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Bioconversion of Lignocellulose into Bioethanol: Process Intensification and Mechanism Research

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Abstract Biofuels produced from lignocellulosic biomass can significantly reduce the energy dependency on fossil fuels and the resulting effects on environment. In this respect, cellulosic ethanol as an alternative fuel has the potential to become a viable energy source in the near future. Over the past few decades, tremendous effort has been undertaken to make cellulosic ethanol cost competitive with conventional fossil fuels. The pretreatment step is always necessary to deconstruct the recalcitrant structures and to make cellulose more accessible to enzymes. A large number of pretreatment technologies involving physical, chemical, biological, and combined approaches have been developed and tested at the pilot scale. Furthermore, various strategies and methods, including multi-enzyme complex, non-catalytic additives, enzyme recycling, high solids operation, design of novel bioreactors, and strain improvement have also been implemented to improve the efficiency of subsequent enzymatic hydrolysis and fermentation. These technologies provide significant opportunities for lower total cost, thus making large-scale production of cellulosic ethanol possible. Meanwhile, many researchers have focused on the key factors that limit cellulose hydrolysis, and analyzing the reaction mechanisms of cellulase. This review describes the most recent advances on process intensification and mechanism research of pretreatment, enzymatic hydrolysis, and fermentation during the production of cellulosic ethanol.

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Abbreviations

SSF	Simultaneous saccharification and fermentation
SSCF	Simultaneous saccharification and co-fermentation
ARP	Ammonia recycle percolation
SAA	Soaking in aqueous ammonia
AFEX	Ammonia fiber explosion
HCW	Hot-compressed water
NMO	*N*-methyl-morpholine-*N*-oxide
EG	Endoglucanase
CBH	Cellobiohydrolase
BG	β-glucosidase
CBR	Conventional batch reactor
MBR	Membrane bioreactor
RBR	Roller bottle reactors
CBM	Cellulose-binding module
CAC	Cellulose accessibility to cellulase
DP	Degree of polymerization
SEC	Size exclusion chromatography
MALLS	Multi-angle laser light scattering

Introduction

The growing demand for energy and diminishing fossil fuel reserves have stimulated tremendous interest in finding alternative renewable energy sources. Lignocellulose, the most abundant renewable biomass produced from photo-

synthesis, has the potential to serve as a sustainable supply of fuels and chemicals [1–6]. Using this renewable carbohydrate source in place of fossil fuels is also one of the most effective ways to fight both the energy crisis and environmental problems caused by carbon dioxide emissions. Over the past few decades, a large number of research efforts have focused on the bioconversion of lignocellulose to produce biofuels, such as ethanol [1, 3]. This process generally involves three main steps: (1) pretreatment, to break down the recalcitrant structures of lignocellulose; (2) enzymatic hydrolysis, to hydrolyze polysaccharides (e.g., cellulose, hemicellulose) into fermentable sugars; and (3) fermentation, to convert sugars into ethanol. As we know, effectively breaking the recalcitrant structure and releasing and degrading the locked polysaccharides are the major barriers to commercializing cellulosic ethanol. As a result, process intensification and mechanism research involved in ethanol production has been increasingly important (Fig. 1).

To produce cellulosic ethanol, breaking the recalcitrant structures through pretreatment is essential to enhance enzymatic hydrolysis of cellulose [3, 7–9]. An effective pretreatment should: (1) disrupt the lignin barrier as well as cellulose crystallinity to allow more facilitated enzymatic attack on cellulose chains, (2) limit the formation of toxic degradation products that inhibit the enzymes or fermentative microorganisms, (3) reduce the loss of sugar components (cellulose and hemicellulose) and maintain other valuable components such as lignin, (4) and minimize capital and operating expenses. Over the last few decades, a wide variety of different technologies have been developed for lignocellulose pretreatment, including common pretreatment technologies and recent new technologies (Fig. 1). However, a comprehensive review of all methods is beyond the scope of this review. Considering there have been some other review articles that referred to the pretreatment process [7–10], herein we will summarize concisely the common pretreatment technologies and concentrate on the most recent new methods used in lignocellulose pretreatment.

During the process of enzymatic hydrolysis (or simultaneous saccharification and fermentation (SSF)), some factors, such as non-cellulose components and sugar products, can restrict the access of cellulases to cellulose and/or diminish enzyme performance. To alleviate these problems, optimizing enzyme composition (multi-enzyme complex) and adding non-catalytic additives (such as surfactants, non-catalytic protein) have been used in many studies [11–15] (Fig. 1). Enzyme recycling is an important strategy to lower enzyme cost [16–19], although enzyme prices have gradually decreased due to intensive research by companies such as Novozymes, Genencor, DSM, and Verenium. At present, the enzyme cost is still the dominating economic barrier to produce ethanol from lignocellulosic biomass.

In recent years, conducting hydrolysis or SSF at high solids loadings has received considerable attention as an essential approach to improve the ethanol concentrations [20–22]. In this respect, fed-batch operation of novel high-solid reactors (e.g., horizontal bioreactor, helical stirring bioreactor) could potentially solve the stirring problem that is caused by high viscosity at high solids loadings [23, 24]. Other reactor designs have also been applied to enzymatic hydrolysis and fermentation to enhance the efficiency of bioconversion, including membrane bioreactors [25–28], ultrasonic airlift reactors [29, 30], and fluidized bed reactors [31]. In addition, to make the SSF (or simultaneous

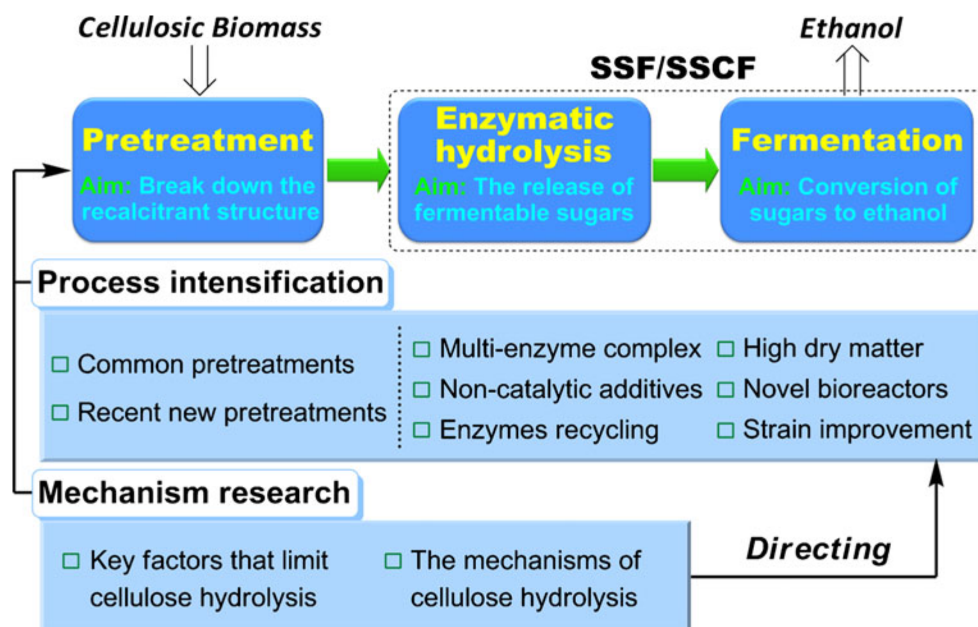

Fig. 1 Schematic flowsheet for the bioconversion of lignocellulosic biomass to ethanol

saccharification and co-fermentation (SSCF)) process more efficient, a lot of attention has been focused on strain improvement, including pentose utilization [32], direct cellobiose or cellulose fermentation [33], and tolerance enhancement [34].

Many researchers have paid great attention to the key factors that limit cellulose hydrolysis and analyzing the reaction mechanisms of cellulase [35–38] (Fig. 1). Currently, a comprehensive understanding of how enzymes hydrolyze lignocellulosic substrates is still a tremendous challenge due to their structural and compositional complexity. However, the above studies were useful to identify the key limited factors, process optimization, and design of novel pretreatment methods. The results also provide insights into the enzyme–substrate interaction and the reaction mechanism of enzymes.

The purpose of this work is to review the most recent advances (mainly between 2007 and 2011) on process intensification of pretreatment, enzymatic hydrolysis, and fermentation in cellulosic ethanol production, as well as to analyze the key factors that limiting cellulose hydrolysis and to summarize concisely the reaction mechanism of enzymatic hydrolysis.

Process Intensification in the Production of Cellulosic Ethanol

Lignocellulose Pretreatment

Common Pretreatment Technologies

The common pretreatment methods are summarized in Table 1. These methods mainly involve acid/base, water, and steam pretreatments. Using acids or bases has long been known to promote hydrolysis for years by removing hemicellulose or lignin.

