. Hydrogen production can be enhanced when dark fermentation, an MFC, and an MEC are integrated together, but optimization is needed for these units. For example, the pH of the effluent discharged from dark fermentation should be adjusted for photosynthetic bacteria and exoelectrogens in the MFC and MEC units, and more intricate aspects underlying the reliable operation of the coupling system need to be identified, which may generate significant costs.
- *Policies*. Like other bioenergy technologies, laws and regulations play an important role in the production of biohydrogen. More investment is needed for fundamental studies and technology innovations targeting low-cost and high hydrogen recovery. Meanwhile, more preferable policies and/or strategies are needed to balance the fraction of renewable energy in the Chinese market to foster the industrialization of biohydrogen in the near future.

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References

1. Levin DB, Pitt L, Love M (2004) Biohydrogen production: prospects and limitations to practical application. Int J Hydrogen Energy 29:173–185
2. Liu BF, Ren NQ, Ding J, Xie GJ, Cao GL (2009) Enhanced photo-H_2 production of *R. faecalis* RLD-53 by separation of CO_2 from reaction system. Bioresour Technol 100:1501–1504
3. Zhu X, Xie XW, Liao Q, Wang YZ, Lee DJ (2011) Enhanced hydrogen production by *Rhodopseudomonas palustris* CQK 01 with ultra-sonication pretreatment in batch culture. Bioresour Technol 102(18):8696–8699
4. Shi XY, Yu HQ (2006) Continuous production of hydrogen from mixed volatile fatty acids with *Rhodopseudomonas capsulate*. Int J Hydrogen Energy 31:1641–1647
5. Tian X, Liao Q, Liu W, Wang YZ, Zhu X, Li J, Wang H (2009) Photo-hydrogen production rate of a PVA-boric acid gel granule containing immobilized photosynthetic bacteria cells. Int J Hydrogen Energy 34(11):4708–4717
6. Liu BF, Xie GJ, Guo WQ, Ding J, Ren NQ (2011) Optimization of photo-hydrogen production by immobilized *Rhodopseudomonas faecalis* RLD-53. Nat Res 2(1):1–7
7. Wang YJ, Liao Q, Wang YZ, Zhu X, Li J (2011) Effects of flow rate and substrate concentration on the formation and H_2 production of photosynthetic bacterial biofilms. Bioresour Technol 102(13):6902–6908
8. Liao Q, Wang YJ, Wang YZ, Zhu X, Tian X, Li J (2010) Formation and hydrogen production of photosynthetic bacterial biofilm under various illumination conditions. Bioresour Technol 101(14):5315–5324
9. Ding J, Liu BF, Ren NQ, Xing DF, Guo WQ, Xu JF (2009) Hydrogen production from glucose by co-culture of *Clostridium butyricum* and immobilized *Rhodopseudomonas faecalis* RLD-53. Int J Hydrogen Energy 34:3647–3652
10. Ren NQ, Wang BZ, Huang JC (1997) Ethanol-type fermentation from carbohydrate in high rate acidogenic reactor. Biotechnol Bioeng 54:428–433
11. Cohen A, van Gemert JM, Zoetemeyer RJ, Breure AM (1984) Main characteristics and stoichiometric spects of acidogenesis of soluble carbohydrate containing wastewater. Proc Biochem 19:228–237
12. Ren N (1994) Principles and controlling strategy of H_2 bio-production in organic wastewater treatment. PhD thesis, Harbin University of Architecture and Engineering, Harbin
13. Chen CC, Lin CY, Chang JS (2001) Kinetics of hydrogen production with continuous anaerobic cultures utilizing sucrose as the limiting substrate. Appl Microbiol Biotechnol 57:56–64
14. Pan CM, Fan YT, Zhao P, Hou HW (2008) Fermentative hydrogen production by the newly isolated *Clostridium beijerinckii* Fanp3. Int J Hydrogen Energy 33(20):5383–5391
15. Zhu DL, Wang GC, Qiao HJ, Cai JL (2008) Fermentative hydrogen production by the new marine Pantoea agglomerans isolated from the mangrove sludge. Int J Hydrogen Energy 33:6116–6123
16. Zhao X, Xing DF, Fu N, Liu BF, Ren NQ (2011) Hydrogen production by the newly isolated *Clostridium beijerinckii* RZF-1108. Bioresour Technol 102(18):8432–8436
17. Wang XJ, Ren NQ, Xiang WS, Guo WQ (2007) Influence of gaseous end-products inhibition and nutrient limitations on the growth and hydrogen production by hydrogen-producing fermentative bacterial B49. Int J Hydrogen Energy 32:748–754
18. Xing DF, Ren NQ, Li QB, Lin M, Wang A (2006) Ethanoligenens harbinense gen nov., sp. nov., isolated from molasses wastewater. Int J Syst Evol Microbiol 56:755–760
19. Niu K, Zhang X, Tan WS, Zhu ML (2010) **C**haracteristics of fermentative hydrogen production with *Klebsiella pneumoniae* ECU-15 isolated from anaerobic sewage sludge. Int J Hydrogen Energy 35(1):71–80
20. Ren NQ, Liu BF, Ding J, Xie GJ (2009) Hydrogen production with *R. faecalis* RLD-53 isolated from freshwater pond sludge. Bioresour Technol 100(1):484–487

21. Wang YZ, Liao Q, Zhu X, Tian X, Wang ZK, Zhang P, Zhang BP (2008) Isolation and identification of photosynthetic bacteria and their hydrogen-producing abilities. Chin J Appl Environ Biol 14(5):673–677
22. Guo WQ, Ren NQ, Wang XJ, Xiang WS, Meng ZH, Ding J, Qu YY, Zhang LS (2008) Biohydrogen production rate from ethanol-type fermentation of molasses in an expanded granular sludge bed (EGSB) reactor. Int J Hydrogen Energy 33(19):4981–4988
23. Li D, Yuan ZH, Sun YM, Kong XY, Zhang Y (2009) Hydrogen production characteristics of the organic fraction of municipal solid wastes by anaerobic mixed culture fermentation. Int J Hydrogen Energy 34:812–820
24. Zhao X, Xing DF, Zhang L, Ren NQ (2010) Characterization and overexpression of a [FeFe]-hydrogenase gene of a novel hydrogen-producing bacterium *Ethanoligenens harbinense*. Int J Hydrogen Energy 35:9598–9602
25. Ren NQ, Lin HL, Zhang K, Zheng GX, Duan ZJ, Lin M (2007) Cloning, expression, and characterization of an acetate kinase from a high rate of biohydrogen bacterial strain *Ethanoligenens* sp. hit B49. Curr Microbiol 55(2):167–172
26. Wang JL, Wan W (2009) Factors influencing fermentative hydrogen production: areview. Int J Hydrogen Energy 34:799–811
27. Zheng XJ, Yu HQ (2005) Inhibitory effects of butyrate on biological hydrogen production with mixed anaerobic cultures. J Environ Manag 74(1):65–70
28. Wang Y, Zhao QB, Mu Y, Yu HQ, Harada H, Li YY (2008) Biohydrogen production with mixed anaerobic cultures in the presence of high-concentration acetate. Int J Hydrogen Energy 33(4):1164–1171
29. Wang B, Wan W, Wang JL (2008) Inhibitory effect of ethanol, acetic acid, propionic acid and butyric acid on fermentative hydrogen production. Int J Hydrogen Energy 33(23):7013–7019
30. Tang J, Yuan Y, Guo WQ, Ren NQ (2011) Inhibitory effects of acetate and ethanol on biohydrogen production of *Ethanoligenens harbinese* B49. Int J Hydrogen Energy. doi:10.1016/j.ijhydene.2011.04.067
31. Wang JL, Wan W (2008) Effect of temperature on fermentative hydrogen production by mixed cultures. Int J Hydrogen Energy 33:5392–5397
32. Mu Y, Zheng XJ, Yu HQ, Zhu RF (2006) Biological hydrogen production by anaerobic sludge at various temperatures. Int J Hydrogen Energy 31:780–785
33. Luo G, Xie L, Zou ZH, Zhou Q, Wang JY (2010) Fermentative hydrogen production from cassava stillage by mixed anaerobic microflora: effects of temperature and pH. Appl Energy 87:3710–3717
34. Ren NQ, Ding J, Ding L, Liu M, Li YF, Bao HX (2004) Effect of Cu^{2+} concentration on hydrogen fermentation by mixed culture. J Harbin Inst Technol (New Ser) 11(1):11–16
35. Wang JL, Wan W (2008) Effect of Fe^{2+} concentration on fermentative hydrogen production by mixed cultures. Int J Hydrogen Energy 33:1215–1220
36. Zhang YF, Liu GZ, Shen JQ (2005) Hydrogen production in batch culture of mixed bacteria with sucrose under different iron concentrations. Int J Hydrogen Energy 30:855–860
37. Liu BF, Ren NQ, Ding J, Xie GJ, Guo WQ (2009) The effect of Ni^{2+}, Fe^{2+} and Mg^{2+} concentration on photo-hydrogen production by *Rhodopseudomonas faecalis* RLD-53. Int J Hydrogen Energy 34:721–726
38. Wang J, Wan W (2008) Influence of Ni^{2+} concentration on biohydrogen production. Bioresour Technol 99:8864–8868
39. Mu Y, Yu HQ (2006) Biological hydrogen production in a UASB reactor with granules. I: physicochemical characteristics of hydrogen-producing granules. Biotechnol Bioeng 94:980–987
40. Guo WQ, Ren NQ, Wang XJ, Xiang WS, Meng ZH, Ding J, Qu YY, Zhang LS (2008) Biohydrogen production from ethanol-type fermentation of molasses in an expanded granular sludge bed (EGSB) reactor. Int J Hydrogen Energy 33:4981–4988
41. Guo WQ, Ren NQ, Chen ZB, Liu BF, Wang XJ, Xiang WS, Ding J (2008) Simultaneous biohydrogen production and starch wastewater treatment in an acidogenic expanded granular

sludge bed reactor by mixed culture for long-term operation. Int J Hydrogen Energy 33:7397–7404
42. Li JZ, Li BK, Zhu GF, Ren NQ, Bo LX, He JG (2007) Hydrogen production from diluted molasses by anaerobic hydrogen producing bacteria in an anaerobic baffled reactor (ABR). Int J Hydrogen Energy 32:3274–3283
43. Ren NQ, Chen ZB, Wang XJ, Hu DX, Wang AJ (2005) Optimized operational parameters of a pilot scale membrane bioreactor for high-strength organic wastewater treatment. Int Biodeterior Biodegrad 56:216–223
44. Chu CF, Ebie Y, Inamori Y, Kong HN (2009) Effect of hydraulic retention time on the hydrogen yield and population of *Clostridium* in hydrogen fermentation of glucose. J Environ Sci 21:424–428
45. Yu HQ, Zhu ZH, Hu WR, Zhang HS (2002) Hydrogen production from rice winery wastewater in an upflow anaerobic reactor by using mixed anaerobic cultures. Int J Hydrogen Energy 27:1359–1365
46. Zhao BH, Yue ZB, Zhao QB, Mu Y, Yu HQ, Harada H, Li YY (2008) Optimization of hydrogen production in a granule-based UASB reactor. Int J Hydrogen Energy 33:2454–2461
47. Ren NQ, Li JZ, Li BK, Wang Y, Liu SR (2006) Biohydrogen production from molasses by anaerobic fermentation with a pilot-scale bioreactor system. Int J Hydrogen Energy 31:2147–2157
48. Wyman CE (1994) Ethanol from lignocellulosic biomass: Technology, economies, and opportunities. Bioresour Technol 50:3–15
49. Wang J (2009) The situation of straw resources and benefit analysis of straw biogas. Environ Prot Circ Econ 29(12):39–41
50. Mosier N, Wyman C, Dale B, Elander R, Lee YY, Holtzapple M, Ladisch M (2005) Features of promising technologies for pretreatment of lignocellulosic biomass. Bioresour Technol 96:673–686
51. Sun Y, Cheng J (2002) Hydrolysis of lignocellulosic materials for ethanol production: a review. Bioresour Technol 83:1–11
52. Ren NQ, Wang AJ, Cao GL, Xu JF, Gao LF (2009) Bioconversion of lignocellulosic biomass to hydrogen: potential and challenges. Biotechnol Adv 27:1051–1060
53. Yang B, Wyman CE (2008) Pretreatment: the key to unlocking low-cost cellulosic ethanol. Biofuels Bioprod Bioref 2:26–40
54. Kumar P, Barrett DM, Delwiche MJ, Stroeve P (2009) Methods for pretreatment of lignocellulosic biomass for efficient hydrolysis and biofuel production. Ind Eng Chem Res 48:3713–3729
55. Fan YT, Zhang YH, Zhang SF, Hou HW, Ren BZ (2006) Efficient conversion of wheat straw wastes into biohydrogen gas by cow dung compost. Bioresour Technol 97:500–505
56. Fan YT, Zhang GS, Guo XY, Xing Y, Fan MH (2006) Biohydrogen-production from beer lees biomass by cow dung compost. Biomass Bioenergy 30:493–496
57. Pan CM, Zhang SF, Fan YT, Hou HW (2010) Bioconversion of corncob to hydrogen using anaerobic mixed microflora. Int J Hydrogen Energy 35(7):2663–2669
58. Cao GL, Ren NQ, Wang AJ (2009) Acid hydrolysis of corn stover for biohydrogen production using *Thermoanaerobacterium thermosaccharolyticum* W16. Int J Hydrogen Energy 34(17):7182–7188
59. Ren NQ, Cao GL, Guo WQ, Wang AJ, Zhu YH (2010) Biological hydrogen production from corn stover by moderately thermophile *Thermoanaerobacterium thermosaccharolyticum* W16. Int J Hydrogen Energy 35:2708–2712
60. Zhu Y, Yang ST (2004) Effect of pH on metabolic pathway shift in fermentation of xylose by *Clostridium tyrobutyricum*. J Biotechnol 110:143–157
61. Xu JF, Ren NQ, Wang AJ, Qiu J, Zhao QL, Feng YJ, Liu BF (2010) Cell growth and hydrogen production on the mixture of xylose and glucose using a novel strain of *Clostridium* sp. HR-1 isolated from cow dung compost. Int J Hydrogen Energy 35(24):13467–13474

62. Long C, Cui J, Liu Z, Liu Y, Long M, Hu Z (2010) Statistical optimization of fermentative hydrogen production from xylose by newly isolated *Enterobacter* sp. CN1. Int J Hydrogen Energy 35(13):6657–6664
63. Ren NQ, Cao GL, Wang AJ, Lee DJ (2008) Dark fermentation of xylose and glucose mix using isolated *Thermoanaerobacterium ihermosaccharolyticum* W16. Int J Hydrogen Energy 33(21):6124–6132
64. Li S, Lai C, Cai Y, Yang X, Yang S, Zhu M, Wang J, Wang X (2010) High efficiency hydrogen production from glucose/xylose by the ldh-deleted *Thermoanaerobacterium* strain. Bioresour Technol 101(22):8718–8724
65. Ren NQ, Wang AJ, Gao LF et al (2008) Bioaugmented hydrogen production from carboxymethyl cellulose and partially delignified corn stalks using isolated cultures. Int J Hydrogen Energy 33:5250–5255
66. Wang AJ, Ren NQ, Shi YJ et al (2008) Bioaugmented hydrogen production from microcrystalline cellulose using co-culture *Clostridium acetobutylicum* X9 and *Ethanoigenens harbinense* B49. Int J Hydrogen Energy 33:912–917
67. Li DM, Chen HZ (2007) Biological hydrogen production from steam-exploded straw by simultaneous saccharification and fermentation. Int J Hydrogen Energy 32(12):1742–1748
68. Liu Y, Yu P, Song X et al (2008) Hydrogen production from cellulose by co-culture of *Clostridium thermocellum* JN4 and *Thermoanaerobacterium thermosaccharolyticum* GD17. Int J Hydrogen Energy 33:2927–2933
69. Fang HHP, Zhang T, Liu H (2003) Biohydrogen production from cellulose in wastewater under thermophilic condition. J Environ Manag 69:149–156
70. Zhang ML, Fan YT, Xing Y et al (2007) Enhanced biohydrogen production from cornstalk wastes with acidification pretreatment by mixed anaerobic cultures. Biomass Bioenergy 31:250–254
71. Fan YT, Zhang YH, Zhang SF, Hou HW, Ren BZ (2006) Efficient conversion of wheat straw wastes into biohydrogen gas by cow dung compost. Bioresour Technol 97:500–505
72. Fan YT, Zhang GS, Guo XY, Xing Y, Fan MH (2006) Biohydrogen-production from beer lees biomass by cow dung compost. Biomass Bioenergy 30:493–496
73. Ren NQ, Xu JF, Gao LF, Xin L, Qiu J, Su DX (2010) Fermentative bio-hydrogen production from cellulose by cow dung compost enriched cultures. Int J Hydrogen Energy 35(7): 2742–2746
74. Cao GL, Ren NQ, Wang AJ, Yao J, Liu LH (2010) Combination of mild chemical pretreatment with biological conversion for enhanced hydrogen production from cornstalk wastes. J Biotechnol 150S:S9
75. Cao GL, Ren NQ, Wang AJ (2010) Effect of lignocellulose-derived inhibitors on growth and hydrogen production by *Thermoanaerobacterium thermosaccharolyticum* W16. Int J Hydrogen Energy 35:13475–13480
76. Li RY, Fang HHP (2009) Heterotrophic photo fermentative hydrogen production. Crit Rev Environ Sci Technol 39(12):1081–1108
77. Logan BE (2009) Exoelectrogenic bacteria that power microbial fuel cells. Nat Rev Microbiol 7(5):375–381
78. Fang HP, Zhu HG, Zhang T (2006) Phototrophic hydrogen production from glucose by pure and co-cultures of *Clostridium butyricum* and and *Rhodobacter sphaeroides*. Int J Hydrogen Energy 31:2223–2230
79. Liu BF, Ren NQ, Tang J, Ding J, Liu WZ, Xu JF, Cao GL, Xie GJ (2010) Bio-hydrogen production by mixed culture of photo-fermentation and dark-fermentation bacteria. Int J Hydrogen Energy 35(7):2858–2862
80. Liu BF, Ren NQ, Xing DF, Ding J, Zheng GX, Guo WQ, Xie GJ (2009) Bio-hydrogen production by immobilized *R. faecalis* RLD-53 using soluble metabolites from ethanol fermentation bacteria *E. harbinense* B49. Bioresour Technol 100:2719–2723
81. Tao YZ, Chen Y, Wu YQ, He YL, Zhou ZH (2007) High hydrogen yield from a two-step process of dark- and photo-fermentation of sucrose. Int J Hydrogen Energy 32:200–206

82. Lo YC, Chen SD, Chen CY, Huang TI, Lin CY, Chang J-S (2008) Combining enzymatic hydrolysis and dark-photo fermentation processes for hydrogen production from starch feedstock: a feasibility study. Int J Hydrogen Energy 33(19):5224–5233
83. Su HB, Cheng J, Zhou JH, Song WL, Cen KF (2009) Improving hydrogen production from cassava starch by combination of dark and photo fermentation. Int J Hydrogen Energy 34:1780–1786
84. Chen CY, Yang MH, Yeh KL, Liu CH, Chang JS (2008) Biohydrogen production using sequential two-stage dark and photo fermentation processes. Int J Hydrogen Energy 33(19):5224–5233
85. Liu H, Grot S, Logan BE (2005) Electrochemically assisted microbial production of hydrogen from acetate. Environ Sci Technol 39(11):4317–4320
86. Liu WZ, Wang AJ, Ren NQ, Zhao XY, Liu LH, Yu ZG, Lee DJ (2008) Electrochemically assisted biohydrogen production from acetate. Energy Fuels 22:159–163
87. Wang AJ, Liu WZ, Ren NQ, Cheng HA (2010) Reduced internal resistance of microbial electrolysis cell (MEC) as factors of configuration and stuffing with granular activated carbon. Int J Hydrogen Energy 35(24):13488–13492
88. Liu WZ, Wang AJ, Cheng SA, Logan BE, Yu H, Deng Y, Van Nostrand JD, Wu LY, He ZL, Zhou JZ (2010) Geochip-based functional gene analysis of Anodophilic communities in microbial electrolysis cells under different operational modes. Environ Sci Technol 44(19):7729–7735
89. Lu L, Xing D, Xie TH, Ren NQ, Logan BE (2010) Hydrogen production from proteins via electro hydrogenesis in microbial electrolysis cells. Biosens Bioelectron 25:2690–2695
90. Wang AJ, Liu WZ, Cheng SA, Xing DF, Zhou JZ, Logan BE (2009) Source of methane and methods to control its formation in single chamber microbial electrolysis cells. Int J Hydrogen Energy 34:3653–3658
91. Hu H, Fan Y, Liu H (2008) Hydrogen production using single chamber membrane-free microbial electrolysis cells. Water Res 42:4172–4178
92. Lu L, Ren NQ, Zhao X, Wang H, Wu D, Xing DF (2011) Hydrogen production, methanogen inhibition and microbial community structures in psychrophilic single-chamber microbial electrolysis cells. Energy Environ Sci 4:1329–1336
93. Sun M, Sheng G, Zhang L, Xia C, Mu Z, Liu X, Wang H, Yu H, Qi R, Yu T, Yang M (2008) An MEC-MFC-coupled system for biohydrogen production from acetate. Environ Sci Technol 42:8095–8100
94. Sun M, Mu ZX, Sheng GP et al (2010) Hydrogen production from propionate in a biocatalyzed system with in situ utilization of the electricity generated from a microbial fuel cell. Int Biodeterior Biodegradation 64:378–382
95. Lu L, Ren N, Xing D, Logan BE (2009) Hydrogen production with effluent from an ethanol-H2-coproducing fermentation reactor using a single-chamber microbial electrolysis cell. Biosens Bioelectron 24:3055–3060
96. Wang AJ, Sun D, Cao GL, Wang HY, Ren NQ, Wu WM, Logan BE (2011) Integrated hydrogen production process from cellulose by combining dark fermentation, microbial fuel cells, and a microbial electrolysis cell. Bioresour Technol 102:4137–4143

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Microbial Fuel Cells in Power Generation and Extended Applications

Wen-Wei Li and Guo-Ping Sheng

Abstract Microbial fuel cells (MFCs) have been progressing at an amazing speed in the past few years, with higher power density but lower cost being continuously achieved. However, most of the studies to date have been conducted at laboratory scale, and many technological and economic barriers remain to be overcome prior to large-scale application of the MFC technique. In recent years, China has been playing an increasingly important role in this field, and has contributed considerably to moving MFCs forward toward large-scale implementations for both power generation and extended applications. Nevertheless, the development of MFCs is still in its infancy, the power density needs to be further improved, the cost reduced and a better understanding gained on the underlying mechanisms of electron generation and flow. All these warrant further investigations at both laboratory and pilot levels, and more cooperation of scientists and engineers from different disciplines and countries. In this review, we highlight the progress achieved to date in MFC technology, especially in China, and discuss the challenges and future opportunities.

Keywords Configuration · Electrode · MFC · Power generation · Power density · Wastewater treatment

Contents

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1 Introduction

Microbial fuel cells (MFCs) have emerged as a promising technology to meet the dual goals of wastewater treatment and energy production in a sustainable way. The idea of recovering electric energy from wastes through microbial metabolism in MFCs has aroused tremendous interest and stimulated intensive research among researchers and engineers all over the world. China in particular, as a country constrained by both severe water pollution and energy crisis, has put great efforts into the development of MFC technologies. In the past few years, China has achieved significant progress in this field, as is shown by the dramatically increased number of publications (Fig. 1). A comparison of the research activities between China and the world clearly show that China is playing an increasingly important role in this field. However, despite intensive studies and much progress, there are significant challenges remaining.

