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Enzymatic hydrolysis of cellulosic biomass

Biofuels (2011) 2(4), 421–450

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Biological conversion of cellulosic biomass to fuels and chemicals offers the high yields to products vital to economic success and the potential for very low costs. Enzymatic hydrolysis that converts lignocellulosic biomass to fermentable sugars may be the most complex step in this process due to substrate-related and enzyme-related effects and their interactions. Although enzymatic hydrolysis offers the potential for higher yields, higher selectivity, lower energy costs and milder operating conditions than chemical processes, the mechanism of enzymatic hydrolysis and the relationship between the substrate structure and function of various glycosyl hydrolase components is not well understood. Consequently, limited success has been realized in maximizing sugar yields at very low cost. This review highlights literature on the impact of key substrate and enzyme features that influence performance, to better understand fundamental strategies to advance enzymatic hydrolysis of cellulosic biomass for biological conversion to fuels and chemicals. Topics are summarized from a practical point of view including characteristics of cellulose (e.g., crystallinity, degree of polymerization and accessible surface area) and soluble and insoluble biomass components (e.g., oligomeric xylan and lignin) released in pretreatment, and their effects on the effectiveness of enzymatic hydrolysis. We further discuss the diversity, stability and activity of individual enzymes and their synergistic effects in deconstructing complex lignocellulosic biomass. Advanced technologies to discover and characterize novel enzymes and to improve enzyme characteristics by mutagenesis, post-translational modification and over-expression of selected enzymes and modifications in lignocellulosic biomass are also discussed.

Enzymatically based cellulosic ethanol production technology was selected as a key area for biomass technology development in the 1980s, and the US Department of Energy (DOE) has actively supported the scale up of ethanol production since the Office of Alcohol Fuels was created in the DOE after the 'energy crisis' of the 1970s. Although biological conversion of cellulosic biomass to fuels and chemicals through **enzymatic hydrolysis** of **cellulose** offers the potential for higher yields, higher selectivity, lower energy costs and milder operating conditions than chemical processes, such technology was judged to be too high risk for industry to pursue at that time [1]. However, application of the emerging field of biotechnology offered the promise for significant advances that could dramatically reduce costs and make cellulosic ethanol competitive. Improvements in dilute acid **pretreatment** and **cellulase** produced by *Trichoderma reesei* discovered during World War II led to most of the historic cellulosic ethanol cost reductions in the 1980s [2–4]. Well-known *T. reesei* Rut C30 was derived at Rutgers University through classical mutagenesis and strain

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Key terms

Enzymatic hydrolysis: Multi-step heterogeneous reaction in which insoluble cellulose is initially broken down at the solid–liquid interface via the synergistic action of endoglucanases and exoglucanases/cellobiohydrolases. This initial reaction is accompanied by further liquid-phase hydrolysis of soluble intermediates, that is, short celluloligosaccharides and cellobiose, which are catalytically cleaved to produce glucose by the action of β-glucosidase.

Cellulose: Predominant polysaccharide that makes up approximately 40–50% of cellulosic biomass in the form of linear fibrils of approximately 30–40 hydrogen-bonded chains of β-(1,4) glucopyranosides with a native degree of polymerization of approximately 10,000–15,000.

Pretreatment: The disruption of the naturally resistant structure of lignocellulosic biomass to make reactive intermediates (e.g., fermentable sugars) to biological processes.

Cellulase: Combination of enzymes that catalyze the reaction of water with cellulose to release shorter chains and ultimately soluble glucose sugar.

Glycoside hydrolases: Enzymes that hydrolyze a glycosidic bond between two adjacent saccharide groups or between a carbohydrate and a noncarbohydrate moiety.

Lignin: Makes up approximately 15–28% of lignocellulosic biomass; it is distinctly different from the other macromolecular components of lignocellulosic biomass. It is an amorphous, cross-linked and 3D polyphenolic polymer that is synthesized by dehydrogenative polymerization of three types of phenyl propanoid units, including monolignols: coniferyl, sinapyl and p-coumaryl alcohol.

Lignocellulosic biomass: Biomass feedstock mainly containing cellulose, hemicelluloses and lignin; usually including agricultural residues, woody crops, herbaceous energy crops and municipal solid wastes.

Genomics: Study of the genomes of organisms. This includes intensive efforts to determine the entire DNA sequence of organisms and fine-scale genetic mapping efforts. Also includes studies of intragenomic phenomena such as heterosis, epistasis, pleiotropy and other interactions between loci and alleles with the genome.

selection from wild strains, such as *T. reesei* QM9414 [5]. Cellulase 150 L produced by Genencor was very effective because of enhanced levels of β-glucosidase [6,501]. Dramatic improvements in reducing glycosyl hydrolase costs by a factor of 20 to 30 was announced recently [7,8].

It is noteworthy that many microorganisms in nature, mostly bacteria and fungi, are capable of producing biomass-degrading enzymes. Cellulolytic microbes may evolve as individual degraders or as part of a 'chain reaction' in microbial communities of some ecosystems. Cellulolytic enzymes secreted by such microbe(s) are classes of **glycoside hydrolases** (GHs), including **lignin**-modifying catalysts in some cases. Enzyme and microbe combinations vary in different biomass-degrading ecosystems depending on the initial biomass source and environmental factors. With emerging biotechnology tools, there is great potential to develop new enzyme sources that offer more desirable enzyme features, including higher specific activities with more balanced synergism, better thermal stability, better resistance to environmental inhibitors and improved combination of various enzymes (e.g., cellulase, hemicellulase, pectinase and proteinase) activities that maximize sugar yields at low cost.

Unfortunately, cellulosic ethanol technologies have not yet been commercialized, at least partly because releasing sugars from naturally recalcitrant cellulosic materials is difficult [9,10]. The result is that high enzyme doses are needed, with the cellulase loadings of approximately 15 FPU per gram cellulose typically used to achieve economically viable sugar yields from pretreated biomass equivalent to approximately 30 g of enzyme per liter of ethanol made. **Figure 1** illustrates the relationship between the cost of enzyme protein production (US$/kg enzyme) and the amount that must be charged for ethanol ($/gallon ethanol) to cover the cost for different enzyme loadings that all achieve the same ethanol yield (data adopted from National Renewable Energy Laboratory report) [11]. Thus, to meet the enzyme cost goal ($0.10/gallon ethanol or less) of the DOE Biomass Program will require that enzymes cost less than $2/kg cellulase protein or strategies must be developed to substantially reduce the loadings needed for high yields, or some of both [12–14]. In addition, mechanisms of action and factors limiting hydrolysis effectiveness are not well known, and consequently limiting in many promising commercial applications [15]. Improving the understanding of the structure and function of both lignocellulosic materials and their degrading enzymes will be invaluable to determining the roles of biomass pretreatment, hydrolysis and enzymes in influencing **lignocellulosic biomass** conversion and in developing appropriate strategies to achieve high rates and yields with low amounts of enzyme.

Enzymatic hydrolysis is influenced by both structural features of cellulose and the mode of enzyme action. Due to the complexity of the cellulose substrate and the cellulase system, the mechanism of hydrolysis of cellulose substrate is still not fully understood, although detailed knowledge of some aspects of enzyme structure, enzyme molecular properties and the ultrastructure of cellulose have been obtained through extensive study over the last few decades. Thus, this paper focuses on a review of the current understanding of key features of the pretreated biomass and glycosyl hydrolases that influence sugar release and suggests opportunities to further advance our understanding of lignocellulosic bioconversion by newly advanced technologies, such as **genomics**, **proteomics** and microscopy.

Substrate-related factors

This section of the review targets updating of recent advances in understanding structural characteristics of biomass and related enzyme features, and providing perspectives towards improvement in substrates for enzymatic hydrolysis. Lignocellulosic biomass has numerous structural features that make it very difficult to deconstruct enzymatically. The majority of biopolymers, including cellulose, **hemicellulose** and lignin, are not just individual units in a plant cell wall but are intimately interconnected [16]. Lignin and carbohydrates (e.g., cellulose and hemicellulose) form lignin–carbohydrate complexes [17]. Recent studies demonstrated that in grasses, polysaccharide–lignin crosslinking is mediated by ferulates attached primarily to arabinoxylans. Ferulated hemicelluloses provide points of growth for lignin via ether bonds that anchor lignin to plant-wall polysaccharides and could

contribute to recalcitrance [18–20]. The complete structure and compositions of lignin, which binds cellulosic fibers together in a composite structure and reduces the accessibility of cellulose to enzymes [21], is still not fully understood. To completely deconstruct these heterogeneous structures in the plant cell wall requires synergistic reactions of enzymes, such as cellulases, hemicellulases, accessory enzymes and lignin-modifying enzymes. Our current knowledge is insufficient to understand the whole picture of enzymatic hydrolysis of cellulosic biomass, and most evidence available to date results from two approaches: purified enzyme(s) acting on purified substrates or mixtures of enzymes acting on thermo-chemically pretreated biomass.

▪ Characteristics of cellulose

The main commercial purpose of enzymatic hydrolysis of cellulosics is to deconstruct cellulose and other carbohydrate polymers into fermentable sugars, including glucose and/or oligomers that can be further converted into valuable products through biological or chemical approaches. Although enzymatic hydrolysis of cellulose is complicated by existence of other components (e.g., hemicellulose and lignin) and their derivatives after pretreatment, it is essential to understand the effects of key features of cellulose itself on the rate and effectiveness of enzymatic hydrolysis.

It is difficult to characterize native cellulose in the plant cell wall, due to its small size and the matrix of polymers (mainly hemicelluloses and lignin) closely interlinked with it. Cellulose can be considered as a composite material built from nanometer-scale microfibrils. Recent studies using advanced imaging techniques, such as atomic force microscopy (AFM), have revealed precise measurements and detailed cellulose surface structure in its native stages. Based on AFM studies of plant cell walls [22–24], the dimensions were measured as 3–5 nm, consistent with the 36-chain model of the cellulose elementary fibril (CEF) based on the proposed cellulose-synthase complexes (the rosettes) that contain 36 cellulose synthases. One of the interesting findings from AFM imaging was macrofibrils only observed on the uppermost layer of the primary cell wall. The macrofibril appeared to consist of a bundle of CEFs that split at the end to form smaller bundles and eventually a single CEF. Each microfibril observed in mature primary cell walls contained only a single CEF and hemicelluloses associated with its surface [25,26]. AFM images of maize cell walls from fresh cells further confirmed this observation [27]. **Figure 2** shows a schematic model of plant cell wall synthesis.