In dilute acid (typically H_2SO_4) pretreatment, most of the hemicellulose can be removed and recovered as soluble sugars [39, 40]. Although little lignin is removed, this process leads to the disruption of lignin that can increase enzyme accessibility to cellulose. Concentrated inorganic acids (e.g., sulfuric acid, phosphoric acid), have also been used in pretreatment processes. Besides the degradation of hemicelluloses, more importantly, data suggested that the crystal structure of cellulose is destroyed completely [41]. The resulting amorphous cellulose can be rapidly converted to glucose at a high rate. However, concentrated acid pretreatment has several major technical obstacles, such as equipment corrosion, sugar–acid separation, sugar decomposition, and acid re-concentration.

Among alkali pretreatment methods, ammonia pretreatments, including ammonia recycle percolation (ARP) [42] and soaking in aqueous ammonia (SAA) [43], have been shown to be effective in improving cellulose digestion. Ammonia has a number of desirable characteristics as a pretreatment reagent, such as its delignification ability, swelling ability, high volatility, and low cost. In the ARP process, aqueous ammonia was passed through a column reactor packed with biomass at high temperatures. High degrees of delignification can be realized due to the reaction of aqueous ammonia with lignin, leading to the cleavage of C–O–C bonds in lignin, as well as ether and ester bonds in the lignin-carbohydrate complex. The removal of lignin and the swelling of the remaining substrate greatly enhances cellulose digestion, although the crystalline structure of cellulose was not changed [44]. The ARP process also solubilized approximately half of the hemicelluloses in the pretreatment hydrolysate.

To reduce hemicellulose loss and water consumption, Kim et al. [43] developed a simpler process termed SAA, in which biomass was treated with aqueous ammonia in a batch reactor under modest reaction conditions (moderate temperatures, atmospheric pressure). Similar cellulose digestion to the ARP process can be achieved by SAA treatment. Due to the retention of hemicellulose in solid during pretreatment, both pentose and hexose sugars were produced in high yields during the subsequent enzymatic hydrolysis by commercial enzymes. This is a desirable feature because the resulting sugar complexes are suitable for the co-fermentation process that can achieve comparable maximum ethanol concentration. It also avoids the separate recovery and processing of xylose from the pretreatment liquid.

Another important category of common pretreatment technologies is steam pretreatment, including un-catalyzed steam explosion [45], acid-catalyzed steam explosion [46, 47], and ammonia fiber explosion (AFEX) [48–51]. In un-catalyzed steam pretreatments, lignocellulosic biomass is heated for several minutes with high-pressure steam without the addition of any chemicals. Then the steam is rapidly vented from the reactor, leading to an explosive discharge of biomass into to a large vessel. Significant amounts of hemicellulose are partially hydrolyzed to mono- and oligosaccharides by the acetic and other acids released during this process. Only a limited amount of lignin is removed during the pretreatment, but the extensive redistribution of lignin occurs on fiber surfaces through the melting and depolymerization/repolymerization reactions [45]. This process provides relatively high cellulose digestibility due to increased enzyme accessibility, which is mainly caused by hemicellulose removal and redistribution of lignin. However, the relatively high operating cost, including the energy consumption and capital equipment, limits commercial use for biomass pretreatment. Some toxic by-products from sugar degradation are also formed during

Table 1 Summary of some common pretreatment technologies and their effects on the chemical composition and physical structures of lignocellulosic biomass

Pretreatment technology	Lignin	Hemicellulose	Accessibility	Crystallinity
Dilute sulfuric acid	▲	■	■	□
Concentrated phosphoric acid	▲	■	■	■
Soaking in aqueous ammonia	■	□	■	□
Ammonia recycle percolation	■	▲	■	□
Lime	■	▲	■	□
Lime+air	■	▲	■	□
Hot water flow through	▲	■	■	□
pH controlled hot water	▲	■	■	□
Steam explosion (auto hydrolysis)	□	■	■	□
Acid-catalyzed steam explosion	□	■	■	□
Ammonia fiber explosion	■	▲	■	■
Wet oxidation	▲	▲	■	□
Milling	□	□	■	■
Ultrasonic pretreatment	□	□	■	▲
Extrusion pretreatment	□	□	■	■
Ethanol/sulfuric acid	□	■	■	□
Fungi	■	□	■	□

■ Major effect, ■ Minor effect, □ Little effect

the pretreatment process due to the harsh conditions (high temperature and high pressure).

To moderate the operating conditions and improve the pretreatment efficiency, some modified explosion pretreatment processes have been developed, including acid-catalyzed steam explosion and ammonia fiber explosion. It was found that the addition of H_2SO_4 (or SO_2) in steam pretreatment can decrease the time and temperature, as well as the formation of inhibitors [46, 47]. In the AFEX process, the lignocellulosic biomass is treated with liquid ammonia at moderate temperatures and high pressure, followed by the rapid release of ammonia. This process has been shown to decrease cellulose crystallinity, expand the fiber structure, and increase the accessible surface area to enzymes. It also depolymerizes or alters lignin structure via ammonia reactions with lignin macromolecules. The combined changes in physical and chemical structures of lignocellulosic biomass allow for improved enzymatic hydrolysis into fermentable sugars [48–50].

Many other common pretreatments have also been shown to be effective for improving the susceptibility of lignocellulosic biomass to enzymatic hydrolysis, such as hot water [52], wet oxidation [53], ultrasonic pretreatment [54–57], extrusion pretreatment [58–60], ethanol extraction [61], and fungi pretreatment [62] (Table 1). For example, hot water pretreatment of biomass is carried out in liquid water at elevated temperatures, which does not require rapid decompression, nor any catalyst or chemicals. Most of the hemicellulose was removed and recovered as soluble sugars, which make the cellulose more accessible and thus enhance enzymatic digestibility of pretreated material [52]. This process avoids the formation of inhibitors when the pH is kept between 4 and 7 during the pretreatment. It also reduces the need for neutralization and conditioning chemicals since acid or base is not added. In general, all these methods have some advantages for biomass pretreatment, but they also present some technical and economic challenges in a given industrial process.

Recent New Pretreatment Technologies

Although many methods have been developed to pretreat biomass over the years, including physical, chemical, physicochemical, and biological pretreatments, further improvements are still required to make the pretreatment process more efficient and economically competitive. Table 2 lists some new pretreatment methods, along with the main principles involved in each process. A combination of chemical,

Table 2 List of recent new pretreatment technologies and the proposed main principles

Pretreatment technology	Proposed main principles	Ref.
Formic acid/aqueous ammonia	Remove most of lignin and hemicellulose; swell the cellulose fibers	[20]
Phosphoric acid/acetone	Remove most of lignin and hemicellulose; destroy the cellulose crystallinity	[41]
Ethanol cooking/ball milling	Activate the lignocellulosic biomass; destroy the cellulose crystallinity	[63]
Microwave/ionic liquid	Enhance the solubility of cellulose; decrease the degree of polymerization of cellulose	[64]
H_2O_2–*Pleurotus ostreatus*	Significantly improve the lignin degradation	[65]
NaOH/urea (low temperature)	disrupt the connections between hemicelluloses, cellulose, and lignin; break down the fiber bundles to small and loose particles	[66]
CO_2-H_2O pretreatment	Remove hemicellulose, swell the plant material	[67]
NMO pretreatment[a]	Dissolve, balloon, or swell the cellulose	[68]
Ionic liquid	Selectively extract lignin from lignocellulose	[69]

[a] *NMO N*-methyl-morpholine-*N*-oxide

physical, and/or biological pretreatment methods appears to be a more efficient approach to enhance enzymatic digestion of lignocellulosic biomass. Several combined pretreatment technologies have been reported recently, including formic acid/aqueous ammonia [20], phosphoric acid/acetone [41], sulfuric acid–free ethanol cooking/ball milling [63], microwave irradiation/ionic liquid [64], and H_2O_2/*Pleurotus ostreatus* pretreatments [65]. Some new strategies (low temperature, high solids) and pretreatment agents (NMO, ionic liquid) have also been used to pretreat lignocellulose for enzymatic hydrolysis [66–69]. These new methods offer many advantages over previously described methods, such as lignocellulose fractionation, high solids, and low enzyme loading in subsequent enzymatic hydrolysis.

In our lab, we applied combined acid and alkali pretreatments (formic acid–aqueous ammonia, dilute sulfuric acid-sodium hydroxide) to enhance cellulose digestion, as well as to fractionate lignocellulose into individual components [20]. This combined pretreatment removed most of the non-cellulosic components, making cellulose more accessible to enzymes that convert it into fermentable sugars. Moreover, the combination of acid and alkali pretreatments resulted in high cellulose content in the substrate, which allowed us to obtain high sugar and ethanol concentrations. Formic acid treatment alone can also fractionate lignocellulose under modest conditions [70, 71]. However, formic acid may cause aggregation to occur in cellulose, decreasing available surface area, resulting in relatively low cellulose digestibility [35]. In formic acid–aqueous ammonia combined pretreatment, a further aqueous ammonia treatment at moderate temperatures enlarges the accessible surface area due to the ammonia-induced swelling action, leading to a high degree of cellulose digestion [35].