The MFC in its basic form is a device that uses bacteria to catalyze electrochemical reactions and generate current. It typically consists of a bacteria-enriched anode, a cathode and a separator between them [1]. A two-chamber or single-chamber design can be adopted, each with specific advantages. A schematic diagram of a typical two-chamber MFC is given in Fig. 2. In such a two-chamber setup, microorganisms oxidize organics in the anode chamber, producing protons and electrons; electrons transfer through an external circuit (with resistor) to the cathode and react with a final electron acceptor (mainly oxygen); the generated protons continuously diffuse via the separator to the cathode chamber to sustain the charge balance. However, researchers found that such two-chamber configurations generally suffer from high aeration cost and are difficult to scale up due to high overpotential. This has lead to the development of single-chamber MFCs [2]. In a single-chamber configuration, the cathode is exposed directly to the open air, which enables more efficient oxygen utilization, no need for aeration, and a compact reactor configuration. Moreover, membrane-less setups have been frequently adopted in single-chamber systems to facilitate proton transfer. However, this also increases the possibility of substrate loss and cathode biofouling. In recent years, MFC configurations have further evolved from simple column or cube reactors toward more bioreactor-type tubular systems [3] and integrated systems [4, 5], attributable to the rapid development of electrode and separator materials [6] and the introduction of the biocathode concept. In a biocathode MFC, cost-effective microorganisms instead of noble metals are used as the catalyst for versatile cathode reduction reactions. Such a strategy has significantly expanded the application scope of MFC technologies [7].

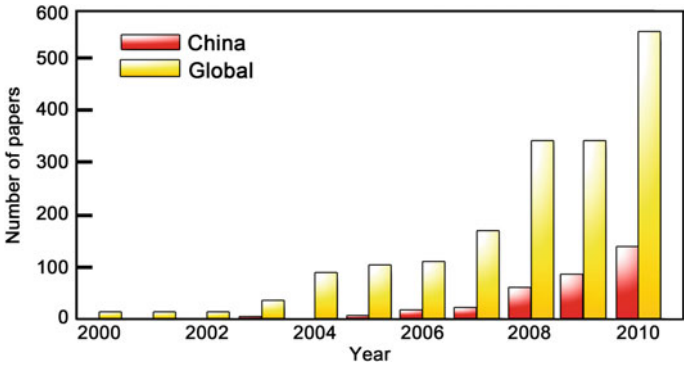

Fig. 1 Number of published articles on MFCs in China and the whole world (data are based on the Scopus search using the keyword "microbial fuel cell")

The reported power output of MFCs at the laboratory scale has improved drastically while the material cost has declined considerably in the past few years. Meanwhile, the potential application scope of MFC has expanded from pure electricity generation to bioremediation, bioproduction and biosensors. Nonetheless, MFC technology is still at a very early stage of development, and several challenges are yet to be addressed if MFC is to become a practical viable technology. The biggest barrier is still the low power output, which now seems to have reached a plateau despite of the recent intensive efforts in configuration, material and microorganism optimization. This low power density is mainly due to the various energy losses in the electrode reaction and electron–proton transfer. Another limiting factor has been the high cost of materials, for example the common use of expensive proton exchange membrane and platinum-loaded cathode. In addition, the significant increase of overpotential with reactor size during scaling up and the gradual biofouling of the separator all present barriers to its practical application. In all, major improvements are required if practical applications of MFC technology are to become feasible. Here, the recent efforts of Chinese researchers are highlighted, and the major obstacles and future opportunities of MFC technology are discussed.

2 Recent Progress for Improved Power Generation

2.1 MFC configuration

2.1.1 Tubular MFCs

Innovation in MFC design has been driven by the desire to increase the power output and scalability. Recently, tubular-type MFCs have become one area of intensive study [3, 8]. A tubular MFC differs from the conventional cube or

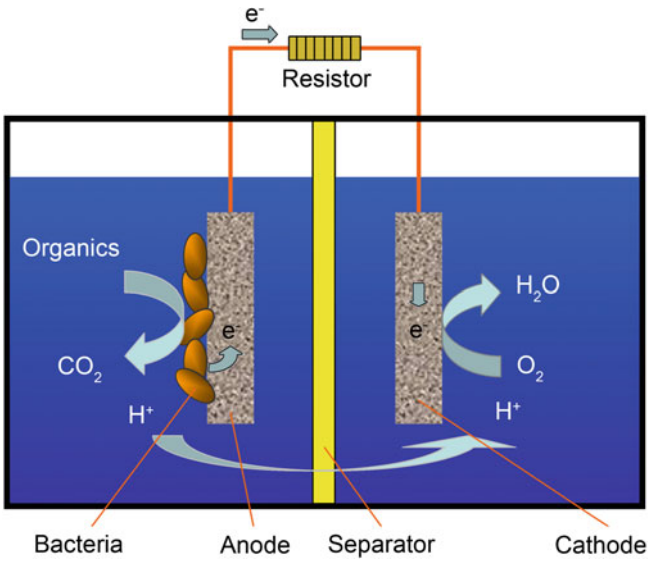

Fig. 2 Schematic diagram of a typical two-chamber MFC

column setup in that tubular-type electrodes are adopted. This design confers several extra benefits, such as great compactness, superior scalability and generally low internal resistance due to the common use of a separator electrode assembly (SEA) that decreases electrode spacing and increases the proton exchange area [8]. You et al. [9] developed a tubular air-cathode MFC with graphite granules packed inside as the anode, which demonstrated a low internal resistance of only 27 Ω and enhanced power generation. In such systems, cathode reactions are usually the limiting factors. To further enlarge the biocathode area, a tubular MFC with graphite fiber packed into the cathode chamber reactor was recently developed [10], and a maximum power density of 15 W/m^3 was achieved. The tubular configuration of MFC reactor enables more flexible choices of separator and electrode materials. Considering the constrained proton transfer by the use of a proton-exchange membrane (PEM), efforts have also been made to substitute the PEM with more porous and cost-effective separators. Zuo et al. (2007) manufactured a SEA by coating a layer of graphite paint and non-precious metal catalyst onto a tubular ultrafiltration membrane, and a high power density of 17.7 W/m^3 was achieved, attributed to improved proton transfer. Likewise, Zhuang et al. [11] developed a tubular SEA-MFC that used canvas cloth as the separator. To enhance its electrical conductivity and catalytic activity, the canvas cloth was coated with a mixture of nickel-based conductive paint and MnO_2. Under the fed-batch operation using brewery wastewater, this tubular MFC generated a maximum power density of 9.87 W/m^3. While the use of coarse-porous separators significantly reduces internal resistance, it also increases oxygen intrusion to anode chamber and substrate leakage to cathode chamber. In an effort to prevent such oxygen

diffusion, Li et al. [12] proposed a innovative overflow-type wetted-wall reactor by integrating a tubular anode chamber reactor and a baffle-chamber reactor. During operation, the anodic medium, after reaction, continuously overflows into the cathode chamber through a baffle and forms a falling-water film on the cathode surface. Nevertheless, this design cannot avoid crossover of substrate and thus still has low overall coulombic efficiency (CE).

2.1.2 Forced-Flow MFCs

Fluid mixing is an important factor of MFC operation, which may affect biofilm formation on the electrode and mass/proton transfer within the reactor chamber. This is especially true in larger-scale systems where mechanical agitation is usually needed to increase the electrochemical reactions. Rabaey et al. [13] found that adding baffles into the granular-bed anode chamber caused a forced flow and increased voltage. It has also been reported that a thick biocathode biofilm was beneficial for restricting oxygen transport. Based on this recognition, Hu [14] designed a baffle-chamber membrane-less MFC by adding a baffle into the reactor chamber to reduce mixing in the vicinity of the cathode and thus facilitate the formation of a thick biofilm. This slight modification of the reactor configuration led to improved CE. The baffle structure was also applied to a single-chamber MFC by setting baffles at the input and output of the reactor, which enabled adequate mixing during continuous operation [15]. Moreover, this configuration decreases the electrode space and offeres more convenience for electrode stacking. Recently, a novel baffle-chamber continuous-flow MFC with tubular air-cathode was designed by Feng et al. [16]. This baffle structure was effective in promoting fluid mixing and mass transfer in the graphite-granule-packed anode chamber and accelerating electrode reactions while maintaining an anaerobic environment. The maximum power density of this system reached 15.2 W/m^3 with an overall internal resistance of only 13.7 Ω, and the average chemical oxygen demand (COD) removal was 88.0% even at a high influent COD load.

Another approach to enhance fluid mixing by forced flow is to adopt upflow configuration. An upflow MFC incorporates the operating mode of upflow anaerobic sludge blanket (UASB) into MFC design [17]. Generally, an upflow was created by feeding influent from the bottom of the anode chamber and discharging the effluent from the top, with partial effluent feeding back to the bottom to form a recirculation. In such a way, adequate anolyte mixing can be achieved without the need for mechanical agitation. A first attempt was made by He and his colleagues [18], who constructed a two-chamber upflow MFC with the cathode chamber placed on top of the anode chamber. This group further improved the configuration by putting a U-shaped cathode chamber inside the anode chamber [19]. Both chambers were filled with granular activated carbon to increase the electrode area while a PEM was assembled as the separator. This setup significantly lowered the internal resistance to 17 Ω, likely due to a reduced ohmic resistance of the MFC at decreased electrode spacing and increased PEM surface area compared with the

conventional up–down configurations. As a consequence, a maximum power density of 29.2 W/m^3 was obtained in this system under a continuous operation mode. While it is necessary to continuously supplement electron acceptors (oxygen or hexacyanoferrate) into the cathode chamber in a two-chamber MFC, a single-chamber air-cathode upflow MFC was found to be more cost-efficient and yield a higher power density of 50.2 W/m^3 at tubular configuration [9].

2.1.3 Stacked Setup

Single MFC units can be assembled as a stack either in series or in parallel to increase the overall output voltage or current [20]. However, stacking multiple MFCs together in series can result in problems such as voltage reversal and contact voltage losses [21]. Liu et al. [22] recently designed a novel stacked configuration by physically bridging two parallel packed MFCs through an extra cation exchange membrane (CEM). This extra CEM facilitated the proton transfer, as the protons can move more conveniently to the cathode chambers that sandwich the anode chamber. The total internal resistance of stacked MFCs was decreased by connecting the circuits of individual reactors in parallel. As a consequence, the maximum specific power density increased. In addition, only an air-saturated buffer was used as the catholyte without addition of catalysts in this system; it thus holds great promise for practical application. In another study, Zhuang and Zhou [23] joined two single MFCs in series via a ball valve, which was employed to control the conductive substrate flow between the twin cells. The individual MFC units shared a feed passage and fuel continuously flowed into each cell through a simple water distribution system. Because energy loss would occur due to parasitic current flow under a substrate cross-conduction effect, the electrode spacing was reduced to effectively lower the voltage loss. By optimizing the connection, a minimal voltage loss of about 26.3% was achieved in such a system. To further lower the voltage loss, Zhong et al. [24] developed a baffle stacking MFC, in which a series of overflow plates and baffles were properly set between the units to minimize the parasitic energy loss caused by the substrate cross-conduction effect. A voltage loss of only 8.1% was obtained. Furthermore, the continuous flow mode of high-strength molasses wastewater in this system is also helpful in avoiding cell reversal caused by fuel starvation.

2.1.4 Mini-MFCs

While most researches have focused on scaling up of MFCs toward practical applications, efforts are also underway to fabricate micro-sized MFCs (mini-MFCs). Due to their small volume and low substrate consumption, mini-MFCs could be especially useful for powering miniature devices and hold great promise for potential application in medical and communication devices and in screening of electricigens as well as electrode materials [25]. Ringeisen et al. [26] fabricated

a mini-MFC with a volume of only 1.2 mL, which produced a high current density of 44 mA/m^2 (normalized to anode area). A high power density of 15 W/m^3 was also obtained in a dual-chamber microfluidic MFC, which contained a vertically stacked 1.5 μL anode chamber and 4 μL cathode chamber [27]. This high power output suggests a high possibility of powering nano-devices using on-chip bioenergy. Despite their good performances in power generation, such MFCs generally suffer from brittleness and a time-consuming and expensive fabrication process. Recently, Chen et al. [28] successfully fabricated a single-chamber mini-MFC array using a microfabrication technique. The MFC array contains eight anodic chambers, each with a uniform volume of 25 μL. Gold was chosen as the anode material because of its high conductivity and compatibility with conventional microfabrication methods. This mini-MFC exhibited excellent power generation performances compared with other microliter-scale MFCs. The current and power densities reached 29 mW/m^2 and 2148 mA/m^2 (normalized to anode area), respectively. Moreover, the fabrication process is relatively simple, and the MFC components can be readily assembled/disassembled, cleaned and reused.

2.2 Electrodes

To develop practical MFC technologies, one of the biggest challenges is to find low-cost, highly efficient, and sustainable electrode materials. Indeed, selection of the proper electrode is of critical importance for MFCs, as the properties of the electrode can affect bacteria adhesion, electron transfer and electrochemical reaction efficiency, and the cost of electrode material constitutes a large proportion of the MFC cost [29]. Although carbon-based materials, such as carbon paper, carbon felt, graphite pad, graphite granule, and graphite fiber, have been commonly employed for both anode and cathode, a variety of low-cost electrode materials and modified materials with favorable properties for anode or cathode reactions are currently under rapid development.

2.2.1 Anodes

Carbon-Based Materials

Anodic electrodes should have a large surface area, good biocompatibility and high chemical stability. In addition, as electrodes, they should ideally also possess high electrical conductivity [30]. Carbon- or graphite-based materials are currently most widely used [29]. Their major advantages lie in their excellent surface properties such as high specific surface area and porosity, and their low cost. You et al. [9] built a tube-type air-cathode MFC that used graphite granules as the anode and a graphite rod as the electron collector, achieving a maximum power density of 50.2 W/m^3. Zhang et al. [31] fabricated a low-cost and high-strength activated carbon fiber (ACF) by using polyvinyl alcohol (PVA) fibers as the

precursor. The PVA-ACFs possess lotus-root-like axially meso- and macroporous structures and a high specific surface area of 2128 m^2/g, which greatly facilitates mass transportation and bacterial adsorption. Using this ACF anode, Liu et al. [32] successfully enriched a large amount of electrochemically active microorganisms for MFC operation. A carbon mesh anode was recently reported [33] to also significantly reduce cost while enhancing electricity production compared to conventional carbon cloth. Granular activated carbon (GAC) was also used as the anode, which as a three-dimension electrode material offer even more available surface than flat-type carbon materials, and thus contribute to high bacterial adhesion and low anode resistance [19, 34].

Composite Electrodes

Despite their good surface properties, however, carbon-based materials mostly have lower conductivity and mechanical durability compared to metal materials like silver, copper, gold, and aluminum, which are also common choices for anode materials. Notably, metal electrodes also have many shortcomings such as low specific surface area and, most of all, relatively high cost. Therefore, one feasible strategy is to combine metal and carbon materials to make a composite electrode. Indeed, metals have been frequently incorporated into electrode design as a current collector from the carbon-based electrode. One good example is the graphite fiber brush anode that consists of a conductive non-corrosive metal rod surrounded by graphite fibers [35]. This unique structure incorporates both the merits of graphite fibers for bacterial adsorption and metal for electron transfer. As a consequence, a high power density of 73 W/m^3 was achieved in an air-cathode MFC. However, pretreatment of the fibers using high-temperature ammonia gas was usually needed to create a more positively-charged surface, which could be costly and unfavorable for large-scale application. To address this, Feng et al. [36] managed to develop a less energy-intensive treatment method that combines the use of heat treatment and acid soaking. The combined heat and acid treatment significantly improved the power production to 1370 mW/m^2, which is 25% higher than that using only acid treatment and 7% higher than only heat treatment. Such a graphite filter brush anode was also recently used by Zhang et al. [37] to enhance power generation in a membrane-less air-cathode MFC, and a maximum power density of 154 W/m^3 was obtained.

Surface Modification

Another approach to improve anode performance is surface modification by coating metal, polymers or even immobilizing mediators onto the electrode surface. Zhang et al. [38] constructed an air-cathode MFC using a mesoporous carbon modified anode, which demonstrated distinctly better performance than a bare carbon paper anode. The peak power density increased by 81%, the startup time of MFC was 68% shorter, and the anode resistance decreased from 300 to 99 Ω. A grapheme-modified stainless steel mesh anode was also recently developed and evaluated for MFC operation for the first time [39]. This novel system delivers a maximum power density of 2668 mW/m^2, which is 18 times larger than that using plain stainless steel mesh and is 17 times larger than that with a polytetrafluoroethylene (PTFE) modified anode. In addition, attempts have also been made to

modify the carbon material with metal. For example, Sun et al. [40] prepared a gold-modified carbon paper anode by homogeneously sputtering a layer of gold onto a carbon paper matrix. This electrode exhibited considerably higher conductivity, electrochemical capability, and biocompatibility, and contributed to a rapid reactor startup and an elevated current output of the MFC.

In addition, nano-materials such as carbon nanotubes (CNTs), due to their unique structure, high conductivity, and high surface-to-volume ratio, have also recently received considerable research interest for potential application in MFCs. However, because of their inherent cellular toxicity [41], CNTs are usually more preferred for electrode modification than direct electrode fabrication. It has been reported that the CNT-polyaniline (PANI) composite material displayed significant enhancement in both mechanical strength and conductivity [42] and showed good compatibility to neutral electrolyte [43]. Qiao et al. [44] successfully introduced such a PANI composite anode material into an *E. coli* inoculated MFC to improve power generation. This enhanced performance could be mainly attributed to the protective effects of PANI and the larger available surfaces and high electron transfer properties of CNTs. Zou et al. [45] constructed a novel polypyrrole (PPy)-coated CNT composite anode by coating the synthesized PPy-CNT solution onto a plain carbon paper. This PPy-CNT modified anode showed distinctly better performance than a plain carbon paper in terms of internal resistance and biocompatibility. The MFC exhibited a good performance even in the absence of any mediator. It is likely that the PPy polymers might contain some molecular units similar to redox mediators and form a redox active biocompatible layer that enhances electron transfer, indicating a great promise of using such composite materials as MFC anode. However, such composites usually have a relatively low CNT content, and thus the surface-to-volume ratio and electron transfer capability may still be limited. Moreover, a simple sprayed thin coat of CNTs are liable to loss during long-term operations. To improve this, Sun et al. [46] fabricated a novel multilayer CNT-modified anode by using a layer-by-layer self-assembly method. This multilayer CNT-modified anode provided a free-standing three-dimensional network structure of interwoven nanotubes, which enabled more specific surface area and a more than four-fold decline in interfacial charge transfer resistance. Operating in an MFC, the system demonstrated a 20% enhancement in power production compared to that with a plain carbon paper anode. In most studies, exoelectrogenic bacteria attach onto the CNT anode to form a biofilm and transfer electrons to the anode. In fact, the electron transfer can be accelerated simply by improving the contact between the bacteria and electrode. This was recently demonstrated by Liang et al. [47], who incorporated CNTs directly into the anodic biofilm matrix to constitute a composite biofilm. Such a strategy enabled more close contact between bacteria and electrode, and thus significantly enhanced the electron transfer rate and improved the MFC performance.

2.2.2 Cathodes

Cathode Materials

The cathode is a highly challenging aspect of MFC design because a three-phase interface of air, catholyte, and electrode is generally involved. In most cases, the cathode is the limiting factor of power generation, thus a careful selection of cathode material and configuration is of critical importance. An MFC cathode should ideally have a high redox potential, high conductivity and strength, good capability for proton capture, a sufficient interfacial area to enable electrochemical reactions, and meanwhile an acceptable manufacturing cost. The bacterial adsorption capability is of more critical importance when it comes to biocathode MFCs. These stringent demands for electrode properties is driving an intensive search for efficient and low-cost cathode materials.

At present, similar materials to the anode are frequently employed in the cathode, such as various carbon-based materials and metals. A combined cathode of graphite fiber brush and graphite granules was recently employed to favor the development of biocathodes and to further improve MFC performance [48]. Another important material being widely used for oxygen reduction in MFC cathodes is activated carbon, which provides even higher surface specific areas than graphite. He et al. [19] built an MFC using a U-shaped cathode chamber, which was filled with GAC and an inserted carbon fiber to constitute a three-dimensional cathode. This configuration resulted in a significant decrease in the cathodic charge-transfer resistance and enhanced power generation. Deng et al. [17] developed an MFC using ACF felt as the cathode, which yielded markedly elevated power density compared with the use of carbon felt or platinum-loaded carbon paper. The ACF felt has an extremely high volumetric specific surface area of 0.6×10^7 m^2/m^3. It is likely that the large surface area of activated carbon compensates for its relatively poor oxygen reduction activity compared to a platinum-catalyzed cathode. This ability for substantially increase power production of the MFC without metal catalysts and relatively low manufacturing cost indicates that activated carbon-based materials could be a promising cathode material for large-scale application.

Cathode Catalysts

A major limitation to the MFC system is the reduction of molecular oxygen by the cathode. Thus, since carbon-based materials mostly have poor catalytic activity, an additional catalyst is required in most cases to enhance the oxygen reduction rate at the cathode. One most commonly used catalyst is platinum (Pt), which is highly reactive but costly and sensitive to poisoning. Several researches suggested that some non-noble metals, such as pyrolysed iron(II) phthalocyanine and cobalt tetramethoxyphenylporphyrin could be potential substitutes for platinum [49]. However, most of these non-noble metals suffer from long-term instability. In the search for novel catalysts, manganese dioxide (MnO_2) was also recently found to significantly improve the catalytic activity of the cathode [50]. To explore into the capability of manganese oxide (MnO_x) as a cathodic catalyst, Liu et al. [51] prepared nano-structured MnO_x of various controlled sizes and

morphologies by an electrochemical deposition method. It was found that MnO_x nano-rods had the best electrochemical activity toward oxygen reduction. When such MnO_x was coated onto a carbon paper cathode, the MFC produced a power density of 772.8 mW/m^3, over three times higher than the plain carbon paper control. Recently, the employment of palladium (Pd) nanoparticles as a low-cost cathodic catalyst has also been documented [52]. The achieved catalytic efficiency of Pd nanoparticles was high, and the cathode coated with Pd nanoparticles showed a distinctly lower overpotential. In an effort to avoid the use of noble metals, the employment of Pt-based alloys has also been recently investigated to decrease cost and poisoning while maintain a high catalytic activity of cathode [53]. Nevertheless, poisoning of these metal catalysts may be inevitable in treatment of composite wastewater and their sustainability might be questionable. In this regard, biocathodes are bringing exciting new promise.

Biocathodes

The application of biocathodes undoubtedly presents a big new step in the development of MFCs. In a biocathode, electrochemically active microorganisms are used as the catalyst to promote cathodic reduction reactions. It confers several advantages over abiotic cathodes, such as low cost, self-sustainability, and the possibility of producing a variety of useful products or removing unwanted compounds [54]. The catalytic performances of biocathodes are directly determined by the amount and activity of the enriched microorganisms, and thus a high surface area of the cathode electrode for bacteria adhesion is invariably needed. You et al. [55] constructed a biocathode by using graphite fiber brush as the cathode material. After 133 h mixed culturing, the charge transfer resistance of the cathode decreased from 188 to 17 Ω, and the MFC generated a maximum power density of 68.4 W/m^3. To further increase the cathode area, a combination of graphite fiber brush and graphite granules cathode was employed by Zhang et al. [48]. This combined application of multiple materials enabled a biocathode of higher bacteria density than when single graphite fiber brush or graphite granules were used.

In addition to increasing the electrode area, efforts have also been devoted to culturing bacteria of high catalytic efficiency. Mao et al. [56] developed a novel biocathode based on the biocatalysis of ferro/manganese-oxidizing bacteria. In this system, iron and manganese oxides were modified onto the GAC cathode to increase the conductivity and promote bacteria activity. This improvement led to a low internal resistance of only 14 Ω and an enhanced power density. In another study, Huang et al. [57] proposed a dual strategy for biocathode enhancement. On the one hand, a combined cathode of granular plate and granular graphite was used to enlarge the cathode surface area to 900 m^2/m^3; on the other, indigenous microbial consortia from a hexavalent chromium (Cr^{6+}) contaminated site were used as the inoculum to improve the overall activity of the biocathode for Cr^{6+} reduction. This proved to be feasible: a power density of 2.4 W/m^3 were achieved in the enhanced system.