In this model, at least three types of cellulose synthases (CesA subunits, α1, α2 and β) are required to spontaneously assemble the rosettes containing 6 × 6 CesA enzymes [28]. Each rosette synthesizes a 36-chain CEF. The estimated dimensions of CEFs are 3 × 5.5 nm based on cellulose Iβ structure, in agreement with direct AFM measurements. A number of CEFs synthesized by rosettes with close proximity may form a bundle, the macrofibril. The deposition of other wall polymers, mostly hemicellulose, during cell growth, causes the macrofibril to split to form single microfibrils with hemicelluloses associated on their surfaces [25,26].

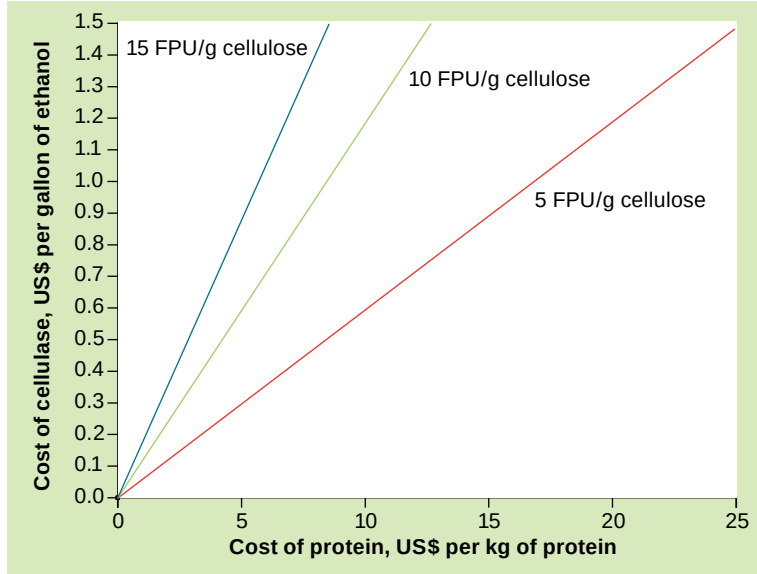

Figure 1. Cost of cellulase for ethanol production versus cost of protein at different loadings that all achieve the same ethanol yield.
Data from [16].

Substrates used in cellulase assays are primarily purified cellulose (e.g., Avicel or Sigmacell) with small proportions of other polysaccharides, mainly hemicelluloses from higher plants. Regardless of the origin and purification methods used in their preparation, the structural characteristics of purified cellulose vary in crystallinity, degree of polymerization (DP) and surface structure, which may significantly affect enzyme hydrolysis.

Crystallinity

Purified celluloses are micrometer-sized particles composed of nanometer-sized microfibrils **(Figure 2)**. Generally, these cellulose particles are believed to consist of crystalline, paracrystalline (disordered) and amorphous structures. Historically, amorphous cellulose has been reported to be rapidly degraded to

Key terms

Proteomics: Large-scale study of proteins, particularly their structures, activities, modifications, localization and interactions of proteins in complexes. Substantial amounts of proteins/enzymes are involved in the lignocellulosic biomass degradation.

Hemicellulose: Make up approximately 20–30% of biomass, exhibit a much broader distribution of sugars and are frequently branched and essentially amorphous polysaccharides. These polysaccharides are usually associated with cellulose, often hydrogen bonded to cellulose. These branched polysaccharides are composed of 1, 4-linked β-D-hexosyl residues.

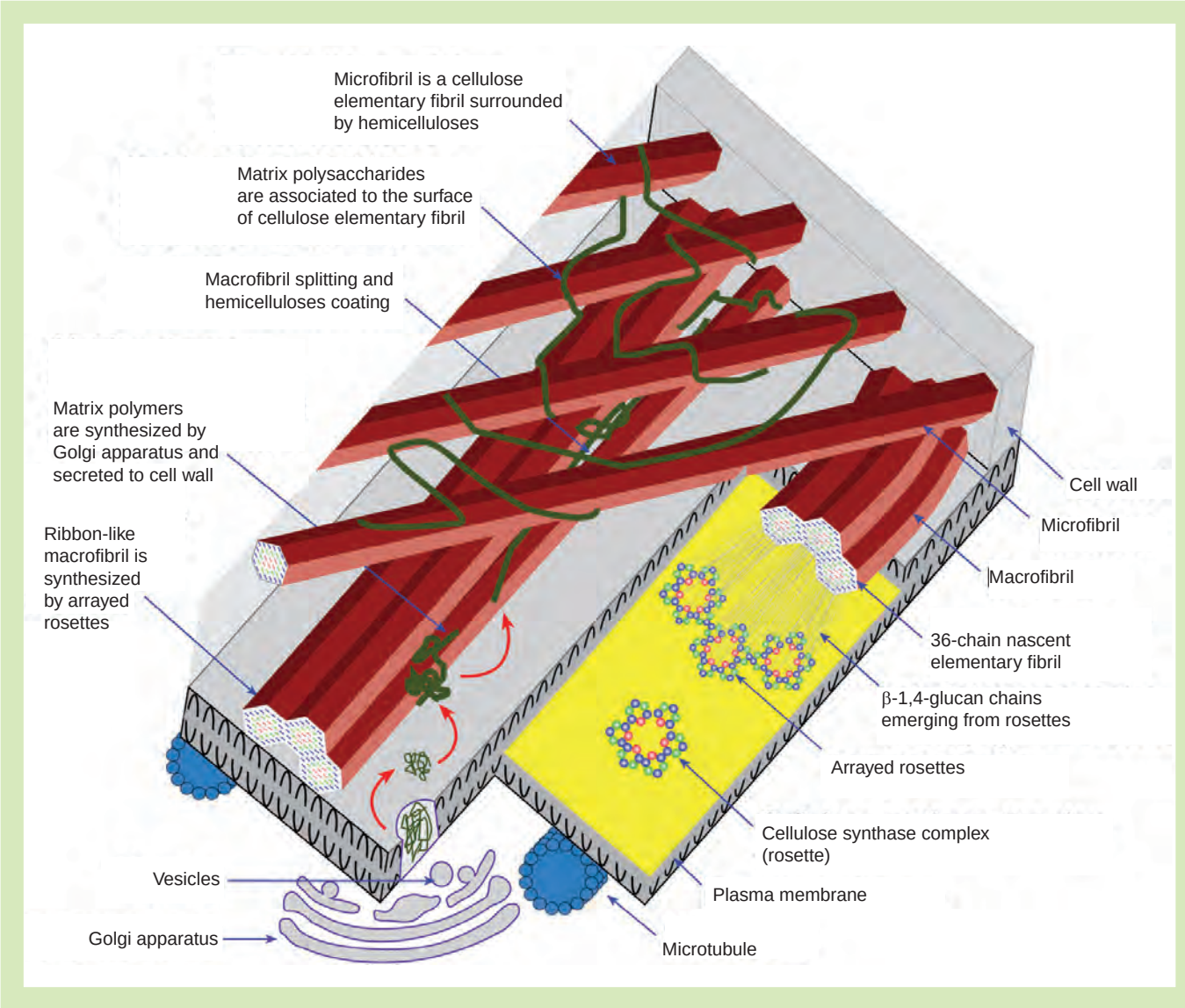

Figure 2. Model of plant cell wall cellulose elementary fibril and its synthesis. The dimensions of cellulose elementary fibril are estimated as 3 × 5.5 nm.
Adapted with permission from [29].

cellobiose by cellulases, while the hydrolysis of crystalline cellulose is much slower. Thus, some authors proposed that hydrolysis rates depended on cellulose crystallinity [29–32]. Although rates have been found to slow with increasing crystallinity of cellulose in some studies [33–35], others found the opposite effect [36–38]. It is expected that crystallinity should increase with cellulose hydrolysis as a result of more paracrystalline and amorphous cellulose removal [38–40]. However, no significant change in crystallinity during cellulose hydrolysis was reported in some studies [41,42]. In some reports, cellulose crystallinity was not considered to affect efficient hydrolysis [37,43–49].

Cellulose crystallinity was also reported to play a role in enzyme adsorption, which can be correlated with hydrolysis rates and yields. Increased hydrolysis rates and yields (>100 times) were shown to be related to the higher capacity of amorphous cellulose than crystalline cellulose for cellulases [35,39,50–55]. Many results showed that enzyme adsorption, including the complete glycosyl hydrolase system, cellulose binding module (CBM) and individual enzyme components, generally declined as cellulose crystallinity increased. Recently, Joeh and co-workers showed that crystallinity greatly impacted the adsorption of Cel7A (CBHI), leading to decreased extent of hydrolysis [55]. Hall and co-workers indicated

that the initial enzymatic hydrolysis rate increased with decreasing crystallinity index, while the adsorbed enzyme concentration stayed constant [42]. In addition, different cellulase components have been shown to have different adsorption capacities and activities for cellulose [50,51]. Endoglucanase I (EGI), known to attack and adsorb preferentially on amorphous cellulose, appeared to have an average adsorption capacity and activity greater than CBHI on both types of cellulose studied. A similar pattern was described for EGI by Ding and Xu [56]. Furthermore, Banka and Mishra observed that crystallinity increased adsorption of a nonhydrolytic protein named fibril-forming protein from *T. reesei* [57]. Such results indicate that cellulose crystallinity has important effects on nonhydrolytic enzyme components, which can be essential to effective enzymatic hydrolysis of cellulose.