Combined cellulose solvent and organic solvent pretreatment has also been developed to separate lignocellulose components using a cellulose solvent (e.g., concentrated phosphoric acid), an organic solvent (e.g., acetone), and water [41, 72]. In this process, the crystalline structure of cellulose was disrupted in concentrated phosphoric acid at moderate temperatures. Subsequently, acetone and water washing are used to remove lignin and hemicellulose sugars, respectively. The combined pretreatment produces highly reactive amorphous cellulose, which has large cellulose accessibility to enzymes and thus can be hydrolyzed quickly with high glucan digestibility yield [72]. Recently, a modified cellulose solvent/organic solvent pretreatment was reported by replacing the organic solvent (acetone) with ethanol for reductions in processing costs [73, 74]. Despite only a limited amount of lignin being removed during pretreatment, high cellulose hydrolysis yields can also obtained even at an ultra-low cellulase loading (1 FPU/g-glucan). This is a very promising feature because low use of costly cellulase would significantly improve the overall economics of cellulosic ethanol production.

Physical-assisted organic solvent or chemical pretreatments, such as sulfuric acid–free ethanol cooking/ball milling [63, 75], hot-compressed water (HCW)/ball milling [76], microwave irradiation/ionic liquid [64], also present some advantages over the corresponding individual pretreatment process. A combined sulfuric acid–free ethanol cooking and ball milling process allow the cellulosic component to be hydrolyzed efficiently. Ethanol cooking produced homogeneous fibrous woods applicable to the milling process. Meanwhile, it avoids the problems associated with the use of strong acid catalysts, including the degradation of monosaccharides and corrosion of reaction vessels [63]. Similarly, partial solubilization of hemicellulose occurred in HCW pretreatment, which reduced the milling time by disrupting the lignocellulose structure, thus avoiding the excess energy requirements for ball milling alone. The combined pretreatment can also reduce enzyme

loading in subsequent hydrolysis, compared to that in the HCW pretreatment alone [76]. Recently, Ha et al. [64] reported a microwave irradiation-assisted ionic liquids pretreatment for accelerated enzymatic hydrolysis. Results suggested that microwave irradiation not only enhanced the solubility of cellulose in ionic liquids but also significantly decreased its degree of polymerization, resulting in significant improvement of cellulose hydrolysis.

Biological pretreatment of lignocellulosic biomass, as an environmental friendly method for lignin removal, has attracted extensive interest in recent years. However, this process has some undesirable disadvantages, including relative low efficiency, considerable loss of carbohydrates, and long residence time. To overcome these limitations, Yu et al. [65] developed two novel combined pretreatments for enzymatic hydrolysis of rice hull by using a mild ultrasonic or H_2O_2 treatment followed by incubation with *P. ostreatus*. The combined pretreatment significantly increased lignin degradation compared to the individual pretreatments. It also shortened the residence time and reduced the loss of carbohydrates. The enhanced delignification was attributed to the structural disruption by ultrasonic or H_2O_2 pretreatment, making it more easy for the hyphae to penetrate into the interior of the biomass.

Besides combined pretreatments, some new strategies such as low temperature and high solids treatments have been introduced to pretreat lignocellulosic biomass. Based on cellulose dissolution in NaOH at low temperature, lignocellulosic material has been pretreated with NaOH/urea solution at cold temperature (e.g., −15°C) to enhance enzymatic hydrolysis [66, 77, 78]. This process disrupts the connections between the principal components (cellulose, hemicelluloses, and lignin) and thus alters the structure of treated biomass to make cellulose more accessible to enzymes. Due to its mild operation conditions, cold NaOH/urea pretreatment has the potential to become an important pretreatment method in lignocellulosic bioethanol production. To reduce the operating cost, some pretreatment processes with high solids loadings have also been developed, including high solids CO_2–H_2O pretreatment [67], high solids alkaline pretreatment [79], and dilute sulfuric acid-steam pretreatment in high solids [80]. These processes significantly reduce chemical inputs and water (or steam) consumption, while the pretreatment efficiency remained at a satisfactory level. Considering the process advantages linked with high solids content, high solids loadings in pretreatment process may attract more attention in the future.

N-methylmorpholine-*N*-oxide (NMO) is an industrial cellulose solvent, and has been used to pretreat high-crystalline cellulose (cotton) under moderate conditions for bioethanol production [68]. In NMO pretreatment, three modes of action of solvent on cellulose, termed as dissolution, ballooning, and swelling, may be involved depending on the NMO content in water solution. Among the three modes of action, the dissolution mode (85% NMO) was found the most effective pretreatment for bioethanol production. The dissolution in NMO might break hydrogen bonds and weaken van der Waals forces between the cellulose chain molecules, leading to high efficiency of enzymatic hydrolysis. High efficiency, relatively moderate conditions, the potential of complete recycling, and the non-toxic and fully biodegradable nature of NMO make it a good alternative for pretreatment of high-crystalline cellulosic biomass [68, 78].

Recently, the use of ionic liquids as solvent for lignocellulose pretreatment has also received much attention [69, 78, 81]. Ionic liquids have the potential to efficiently dissolve high-crystalline cellulose as well as lignocellulosic materials [82, 83]. The regenerated cellulose produced by rapid precipitation of solution with an anti-solvent (e.g., water) has demonstrated a great improvement in enzymatic hydrolysis. In addition to being a cellulose solvent, ionic liquids also possess high solubility of lignin (e.g., [Emim][CH_3COO]), and have been employed to improve enzymatic hydrolysis of cellulose [69]. Although this solvent did not dissolve the lignocellulosic materials due to its low cellulose solubility, it can facilitate lignin extraction to achieve high cellulose digestibility. When 40% of the lignin was removed, the crystallinity index of cellulose decreased below 45%, resulting in >90% of the cellulose being hydrolyzed. Meanwhile, the removed lignin nearly remained unchanged, thereby providing an unadulterated source of raw material for use [69]. Current efforts to develop ionic liquid pretreatments have not yet been economically viable, and further research is needed to improve performance before industrial-scale application.

Enzymatic Hydrolysis and Fermentation

Multi-Enzyme Complex

During enzymatic hydrolysis of pretreated lignocellulosic biomass, cellulose is gradually degraded into glucose under the actions of a series of enzymes with different functions. Most commercial cellulases produced by *Trichoderma reesei*, one of the most well-known cellulase-producing fungi, are comprised of endoglucanase (EG, EC 3.2.1.4), exoglucanase or cellobiohydrolase (CBH, EC 3.2.1.91), and β-glucosidase (BG, EC 3.2.1.21). All these enzymes work synergistically to hydrolyze cellulose by creating new accessible sites for each other. However, *T. reesei* secretes low levels of BG activity, leading to the incomplete conversion of cellobiose to glucose and inhibition of CBH. Therefore, supplemental β-glucosidase, such as Novozyme 188, is often required to

achieve complete conversion of cellobiose to glucose (Table 3) [11, 13].

Hemicellulose and possibly pectin are thought to restrict access of cellulases to the cellulose surface in pretreated biomass, especially in the alkali-treated (e.g., SAA, AFEX) materials. It has been shown that addition of xylanases and pectinases can degrade these non-cellulosic saccharides and thus increase cellulose conversion [11–13, 84]. Furthermore, xylobiose and higher degree of polymerization (DP) xylooligomers were recently found to inhibit enzymatic hydrolysis of glucan, xylan, and pretreated lignocellulose [13]. A comparison among different carbohydrate-based enzyme inhibitors showed that xylooligomers were more inhibitory to cellulase than either xylan or xylose, or for that matter either glucose or cellobiose [85]. The results provide further evidence that supplementation with xylanase and β-xylosidase are necessary to improve cellulose conversion efficiency. To obtain a more efficient hydrolysis of hemicellulose, some accessory enzymes, like α-L-arabinofuranosidase [86, 87] and feruloyl esterases [88], have also been used to remove side groups (e.g., arabinofuranosyl and feruloyl substitutions) that limit the access of xylanases to the xylan backbone.