Photocatalytic Cathodes

Another relatively new type of cathode is the photocatalytic cathode, which is also attracting increasing attention in MFCs for energy generation and pollutant degradation. Semiconductor photocatalysts have long been applied to catalyzing electron transfer in photoelectrochemical cells, which convert solar energy into electricity [58]. Lu et al. [59] incorporated a semiconductor mineral of natural rutile as the cathodic catalyst into an MFC design. This novel design led to additional photoelectrochemical reactions at the cathode, which significantly enhanced electron transfer to the terminal electron acceptors in the MFC and enabled an almost doubled power density (12.03 W/m^3) under light irradiation compared to that in the dark. Ding et al. [60] evaluated the performance of a rutile-modified photocatalytic cathode in wastewater treatment. Rapid reduction of methyl orange with concomitant electricity production was achieved when exposing the cathode to visible light illumination. The internal resistance of the rutile-cathode MFC decreased significantly from 1378 Ω in the dark to 443.4 Ω in the light, demonstrating the practical feasibility of applying rutile as an efficient photocatalyst to enhance the cathodic electron transfer process. By constructing a TiO_2-coated paper photocatalytic cathode, Yuan et al. [61] also significantly lowered the cathodic electron-transfer resistance in an MFC and considerably accelerated *p*-nitrophenol degradation.

2.3 Separators

Although the development and success of membrane-less air-cathode MFCs have demonstrated that, at least in some situations, a highly efficient MFC without the use of a proton exchange membrane (PEM) is possible [2], the need to prevent substrate and oxygen crossover between the MFC chambers and to place the electrodes closely together means that some kind of separator is ultimately indispensible for efficient and sustainable operation of MFCs [6]. Conventional PEMs have many drawbacks such as constrained proton diffusion, high internal resistance, limited mechanical strength, and a high cost, which presents an important bottleneck to MFC application. In the past few years, a variety of separator materials have been investigated to substitute PEMs and to alleviate these limitations. These materials include anion exchange membranes, bipolar membranes, ultrafiltration/microfiltration membranes, micro-porous fabricates and some composite separators [6, 62]. Among the numerous options, the porous fabricates like cloth and glass fibers seem to be more attractive for practical use due to their excellent filterability, durability and, above all, low cost. Zhuang et al. [11] developed a separator–cathode assembly (SCA) using canvas cloth instead of membrane as separator material. A mixture of conductive nickel (Ni)-based paint and MnO_2 catalyst was coated onto the separator to increase the electrical conductivity and catalytic activity of SCA. In fed-batch mode, the tubular air-chamber MFCs equipped with the Ni-SCA generated maximum power densities of

9.87 W/m^3. By replacing the canvas cloth with a highly waterproof and durable Gore-Tex®cloth, Zhuang and his colleagues further raised the power density to 11 W/m^3 [63]. A comparison of the manufacturing cost of different SCAs showed that only 198.1 USD/m^3 is needed to construct such a cloth-based SCA, which is only a half the cost of membrane cathode assemblies. Zhang et al. [64] designed a SCA using non-biodegradable glass fiber as the separator material; the MFC generated a power density of 70 W/m^3 at a 2-cm electrode spacing. The employment of a double SCA further increased the power density to 150 W/m^3 at 2-cm electrode spacing and dramatically to 696 W/m^3 at 0.3-cm spacing. This enhancement was mainly attributed to the extremely low ohmic resistance of 2.2 Ω in such a SCA configuration. Recently, Zhang et al. [37] also applied glass fiber separators in a scalable MFC configuration to produce a maximum power density of 154 W/m^3, implying a high suitability of such materials for practical use in MFCs.

It is worthwhile to note that poor contact between the separator and the cathode in a SCA could significantly impair the system performance [6]. Therefore, an appropriate application of hydrogel [10] or a supporter [64] is essential to bind the separator and cathode together and to avoid possible deformation of separator in practical application.

2.4 Microbiology

In the past few years, there has been a dramatic increase in the reported number of microorganisms that can generate electricity in MFCs. Apart from several frequently-reported bacterial families such as *Shewanella*, *Geobacter* and *Proteobactor*, some new strains have also been recently discovered to effectively catalyze electrochemical reactions. Nevertheless, the categories of exoelectrogens are still relatively small compared to the vast diversity of microorganisms, and many of the known exoelectrogen strains show low electricity-producing ability. From the point of view of the biocathode, the community structure, metabolism pathways, and interactions of many biocathode bacteria remain unclear, which limits the biocathode capability and application. Therefore, to further promote power generation, highly efficient microorganisms need to be further explored, and the metabolic and catalytic activities of bacteria need to be enhanced. These all require a more fundamental understanding of the intracellular and extracellular electron transfer processes.

2.4.1 Bacteria Screening/Enrichment

The power generation performance of an MFC depends heavily on the enrichment degree and activity of exoelectrogenic bacteria. Thus, screening or enrichment of competitive exoelectrogens that can rapidly oxidize organic matter and transfer electrons to the anode is of vital importance for the establishment of an efficient

and robust MFC system. Since many of the exoelectrogens belong to dissimilar metal-reducing bacteria, Wang et al. [65] proposed a rapid selection strategy for an anodophilic consortium through serial dilution and regrowth of bacteria using poorly crystalline Fe(III) oxide, which selectively offers a favorable environment for anodophilic consortium growth. The MFC inoculated with this selected consortium showed distinctly higher power density and CE than those with original biofilm and activated sludge. Meanwhile, the start-up period of the MFC was also significantly shortened. However, this method of dilution and regrowth can only screen one major category of bacteria each time and is unsuitable for isolation of species with low population density. Hou et al. [66] developed a microfabricated MFC array to achieve high throughput screening of electrochemically active microbes. The MFC array consists of 24 integrated anode and cathode chambers to support direct and parallel comparison of bacterial activities. Moreover, the small volume of the MFC array enabled a rapid and sensitive reaction process. Using this array, several *Shewanella* strains capable of high electricity generation were successfully identified and characterized. More recently, Chen et al. [28] managed to build a microliter-level MFC array by using the simpler photolithography technique, which offers more flexibility and economic benefits for the implement of such rapid-screening technologies. Nevertheless, the available techniques for rapid screening of exoelectrogens are still few, while the sensitivity and applicable scope of the established screening/enrichment techniques are yet to be further improved. It is worth mentioning that biocathode microbiology is also an important area to be explored. In light of the high versatility of biocathodes, usually bacteria with strong capabilities not only in electron transfer but also in catalyzing the degradation of specific compounds are desired [57]. This also calls for the development of efficient and reliable screening/enrichment techniques.

2.4.2 Consortia Interactions

Notwithstanding the advantages of selected pure strains for power generation and specialized purposes, it should be recognized that a mixed culture could be ultimately more desirable from a standpoint of practical wastewater treatment, due to a higher flexibility, robustness and suitability for composite wastewater, and usually higher electricity-producing performances. Therefore, an investigation into the interactions among different consortia is highly desired. Indeed, the substantial functional diversity of anode biofilm is closely associated with its phylogenetic diversity. A synergistic relationship between photosynthetic bacteria and heterotrophic exoelectrogenic bacteria was found to exist in a self-sustained phototrophic MFC [67]. Synergistic interactions between anode-attached and planktonic bacteria were also recently demonstrated in an MFC for simultaneous electricity generation and sulfide removal [68]. However, it remains unclear how these different populations coexist and affect each other. This warrants a deeper investigation.

2.5 Process Enhancement Techniques

In biological processes, various physical or chemical enhancement technologies are usually applied to promote bioactivity and degradation performance. Such strategies have also been recently investigated in MFC systems to improve biocatalytic activity and accelerate electrochemical reactions.

Since the anodic potential to some extent determines the degradation rate of the substrate in the anodic chamber, it is expected that the anodic potential may also affect the growth and activity of anodic microorganisms. An improved enrichment of acetate-oxidizing microorganisms on the anode at a positive-poised potential was first observed by Finkelstein et al. [69] in a benthic MFC. To obtain a more comprehensive understanding of this enhancement, the effect of anodic positive potential on start-up time and performance of the MFC was investigated by Wang et al. [70]. The application of a +200 mV (versus Ag/AgCl) poised potential on the anode was found to shorten the startup time from 59 to 35 days compared to the control. In this process, a higher voltage output and a lower charge transfer resistance were obtained, demonstrating that the applied positive potential could increase the electrochemical activity of anodic microorganisms. This enhancement was thought to mainly resulted from an increased driving force of substrate oxidation that favors bacteria growth and bacteria adhesion onto the positively-charged anode surface. In addition to constant additional voltage application, a transient external voltage (lasting for only 10 min) was also recently found to promote the bioanode performance of MFCs [71]. The enhancement effects were thought to be mainly attributed to an improved bioanode catalytic activity as well as an accelerated bacteria adhesion. Notably, employing a transient voltage, which requires only small amount of energy input, could be economically more attractive than a permanent voltage application. Such external voltage enhancement techniques offer a possible new avenue for MFC improvement, but the underlying mechanism is yet to be elucidated and further optimization of the specific parameters is needed. Apart from external voltage application, an external magnetic field was also recently found to improve MFC performance. Li et al. [72] applied a 100 mT magnetic field to MFCs, and found it significantly decreased the internal resistance and enhanced the power generation. The magnetic field is suspected to have directly accelerated the growth and improved the bioelectrochemical activity of a *Shewanella* strain at the anode through an oxidative stress mechanism. The exact mechanism likewise needs further in-depth investigation.

In addition, direct chemical dosing may provide another feasible approach to enhancing MFC performance. Wen et al. [73] investigated the possibility of promoting intracellular electron transfer through biosurfactant addition, because surfactants are known to readily change the cell membrane ultrastructure to create trans-membrane channels. As expected, the addition of rhamnolipid as a biosurfactant increased the permeability of bacteria cells, leading to substantially lowered electron transfer resistance and a 12.5-fold increase in power density (from 22 to 275 W/m^3).

All these efforts have opened up new opportunities for improving power generation of MFCs. However, we still have no clear idea about the detailed interactions between the physical and chemical factors and the microbial properties. Indeed, one of the biggest barriers in the road of advancing MFC technology is our lack of understanding on the ecology of the microbial communities that produce and transfer electricity and their interactions with the external environment. This is exactly where the greatest uncertainty in MFC design lies. Therefore, one of our first tasks is to get a deeper understanding of such processes so that we can have a better handle on microbial behavior regulation by applying appropriate enhancement techniques. Meanwhile, more efficient and economically-viable approaches for MFC enhancement are still to be pursued.

3 Extended Applications

MFCs were originally designed to produce power from organic matters under bacterial catalysis, but there has been a rapid evolution in these systems in recent years, resulting in a variety of possibilities for extended applications. In the light of the many remaining limitations to further improvement of power output and scale-up, some researchers have argued that the greatest value of MFC technology may not be the production of electricity but the ability to efficiently degrade wastes and recover valuable products [74, 75]; this may open up new fields of MFC application.

3.1 Enhanced Wastewater Treatment

One of the most promising directions of practical MFC application is for wastewater treatment and bioremediation [76]. MFCs for wastewater treatment show several obvious advantages compared with conventional biological treatment processes, such as very low sludge production, high capability to remove a wide range of refractory organics and other contaminants, energy generation, and possible low cost because the power output from MFCs may ultimately compensate for part of the energy consumption [74]. Indeed, there are already some successes in implementing MFCs for enhanced wastewater treatment and bioremediation in real-world scenarios, but mostly at small scales.

3.1.1 Refractory Substance Degradation

Success in using various readily degradable organics, such as glucose, acetate and sucrose, to fuel MFCs has been intensively documented [77], while the exploration of MFCs for degradation of bio-refractory substance is at the very beginning.

Contaminants often persist in the environment due to a lack of suitable electron acceptors, whereas the electrodes of MFCs could be a highly attractive option, because they can provide a sustainable clean electron sink for the degradation or conversion of harmful environmental contaminants [78]. Thus, it is widely believed that MFCs could be a practical viable technology for treatment of toxic and complex matters that are not readily treated by conventional methods [79]. Mu et al. [80] used a dual-chamber MFC to decolorize Acid Orange 7, achieving a maximum decolorization rate of 2.64 mol/m^3/d at the cathode and even more when external power was supplied. Liu et al. [32] also achieved efficient methyl orange reduction at a pseudo-first-order decolorization rate kinetic constant of 0.05 h^{-1} and a 20% further improvement after modifying the cathode with thionine. Zhu and Ni [81] managed to construct an MFC–Fenton system for enhanced azo dye degradation by incorporating an electro-Fenton process into MFC design. In this system, the electrons produced from a microbial reaction were utilized to drive electro-Fenton reactions for *p*-nitrophenol degradation in the MFC cathode, which enabled a continuous hydrogen peroxide (H_2O_2) generation and a high oxidation efficiency. However, an acid environment is required in that case, which limited its application. In a more recent study, Feng et al. [82] further improved this process to make it adaptable to neutral pH conditions. A PPy/AQDS-modified anode was used to lower electron transfer resistance. Moreover, a mineral iron oxide instead of Fe^{2+} was used as the iron source, so that the iron reagent could be easily recycled. With these improvements, the H_2O_2 production at the cathode was significantly enhanced, leading to rapid mineralization of Orange II at a rate of 0.145 h^{-1}. Antibiotics are another group of bio-refractory compounds that are widely present in water. Wen et al. [83] studied the possibility of degrading penicillin by an MFC. It was interesting to find that the glucose–penicillin mixtures played an active role in the production of electricity, which might have resulted from an enhanced permeability of microbial cell membranes in the presence of penicillin and thus an accelerated electron transfer.

In addition to the organic compounds, the biomass is also an important refractory substance that is proposed to fuel MFCs. Anaerobic sludge has been demonstrated to be a feasible substrate for MFCs [14]. Significant sewage sludge degradation accompanied by power generation was reported by Jiang et al. [84] in a two-chamber MFC using potassium ferricyanide as the electron acceptor. After 250 h operation, the total COD (TCOD) of sludge was reduced by 46.4% from an initial TCOD of 10,850 mg/L. Recently, blue-green algae was also tested as the feedstock for a tubular MFC [85]. Over 78.9% of the TCOD and 80.0% of soluble COD (SCOD), and 91.0% of total nitrogen (TN) were removed within 12 days. Moreover, more than 90% of microcystins released from blue-green algae were removed, exhibiting a potential means of power generation from blue-green algae coupled with removal of algal toxins. This capability of removing toxicants can be very valuable in biological processes. For example, experiments have shown that MFCs are able to remove fermentation inhibitors that accumulate during cellulosic biomass pretreatment [86]. This removal of the inhibitors would undoubtedly allow for more efficient subsequent fermentation processes.

In the continuing efforts to promote MFC applications, some researchers have tried the use of low-cost and abundantly available lignocellulose biomass for direct power generation. The use of raw corn stover as a substrate for electricity generation was recently demonstrated by Wang et al. [87] in a single-chamber MFC with bio-augmented mixed culture, although the power output was much less than with glucose substrate. It should be noted that when lignocellulose is used as the substrate for electricity production, a microbial community with both cellulolytic and exoelectrogenic activities is required. For this purpose, Zang et al. [88] inoculated a special category of rumen microorganisms in an MFC to directly recover electricity from *Canna indica*, a lignocellulosic aquatic plant rich in cellulose, hemicellulose, and lignin. Approximately 46% of cellulose, 61% of hemicellulose, and 22% of lignin were degraded in this system, indicating the feasibility of decomposing complex lignocellulosic biomass in MFCs.

3.1.2 Metal and Nutrient Removal

MFCs have also been frequently used for in-situ clean-up of metal pollutants and nutrients. Indeed, the introduction of biocathodes into MFCs has led to remarkable developments in bioremediation. By using a tubular biocathode MFC, Huang et al. [10] achieved a high Cr^{6+} reduction rate of 12.4–20.6 mg/g-VSS/h at the cathode (VSS: volatile suspended solid), with a pseudo-first-order removal kinetic constant of 0.451 h^{-1}. Li et al. [89] reported a high-rate photocatalytic-cathode MFC where a photocatalytic process was integrated into cathodic reactions by modifying rutile on the cathode. A high Cr^{6+} removal rate of 97% was achieved under light irradiation within 26 h, which was 1.6 times higher than that obtained by the dark control. A system with even higher performance was developed by Liu et al. [90] in a biocathode MFC, where a complete reduction of Cr^{6+} was achieved within only 4 h, attributed to the electrogenerated H_2O_2 driven by iron-reducing bacteria. Besides Cr^{6+} removal, an MFC was also recently shown to effectively remove Cu^{2+} from aqueous solution through cathodic reduction [91]. A very high Cu^{2+} removal efficiency (>99%) was obtained at an initial Cu^{2+} concentration of 196.2 mg/L, leaving a final effluent Cu^{2+} concentration below the USEPA standards for drinking water. Moreover, the Cu^{2+} was directly reduced to cuprous oxide and metal copper and deposited on the cathode, which favored a subsequent recovery.

MFCs for nitrogen removal present another area of research interest for ammonia-rich wastewaters [92]. You et al. [93] developed a biocathode MFC for cathodic nitrification. It is interesting to find that the additional protons produced in the nitrification process contributed markedly to electricity generation by lowering the ohmic resistance and buffering the pH, and meanwhile enabled a phosphate-buffer-free operation of the MFC. This may confer significant benefits to practical wastewater treatment, as the dosing of the pH buffer could be economically unfavorable. Based on this process, a novel coupled setup comprising an comprising oxic-biocathode MFC and an anoxic-biocathode MFC was recently proposed to simultaneously remove carbon and nitrogen [94]. With this coupled

MFC system, the maximum removal rates of COD, NH_4^+-N, and TN reached 98.8, 97.4, and 97.3% respectively, accompanied by a power output of 14 W/m^3 in the oxic-biocathode MFC and 7.2 W/m^3 in the other MFC.

3.1.3 Practical Wastewater Treatment

Inspired by the hope of simultaneous pollutant removal and energy recovery in practical systems, MFC setups have also been incorporated into various conventional anaerobic/aerobic processes for practical wastewater treatment. The effectiveness of MFCs for treating beer brewery wastewater was first demonstrated by Feng et al. [95] using an air-cathode MFC, which achieved a power density of up to 528 mW/m^2 (normalized to anode surface) and COD removal of 87–98% depending on the influent COD concentration. The suitability of brewery wastewater as the substrate for MFC was also recently shown in a continuous-operation MFC [96], with a maximum power density of 24.1 W/m^3 obtained. Huang et al. [97] investigated the performance of an MFC for treatment of paper recycling plant wastewater, which contained both soluble organics and refractory particulate matter such as cellulose. The SCOD and TCOD removal reached 73% and 76% respectively, and the cellulose was almost completely degraded. The researchers recently developed another efficient MFC system by integrating an MFC with the electro-oxidation process for coking wastewater treatment [98]. Driven by the electro-oxidation reactions, the removal rates of TCOD and TN reached 82% and 68% respectively, which present an approximately 30% improvement in both indexes over a pure MFC system. Zhang et al. [99] used an upflow air-cathode MFC to treat landfill leachates, which had a complex composition of dissolved organic matter, inorganic macro-components, heavy metals, and xenobiotic compounds. Despite the highly recalcitrant property of such wastewater, a considerable removal of COD and NH_4^+-N and a maximum power density of 12.8 W/m^3 were obtained in this system, suggesting a good capability of MFCs for pollutant removal.

Another commonly-cited advantage of MFCs for wastewater treatment is their capability to offset, at least partially, the energy consumption of conventional activated sludge processes. This has been experimentally shown by several studies [5, 100]. In an attempt to reduce operating cost, Liu et al. [5] managed to integrate an MFC into a sequencing batch reactor (SBR) system for enhanced COD removal and power generation. In this SBR-MFC system, the MFC accounted for 12% of the reactor volume but contributed to 18.7% of the total COD removal, while a maximum power density of 2.34 W/m^3 was achieved, suggesting a good possibility for enhancing the performance and economics of existing activated sludge processes.

In addition to direct organic degradation, MFCs could also be employed as a post-treatment step for anaerobic digestion. On the one hand, these effluents often contain volatile fatty acids (VFAs) that require further treatment to meet the discharge standard. On the other, MFCs have good capability to utilize VFAs for

electricity generation [101]. However, the acid nature of the fermentation liquor may be detrimental to anodic microorganisms, and the feasibility of MFCs for anaerobic effluent polishing are yet to be experimentally demonstrated.

In most of the above cases, the electricity produced by the microbes is trivial compared to the increased rates of contaminant degradation and bioremediation. These extended values and potentials of MFC are worth pursuing, but need further exploration.

3.2 Other Potential Applications

3.2.1 Power Sources for Low-Power Devices

Despite the great promise of MFC technologies for power generation and wastewater treatment at large scales, most of these envisaged applications are currently still unfeasible and significant improvements are required. One of the most likely areas to see practical application of MFCs in the near future, if any, would be utilizing the limited MFC-derived electricity for low-power devices.

One most attractive attribute of MFCs is powering off-grid devices at remote locations, such as in lakes and seas. The first demonstration that an MFC could power a practical device was a meteorological buoy for remote monitoring, which continuously obtained its entire power from a benthic MFC [102]. Another case is a benthic MFC that powered an ultrasonic receiver for real-time tracking of acoustically tagged green sea turtles and collected data on their behavior [103], which successfully operated for several years with no power decrease. To further optimize the configuration of benthic MFCs, Li et al. [104] recently investigated the impacts of electrode shape on power generation performance, and found that the column-type electrode offered superior properties and enabled a lower internal resistance.

Another unusual but quite possible application of MFCs is to power implanted medical devices, which may hopefully provide indefinite power and remove the need for surgery to replace batteries. For example, Siu and Mu [105] developed a microfabricated polydimethylsiloxane MFC with flexible and biocompatible structure. This MFC could use glucose and oxygen in the blood for power generation, and thus could serve as a potential power source for implanted chemical devices. However, implanting an MFC into the blood might carry risks of thrombosis and body rejection reactions. Considering that the large intestine might be a more suitable site for MFC implantation, Han et al. [106] managed to develop an MFC that simulated the environmental features of the transverse colon. Stable power generation with an average voltage of 308 mV (at 500 Ω internal resistance) was obtained, and the maximum power density reached 73.3 mW/m^2 (normalized to anode surface) in this system. This demonstrated that an MFC located in the large intestine could be a promising power source for implanted medical devices.

Notwithstanding all these potential applications, low power output remains the most challenging obstacle to their practical use. The involvement of a capacitor in an MFC power system is considered a possible solution, enabling continuous energy storing while being intermittently discharged at higher voltage. This raises the need for development and cooperation in disciplines such as biology and materials.

3.2.2 MFC Biosensors

The impacts of the ambient environment (such as organic types and content, toxicants, pH, and temperature) on the activities of microorganisms can be reflected by their metabolism and proliferation in MFCs, which would ultimately alter the current generation. Therefore, an MFC can offer valuable real-time information on the rate and nature of biodegradation process, and has been proposed to work as a sensitive biosensor for environmental and bioprocess monitoring [107]. It possesses several potential advantages over conventional biosensors, such as high sensitivity and accuracy, applicability for remote or dangerous sites, and self-sustained operation. Chang et al. [108] invented an MFC sensor in which the current signal generated by the MFC was amplified to sensitively reflect the biochemical oxygen demand in a continuous mode. An MFC biosensor for in-situ assessment of microbial activity was also recently explored [109]. In addition to monitoring, it should be noted that the capability of bacteria to donate electrons to an external circuit with a controllable resistance may ultimately make the MFC process amenable to real-time control. These are relatively new but highly promising areas of MFC application for which research interest is also rising in China, although relevant reports are still scarce to date.

3.2.3 Driving Synthesis Processes

How to utilize in situ the limited electrical power generated from MFCs is an important issue for MFC development. Aside from their potential use as power sources for low-power devices and biosensors, several studies have demonstrated that it is also possible to use this energy to drive other bioprocesses. One such bioprocess could be microbial electrosynthesis: the synthesis process catalyzed by active microorganisms in microbial electrochemical cells, which is capable of producing a variety of useful chemical compounds such as hydrogen, ethanol, methane, and H_2O_2 in a cost-effective way [110]. Usually, a small amount of energy is needed to drive the thermodynamically unfavorable reactions, which necessitates an external power supply. One way to address this is utilizing bioelectricity from MFCs. Sun et al. [4] designed a microbial electrolysis cell (MEC)-MFC coupled system, in which an MFC was used as an assistant power source to drive H_2 production in an MEC. Without any external power supply, considerable hydrogen production was achieved in this coupled system.

The highest hydrogen production rate and hydrogen yield reached 14.9 mL/L/d and 1.60 mol-H_2/mol-acetate, respectively. The researchers further improved this system by offering an optimal input voltage by using a MFC stack as the power supply and by adjusting the loading resistance of the MFC [111]. These results clearly suggest a good feasibility of such a coupled system for self-sustained hydrogen production.

