Toward a Fundamental Understanding of Cellulase-Lignin Interactions in the Whole Slurry Enzymatic Saccharification Process

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Abstract

Lignocellulosic biomass is a promising feedstock for sustainable production of non-food building-block sugars. This bioconversion process is preferentially carried out through the whole slurry enzymatic saccharification of the pretreated lignocellulosic substrates. However, dissolved lignin, residual lignin, and lignin-derived phenolic molecules in the pretreated biomass slurry can all trigger the decrease in activity and stability of cellulases, as well as the unfeasible enzyme recyclability. The hydrolyzing efficiencies can be considerably hindered by the lignin-induced nonproductive binding of cellulases through various mechanisms. Three major non-covalent forces, *i.e.*, hydrophobic, electrostatic, and hydrogen bonds interactions, can occur between the amino acid residues incellulases and the functional groups in lignin. Various strategies such as enzyme engineering, substrate modification, additive blocking have been intensively developed to minimize the cellulase-lignin interactions. To investigate the impacts and benefits of different mechanisms and processes this article provides a systematic overview of the current opinions about the nonproductive binding of cellulase to lignin. Through better understanding of their interactions it is our hope that the enzyme binding groups in lignin could be properly quenched through new pretreatment method and/or biochemical processing to increase the efficiency of cellulose bioconversion.

Keywords: lignin, cellulase, hydrolysis, non-covalent interactions, cellulose binding modules, blocking additives

Contents

1. Introduction

Bioconversion of lignocellulose through the sugar platform has long been regarded as a promising approach to produce non-food based building block chemicals in a biomass-to-biofuel biorefinery [1,2]. Direct conversion of the lignocellulosic biomass (*i.e.*, agricultural and forestry residues) through using only the polysaccharide hydrolysases or microorganisms, however, has not yet been technologically or economically feasible in large scale [3]. This challenge is mainly attributed to the well-known biomass recalcitrance to enzyme hydrolysis. The plant cell wall has a multilayered micro-scale architecture which protects the plant cells from enzymatic degradation [4].Cellulose,the major polysaccharides (30~50 wt%), existing in the plant cell wall in the form of highly crystallized microfibrils, is embedded in a complex matrix composed of hemicelluloses (10~40 wt%) and lignin (5~30 wt%) [5]. Since hemicelluloses are enzymatically digestible, we discussed only the lignin in this paper for its severe impacts on cellulase efficiency.

To allow the cellulases access the target substrates, many pretreatment technologies have been developed to destruct the cell wall by removing or relocating the recalcitrant components in the wall matrix [3]. Thermochemical pretreatment processes are currently the preferred option for full-scale application [6]. During the pretreatment process, the native structure of lignocellulose is decomposed, and the structurally modified lignin and/or small lignin-derived molecules are released into the pretreatment liquor [7,8]. These substances together with the residual lignin in solids can still inhibit cellulase through the so-called nonproductive binding (as illustrated in Figure 1) [4,9]. Lignin-induced binding has been widely accepted as a major reason for cellulase inactivation and the poor recyclability of the used enzymes [4,9].

Insert Figure 1 here

Since 1990s, the roles of lignin in enzymatic hydrolysis of cellulose, especially the interactions between lignin moieties and cellulases, have been extensively investigated [4,10]. The nonproductive binding has been concerned in nearly all the newly developed biomass pretreatment methods [11]. In addition, various chemical, biochemical and genetic tools have been employed for stabilizing or even activating cellulase in presence of lignin [12-15].The cellulase-lignin binding have been reviewed extensively from different aspects, such as the overall state-of-the-art processes [16,17], substrate accessibility [18], enzyme mechanisms[19], protein engineering [20], and enzyme recyclability [21].With the recent increased attention paid to the whole slurry biomass saccharification [22], in which the pretreated substrate is hydrolyzed together with spent liquor without washing (to completely uses of the sugars for high product yield), the importance of the interactions between the cellulases and pretreatment products have been re-emphasized. This paper summarizes the most recent research progress on the fundamental mechanisms correlating the cellulase-lignin interactions with the status and structures properties of lignin; the synergistic action of cellulases; and the rapidly developed stabilization strategies. We hope this paper can contribute to the future development of new generation bioconversion techniques through whole slurry saccharification.

2. General description of nonproductive cellulase binding on lignin

Enzymatic hydrolysis of cellulose is a synergetic reaction carried out by the cellulase complex as illustrated in Figure 2A. The hydrolyzing process starts from the specific binding of β-1,4-endoglucanases (EGs, EC 3.2.1.4) on the substrate and the random cleavage of β-1,4-glucosidic bonds in the amorphous region [20]. The β-1,4-cellobiohydrolases (CBHs, EC 3.2.1.91) progressively release soluble cellobiose from the newly formed chain ends. Meanwhile, the β-glucosidases (BGs, EC 3.2.1.21) subsequently hydrolyze cellobiose to glucose, which eliminates the inhibition of EG-CBH synergy by the accumulated cellobiose

[20,23]. At least eight EG isozymes have been found in *Trichoderma reesei* cellulase, in which Cel7B (EGI), Cel5A (EGII), Cel61A (EGIV), Cel45A (EGV) and Cel74A (EGVI) have the cellulose binding modules (CBMs), while Cel12A (EGIII) and Cel61B (EGVII) have only the catalytic modules (CMs) [24]. The two CBHs, Cel7A (CBHI) and Cel6A (CBHII) both have CBMs, and can attack the reducing end and non-reducing end of the cellulose chains, respectively[23]. BGs do not contain CBMs and their activities in *T. reesei* cellulase are low and cannot meet the requirement in the biorefinery; *Aspergillus niger* BGs are supplemented in some commercial cellulases to improve the synergetic effects of enzymatic hydrolysis.