The second strategy for improving enzyme cocktails is to construct enzyme mixtures de novo, in which the individual components and their relative proportions can be controlled. For this purpose, Banerjee et al. [89–91] recently developed a high-throughput platform termed Great Lakes Bioenergy Research Center Enzyme Platform (GENPLAT) to analyze and optimize synthetic enzyme mixtures. This platform includes the production of individual purified enzymes, statistical experimental design, and automated liquid handling for cellulose digestion and sugars assays. The GENPLAT system provides an efficient method to optimize multi-enzyme mixtures, identify superior forms of core enzymes, and discover new accessory enzymes [89]. It can also be employed to rapidly optimize enzyme mixtures tailored to different pretreatments and feedstocks [91]. For example, statistical models of AFEX pretreated corn stover indicated an optimum enzyme cocktail for glucose release of 35% cellobiohydrolase 1 (CBH1), 4% cellobiohydrolase 2 (CBH2), 26% endo-β 1, 4-glucanase 1 (EG1), 12% β-glucosidase (BG), 19% endo-β 1,4-xylanase 3 (EX3), and 4% β-xylosidase (BX) at a protein loading of 15 mg/g-glucan. For NaOH-pretreated corn stover, the optimal proportions of CBH1 and EG1 increased to 49% and 34%, while BG and EX3 decreased to 5% and 4%, respectively [91].

Besides the free multi-enzyme system, another cellulose-degrading system is the cellulosome that is produced by anaerobic bacteria or fungi [92–94]. In general, cellulosomes are composed of scaffoldin and catalytic subunits. The scaffoldin subunit contains multiple cohesin modules, thereby enabling different enzyme subunits to be integrated into the cellulosome complex. It also serves to target the cellulosomal enzymes as well as the entire cell to the cellulosic substrate [93]. This modular architecture led to the proposal of constructing "designer cellulosomes" in vitro, which comprise recombinant chimeric scaffoldin and selected dockerin-containing enzyme hybrids. The composition and distribution of each enzyme in the cellulosome can be controlled by simply mixing them in solution together with the chimeric scaffoldin [92, 95, 96]. If the construction displayed high efficiency for cellulose degradation, they have the potential to be applied for cellulosic ethanol production. However, it is still a challenge for

Table 3 Summary of some multi-enzyme complex and the role of supplemental enzymes

Multi-enzyme complex [a]	The role of supplemental enzymes	Ref.
Cellulase+*β-glucosidase*	Eliminate the inhibition of cellobiose to cellulase	[11–13]
Cellulase+β-glucosidase+*xylanases*	Hydrolyze the xylan and make the cellulose more accessible to cellulase	[11, 12, 84]
Cellulase+β-glucosidase+*pectinases*	Remove the pectin that coat cellulose fibers	[11, 12]
Cellulase+*xylanase*+*β-xylosidase*	Hydrolyze the xylan and eliminate the inhibition of xylobiose and higher xylooligomers	[13]
Cellulase+endoxylanase+*α-L-arabinofuranosidase*	Remove the arabinofuranosyl group that limits the access of xylanases to xylan backbone	[86, 87]
CBH1+ CBH2+EG1+ BG+EX3+BX	Construct high efficient enzyme system by optimizing the 'core' purified enzymes	[89, 91]
Core set+Cel61A+α-Glr+Abf2+Cel5A+EX2+Cel12A	Construct more efficient multi-enzymes system by optimizing the 'core' and 'accessory' enzymes	[90]

CBH1 cellobiohydrolase 1; *CBH2* cellobiohydrolase 2; *EG1* endo-β 1,4-glucanase 1; *BG* β-glucosidase; *EX3* endo-β 1,4-xylanase 3; *BX* β-xylosidase; *Core set* CBH1+ CBH2+EG1+ BG+EX3+BX; *Cel61A* endo-β 1,4-glucanase 4; *α-Glr* α-glucuronidase; *Abf2* arabinosidase 2; *Cel5A* endo-β 1,4-glucanase 2; *EX2* endo-b-1,4-xylanase 2; *Cel12A* endo-β 1,4-glucanase 3

[a] The supplemental enzymes are rendered in italics

currently reported cellulosomes to hydrolyze lignocellulosic biomass.

Non-Catalytic Additives

Although better pretreatments and a more effective enzymes system are needed to reduce the enzyme requirements for lignocellulosic biomass saccharification, the addition of various non-catalytic compounds such as surfactants, polymers, and protein have also been shown to significantly improve enzyme performance and thus increase sugar yields and/or lower enzymes loading (Fig. 2) [14, 17, 97]. Such additives were thought to impede deactivation and/or nonproductive binding, to increase cellulose accessibility, and/or to enhance enzyme activity. In previous studies, nonionic surfactants like Tween 20 or Tween 80 were often used to prevent irreversible cellulase adsorption on lignin by occupying the binding sites, resulting in an enhanced cellulose hydrolysis [17, 98, 99]. Meanwhile, supplementing surfactants during hydrolysis could facilitate enzyme recycling and thus reduce enzyme cost [17]. Polyethylene glycol (PEG) was also shown to be an effective additive for the enhancement of enzymatic hydrolysis [97, 100]. In lignin-containing substrates, Börjesson et al. [100] suggested the binding of PEG to lignin via hydrophobic interaction and hydrogen bonding reduced unproductive binding of enzymes and thereby increased cellulose conversion. Subsequent research indicated that increases in cellulase activity and stability were the dominating reason for the enhanced cellulose (Avicel PH101) conversion [97]. More than 90% of enzyme activity remained in the solution after 48 h hydrolysis. Another common additive is bovine serum albumin (BSA) [101]. BSA has a great affinity for lignin-containing substrates, and prevents adsorption of cellulase and particularly β-glucosidase on lignin, thus making more of the enzyme available for hydrolysis. Similar to PEG, BSA can also improve the stability of cellulases [102].

Recently, a class of non-catalytic proteins, including expansins [103, 104], expansin-like protein [15, 105], and swollenins [106, 107], has been shown to modify cellulose and enhance its hydrolysis by cellulase. Plant expansins and expansin-like protein are known to bind to complex polysaccharides and loosen the cell-wall structure. Although the exact mechanism of this process is not fully understood, it has been proposed that they disrupt the hydrogen bonding between cellulose microfibrils and between cellulose and other cell-wall polysaccharides. The loose or disrupted structure will lead to an enhanced enzymatic hydrolysis of cellulose by enabling it to be more accessible to cellulase. For instance, Kim et al. [15] found that the BsEXLX1 protein from *Bacillus subtilis* had functional homology to plant expansins and exhibited significant synergistic effect with cellulase. When they added 0.012 FPU cellulase and 300 mg of BsEXLX1 per gram cellulose (filter paper), the sugar yield was 5.7-fold greater than that obtained when cellulase alone was used. On the other hand, some hydrolytic enzymes like pectinases could synergistically enhance expansin-induced wall extension in vitro, suggesting that the pectin network may restrict the access of expansin to substrate [103]. Swollenin (a fungal protein) has the sequence similarity to expansins. Unlike expansin, it has a cellulose-binding module (CBM) domain connected by a linker region to expansin-like domain. Swollenin also exhibits disruptive activity on cellulosic materials without hydrolytic activity.

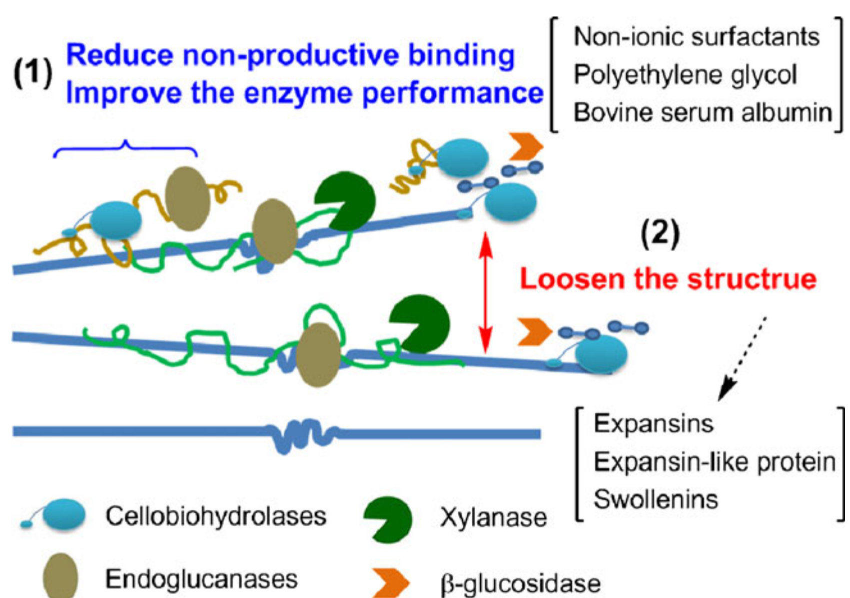

Fig. 2 Overview of the roles of non-catalytic additives on the enhancement of cellulose hydrolysis (*blue line* cellulose microfibrils; *green line* hemicellulose; *yellow line* lignin)

Chen et al. [106] reported a swollenin protein (designated *Afswo1*) produced from *Aspergillus fumigatus*. This protein can disrupt crystalline cellulose (Avicel) into smaller particles and thereby facilitate its enzymatic hydrolysis. Current studies have demonstrated the possible action of these non-catalytic proteins on pure cellulose hydrolysis; however, little information is available on their exact action and synergism with multi-enzymes in lignocellulose hydrolysis. Further studies are need for successful application of these proteins in lignocellulosic ethanol production.