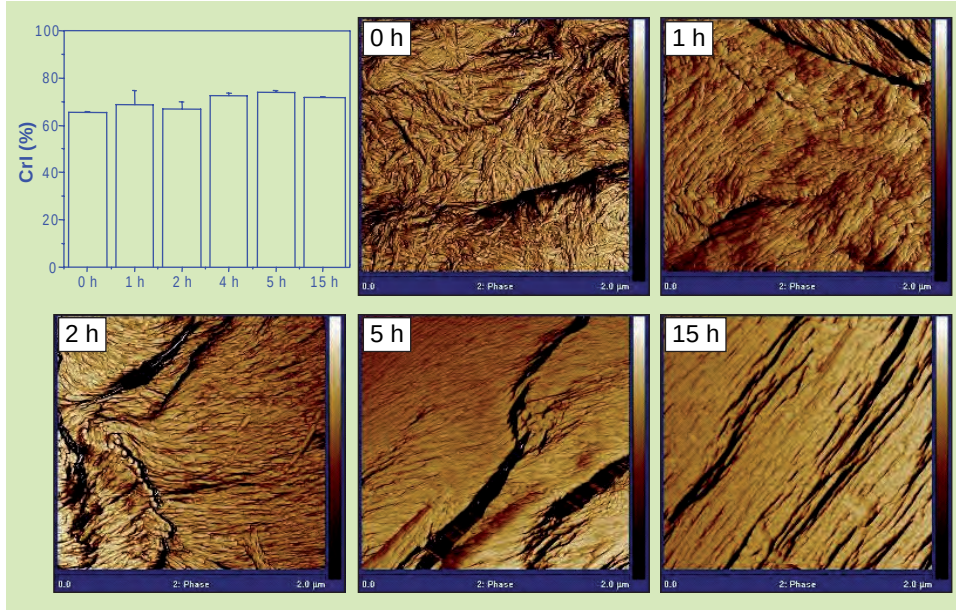

Figure 3. Features of solids resulting from interrupted hydrolysis of Avicel cellulose. Crystallinity index by x-ray diffractometer and atomic force microscopy for interrupted hydrolysis samples at 0, 1, 2, (4), 5 and 15 h.

Cellulose crystallinity may not only affect cellulase adsorption but may also impact the effectiveness of adsorbed cellulase components. The literature has shown that cellulose crystallinity affects the synergism among cellulase components [42,51,58–66]. Hoshino *et al.* found increased synergism between CBHI and endoglucanase II (EGII) from *T. reesei* with increased crystallinity and the highest synergism between CBHII and EGII at a crystallinity index approximately 1.0. In another study, Igarashi and co-workers showed that nature of the crystalline cellulose polymorph affected the hydrolytic activity of adsorbed CBHI [67–69]. Moreover, Mizutani *et al.* [70] and Gama and Mota [71] showed that the impact of surfactant in enhancement of saccharification is influenced by the crystallinity of pure cellulose.

A few studies investigated the relationship between cellulase processivity and crystallinity. The processivity of CBHI, a dominant enzyme of the *Trichoderma* system, was shown to be affected by cellulose crystallinity. A rough estimate of processivity, determined by the ratio of cellobiose to glucose, was reported to be 23 and 14 cellobiose units for bacterial microcrystalline cellulose (BMCC, CrI ~>85%) and amorphous cellulose, respectively [72]. In another study, processivity values for CBHI from *T. reesei* were reported to be 88 ± 10, 42 ± 10 and 34 ± 2.0 cellobiose units for bacterial cellulose CrI ~88), BMCC (CrI ~92) and endoglucanase-pretreated bacterial cellulose (unknown CrI), respectively [73]. Further studies are needed to confirm and elucidate the influence of crystallinity on the effectiveness of processive or pseudo-processive enzymes from various microorganisms.

Degree of polymerization

Several studies and literature reviews discuss the change in DP of insoluble and soluble cellulose after hydrolysis with a complete set of cellulases or its purified components [43,44,51,52,74–80]. However, the understanding of the impact of cellulose chain length on hydrolysis is still limited. Sinistyn *et al.* showed that reduction in DP of cotton linters by γ-irradiation, while keeping crystallinity index constant, had negligible impact on the hydrolysis rate [35]. A recent kinetic study by Zhang and Lynd [81] found that a decrease in cellulose DP had less effect in accelerating hydrolysis than an increase in accessibility of β-glycosidic bonds as generally measured by the maximum amount of cellulase adsorbed on cellulose. For soluble cellulose, Nidetzky *et al.* showed that the initial velocity of cello-oligosaccharides degraded by CBHI increased with DP up to cellohexose and then remained constant [82]. Similar effects of DP of soluble cellodextrins on CBHII and EGI activity were also reported [51]. Furthermore, β-glucosidase activity was reported to decrease as DP was reduced [83,84]. However, no information is available on the effect of insoluble cellulose DP on the catalytic efficiency of cellulases except that higher DP could result in higher synergy between CBHI and EGI [65,81,85,86]. Furthermore, cellulose DP may affect the processivity index, and full

processivity of CBHI may not be realized with short cellulose chains [61]. Limited information is available in the literature on the effect of cellulose DP on cellulase adsorption. Kaplan *et al.* showed a significant drop in cellulase adsorption resulting in reduced hydrolysis, with a change in DP along with some ring openings of cotton cellulose due to withering; however, crystallinity was not affected much [87]. Given the typically large amount of CBHI in cellulase (>65%) and its preferences [82,88–91], one could quickly conclude that DP reduction should improve hydrolysis effectiveness by making more ends available to CBHI and be a promising target to enhance hydrolysis rates and yields, provided the enzyme formulation is adjusted to take advantage of the lower DP.

Accessible surface for cellulase

Cellulose accessibility to cellulases is limited by the structure of cellulose microfibrils that are believed to be nanometer-sized **(Figure 2)**. Crosslinking among chains of cellulose fibers, coupled with their being imbedded in a matrix of polysaccharides involving lignin and other polymers, provides extra rigidity in native plant cell walls but complexity for enzymatic digestion [92]. Although extensive modification may occur during cellulose purification, the diameter of cellulose microfibrils may remain approximately 3–5 nm in plant cell walls, the same as in the original source, but the length of these microfibrils may be significantly reduced to several hundred nanometer **(Figure 2)**. The accessibility of cellulose to cellulases may refer to two levels of limitations, with one being the face of crystalline cellulose available to cellulases binding, with the carbohydrate-binding module of CBH I attaching to only the hydrophilic face [93–95]. The second limitation is the anatomical structure of the plant cell wall, which may also affect accessibility for cellulases, specifically the pores existing in the plant cell walls that allow cellulases to enter into the 'boxes' of plant tissue to access the surface of cellulose microfibrils. One of the impacts of pretreatment could be to enlarge pore sizes to enhance cellulase penetration into biomass.

Based on the premise that enzymatic hydrolysis of cellulose is a surface reaction, available surface area of cellulose for cellulase attack should be one of the most influential structural features of biomass that influences cellulase adsorption on the cellulose surface and subsequent enzymatic breakdown [16,96–101]. Many papers have discussed available pore volume and specific surface area in this context [51,44]. Accessibility can be also correlated to other substrate-related factors, such as cellulose crystallinity or depolymerization. However, some studies offered evidence of other substrate features, including pore volume [44,101–105] and particle size [37,103,106–108] affecting cellulose hydrolysis. Nevertheless, because some bacterial cellulases, such as cellulosome, a multienzyme complex with a size approximately 100 times that of individual fungal cellulases, hydrolyze cellulose at a higher rate than fungal cellulases, micropores appear to be less important [50,109]. Furthermore, it was observed that cellulase components did not penetrate into the pores [110], and no relation was observed between pore volume and digestibility [111]. A limited to negligible effect of particle size on cellulose adsorption [112–114] and cellulose hydrolysis [47,115] was reported, but the possibility of an increase in the rate of cellulose fragmentation with smaller particles cannot be ruled out. In contrast, it was shown that larger particles could be inhibitory to effective hydrolysis [116]. On the other hand, various studies on the effect of DP and crystallinity on enzymatic digestibility demonstrated that susceptibility of pretreated substrates to enzymatic hydrolysis could not be easily predicted from differences in cellulose DP or crystallinity [37,117], possibly due to the complexity of real cellulosic substrates. However, accessible surface area can provide a useful perspective on these features and help identify characteristics that can be changed by pretreatment.

Change in cellulose reactivity & enzyme functionality with conversion

The dramatic decline in overall enzymatic hydrolysis rates and rates per amount of adsorbed enzyme as hydrolysis progresses is responsible for low yields, and long processing times cannot be attributed to just product inhibitory effects. However, the mechanism still remains unclear [118,119]. In addition to enzyme-related factors, such as thermal instability of cellulases [120–123], products inhibition [120,124–128], enzyme inactivation [125,129–135], enzyme slowing down/stopping [136], substrate-related factors, including substrate transformation into a less digestible form [137], and the heterogeneous structure of the substrate [137,138], have been proposed to account for such phenomena. At one time, the drop in rate was explained by declining substrate reactivity as the more easily reacted material was thought to be consumed preferentially [137], but other reports concluded that substrate reactivity was not the principal cause of the long residence time required for good cellulose conversion [136].

'Interrupt' and 'restart' experiments were conducted to identify factors that control cellulose hydrolysis [136–141]. A new restart approach, involving proteinase treatment to remove cellulases followed by proteinase inhibitors to deactivate the proteinase before restarting cellulose hydrolysis at the original conditions, was developed to understand reactivity loss during the dynamic process of enzymatic hydrolysis of cellulose [142]. The resulting hydrolysis rate and the rate

per adsorbed enzyme of Avicel were nearly constant with changing conversion for these restart experiments, but declined in continual hydrolysis. Thus, the drop in hydrolysis rate for continual cellulose digestion could not be attributed to changes in substrate reactivity, while other enzyme-related effects such as enzymes slowing down by getting 'stuck' or 'jamming' could be responsible [142,143]. For this restart approach, the cellulose CrI increased slightly with cellulose conversion to approximately 80% within 5 h [144]. AFM images of the interrupted hydrolyzed Avicel showed that the somewhat rough surface of the original Avicel became smoother and flatter as enzymatic hydrolysis progressed **(Figure 3)**. The new 'restart' approach allows one to revisit many aspects of enzymatic hydrolysis of cellulose needed to advance the understanding of the dynamic interactions between enzyme and cellulose.