Insert Figure 2 here

Lignin physiologically deposited in the middle lamella can physically block the productive binding of cellulases to cellulose fibrils in the inner secondary wall (see the left column, Figure 2B, [6,16,25,26]. During thermochemical pretreatment, a portion of the lignin could be dissolved in the pretreatment liquor and is denoted as the dissolved lignin (DL) in this paper. Lignin extraction usually accompanies with depolymerization through which various amounts of the lignin-derived phenolic molecules (LDPMs) are generated [27]. Harsh pretreatment conditions, *i.e.*, high chemical doses and/or cooking conditions, can lead to lignin condensation/re-precipitation during the cooling process of the pretreated slurry [28,29]. The undissolved structural lignin together with condensed and re-precipitated lignin is referred to the residual lignin (RL). DL, LDPMs, and RL can all cause negative impacts to enzymatic hydrolysis, *i.e.*, unspecific enzyme adsorption; decreases in enzyme activity and stability; and reduced enzyme recyclability [6,16,25,26]. On the other hand, hydrophilic derivation of lignin moieties could reduce the inhibitory effects or even stimulate cellulase activity [30-32]. More detailed recent findings of the formation and impacts of different DL, LDPMs, and RL

to enzymatic hydrolysis are provided in Table 1.

Insert Table 1 here

Cellulase binding onto lignin does not always reduce the efficiency of hydrolysis (quantified by the cellulose enzymatic digestibility, CED, %), which can be affected by many different parameters. Although it is unlikely to obtain a conclusive statement only from the previous studies presented in Table 1, some general patterns have been found, *i.e.,* (1) RL is more harmful to cellulase than native lignin or DL; (2) introducing hydrophilic, charged groups could switch the role of lignin from inhibitor to activator; (3) phenolic hydroxyl groups react differently to hydrolysis when those groups are in RL or lignin oligomers than in LDPMs; and (4) mono-cellulase showed different affinities to lignin; and thereby total activity of cellulases are only inhibited when their synergistic action is disturbed.

3. Interactions between lignin and cellulase enzymes

The governing non-covalent interactions between cellulase and lignin include the hydrophobic interactions, electrostatic interactions, and hydrogen binding forces. A schematic illustration is shown in Figure 1. It should be noted that all the three interactions are comprised in the binding of cellulase and lignin. Obviously, these interactions occur due to the change in physiochemical properties of the lignin after pretreatment. The following sections discuss the formation mechanisms, impacts on hydrolysis, and minimization strategies of the three interactions.

3.1. Hydrophobic interactions

Hydrophobic interactions have been proposed as a major feature in the nonproductive

binding of cellulase to DL or RL [39]. Direct measurement of adhesion forces between kraft lignin and cellulase by Atomic Force Microscopy (AFM) showed a strong attractive force during the engaging of a hydrophobic functionalized AFM tip and the silicon wafer immobilized *T. reesei* cellulase [39]. Molecular interactions between cellulase and hydrophobic tips were 13% and 43% higher than those with tips carrying -COOH and -OH groups, respectively [39]. Rojas et al.[40,41] combined AFM and quartz crystal microgravimetry (QCM) to examine the interactions between cellulase and different lignin films and demonstrated the dominating effect of hydrophobic interactions on the lignin affinity of enzymes, while electrostatic interactions exhibit a minor effect in the reaction [40]. Kinetic analysis based on QCM showed that Cel7B binding to lignin films only fitted the two-site transition model, which suggests that Cel7B can bind reversibly to two distinct sites in a lignin surface at different adsorption rates [42]. In another word, there are at least two binding sites on cellulase protein surface for binding with the lignin.

Among the intra-structure of cellulase (*i.e.*, EG and CBH), CBM has been considered as a major binding domain to lignin. Three tyrosine residues (*i.e.*, Y5, Y31 and Y32) dominate the specific interaction between TrCel7A CBM and the hydrophobic surface of cellulose (*i.e.*, I_α and I_β) [43,44]. In CBM of Cel6A, the hydrophobic surface is composed of W5, Y31 and Y32. Hydrogen bonds between the -OH groups of cellulose and the planar modif (*i.e.*, Y5, Y31, Y32, Q7, Q34 and N29) might drive the processivity of Cel7A CBM on the hydrophobic surface of cellulose [44]. Introducing a more hydrophobic amino acid (*i.e.*, tryptophan) to Y31position resulted in increased binding of the CBM to microcrystalline cellulose and enzyme residual lignin (ERL) [45]. In accordance, mutation Y32A (A = alanine) decreased the CBM affinity to cellulose and lignin. This result suggested that hydrophobic interactions between CBM and lignin are formed at the same aromatic amino acids that dominate the CBM-cellulose interaction [45]. A more recent work on atomic-detail molecular dynamics

simulation confirmed that lignin binds preferentially to the tyrosine residues of *Tr*Cel7A CBM that are critical for cellulose binding [26]. TrCel7A CBM mutants linking with a *T. emersonii* Cel7A CM were engineered by Strobel et al. [14,46]. Both cellulose and lignin affinity were greatly decreased by adding hydrophobic or positively charged residues onto the planar face of the CBM [14].Interestingly, the lignin affinity can be more selectively changed by engineering the number of *O*-linked saccharides in the peptide linker which determines the linker's hydrophobicity [46]. Obviously, glycosylation patterns of the linker play an important role in cellulase-lignin nonproductive binding.

The CMs of cellulase can also bind with lignin but with significantly weaker affinity. For example, the binding ratios of Cel7A-CM and Cel7B-CM account for 28% and 19% of those for respective intact enzymes under the same conditions [47]. Cel7A core exhibiting lower affinity towards lignocellulose films than the native protein [41]. Viikari's group [48,49] found that complementation of *T. aurantiacus* Cel7A and Cel5A lacking CBM with intact CtCel6A improved the hydrolysis at high substrate consistency. The use of core cellulases reduced the nonproductive binding and increased the enzyme recyclability [48,49]. The CMs of TrCel7A and Cel5A differed essentially in binding lignin; *i.e.*, Palonen et al. [50] measured a high affinity of Cel5A-CM to isolated alkaline lignin, whereas Cel7A-CM did not adsorb on the same material. The different affinities could be due to the difference in CM configuration: the active site of Cel5A-CM is more opened to the environment than the tunnel-shaped active site of Cel7A-CM [50].