Enzyme Recycling and Reuse

Lower enzyme cost has long been pursued for the industrialization of cellulosic bioethanol. Besides improving enzyme performance, as reviewed above, through constructing multi-enzyme systems or supplementing with non-catalytic additives, recycling of enzymes has also been explored as an effective way of reducing the high cost of enzymes [18, 108, 109]. After enzymatic hydrolysis of lignocellulosic substrates, some enzymes remain free in the hydrolysate, while others are bound to the residual substrates. Several strategies have been developed to recover and reuse the enzymes, including ultrafiltration, readsorption onto fresh substrates, and enzyme immobilization (Fig. 3). Previous studies showed that binding of cellulase to the substrate, especially the nonreversible adsorption to lignin, could lead to loss of enzymes during the recycling process. Qi et al. [109] reported that alkali-treated wheat straw (3.6% lignin) showed better recycling efficiency than acid-treated samples (20.5% lignin), indicating the detrimental effect of lignin on the enzyme recycling. Therefore, reducing lignin content or adding some nonionic surfactants is beneficial to enhance recovery of enzymes [18, 109].

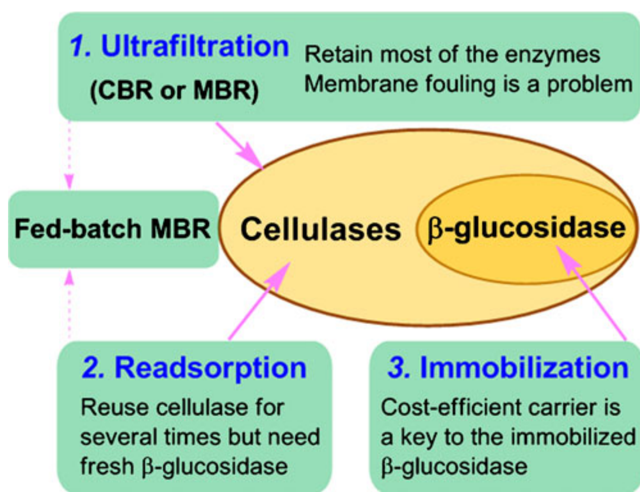

Fig. 3 The strategies of enzymes recycling and their main characteristics

Ultrafiltration has been proven to be capable of recovering cellulases and β-glucosidase. For example, in a CBR, approximately 66.6% of the cellulases and 88% of cellobiase were recycled using this approach after the hydrolysis of ammonia fiber explosion-treated corn stover [18]. Furthermore, ultrafiltration has the capacity to continuously remove sugars and other small compounds that may potentially inhibit enzyme activity. For this reason, a membrane bioreactor (MBR) may provide some advantages in performing enzymatic hydrolysis [26]. The MBR combines product inhibitor removal and cellulase reuse in one device, which facilitates operation and reduces equipment costs by eliminating an extra reactor. Another advantage of MBR is that the enzymatic hydrolysis rate increases because product inhibition is lowered compared with that in a conventional batch reactor. In the fed-batch MBR, fresh substrate was supplemented five times at the rate of 10 g cellulose per liter each time, resulting in a total cellulose loading of 7%. After 60 h, the enzyme utilization efficiency was 3.13-fold of MBR [26]. Thereby, an additional process, like reverse osmosis, is required on the downstream processes to concentrate the sugar products.

Cellulases have relatively high stability and high affinity for cellulose. After hydrolysis, most cellulases are free in the hydrolysate. Therefore, free cellulases can be recovered for further use by readsorption onto fresh substrates and subsequent microfiltration. One of the most important factors that limit enzyme recovering in the readsorption process is the adsorption of cellulases to lignin. Tu et al. [108] found that 90% of the added cellulases remained free in the liquid phase after hydrolysis of Avicel, while only 51% remained unbound in the case of the ethanol-pretreated mixed softwood (EPMS, 6.0% lignin). As a result, approximately 76% and 51% of the cellulases could be recovered, respectively. To reduce the nonproductive adsorption, nonionic surfactants, such as Tween 80, were added to increase free enzymes in the supernatant [110]. In this case, the enzymes (e.g., Spezyme CP) can be used in four successive rounds of enzyme recycling, achieving an above 80% hydrolysis yield after the fourth round. Compared to ultrafiltration, enzyme recycling by readsorption is unable to recover β-glucosidase because it does not typically bind to the cellulosic substrate. Therefore, the supplementation of fresh β-glucosidase is necessary to subsequent rounds of hydrolysis.

Immobilization of enzymes is an important approach for enzyme recycling. Among the multi-enzyme complex for cellulosic hydrolysis, β-glucosidase is frequently used to supplement cellulase preparations for hydrolysis of cellobiose to glucose. In recent years, some inert materials, such as chitosan–alginate composite [111], chitosan–clay composite [112], Eupergit C [113], mesoporous silicates [114], silica gel and Kaolin [115], had been identified as

efficient carriers for immobilization of commercial β-glucosidase via physical adsorption or covalent binding. Compared to free cellobiase, immobilized cellobiase had improved thermal stability and mechanical strength. It could be reused several times in a continuous reaction packed column. Moreover, immobilized cellobiase is readily separated from the substrate and products, which can simplify subsequent purification processes. In addition, several studies also applied immobilized cellulases to improve hydrolysis efficiency of microcrystalline cellulose [116, 117]. Combined with cellulose pretreatments, such as ball milling and ionic liquids, immobilized cellulases also exhibited a relatively high efficiency in cellulose degradation. However, few studies have used lignocellulosic biomass as substrates and none have been done at high solids loadings. The use of immobilized cellulases for lignocellulose hydrolysis is still a technically difficult task because immobilization may hinder enzyme access to insoluble cellulose. Multi-enzymes complex that are required for hydrolysis of lignocellulose also increase the difficulty in immobilization process.

High Dry Matter Operation

For lignocellulosic ethanol production, one obvious target for process optimization is to achieve efficient conversion of polysaccharide substrates to fermentable sugars. Another objective is to increase the final ethanol concentration, to reduce costs of downstream processing. Ethanol recovery by distillation is economically viable on an industrial scale when the fermentation broth contains more than 4% (*w*/*w*) ethanol [20]. High concentrations of fermentable sugars are required to obtain high ethanol concentrations. A promising approach to increase sugar and ethanol concentrations involves operating enzymatic hydrolysis and subsequent fermentation at high substrate concentrations. To reach an ethanol concentration higher than 4% (*w*/*w*), for most types of lignocellulosic materials, will require dry mass concentrations above 20%. [118]. However, using high substrate concentrations in hydrolysis or SSF systems poses two main problems: (1) high viscosity prevents efficient mixing and mass transfer, resulting in more power consumption, poor solid and enzyme distribution, and localized product build-up; (2) high concentrations of end-products (such as glucose, xylose, or ethanol) and other compounds (such as lignin, furfural, or organic acids) inhibit cellulolytic enzyme and fermentative yeast. To decrease these negative effects and maximize the ethanol concentration, some effective strategies have been suggested for enzymatic hydrolysis or SSF at high substrate concentrations.

One way to maximize dry matter levels is to remove non-cellulosic material by some type of pretreatment [20]. Removing hemicellulose and lignin results in higher cellulose contents in the substrate, which facilitates subsequent SSF operating at high cellulose content to obtain high ethanol concentration. For example, an SAA pretreatment resulted in a substrate of relatively low cellulose content that produced only 12.3 g/L ethanol at 7.5% dry matter loading. Conversely, a combined acid and alkali (formic acid–aqueous ammonia) pretreatment increased ethanol production to 29.4 g/L at a dry matter loading of 7.5 wt. %. Because the lignocellulosic biomass (corncob) was treated by alkali after acid treatment, no furfural and HMF were detected by HPLC. Moreover, the density of the substrate increased after the acid/alkali pretreatment, and there was no increase in the viscosity of the slurry. Thus, the substrate mixture was easier to stir, even when operated at high dry mass concentration. When SSF was carried out at a high substrate concentration of 19%, a high ethanol concentration of 69.2 g/L was obtained, which was almost 2.2-fold higher than that for the substrate mixture with 7.5% solid content [20]. The high ethanol concentration exceeds the technical and economic limits of industrial-scale alcohol distillation.