▪ Derived insoluble matter distribution

Cellulose, hemicelluloses and lignin are the major polymers in the plant cell walls, and any change in or removal of these components would be expected to consequently affect enzymatic digestibility. However, experimental results have been rather inconsistent. Grohmann *et al.* and others showed direct relationships between hemicellulose removal and glucose yields from cellulose [111,145–150], but other reports do not support a role for hemicellulose removal in changing cellulose digestibility [151–154]. Similarly, conflicting conclusions have been reached on the importance of lignin removal in enhancing cellulose conversion [102,155–157]. All plant cell wall constituents are modified to different extents by pretreatments, depending on the technologies and conditions applied, making it challenging to deduce whether altering cellulose microfibrils, removing hemicelluloses, modifying or relocating lignin, or other effects on the substrate are responsible for improving enzyme effectiveness.

Hemicellulose

The enzymatic digestion of cellulose has been shown to significantly improve with hemicellulose removal, thereby suggesting that hemicellulose provides the key barrier to cellulose breakdown by enzymes [157]. However, simultaneous lignin alteration during pretreatment can confound the role of hemicellulose solubilization and modification [102,155,158,159]. From a more applied perspective, some pretreatments such as ammonia fiber expansion (AFEX) produce highly digestible cellulose without removing any significant amounts of hemicellulose [160–162], although AFEX may modify the chemistry of hemicelluloses. Less attention has been given to the degree of acetylation of the substrate. Hemicellulose chains are extensively acetylated in many types of biomass, and deacetylation was reported to triple cellulose digestibility, with some differences reported in the degree of removal needed to be effective [163,164]. One study showed that this effect appeared to become less important beyond removal of 75% of the acetyl groups, while another study revealed continued improvements up to full removal of hemicellulose [156,165]. Grohmann and co-workers showed that removing acetyl esters from aspen wood and wheat straw made them five to seven times more digestible. Kong *et al.* observed a major effect on cellulose digestibility by the removal of acetyl content of aspen wood while preserving lignin and polysaccharides [165]. Chang and Holtzapple applied similar methods as above but showed that removal of acetyl bonds is less important than crystallinity reduction and/or lignin removal [166]. In addition, a study by Weimer *et al.* suggested that intimate association of xylan and cellulose does not inhibit biodegradability of polysaccharides [167]. Removing hemicellulose also removes acetyl groups and usually alters the form of lignin left, making it difficult to isolate the factors most influential in improving performance. Unfortunately, it is still debatable whether hemicellulose removal or the breakdown of the cross-linked network of polysaccharides and bonds among them is responsible for enhanced digestion of cellulose in pretreated biomass.

Studies such as these lead us to believe that even if cellulose is made completely accessible to enzymes, they would not be able to hydrolyze cellulose unless the network of biomass components including hemicellulose and acetylation is disrupted [168–170]. Jeoh and co-workers observed increased cellulose accessibility, as measured by the adsorption of fluorescent labeled CBHI, and an increase in hydrolysis with the extent of xylan removal [55,171]. Pan *et al.* suggested that acetyl groups in pulp might restrict cellulase accessibility to cellulose by inhibiting productive binding, which might be caused by increased diameter of cellulose and/or changed hydrophobicity [168]. However, there is not much evidence as to whether selective hemicellulose removal or deacetylation impact cellulase adsorption. In addition, because recent results showed xylooligomers are strong inhibitors of cellulase, their release during enzymatic hydrolysis could substantially slow hydrolysis, making their removal in pretreatment important [172,173]. Deacetylation may indirectly affect cellulase effectiveness in enzymatic hydrolysis of lignocellulosics because the removal of acetyl and other substituents from xylan could increase xylan digestibility by xylanase [156,174–178] and result in increased cellulose digestibility [179–182]. Thus, although its role in enhancing cellulose digestion is ambiguous, xylan removal during pretreatment may be desirable for a number of economic and technical

reasons such as higher recovery of xylose, less inhibition by xylooligomers and less need for hemicellulose degrading and accessory enzymes [183–185].

Lignin

Lignin binds cellulosic fibers together in a composite structure with excellent properties, but also reduces the accessibility of cellulose to enzymes [21]. Various studies reported cellulose hydrolysis was improved with increasing lignin removal, although differences were reported in the degree of lignin removal needed [44,102,157,186]. The ratio of syringyl to guaiacyl moieties in lignin was also considered to have important effects on digestibility [187], yet the importance of lignin in limiting hydrolysis has been difficult to determine. One of the most significant limitations is the effect of lignin on fiber swelling and its resulting influence on cellulose accessibility [116,188,189]. Lignin has been claimed to depolymerize and then repolymerize during hemicellulose hydrolysis by pretreatment, although no doubt in a different morphology that could change its impact on cellulose digestion [117,190–192]. The removal of lignin not only increased cellulose accessibility but also allowed more cellulase action [157]. Lignin and its derivatives were reported to precipitate and bond with protein [16], and condensed lignin was reported to adsorb protein from aqueous solutions [193]. Thus, it appears that lignin could physically and chemically resist cellulose attack by enzymes. Lignin not only plays a very important role in irreversible cellulase absorption but also acts as a barrier to cellulase, limiting hydrolysis efficacy [194]. Thus, lignin removal may both open more space for enzymes and reduce enzyme nonspecific absorption on lignin [157]. Low levels of lignin have been shown to enhance cellulose hydrolysis due to a physical separation of microcellulose fibrils enhancing cellulase access/activity [16,55,157]. Lignin modifications in transgenic biomass have resulted in decreased recalcitrance to saccharification with improved fermentable sugar yield [18].

Our recent findings suggest that enzymatic digestibility of cellulose is related to both hemicellulose and lignin removal. For example, similarly high enzymatic digestion was observed for corn stover pretreated at optimized conditions for several CAFI pretreatments, even though hemicellulose and lignin removal varied considerably among the solid residues from each pretreatment technology [21,195]. Relocation and/or modification of lignin on the solid substrates could lead to lower degree of observed final lignin removal [16]. Although some demonstrated a linear relationship between lignin removal and cellulose digestion, others showed little or no effect of lignin removal on cellulose digestibility [196]. For example, AFEX-pretreated solids, in which hemicellulose and lignin removal were very limited, needed less cellulase to achieve similar hydrolysis performance than dilute acid pretreated solids, which had little hemicellulose left and most of the original lignin content [160]. Overall, lignin modification seems more important than hemicellulose dissolution, with the latter perhaps just providing a convenient marker of lignin alterations that improve cellulose digestibility [194].

Such information leads us to believe that lignin modification is vital to enhance cellulose digestibility and that lignin removal provides even greater benefits [157]. Removing lignin enhances cellulose accessibility and reduces nonproductive binding of enzymes, thus improving enzymatic hydrolysis performance [16]. Interestingly, recent lignin-blocking technology brings new insight into disrupting the original cellulose–lignin–hemicellulose structure and fully liberating highly susceptible cellulose and hemicellulose for enzymes to hydrolyze, rather than pursue complete removal of lignin and its derived compounds [16,197].

Although literature regarding the effect of lignin on cellulose hydrolysis is abundant, the role of lignin in enzymatic hydrolysis of heterogeneous cellulosic biomass is still unclear. However, it may be advantageous to remove lignin before hydrolysis to enhance the technical and economic prospects of cellulose saccharification, because lignin will lead to less available enzyme due to unproductive binding, may be inhibitory to fermentation [50] and may cause mixing problems at higher solids loadings [198,199]. It is not clear whether lignin removal or disruption of its tight association with carbohydrates is necessary. Grabber and co-workers suggested that inhibition of fungal hydrolases is not affected by the composition of lignin [200]. However, lignin concentration and its crosslinking with feruloylated xylans greatly affect degradability of cell walls [201,202]. The negative impact of lignin concentration on cell wall digestibility of tobacco stems was observed by Sewalt *et al.* in another study [203].

Ooshima and co-workers observed that dilute acid pretreatment at higher temperatures led to shrinking and agglomeration of lignin that increased cellulase adsorption on cellulose [134]. Similar observations of lignin melting and its relocation are affirmed by others as well [204,205]. In a recent study, Selig *et al.* explained that droplets of lignin, formed during high temperature dilute acid or water only pretreatment, may migrate to the surface and impede cellulase adsorption on cellulose [206]. Yuldashev *et al.* observed that the amount of cellulase on the surface of cotton stalks (cellulose: 44% and lignin: 26.4%) was lower than the milled cotton stalks (cellulose: 92% and lignin: 0.6%), leading to a drop in conversion; however, lignin did not inactivate free or bound enzymes [207]. In another study, Ishihara

and co-workers showed that lignin slows down enzyme adsorption but does not restrict the conversion of carbohydrates in steamed shirakamba wood [208]. Limited delignification of wheat straw by sodium hydroxide was shown to result in increased cellulase adsorption by Estrada *et al.* [209]. Conversely, Mooney *et al.* studied the effect of amount of lignin in four different types of pulp on cellulase adsorption and concluded that the proportion of lignin did not influence cellulase adsorption [210]. However, Selig and co-workers showed that lignin droplets deposited on cellulose may interact with water and form a boundary layer impeding cellulase movement [205]. Furthermore, lignin linkages with cellulose may presumably impede the processive action of cellulase. Although lignin may reduce the amount of active enzyme available for cellulose hydrolysis, its impact on the effectiveness of adsorbed cellulase still requires clarification.