3.2. Electrostatic interactions

Electrostatic interactions between cellulase and lignin have been deduced by many researchers according to the pH dependent binding behaviors [45,51]. In general, enzymes carry a net positive charge at a pH below their isoelectric point (pIs), which can form electrostatic

attraction with the dissociated acidic groups in lignin such as $–SO_3^-$ (pKa ~2.0), $–COO^-$ (pKa ~4.0). Under regular hydrolyzing conditions (pH 4.8), Cel7A (pI 3.5~4.2) possesses weak negative net charge; Cel6A (pI4.6~5.0) is nearly electrically neutral; while Cel6A (pI 5.1~6.3), Cel5A (pI 5.5~6.1) and Cel12A (pI 5.1~7.4) are positively charged [52]. Total protein binding affinity of *T. reesei* cellulase on isolated lignin strongly depends on the pH and ionic strength [51]. At a higher pH, *e.g.,* 6.0, less enzymes were nonproductively bound on the lignin, which was presumably due to the repulsive electrostatic interactions [51]. Replacement of a polar residue (Q2) in Cel7A CBM with positively charged lysine increased the binding selectivity toward lignin; in contrast, negatively charged glutamic acid increased the specificity of cellulose in presence of lignin [14]. Particular concerns of the electrostatic interactions have been emphasized in the case of highly charged lignin in lignocellulosic substrates or dissolved out [53,54]. Zhu et al.[54]reported the optimal pHs were 5.2–5.7 for Celluclast 1.5L and 5.5–6.2 for CtecII towards several different substrates undergone dilute acid, alkaline, or sulfite pretreatment. Zeta potential analysis showed an elevated pH significantly increased the negative charge on lignin surface, which promoted the hydrophilicity of lignin and increased its Coulombic repulsion against the negatively charged cellulases[53].

Although electrostatic forces contribute to the binding, they do not dominate protein binding in all conditions. An obvious fact is that Cel7A strongly binds lignin under a pH above its pI, which suggests the Coulombic repulsion can be overcome by the hydrophobic interactions [45]. Direct evidences are also available from the measurement of AFM attractive force between cellulase and functional groups [39] and the QCM adsorption studies varying with lignin and pH [40,41]. Charge engineering of cellulase could be achieved by chemically derivatization of enzyme (e.g. succinylation and acetylation) [15] or non-chemically with additives (*e.g.*, polymers, metal ions) [13,55]. The effectiveness will be described in subsequent sections.

3.3. Hydrogen bonding

Hydrogen bonding is a more frequently discussed force in explaining the denatureof cellulase exposed to the dissolved phenolic compounds [27,33,56]. Almost all thermochemical pretreatments can release phenolic compounds into the spent liquor from the extracts (e.g., tannic acids) and lignin (*e.g.*, *p*-coumaric acid) [33,56]. During the hydrolysis of the whole hot-water pretreated bagasse slurry the dissolved phenolics strongly deactivated the endoxylanase and total cellulase activity, and also inhibited the activities of β-glucosidase and xylanase [27]. Ximenes et al. [57] and Mhlongo et al. [56] identified that tannic acids caused the strongest inhibition to hydrolysis. Moderate or minimal inhibition by lignin-derived phenolics, such as the hydroxy-cinnamic, *p*-coumaric acid, ferulic acid, 4-hydroxybenzoic acids, vanillin, have also been verified and showed different susceptibilityto monocomponent cellulases [56-58]. Xu et al. [58] found that oligomeric phenolics initiated stronger inhibition on enzymatic cellulolysis than simple phenolics. Tian et al. [33] reported that the activation of cellulase by ferulic acid, *p*-coumaric acid and salicylic acid at specific concentration ranges. Zhao and Chen [32] identified the small phenolic compounds identified in steam exploded corn stover and concluded that phenolic aldehydes are strong inhibitors, but phenolic acids offer slight stimulation in a specific concentration range, *e.g.*, 2~4 g/L.

The interactions between proteins and phenolic compounds are generally attributed to reversible non-covalent hydrogen bonds and irreversible covalent bonds between the amino acid residues and the phenolic hydroxyls [59]. Fluorescence analysis carried out by Tian et al. [33] showed that the salicylic acid interacted non-covalently with cellulase, whereas *p*-coumaric acid and ferulic acid reacted covalently with the cellulase. Circular dichroism analysis further demonstrated that the phenolic acids can destroy the α-helix structure and increase β-sheet and random coil contents in enzymes [33].

Hydrogen bonding between cellulases and lignin or lignocellulosic substrates have also been recently concerned [34]. Their hydrogen bonding affinity correlated positively with the phenolic hydroxyl content but negatively with the aliphatic hydroxyl content [38,60]. However, blocking phenolic hydroxyl groups by hydroxypropylation did not significantly change the binding behavior of cellulase on lignin. In other words, phenolic hydroxyl was not a determining factor in enzyme binding on lignin [34]. AFM studies confirmed that hydrogen bonding contributed to, but not dominated the attraction force in cellulase binding to lignin [39]. Site-directed mutagenesis studies showed hydrogen bonding is more important for binding to cellulose than to lignin [46].

3.4. Affinity order - How lignin disturbs the synergistic reactions

Owing to the difference in affinity, monocomponent cellulases preferentially bind onto the lignin, which would interrupt the synergy of hydrolysis and consequently lower the glucose yield from hydrolysis. When comparing the two major components (*i.e.*, Cel7A, 60~75% and Cel7B, 6~10%, respectively) in *T. reesei* cellulase, Börjesson et al. [47] found that the binding affinity of Cel7B to steam-pretreated spruce lignin was stronger than that of Cel7A. An explanation of this phenomenon was that the Cel7B has a more hydrophobic rough surface on the flat face of CBM, which gives a higher affinity to lignin [47]. QCM studies confirmed the results and further demonstrated that the Cel7A could penetrate the lignocellulose films while Cel7B only exhibited activity on the surface of film [41]. For steam exploded wheat straw, Cel7A showed a lower affinity to lignin, but a higher affinity to cellulose than Cel6A and Cel7B [61]. Of the cellobiohydrolases on steam-exploded wood, TrCel6A also remained clearly less bound than TrCel7A [62]. On spruce ERL, however, alkaline lignin and CEL lignin, the relative affinity of Cel7A was significantly higher than Cel5A [50]. The influence of preferential binding of CBH or EG is illustrated in Figure 2B and

2C. Lack of CBH would lead to insufficient depolymerization of cellulose in the amorphous regions, releasing oligomers or segments (Figure 2B). When EG was absent, on the other hand, the hydrolysis would be very slow and eventually terminated due to the gradual inactivation of bound CBH on cellulose chains (Figure 2C).