Use of the fed-batch mode could potentially solve the agitation problem caused by high viscosity at high solids loadings [21, 119, 120]. In fed-batch mode, substrate materials are continuously degraded to soluble sugars at relatively low initial concentrations, reducing the viscosity of the slurry mixture. Therefore, substrate feeding at intervals can be utilized to increase the cumulative insoluble solids level during saccharification and fermentation. Another advantage of the fed-batch mode is that it mitigates the inhibitory effects of sugars and other compounds on the enzymes and fermentative microorganisms [119]. Fed-batch mode can result in lower concentrations of these inhibitory compounds, as all the substrate is not added at the same time. This allows the microorganism to convert some of the inhibitory compounds into compounds with lower inhibition over time (i.e., glucose can be fermented into ethanol) [20]. For fed-batch SSCF, the low glucose level also favored a higher conversion of pentose and hexose sugars to ethanol [121]. Furthermore, as cellulose is hydrolyzed, enzymes should be released back into the liquid. Therefore, fed-batch mode can utilize feeding fresh substrate to adsorb and reuse the free enzyme. In view of these advantages, many studies have employed the fed-batch mode for hydrolysis and SSF with high substrate concentrations. We conducted fed-batch SSF experiments started with an initial 19% dry mass, achieving a final substrate concentration of 25% by feeding another 6% solids during the first 24 h [20]. This fed-batch SSF produced 84.7 g/L ethanol, compared to 62.7 g/L in batch SSF. Lu et al. [122] used a fed-batch separate enzymatic hydrolysis and fermentation approach to achieve high ethanol yields

(49.5 g/L) at a 30% cumulative insoluble solids loading. This study also showed that washing the pretreated material results in enhanced conversion of cellulose due to removal of inhibitory compounds.

Another strategy for achieving high solids concentrations is to utilize alternative bioreactor designs for lignocellulose processing (Table 4). At high solids loadings (≥15%), mixing via conventional shaking and stirring is ineffective. Several novel mixing modes, including gravitational tumbling in roller bottle reactors (RBRs) [22, 123], horizontal rotating shaft with paddlers [23, 123], and stirring with helical impellers [24], have been designed and applied to saccharification or SSF under high solids loadings.

Roche et al. [22] compared small-scale enzymatic saccharification vessels with three different mixing mechanisms: shaking, gravitational tumbling, and hand stirring. Results indicated that gravitational tumbling in the RBRs provided sufficient mixing throughout the entire reaction vessel, thus mitigating mass transfer limitations. For mixing by horizontally rotating shaft with paddles, up to 40% (*w*/*w*) solid concentration of lignocellulose can be achieved. When the solids loading was 35% (*w*/*w*), the ethanol concentration reached 48 g/kg (approximately 62 g/L) after 96 h SSF operation [23]. This unique mixing mode was scaled up to a pilot bioreactor of 11 m^3. Recently, Zhang et al. [24] reported a bioreactor with a novel helical impeller for SSF at high solids loadings of 15~30% (*w*/*w*) of steam explosion pretreated corn stover. They found that the helical stirring system had better performances in terms of ethanol concentration and energy consumption, compared to a Rushton impeller stirring. At the highest solids loading of 30%, the ethanol concentration reached 40.0 and 64.6 g/L after 72 h SSF process, at enzyme dosages of 7 and 30 FPU/g dry mass, respectively. Mixing energy consumption was 58.6% of the total thermal energy of the ethanol produced.

Novel Bioreactors

To overcome the mixing problem at high solids loadings, as mentioned above, some novel reactors were designed and applied to bioconversion of lignocellulosic biomass to ethanol (Table 4) [23, 24, 124]. Among these reactors, horizontal bioreactor has proven to be a more effective system than conventional tank reactor at high solids loadings [23, 123, 124]. In horizontal bioreactor, the rotating paddles (or impellers) provide sufficient mixing at very low rotation rates, thus requiring much less power consumption. Supplemental scraping blades can be used to avoid the dead zone formation, keep the reactor surface clear, and thereby improve the heat-transfer characteristics [124]. During enzymatic saccharification of pretreated corn stover, viscosity measurements reveal that the viscosity decreases rapidly during the first 8 h and becomes approximately constant after 96 h. At 25% solids loading, the specific power consumption at 2 rpm is only 0.56 kW/m^3, which is about half of the lower limit of the typical energy requirement (1–5 kW/m^3) at industrial scale [124].

Li et al. [125] found that continuous ultrasonic irradiation can improve the enzymatic saccharification of the waste papers. To obtain homogeneous dispersion at higher solids loading, an ultrasonic airlift reactor that contained an external loop airlift bubble column was designed [29, 30]. Compared to the stirred tank reactor, the airlift bubble column was shown to be more effective in the enhancing cellulose hydrolysis. Another type of reactor is the membrane bioreactor, which combines the reaction with a simultaneous separation [25, 126]. Membrane technology has two main advantages for cellulose saccharification:

Table 4 Some novel bioreactors and their advantages for conversion of lignocellulosic biomass into ethanol

Bioreactors	Operating at high solids or not	Main advantages	Ref.
Horizontal rotating bioreactor	Yes	Provide sufficient mixing at low rotation rates, thus reduce the power consumption	[23, 123, 124]
Scraped surface bioreactor	Yes	Provide effective mixing and prevent particle settling, while requiring low rotation rates and low power inputs; keep the reactor surface clear that improve heat-transfer.	[124]
Helical stirring bioreactor	Yes	Obtain higher ethanol concentration and lower energy consumption than those of the Rushton impeller stirring	[24]
Ultrasonic airlift reactor	No	Obtain higher ultimate sugar concentrations and the apparent kinetic constant than those of the stirred tank reactor	[29, 30]
Ultrafiltration membrane bioreactor	No	Have the capacity of recycling most of the enzymes and removing inhibitory products, thus reduce the enzymes cost and improve cellulose hydrolysis	[25–28]
Fluidized bed reactor	No	Operate continuously for anaerobic ethanol fermentation as well as continuous ethanol removal	[31]

removing the product to prevent feedback inhibition and allowing for recycle of enzymes [26]. The performance of membrane reactors depends on the rate of product formation and product removal, the feeding rate of substrate, enzyme and buffer, and membrane fouling [25]. Among these factors, membrane fouling is a serious problem when lignocellulosic substrates are used at high concentrations, since small solid particles can easily enter the pore of the membrane. Therefore, efficient membranes washing, developing new strategy or membrane materials are required to prevent membrane fouling.

Strain Improvement

Mixed sugars (hexose and pentose) fermentation is one of the prerequisites to achieve high ethanol yields from lignocellulosic materials. However, some commonly used organism in the ethanol industry cannot naturally ferment pentose sugars. Over the last few decades, many researches have been devoted to the development of efficient pentose (especially xylose) fermenting microorganisms, such as recombinant *Saccharomyces cerevisiae*, *Zymomonas mobilis*, *Escherichia coli* strains [32, 127–130] (Fig. 4). In naturally xylose-utilizing strains, D-xylose is isomerized to D-xylulose by xylose reductase–xylitol dehydrogenase (XR-XDH) or xylose isomerase (XI). Xylulose is then phosphorylated to xylulose 5-phosphate by xylulokinase (XK) and further metabolized through the pentose phosphate pathway [32]. Therefore, one of the most important strategies for xylose fermentation is to introduce the XR-XDH (encoded by *XYL1* and *XYL2* genes) pathway or the XI (encoded by *xylA* genes) pathway from bacteria or fungi, respectively [32, 131–133]. Since the low XK activity in the wild-type *S. cerevisiae*, the expression of additional *XKS1* genes (coding for XK) were also required in recombinant strains [131, 132]. In addition, the transport of xylose in *S. cerevisiae* is one of the rate-controlling steps for the fermentation of xylose to ethanol, at least at low xylose concentrations, because it occurred through non-specific hexose transporters. Recently, some heterologous xylose transporters have been expressed in recombinant *S. cerevisiae*, such as Gxf1 [134], Sut1 [135], Trxlt1 [136], At5g5920 [137], to improve xylose uptake. Runquist et al. [138] further compared three heterologous xylose transporters (Gxf1, Sut1, At5g5920) under identical conditions. Results showed the Gxf1 transporter had the highest transport capacity and the highest xylose utilization rate. A direct relationship was also found between transport kinetics and xylose utilization at xylose concentrations of 0~15 g/L. Besides the strategies mentioned above, the other modifications, such as arabinose utilization [139, 140], galactose utilization [141], and engineering redox metabolism [142], had also been made to improve the performance of pentose fermentation.