Derived soluble matter distribution effects

Much attention has been paid to removing hemicellulose and lignin from biomass solids as obvious physical barriers to cellulose access by enzymes, but little work has been devoted to understanding how soluble matter (e.g., sugar, sugar oligomers, sugar degradation products and lignin-derived compounds) released during pretreatment and enzymatic hydrolysis affect enzymatic hydrolysis of cellulose. In addition, in most research, pretreated cellulosic biomass solid was separated from the hydrolyzate and thoroughly washed to get a clear-cut evaluation of the effect of pretreatment on cellulose digestibility independent of dissolved inhibitors. On the other hand, enzymatic hydrolysis of pretreated whole slurry, including both pretreated solids and liquor (at least partially if not all of the liquor), will likely be necessary to lower capital and operating costs. Even with washed pretreated solids, the concentration of soluble matter released from the pretreated solids during enzymatic hydrolysis becomes more significant as the solid loadings increase. However, it was reported that cellulose conversion by enzymatic hydrolysis was reduced when pretreated solids were not washed [211], pretreatment hydrolyzate was added back to the pretreated solids [212] or the whole slurry (i.e., pretreated solids and hydrolyzate) was enzymatically hydrolyzed [213–217]. These results suggest that compounds in the pretreatment hydrolyzate have inhibitory effects on enzymatic hydrolysis of cellulose.

Research revealed that some compounds in the pretreatment hydrolyzate, which usually contains soluble lignin, oligomeric sugars primarily from hemicellulose, sugars, and lignin degradation products, had profound inhibitory effects on cellulase and microbial activities [175,218–221]. Kim *et al.* showed that effluent from ammonia-recycled percolation pretreatment of corn stover, containing xylooligomers, soluble lignin, sugar and lignin degradation products, inhibited cellulase and microbial activity significantly [222]. However, little was known about exactly which soluble compounds from hemicellulose, lignin and other biomass components affect enzymatic hydrolysis, or how they acted. However, recent work by Kumar and Wyman revealed that xylo-oligomers strongly inhibit cellulase action [176], and a follow-on study showed that inhibition by xylooligomers was stronger than by glucose or cellobiose, with longer chained xylooligomers having the greatest impacts [175]. This research also showed that soluble xylooligomers had strong inhibitory effects on cellulases and such effects increased with concentration [175]. It was also reported that soluble products from xylan, including xylooligosaccharides and xylose, are significantly more inhibitory to glucan hydrolysis even though xylan and pectin inhibited glucan hydrolysis [223]. Xylooligomers were found to significantly inhibit cellulase adsorption onto Avicel (Shi *et al.*, Unpublished Data). Other sugars from hemicellulose, such as mannose and galactose, were found to be inhibitory to cellulases and β-glucosidase [224]. Another study identified sugars in pretreatment hydrolyzate as the primary inhibitor to enzymatic hydrolysis of dilute acid pretreated whole slurry, while other soluble compounds, including acetic acid, phenolic compounds and furans, only slightly inhibited enzymatic cellulose hydrolysis [225].

Soluble lignin derivatives were reported to affect not only microorganisms but also enzymes such as cellulases and β-glucosidases [226–238]. Mendels and Reese found that substituted phenols had moderate inhibition on cellulases [233]. Panagiotou and Olsson tested multiple compounds (including furans, phenols and low molecular weight acids) and reported formic acid as the strongest inhibitor to cellulases by complete inactivation of enzymes [239]. Because the lignin preparation used in the study partially dissolved, the observed inhibition of cellulases was believed to be due to not only cellulase adsorption on the major particulate lignin component but also on solubilized small-molecular lignin compounds [226]. Soluble phenol compounds, including vanillin, syringaldehyde, *trans*-cinnamic acid and hydroxybenzoic acid, were reported to inhibit cellulose hydrolysis in wet cake by endo- and exo-cellulases, and cellobiose hydrolysis by β-glucosidase [223]. In this study, vanillin showed strongest inhibition on the mixture of Spezyme CP and Novozyme 188, while hydroxybenzoic acid had the greatest inhibition of these individual commercial enzymes. On the other hand, soluble lignin degradation aldehydes (vanillin, syringaldehyde and 4-hydroxybezaldehyde) or corresponding carboxylic acids were reported to have minor inhibitory effects on cellulases [221].

Enzyme-related factors

Enzymatic hydrolysis of cellulose, typically characterized by an insoluble reactant (cellulosic substrate) and a soluble catalyst (enzymes), is not only influenced by structural features of the solid substrate but also by enzyme-related factors, such as enzyme source, product inhibition, thermal inactivation, activity balance for synergism, specific activity, nonspecific binding, enzyme processibility and enzyme compatibility. Due to the complexity of both the cellulose substrate and the cellulase system, the mechanism of cellulose hydrolysis is still not completely understood, although some knowledge of enzyme structure, enzyme molecular properties, fibers and cellulose ultrastructure has been obtained through extensive study over the decades. Since many enzyme-related factors have been extensively reviewed [240–243], we will focus more on the enzyme source, enzyme-specific interaction with cellulosic substrates, synergistic effects of glycosyl hydrolases and strategies to improve enzyme effectiveness.

▪ Features of glycosyl hydrolases from different microbes

In order to significantly improve the efficiency of enzymatic hydrolysis of cellulosic biomass and lower costs, approaches have been taken to find more robust enzymes and advance the understanding of enzyme interactions with cellulosic biomass. Different sets of hydrolytic enzymes, such as cellulases, hemicellulases, accessory enzymes to attack hemicellulose debranching, phenolic acid esterases and ligninases for lignin degradation/modification are required for complete deconstruction of the various components of lignocellulosic biomass [244]. However, it is not well known how the glycosyl hydrolases and their associated enzymes/proteins function together to breakdown lignocellulosic biomass. Diverse microorganisms, including bacteria and fungi, can produce various glycosyl hydrolases for biomass conversion and deconstruction. In nature, lignocellulosic biomass is completely deconstructed by a mixture of glycosyl hydrolases from various microbes in specific communities, such as the hindgut of termite, the rumen of cows, various lignocellulosic biomass composts and the extreme environmental niches. Those anaerobic or aerobic microbial communities may consist of only bacteria, only fungi, or bacteria and fungi together [245]. Selected microbial strains that have been explored for various glycosyl hydrolase applications and their characteristics are listed in Table 1.

These microbes were isolated from different environmental niches and grouped into aerobic or anaerobic bacteria or fungi on the basis of their growth conditions. The glycosyl hydrolases have evolved different properties such as thermal, acid or alkaline tolerance under unusual culture environments. Based on their protein structures, the glycosyl hydrolases are further classified into four groups: multienzyme complex (cellulosome) systems, noncomplex cellulase systems, and hemicellulase and ligninase systems. Since the cellulosome system in the anaerobic thermophilic bacterium *Clostridium thermocellum* was first identified in the early 1980s by Bayer, Lamed and their colleagues [246,247], substantial progress has been realized in understanding the protein complex, characteristics, genes governing formation of protein complexes, diversity and their interaction with plant cell walls. So far, the cellulosome system is found only in anaerobic microbes. Many elegant reviews have discussed these complex cellulase systems [248–253]. Cellulosomes have several unique features: efficient nanomachines to deconstruct plant cell wall polysaccharides, molecular integration of cellulases and hemicellulases into cellulosomal multienzyme complexes resulting from high-affinity interaction between type I dockerin domains of the modular enzymes and type I cohesion modules of a noncatalytic scaffoldin, and a scaffoldin-borne carbohydrate binding module (CBM) to attach to plant cell walls [252].

In contrast, noncomplex glycosyl hydrolases are found in all microbes, even those with cellulosomal systems. In this review, properties of the noncomplex glycosyl hydrolases and their interaction with celluloses will be discussed in more detail. Noncomplex glycosyl hydrolases have been extensively studied in the several filamentous fungi. Among these, *Trichoderma* and *Aspergillus* strains are well developed for industrial glycosyl hydrolase production with enzyme production conditions extensively optimized. Generally, *T. reesei* secreted at least two cellobiohydrolases (CBHI and CBHII), five to six endoglucanases (EGI, EGII, EGIII, EGIV, EGV, and EGVI), β-glucosidase (BGL I and II), two xylanases and various accessory hemicellulases [254]. The effectiveness of cellulase components acting on insoluble substrates, and especially crystalline cellulose, is affected by the proportion of these components, with some ratios being particularly effective due to their synergistic action [60,66]. Although the CBHI:EGI ratio of commercial cellulase preparations from *T. reesei* is typically approximately 4–5:1, recent studies suggested that the optimal enzyme ratio is affected by both pretreatment conditions and feedstock sources [255,256]. Although several studies validate that CBHI and EGI share common sites on cellulose, CBHI has higher binding capacity and affinity than EGI, and CBHII has separate binding sites than CBHI [56,257]. However, the influence of the molar ratio of these components on binding and/or of bound enzyme on synergism has

Table 1. Selected bacterial and fungal strains for glycosyl hydrase production.