Temperature plays an important role in the preferential binding properties of cellulase isozymes onto lignin. Lignin-bound cellulases are more readily denatured at an elevated temperature, *i.e.*, 50°C.Heat-induced inactivation may connect the nonproductive binding of cellulase enzymes with the destroying of their synergistic actions together. For instance, after 48-h adsorption at 4°C, ca.55% of the initial total EGs activities and 65% of Cel7A, respectively, were retained in forms of bound protein on acid hydrolysis residues [62]. At 45°C, only ca. the active Cel7A bound on residues occupied only 8% of the total activity while all bound EGs were denatured [62]. The impacts of temperature could be different for various lignin samples. Tu et al. [63]found the equilibrium constants for cellulase binding on ERL from ethanol-pretreated lodgepole pine was 2.6-fold higher at 45°C than the constants measured at 4°C. However, the constant for ERL from steam-exploded substrates was slightly lower at 45°C [63]. Temperature sensitivity of cellulase binding on lignin was reduced by fusion of CBM from *Tr*Cel7A with the core domain from thermostable *Talaromycesemersonii* Cel7A [64]. The obtained *Te*Cel7A-CBM1 was more tolerant to lignin than native *Tr*Cel7A at elevated temperature [64].

Furthermore, *A. niger* β-glucosidase (pI~4.0) showed minimal deactivation by isolated lignin owing to the lack of CBM [51,62,65,66]. According to Machado et al.[65], no significant binding of *A. niger* β-glucosidase on Avicel or pretreated bagasse was discovered. The activity of AnCel3A decreased continuously during hydrolysis of lignocellulose, which is probably due to the end-product inhibition [62]. Interestingly, Yarbrough et al. [51] recently reported a strong binding affinity of basic β-glucosidase to insoluble lignin. They proposed

that basic β-glucosidases could be used to displace CBM interactions with lignin [51].

4. Strategies to minimize cellulase-lignin interactions

Three major strategies have been applied to minimize the inhibitory effects of lignin to cellulase, *i.e.*, developing weak lignin-binding enzymes [14]; conducting post-delignification or modification; and blocking nonproductive binding by exogeneous additives [12]. The following section provides a brief overview of those strategies.

4.1. Enzyme engineering

To overcome the impacts of lignin-cellulase interaction the structure and properties of enzyme can be modified through both the chemical and genetic routes. Rational charge engineering of *T. reesei* cellulase cocktail has recently been demonstrated by Nordwald et al. [15]. The ratio of negative-to-positive surface charges was increased after succinylation or acetylation of primary amine groups in enzymes. Succinylation resulted in more than twofold of increase in hydrolyzing the mixture of Avicel with 1 wt% lignin (2.7-fold reduction in apparent K_m) [15]. Strobel and coworkers [14,46] screened the TrCel7A CBM mutants with a*Te*Cel7A CM for selective decrease in cellulose or lignin affinity. The addition of hydrophobic or positively charged residues in CBM mutations decreased the specificity for cellulose. Lignin affinity was tunable by alternating the predicted glycosylation patterns of linker. A mutant was obtained with full cellulose affinity but 2.5-fold less lignin affinity, which generated 40% more glucose from acid-pretreated grass without removing lignin from the substrates [14]. Rahikainen's group [64] focused on fusion enzymes engineered from TrCel7A-CBM1 and TeCel7A-CBM3. They found that the *Te*Cel7A-CBM1 was most lignin-tolerant among the studied enzymes, whereas *Tr*Cel7A was most susceptible to lignin especially at 55°C. Increase in temperature leads to increased inhibitory effect of

supplemented lignin in hydrolyzing Avicel [64].

In terms of endoglucanases, Rahikainen et al. [67] investigated the fusions of *Melanocarpus albomyces* endoglucanaseCel45A (*Ma*Cel45A) with *Tr*Cel7A-CBM1. Binding capacity of*Ma*Cel45A to lignin is a function of pH, in either presence or absence of the CBM1; the CBM increased binding of *Ma*Cel45A to the isolated lignins only at high pH. Hydrophobic interactions and electrostatic interactions contributed to nonproductive enzyme binding. Scottet al. [68]filed a patent on constructing linker variants to obtain lignin resistant *Tr*cel6A by altering linker amino acids (*i.e.*, Arg or Ser to Glu or Thr, respectively). The ratio of Thr/Ser was increased and the isoelectric point of the linker peptide was changed. The linker-engineered cellulase showed decreased lignin affinity and increased activity [68].

4.2. Substrate engineering

Post-delignification or hydrophilic lignin modification has been suggested to reduce the recalcitrance of pretreated biomass. Alkaline hydrogen peroxide post-treatment was the most frequently performed strategy through which a significant portion of residual lignin could be removed. More hydrophilic substrates were generated after introducing carboxylic acid groups [69].The increased number of acidic groups would provide more electric repels between lignin and cellulase; and their hydrophobic binding is correspondingly reduced [70]. Likewise, sulfonation could also efficiently enhance the hydrophilic properties of residual lignin in substrates. The increase in water swelling of substrate would ensure the improvement in enzyme penetration into the cell wall [71].

Etherification (*e.g.*, hydroxypropylation) could generate lignin that is less detrimental to cellulose hydrolysis than the unmodified compound[60]. Pan et al.,[34,72]found that carboxylation and sulfonation promoted the hydrophilicity by approximately 22-30%, resulting in the decrease of the lignin inhibition by approximately 76-96%. As a comparison, blocking

phenolic hydroxyl group via hydroxypropylation reduced the inhibitory effect of lignin by 65-91%[34].