The coupling of an MFC with other photochemical and electrochemical processes was also recently reported. Yuan et al. [61] designed a novel MFC-photoelectrocatalysis cell coupled system to effectively reduce*p*-nitrophenol through a photocatalytic oxidation process assisted by power from an MFC. In such a system, there exists a synergistic effect between the electrochemical and photocatalytic oxidation processes: the additional electrons provide by the MFC can reduce the recombination of photogenerated electrons and holes in the photoelectrocatalysis cell and increase photocatalytic efficiency, while the efficient consumption of electrons at the MFC cathode in turn favors more power generation. As a consequence, a high *p*-nitrophenol degradation rate was obtained, which was twice the sum of the rates by the individual photocatalytic and electrochemical methods. In another study, the MFC electricity was used to promote H_2O_2 production through cathodic reduction of oxygen, and a maximum H_2O_2 concentration of 62.21 mmol/L was obtained [112], which is comparable to that in some electrolysis systems [113].

4 Challenges and Future Opportunities

Scientific research has advanced MFC technologies at an amazing pace in the past decade. Despite the great progress achieved so far, however, we are still far from seeing MFCs implemented in real system at large scales. One of the biggest challenges of MFC technologies to date is to bring them out of the laboratory and engineer these technologies to practical large-scale systems. To address this, a balanced consideration of the technological and economic factors is needed. On the other hand, the many new discoveries and the broadening cooperation between multiple disciplines are opening up new opportunities to move MFCs toward practical application.

4.1 Challenges To Large-Scale Application

4.1.1 Further Power Output Lifting

The power densities of MFC achieved so far are still far from adequate for any commercialized application. Thus, a further raise of the power output is necessary. However, there are several major bottlenecks. One of the most highlighted

obstacles is the high voltage losses, also called overpotential [1]. Several reasons contribute to this overpotential. First, the oxidation or reduction reactions at the electrode incur activation overpotential. Addition of chemicals or employing biological catalysts can decrease but will never eliminate the activation overpotential. Second, the transfer of electrons through an electrical circuit and ions through the electrolyte result in ohmic loss. Finally, the supply of substrate or discharge of protons may become limited at high current density or insufficient mixing, leading to concentration overpotential. All these add up to voltage losses and thus decrease the power output [110]. Intensive investigations have focused on these aspects in efforts to reduce the overall voltage losses; for example, searching for more conductive materials, adopting highly-active catalysts and removing separation membranes. However, there is a trade-off between the properties and costs of the materials in most cases. A membrane-less operation can accelerate proton transfer and reduce anodic inhibition at high pH, but it also incurs the risk of oxygen intrusion and substrate crossover. One possible solution is to use coarse-porous separators in place of conventional PEMs, but these materials vary significantly in their properties and mostly may not be able to resist hydraulic pressure. This makes separator design difficult.

Although MFC technologies are also progressing toward low-power utilization, such as powering implanted medical devices and on-chip instruments, they are still in the infancy stage. In addition, high current density should be paid more attention, because it may result in excessive ohmic heating. More detailed study on optimizing the electrode allocations, power density, and operating stability is necessary before further applications are possible. Moreover, further studies on implantation rejection, microbe leakage, and cytotoxicity are needed to investigate whether mini-MFCs are suitable for practical medical applications.

One common practice to raise overall power output is to connect multiple MFCs as a stack either in series or in parallel [20]. However, voltage reversal may occur in such stacked systems due to different resistances between stack units or substrate starvation during operation. The voltage loss is proposed to be a consequence of parasitic current flow due to the substrate cross-conduction effect when fuel cell arrays share the same electrolyte but have inappropriate connection with each other. Another crucial parameter to be considered is to reduce the internal resistance in order to minimize the number of MFCs needed. Thus, key developments are still required to create highly conductive and scalable scaffolds with suitable properties for stacking.

4.1.2 Scaling Up

Scale-up of MFCs for practical wastewater treatment and energy production is an important issue. However, at the present stage, full-scale implementation of this technology is not straightforward because of several technological and economic barriers. Scaling up an MFC involves more than just linearly scaling up all reactor dimensions and increasing the surface area of electrodes. There are many problems

associated with internal resistance, substrate diffusion, proton transfer, and mechanical strength. For example, the overpotentials may increase with size due to increased electrode spacing and longer distance for proton transfer, the substrate and proton diffusion would be hindered at a decreased mixing (or it may unfavorably lead to biomass detachment and increased energy consumption), the separator and air-cathode may be vulnerable and have more leakage under increased hydrostatic pressure, and there are the economic limitations. For these reasons, currently the large MFCs generally show poorer performances than smaller reactors [5, 114]. In order to minimize the internal electrical resistances at the electrodes, it is believed that an arrangement of multiple small reactors in a stack might ultimately be the most effective design. Indeed, baffled and stacked types of MFCs may be advantageous because of not only the possible lower overpotential but also more flexibility for setup and operations. Nevertheless, our knowledge in this aspect is still rather limited.

While many of the problems encountered can be addressed in laboratory studies, other issues remain that ultimately require field testing. For example, pilot-scale studies are needed to address questions like how well materials can perform at larger scale, how the materials would be influenced by environmental conditions, and how to offer proper maintenance on aeration and to control electrode fouling. So far, there are already several pilot-scale MFC facilities running [29], which are expected to offer valuable information on practical scaling-up of MFCs.

4.1.3 Long-Term Stability

For the MFC to become a commercially-viable technology, sufficient attention should also be paid to process stability. Currently there are several factors that unfavorably affect the long-term stability of MFC processes. First, the separator performance would change over time due to biofilm growth or biofouling [22]. Second, there is the possibility of cathode fouling due to biofilm development and electrode material clogging by suspended solids. These two factors would lead to decreased mass and iron diffusion. Last, catalyst poisoning may occur due to the complex nature of real wastewater. All these would ultimately impair the system performance and lead to a decrease in electricity generation or pollutant removal. Thus, these factors that lead to MFC instability during long-term operation and the possible countermeasures all warrant further investigation.

4.1.4 Ecological Barriers

Cost is also a critical factor to consider in practical applications of MFCs. Specifically, the costs of electrode materials, catalysts, separators, buffer agent and the operations all need to be further reduced to enable commercialization of this technology.

While the properties of the electrode affect the performance of MFCs, it is worth noting that the economic factor is still a critical criteria for electrode design [30].

Even though silver or gold electrodes might be the best choices to reduce resistivity, they are too expensive for practical use on large scales. In contrast, relatively low-cost carbon cloth is commonly found suitable for biomass attachment, but the incorporation of a current collector such as stainless steel or coating of some modifiers are usually still needed to reduce charge transfer resistance. This may considerably increase the overall cost [115]. Nevertheless, the cost of carbon cloth at fuel-cell grade is still too high for practical use [29], and some graphite fiber materials seem to be a more desirable choice [63], but the performances of such materials are still to be further ascertained and optimized. In addition, chemical treatment, such as ammonia gas treatment, has also been shown to improve electrode performance, but this also increases the cost. Recently, Wang et al. [33] proposed a heat-treatment method for improving carbon mesh materials, which showed comparable performances but at a much lower cost. In another study, Zhu et al. [116] recommended a combined treatment of activated carbon filter by nitric acid soaking and EDTA treatment, which also achieved good effects. Currently, there remains a need for developing low-cost and high-efficiency electrode materials as well as treatment methods.

Likewise, low-cost alternatives to costly precious metal catalysts (e.g. Pt), and development of cheap separators should be considered. However, such reported alternatives are still scarce, while their performances and costs must still be further improved to meet the need of practical implementation [62, 63]. Notably, the introduction of biocathodes is bringing new opportunities. On the one hand, the microorganisms, as the biocathode catalyst, offer a cost-effective and self-sustainable way to catalyze oxygen reduction, although their catalytic capabilities are still generally lower than those of metals at the present stage [29]. On the other, the development of biofilms on the cathode can effectively alleviate oxygen intrusion and thus enable the use of a low-cost porous separator. Nevertheless, it is yet to be seen whether the biocathode can achieve better performances and maintain a sufficient operating stability.

While the use of a buffer to reduce pH gradient in MFC is common at laboratory scale [95, 97], it has also become a big challenge for practical wastewater treatment because a continuous addition of chemicals would be economically prohibitory [117]. A possible way to prevent this pH gradient is either to remove the membrane or to pump the anolyte to the cathode for neutralizing. But such methods also cause substrate crossover and may lead to unwanted side reactions, and meanwhile incur additional energy consumption for pumping. In addition, the low conductivity of wastewater in practice would also present another challenge for proton transfer, as salt addition is not possible because of economic issues and discharge regulation limitations. Meanwhile, how to improve the degradation efficiency of low-cost and complex substrates like lignocellulosic biomass presents another challenge. Thus, care should be taken when designing an MFC system on the merits of both technological and economic considerations. In all, there are compromises on MFC performance based on cost, and both performance and economic feasibility still need considerable improvements to enable practical MFC implementation.

4.2 Future Directions

4.2.1 Microbiology Insight and Manipulation

It is quite possible that MFCs will one day be used as a stand-alone technology for power generation and waste treatment, but the capabilities of such technologies are currently limited by a couple of technological and economic challenges. In fact, we are not yet at the upper limits of maximum power densities of MFCs. To overcome these challenges and limitations, we need a better understanding of the bacteria that metabolize various substrates and transfer electrons to the anode [118]. Moreover, an in-depth knowledge of the acclimatization of the communities in MFCs and their response to environmental disturbances would reduce the perceived risks and accelerate the startup of MFCs. Thus, it is crucial to investigate the biochemical pathways and bacterial physiology of electricigens. With this deep insight, it would then be possible for us to derive more quantitative methods for rationally designing the composition of microbial communities, and to offer a better manipulation of the biological and electrochemical processes at molecular levels. Specifically, mining of functional genes and effective metabolic engineering approaches would allow better manipulation of the electron flow and the creation of a highly catalytic interface between electrode and microorganisms.

4.2.2 Process Modeling and Control

Modeling provides another useful tool for exploring the underlying scenario of substrate metabolism and electron flow and also a reliable foundation for process monitoring and control. Indeed, mathematical modeling does have the potential to inform an MFC process; however, the many uncertainties in the function of microorganisms may present the greatest barrier [119]. Mathematical modeling can be suitably employed to reduce the burden on laboratory-based testing and characterization. There are only a few modeling investigations of MFCs [120]. A more reliable model based on the microbial metabolism and biochemical process should be considered, but the processes are too complex. Recently, a much simpler electrochemical model of MFCs was established based on the polarization curve [121], which revealed that the most important factors influencing MFCs were reaction kinetic loss and mass transport loss. A better control of the kinetics would thus be possible according to this study.

In the light of the instability of MFCs during long-term operation, it is necessary to offer in-time monitoring and control of the MFC system. In this regard, MFCs have a unique advantage because the electricity output can hopefully serve as a useful indicator of the system operation status. With a better understanding of the relationships between operation conditions, microbial activity, and electrical responses through modeling analysis, a real-time monitoring and control of the MFC's operation would be possible.

4.2.3 Integration with Other Processes

It should be admitted that each process has its inherent merits and demerits; such is the case with MFC technology. Thus, it is highly recommended that an MFC can be incorporated with other processes for improved power generation or extended applications. In addition, a flexible combination of such processes would enable the system to efficiently cope with various specific conditions. For example, Zhuang et al. [122] successfully integrated a Fenton reaction unit into MFC design to realize in-situ Fenton-enhanced electricity generation, Liu et al. [5] built a SBR-MFC system for cost-effective treatment of wastewater, while Sun et al. [4] achieved self-sustainable hydrogen production in a MEC-MFC coupled system without the need of an external power supply. With continuing advances and multi-disciplinary interaction and the development of more coupled systems, enhanced power generation and a further expanded application of MFCs can be expected in the future.

5 Conclusions

The capacity of MFCs to extract energy from various substrates and for other potential extended applications implies a great promise of MFC as a sustainable and low-cost technology. Power densities have seen rapid improvement in the past few years, more cost-effective materials are now available, the range of applications has been considerably expanded, and pilot tests are well underway. Nevertheless, we are still a long way from seeing MFCs implemented in real-world large-scale systems. The capital costs need to be further reduced, reactor designs and materials optimized, control systems implemented, and a better understanding of the instability sought. This requires a more fundamental understanding of the biological communities that metabolize waste and transfer electrons, and a higher degree of interactions between diverse disciplines such as microbiology, electrochemistry, photochemistry, materials science, electrical engineering, and environmental engineering.

Driven by increasing concerns over energy and environment due to rapid economic development and urbanization, China has started to take an active and increasingly important role in pushing MFC technology forward toward practical application. Some research areas in China are now leading the world, such as new materials and integrated applications. Nevertheless, continuing efforts and more investment in this area are needed to make MFC technology ultimately commercially available one day.

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References

1. Logan BE, Hamelers B, Rozendal RA, Schrorder U, Keller J, Freguia S, Aelterman P, Verstraete W, Rabaey K (2006) Microbial fuel cells: methodology and technology. Environ Sci Technol 40(17):5181–5192
2. Liu H, Logan BE (2004) Electricity generation using an air-cathode single chamber microbial fuel cell in the presence and absence of a proton exchange membrane. Environ Sci Technol 38:4040–4046
3. Rabaey K, Clauwaert P, Aelterman P, Verstraete W (2005) Turbular microbial fuel cells for efficient electricity generation. Environ Sci Technol 39:8077–8082
4. Sun M, Sheng GP, Zhang L, Xia CR, Mu ZX, Liu XW, Wang HL, Yu HQ, Qi R, Yu T, Yang M (2008) An MEC-MFC-coupled system for biohydrogen production from acetate. Environ Sci Technol 42(21):8095–8100
5. Liu XW, Wang YP, Huang YX, Sun XF, Sheng GP, Zeng RJ, Li F, Dong F, Wang SG, Tong ZH, Yu HQ (2011) Integration of a microbial fuel cell with activated sludge process for energy-saving wastewater treatment: taking a sequencing batch reactor as an example. Biotechnol Bioeng 108:1260–1267
6. Li WW, Sheng GP, Liu XW, Yu HQ (2011) Recent advances in the separators for microbial fuel cells. Bioresour Technol 102(1):244–252
7. Harnisch F, Schröder U (2010) From MFC to MXC: chemical and biological cathodes and their potential for microbial bioelectrochemical systems. Chem Soc Rev 39:4433–4448
8. Kim JR, Premier GC, Hawkes FR, Dinsdale RM, Guwy AJ (2009) Development of a tubular microbial fuel cell (MFC) employing a membrane electrode assembly cathode. J Power Sources 187(2):393–399
9. You S, Zhao Q, Zhang J, Jiang J, Wan C, Du M, Zhao S (2007) A graphite-granule membrane-less tubular air-cathode microbial fuel cell for power generation under continuously operational conditions. J Power Sources 173(1):172–177
10. Huang LP, Chai XL, Cheng SA, Chen GH (2011) Evaluation of carbon-based materials in tubular biocathode microbial fuel cells in terms of hexavalent chromium reduction and electricity generation. Chem Eng J 166(2):652–661
11. Zhuang L, Zhou S, Wang Y, Liu C, Geng S (2009) Membrane-less cloth cathode assembly (CCA) for scalable microbial fuel cells. Biosensors Bioelectron 24(12):3652–3656
12. Li Z, Zhang X, Zeng Y, Lei L (2009) Electricity production by an overflow-type wetted-wall microbial fuel cell. Bioresour Technol 100(9):2551–2555
13. Rabaey K, Ossieur W, Verhaege M, Verstraete W (2005) Continuous microbial fuel cells convert carbohydrates to electricity. Water Sci Technol 52:515–523
14. Hu Z (2008) Electricity generation by a baffle-chamber membraneless microbial fuel cell. J Power Sources 179(1):27–33
15. Li Z, Yao L, Kong L, Liu H (2008) Electricity generation using a baffled microbial fuel cell convenient for stacking. Bioresour Technol 99(6):1650–1655
16. Feng YJ, Lee H, Wang X, Liu YL, He WH (2010) Continuous electricity generation by a graphite granule baffled air-cathode microbial fuel cell. Bioresour Technol 101(2):632–638
17. Deng Q, Li XY, Zuo JE, Ling A, Logan BE (2010) Power generation using an activated carbon fiber felt cathode in an upflow microbial fuel cell. J Power Sources 195(4): 1130–1135
18. He Z, Minteer SD, Angenent LT (2005) Electricity generation from artificial wastewater using an upflow microbial fuel cell. Environ Sci Technol 39(14):5262–5267
19. He Z, Wagner N, Minteer SD, Angenent LT (2006) An upflow microbial fuel cell with an interior cathode: assessment of the internal resistance by impedance spectroscopy. Environ Sci Technol 40(17):5212–5217
20. Aelterman P, Rabaey K, Pham HT, Boon N, Verstraete W (2006) Continuous electricity generation at high voltages and currents using stacked microbial fuel cells. Environ Sci Technol 40(10):3388–3394

21. Oh SE, Logan BE (2007) Voltage reversal during microbial fuel cell stack operation. J Power Sources 167:11–17
22. Liu Z, Liu J, Zhang S, Su Z (2008) A novel configuration of microbial fuel cell stack bridged internally through an extra cation exchange membrane. Biotechnol Lett 30(6): 1017–1023
23. Zhuang L, Zhou S (2009) Substrate cross-conduction effect on the performance of serially connected microbial fuel cell stack. Electrochem Commun 11(5):937–940
24. Zhong C, Zhang B, Kong L, Xue A, Ni J (2010) Electricity generation from molasses wastewater by an anaerobic baffled stacking microbial fuel cell. J Chem Technol Biotechnol 86(3):406–413
25. Wang HY, Bernarda A, Huang CY, Lee DJ, Chang JS (2011) Micro-sized microbial fuel cell: a mini-review. Bioresour Technol 102(1):235–243
26. Ringeisen BR, Henderson E, Wu PK, Pietron J, Ray R, Little B, Biffinger JC, Jones-Meehan JM (2006) High power density from a miniature microbial fuel cell using *Shewanella oneidensis* DSP10. Environ Sci Technol 40(8):2629–2634
27. Qian F, Baum M, Gu Q, DE Morse (2009) A 1.5μL microbial fuel cell for on-chip bioelectricity generation. Lab Chip 9(21):3076–3081
28. Chen YP, Zhao Y, Qiu KQ, Chu J, Lu R, Sun M, Liu XW, Sheng GP, Yu HQ, Chen J, Li WJ, Liu G, Tian YC, Xiong Y (2011) An innovative miniature microbial fuel cell fabricated using photolithography. Biosensors Bioelectron 26(6):2841–2846
29. Logan BE (2010) Scaling up microbial fuel cells and other bioelectrochemical systems. Appl Microbiol Biotechnol 85:1665–1671
30. Zhou M, Chi M, Luo J, He H, Jin T (2011) An overview of electrode materials in microbial fuel cells. J Power Sources 196(10):4427–4435
31. Zhang SJ, Yu HQ, Feng HM (2006) PVA-based activated carbon fibers with lotus root-like axially porous structure. Carbon 44(10):2059–2068
32. Liu RH, Sheng GP, Sun M, Zang GL, Li WW, Tong ZH, Dong F, Hon-Wah Lam M, Yu HQ (2010) Enhanced reductive degradation of methyl orange in a microbial fuel cell through cathode modification with redox mediators. Appl Microbiol Biotechnol 89(1):201–208
33. Wang X, Cheng S, Feng Y, Merrill MD, Saito T, Logan BE (2009) Use of carbon mesh anodes and the effect of different pretreatment methods on power production in microbial fuel cells. Environ Sci Technol 43(17):6870–6874
34. Jiang D, Li B (2009) Granular activated carbon single-chamber microbial fuel cells (GAC-SCMFCs): a design suitable for large-scale wastewater treatment processes. Biochem Eng J 47(1–3):31–37
35. Logan BE, Cheng SA, Watson V, Estadt G (2007) Graphite fiber brush anodes for increased power production in air-cathode microbial fuel cells. Environ Sci Technol 41(9):3341–3346
36. Feng Y, Yang Q, Wang X, Logan BE (2010) Treatment of carbon fiber brush anodes for improving power generation in air-cathode microbial fuel cells. J Power Sources 195(7): 1841–1844
37. Zhang XY, Cheng SA, Liang P, Huang X, Logan BE (2011) Scalable air cathode microbial fuel cells using glass fiber separators, plastic mesh supporters, and graphite fiber brush anodes. Bioresour Technol 102(1):372–375
38. Zhang Y, Sun J, Hou B, Hu Y (2011) Performance improvement of air-cathode single-chamber microbial fuel cell using a mesoporous carbon modified anode. J Power Sources 196(18):7458–7464
39. Zhang YZ, Mo GQ, Li XW, Zhang WD, Zhang JQ, Ye JS, Huang XD, Yu CZ (2011) A graphene modified anode to improve the performance of microbial fuel cells. J Power Sources 196(13):5402–5407
40. Sun M, Zhang F, Tong ZH, Sheng GP, Chen YZ, Zhao Y, Chen YP, Zhou SY, Liu G, Tian YC, Yu HQ (2010) A gold-sputtered carbon paper as an anode for improved electricity generation from a microbial fuel cell inoculated with *Shewanella oneidensis* MR-1. Biosensors Bioelectron 26(2):338–343

41. Wu Z, Feng W, Feng Y, Liu Q, Xu X, Sekino T, Fujii A, Ozaki M (2007) Preparation and characterization of chitosan-grafted multiwalled carbon nanotubes and their electrochemical properties. Carbon 45(6):1212–1218
42. Mottaghitalab V, Spinks GM, Wallace GG (2005) The influence of carbon nanotubes on mechanical and electrical properties of polyaniline fibers. Synth Met 152(1–3):77–80
43. Liu J, Tian S, Knoll W (2005) Properties of polyaniline/carbon nanotube multilayer films in neutral solution and their application for stable low-potential detection of reduced–nicotinamide adenine dinucleotide. Langmuir 21(12):5596–5599
44. Qiao Y, Li CM, Bao SJ, Bao QL (2007) Carbon nanotube/polyaniline composite as anode material for microbial fuel cells. J Power Sources 170(1):79–84
45. Zou Y, Xiang C, Yang L, Sun LX, Xu F, Cao Z (2008) A mediatorless microbial fuel cell using polypyrrole coated carbon nanotubes composite as anode material. Int J Hydrogen Energy 33(18):4856–4862
46. Sun JJ, Zhao HZ, Yang QZ, Song J, Xue A (2010) A novel layer-by-layer self-assembled carbon nanotube-based anode: preparation, characterization, and application in microbial fuel cell. Electrochim Acta 55(9):3041–3047
47. Liang P, Wang HY, Xia X, Huang X, Mo YH, Cao XX, Fan MZ (2011) Carbon nanotube powders as electrode modifier to enhance the activity of anodic biofilm in microbial fuel cells. Biosensors Bioelectron 26(6):3000–3004
48. Zhang GD, Zhao QL, Jiao Y, Zhang JN, Jiang JQ, Ren NQ, Kim BH (2011) Improved performance of microbial fuel cell using combination biocathode of graphite fiber brush and graphite granules. J Power Sources 196(15):6036–9041
49. Zhao F, Harnisch F, Schröder U, Scholz F, Bogdanoff P, Herrmann I (2005) Application of pyrolysed iron (II) phthalocyanine and CoTMPP based oxygen reduction catalysts as cathode materials in microbial fuel cells. Electrochem Commun 7(12):1405–1410
50. Zhang L, Liu C, Zhuang L, Li W, Zhou S, Zhang J (2009) Manganese dioxide as an alternative cathodic catalyst to platinum in microbial fuel cells. Biosensors Bioelectron 24(9):2825–2829
51. Liu XW, Sun XF, Huang YX, Sheng GP, Zhou K, Zeng RJ, Dong F, Wang SG, Xu AW, Tong ZH, Yu HQ (2011) Nano-structured manganese oxide as a cathodic catalyst for enhanced oxygen reduction in a microbial fuel cell fed with a synthetic wastewater. Water Res 44(18):5298–5305
52. Huang YX, Liu XW, Sun XF, Sheng GP, Zhang YY, Yan GM, Wang SG, Xu AW, Yu HQ (2011) A new cathodic electrode deposit with palladium nanoparticles for cost-effective hydrogen production in a microbial electrolysis cell. Int J Hydrogen Energy 36(4): 2773–2776
53. Zhang JN, You SJ, Yuan YX, Zhao QL, Zhang GD (2011) Efficient electrocatalysis of cathodic oxygen reduction with Pt–Fe alloy catalyst in microbial fuel cell. Electrochem Commun 13(9):903–905
54. He Z, Angenent LT (2006) Application of bacterial biocathodes in microbial fuel cells. Electroanalysis 18(19–20):2009–2015
55. You SJ, Ren NQ, Zhao QL, Wang JY, Yang FL (2009) Power generation and electrochemical analysis of biocathode microbial fuel cell using graphite fibre brush as cathode material. Fuel Cells 9(5):588–596
56. Mao Y, Zhang L, Li D, Shi H, Liu Y, Cai L (2010) Power generation from a biocathode microbial fuel cell biocatalyzed by ferro/manganese-oxidizing bacteria. Electrochim Acta 55(27):7804–7808
57. Huang L, Chen J, Quan X, Yang F (2010) Enhancement of hexavalent chromium reduction and electricity production from a biocathode microbial fuel cell. Bioproc Biosys Eng 33(8):937–945
58. Aruchamy A, Aravamudan G, Subba Rao G (1982) Semiconductor based photoelectrochemical cells for solar energy conversion- An overview. Bull Mater Sci 4(5):483–526
59. Lu A, Li Y, Jin S, Ding H, Zeng C, Wang X, Wang C (2009) Microbial fuel cell equipped with a photocatalytic rutile-coated cathode. Energy Fuels 24(2):1184–1190