Name	Enzymes types		Ref.
Bacteria (aerobic)			
Acidothermus cellulolyticus	NC/HC	T	[375]
Bacillus sp.	NC/HC	M/AT	[376]
Bacillus pumilus	NC/HC	M/AT	[377,378]
Bacillus substilis	NC/HC	M/T	[379]
Bacillus agaradhaerens JAM-KU023	NC/HC	T/A	[380]
Brevibacillus sp. strain JXL	NC/HC	T	[381]
Cellulomonas flavigena	NC/HC	T/AT	[382]
Cellulomonas fimi	NC/HC	M	[273,383]
Geobacillus thermoleovorans	NC/HC	T/AT	[384]
Paenibacillus campinasensis BL11	NC/HC	T	[385]
Paenibacillus strain B39	NC	T	[386]
Streptomyces sp.	NC/HC	M/T	[387]
Thermoactinomyces sp.	NC/HC	T	[388]
Thermomonospora curvata	NC/HC	T	[389]
Thermomonospora fusca	NC/HC	T	[390]
Bacteria (anaerobic)			
Acetivibrio cellulolyticus	Cellulosome/NC	M	[391]
Bacteroides cellulosolvens	Cellulosome	M	[267]
Clostridium acetobutylicum	Cellulosome	M	[392]
Clostrium cellulolyticum	Cellulosome/NC	M	[393]
Clostrium cellulovorans	Cellulosome/NC	M	[394]
Clostrium josui	Cellulosome	M	[395]
Clostrium papyrosolvens	Cellulosome	M	[396]
Clostrium thermocellum	Cellulosome/NC	T	[246]
Ruminococcus albus	Cellulosome	M	[397]
Ruminococcus flavefaciens	Cellulosome	M	[398]
Filamentous fungi (aerobic)			
Acremonium cellulolyticus	NC/HC	M	[399]
Acrophialophora nainiana	HC/HC	M	[400]
Aspergillus acculeatus	NC/HC	M	[401,402]
Aspergillus fumigatus	NC/HC	M/T	[403]
Aspergillus niger	NC/HC	M	[404]
Aspergillus oryzae	NC/HC	M	[405]
Fusarium solani	NC/HC	M	[406]
Humicola grisea var. *thermoidea*	NC/HC	T	[407]
Irpex lacteus	NC/HC/LN	M	[408]
Penicillium funmiculosum	NC/HC	M	[409]
Penicillium atrovenetum	NC/HC	T	[410]
Penicillium citrinum	NC/HC	M	[411]
Phanerochaete chrysosporium	NC/HC/LN	M	[412]
Schizophyllum commune	NC/HC	M	[413]
Sclerotium rolfsii	NC/HC	M	[414,415]
Sporotrichum cellulophilum	NC/HC	T	[501]
Talaromyces emersonii	NC/HC	T	[416]
Thielavia terrestris	NC/HC	T	[502]
Trichoderma koningii	NC/HC	M	[406]
Trichoderma. reesei	NC/HC	M	[406,417]
Trichoderma viride	NC/HC	M	[418]

AT: Alkali tolerant; HC: Hemicellulase; LN: Ligninase; M: Mesophilic; NC: Noncomplexed cellulase; T: Thermophilic.

Table 1. Selected bacterial and fungal strains for glycosyl hydrase production (cont.).

Name	Enzymes types		Ref.
Anaerobic fungi			
Anaeromyces elegans	NC/HC	M	[419]
Anaeromyces mucronatus	NC/HC	M	[420]
Caecomyces CR4	NC/HC	M	[421]
Neocallimastic frontalis	Cellulosome	M	[422]
Neocallimastic hurleyensis	Cellulosome	M	[423]
Neocallimastic patriciarum	Cellulosome	M	[424]
Orpinomyces joyonii	Cellulosome	M	[425]
Orpinomyces PC-2	Cellulosome	M	[426,427]
Piromyces communis	Cellulosome	M	[428]
Piromyces equi	Cellulosome	M	[429]
Piromyces E2	Cellulosome	M	[430]

AT: Alkali tolerant; HC: Hemicellulase; LN: Ligninase; M: Mesophilic; NC: Noncomplexed cellulase; T: Thermophilic.

received little attention. Nidetzky *et al.* showed that competitive rather than synergistic binding is observed for cellulase components [258]. On the other hand, Jeoh and co-workers concluded that the presence of Cel5A (endocellulase) of *Thermomonospora fusca* increased binding of an exocellulase and an endocellulase [259,260].

The rationale for both strongly and weakly binding enzymes is still unclear. Typical cellulases contain carbohydrate-binding modules (CBMs) [261] that are beneficial for enzyme efficiency by adhering to and sometimes possibly disrupting the substrate. CBMs from different enzymes and different taxonomic origins have been classified into families with similar amino acid sequences and 3D structures. CBMs of *T. reesei* CBH1, CBHII and EGI have aromatic residues that are critical for the binding of a CBM onto crystalline cellulose. Structural studies indicate that the spacing of the three aromatic residues coincides with the spacing of every second glucose ring on a glucan chain. Therefore, it has been postulated that the aromatic amino acids of the CBMs form van der Waals' interactions and aromatic ring polarization interactions with the pyranose rings exposed on the surface of cellulose [262]. It was reported that the CBHI-CBM was capable of interacting with approximately ten cellobiose units (20 glucose units), and its catalytic core with approximately 36–54 cellobiose units [263]. Cellulases processivity, which involves CBM and catalytic domains of cellulases, was studied in some recent reports [264,265]. It is vital to understand how features of individual cellulase components and their synergism dynamically change as enzymatic hydrolysis of cellulose progresses.

▪ Synergistic enzyme effects on overall degradation processes

Synergistic phenomena are widely observed in cellulose hydrolysis, with many forms reported and proposed, including endoglucanase with exoglucanase, exoglucanase with exoglucanase, endoglucanase with endoglucanase [266], exoglucanase or endoglucanase with β-glucosidase [267,268], catalytic domain with CBM [269] or two catalytic domains [270], cellulose-enzyme-microbe synergism [271] and spatial synergism for cellulase complexes (i.e., the cellulosome of *C. thermocellum*) [241]. Such synergisms depend on cellulase sources or even substrate features. For example, synergism between the catalytic domain and CBM was reported for CenA of *Cellulomonas fimi* on cotton fibers but was not observed on bacterial microcrystalline cellulose (BMCC) [269]. Endo–endo type synergism was only reported in fungal cellulases of *Gloeophyllum sepiarium* and *Gloeophyllum trabeum* [266]. Cell–cellulase–cellulose synergism has been shown for some cellulolytic microorganisms such as *C. thermocellum* that have tightly cell-associated cellulase systems.

Extensive study of synergisms in noncomplex cellulases showed that they act in a synergistic or cooperative manner. The synergism among different cellulases depends on several factors including the nature of the substrate, enzyme compositions and concentration, cellulase affinity for substrate, component stereospecificity and the enzyme to substrate ratio. The synergistic interaction between cellulolytic components of *T. reesei* was reported high on crystalline cellulose but decreased as substrate concentration increased [272]. Endo–exo and exo–exo synergism was reported to be influenced by the nature of the substrate such as DP [272]. Studies of the size distribution during hydrolysis of BMCC and acid-swollen cellulose also showed that the behavior of endoglucanases and cellobiohydrolases (e.g., purified CenA, CenC and CbhA, and CbhB from *C. fimi*) varied with different substrates [273].

Complex cellulosomes consist of many cellulases and hemicellulases that function synergistically to degrade celluloses. For example, the cellulosomal subunits of *C. thermocellum* include 12 endoglucanases,

two cellobiohydrases, two exoglucanases, six xylanases, one chitinase, one lichenase and one mannanase [265]. Besides cellulosomes, noncellulosomal cellulases have been found in anaerobic microbes. However, attention to these noncellulosome cellulases is limited even though many noncellulosomal cellulases have been identified in cellulosomal microbes. Noncellulosomal cellulases may act synergistically with cellulosomes for cellulose degradation [240].

One of the major challenges for cellulase researchers is to elucidate the synergistic interactions between individual components [274]. In order to determine the degree of synergism between cellulase components, it is imperative that each component be purified to homogeneity, but aggregates and enzyme–enzyme complexes between cellobiohydrolases and endoglucanases are extremely difficult to break into their constituent parts. Such complexity has been shown in cellulase from *T. reesei* [275]. On the other hand, some cellulase components, such as endoglucanases, are quite difficult to purify to homogeneity. More detailed models of cellulose degradation depend on firm knowledge of the kinetics and substrate specificities of individual cellulases. This area has been dramatically improved by introduction of a low molecular mass chromogenic 4-methylumbelliferyl-β-glycosides and by the expression of cloned cellulases genes in heterologous expression systems, such as *Saccharomyces cerevisiae*, which eliminate the problem of cross-contamination of enzyme purified to homogeneity [276–278]. We also need to consider the potential impacts of glycosylation on functions of glycosyl hydrolases in the heterologous expression systems. Furthermore, because synergistic effects between cellulases are influenced by the nature of the substrate, such as chemical composition, degree of crystallinity, DP and solubility, it is often challenging to compare research results in the literature using different substrates. Therefore, establishing a set of cellulose model substrates and widely employing such model substrates in the research community would certainly facilitate comparisons and help in understanding mechanisms and kinetics of cellulose hydrolysis by cellulases. In addition, results from such studies would provide experimental evidence to validate computational simulations of synergetic interactions among enzymes/proteins and lignocellulosic biomass, a new research area to determine molecular dynamics of those complex interactions [279–284].

Besides synergism among cellulase components, the synergetic effects of various glycosyl hydrolases (e.g., core cellulases and enzymes involved in hemicellulose and lignin degradation) on lignocellulosic degradation have been evaluated [192,250,268,285–288]. Natural synergism for lignocellulose degradation is very common, with examples being cellulolytic systems in insect hindguts [289–291], rumen microbial communities [292] and various environmental composts. Those synergetic systems can consist of just bacterial or fungal communities or communities of bacteria with fungi, and may include some contributions by the host. **Metagenomic** and functional analysis of hindgut microbiota of a wood-feeding higher termites showed the presence of a large, diverse sets of bacterial genes for cellulose and xylan hydrolysis [293]. Brulc *et al.* examined the gene-centric metagenomics of the complex fiber-adherent bovine rumen microbiome and compared it with termite hindgut microbiota [294]. The study indicated fundamental differences in the GH content that appeared to be diet driven for either bovine rumen (forages and legumes) or termite hindgut (wood). Both studies suggested that *Clostridium* cellulosomes are rarely present in both synergetic communities. In contrast, *Clostridium* species were major players in microbial communities in cellulolytic enrichment cultures from thermophilic compost [295]. Furthermore, fungal species found in enriched cultures were *Piromyces* species that produced cellulosomes as well. Interestingly, *celY*, encoding the noncellulosomal glycosyl hydrolase family 48 along with its cellulosomal systems, previously observed in *Clostridium stercorarium* was found in *Clostridium straminisolvens* and *Clostridium clariflavum* [296,297], and *C. thermocellum* [298]. *Clostridium stercoraium* produced cellulosomes with a large number of hemicellulases but only two noncellulosomal cellulases, GH9 endoglucanase CelZ and GH48 exoglucanase celY, that synergistically degrade crystalline cellulose in biomass. This suggests that a stand-alone cellulosome may not be sufficient to degrade complex lignocellulosic biomass, and additional glycosyl hydrolases may be required for complete degradation. This hypothesis is supported by results from metagenome studies of cellulolytic enrichment cultures from different composts [295,299,300], in which a series of noncellulosomal bacteria coexisted with cellulosomal *Clostridium* species. Therefore, the study of synergetic microbial communities may lead to potential breakthroughs in lignocellulosic conversion. Besides glycosyl hydrolases, associated proteins/enzymes and reagents may also play important roles in lignocellulosic biomass conversion. Thus far, tool-kits are insufficient to quantify the contribution of individual components to synergisms, further impeding improvements in enzyme characteristics.

