Enzymatic hydrolysis of cellulosic biomass for the production of biofuels, A Review

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***Abstract*— 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.**

***Index Terms*— Enzymatic hydrolysis, Cellulose, Pretreatment, Cellulase, Lignin.**

I. INTRODUCTION

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 crises 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. 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. 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.

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 ultra structure 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.

II. 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 [2]. Lignin and carbohydrates (e.g., cellulose and hemicellulose) form lignin–carbohydrate complexes [3]. 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 [4–5]. The complete structure and compositions of lignin, which binds cellulosic fibers together in a composite structure and reduces the accessibility of cellulose to enzymes [6], 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

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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.

Crystallinity

Purified celluloses are micrometer-sized particles composed of nanometer-sized microfibrils**.** 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 cellobiose by cellulases, while the hydrolysis of crystalline cellulose is much slower. Thus, some authors proposed that hydrolysis rates depended on cellulose crystallinity [7–10]. Although rates have been found to slow with increasing crystallinity of cellulose in some studies [11–13], others found the opposite effect [14–16]. It is expected that crystallinity should increase with cellulose hydrolysis as a result of more paracrystalline and amorphous cellulose removal [16-18]. However, no significant change in crystallinity during cellulose hydrolysis was reported in some studies [19,20]. In some reports, cellulose crystallinity was not considered to affect efficient hydrolysis [21–27]. In addition, different cellulase components have been shown to have different adsorption capacities and activities for cellulose [28,29]. 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 [30]. Furthermore, Banka and Mishra observed that crystallinity increased adsorption of a nonhydrolytic protein named fibril-forming protein from *T. reesei* [31]. Such results indicate that cellulose crystallinity has important effects on nonhydrolytic enzyme components, which can be essential to effective enzymatic hydrolysis of cellulose. 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 [32–40]. 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 [41–43].

Accessible surface for cellulase

Cellulose accessibility to cellulases is limited by the structure of cellulose microfibrils that are believed to be nanometer-sized **(Figure 1).** 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 [44]. 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 1)**. 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 [45–47]. 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. 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 [48–52] and particle size [53–55] affecting cellulose hydrolysis.

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 [56,57]. In addition to enzyme-related factors, such as thermal instability of cellulases [58–61], products inhibition [58,62–66], enzyme inactivation [63,67–73], enzyme slowing down/stopping [74], substrate-related factors, including substrate transformation into a less digestible form [75], and the heterogeneous structure of the substrate [75,76], 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 [75], but other reports concluded that substrate reactivity was not the principal cause of the long residence time required for good cellulose conversion [74].

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 [77–82], but other reports do not support a role for hemicellulose removal in changing cellulose digestibility [83–86]. Similarly, conf licting conclusions have been reached on the importance of lignin removal in enhancing cellulose conversion [87–89]. 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

Hemicelluloses are a heterogeneous group of polysaccharides with the b-(1→4)- linked backbone structure of pentose (C5) sugars, such as xylose and arabinose, and hexose (C6) sugars, including mannose, galactose and glucose as the repeating units, which have the same equatorial configuration at C1 and C4, as illustrated in **Fig. 2** . The structural similarity of hemicelluloses to the b-1,4-glycosidic bonds of the cellulose molecule benefits from a conformational homology, which can lead to a strong non-covalent association with cellulose microfibrils. Unlike cellulose which is crystalline and resistant to degradation, hemicelluloses are random and amorphous, and thus easily hydrolyzed to monomer sugars. However, hemicelluloses are embedded and interact with cellulose and lignin, which significantly increase the strength and toughness of plant cell walls. Xyloglucan and xylans are major hemicelluloses in plant biomass. Xyloglucan is abundant in the primary walls, with the oligosaccharide composed of xylose (X) and glucose (G) with various side chains, XXXG or XXGG for vascular plants including grain crops, as the repeating unit. Xylans are polysaccharides with b-(1→4)-linked xylose residues as a backbone, which are often acetylated at the O-3 position of xylose residues and/or modified by a-(1→2)-linked glucuronosyl and 4-O-methyl glucuronosyl residues. Xylans, also known as glucuronoxylans, are the dominant noncellulosic polysaccharide in the secondary walls of dicots. The major sugars in the hydrolysate of hemicelluloses are therefore xylose, arabinose, glucose and galactose.