60. Ding H, Li Y, Lu A, Jin S, Quan C, Wang C, Wang X, Zeng C, Yan Y (2010) Photocatalytically improved azo dye reduction in a microbial fuel cell with rutile-cathode. Bioresour Technol 101(10):3500–3505
61. Yuan SJ, Sheng GP, Li WW, Lin ZQ, Zeng RJ, Tong ZH, Yu HQ (2010) Degradation of organic pollutants in a photoelectrocatalytic system enhanced by a microbial fuel cell. Environ Sci Technol 44(14):5575–5580
62. Tang XH, Guo K, Li HR, Du ZW, Tian JL (2010) Microfiltration membrane performance in two-chamber microbial fuel cells. Biochem Eng J 52(2–3):194–198
63. Zhuang L, Feng C, Zhou S, Li Y, Wang Y (2011) Comparison of membrane- and cloth-cathode assembly for scalable microbial fuel cells: construction, performance and cost. Process Biochem 45(6):929–934
64. Zhang X, Cheng S, Wang X, Huang X, Logan BE (2009) Separator characteristics for increasing performance of microbial fuel cells. Environ Sci Technol 43(21):8456–8461
65. Wang AJ, Sun D, Ren NQ, Liu C, Liu WZ, Logan BE, Wu WM (2010) A rapid selection strategy for an anodophilic consortium for microbial fuel cells. Bioresour Technol 101(14): 5733–5735
66. Hou H, Li L, Cho Y, de Figueiredo P, Han A (2009) Microfabricated microbial fuel cell arrays reveal electrochemically active microbes. PLoS ONE 4(8):e6570
67. He Z, Kan J, Mansfeld F, Angenent LT, Nealson KH (2009) Self-sustained phototrophic microbial fuel cells based on the synergistic cooperation between photosynthetic microorganisms and heterotrophic bacteria. Environ Sci Technol 43(5):1648–1654
68. Sun M, Tong ZH, Sheng GP, Chen YZ, Zhang F, Mu ZX, Wang HL, Zeng RJ, Liu XW, Yu HQ, Wei L, Ma F (2010) Microbial communities involved in electricity generation from sulfide oxidation in a microbial fuel cell. Biosensors Bioelectron 26(2):470–476
69. Finkelstein DA, Tender LM, Zeikus JG (2006) Effect of electrode potential on electrode-reducing microbiota. Environ Sci Technol 40(22):6990–6995
70. Wang X, Feng YJ, Ren Nq, Wang Hm, Lee H, Li N, Zhao QL (2009) Accelerated start-up of two-chambered microbial fuel cells: effect of anodic positive poised potential. Electrochim Acta 54(3):1109–1114
71. Sun M, Mu ZX, Sheng GP, Liu XW, Zhang L, Xia CR, Wang HL, Tong ZH, Yu HQ (2011) Effects of a transient external voltage application on the bioanode performance of microbial fuel cells. Electrochim Acta 55(9):3048–3054
72. Li WW, Sheng GP, Liu XW, Cai PJ, Sun M, Xiao X, Wang YK, Tong ZH, Dong F, Yu HQ (2011) Impact of a static magnetic field on the electricity production of Shewanella-inoculated microbial fuel cells. Biosensors Bioelectron 26(10):3987–3992
73. Wen Q, Kong FY, Ren YM, Cao DX, Wang GL, Zheng HT (2010) Improved performance of microbial fuel cell through addition of rhamnolipid. Electrochem Commun 12(12): 1710–1713
74. Oh ST, Kim JR, Premier GC, Lee TH, Kim C, Sloan WT (2010) Sustainable wastewater treatment: how might microbial fuel cells contribute. Biotechnol Adv 28(6):871–881
75. Franks AE, Nevin KP (2010) Microbial fuel cells, a current review. Energies 3(5):899–919
76. Lefebvre O, Uzabiaga A, Chang I, Kim B–H, Ng H (2011) Microbial fuel cells for energy self-sufficient domestic wastewater treatment–a review and discussion from energetic consideration. Appl Microbiol Biotechnol 89(2):259–270
77. Pant D, Van Bogaert G, Diels L, Vanbroekhoven K (2010) A review of the substrates used in microbial fuel cells (MFCs) for sustainable energy production. Bioresour Technol 101(6):1533–1543
78. Zhang T, Gannon SM, Nevin KP, Franks AE, Lovley DR (2010) Stimulating the anaerobic degradation of aromatic hydrocarbons in contaminated sediments by providing an electrode as the electron acceptor. Environ Microbiol 12(4):1011–1020
79. Huang L, Cheng S, Chen G (2010) Bioelectrochemical systems for efficient recalcitrant wastes treatment. J Chem Technol Biotechnol 86(4):481–491
80. Mu Y, Rabaey K, Rozendal RA, Yuan Z, Keller J (2009) Decolorization of azo dyes in bioelectrochemical systems. Environ Sci Technol 43(13):5137–5143

81. Zhu X, Ni J (2009) Simultaneous processes of electricity generation and p-nitrophenol degradation in a microbial fuel cell. Electrochem Commun 11(2):274–277
82. Feng C, Li F, Liu H, Lang X, Fan S (2010) A dual-chamber microbial fuel cell with conductive film-modified anode and cathode and its application for the neutral electro-Fenton process. Electrochim Acta 55(6):2048–2054
83. Wen Q, Kong F, Zheng H, Cao D, Ren Y, Yin J (2011) Electricity generation from synthetic penicillin wastewater in an air-cathode single chamber microbial fuel cell. Chem Eng J 168(2):572–576
84. Jiang J, Zhao Q, Zhang J, Zhang G, Lee D-J (2009) Electricity generation from bio-treatment of sewage sludge with microbial fuel cell. Bioresour Technol 100(23):5808–5812
85. Yuan Y, Chen Q, Zhou SG, Zhuang L, Hu P (2011) Bioelectricity generation and microcystins removal in a blue-green algae powered microbial fuel cell. J Hazard Mater 187(1–3):591–595
86. Borole A, Mielenz J, Vishnivetskaya T, Hamilton C (2009) Controlling accumulation of fermentation inhibitors in biorefinery recycle water using microbial fuel cells. Biotechnol Biofuels 2(1):7
87. Wang X, Feng Y, Wang H, Qu Y, Yu Y, Ren N, Li N, Wang E, Lee H, Logan BE (2009) Bioaugmentation for electricity generation from corn stover biomass using microbial fuel cells. Environ Sci Technol 43(15):6088–6093
88. Zang GL, Sheng GP, Tong ZH, Liu XW, Teng SX, Li WW, Yu HQ (2010) Direct electricity recovery from *canna indica* by an air-cathode microbial fuel cell inoculated with rumen microorganisms. Environ Sci Technol 44(7):2715–2720
89. Li Y, Lu A, Ding H, Jin S, Yan Y, Wang C, Zen C, Wang X (2009) Cr(VI) reduction at rutile-catalyzed cathode in microbial fuel cells. Electrochem Commun 11(7):1496–1499
90. Liu L, Yuan Y, Li F-b, Feng CH (2011) In situ Cr(VI) reduction with electrogenerated hydrogen peroxide driven by iron-reducing bacteria. Bioresour Technol 102(3):2468–2473
91. Tao HC, Liang M, Li W, Zhang LJ, Ni JR, Wu WM (2011) Removal of copper from aqueous solution by electrodeposition in cathode chamber of microbial fuel cell. J Hazard Mater 189(1–2):186–192
92. Virdis B, Rabaey K, Yuan Z, Keller J (2008) Microbial fuel cells for simultaneous carbon and nitrogen removal. Water Res 42(12):3013–3024
93. You SJ, Ren NQ, Zhao QL, Kiely PD, Wang JY, Yang FL, Fu L, Peng L (2009) Improving phosphate buffer-free cathode performance of microbial fuel cell based on biological nitrification. Biosensors Bioelectron 24(12):3698–3701
94. Xie S, Liang P, Chen Y, Xia X, Huang X (2010) Simultaneous carbon and nitrogen removal using an oxic/anoxic-biocathode microbial fuel cells coupled system. Bioresour Technol 102(1):348–354
95. Feng Y, Wang X, Logan B, Lee H (2008) Brewery wastewater treatment using air-cathode microbial fuel cells. Appl Microbiol Biotechnol 78(5):873–880
96. Wen Q, Wu Y, Zhao L, Sun Q (2010) Production of electricity from the treatment of continuous brewery wastewater using a microbial fuel cell. Fuel 89(7):1381–1385
97. Huang L, Logan B (2008) Electricity generation and treatment of paper recycling wastewater using a microbial fuel cell. Appl Microbiol Biotechnol 80(2):349–355
98. Huang L, Yang X, Quan X, Chen J, Yang F (2010) A microbial fuel cell–electro-oxidation system for coking wastewater treatment and bioelectricity generation. J Chem Technol Biotechnol 85(5):621–627
99. Zhang JN, Zhao QL, You SJ, Jiang JQ, Ren NQ (2008) Continuous electricity production from leachate in a novel upflow air-cathode membrane-free microbial fuel cell. Water Sci Technol 57(7):1017–1021
100. Cha J, Choi S, Yu H, Kim H, Kim C (2009) Directly applicable microbial fuel cells in aeration tank for wastewater treatment. Bioelectrochemistry 78(1):72–79
101. Teng SX, Tong ZH, Li WW, Wang SG, Sheng GP, Shi XY, Liu XW, Yu HQ (2010) Electricity generation from mixed volatile fatty acids using microbial fuel cells. Appl Microbiol Biotechnol 87(6):2365–2372

102. Tender LM, Gray SA, Groveman E, Lowy DA, Kauffman P, Melhado J, Tyce RC, Flynn D, Petrecca R, Dobarro J (2008) The first demonstration of a microbial fuel cell as a viable power supply: powering a meteorological buoy. J Power Sources 179(2):571–575
103. Reimers CE, Girguis P, Stecher HA, Tender LM, Ryckelynck N, Whaling P (2006) Microbial fuel cell energy from an ocean cold seep. Geobiology 4(2):123–136
104. Li JH, Fu YB, Liu J, Li AL, Ma DD (2009) Effect of electrode shape on power and internal resistance in benthic microbial fuel cell material on marine sediment. Adv Mater Res 79–82:2195–2198
105. Siu CPB, Mu C (2008) A microfabricated PDMS microbial fuel cell. J Microelectromechan System 17:1329–1341
106. Han Y, Yu C, Liu H (2010) A microbial fuel cell as power supply for implantable medical devices. Biosensors Bioelectron 25(9):2156–2160
107. Peixoto L, Min B, Martins G, Brito AG, Kroff P, Parpot P, Angelidaki I, Nogueira R (2011) In situ microbial fuel cell-based biosensor for organic carbon. Bioelectrochemistry 81(2):99–103
108. Chang IS, Jang JK, Gil GC, Kim M, Kim HJ, Cho BW, Kim BH (2004) Continuous determination of biochemical oxygen demand using microbial fuel cell type biosensor. Biosensors Bioelectron 19(6):607–613
109. Tront JM, Fortner JD, Plötze M, Hughes JB, Puzrin AM (2008) Microbial fuel cell biosensor for in situ assessment of microbial activity. Biosensors Bioelectron 24(4): 586–590
110. Rabaey K, Rozendal RA (2010) Microbial electrosynthesis–revisiting the electrical route for microbial production. Nat Rev Microbiol 8(10):706–716
111. Sun M, Mu ZX, Sheng GP, Shen N, Tong ZH, Wang HL, Yu HQ (2010) Hydrogen production from propionate in a biocatalyzed system with in situ utilization of the electricity generated from a microbial fuel cell. Int Biodeterior Biodegrad 64(5):378–382
112. You SJ, Wang JY, Ren NQ, Wang XH, Zhang JN (2010) Sustainable conversion of glucose into hydrogen peroxide in a solid polymer electrolyte microbial fuel cell. ChemSusChem 3(3):334–338
113. Panizza M, Cerisola G (2008) Electrochemical generation of H_2O_2 in low ionic strength media on gas diffusion cathode fed with air. Electrochim Acta 54(2):876–878
114. Cheng S, Logan BE (2011) Increasing power generation for scaling up single-chamber air cathode microbial fuel cells. Bioresour Technol 102(6):4468–4473
115. Rozendal RA, Hamelers HVM, Rabaey K, Keller J, Buisman CJN (2008) Towards practical implementation of bioelectrochemical wastewater treatment. Trends Biotechnol 26(8):450–459
116. Zhu NW, Chen X, Zhang T, Wu PX, Li P, Wu JH (2011) Improved performance of membrane free single-chamber air-cathode microbial fuel cells with nitric acid and ethylenediamine surface modified activated carbon fiber felt anodes. Bioresour Technol 102(1):422–426
117. Huang L, Regan JM, Quan X (2011) Electron transfer mechanisms, new applications, and performance of biocathode microbial fuel cells. Bioresour Technol 102(1):316–323
118. Logan BE (2009) Exoelectrogenic bacteria that power microbial fuel cells. Nat Rev Microbiol 7(5):375–381
119. Osman MH, Shah AA, Walsh FC (2010) Recent progress and continuing challenges in bio-fuel cells. Part II: microbial. Biosensors Bioelectron 26(3):953–963
120. Torres CI, Marcus AK, Parameswaran P, Rittmann BE (2008) Kinetic experiments for evaluating the Nernst-Monod model for anode-respiring bacteria (ARB) in a biofilm anode. Environ Sci Technol 42(17):6593–6597
121. Wen Q, Wu Y, Cao D, Zhao L, Sun Q (2009) Electricity generation and modeling of microbial fuel cell from continuous beer brewery wastewater. Bioresour Technol 100(18): 4171–4175
122. Zhuang L, Zhou SG, Li YT, Liu TL, Huang DY (2010) In situ Fenton-enhanced cathodic reaction for sustainable increased electricity generation in microbial fuel cells. J Power Sources 195(5):1379–1382

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Fuels and Chemicals from Hemicellulose Sugars

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Abstract Industrial processes of lignocellulosic material have made use of only the hexose component of the cellulose fraction. Pentoses and some minor hexoses present in the hemicellulose fraction, which may represent as much as 40% of lignocellulosic biomass, have in most cases been wasted. The lack of good methods for utilization of hemicellulose sugars is a key obstacle hindering the development of lignocellulose-based ethanol and other biofuels. In this chapter, we focus on the utilization of hemicellulose sugars, the structure of hemicellulose and its hydrolysis, and the biochemistry and process technology involved in their conversion to valuable fuels and chemicals.

Keywords Chemicals · Fuels · Hemicelluloses · Hydrolysis · Sugars

Contents

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1 Introduction

In the 21st century, human society will face great obstacles of resource shortages and environmental pollution. After 28 years of rapid development, since 1993 China has become a huge oil-consuming and net oil-importing country [1, 2], and is now facing a very serious energy shortage problem. Thus, the Chinese government is encouraging ethanol use as an alternative transportation fuel by introducing fuel ethanol production and distribution in several provinces [3–5]. By 2020, the total utilization of fuel ethanol (based on grain or non-grain) will reach 10 million metric tonnes, according to 'A Long and Mid-Term Planning for Renewable Energy Plan' issued by the Chinese National Development and Reform Commission [6, 7]. Although the current emphasis is on ethanol production from corn and other grains, China has huge quantities of low-cost lignocellulosic biomass that could significantly expand ethanol production volume and reduce feedstock costs. The key now is to successfully commercialize current technologies to reduce the cost of lignocellulosic biomass processing to ethanol. However, three obstacles still hinder the development of lignocellulosic ethanol: inefficient pretreatment measures, high cost of cellulase, and lack of good methods for utilization of hemicellulose sugars [8–10]. In this chapter, we focus on different methods for hemicellulose sugar utilization.

Industrial processes of lignocellulosic material have made use of only the hexose component of the cellulose fraction. Pentoses and some minor hexoses present in the hemicellulose fraction, which may comprise as much as 40% of the lignocellulosic material (Table 1), have therefore in most cases been wasted. Without a profitable use for the hemicellulose fraction, bioethanol is too expensive to compete in commercial markets [12]. Therefore, to foster the commercial production of lignocellulosic ethanol, conversion of the hemicelluloses into fermentable sugars and then to fuels or chemicals is essential. This chapter deals with the structure and availability of these hemicellulose sugars, and with the biochemistry and process technology involved in their conversion to fuels and chemicals.

2 Hemicellulose Structure and Hydrolysis

2.1 Hemicellulose Structure

Hemicellulose is often described as plant cell-wall polysaccharides that are associated with cellulose in lignified tissues. The close association of hemicellulose with cellulose and lignin contributes to cell-wall rigidity and flexibility. The majority of the hemicellulose polysaccharides are derived from cell-wall middle lamella. Some of the non-starch, non-cellulose polysaccharides, excluding pectic materials, which are known as pentosans, are sometimes also considered

Table 1 Composition of various agricultural lignocellulosic biomass (adapted from [11])

	Composition (%, dry basis)		
	Cellulose	Hemicellulose	Lignin
Corn fiber[a]	15	35	8
Corn cob	45	35	15
Corn stover	40	25	17
Rice straw	35	25	12
Wheat straw	30	50	20
Sugarcane bagasse	40	24	25
Switchgrass	45	30	12
Coastal bermuda grass	25	35	6

[a] Contains 20% starch

hemicelluloses [13]. Hemicelluloses are heterogeneous polymers of pentoses (xylose, arabinose), hexoses (mannose, glucose, galactose), and sugar acids. Unlike cellulose, hemicelluloses are not chemically homogeneous [11]. The most relevant hemicelluloses are xylans and glucomannans; hardwood hemicelluloses contain mostly xylans, whereas softwood hemicelluloses contain mostly glucomannans [14].

Xylans are the main hemicellulose components of secondary cell walls, constituting about 20–30% of the biomass of hardwoods and herbaceous plants. In some tissues of grasses and cereals, xylans can account for up to 50% [15]. Xylans are usually available in huge amounts as byproducts of forest, agriculture, pulp and paper industries. Xylans are heteropolysaccharides with homopolymeric backbone chains of 1,4-linked β-D-xylopyranose units. Besides xylose, xylans may contain arabinose, glucuronic acid or its 4-O-methyl ether, and acetic, ferulic, and p-coumaric acids. The frequency and composition of branches are dependent on the source of xylan [11]. The backbone consists of O-acetyl, α-L-arabinofuranosyl, α-1,2-linked glucuronic or 4-O-methylglucuronic acid substituents. However, unsubstituted linear xylans have also been isolated from some agricultural residues. Xylans can thus be categorized as linear homoxylan, arabinoxylan, glucuronoxylan, and glucuronoarabinoxylan. About 80% of the xylan backbone is highly substituted with monomeric side-chains of arabinose or glucuronic acid linked to O-2 and/or O-3 of xylose residues, and also with oligomeric side chains containing arabinose, xylose, and sometimes galactose residues (Fig. 1).

2.2 Hemicellulose Hydrolysis

Hemicellulose can be converted to various products, using biological or chemical methods. However, these often require prior hydrolysis of the polysaccharides to their sugar constituents. The yield, rate of hydrolysis, and type of sugar recovered depend on the hydrolysis method, as well as the source of substrate and its composition [10].

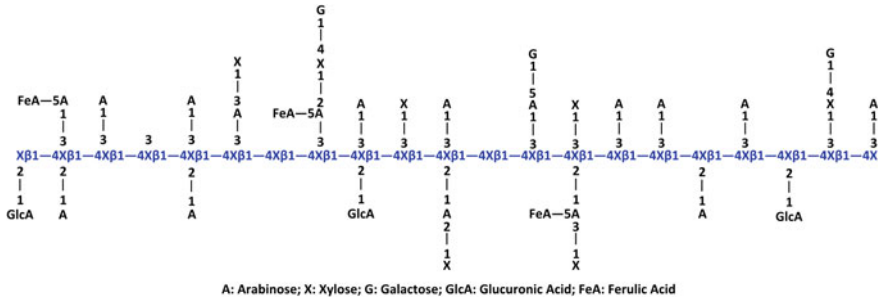

Fig. 1 Schematic structure of corn fiber heteroxylan (modified from [11, 16])

In general, hemicellulose can be hydrolyzed to its sugar constituents by chemical or enzymatic processes. The chemical hydrolysis of hemicelluloses is much easier to accomplish than the hydrolysis of cellulose, due to the heterogeneous structure and composition of hemicelluloses and its low degree of polymerization and low crystallinity [17]. Generally, the first step of hemicellulose-based fuel or chemical production is lignocellulose pretreatment, and different pretreatment methods have been developed. In most cases, hemicelluloses are commonly removed during the initial stage of biomass processing, aiming to reduce structural constraints for further enzymatic cellulose hydrolysis (Fig. 2) [18, 19]. The main process options for the selective fractionation of hemicelluloses from biomass include the use of acids, water (liquid or steam), organic solvents, and alkaline agents (Table 2) [20]. The last two are not selective towards hemicellulose as they also remove lignin, which in turn can hinder the fermentation or bioconversion process of the hemicellulose sugars. Therefore, acid/water/steam pretreatments are the most commonly applied technologies, yielding a selective solubilization of hemicelluloses and producing hemicellulose-rich liquids totally or partially hydrolyzed to oligomeric and monomeric sugars and cellulose-enriched solids for further bioprocessing.

Many acids are known to be good hydrolytic agents. The common method of acid hydrolysis uses dilute acid [21]. Dilute sulfuric acid hydrolysis has proved to be a favorable method for pretreatment before enzymatic hydrolysis and also for the conversion of hemicellulose to sugars [22–24]. One of the earliest commercial hydrolysis processes was a dilute sulfuric acid process carried out at a relatively low temperature for a prolonged period of time. Recently, a great deal of research has examined the dilute acid hydrolysis of woods and agricultural residues to produce sugars. During acid hydrolysis of hemicellulose, pentoses are degraded rapidly to furfural and condensation byproducts [21]. These compounds may also inhibit the later fermentation processes, leading to lower yield and productivity, and a prior detoxification treatment might therefore be required. In order to prevent the decomposition of sugars, especially pentose, a more dilute acid, a shorter reaction time, a lower temperature, and the rapid removal of hydrolytic agents are required.