Key term

Metagenomics: Study of genetic material recovered directly from environmental samples, where the microorganisms are not easily cultured in laboratory or simply studied in their natural environment. In addition, metagenomics allows researchers to look at the genomes of all of the microbes in an environment at once, providing a 'meta' view of the whole microbial community and the complex interactions within it, such as lignocellulosic biomass degradation and conversion in certain environmental composites.

▪ Advanced technologies for discovery, characterization & over-expression of glycosyl hydrolases

DNA sequence technology for whole genome sequence of biocatalystic microbes

The next-generation of DNA sequencing has the potential to dramatically accelerate lignocellulosic biomass conversion research by enabling inexpensive, routine and widespread comprehensive analyses of genomes, transcriptiomes and interactomes. To date, it has been applied to determine the whole genome sequence related to lignocellulosic biomass production and microbial systems for lignocellulosic biomass conversion. Recent progress in whole genome sequencing of cellulosic biofuel crops has been reported in several publications [301–305]. Such genome sequence information provides the foundation for improvements in plant oil and lignocellulosic biomass production in selected biofuel crops and especially regulation of complex lignocellulosic biomass formation. Genome sequences also provide a foundation to examine the potential of heterologous expression of microbial glycosyl hydrolases in biofuel crops, as discussed in a later section.

As noted above, both eukaryotic and prokaryotic microbes can produce glycosyl hydrolases. The eukaryotic microbes mainly consist of various fungal species, with the most common being filamentous fungi. The genome sequence of *T. reesei*, which is widely used for commercial production of cellulases and hemicellulases, was recently determined [306]. In addition, genome sequences have been determined for several other filamentous fungi, such as *Phanerochaete chrysosporium* [307], *Aspergillus niger* [308], *Aspergillus fumigatus* [309], *Aspergillus nidulans* [310], *Aspergillus oryzae* [311], *Fusarium graminearum* [312], *Magnaporthe grisea* [313], *Neurospora crassa* [314], *Penicillium chrysogenum* [315] and *Ustilago maydis* [316]. Such genome sequence data allow examination of carbohydrate-active enzymes (CAZymes) categorized into different classes and families that include GHs, glycosyltransferases, polysaccharide lyases, carbohydrate esterases and carbohydrate-binding modules [601]. Those enzymes cleave, build and rearrange oligo- and poly-saccharides and play a central role in fungal metabolisms, which are crucial for biomass degradation. Each enzyme class has different families. Gylcosyl hydrolases, such as cellulolytic and hemicellulose-degrading enzymes, are classified with GHs and key enzymes for lignocellulosic biomass degradation. So far, 118 families of GHs have been identified in all biological systems, a number that will grow as more genes are sequenced. More than 60 of them have been found in the filamentous fungi mentioned above. The 36 families of GHs found in all those filamentous fungi are GH1, 2, 3, 5, 6, 7, 10, 11, 13, 15, 16, 17, 18, 27, 31, 32, 36, 37, 38, 43, 47, 51, 53, 54, 55, 61, 63, 67, 72, 75, 79, 81, 92, 105 and 114. The exocellobiohydrolase I and II and EG I, II, III and IV belong to the GH families GH5, 6, 7, 12 and 61, while the hemicellulose-degrading enzymes belong to the GH families GH10, 11, 26, 29, 43, 51, 53, 54, 62, 67, 74 and 95. Sizes of CAZyme families for the 23 fungal genomes with complete sequences available, are summarized by class in **Table 2**.

P. chrysosporium, a white rot fungus that efficiently degrades lignin, has the lowest number of genes encoding GHs, glycosyltransferases and carbohydrate esterases among the filamentous fungi. *T. reesei*, an efficient plant polysaccharide degrader and an important model of biomass degradation systems, has surprisingly few genes encoding GHs compared with other filamentous fungi. Thorough inspection of the *T. reesei* genome revealed only seven genes encoding well-known cellulases (endoglucanases and cellobiohydrolases), 16 hemicellulase genes and five genes for pectin breakdown enzymes [306], the smallest set of genes for glycosyl hydrolases among plant cell wall degrading fungi.

Rapid improvements in DNA sequencing technology also provide powerful tools for metagenomics. Metagenomics, a relatively new field of genetic research, enables studies of organisms that are not easily cultured in a laboratory as well as studies of organisms in their natural environment. Functional metagenomics has been applied to examine cellulolytic systems in insects [289–291], rumen microbial communities [292,293] and various environmental composts (e.g., a switchgrass-adapted compost community) [317], thermophilic biocompost [295] and agricultural soils [318]. All communities contain different family sizes but all have the families GH1, 2, 3, 5, 8, 10, 28, 35, 38, 42, 43 and 53.

With the significant interest in identification of novel enzymes for lignocellulosic biomass degradation and conversion, functional metagenomics studies are being rapidly extended to expand the CAZyme families [319–323]. Although genes encoding the diversity of CAZyme families have been identified via whole genome sequencing and metagenomics, detection of an open reading frame alone does not warrant actual production of protein, nor does it readily indicate the spatial and temporal expression of the gene in the biosystem. Therefore, new technologies in protein identification and characterization will be crucial for improvements in enzyme production, pathway optimization and biofuels crops.

Mass spectrometry technology for secretomes & subcellular organelle proteomics of biocatalystic microbes

Mass spectrometry is a promising tool to assess relatively abundant proteins, provided that the biosystem

Table 2. Sizes of CAZyme families by class identified in genome databases of 23 fungi.

Name	Glycoside hydrolase	Glycosyl-transferase	Polysacch-aridelyase	Carbohydrate esterase	Carbohydrate-binding module
Filamentous fungi					
Aspergillus fumigatus	263	103	13	29	55
Aspergillus niger	248	114	8	25	38
Aspergillus oryzae	303	119	23	30	34
Aspergillus nidulans	252	90	21	33	41
Fusarium graminearum	243	110	20	42	61
Magnaporthe grisea	232	94	5	47	65
Neurospora crassa	174	78	4	22	42
Penicillium chrysogenum	219	102	9	22	49
Phanerochaete chrysosporium	166	57	14	14	N/A
Podospora anserine	230	89	7	49	97
Trichoderma reesei	200	103	3	16	36
Single cell fungi					
Candida albicans	58	69	0	3	4
Candida dubliniensis	49	69	0	3	10
Candida glabrata	39	79	0	3	12
Cryptococcus neoformans	77	66	3	7	12
Saccharomyces cerevisiae	50	68	0	3	12
Schizosaccharomyces pombe	51	61	0	5	8
Debaryomyces hansenii	74	74	0	3	11
Eremothecium gossypii	44	57	0	2	9
Kluyveromyces lactis	46	62	0	2	11
Lachancea thermotolerans	45	59	1	2	11
Pichia pastoris	39	60	1	2	16
Pichia stipitis	53	67	0	4	7

or community is not too complex and has been sampled deeply enough at the molecular level. In the last decade, mass spectrometry for proteomics has progressed radically and is now on par with most genomic technologies in high throughput and comprehensiveness [324,325]. So far, only a few proteomic studies have directly examined proteins and enzymes in the biosystems or communities involved in lignocellulosic biomass degradation under realistic environmental conditions. However, proteomics have been applied to examine production and dynamics of complex and noncomplex glycosyl hydrolases in different microbes rapidly. Recently, proteome analysis of fungal and bacterial involvement in leaf litter decomposition was conducted for a co-culture of two model organisms, *Pectobacterium carotovorum* (Gram-negative bacterium) and *A. nidulans* (filamentous fungus), in culture with beech litter [326]. Proteome analysis revealed that most of the extracellular biodegradative enzymes (proteases, cellulases and pectinases) in the culture were secreted by the fungus, while the bacterium produced only low levels of pectinases. Proteomic studies were also employed to examine lignocelluloses-degrading enzymes secreted by the white-rot softwood degrading fungus *Phanerochaete carnosa* grown on spruce and microcrystalline cellulose [327] and the cellulosome composition of *C. thermocellum* grown on the diluted-acid pretreated switchgrass [328]. Proteins identified in the extracellular filtrates of *P. carnosa* included GH2, 3, 5, 6, 7, 10, 11, 13, 15, 16, 18, 31, 35, 43, 47, 55, 61, 92, glucuronoyl esterase (CE1), pectin esterase (CE8), polysaccharide lyase (PL14) and proteins corresponding to glyoxal oxidases, monooxygenase P450, peroxidases and multicopper oxidase. Results from *C. thermocellum* suggested that a coordinated substrate-specific regulation of the cellulosomal subunit composition occurred to better suit the organism's need for growth under specific carbon source conditions. Furthermore, the development of methods for effective sample preparation for the extracellular proteome of the extreme thermophile *Caldicellulosiruptor saccharolytucus* suggested that two levels of sample purification were necessary to effectively desalt the sample and provide sufficient protein identification [329]. More recently, mass spectrometry proteomics in combination with transcriptomics and genomics provided a powerful system to examine regulation of glycosyl hydrolase production under different culture conditions [330–332].