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 [89]. However, simultaneous lignin alteration during pretreatment can confound the role of hemicellulose solubilization and modification [87,90,91]. From a more applied perspective, some pretreatments such as ammonia fiber expansion (AFEX) produce highly digestible cellulose without removing any significant amounts of hemicellulose [92–94], 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 [95,96]. 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 [97]. 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 [97]. 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 [98]. In addition, a study by Weimer *et al.* suggested that intimate association of xylan and cellulose does not inhibit biodegradability of polysaccharides [99]. 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.

Lignin

Lignin binds cellulosic fibers together in a composite structure with excellent properties, but also reduces the accessibility of cellulose to enzymes. Various studies reported cellulose hydrolysis was improved with increasing lignin removal, although differences were reported in the degree of lignin removal needed [100]. The ratio of syringyl to guaiacyl moieties in lignin was also considered to have important effects on digestibility [101], 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 [102,103]. 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 [104–106]. The removal of lignin not only increased cellulose accessibility but also allowed more cellulase action. Lignin and its derivatives were reported to precipitate and bond with protein and condensed lignin was reported to adsorb protein from aqueous solutions [107]. 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 [108]. Thus, lignin removal may both open more space for enzymes and reduce enzyme nonspecific absorption on lignin. Low levels of lignin have been shown to enhance cellulose hydrolysis due to a physical separation of microcellulose fibrils enhancing cellulase access/activity. Lignin modifications in transgenic biomass have resulted in decreased recalcitrance to saccharification with improved fermentable sugar yield.

III. 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 [109], pretreatment hydrolyzate was added back to the pretreated solids [110] or the whole slurry (i.e., pretreated solids and hydrolyzate) was enzymatically hydrolyzed [111–115]. These results suggest that compounds in the pretreatment hydrolyzate have inhibitory effects on enzymatic hydrolysis of cellulose.

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 [116–119], 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 [120]. 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 [121]. Selected microbial strains that have been explored for various glycosyl hydrolase applications and their characteristics. These microbes were isolated from different environmental niches and grouped into aerobic or anaerobic bacteria or fungi on the basis of their growth conditions (**Table 1**). 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 [122,123], 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 [124].

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, exoglucanase or endoglucanase with b-glucosidase, catalytic domain with CBM or two catalytic domains, cellulose-enzyme-microbe synergism and spatial synergism for cellulase complexes (i.e., the cellulosome of *C. thermocellum*). 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). Endo–endo type synergism was only reported in fungal cellulases of *Gloeophyllum sepiarium* and *Gloeophyllum trabeum*. Cell–cellulase– cellulose synergism has been shown for some cellulolytic microorganisms such as *C. thermocellum* that have tightly cell-associated cellulase systems.

IV. 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.

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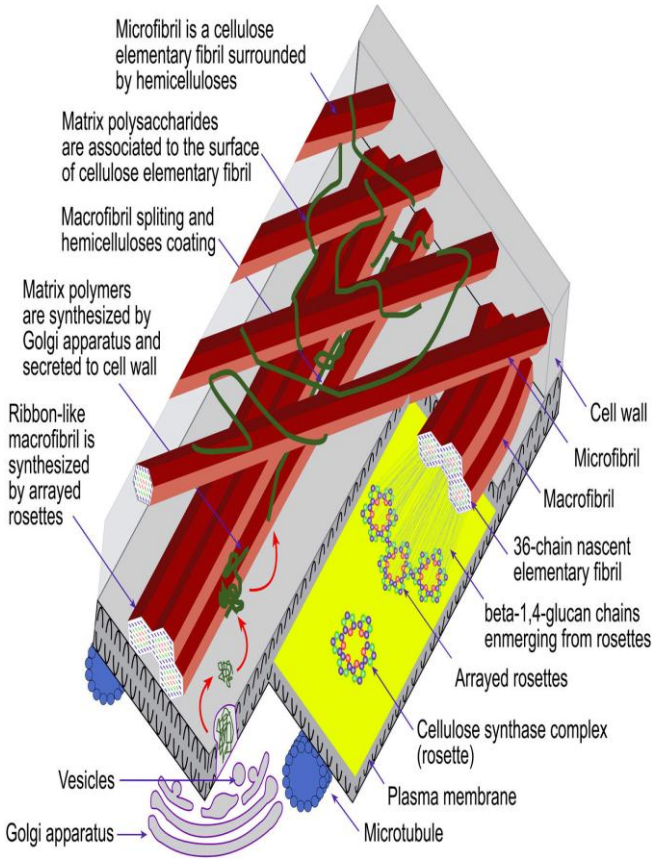

Fig 1. Model of plant cell wall cellulose elementary fibril and its synthesis. The dimensions of cellulose elementary fibril are estimated as 3 × 5.5 nm. (Adapted from Yang, Dai, Ding & Wyman).