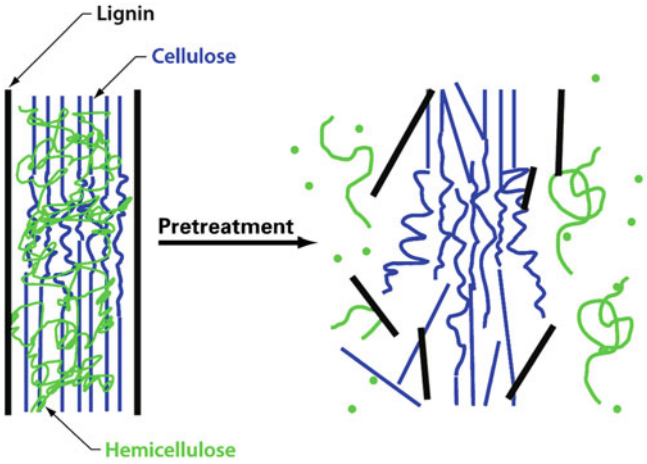

Fig. 2 Schematic representation of lignocellulosic biomass pretreatment (modified from [18, 19]). Pretreatment of lignocellulose by different methods removes hemicellulose and lignin from this matrix of polymers in which cellulose exists before hydrolysis

Table 2 Methods for pretreatment of lignocellulosic biomass (adapted from [20])

Method	Example
Thermo-mechanical	Grinding, milling, shearing, extruder
Autohydrolysis	Steam pressure, steam explosion, supercritical carbon dioxide explosion
Acid treatment	Dilute acid (H_2SO_4, HCl), concentrated acid (H_2SO_4, HCl)
Alkali treatment	Sodium hydroxide, ammonia, alkaline hydrogen peroxide
Organic solvent treatment	Methanol, ethanol, butanol, phenol

Apart from the chemical hydrolysis of hemicelluloses to monomer sugars, the most promising method is using enzymes, i.e., hemicellulases. Moreover, hemicellulases can facilitate cellulose hydrolysis by exposing the cellulose fibres, thus making them more accessible [25]. Because hemicelluloses are heterogeneous, with different constituents linked by different types of bonds, the enzymatic hydrolysis requires several enzymes. Each enzyme attacks one or more types of bonds and all are identified as hemicellulases (Fig. 3) [18, 26]. A wide range of microorganisms produce different types of hemicellulases in response to the different types of hemicellulose in their environments [25]. In general, microorganisms can be divided into three groups according to their strategies for hydrolyzing hemicellulose [25]: (1) complete hydrolysis to monosaccharides and disaccharides by several and synergic extracellular hemicellulases; (2) partial extracellular hydrolysis to oligosaccharides further hydrolyzed by cell-associated or intracellular hemicellulases; (3) hydrolysis by cellulosomes, extracellular cell-associated multienzyme complexes, harbouring cellulases and hemicellulases. In combination, the hemicellulase enzymes that work synergistically, and, in some cases, are organized in complexes,

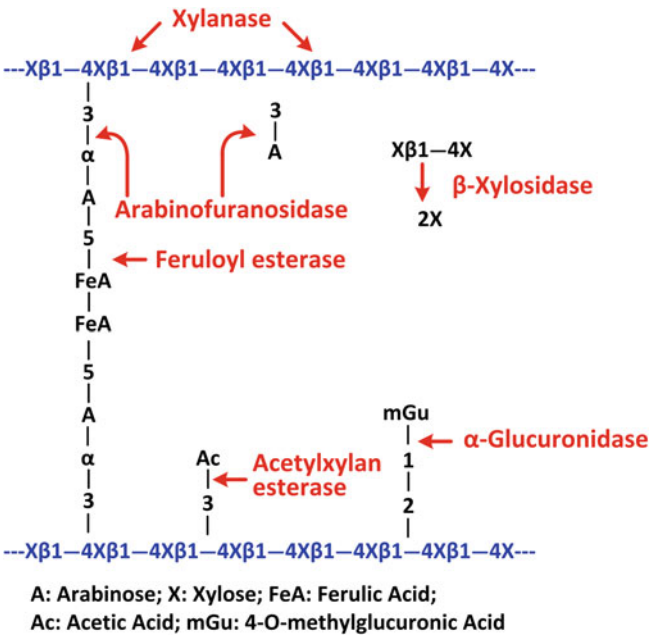

Fig. 3 Complex mixture of enzymes for degrading hemicelluloses (modified from [18, 26]). The example depicted is cross-linked glucuronoarabinoxylan. The complex composition and structure of hemicellulose require multiple enzymes to break down the polymer into sugar monomers, primarily xylose, but other pentose and hexose sugars are also present in hemicelluloses. A variety of debranching enzymes (*red*) act on diverse side chains hanging off the xylan backbone (*blue*). These debranching enzymes include arabinofuranosidase, feruloyl esterase, acetylxylan esterase, and α-glucuronidase (see Table 3). As the side chains are released, the xylan backbone is exposed and made more accessible to cleavage by xylanase. *β*-Xylosidase cleaves xylobiose into two xylose monomers; this enzyme can also release xylose from the end of the xylan backbone or a xylo-oligosaccharide

Table 3 Glycoside hydrolase (GH) and carbohydrate esterase (CE) enzyme families for degrading hemicelluloses (adapted from [18])

Enzyme	Enzyme families
Endoxylanase	GH5, 8, 10, 11, 43
β-Xylosidase	GH3, 39, 43, 52, 54
α-L-Arabinofuranosidase	GH3, 43, 51, 54, 62
α-Glucuronidase	GH4, 67
α-Galactosidase	GH4, 36
Acetylxylan esterase	CE1, 2, 3, 4, 5, 6, 7
Feruloyl esterase	CE1

hydrolyze hemicellulose to its constituent sugars; that is to say, some of these enzymes are multifunctional, with catalytic domains belonging to different enzyme families. Their great diversity and that of other enzymes involved in hemicellulose degradation present a remarkably complicated enzymatic system whose more thorough analysis may yield a greater understanding of hemicellulosic degradation.

3 Fuels and Chemicals from Hemicellulose Sugars

3.1 Ethanol

Ethanol is the most commonly employed liquid biofuel, either as a fuel or as a gasoline enhancer. The demand for fuel ethanol is expected to increase greatly as a safer alternative to methyl tertiary butyl ether (MTBE), the most common additive to gasoline used to provide cleaner combustion [8]. Ethanol as a fuel additive can be utilized to elevate the oxygen content of gasoline, allowing a better oxidation of hydrocarbons and reducing tailpipe emissions of carbon dioxide and unburned hydrocarbons into the atmosphere [27].

The production of fuel ethanol from hemicellulosic hydrolysates is essential for the economic success of lignocellulosic ethanol [14]. Low-cost feedstocks for production of ethanol can be found from various waste and under-utilized lignocellulosic agricultural residues. These lignocellulosic feedstocks containing hemicellulose generate a mixture of sugars when pretreated alone or in combination with enzymatic hydrolysis, and the utilization of hemicellulosic sugars, especially xylose, is essential for efficient and cost-effective conversion of lignocellulosic materials to ethanol. The microbial conversion of hexose into ethanol is well established, while the ability of ethanol fermentation using pentose by these microorganisms is somewhat less so.

The yeasts *Pachysolen tannophilus*, *Candida shehatae*, *Pichia stiptis*, and *Kluveromyces marxianus* have been studied extensively for their capability to ferment xylose to ethanol [28]. Other yeasts, such as recombinant *Saccharomyces cerevisiae*, were also investigated for their xylose-fermenting ability [29, 30]. However, commercial utilization of these yeasts for ethanol production from xylose is restricted by their slow fermentation rate, low ethanol tolerance, difficulty in controlling the optimal oxygen supply rate, and sensitivity to inhibitors generated during pretreatment and hydrolysis of lignocellulosic substrates [31, 32]. Bacterial species, including *Clostridium thermohydrosulfuricum, Clostridium thermocellum, Clostridium thermosaccharolyticum*, recombinant *Escherichia coli*, recombinant *Zymomonas mobilis*, recombinant *Klebsiella planticola*, and several pentose-utilizing fungal species like *Fusarium oxysporum*, *Neurospora crassa, Mucor* sp., and *Chalara parvispora*, have been extensively reviewed for their capability to convert xylose to ethanol [33]. Several representatives of these microorganisms have different natural characteristics that can be regarded as either advantages or disadvantages in processes of ethanol production from hemicellulosic sugars (Table 4).

In China, different microorganisms, including yeasts, bacteria and fungi, have been used and optimized for ethanol fermentation, using both pure xylose and hemicellulose-derived sugar mixtures [34–40]. Zhao and Xia [40] detoxified the acid hydrolyzate of corn stover (the most favored lignocellulosic resource for large-scale ethanol production) by rotoevaporation and lime neutralization, and

Table 4 Characteristics of the most relevant microorganisms considered for ethanol production from hemicelluloses (adapted from [14])

Characteristics	Microorganism			
	E. coli	*Z. mobilis*	*S. cerevisiae*	*P. stipitis*
D-Glucose fermentation	+	+	+	+
Other hexose utilization (D-galactose andD-mannose)	+	–	+	+
Pentose utilization (D-xylose andL-arabinose)	+	–	–	+
Direct hemicellulose utilization	–	–	–	w
Anaerobic fermentation	+	+	+	–
Mixed-product formation	+	w	w	w
High ethanol productivity (from glucose)	–	+	+	w
Ethanol tolerance	w	w	+	w
Tolerance to lignocellulose-derived inhibitors	w	w	+	w
Osmotolerance	–	–	+	w
Acidic pH range	–	–	+	w
GRAS microorganism	–	+	+	+

+ Positive, – negative, *w* weak, *GRAS* generally recognized as safe

then using the pentose-rich hydrolyzate as substrate for ethanol production by cells of recombinant *S. cerevisiae* immobilized by Ca-alginate. An ethanol concentration of 31.1 g/L and a corresponding ethanol yield on fermentable sugars of 0.406 g/g were obtained within 72 h in batch fermentation of the detoxified hydrolyzate with immobilized cells. Sugarcane bagasse, the most abundant agricultural material in southern China, was recently used as a substrate for production of ethanol using *P. tannophilus* [34]. The detoxification of the sugarcane bagasse hemicellulose hydrolyzate used electrodialysis, which decreased the loss of sugar and increased acetic acid removal, leading to better fermentability. A batch culture with electrodialytically pretreated hydrolyzate as substrate was developed, giving 21 g/L ethanol with a yield of 0.35 g/g sugar and productivity of 0.59 g/(L h). Chen et al. [37] performed a laboratory xylose/cellulose fractionation and separate fermentation (XCFSF) process for ethanol production. Three xylose/cellulose fractionation strategies, dilute sulfuric acid hydrolysis and detoxification, lime pretreatment and xylanase hydrolysis, bio-treatment with *Phanerochaete chrysosporium* and xylanase hydrolysis, were applied to corn cobs. The xylose was dissolved in acid and the enzymatic hydrolyzates was fermented to ethanol by *C. shahatae*, while the cellulose remaining in solid residues was converted to ethanol by simultaneous saccharification and fermentation with *S. cerevisiae*. Finally, using the three fractionation methods, 70.40, 52.87, and 39.22% of hemicellulose and 89.77, 84.30, and 71.90% of cellulose in corn cobs were converted to ethanol, respectively.

Table 5 Microbial production of xylitol using different hemicellulosic substrates

Raw material	Strain	Xylitol yield (g/g)[a]	Xylitol productivity (g/(L h))	Reference
Brewer's spent grain hemicellulose hydrolyzate	*Candida guilliermondii*	0.73	0.52	[47]
Eucalyptus globulus wood hemicellulose hydrolyzate	*Debaryomyces hansenii*	0.84	0.53	[50]
Hardwood hemicellulose hydrolyzate	*Pachysolen tannophilus*	0.63	0.41	[51]
Glucose–xylose mixtures	*Escherichia coli* (recombinant)	0.63	–	[53]
Glucose–xylose mixtures	*Saccharomyces cerevisiae* (recombinant)	0.95	0.40	[54]
Glucose–xylose mixtures	*Corynebacterium glutamicum* (recombinant)	–	7.90	[55]
Corncob hemicellulose hydrolyzate	*Candida tropicalis*	0.70	0.95	[58]

[a] Means g xylitol per g xylose

3.2 Xylitol

Xylitol, a naturally occurring five-carbon sugar alcohol, has attracted worldwide interest because of its unique properties and huge potential. Xylitol has important applications in pharmaceuticals and food industries due to its high sweetening properties, lower energy value, non- and anti-carcinogenicity property, and microbial growth inhibition capacity [41]. Xylitol has been used as a sugar substitute in dietary foods, especially those for insulin-deficiency diabetics. Due to its anti-carcinogenicity, pleasant taste, and no unpleasant aftertaste, xylitol has been widely applied in the dental industry.

Xylitol can be produced by either chemical synthesis or fermentation. Industrially, it is produced by catalytic hydrogenation of xylose under high temperature and high pressure conditions. Compared with traditional chemical synthesis, the microbial fermentation process is easier to control, and also the in-vitro enzyme-based conversion has higher selectivity [42]. With the increase of interest in exploring more environment-friendly and economic xylitol production methods, the biosynthesis of xylitol by microorganisms is becoming increasingly popular.

Many studies have investigated the use of the hemicellulose portion of agricultural residues like corn stover [43], rice straw [44], sugarcane bagasse [45], eucalyptus [46], spent grain [47], and corncob [48] for xylitol production. Among the various agricultural crop residues, corncob is one of the most abundant feedstocks for xylitol production in China. Xylose can be converted to xylitol by several microorganisms including *Candida guilliermondii* [47, 49], *Debaryomyces*

hansenii [50], *Pachysolen tannophilus* [51], *Candida tropicalis* [52], recombinant *E. coli* [53], recombinant *S. cerevisiae* [54], and recombinant *Corynebacterium glutamicum* [55]. Among these microorganisms, *C. tropicalis* ferments xylose to xylitol with good yield and productivity (Table 5).

In China, the xylitol bioconversion process using hemicellulose sugars as substrates has been successfully developed [44, 56–60]. Fang et al. [56] investigated the influences of xylose, glucose, arabinose, fructose and the inhibitors from corncob hemicellulosic hydrolyzate on xylitol production by *Candida* sp. It was found that the optimum initial xylose concentration in the culture media was about 100 g/L and the inhibitions of acetic acid and furfural in the hydrolyzate increased gradually when their concentrations exceeded 1 g/L. Ding et al. [57] studied the effect of different aeration conditions on xylitol production from corncob hemicellulose hydrolyzate by *Candida* sp. ZU04, and found that the two-phase aeration process was more effective than one-phase aeration for xylitol production. Cheng et al. [58] studied the effects of glucose and acetate concentrations in the hydrolyzate on xylitol production by *C. tropicalis* W103. It was found that glucose in corncob hydrolyzate promoted the growth of *C. tropicalis* while acetate at high concentration was inhibitory. In another study, *C. tropicalis* cells were immobilized in Ca-alginate beads and used for xylitol production from corncob hydrolyzates [59]. In 10 repeated batches (30 days) fermentation, the average xylitol yield was 73.7%. The immobilized cells have high density, steady fermentability and good resistance to inhibitors in the hydrolyzates. Deng et al. [44] employed the ammonia steeping strategy to pretreat the rice straw, and converted the hydrolyzate to xylitol by *C. tropicalis* directly. Experimental results showed that the content of toxic compounds created in the hydrolysis process, such as acetic acid and phenolic compounds, was greatly reduced and the fermentation of the hydrolyzate was enhanced. Xylitol fermentation was investigated in flasks and a 2-L bioreactor. The xylitol yield and volumetric productivity were 0.746 g/g and 0.686 g/(L h) in the lab-flask fermentation. The corresponding results conducted in bioreactor fermentation were 0.689 g/g and 0.697 g/(L h), respectively.

3.3 2,3-Butanediol, Methyl ethyl ketone, and 2-Butanol

When evaluating the benefits of 2,3-butanediol (2,3-BD) production, consideration must be given to the following facts. 2,3-BD is less toxic to microbial systems than other alcohol products, and its energy content is comparable with other microbial liquid fuels. An equimolar mixture of ethanol and 2,3-BD has an energy content of 27,700 kJ/kg, while pure ethanol, 2,3-BD, and methanol contain 29,100, 27,200, and 22,100 kJ/kg respectively [61]. Therefore, 2,3-BD can be used as an alternative to traditional ethanol. Additionally, 2,3-BD may serve as a feedstock for the production of numerous valuable chemicals, including printing inks, perfumes, fumigants, moistening and softening agents, explosives, plasticizers, foods, and pharmaceuticals [62–65].

2,3-BD is a fermentation product of a number of bacteria such as *Klebsiella* sp. (*K. pneumoniae* or *K. oxytoca*) and *Panebacillus polymyxa*, which are considered of industrial importance in the production of 2,3-BD [66, 67]. The advantage of 2,3-BD production using bacteria is that the species are easy to cultivate and they grow rapidly in a simple medium and metabolize all of the major sugars present in hemicellulose and cellulose hydrolyzates into 2,3-BD [68, 69]. 2,3-BD fermentations using hemicellulosic materials were reported as early as the 1980s [70, 71]. Yu et al. [72, 73] successfully developed the processes using both acid and enzymatic hydrolyzed wood hemicellulose for 2,3-BD production by *K. pneumoniae*. 2,3-BD yields of 0.4–0.5 g/g were obtained from hydrolyzed wood hemicellulose. The authors proposed that such high yields were partially due to the ability of *K. pneumoniae* to simultaneously ferment uronic acids, such as D-glucuronic and D-galacturonic acid, present in the wood samples. Other compounds in wood (such as furfural and lignin derivatives) were inhibitory to the bacteria if the wood hydrolyzate was added at a high concentration [74, 75]. A more efficient approach using woody biomass for 2,3-BD production was solid-state fermentation (SSF) with addition of culture filtrates of *Trichoderma harzianum* as a source of hydrolytic enzymes [76, 77]. Corn fiber, a hemicellulose-rich byproduct in corn processing, was also successfully used as a substrate for 2,3-BD production by Saha and Bothast [78]. They isolated a 2,3-BD-producing *Enterobacter cloacae* from decaying wood/corn soil samples, which was found to utilize sugars from acid plus enzyme-saccharified corn fiber and produced 2,3-BD (0.35 g/g available sugars). The strain was also able to produce 2,3-BD from dilute acid-pretreated corn fiber by SSF (0.34 g/g theoretical sugars).

In China, pure xylose, glucose–xylose mixtures, and hemicellulose-derived sugar mixtures have been used and optimized for 2,3-BD fermentation [79–84]. Cheng et al. [80] detoxified the acid hydrolyzate of corncob (hemicellulose components rich stream) by sequentially boiling, over-liming, and adsorbing the hydrolyzate onto activated charcoal, and then using the pentose-rich hydrolyzate as substrate for 2,3-BD production by *K. oxytoca*. Under optimum conditions, a maximal 2,3-BD concentration of 35.7 g/L was obtained after 60 h of fed-batch fermentation, giving a yield of 0.5 g/g reducing sugar and a productivity of 0.59 g/(L h). Corncob molasses, a waste byproduct in xylitol production that contained high concentrations of hemicellulose-derived arabinose and xylose, was recently reused as a substrate for production of 2,3-BD using *K. pneumoniae* [82]. The maximum 2,3-BD concentration was 78.9 g/L after 61 h of fed-batch fermentation. This approach, using corncob molasses for 2,3-BD production, not only cut down the feedstock cost, but also provided a method of exploiting xylitol industry byproducts, which could reduce environmental pollution. Ji et al. [79] successfully developed an economical industrial medium for glucose–xylose co-fermentation using a metabolic engineered *K. oxytoca* [85, 86]. However, when glucose and xylose were present at the same time, xylose consumption generally did not commence until glucose was depleted, i.e., sugars were sequentially consumed resulting in several exponential growth phases that were separated by intermediate lag phases, developing so-called diauxic growth [87]. This would hinder the possibility of utilizing the hemicellulose

hydrolyzates as carbon sources for efficient 2,3-BD production. Aiming at simultaneous consumption of glucose and xylose, Ji et al. [84] further constructed an engineered *K. oxytoca* harboring the CRP(in) phenotype which could metabolize glucose and xylose mixtures simultaneously. As a consequence of the co-metabolism of glucose and xylose, the total sugars could be consumed faster, leading to higher growth rate and 2,3-BD productivity by the recombinant compared to the parent strain. This recombinant has the potential to produce 2,3-BD directly from the hemicellulose hydrolyzates.

The above biologically produced 2,3-BD from the hemicellulose may also be further converted to methyl ethyl ketone (MEK, also called 2-butanone), which is an industrial solvent and may find use as a liquid fuel additive by acid-catalyzed dehydration [88]. This reaction can be carried out using catalysts such as alumina or sulfuric acid, resulting in a >95% yield [89, 90]. This reaction mechanism involves a hydride shift which could also be made possible by an enzyme catalyzing process. Biosynthesis of MEK from 2,3-BD using a diol dehydratase (EC 4.2.1.28) obtained from *Lactobacillus brevis* has been previously reported [91]. This method would be a promising approach for MEK biosynthesis in the future. In a more recent paper of Wang et al. [92], dehydration of pretreated 2,3-BD fermentation broth to MEK using dilute sulphuric acid as catalyst was conducted. The >95% yield of MEK was then distilled from the broth. As the boiling point of 2,3-BD was greater than that of MEK (180 vs. 79°C), MEK could be distilled from the broth more easily. The designed 2,3-BD–MEK coupling production process would reduce the energy consumption for the downstream processing of 2,3-BD fermentation, and achieve the production of the 2,3-BD value-added derivative at the same time. Recently, besides the homogeneous catalysis process for MEK production from 2,3-BD, great attention has been paid to the heterogeneous catalysis process using solid acid as catalyst, such as the zeolites of NaY and ZSM-5, and also the metal-modified zeolites, as this means reduced consumption of sulphuric acid and its possible pollution [93–95].

To further improve the added value of the hemicellulose-derived 2,3-BD, the coupled MEK produced could be further hydrogenated to 2-butanol, a novel biofuel. Butanol, which has four isomers, n-butanol, 2-butanol, iso-butanol, and t-butanol, contains more energy, is more hydrocarbon-like, and blends easier with gasoline than ethanol. Furthermore, butanol does not absorb moisture from air; without moisture, butanol causes no corrosion. Butanol and butanol–gasoline blends can be transported through existing pipelines, without expensive trucking. Major oil companies are now showing more interest in butanol than ethanol [96].

Among the four butanol isomers, n-butanol is one of three products of acetone–butanol–ethanol (ABE) fermentation that predated the petrochemical industry. Acetone, butanol and ethanol are produced in the ratio of 3:6:1. The high toxicity of butanol towards the bacterial cells that produce it limits the final concentration of butanol to 14 g/L [97]. The industry disappeared after petro-processing became popular. The process suffers from low yield, high cost of separation of co-products, and handling in strictly anaerobic conditions. Recent interest in biofuels has

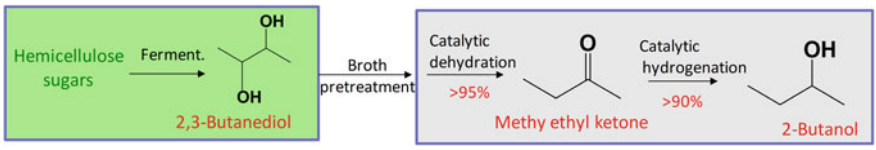

Fig. 4 An integrated 2,3-butanediol (2,3-BD)–methyl ethyl ketone (MEK)–2-butanol coupled production route using hemicellulose sugars as substrates. The process can be divided into three steps: (1) fermentation to convert hemicellulose sugars to 2,3-BD (~50% yield, 40%); (2) Chemical dehydration of 2,3-BD to form MEK (>95% yield), using dehydration catalysts sulfuric acid, acidic oxides, zeolites, etc.; (3) hydrogenation of MEK to produce 2-butanol (>90% yield), using hydrogenation catalysts mixed oxides, Raney Ni, Pd/C, Ru/oxides, supported Cu, supported Fe, etc.

promoted renewed studies of ABE fermentation. A higher concentration of 20 g/L has reportedly been achieved [98]. Iso-butanol exists in very small quantities in the "fusel oil" of beverage alcohol fermentation. Genetically modified microbial cells have achieved higher yields. It is not as toxic as *n*-butanol but the final concentration is still reported to be no more than about 60 g/L [99, 100]. *t*-Butanol is a product of petrochemical processing. No known biological process can produce t-butanol at present. 2-Butanol could be made from fermentation using hemicellulose-derived pentoses (C5) and also hexoses (C6) to produce the intermediate 2,3-BD that has an inhibitory effect only after the concentration exceeds 11% [65]. The process for bio-based 2-butanol production can be divided into three steps: (1) fermentation to convert sugars to 2,3-BD (~50% yield, 40%); (2) chemical dehydration of 2,3-BD to form MEK, a marketable intermediate product (>95% yield); (3) hydrogenation of MEK to produce 2-butanol (theoretically, 100 g of MEK can produce 103 g of 2-butanol; the practical yield is 90% of the theoretical). Therefore, 100 g of C5 or C6 sugars from hemicelluloses hydrolysis will yield 35.2 g of 2-butanol, or approximately 1 ton of 2-butanol from 3 tons of sugars from starch, cellulose and hemicellulose, or 100 gallons of 2-butanol per ton of sugars. That is to say, the bio-based 2-butanol route is more competitive than the traditionally used n-butanol or iso-butanol fermentation process, as the latter two butanol isomer fermentations not only have a lower final product concentrations but also a relatively lower yields from the sugars. The coupled bio-based 2-butanol production route from lignocelluloses using fermentatively-produced 2,3-BD as intermediate is therefore economical to some extent (Fig. 4). In addition, the fermentatively produced 2,3-BD could be directly catalyzed to 2-butanol using a heterogeneous catalyst system that can function both as an acid catalyst and as a hydrogenation catalyst [101]. With emerging synthetic biology tools, the 2,3-BD biosynthetic pathway and the 2,3-BD to 2-butanol pathway existing in some lactic acid bacteria could be constructed into a new minimal genome chassis to form a homo-fermentative 2-butanol-producing strain. The de novo biosynthesis of 2-butanol would then be achieved [102–105].