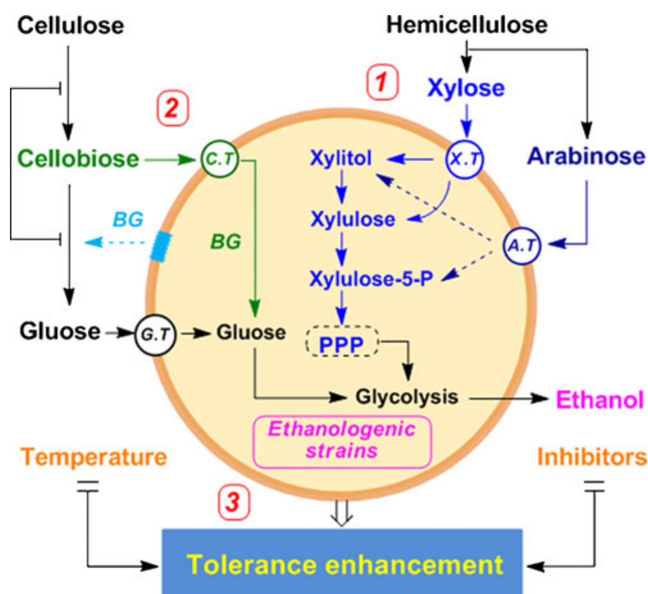

Fig. 4 Three main strategies for strain improvement in cellulosic ethanol production. (*1*) Pentose utilization; (*2*) direct cellobiose fermentation; (*3*) tolerance enhancement

Ethanologenic microorganisms that possess direct cellobiose fermentation capability are very useful for the conversion of cellulosic biomass into ethanol (Fig. 4). For this purpose, one approach is to introduce both cellobiose transporter and intracellular β-glucosidase into microorganisms. Recently, Galazka et al. [33] reported a novel recombinant *S. cerevisiae* engineered with a high-affinity cellodextrin transport system and an intracellular β-glucosidase. Results showed that this recombinant yeast could rapidly grow on cellodextrins and convert them to ethanol. In addition, intracellular hydrolysis of cellobiose also minimizes glucose inhibition on xylose uptake during xylose fermentation, allowing co-consumption of cellobiose and xylose [143, 144]. The other approach is to produce extracellular β-glucosidase from ethanologenic microorganisms [145]. In this respect, heterologous β-glucosidases genes with different origin have been successfully cloned and expressed in *S. cerevisiae* strains, including those from *Saccharomycopsis fibuligera* [146, 147], *Issatchenkia orientalis* [148]. Furthermore, to reduce the cellulosic ethanol production cost, a promising strategy termed as "consolidated bioprocessing" has been proposed, which involves production of all the cellulolytic enzymes (EG, CBH, BG) and fermentation of resulting sugars to ethanol [149–151]. Considerable success has been achieved enabling growth of recombinant *S. cerevisiae* on pure cellulose [152, 153]. However, it is still a challenge for these ethanologenic microorganisms to directly utilize lignocellulosic biomass at industrial scale.

Another strategy for improving fermentation performance of microorganisms is to enhance their tolerance to environmental stress, including thermo-tolerance and inhibitors (e.g., ethanol, acids, furans, phenolics) tolerance [34, 154–156]. Various effective strategies, such as random mutagenesis [157], genome shuffling [158–161], artificial transcription factor engineering [162], global transcription machinery engineering [163–165], error-prone whole genome amplification (ep-WGA) [166], have also been developed for this purpose. Among these, genome shuffling is one of the efficient tools to construct combinatorial libraries of complex progeny from a few previously selected parental strains. This technique has been successfully used to improve the acid tolerance in *Lactobacillus* [160, 161], degradation of pentachlorophenol in *Sphingobium chlorophenolicum* [167], and thermo-tolerance and ethanol tolerance in *S. cerevisiae* [158]. For instance, Shi et al. [158] obtained an improved *Saccharomyces* yeast strain F34 which can effectively ferment glucose up to 48°C within 48 h, while maintaining high-cell viability up to 55°C and tolerating 25% (*v*/*v*) ethanol stress. In general, each of these methods has its own advantage as well as applicable limitation for multi-tolerance improvement in cellulosic ethanol industry. It is thus desirable that combining these strategies or developing new tools to engineer the complex phenotypes of microorganisms in future studies.

Mechanism Research in the Production of Cellulosic Ethanol

Key Factors That Limit Cellulose Hydrolysis

A large number of research efforts have focused on identifying limiting factors in enzymatic hydrolysis of lignocellulosic biomass [36, 37]. The main factors can be divided into three general categories: (1) substrate characteristics, like lignin barrier and accessible surface area; (2) adsorption and synergism of enzymes; and (3) inhibitory compounds, such as sugars and organic acids (Fig. 4). The US Department of Energy had created a joint research agenda for breaking the biological barriers to cellulosic ethanol, in which one of the key research goals is to identify and quantify the relative importance of potentially limiting factors in bioconversion by using biological, mathematical, imaging, and other analytical tools [168]. These detailed studies will help us better understand mechanisms of action and apply various pretreatments and enzyme systems to enhance lignocellulose bioconversion to ethanol (Fig. 5).

Substrate Characteristics

It is now accepted that substrate characteristics can limit the rate and the degree of enzymatic hydrolysis of lignocellulose. The effects of several physicochemical properties (e.g., lignin content, hemicellulose content, crystallinity, degree of polymerization, accessible surface area, and particle size), can be modified by pretreatments, and their effects on lignocellulose hydrolysis have been investigated [35, 169–172]. However, these factors are often interrelated due to the structural complexity of biomass. For example, it is not clear which characteristic is the major determinant controlling cellulose degradation, since factors such as degree of polymerization and cellulose crystallinity have yielded contradictory results in different hydrolysis systems. Focusing on these issues, recent studies have developed some new methods and tools to systematically assess the effects of substrate properties.

In our lab, we employed partial least squares (PLS) regression to identify key factors limiting the rate and extent of cellulose digestion. In this study, multilevel

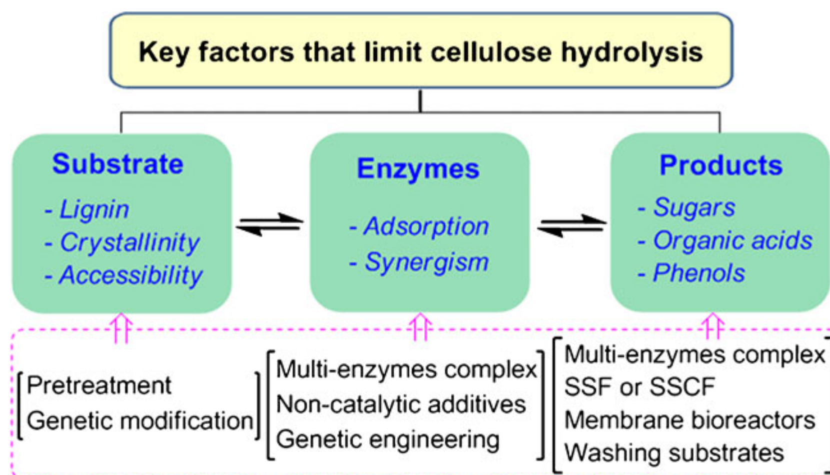

Fig. 5 The key factors that limit cellulose hydrolysis and the corresponding strategies or methods to minimize their influences

structures of lignocelluloses were quantified after different chemical pretreatments [35]. According to the PLS results, the most important factor for cellulose digestion was accessible interior surface area, followed by delignification and the destruction of the hydrogen bonds. Other properties, including the cellulose crystallinity, and hemicellulose content, had less effect on the extent of cellulose hydrolysis. Besides the PLS analysis as mentioned above, the other statistical methods such as neural network were also applied to analyze the relationship between enzymatic digestibility and structural characteristics of lignocelluloses [173]. Results showed that the feed-forward back-propagation neural networks can correlate well (R^2>0.9 for most cases) with biomass structural features with the sugar slopes and intercepts. The interaction of glucan and xylan hydrolysis can also be investigated by increasing the dimensionality of the neural network input matrix. For poplar wood samples, the model suggested that glucan hydrolysis affected the last stage of xylan digestion, while xylan hydrolysis had no influence on glucan saccharification [173].

In earlier work, lignin was found to decrease the hydrolysis yields of cellulose due to its physical barrier and nonproductive adsorption of cellulases. Zhu et al. [38] further demonstrated that delignification could significantly increase the ultimate extent of cellulose hydrolysis, but not the initial hydrolysis rate. On the other hand, high-lignin and low-crystallinity poplar wood allowed enzyme to efficiently cleave cellulose during short hydrolysis periods. Rollin et al. [174] also proposed that increasing cellulose accessibility is more important than removing lignin. The phosphoric acid/ethanol treated substrate retained a large lignin fraction, but still had greatly increased cellulose accessibility that may be due to its low-crystallinity, resulting in rapid hydrolysis rates. Recently, the definite correlations between enzymatic digestibility of cellulose and the lignin content have been identified by our lab [35]. Data suggested that the cellulose digestion at 2 and 24 h were improved with decreased lignin content. However, the pretreated lignocellulosic samples with small amount of lignin (<10%) can also exhibit high enzymatic digestibility at 24 h. Therefore, delignification combined with decrystallization (or increasing cellulose accessibility) shows great benefits for shorter hydrolysis times. For longer hydrolysis periods, delignification alone is sufficient, but the complete removal of lignin might not be required to improve cellulose digestion.