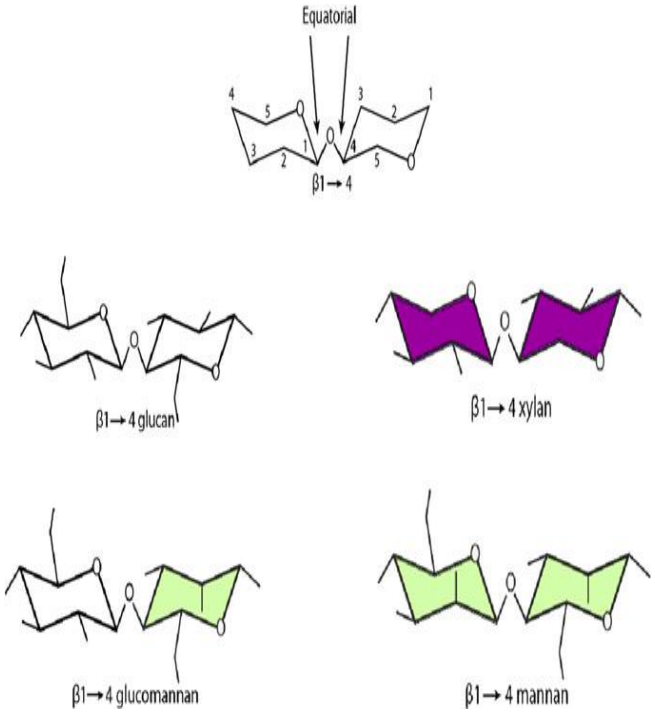

Fig. 2. Repeating units of hemicelluloses.

Name	Enzymes types
(Bacteria (aerobic)	
Acidothermus cellulolyticus	NC/HC
Bacillus sp. *Bacillus pumilus* *Bacillus substilis* *Bacillus agaradhaerens* JAM-KU023	NC/HC NC/HC NC/HC NC/HC
Brevibacillus sp. strain JXL	NC/HC
Cellulomonas flavigena	NC/HC
Cellulomonas fimi	NC/HC
Geobacillus thermoleovorans	NC/HC
Paenibacillus campinasensis BL11 *Paenibacillus strain* B39	NC/HC NC
Streptomyces sp.	NC/HC
Thermoactinomyces sp.	NC/HC
Thermomonospora curvata *Thermomonospora fusca*	NC/HC NC/HC
Bacteria (anaerobic)	
Acetivibrio cellulolyticus	Cellulosome/NC
Bacteroides cellulosolvens	Cellulosome
Clostridium acetobutylicum *Clostrium cellulolyticum* *Clostrium cellulovorans* *Clostrium josui* *Clostrium papyrosolvens* *Clostrium thermocellum*	Cellulosome Cellulosome/NC Cellulosome/NC Cellulosome Cellulosome Cellulosome/NC
Ruminococcus albus *Ruminococcus flavefaciens*	Cellulosome Cellulosome
Filamentous fungi (aerobic)	
Acremonium cellulolyticus	NC/HC
Acrophialophora nainiana	HC/HC
Aspergillus acculeatus *Aspergillus fumigatus* *Aspergillus niger* *Aspergillus oryzae*	NC/HC NC/HC NC/HC NC/HC
Fusarium solani	NC/HC
Humicola grisea var. thermoidea	NC/HC
Irpex lacteus	NC/HC/LN
Penicillium funmiculosum *Penicillium atrovenetum* *Penicillium citrinum*	NC/HC NC/HC NC/HC
Phanerochaete chrysosporium	NC/HC/LN
Schizophyllum commune	NC/HC
Sclerotium rolfsii	NC/HC
Sporotrichum cellulophilum	NC/HC
Talaromyces emersonii	NC/HC
Thielavia terrestris	NC/HC
Trichoderma koningii *Trichoderma. reesei* *Trichoderma viride*	NC/HC NC/HC NC/HC
Anaerobic fungi	
Anaeromyces elegans *Anaeromyces mucronatus*	NC/HC NC/HC
Caecomyces CR4	NC/HC
Neocallimastic frontalis *Neocallimastic hurleyensis* *Neocallimastic patriciarum*	Cellulosome Cellulosome Cellulosome
Orpinomyces joyonii *Orpinomyces* PC-2	Cellulosome Cellulosome
Piromyces communis *Piromyces equi* *Piromyces* E2	Cellulosome Cellulosome Cellulosome
HC: Hemicellulase; LN: Ligninase; NC: Noncomplexed cellulase	

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