Delignification and lignin modification can also be achieved by using laccase/mediator [73]. For example, over half of the RL(especially G Units) was lost from eucalypt woodafter laccase/methyl syringate treatment; as a result, the glucose yield was increased from 40% to 55% [74]. Laccase was also capable of oxidizing phenolic compounds from pretreated substrates and therefore improve lignocellulolytic enzyme efficiency [75,76]. Moilanen et al. [77]discussed the role of laccase and mediator in the enzymatic hydrolysis of the steam pre-treated spruce and found both lignin and cellulose was oxidized. Laccase modification reduced the binding of EG Cel5A and CBH Cel7A on lignin, which consequently promoted the cellulose conversion degree. [77]. In addition, laccase treatment has more often been used as a detoxification strategy for enhancing the fermentability of the pretreated biomass slurry. After laccase treatment, the lag phase of yeast was reduced and the cell viability in SSF was promoted, which finally enhanced the ethanol yield(by 22%)[78,79].

4.3. Blocking with polymeric additives

Blocking strategies based on addition of a variety of chemicals (See Fig.3) are more attractive due to their operation feasibility. The effects of surfactants on enzymatic hydrolysis of lignocellulosic materials have been extensively investigated since1980s [80,81]. In general, non-ionic surfactants/polymers such as Tween, PEG, and Triton X100 stabilize and activate cellulase activity; and anionic and cationic surfactants deactivated enzymes even at a very low concentration [81,82]. Non-ionic surfactants/polymers generally protect cellulase from denaturing by heat and shear force [83].For example, addition of PEG6000 prevented the precipitation of cellulase under hydrolysis conditions without any substrate [84]. These chemicals can lower the surface tension of lignocellulose, which allows the substrate more

accessible to cellulase [85,86]. Meanwhile, the chemicals can also eliminate the nonproductive binding cellulase to lignin due to the effects that the hydrophobic part of the surfactant can bind to the RL through hydrophobic interactions to prevent cellulase adsorption[83,87]. During the past decades, the efficiency of non-ionic surfactants has been examined repeatedly by a great many research groups. Therefore, only the most recent opinions are discussed in this study and have been summarized in Table 2.

Insert Figure 3 here

Insert Table 2 here

PEG has been found to improve both the catalytic activity and the thermal stability of CBHI [102]. The *T. reesei* CICC 13052 CBHI interacted with PEG through hydrophobic forces and the hydrogen bonds. Two key amino acids, Tyr 171 and Asn 184, contributed to CBHI activity enhancement by PEG [92]. On the lignin-blocking function, the binding constant of PEG on pretreated biomass was 10-times higher than the constant for cellulase-lignin binding [93]. However, PEG chemicals show minimal effect on EG and BG [102]

Amphiphilic lignin derivatives (ALDs) are synthesized as a special cellulase stabilizer, which can enhance both the hydrolytic activity and the stability of cellulases [99,103,104]. ALD generated from lignin coupled with epoxylated PEG directly associated with Cel6A, whereas PEG 4000 was not [97]. Furthermore, the ALDs could maintain cellulase activity at a high level even after the fourth cycle of hydrolysis, while the efficiency was significantly lost after repeated use in absence of the additives [105].

Ionic surfactants/polymers have also been investigated to surpass the previous early

attempts, which mainly on the inhibiting effects of lignin to enzymatic hydrolysis. Lou et al. [30] investigated the use lignosulfonates (LSs), an industrial anionic surfactant, and evidenced the strong inhibitory effect of LS to enzymatic hydrolysis of pure cellulose. The authors demonstrated that almost all the tested LSs with varied sulfonation degrees and molecular weights (MW) could enhance the enzymatic conversion of pretreated lignocellulose [30,31]. Lu et al. [106,107] developed a "patching/bridging" strategy based on cationic polyacrylamide (CPAM) to minimize the charge repulsion between fiber and enzyme by reducing their zeta potential on the particle surface. The authors demonstrated that CPAM indirectly promoted enzyme binding to the substrates hence increased the rate of hydrolysis [106].

4.4. Protein and peptides

The use of bovine serum albumin (BSA) as lignin-block additives was first reported by Yang and Wyman in 2006 [12]. Several other exogenous non-enzymatic proteins, such as peptone, protein digest, and yeast extract, have also been studied as a substitution of pure BSA owing to their advantages in cost and availability [94,108]. The efficiency of proteineous additives was summarized in Table 2.

Nonplantexpansin-like proteins (EXLX) act synergistically with cellulase but exhibit no hydrolytic activity has received a great deal of attention in recent years [109]. *Bacillus subtilis* EXLX1 showed significantly higher affinity tolignin than to cellulose and could serve as a lignin blocker in the enzymatic hydrolysis of lignocellulose [95]. EXLX proteins also synergistically enhanced the activity of several cellulases; however, the synergism merely slightly beyond the nonspecific blocking effect of BSA [110].

Enhancement of enzymatic digestion of plant biomass by three non-glycoside hydrolase Proteins were reported by Su et al [111]. These hydrolases are all small proteins heterologously produced in *E. coli*., which indicates a promising direction for rationally design of

lignin-blocking polypeptides. Nakazawa et al. [96] designed and in vivo synthesized a series of capping peptides molecules by molecular evolution. These artificial peptides showed higher affinity to the lignin-rich biomass than to the native biomass or pure cellulose. The preferred peptide (SSLQAHKPHHLR) promoted the production of reducing sugars from acid pretreated grass by 90% which significantly surpassed that with addition of BSA as control [96]. Another group identified the lignin-binding peptides processing characteristic sequences of HFPSP based on the phage display technique [112]. The additional outer determined the binding affinity of the peptides to lignin. Replacing Phe_7 with Ile_7 in a 12-mer peptide (HFPSPIFQRHSH) decreased the affinity of the peptide to softwood lignin while did not affected its binding with hardwood lignin. Obviously, the lignin-binding peptide is capable of recognizing the structural differences between different lignin structures [112].