3.4 Organic Acids

Because of their multifunctional groups, organic acids are extremely useful as food additives, raw materials for the manufacture of biodegradable plastics, starting materials for the chemical industry, and other applications [106, 107]. Organic acids constitute a key group of chemicals, and most of them can be produced by microorganisms. Among the twelve sugar-derived building-block chemicals identified by the US Department of Energy, nine are organic acids [108]. The market for bio-based organic acids is huge. One example is fumaric acid; at present, the annual production of fumaric acid is about 12,000 tonnes, but by creating new economical production processes, the petroleum-derived commodity chemical maleic anhydride could be replaced by fumaric acid, and the market for fumaric acid will be greater than 200,000 tonnes annually [109]. Owing to price limitations, most of the bio-based organic acids are still uncompetitive compared to the corresponding petroleum-derived products. Low-cost availability is an obvious prerequisite for the biological organic acid industry; one way to achieve this goal is to utilize inexpensive substrates, such as hemicellulose hydrolyzates.

Lactic acid is one of the organic acids that could be produced from hemicellulose. It is the most widely utilized organic acid in the food, pharmaceutical, and chemical industries. Recently, more attention has been paid on lactic acid production because of the development of polylactic acid (PLA), which is 100% biodegradable, and has been approved to be used in food and drugs. Lactic acid exists in two isomeric forms, D-lactic acid and L-lactic acid, and optically pure lactic acid is important in the formation of PLA with desirable mechanical properties.

L-Lactic acid can be naturally produced by bacteria and fungi; most lactic acid-producing strains can convert glucose to lactic acid, and some are even capable of utilizing hemicellulose-derived pentoses (Table 6). *Lactobacillus pentosus* [110], *Lactobacillus bifermentans* [111], and *Rhizopus oryzae* [112, 113] have been reported to convert hemicellulose-derived xylose and glucose to lactic acid. However, compared with glucose, due to the difficulties in xylose metabolism, and the inhibitors presented in the hemicellulose hydrolyzate, such as furfural and acetic acid, which are generated during pretreatment, the productivity of lactic acid is usually poor when using hemicellulose hydrolyzate as substrate. Reungruglikit and Hang [112] described the conversion of xylose into lactic acid by *R. oryzae*, and obtained a yield of 299.4 g/kg dry material after 48 h fermentation; such productivity is equivalent to 0.31 g/(L h) compared with 2.5 g/(L h) achieved using glucose. Most studies on methods to improve lactic acid production have been concerned with the detoxification of the hydrolyzate, strain improvement, or process optimization. Walton et al. [114] isolated a moderately thermophile organism, named *Bacillus coagulans* MXL-9, which was found to have high tolerance for inhibitors such as acetic acid, and showed it to be an excellent lactic acid producer using hardwood hemicellulose hydrolyzate as substrate. Wee et al. [115] reported that the inhibition of fermentation could be reduced by

Table 6 Fermentative production of lactic acid using different hemicellulosic substrates

Raw material	Strain	Lactic acid yield (g/g)	Lactic acid concentration (g/L)	Reference
Wheat bran hemicellulose hydrolyzate	*Lactobacillus bifermentans*	0.47	41.30	[111]
Wood hemicellulose hydrolyzate	*Rhizopus oryzae*	0.26	19.13	[113]
Wheat straw hemicellulose hydrolyzate	*Lactobacillus pentosus*	0.90	6.70	[116]
Trimming vine shoots hemicellulose hydrolyzate	*Lactobacillus pentosus*	0.78	46.00	[117]
Barley bran hemicellulose hydrolyzate	*Lactobacillus pentosus*	0.57	33.70	[118]
Corncob hemicellulose hydrolyzate	*Lactobacillus brevis*	0.69	39.10	[123]
Corncob molasses	*Bacillus* sp.	0.81	74.70	[124]

approximately 20% by direct adaptation of *Enterococcus faecalis* to wood hydrolyzate-based medium. Garde et al. [116] observed that neither *L. pentosus* nor *L. brevis* was able to fully utilize the released sugars of hemicellulose after acid treatment; however, lactic acid production could be increased to 95% of the theoretical maximum yield by co-inoculation of the two strains.

In China, pure xylose, glucose–xylose mixture, and hemicellulose-derived sugar mixtures have been used for lactic acid production [119–124]. More attention has been paid to strain improvement for xylose utilization. *R. oryzae* RLC41-6 mutated by means of low-energy ion beam implantation was found to be an efficient L-lactic acid producer from xylose; under optimal conditions, L-lactic acid yield of 0.77 g/g was achieved [119]. Bai et al. [120] obtained an adapted *R. oryzae* strain HZS6, which had a significantly improved utilization of sugars from corncob hydrolyzate; the final L-lactic acid concentration, yield, and productivity were twice that of its parental strain. Guo et al. [123] isolated two strains of *L. brevis* from sour cabbage, which exhibited the potential to completely utilize various sugars contained in hemicellulose hydrolyzate, and was strongly resistant to the fermentation inhibitors such as furfural; 39.1 g/L of lactic acid was finally achieved from dilute acid hydrolyzate of corncobs which contained 46.4 g/L xylose, 4.0 g/L glucose, and 6.5 g/L arabinose. Wang et al. [124] used corncob molasses, containing a high xylose content, for L-lactic acid production via a newly isolated xylose-utilizing *Bacillus* sp. XZL9. A high concentration of L-lactic acid (74.7 g/L) was obtained from corncob molasses (total sugars 91.4 g/L) under fed-batch fermentation.

Fumaric acid is another organic acid that can be obtained from hemicellulose sugars. It is widely used in the food industry without limitations and is a valuable intermediate for preparing edible products such as L-malic acid and L-aspartic acid. With a double bond and two carboxylic groups, fumaric acid is an important intermediate for polymer production, such as in the manufacture of synthetic resins and biodegradable polymers. At present, all commercial fumaric acid is produced from

maleic acid (or maleic anhydride), obtained from benzene oxidation [125]. However, fumaric acid can also be accumulated by microorganisms, particularly those belonging to the genus *Rhizopus*. Fumaric acid production by fermentation was operated in the USA as early as the 1940s, but later this process was discontinued and replaced by chemical synthesis due to its low productivity and the cheap availability of petroleum-derived feedstock. However, today, people are more concerned about natural products, especially in the food and pharmaceutical industry, and thus fermentative fumaric acid production is urgently needed [106, 126].

The economics of the fermentation route is still less favorable than the petrochemical route, in particular the raw material costs. Many studies have been conducted on the potential of utilizing cheap raw materials, such as lignocellulose resources. Liao et al. [127] developed a three-step fermentation process for fumaric acid production by *R. oryzae* from animal manure hydrolyzate, which was rich in glucose and xylose; under optimal conditions, a fumaric acid yield of 31%, and a chitin content of 0.21 g/g biomass was reached. Fumaric acid can also be produced from pure xylose. Kautola and Linko [128] used immobilized *Rhizopus arrhizus* to ferment xylose; when the initial xylose concentration was 100 g/L, a fumaric acid productivity of 0.087 g/(L h) was obtained. Fumaric acid production using hemicellulose hydrolyzate directly as substrate has also been studied. Woiciechowski et al. [129] investigated the performance of different strains in fumaric acid production with wood chips hydrolyzate as substrate; two *R. arrhizus* strains showed the highest amount of fumaric acid accumulation: 5.085 g/L of fumaric acid could be generated from 56.55 g/L sugar. However, the utilization rate of the hemicellulose-derived sugars, especially pentose, are rather slow compared with using glucose as substrate. To overcome this obstacle, a novel strategy to utilize hemicellulose-derived sugars was developed during fumaric acid production from lignocellulose. Liu et al. [130] observed that xylose was more suitable for *R. arrhizus* to accumulate biomass than glucose. Tai et al. [131] further observed that hemicellulose was an excellent substrate for the growth of fumaric acid-producing fungus, and that chitosan accumulated on the cell membrane. Depending on the pretreatment methods, sugars contained in the hemicellulosic fraction could be separated from the cellulose fraction. A two-stage corn straw utilization strategy for fumaric acid production was thus developed by Xu et al. [132] (Fig. 5). Corn straw was first pretreated by dilute sulphuric acid to obtain a xylose-rich liquid (hemicellulose hydrolyzate) that could be used as a carbon source to increase fungal biomass and chitosan accumulation; in addition the residue of corn straw was digested by enzyme to obtain a glucose-rich liquid that could be used for fumaric acid production. Under optimal conditions, fumaric acid production was up to 27.79 g/g, with yield of 0.35 g/g and productivity of 0.33 g/(L h).

Succinic acid is another organic acid that could be generated from hemicellulose sugars. It is a valuable four-carbon intermediate that can be used as the precursor of many important industrial chemicals: 1,4-butanediol, tetrahydrofuran, N-methyl pyrrolidinone, 2-pyrrolidinone, and several others. Furthermore, the demand for succinic acid extends to the synthesis of biodegradable polymers such as polybutyrate succinate (PBS) and polyamides [133]. Currently, succinic acid is

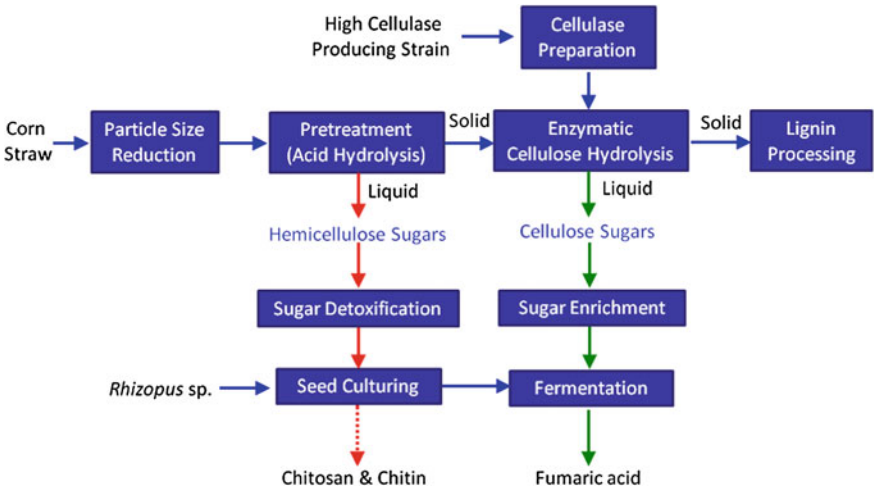

Fig. 5 Two-stage utilization of the hemicellulose and cellulose components of corn straw for fumaric acid production

mainly manufactured by a chemical route, hydrogenation of maleic anhydride to succinic anhydride, followed by hydration to succinic acid. However, recent analysis showed that the cost of fermentative production of succinic acid from renewable resources is getting closer to the petroleum-based process, and bio-based succinic acid production also involves CO_2 fixation (theoretically 1 mol CO_2 per mole succinic acid produced); fermentative production of succinic acid is therefore becoming more and more competitive [134].

Many different microorganisms such as fungi, yeast, and bacteria have been chosen for succinic acid production. Among them, *Actinobacillus succinogenes*, *Anaerobiospirillum succiniciproducens*, *Mannheimia succiniciproducens*, and recombinant *E. coli* have been intensively studied due to their ability to produce a large amount of succinic acid. Recently, more efforts have been put into succinic acid production from hemicellulose. Lee et al. [135] isolated a new succinic acid-producing bacterium, *M. succiniciproducens* MBEL 55E, from bovine rumen, and found that this strain can utilize xylose as well as glucose. Batch and continuous fermentation strategies were then developed for succinic acid production from wood hydrolyzate. In batch cultures, the final succinic acid concentration, yield, and productivity were 11.73 g/L, 56%, and 1.17 g/L respectively [136].

In China, fermentative succinic acid production using hemicellulose sugars as substrate has also attracted great attention. Zheng et al. [137] suggested that corn straw could be the most suitable material for succinic acid production by *A. succinogenes* after comparing kinds of raw material straw, and found that cell growth and succinic acid production were inhibited when the initial sugar concentration was higher than 60 g/L. Chen et al. [138] optimized the hydrolysis conditions of corn fiber pretreatment, and indicated that the effective method for removing fermentation inhibitors, especially furfural, was to use $CaCO_3$

neutralization combined with activated carbon adsorption after dilute acid hydrolysis. The final succinic acid yield was greater than 72% with the detoxified hydrolyzate as the carbon source in anaerobic culture. Yu et al. [139] developed a process for succinic acid production from corncob hydrolyzate by *A. succinogenes* without detoxification, involving medium optimization, selecting yeast extract as the nitrogen source, and $MgCO_3$ as the neutralization agent. A total of 23.64 g/L succinic acid with a yield of 0.58 g/g could be obtained.

In summary, apart from the above three organic acids, other organic acids such as itaconic acid, xylonic acid, etc. can also be obtained from hemicellulose sugars [140, 141]. However, to improve the utilization efficiency of hemicellulose sugars, apart from the measures traditionally used such as process development of raw material pretreatment, strain improvement, and fermentation optimization, future research focus should be placed on elucidating the mechanism of metabolism of different sugars which exist in the hemicellulose hydrolyzates. In addition, the strain tolerance of inhibitors should be enhanced based on the analysis of the strain response mechanisms.

3.5 Others

Besides the fuels and chemicals mentioned above, hemicellulose sugars can be converted to other industrially significant products such as single cell protein (SCP), furfural, methane gas, etc.

Qu et al. [142] extracted steam-exploded cellulosic biomass with hot water and obtained a liquor containing 16.0–47.7 g/L of total sugar. This is known as steam-exploded hemicellulose autohydrolyzate liquor (SEHAL). A mutant yeast *Trichosporon cutaneum* capable of direct utilization of oligomers of xylose was then used to produce SCP from SEHAL. This mutant can grow quickly in the medium with an increased protein content of 47.0%. By continuous fermentation with a dilution rate of 0.33 h^{-1}, a cell concentration of 10.5 g/L, a cell mass yield of 0.72 g/g total sugar, and a cell productivity of 3.0 g/(L h) were obtained. Chen et al. [143] obtained a total hemicellulose sugar concentration of 87.6 g/L through optimizing the sugar leaching process for steam-exploded straw. By using a repeated fed-batch fermentation strategy, a biomass concentration of 45.0 g/L and a productivity of 4.4 g/(L h) were obtained. Finally, SCP was separated from the broth by spray drying. In a more recent study, high enzyme activity of cellulase and SCP were co-produced from steam-exploded cornstalk using a mixed fermentation by *Trichoderma reesei* and *C. tropicalis*. In this strategy, the hemicellulose and cellulose fractions were both used sufficiently [144]. Similarly, corn stalk pretreated with diluted sulfuric acid was degraded with *Trichoderma* sp., and then converted successfully to SCP using the same yeast [145]. The co-fermentation by *Trichoderma* sp. and yeast degraded 70% of the total cornstalks and 60% of the cellulose content. This is an efficient and low-cost method for converting

lingocellulosics into protein-rich feed and has made possible the improvement of the nutritional value of cornstalks.

Apart from SCP, the hemicellulose fraction in lignocellulosics has also been used for furfural and methane gas production [146, 147]. The lignocellulosic substrate such as corncob or rice straw is widely distributed in China. The hemicellulosic fraction can first be hydrolyzed to pentose-rich sugars using sulfuric acid as the catalyst. The sugars can be further converted into furfural [148, 149]. The residues rich in cellulose can be further converted to ethanol by simultaneous saccharification and fermentation [150]. Rice straw is a widely available lignocellulosic resource in China. Pilot scale anaerobic digestion of rice straw to convert both hemicellulose and cellulose to methane gas has been developed [151, 152].

4 Conclusions and Perspectives

The hemicellulose fraction represents as much as 40% of total lignocellulosic materials. In the development of industrial production of ethanol as a biofuel, it is crucial to have efficient and cost-effective methods to convert sugars derived from hemicellulose into either additional ethanol or other valuable byproducts. At present, the conversion of hemicellulosic substrates to fermentable sugars is still a key obstacle hindering hemicellulose usage. It is important to develop a suitable pretreatment method, minimizing the formation of inhibitory compounds for fermentation organisms, so that the hemicellulose fraction can be utilized for production of fuels and chemicals by fermentation. In addition, there is still a need for further development of efficient and cost-effective methods for production of enzymes for hemicellulose conversion on an industrial scale. Additional challenges to the process economics of hemicellulose sugars conversion will include the development of robust efficient microorganisms to ferment hemicellulose sugars, and also methods for cost-effective recovery of fermentation products.

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References

1. Qu YB, Zhu M, Liu K, Bao X, Lin J (2006) Studies on cellulosic ethanol production for sustainable supply of liquid fuel in China. Biotechnol J 1:1235–1240
2. Fang X, Shen Y, Zhao J, Bao XM, Qu YB (2010) Status and prospect of lignocellulosic bioethanol production in China. Bioresour Technol 101:4814–4819
3. Yang B, Lu Y (2007) The promise of cellulosic ethanol production in China. J Chem Technol Biotechnol 82:6–10

4. Tan TW, Shang F, Zhang X (2010) Current development of biorefinery in China. Biotechnol Adv 28:543–555
5. Tan TW, Yu JL, Lu JK, Zhang T (2010) Biofuels in China. Adv Biochem Eng Biotechnol 122:73–104
6. Li ZJ, Ji XJ, Kan SL, Qiao HQ, Jiang M, Lu DQ, Wang J, Huang H, Jia HH, Ouyang PK, Ying HJ (2010) Past, present, and future industrial biotechnology in China. Adv Biochem Eng Biotechnol 122:1–42
7. Zhong C, Cao YX, Li BZ, Yuan YJ (2010) Biofuels in China: past, present and future. Biofuels Bioprod Bioref 4:326–342
8. Gong CS, Cao NJ, Du J, Tsao GT (1999) Ethanol production from renewable resources. Adv Biochem Eng Biotechnol 65:207–241
9. Wyman CE (2007) What is (and is not) vital to advancing cellulosic ethanol. Trends Biotechnol 25:153–157
10. Yang B, Wyman CE (2008) Pretreatment: the key to unlocking low-cost cellulosic ethanol. Biofuels Bioprod Bioref 2:26–40
11. Saha BC (2003) Hemicellulose bioconversion. J Ind Microbiol Biotechnol 30:279–291
12. Wyman CE (1999) Biomass ethanol: technical progress, opportunities, and commercial challenges. Ann Rev Energ Environ 24:189–226
13. Gong CS, Chen LF, Flickinger MC, Tsao GT (1981) Conversion of hemicellulose carbohydrates. Adv Biochem Eng Biotechnol 20:93–118
14. Gírio FM, Fonseca C, Carvalheiro F, Duarte LC, Marques S, Bogel-Łukasik R (2010) Hemicelluloses for fuel ethanol: a review. Bioresour Technol 101:4775–4800
15. Ebringerova A, Hromadkova Z, Heinze T (2005) Hemicellulose. Adv Polym Sci 186:1–67
16. Saulnier L, Marot C, Chanliaud E, Thibault JF (1995) Cell wall polysaccharide interactions in maize bran. Carbohydr Polymers 26:279–287
17. Lee YY, Iyer P, Torget RW (1999) Dilute-acid hydrolysis of lignocellulosic biomass. Adv Biochem Eng Biotechnol 65:93–115
18. DOE US (2006) Breaking the biological barriers to cellulosic ethanol: a joint research agenda, DOE/SC-0095. U.S. Department of Energy Office of Science and Office of Energy Efficiency and Renewable Energy. www.doegenomestolife.org/biofuels
19. Mosier N, Wyman C, Dale B, Elander R, Lee YY, Holtzapple M, Ladisch M (2005) Features of promising technologies for pretreatment of lignocellulosic biomass. Bioresour Technol 96:673–886
20. Saha BC, Bothast RJ (1997) Enzymes in lignocellulosic biomass conversion. In: Saha BC, Woodward J (eds) Fuels and chemicals from biomass. American Chemical Society, Washington, D.C
21. Jin Q, Zhang H, Yan L, Huang H (2010) Dilute acid hydrolysis reaction of biomass hemicellulose. Prog Chem 22:654–661 (in Chinese)
22. Jin Q, Zhang H, Yan L, Qu L, Huang H (2011) Kinetic characterization for hemicellulose hydrolysis of corn stover in a dilute acid cycle spray flow-through reactor at moderate conditions. Biomass Bioenerg 35:4158–4164.
23. Cheng KK, Cai BY, Zhang JA, Ling HZ, Zhou YJ, Ge JP, Xu JM (2008) Sugarcane bagasse hemicellulose hydrolysate for ethanol production by acid recovery process. Biochem Eng J 38:105–109
24. Yan L, Zhang H, Chen J, Lin Z, Jin Q, Jia H, Huang H (2009) Dilute sulfuric acid cycle spray flow-through pretreatment of corn stover for enhancement of sugar recovery. Bioresour Technol 100:1803–1808
25. Shallom D, Shoham Y (2003) Microbial hemicellulases. Curr Opin Microbiol 6:219–228
26. Selinger LB, Forsberg CW, Cheng KJ (1996) The rumen: a unique source of enzymes for enhancing livestock production. Anaerobe 2:263–284
27. Hill J, Nelson E, Tilman D, Polasky S, Tiffany D (2006) Environmental, economic, and energetic costs and benefits of biodiesel and ethanol biofuels. Proc Natl Acad Sci USA 103:11206–11210

