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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].

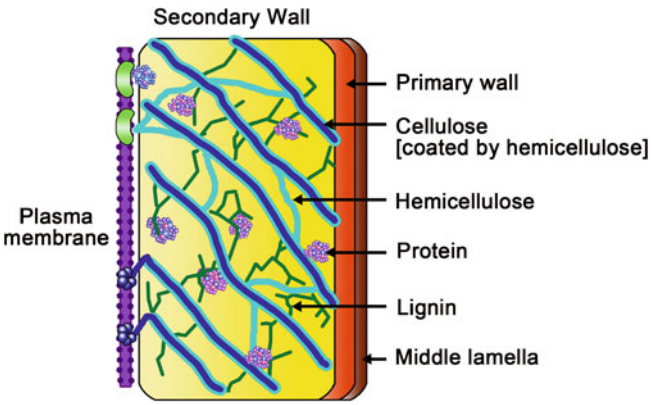

Fig. 1 Schematic diagram of plant cell walls

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.

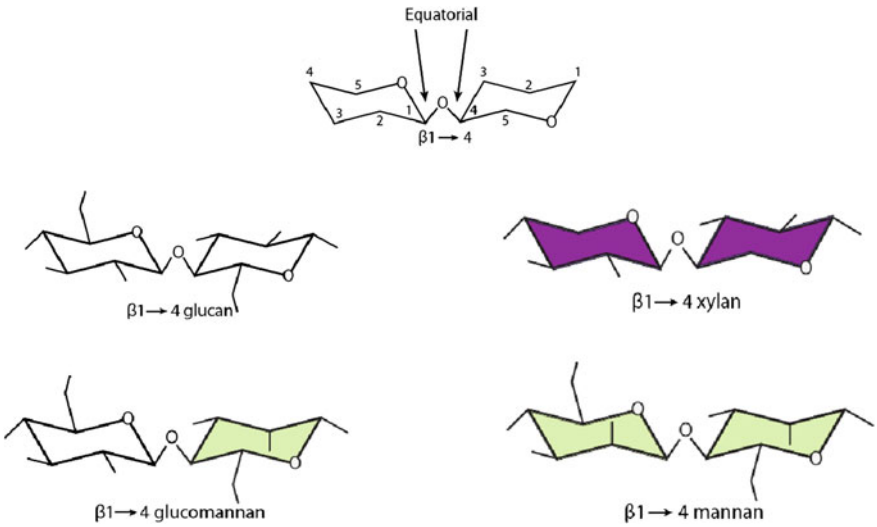

Fig. 2 Repeating units of hemicelluloses (Reprinted from [12] with permission)

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

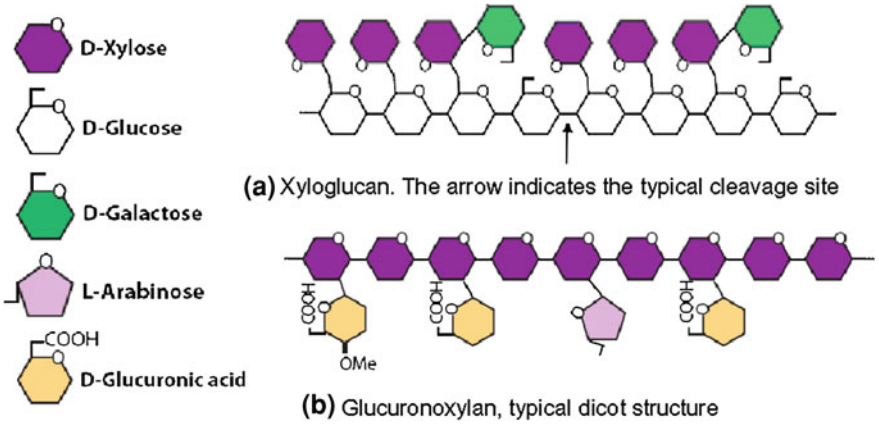

Fig. 3 Diagram of xyloglucan and xylans (Reprinted from [12] with permission)