4.5. Complexation of lignin with specific divalent metal ions

This strategy was developed by Liu et al. [13,55] for stabilizing the activity of cellulase exposed to both DLs and RLs with charged moieties, through the formation of lignin-metal complexes as supported by the experimental evidences provided by the following studies. Barsberg et al. [113] found a significant level of calcium in wheat straw ERL after wet oxidation pretreatment. The calcium-containing ERL showed minimal inhibition to cellulase, suggesting that the lignin-calcium complexes could reduce the enzyme-lignin interactions [113]. In the latest report of Vasconcellos et al. [114], various divalent metal ions were tested for promoting the efficiency of hydrolysis of an acid-pretreated bagasse whole slurry by using a homemade *A. niger* (hemi)cellulolytic enzyme. The stabilization effects of Mg^{2+} were reconfirmed by examining residual enzyme activity after 72-h hydrolysis. Furthermore, the addition of Mn^{2+} (10mM) significantly maintained the enzyme activity in the hydrolysates, resulting in approximately 110% of the residual EG activity after hydrolysis, and a 34%

increase of glucose production [114]. In another work conducted by Akimkulova et al.[115], Mg^{2+} was selected from a number of metal ions for blocking the nonproductive binding of cellulase onto residual lignin of dilute acid pretreated wheat straw. The optimal concentration of Mg^{2+} was 1 mM, but the increment in cellulose conversion was dependent on pH, time and cellulase loading [115].

The formation of lignin-metal complexes may reduce the enzyme binding sites, change the charge balance on substrate and thereafter weaken the hydrogen bonding and the electrostatic attraction between lignin and enzyme [13,113-115]. Akimkulova et al. [115] suggested that phenolic hydroxyl group was the main active site blocked by Mg^{2+} by comparing isolated lignins with tannic acid and a series of lignin model compounds. The interaction strength between Mg^{2+} and phenolic group of monolignol followed an order of p-hydroxyphenyl (H) >guaiacyl (G) >syringyl (S) [115]. Inspired by the role of metal ions, several novel pretreatment methods were developed based on the use of metal compounds such as thermal calcium hydroxide, microwave-assisted calcium chloride, surfactant-assisted metal chloride [25,116,117].

5. Conclusions

Thermochemical pretreatment of biomass produces dissolved lignin, residual lignin and lignin-derived phenolic molecules which could nonproductively bind to cellulases, resulting in enzyme inactivation, hydrolysis hinderance and unfeasible enzyme recyclability. The binding of cellulase to lignin is governed by hydrophobic interaction, electrostatic interaction and hydrogen bonding. The contribution of the three non-covalent forces strongly depends on both the enzymatic characteristics (*i.e.*, cellulose binding modules, key amino acid residues, and net surface charge) and the physiochemical properties of lignin (*i.e.*, hydrophobicity, charged groups, phenolic hydroxyl groups, and molecular weight). Lignin preferentially

binds to monocomponent cellulases, disturbs their synergistic action, and finally slows down the overall cellulose conversion. Through this review, better understandings on the cellulase-lignin interactions and the mechanisms of various elimination strategies have been obtained, leading to new research subjects toward a more economical cellulosic biorefinery through the sugar platform.

Minimization of nonproductive cellulase-lignin interactions could be achieved through enzyme engineering; substrate modification (*i.e.*, sulfonation, hydroxypropylation, and enzymatic oxidation); and addition of blocking chemicals (*i.e.*, surfactant/polyelectrolyte, protein/peptide, and metals). It is still not yet clear how the binding of cellulases with lignin consequently results in the decrease of catalytic efficiency through a more fundamental aspect. As discussed in this paper, binding may not be the direct cause of cellulase inactivation; and partial inactivation of an individual enzyme does not represent the invalidation of synergistic action. More research efforts are needed to elucidate those relationships, including establishment of appreciate analytical tools for evaluating the loss of synergy due to lignin inhibition.

Enzyme engineering works could target on overexpression of the key lignin-sensitive isozyme that determines the efficiency of synergistic action. Critical peptides could be heterologously expressed in cellulases producers. According to the new findings, cellulase activity can be well stabilized and hence be recycled after hydrolysis in the presence of lignin preferred sequence. The effectiveness of the reported peptide sequence should further be examined by using diverse lignin samples. Chemical modification to produce engineering lignin-resistant cellulases could be desired and is related to the development of enzyme post-processing. However, the costs and the storage stability of the final products must be taken into consideration.

New generation pretreatment methods, such as SO_2-asissted steam explosion and sulfite

cooking, have shown their robustness in improving the hydrophobicity of the various biomass substrates, while the appropriate environmental control techniques should be developed. Novel lignin derivation technologies with similar functions could be applied in biomass pretreatment. For example, lignin in the biomass can be grafted into water-soluble amphiphilic polymers during pretreatment. The residues can be readily digested by cellulases and hemicellulases even without intermediate washing. According to previous studies, the lignin-based amphiphiles would offer additional benefits to the hydrolysis and fermentation processes. Integrated production of biofuels and lignin-based value-added products can be an attractive alternative for future biorefinery.

Finally, lignin blocking additives would continuously receive attention because of the potential to improve the activities and recyclability of the enzyme. Non-hydrolytic proteins (especially the yeast extracts) are the most promising candidate for binding with lignin before the introduction of the cellulase. Yeast extracts would be available from the growth of fermentation microbes as well. It however needs more research on whether the lignin-adsorbed yeast extracts support the fermentation. In addition, attention may be paid on metal ions that can be generated from industrial wastewaters, which contains considerable amount of calcium and magnesium ions, as well as many other process transition metal ions. Identification of their roles in biorefining processes can help establish a more efficient and environmental-friendly biorefinery.

Nomenclature

ALD: Amphiphilic lignin derivatives

BG: β-glucosidase

BSA: bovine serum albumin

CBH: β-1,4-cellobiohydrolase

CBM: cellulose binding module

CED: celluloseenzymaticdigestibility

CM: catalytic module

CPAM: cationic polyacrylamide

DL: dissolved lignin

EG: β-1,4-endoglucanase

ERL: enzyme residual lignin

EXLX: nonplant expansin-like proteins

LDPMs: lignin-derived phenolic molecules

RL: residual lignin

PEG: polyethylene glycol

QCM: quartz crystal microgravimetry

TrCel7A: *Trichoderma reesei* Cel7A

TeCel7A-CBM1: cellulose binding module (Family 1) in the native *T. emersonii* Cel7A

Tween: polysorbates

Acknowledgements

The financial supports of the Fundamental Research Funds for the Central Universities (No. x2qsD2142050), the Foundation of State Key Laboratory of Pulp and Paper Engineering (No.2015QN03), Hong Kong General Research Fund - Early Career Scheme (No. 25201114), Open Project of State Key Laboratory of Urban Water Resource and Environment (No. HC201410), and Guangdong-Hong Kong jointed innovation program (No. 2014B050505019) are gratefully acknowledged.