To efficiently hydrolyze cellulosic biomass, cellulases must firstly be able to access the glycosidic bonds in cellulose chains, which are tightly packed in the form of insoluble microfibrils. Therefore, substrate accessibility (or accessible surface area) has long been recognized as an important factor in the enzymatic hydrolysis of lignocellulose. Various methods have also been used to measure cellulose accessibility by using molecular probes (such as polyethylene glycol) [35], non-hydrolytic proteins [175, 176], or cellulases (monocomponent or complex) [177–180]. Huang et al. [35] used polyethylene glycol in column solute exclusion technique to measure the accessible surface area of corn cobs after different chemical pretreatments. Data showed that the accessible surface area increased from 17.6 to 57.4 m^2/g after sodium hydroxide pretreatment, which also led to the highest digestibility. A good positive correlation is seen between digestion yields (2 and 24 h) of cellulose and accessible surface area to the reporter molecules of 5.1-nm diameter. Zhang and co-workers [174, 175] constructed a non-hydrolytic fusion protein termed as TGC, which contains a green fluorescent protein and a CBM, and employed it to quantitatively determine cellulose accessibility to cellulase (CAC). For lignocellulosic biomass, BSA can be used to block the lignin absorption sites before the TGC adsorption measurement. Using this method, they found that the phosphoric acid/ethanol pretreatment caused a 16-fold increase in CAC, while soaking in aqueous ammonia only induced a 1.4-fold increase in CAC [174]. Another non-hydrolytic protein composed of carbohydrate-binding module and cyan fluorescent protein was also proven to be a powerful tool to estimate the initiation sites of hydrolysis and saccharification yields of chemical pretreated lignocellulose [176].

Cellulases Adsorption

To directly determine cellulose accessibility and understand the mechanism of enzyme actions, cellulase adsorption has been extensively studied using both commercial and purified enzyme preparations. Jeoh et al. [177] reported a direct method for measuring the cellulose accessibility by probing cellulase binding and activity using a fluorescence-labeled and purified *T. reesei* cellobiohydrolase (Cel7A). It was shown that the substrate with high concentrations of bound Cel7A exhibited increased degree of cellulose conversion. The pretreatment severity, drying after pretreatment, and cellulose crystallinity were found to directly influence cellulose accessibility. For instance, the bound Cel7A concentrations on the amorphous celluloses were significantly higher than that on its crystalline forms, leading to high hydrolysis rates and sugar yields. Recently, Várnai et al. [178] provided a more rounded analysis of enzyme adsorption on microcrystalline cellulose and pretreated lignocellulosic materials by using the individual cellulases (TrCel7A, TrCel6A and TrCel5A), xylanase (TrXyn11) and *Aspergillus niger* β-glucosidase (AnCel3A). Cellulases adsorbed quickly at early stages of the hydrolysis and remained bound throughout the hydrolysis of two different types of substrates (Avicel- and steam -pretreated spruce). However, the bound cellulases could be desorbed from the

catalytically delignified spruce when the hydrolysis degree reached 80%. Commercial enzyme preparations were often used to investigate adsorption behavior. Kumar et al. [179] compared cellulases adsorption for pure cellulose (Avicel) and lignocellulosic materials pretreated by leading pretreatments (AFEX, ARP, controlled pH, dilute acid, lime, and SO_2 pretreatments). The cellulase adsorption capacity decreased in the following order: lime (133.6 mg/g solids)> SO_2 (124.8 mg/g solids)>ARP (113.8 mg/g solids)>controlled pH (101.7 mg/g solids)>AFEX (99.7 mg/g solids)> dilute acid (90.7 mg/g solids)>Avicel (84 mg/g solids). Data suggested that the 24-h glucan hydrolysis rate had a strong relationship to cellulase adsorption capacities (R^2>0.75).

Inhibitory Compounds and Ions

Some released compounds (or ions) such as sugar products [25, 85], organic acid [181], phenols [182], and ash [183] have been shown to inhibit cellulase activity. Among the sugar inhibitors, besides the cellobiose and glucose, xylooligomers also showed strong inhibition of cellulase activity [85]. Compared with the potential organic acid inhibitory compounds, formic acid had the most severe influence on the hydrolysis rate, resulting in a complete enzyme inactivation [181]. In addition, some cations, like K^+, Mg^{2+}, Ca^{2+}, and Al^{3+}, also exhibited inhibition of cellulases at different levels, except for the stimulation of Ca^{2+} and Mg^{2+} on β-glucosidase [183]. Therefore, removing these inhibitory compounds is necessary to improve the enzyme performance. In this respect, some common methods, including membrane bioreactors, multi-enzyme complex, washing with water (or alkali solution) after pretreatment, and biodegradation of inhibitors have been developed to reduce the inhibitory effect on enzymes and microbe systems.

The Mechanisms of Cellulose Hydrolysis

Much work has been carried out to understand enzyme–substrate interactions, and several models of cellulose hydrolysis have been developed over the last few decades [184]. Cellulose hydrolysis generally involves three main steps: (1) adsorption of cellulases onto the substrate; (2) formation of enzyme–substrate complex, and (3) synergistical hydrolysis of the β-glycosidic bonds. It is widely accepted that three types of cellulases, termed as EG, CBH, and BG, act synergistically to degrade crystalline cellulose. EG randomly cleaves internal glycosidic bonds of the cellulose molecule, resulting in a rapid decrease in the degree of polymerization and generating new chain ends. CBH acts upon these chain ends to release soluble cellobiose or glucose. The third enzyme, β-glucosidases, hydrolyzes cellobiose into glucose, thereby preventing end-product inhibition of cellobiohydrolases [37, 184]. For lignocellulosic biomass, the presence of hemicellulose and lignin further increased the complexity of the hydrolysis system. Incorporating all these factors into a single model is cumbersome and highly complicated; therefore, most models contain simplified representations of the cellulases and/or the substrates. Besides some empirical models, Michaelis–Menten-based and more complex mechanistic models have been used to describe enzymatic hydrolysis of pretreated lignocellulosic biomass. Under certain conditions, these models fit the experimental data well and provide critical insights into the hydrolysis mechanisms; however, some still employ unrealistic assumptions. Brown et al. [185] tested six different mechanistic models against the initial hydrolysis rate of AFEX-treated wheat straw. Among these two- and three-parameter models, the HCH-1 model best fit the experimental data. The results also suggested that all the three-parameter models fit the data better than the two-parameter models. Levine et al. [186] reported a detailed mechanistic model of enzymatic hydrolysis of cellulose, which incorporated independent enzyme adsorption and complexation steps. This model achieved good agreement with the experimental data at a sufficiently high cellulose surface area.

To track the hydrolysis process, soluble sugar release [187] and molecular weight of residues [188] have often been measured by using HPLC or DNS method. Compared to sugars analysis, the characterization of residual substrate can provide a more direct understand of enzyme-cellulose interactions. According to the structural changes, the mechanism of EG and CBH can be extrapolated. Pala et al. [188] determined the cellulose DP before and after the enzymatic treatment. Based on the DP changes, it can be speculated that CBH digestion followed a layer-by-layer manner. In other words, CBH could absorb onto a chain and cleave it until it was completely degraded. Chen et al. [189] employed size exclusion chromatography (SEC) and X-ray diffraction to monitor the change in molecular weight and crystallinity of pure celluloses. SEC results showed that EG decreased molecular weight rapidly while CBH resulted in a slower molecular weight loss. By using SEC combined with online MALLS (SEC-MALLS), more physical parameters of insoluble solid residues can be determined, such as absolute molecular weight, molecular weight distribution, degree of polymerization, and the radii of gyration. Therefore, this technique may be an effective tool to elucidate further details of the reaction mechanism of cellulase.

Future Prospects

Cellulosic ethanol produced from lignocellulose has attracted a lot of development efforts in process intensifi-

cation and mechanism research. However, many technological problems still remain unresolved, and our understanding of how enzymes can hydrolyze lignocellulosic substrates with high efficiency is far from comprehensive. Two technical approaches seem promising to realize efficient cellulose degradation: (1) breaking the recalcitrant structures via pretreatments, and (2) constructing highly efficient hydrolytic enzyme systems. Both approaches are necessary for successful commercialization of cellulosic ethanol. Many current pretreatment technologies have been demonstrated to facilitate cellulose hydrolysis; however, most of them have only been tested at the laboratory scale. The technical and economic feasibility of these processes still need to be assessed, especially for the new pretreatment technologies. To improve enzyme performance, optimizing enzyme composition and adding non-catalytic additives have been used in many studies. Biological and genetic engineering might provide a more powerful tool to construct highly efficient hydrolytic enzymes. Much was learned concerning the limiting substrate and enzyme characteristics, adsorption and catalysis kinetics, and enzymes-substrates interactions. An integrated experimental and computational approach provides us new insights into the action mechanisms of enzymes.

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