All glycosyl hydrolases are secreted into the external environment for lignocellulosic biomass degradation. Most of those glycosyl hydrolase proteins undergo post-translation modification with glycosylation playing important roles in enzyme function, stability and interaction with lignocellulose. During the last decade, mass spectrometry methods have also been developed to analyze glycoproteins in different organisms [333–338]. The methods were mainly employed to analyze *N*- and *O*-glycosylation sites and glycan structures in several glycosyl hydrolases (endoglucanase I, II and exoglucanase I, II), mostly from *T. reesei* [339–342]. Some evidence indicated that glycosylation also occurs in bacterial cellulosomes [343,344]. More and more studies indicate that glycosylation plays an important role in glycosyl hydrolase function and biomass conversion and is influenced by different organisms and culture conditions [345–347]. However, thus far, no studies have globally examined glycosylation in the large GH families involved in lignocellulosic biomass degradation. Complete genome sequences of several glycosyl hyrodrases producing filamentous fungi will lay an important foundation to globally study the glycosylation of glycosyl hydrolases and its effects on lignocellulosic degradation.

Gene transfer technologies for improvement of glycosyl hydrolase production in both homologous & heterologous organisms

Genetic engineering technology has been drastically improved for both microbial systems and higher plants, especially with the development of *Agrobacterium*-mediated transformation of higher plants 30 years ago [348]. This method has been widely applied to functional genomics in higher plants for large-scale targeted and random mutagenesis to provide one of the most effective strategies to understanding gene functions and improve productivity and quality of various lignocellulosic biomasses [349–354]. Later, this method was adapted to fungal transformations and as a tool for functional genomics in fungi [355,356]. Concurrently, other transformation methods have been developed and adapted to both plant and fungal transformations, such as chemical-mediated protoplast transformation [357] and biolistic nuclear transformation [358,359]. These effective gene transfer systems allow us to examine the potential for improvement of glycosyl hydrolase production in both homologous and heterologous systems.

Research on homologous systems is needed to optimize glycosyl hydrolase production in known production hosts, where the endogenous glycosyl hydrolases have been identified and characterized. Traditionally, various mutagenesis methods, such as chemical or physical (UV light or x-ray) induction [360–363], have been applied, but with advances in gene transfer technology, the potential regulation of glycosyl hydrolase production can be determined by gene deletion or over-expression. For example, deletion of the glucose repressor gene *Cre1* from *T. reesei* improved cellulase production [364,365] and similar responses have been observed for deletion of the *ACE1* gene, which also affects xylanase expression in *T. reesei* [366]. Besides the negative regulation of transcriptional factors ACEI and CREI, expression of cellulases and xylanases was also positively regulated by transcriptional factors XYR1, ACE2 and HAP2/3/5. Recent completion of the genome sequences of several filamentous fungal species including two glycosyl hydrolase-producing strains of *T. reesei* [306] and *A. niger* [308], and broad studies of glycosyl hydrolase production in those hosts can enhance strain improvements for glycosyl hydrolases production by genetic engineering.

In heterologous systems, over-expression of glycosyl hydrolases has been evaluated in different fermentative microbes such as bacteria, yeast and filamentous fungi [367–369], and in higher plants with or without tissue-specific targeting [370–372]. Several recent publications have reviewed detailed strategies for heterologous expression of glycosyl hydrolases in higher plants [373,374]. The ultimate goal is to realize economic production of glycosyl hydrolases.

Future perspective

For lignocellulosics, cellulase adsorption and efficacy cannot be simply related to a few substrate features. Thus, hemicellulose and lignin removal, deacetylation, decrystallization, accessible surface area and the nature of different cellulase components could all affect access of enzymes to substrate and their effectiveness once they attach. Yet, some of these factors are likely more influential than others, and a concerted effort is needed to understand fundamental physical and chemical features of lignocellulosic biomass that impede glycosyl hydrolase access to carbohydrates and slow the rate of biomass deconstruction into fermentable sugars. Understanding factors that control interactions between lignocellulosic biomass and glycosyl hydrolases as well as inhibitory compounds that are either natural biomass compounds released during deconstruction or formed by degradation of sugars and other biomass constituents in up-stream processing would be invaluable in identifying better pretreatments and enzyme systems to lower the cost of biomass conversion to meet industrial needs. For example, understanding how pretreated cellulosic biomass reactivity changes with conversion and structure and the effects of enzyme–substrate interactions on

sugar release could suggest advanced technologies with lower costs. Improved analytical methods are needed to better characterize biomass composition and structure and interactions between biomass, enzymes and other compounds, and to follow the details of biomass deconstruction. Results from such research can guide further optimization of glycosyl hydrolases production in both homologous and heterologous systems. Further advanced biotechnologies are crucial for discovery and characterization of new enzymes and improvement of the enzyme characteristics and production in homologous or heterologous systems and ultimately lead to low-cost conversion of lignocellulosic biomasses into fuels and chemicals.

Executive summary

- Advancements in pretreatment and cellulase technologies have contributed significantly to historical cost reductions for biological conversion of cellulosic biomass to ethanol and other products.
- A key to lower cellulosic ethanol cost is to reduce enzyme usage and costs.
- Emerging biotechnology tools offer great promise in discovery of new enzyme sources with desirable features.
- Improving the understanding of the structure and function of both lignocellulosic materials and their degrading enzymes will be invaluable in determining the roles of biomass pretreatment, hydrolysis and enzymes in influencing cellulose conversion and in targeting appropriate strategies to enhance rates and yields.

Substrate-related factors that affect enzymatic hydrolysis of cellulosics

- Characteristics of cellulose:
  - Advanced imaging techniques provide new insights into plant cell wall structure and changes during hydrolysis, as well as new understanding of enzyme–substrate and enzyme–enzyme interactions;
  - Cellulose characteristics (e.g., size, structure, crystallinity, degree of polymerization and accessible surface area) were shown to affect cellulase adsorption, synergism and processivity;
  - The mechanism of the rapid decline in hydrolysis rate with cellulose conversion during enzymatic hydrolysis remains unclear, and innovative research is needed to shed new light on what slows cellulase action.

Derived insoluble matter distribution effects

- Deacetylation was reported to improve cellulose digestibility.
- Hemicellulose (e.g., xylan) and lignin removal appeared to improve cellulose digestibility, but some pretreatment methods are effective without removing either (e.g., ammonia fiber expansion).
- Lignin modification rather than complete removal by thermal pretreatment could result in highly accessible cellulose while the remaining solid lignin could cause nonspecific binding of cellulases.
- Hemicellulose removal is favorable in regards to reducing inhibitory effects of hemicellulose sugars (e.g., xylan mono/oligomers) on cellulases.

Derived soluble matter distribution effects

- The inhibitory effects of soluble compounds released in thermochemical pretreatments are not well understood.
- Some xylan and lignin derivatives, especially xylan oligomers, were reported to show different degrees of inhibition of enzymatic hydrolysis of cellulose.
- Identification of soluble compounds derived from thermal pretreatment that account for the greatest inhibition effects and strategies to reduce or eliminate their deleterious effects needs further investigation.

Enzyme-related factors that affect enzymatic hydrolysis of cellulosics

- Features of glycosyl hydrolases from different microbes:
  - Lignocellulosic biomass is naturally deconstructed by glycosyl hydrolase mixtures from various microbes in specific communities.
  - Glycosyl hydrolases from different microbes have different functional features that potentially can become new sources of effective enzymes.

Enzyme synergy effects on overall degradation process

- Many forms of enzyme synergism have been observed among cellulases.
- Enzyme synergism depends on enzyme sources and substrate features.
- Producing purified enzymes in heterologous systems and establishing standard substrates would improve the understanding of enzyme synergism.
- Study of synergetic microbial communities may lead to potential breakthroughs in lignocellulosic biomass conversion.

Advanced technologies for discovery, characterization, & over-expression of glycosyl hydrolases

- DNA sequence technology for whole genome sequencing of biomass feedstocks, biocatalystic microbes, metagenomics, and functional genomics and metagenomics, can build a foundation for advances in biomass production, enzyme categorization and applications.
- Mass spectrometry technology has been applied to examine secretomes and subcellular organelle proteomics of biocatalystic microbes and protein glycosylation.
- Gene transfer technologies improve glycosyl hydrolase production in both homologous and heterologous organisms.
- Cellulase engineering through directed evolution, rational design, post-translational modifications, and their combination may greatly increase cellulase performance and dramatically decrease enzyme use.

Financial & competing interests disclosure

Bin Yang's research is sponsored by the Center for Bioproducts and Bioenergy and Department of Biological Systems Engineering at Washington State University. Ziyu Dai's research was funded by the Biomass Program of the US Department of Energy. Pacific Northwest National Laboratory is operated by Battelle Memorial Institute, Pacific Northwest Division, for US Department of Energy under contract No: DE-AC05-76RL01830. Shi-You Ding is supported by the US Department of Energy, the Office of Science, Office of Biological and Environmental Research through the BioEnergy Science Center, a DOE Bioenergy Research Center. Charles Wyman is supported by the Ford Motor Company Chair in Environmental Engineering at the Center for Environmental Research and Technology of the Bourns College of Engineering at UCR; the BioEnergy Science Center, a DOE Bioenergy Research Center, supported by the Biological and Environmental Research Office in the DOE Office of Science, contract DE-AC05-00OR22725, and subcontract 4000063616; the University of California at Riverside; the USDA National Research Initiative Competitive Grants Program, contract 2008-35504-04596; the Defense Advanced Research Projects Agency through subcontract SUB-226-UCR1 from Logos Technologies supported by contract HR0011-09-C-0075; the Defense Advanced Research Projects Agency and Army Research Lab through Defense Science Office Cooperative Agreement W911NF-09-2-0010 and subcontract 09-005334-000 to the University of Massachusetts, Amherst; and the DOE Office of the Biomass Program, contract DE-FG36-07GO17102. Dr Wyman is also a co-founder, SAB chair, and Chief Development Officer of Mascoma Corporation. The authors have no other relevant affiliations or financial involvement with any organization or entity with a financial interest in or financial conflict with the subject matter or materials discussed in the manuscript apart from those disclosed.

No writing assistance was utilized in the production of this manuscript.

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