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Captions of Figures and Tables

Figure 1 Schematic illustration of cellulase-lignin interactions forming dependent on lignin alteration during biomass pretreatment.

Figure 2 Illustrations of cellulase productive/nonproductive binding in presence of lignin.

Figure 3 Illustrations of blocking cellulase-lignin binding with additives.

Table 1 Nonproductive binding and denaturing of cellulase by lignin from various hydrothermal pretreatments.

Table 2 Efficiency of various polymers for minimizing nonproductive cellulase-lignin binding.

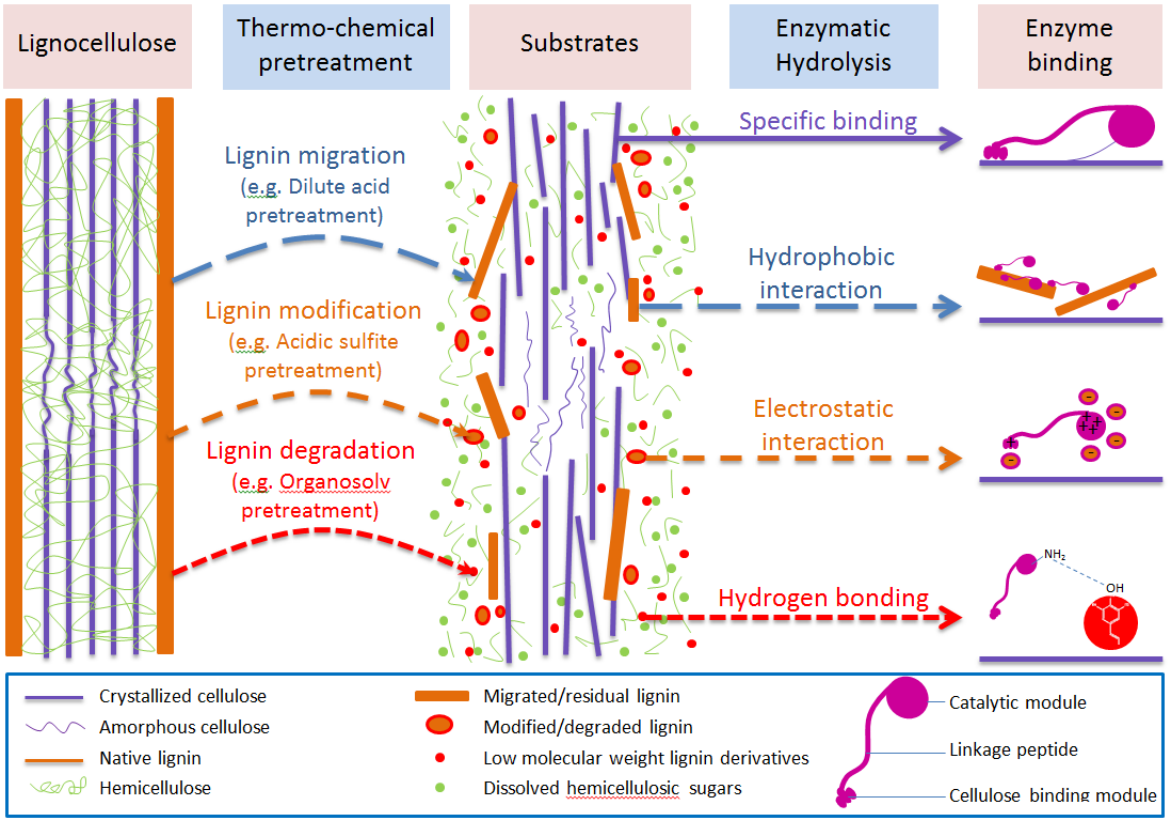
Lignocellulose
Thermo-chemical pretreatment
Substrates
Enzymatic Hydrolysis
Enzyme binding
Lignin migration
(e.g. Dilute acid pretreatment)
Lignin modification
(e.g. Acidic sulfite pretreatment)
Lignin degradation
(e.g. Organosolv pretreatment)
Specific binding
Hydrophobic interaction
Electrostatic interaction
Hydrogen bonding
Crystallized cellulose
Amorphous cellulose
Native lignin
Hemicellulose
Migrated/residual lignin
Modified/degraded lignin
Low molecular weight lignin derivatives
Dissolved hemicellulosic sugars
Catalytic module
Linkage peptide
Cellulose binding module