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

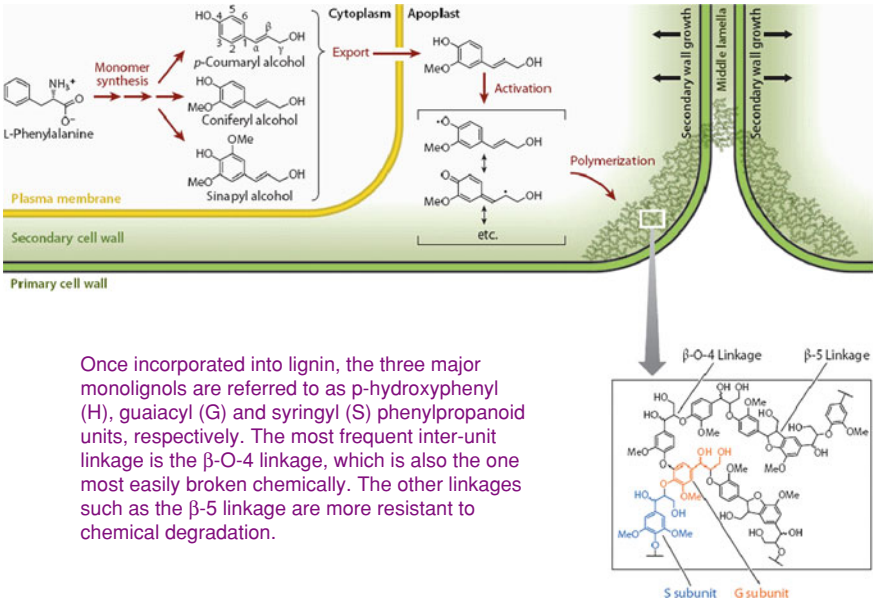

Fig. 4 Schematic diagram of lignin biosynthesis (Adapted from [13] with modifications)

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].

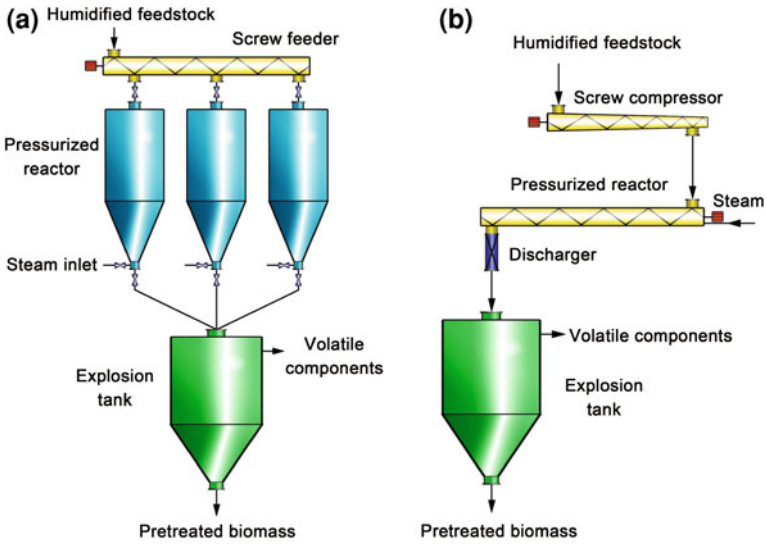

Fig. 5 Process diagram of steam explosion. Batch process (**a**), and continuous mode (**b**)

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

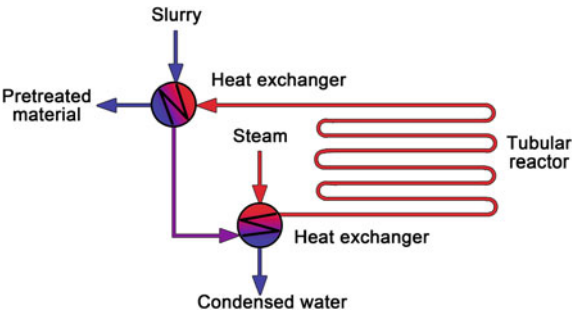

Fig. 6 Schematic diagram of the LHW process

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

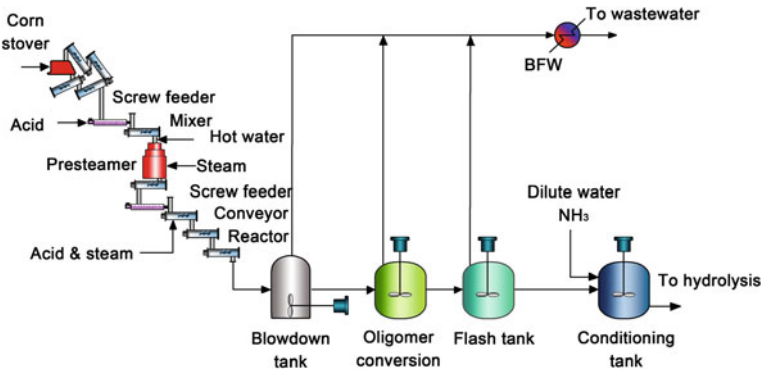

Fig. 7 Process diagram of the NREL projected dilute-acid pretreatment of corn stover [28]