28. Chandel AK, Chan EC, Rudravaram R, Narasu ML, Rao LV, Ravindra P (2007) Economics and environmental impact of bioethanol production technologies: an appraisal. Biotechnol Mol Biol Rev 2:14–32
29. Wang Y, Shi WL, Liu XY, Shen Y, Bao XM, Bai FW, Qu YB (2004) Establishment of a xylose metabolic pathway in an industrial strain of *Saccharomyces cerevisiae*. Biotechnol Lett 26:885–890
30. Liu EK, Hu Y (2010) Construction of a xylose–fermenting *Saccharomyces cerevisiae* strain by combined approaches of genetic engineering, chemical mutagenesis and evolutionary adaptation. Biochem Eng J 48:204–210
31. Du Preez JC (1994) Process parameters and environmental factors affecting D-xylose fermentation by yeasts. Enzyme Microb Technol 16:944–956
32. Hahn–Hagerdal B, Jeppsson H, Skoog K, Prior BA (1994) Biochemistry and physiology of xylose fermentation by yeasts. Enzyme Microb Technol 16:933–943
33. Lin Y, Tanaka S (2006) Ethanol fermentation from biomass resources: current state and prospects. Appl Microbiol Biotechnol 69:627–642
34. Cheng KK, Ge JP, Zhang JA, Ling HZ, Zhou YJ, Yang MD, Xu JM (2007) Fermentation of pretreated sugarcane bagasse hemicelluloses hydrolysate to ethanol by *Pachysolen tannophilus*. Biotechnol Lett 29:1051–1055
35. Zhang ZH, Qu YB, Zhang X, Lin JQ (2008) Effects of oxygen limitation on xylose fermentation, intracellular metabolites, and key enzymes of *Neurospora crassa* AS3.1602. Appl Biochem Biotechnol 145:39–51
36. Zhao L, Zhang X, Tan TW (2008) Influence of various glucose/xylose mixtures on ethanol production by *Pachysolen tannophilus*. Biomass Bioenerg 32:1156–1161
37. Chen YF, Dong BY, Qin WJ, Xiao DG (2010) Xylose and cellulose fractionation from corncob with three different strategies and separate fermentation of them to bioethanol. Bioresour Technol 101:6994–6999
38. Liu TJ, Lin L, Sun ZJ, Hu RF, Liu SJ (2010) Bioethanol fermentation by recombinant *E. coli* FBR5 and its robust mutant FBHW using hot–water wood extract hydrolyzate as substrate. Biotechnol Adv 28:602–608
39. Zhang XR, Shen Y, Shi WL, Bao XM (2010) Ethanolic cofermentation with glucose and xylose by the recombinant industrial strain *Saccharomyces cerevisiae* NAN–127 and the effect of furfural on xylitol production. Bioresour Technol 101:7093–7099
40. Zhao J, Xia LM (2010) Ethanol production from corn stover hemicellulosic hydrolysate using immobilized recombinant yeast cells. Biochem Eng J 49:28–32
41. Edgar WM (1998) Sugar substitutes, chewing gum and dental caries—a review. Br Dent J 184:29–32
42. Chen X, Jiang ZH, Chen SF, Qin WS (2010) Microbial and bioconversion production of D-xylitol and its detection and application. Int J Biol Sci 6:834–844
43. Mancilha IM, Karim MN (2003) Evaluation of ion exchange resins for removal of inhibitory compounds from corn stover hydrolyzate for xylitol fermentation. Biotechnol Prog 19:1837–1841
44. Deng LH, Wang YH, Zhang Y, Ma RY (2006) The enhancement of ammonia pretreatment on the fermentation of rice straw hydrolysate to xylitol. J Food Biochem 31:195–205
45. Carvalho W, Santos JC, Canilha L, Silva SS, Perego P, Converti A (2005) Xylitol production from sugarcane bagasse hydrolysate: metabolic behaviour of *Candida guilliermondii* cells entrapped in Ca-alginate. Biochem Eng J 25:25–31
46. Canilha L, Almeida SJB, Solenzal AIN (2004) Eucalyptus hydrolysate detoxification with activated charcoal adsorption or ionexchanger resins for xylitol production. Process Biochem 39:1909–1912
47. Solange IM, Giuliano D, Ines CR (2005) Influence of the toxic compounds present in brewer's spent grain hemicellulosic hydrolysate on xylose-to-xylitol bioconversion by *Candida guilliermondii*. Process Biochem 40:3801–3806
48. Farooq L, Mohammad IR (2001) Production of ethanol and xylitol from corncobs by yeasts. Bioresour Technol 77:57–63

49. Roberto IC, de Mancilha IM, Sato S (1999) Influence of k_La on bioconversion of rice straw hemicellulose hydrolysate to xylitol. Bioprocess Biosyst Eng 21:505–508
50. Parajó JC, Domínguez H, Domínguez JM (1997) Improved xylitol production with *Debaryomyces hansenii* Y-7426 from raw or detoxified wood hydrolysates. Enzyme Microb Technol 21:18–24
51. Converti A, Perego P, Domínguez JM (1999) Xylitol production from hardwood hemicellulose hydrolyzates by *Pachysolen tannophilus*, *Debaryomyces hansenii*, and *Candida guillermondii*. Appl Biochem Biotechnol 82:141–151
52. Walther T, Hensirisak P, Agblevor FA (2001) The influence of aeration and hemicellulosic sugars on xylitol production by *Candida tropicalis*. Bioresour Technol 76:213–220
53. Cirino PC, Chin JW, Ingram LO (2006) Engineering *Escherichia coli* for xylitol production from glucose-xylose mixtures. Biotechnol Bioeng 95:1167–1176
54. Lee WJ, Kim MD, Yoo MS, Ryu YW, Seo JH (2003) Effects of xylose reductase activity on xylitol production in two-substrate fermentation of recombinant *Saccharomyces cerevisiae*. J Microbiol Biotechnol 13:725–730
55. Sasaki M, Jojima T, Inui M, Yukawa H (2010) Xylitol production by recombinant *Corynebacterium glutamicum* under oxygen deprivation. Appl Microbiol Biotechnol 86: 1057–1066
56. Fang XN, Huang W, Xia LM (2004) Xylitol production from corn cob hemicellulosic hydrolysate by *Candida* sp. Chin J Biotechnol 20(2):295–298 (in Chinese)
57. Ding XH, Xia LM (2006) Effect of aeration rate on production of xylitol from corncob hemicellulose hydrolysate. Appl Biochem Biotechnol 133:263–270
58. Cheng KK, Zhang JA, Ling HZ, Ping WX, Huang W, Ge JP, Xu JM (2009) Optimization of pH and acetic acid concentration for bioconversion of hemicellulose from corncobs to xylitol by *Candida tropicalis*. Biochem Eng J 43:203–207
59. Huang W, Fang XN, Xia LM (2004) Production of xylitol from hemicellulose hydrolysate by immobilized *Candida tropicalis* cells. Chem Ind Forest Prod 24(1):29–33 (in Chinese)
60. Cheng H, Wang B, Lv J, Jiang M, Lin S, Deng Z (2011) Xylitol production from xylose mother liquor: a novel strategy that combines the use of recombinant *Bacillus subtilis* and *Candida maltose*. Microb Cell Fact 10:5
61. Magee RJ, Kosaric N (1985) Bioconversion of hemicellulosics. Adv Biochem Eng Biotechnol 32:61–93
62. Garg S, Jain A (1995) Fermentative production of 2,3-butanediol: a review. Bioresour Technol 51:103–109
63. Syu MJ (2001) Biological production of 2,3–butanediol. Appl Microbiol Biotechnol 55:10–18
64. Celińska E, Grajek W (2009) Biotechnological production of 2,3–butanediol–current state and prospects. Biotechnol Adv 27:715–725
65. Ji XJ, Huang H, Ouyang PK (2011) Microbial 2,3-butanediol production: a state-of-the-art review. Biotechnol Adv 29:351–364
66. Maddox IS (2008) Microbial production of 2,3-butanediol. In: Rehm HJ, Reed G (eds) Biotechnology, 2nd edn. Wiley-VCH Verlag GmbH. Weinheim
67. Yu B, Sun J, Bommareddy RR, Song L, Zeng AP (2011) Novel (2R, 3R)-2,3-butanediol dehydrogenase from potential industrial strain Paenibacillus polymyxa ATCC 12321. Appl Environ Microbiol 77:4230–4233
68. Jansen NB, Tsao GT (1983) Bioconversion of pentoses to 2,3-butanediol by *Klebsiella pneumoniae*. Adv Biochem Eng Biotechnol 27:85–99
69. Chandel AK, Singh OV, Rao LV (2010) Biotechnological applications of hemicellulosic derived sugars: State-of-the-art. In: Singh OV, Harvey SP (eds) Sustainable Biotechnology. Springer Netherlands, Amsterdam
70. Frazer FR, McCaskey TA (1989) Wood hydrolyzate treatments for improved fermentation of wood sugars to 2,3-butanediol. Biomass 18:31–42
71. Grover BP, Garg SK, Verma J (1990) Production of 2,3-butanediol from wood hydrolysate by *Klebsiella pneumoniae*. World J Microbiol Biotechnol 6:328–332

72. Yu EKC, Deschatelets L, Tan LUL, Saddler JN (1985) A simple method for xylanase preparation used for the hydrolysis and fermentation of hemicellulose to butanediol. Biotechnol Lett 7:425–430
73. Yu EKC, Levitin N, Saddler JN (1982) Production of 2,3-butanediol by *Klebsiella pneumoniae* grown on acid hydrolyzed wood hemicellulose. Biotechnol Lett 4:741–746
74. Frazer FR, McCaskey TA (1991) Effect of components of acid-hydrolysed hardwood on conversion of D-xylose to 2,3-butanediol by *Klebsiella pneumoniae*. Enzyme Microb Technol 13:110–115
75. Nishikawa NK, Sutcliffe R, Saddler JN (1998) The effect of wood-derived inhibitors on 2,3-butanediol production by *Klebsiella pneumoniae*. Biotechnol Bioeng 31:624–627
76. Yu EKC, Deschatelets L, Louis-Seize G, Saddler JN (1985) Butanediol production from cellulose and hemicellulose by *Klebsiella pneumoniae* grown in sequential coculture with *Trichoderma harzianum*. Appl Environ Microbiol 50:924–929
77. Yu EKC, Saddler JN (1985) Biomass conversion to butanediol by simultaneous saccharification and fermentation. Trends Biotechnol 3:100–104
78. Saha BC, Bothast RJ (1999) Production of 2,3-butanediol by newly isolated *Enterobacter cloacae*. Appl Microbiol Biotechnol 52:321–326
79. Ji XJ, Huang H, Du J, Zhu JG, Ren LJ, Li S, Nie ZK (2009) Development of an industrial medium for economical 2,3-butanediol production through co-fermentation of glucose and xylose by *Klebsiella oxytoca*. Bioresour Technol 100:5214–5218
80. Cheng KK, Liu Q, Zhang JA, Li JP, Xu JM, Wang GH (2010) Improved 2,3-butanediol production from corncob acid hydrolysate by fed-batch fermentation using *Klebsiella oxytoca*. Process Biochem 45:613–616
81. Si Y, Xia LM (2010) Fermentative production of 2,3-butanediol from corn stover hydrolysate. Food Ferment Ind 36(2):26–29 (in Chinese)
82. Wang AL, Wang Y, Jiang TY, Li LX, Ma CQ, Xu P (2010) Production of 2,3-butanediol from corncob molasses, a waste by-product in xylitol production. Appl Microbiol Biotechnol 87:965–970
83. Yang Y, Zhang YC, Sun YF, He LF, Jiang Y (2010) Study on production of 2,3-butanediol from straw paper pulp hydrolysate fermentation by *Klebsiella pneumoniae*. Renew Energ Resour 28(2):53–58 (in Chinese)
84. Ji XJ, Nie ZK, Huang H, Ren LJ, Peng C, Ouyang PK (2011) Elimination of carbon catabolite repression in *Klebsiella oxytoca* for efficient 2,3-butanediol production from glucose–xylose mixtures. Appl Microbiol Biotechnol 89:1119–1125
85. Ji XJ, Huang H, Li S, Du J, Lian M (2008) Enhanced 2,3-butanediol production by altering the mixed acid fermentation pathway in *Klebsiella oxytoca*. Biotechnol Lett 30:731–734
86. Ji XJ, Huang H, Zhu JG, Ren LJ, Nie ZK, Du J, Li S (2010) Engineering *Klebsiella oxytoca* for efficient 2,3-butanediol production through insertional inactivation of acetaldehyde dehydrogenase gene. Appl Microbiol Biotechnol 85:1751–1758
87. Görke B, Stülke J (2008) Carbon catabolite repression in bacteria: many ways to make the most out of nutrients. Nat Rev Microbiol 6:613–624
88. van Haveren J, Scott EL, Sanders J (2008) Bulk chemicals from biomass. Biofuels Bioprod Bioref 2:41–57
89. Emerson RR, Flickinger MC, Tsao GT (1982) Kinetics of dehydration of aqueous 2,3-butanediol to methyl ethyl ketone. Ind Eng Chem Prod Res Dev 21:473–477
90. Tran AV, Chambers RP (1987) The dehydration of fermentative 2,3-butanediol into methyl ethyl ketone. Biotechnol Bioeng 29:343–351
91. Speranza G, Manitto P, Fontana G, Monti D, Galli A (1996) Evidence for enantiomorphic-enantiotopic group discrimination in diol dehydratase-catalyzed dehydration of meso-2,3-butanediol. Tetrahedron Lett 37:4247–4250
92. Wang D, Wang FQ, Wang JH (2000) Production of MEK by fermentation. Fine Spec Chem 15(9):19–20 (in Chinese)
93. Lee J, Grutzner JB, Walters WE, Delgass WN (2000) The conversion of 2,3-butanediol to methyl ethyl ketone over zeolites. Stud Surf Sci Catal 130:2603–2608

94. Huang H, Yan J, Ji XJ, Li S, Hu YC. A method for methyl ethyl ketone production. Chinese Patent: 200810122489.4, June 20, 2008
95. Zhang JA, Xie Y, Cheng KK, Zhou YJ, Liu HJ, Liu DH. A high efficient method for converting 2,3-butanediol to methyl ethyl ketone. Chinese Patent: 200910083730.1, May 8, 2009
96. Ni Y, Sun Z (2009) Recent progress on industrial fermentative production of acetone-butanol-ethanol by *Clostridium acetobutylicum* in China. Appl Microbiol Biotechnol 83:415–423
97. Lee SY, Park JH, Jang SH, Nielsen LK, Kim J, Jung KS (2008) Fermentative butanol production by clostridia. Biotechnol Bioeng 101:209–228
98. Chen CK, Blaschek HP (1999) Acetate enhances solvent production and prevents degeneration in *Clostridium beijerinckii* BA101. Appl Microbiol Biotechnol 52:170–173
99. Atsumi S, Hanai T, Liao JC (2008) Non-fermentative pathways for synthesis of branched-chain higher alcohols as biofuels. Nature 451:86–89
100. Zhang KC, Sawaya MR, Eisenberg DS, Liao JC (2008) Expanding metabolism for biosynthesis of nonnatural alcohols. Proc Natl Acad Sci USA 105:20653–20658
101. Manzer LE. Method of making 2-butanol. US 7754928B2, Jul 13, 2010
102. Siemerink MAJ, Kuit W, Lopez Contreras AM, Eggink G, van der Oost J, Kengen SWM (2011) Heterologous expression of an acetoin reductase leads to D-2,3-butanediol production in *Clostridium acetobutylicum*. Appl Environ Microbiol 77:2582–2588
103. Bramucci MG, Flint D, Miller ES, Nagarajan V, Sedkova N, Singh M, Van Dyk TK. Method for the production of 2-butanol, US 20080274525 A1, April 28, 2008
104. Burk MJ, Pharkya P, Burgard AP. Microorganisms for the production of methyl ethyl ketone and 2-butanol. US 20100184173 A1, November 13, 2009
105. Osterhout RE, Niu W, Burgard AP. Microorganisms and methods for carbon-efficient biosynthesis of MEK and 2-butanol. US 20110008858 A1, June 10, 2010
106. Tsao GT, Cao NJ, Du J, Gong CS (1999) Production of multifunctional organic acids from renewable resources. Adv Biochem Eng Biotechnol 65:243–280
107. Nigam P (2009) Production of organic acids from agro-industrial residues. In: Nigam P, Pandey A (eds) Biotechnology for agro-industrial residues utilization. Springer Netherlands, Amsterdam
108. Werpy T, Petersen G (2004) Top value added chemicals from biomass, Volume 1: results of screening for potential candidates from sugars and synthesis gas. U.S. Department of Energy. http://www1.eere.energy.gov/biomass/pdfs/35523.pdf
109. Sauer M, Porro D, Mattanovich D, Branduardi P (2008) Microbial production of organic acid: expanding the markets. Trends Biotechnol 26:100–108
110. Zhu YM, Lee YY, Elander RT (2007) Conversion of aqueous ammonia-treated corn stover to lactic acid by simultaneous saccharification and cofermentation. Appl Biochem Biotechnol 137:721–738
111. Givry S, Prevot V, Duchiron F (2008) Lactic acid production from hemicellulosic hydrolyzate by cells of *Lactobacillus bifermentans* immobilized in Ca-alginate using response surface methodology. World J Microbiol Biotechnol 24:745–752
112. Ruengruglikit C, Hang YD (2003) L(+)-lactic acid production from corncobs by *Rhizopus oryzae* NRRL–395. Food Sci Technol 36:573–575
113. Woiciechowski AL, Soccol CR, Ramos LP, Pandey A (1999) Experimental design to enhance the production of L-(+)-lactic acid from steam-exploded wood hydrolysate using *Rhizopus oryzae* in a mixed-acid fermentation. Process Biochem 34:949–955
114. Walton SL, Bischoff KM, van Heiningen ARP, van Walsum GP (2010) Production of lactic acid from hemicellulose extracts by *Bacillus coagulans* MXL-9. J Ind Microbiol Biotechnol 37:823–830
115. Wee YJ, Yun JS, Park DH, Ryu HW (2004) Biotechnological production of L-lactic acid from wood hydrolyzate by batch fermentaion of *Enterococcus faecalis*. Biotechnol Lett 26:71–74

116. Garde A, Jonsson G, Schmidt AS, Ahring BK (2002) Lactic acid production from wheat straw hemicellulose hydrolysate by *Lactobacillus pentosus* and *Lactobacillus brevis*. Bioresour Technol 81:217–223
117. Bustos G, Moldes AB, Cruz JM, Domínguez JM (2005) Influence of the metabolism pathway on lactic acid production from hemicellulosis trimming vine shoots hydrolyzates using *Lactobacillus pentosus*. Biotechnol Prog 21:793–798
118. Moldes AB, Torrado A, Converti A, Domínguez JM (2006) Complete bioconversion of hemicellulosic sugars from agricultural residues into lactic acid by *Lactobacillus pentosus*. Appl Biochem Biotechnol 135:219–227
119. Yang Y, Fan Y, Li W, Wang D, Wu Y, Zheng Z, Yu Z (2007) Optimization of L-lactic acid production from xylose with *Rhizopus oryzae* mutant RLC41-6 breeding by low-energy ion implantation. Plasma Sci Technol 9:638–642
120. Bai DM, Li SZ, Liu ZL, Cui ZF (2008) Enhanced L-lactic acid production by an adapted strain of *Rhizopus oryzae* using corncob hydrolysate. Appl Biochem Biotechnol 144:79–85
121. Wang P, Li J, Wang L, Tang M, Yu Z, Zheng Z (2009) L-lactic acid production by co-fermentation of glucose and xylose with *Rhizopus oryzae* obtained by low-energy ion beam irradiation. J Ind Microbiol Biotechnol 36:1363–1368
122. Guo Y, Yan Q, Jiang Z, Teng C, Wang X (2010) Efficient production of lactic acid from sucrose and corncob hydrolysate by a newly isolated *Rhizopus oryzae* GY18. J Ind Microbiol Biotechnol 37:1137–1143
123. Guo W, Jia W, Li Y, Chen S (2010) Performances of *Lactobacillus brevis* for producing lactic acid from hydrolysate of lignocellulosics. Appl Biochem Biotechnol 161:124–136
124. Wang L, Zhao B, Liu B, Yu B, Ma C, Su F, Hua D, Li Q, Ma Y, Xu P (2010) Efficient production of L-lactic acid from corncob molasses, a waste by-product in xylitol production, by a newly isolated xylose utilizing *Bacillus* sp. strain. Bioresour Technol 101:7908–7915
125. Gao Z, Zhang K, Huang H, Li S, Wei P (2009) Fumaric acid production by *Rhizopus* sp. Prog Chem 21(1):251–258 (in Chinese)
126. Roa Engel CA, Straathof AJJ, Zijlmans TW, van Gulik WM, van der Wielen LAM (2008) Fumaric acid production by fermentation. Appl Microbiol Biotechnol 78:379–389
127. Liao W, Liu Y, Frear C, Chen SL (2008) Co-production of fumaric acid and chitin from a nitrogen-rich lignocellulosic material–dairy manure–using a pelletized filamentous fungus *Rhizopus oryzae* ATCC 20344. Bioresour Technol 99:5859–5866
128. Kautola H, Linko K (1989) Fumaric acid production from xylose by immobilized *Rhizopus arrhizus* cells. Appl Microbiol Biotechnol 31:448–452
129. Woiciechowski AL, Soccol CR, Ramos LP, Affonso LF (2001) Screening of several *Rhizopus* strains to produce fumaric acid by biological conversion of hemicellulosic hydrolysates obtained by steam explosion. In: Proceedings V Simpósio de Hidrólise Enzimática de Biomassa. Universidade Estadual de Maringá, Maringá, Brasil
130. Liu N, Li S, He H, Wu H, Huang H, Ji SY (2008) Stepped utilization of xylose and glucose in fermentation of fumaric acid by *Rhizopus arrhizus*. Chin J Process Eng 8(4):794–797 (in Chinese)
131. Tai C, Li S, Xu Q, Ying H, Huang H, Ouyang PK (2010) Chitosan production from hemicellulose hydrolysate of corn straw: impact of degradation products on *Rhizopus oryzae* growth and chitosan fermentation. Lett Appl Microbiol 51:278–284
132. Xu Q, Li S, Fu YQ, Tai C, Huang H (2010) Two-stage utilization of corn straw by *Rhizopus oryzae* for fumaric acid production. Bioresour Technol 101:6262–6264
133. McKinlay JB, Vieille C, Zeikus JG (2007) Prospects for a bio-based succinate industry. Appl Microbiol Biotechnol 76:727–740
134. Yu C, Cao Y, Zou H, Xian M (2011) Metabolic engineering of *Escherichia coli* for biotechnological production of high-value organic acids and alcohols. Appl Microbiol Biotechnol 89:573–583
135. Lee PC, Lee SY, Hong SH, Chang HN (2002) Isolation and characterization of a new succinic acid-producing bacterium, *Mannheimia succiniciproducens* MBEL55E, from bovine rumen. Appl Microbiol Biotechnol 58:663–668

136. Kim DY, Yim SC, Lee PC, Lee WG, Lee SY, Chang HN (2004) Batch and continuous fermentation of succinic acid from wood hydrolysate by *Mannheimia succiniciproducens* MBEL55E. Enzyme Microb Technol 35:648–653
137. Zheng P, Dong JJ, Sun ZH, Ni Y, Fang L (2009) Fermentative production of succinic acid from straw hydrolysate by *Actinobacillus succinogenes*. Bioresour Technol 100:2425–2429
138. Chen K, Jiang M, Wei P, Yao J, Wu H (2010) Succinic acid production from acid hydrolysate of corn fiber by *Actinobacillus succinogenes*. Appl Biochem Biotechnol 160:477–485
139. Yu J, Li ZM, Ye Q, Yang Y, Chen S (2010) Development of succinic acid production from corncob hydrolysate by *Actinobacillus succinogenes*. J Ind Microbiol Biotechnol 37:1033–1040
140. Willke T, Vorlop KD (2001) Biotechnological production of itaconic acid. Appl Microbiol Biotechnol 56:289–295
141. Li ZY, Nie ZK, Ji XJ, Huang H (2010) Progress in xylonic acid production and application. Chem Ind Eng Prog 29(8):1525–1529 (in Chinese)
142. Qu YB, Chen HZ, Gao PJ (1992) SCP production from steam exploded hemicellulose autohydrolysate by *Trichosporon cutaneum*. J Ferment Bioeng 73:386–389
143. Chen HZ, Liu J, Li ZH (1999) Production of single cell protein by fermentation of extracts from hemicellulose autohydrolysis. Eng Chem Metall 20(4):428–431 (in Chinese)
144. Wang CH, Ding YQ, Xiao CF, Hua W, Sun NX (2001) Production of high enzyme activity SCP from cellulose material. Ind Microbiol 31(1):30–33 (in Chinese)
145. Liu JH, Chen QS, Chen JY, Yan YL, Zhang XL, Pang GC (2001) Research on biotransformation to single cell proteins by using diluted sulfuric acid solution to pretreat corn stalk. J Tianjin Univ Commun 21(3):1–5 (in Chinese)
146. Gunaseelan N (1997) Anaerobic digestion of biomass for methane production: a review. Biomass Bioenerg 13:83–114
147. Mamman AS, Lee JM, Kim YC, Hwang IT, Park NJ, Hwang YK, Chang JS, Hwang JS (2008) Furfural: Hemicellulose/xylose derived biochemical. Biofuels Bioprod Bioref 2:438–454
148. Li ZS, Yi WG (2010) Study on furfural preparation from corn cob. Fine Chem Intermed 40(4):53–55 (in Chinese)
149. Gao LF, Xu HB, Zhang Y, Cao HB (2010) Optimization on production process of furfural by high-temperature dilute-acid hydrolysis of corncobs. Chin J Process Eng 10(20):292–297 (in Chinese)
150. Sun YD, Sun R, Jiang JX, Zhu LW (2008) Study on conversion process for furfural residue manufacture to ethanol by simultaneous saccharification and fermentation. Mod Chem Ind 28(12):48–52 (in Chinese)
151. Chen L, Zhao LX, Dong BC, Wan XC, Gao XX (2010) The status and trends of the development of biogas plants for crop straws in China. Renew Energ Resour 28(3):145–148 (in Chinese)
152. Zhang W, Li XJ, Pang YZ, Cai LP (2008) A pilot study on mesophilic dry anaerobic digestion of rice straw. J Agro-Environ Sci 27(5):2075–2079 (in Chinese)

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