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

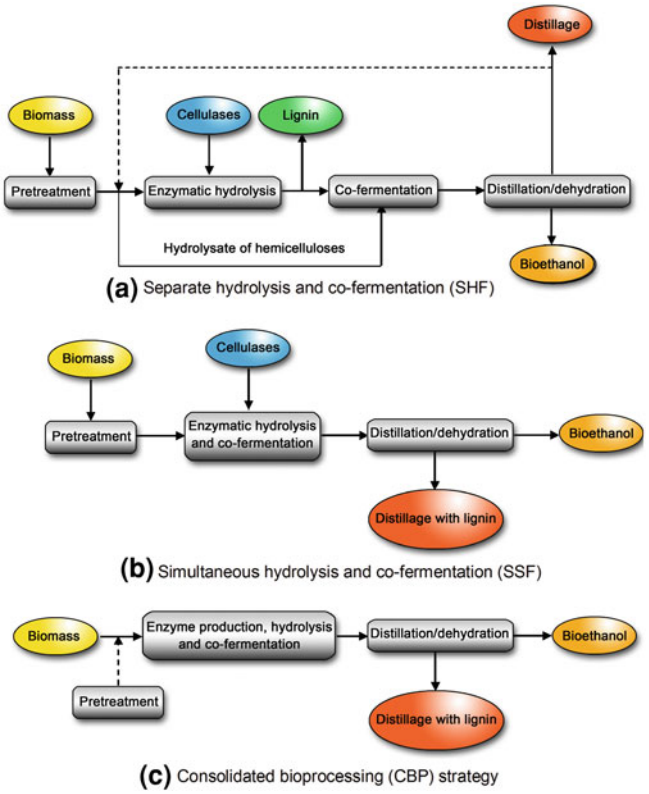

Fig. 8 Process engineering strategies for bioethanol production

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.

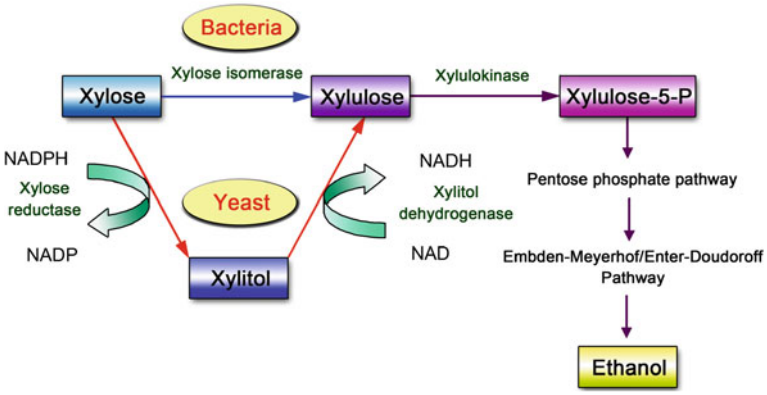

Fig. 9 Simplified xylose-metabolizing pathways in bacteria and yeast (Adapted from [60])

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

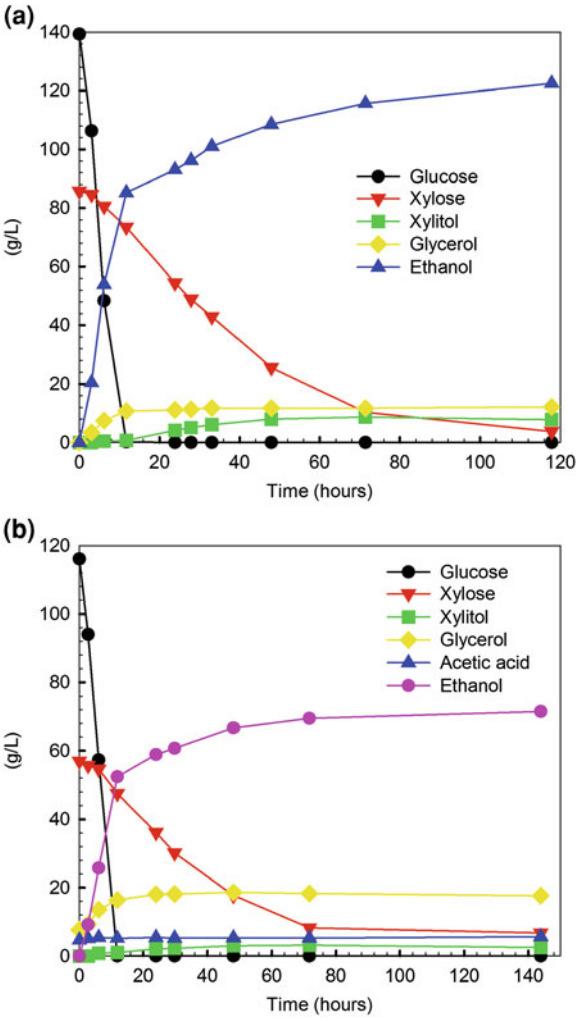

Fig. 10 Co-fermentations of glucose and xylose in simulated medium (**a**) and wheat straw hydrolysate (**b**) by the recombinant *S. cerevisiae* 424A(LNH-ST)

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.

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