Figure 1

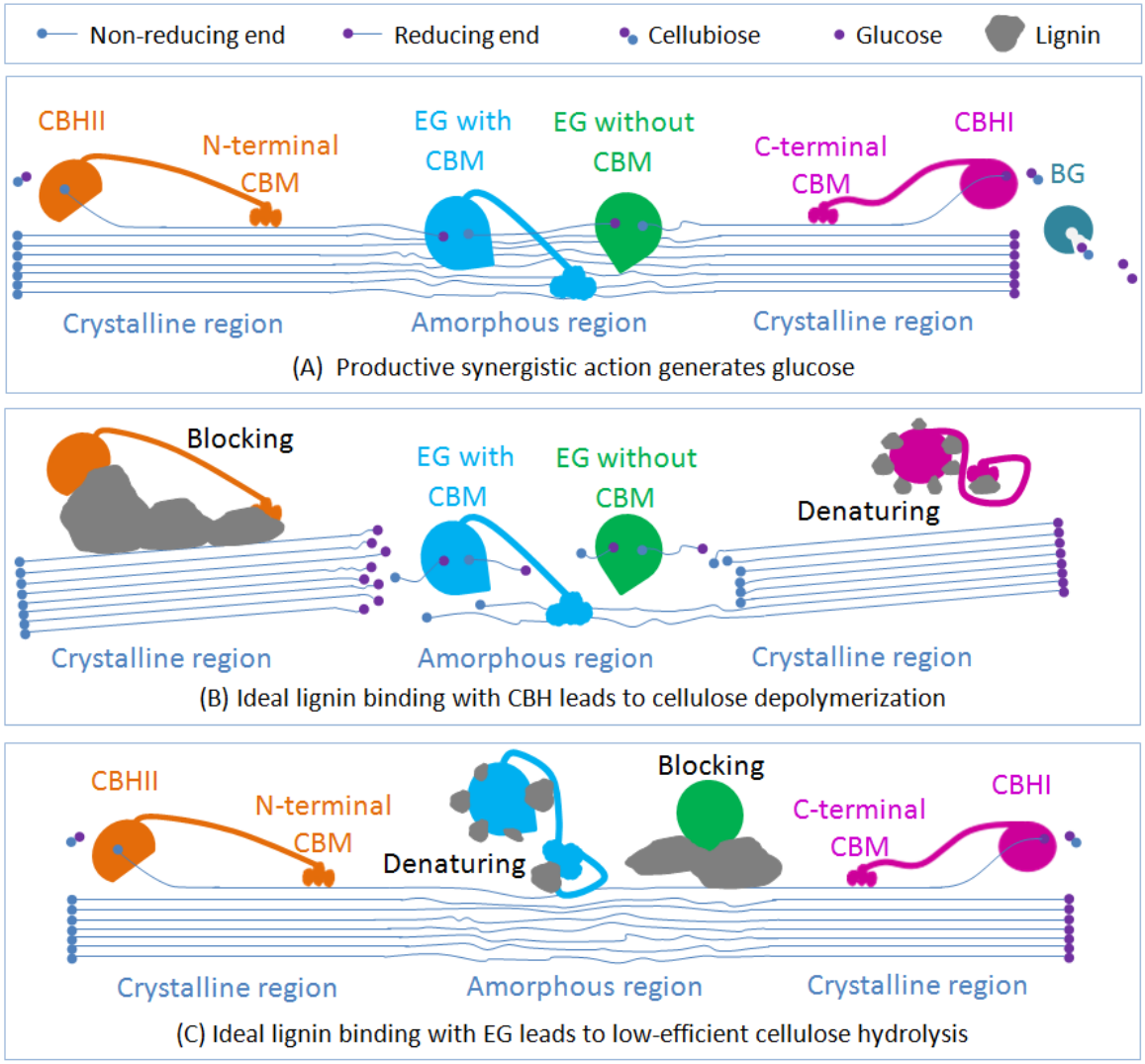
Non-reducing end
Reducing end
Cellubiose
Glucose
Lignin
CBHII
N-terminal CBM
EG with CBM
EG without CBM
C-terminal CBM
CBHI
BG
Crystalline region
Amorphous region
Crystalline region
(A) Productive synergistic action generates glucose
Blocking
EG with CBM
EG without CBM
Denaturing
Crystalline region
Amorphous region
Crystalline region
(B) Ideal lignin binding with CBH leads to cellulose depolymerization
CBHII
N-terminal CBM
Denaturing
Blocking
C-terminal CBM
CBHI
Crystalline region
Amorphous region
Crystalline region
(C) Ideal lignin binding with EG leads to low-efficient cellulose hydrolysis

Figure 2

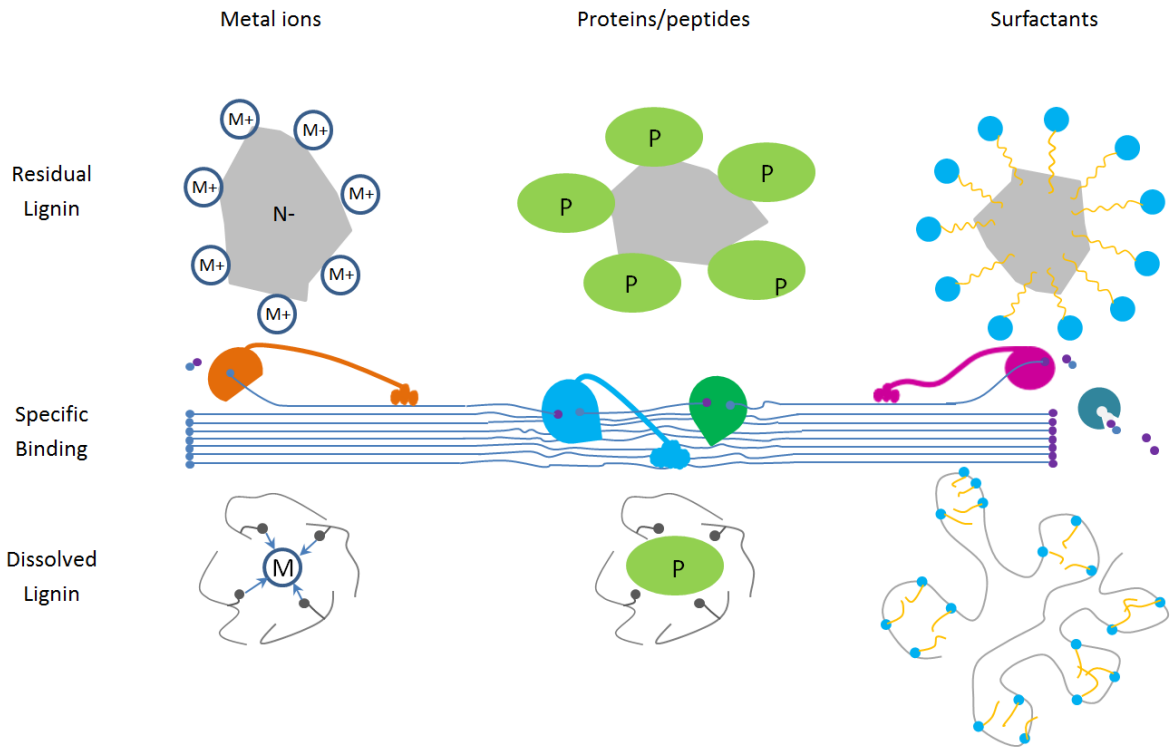
Metal ions
Proteins/peptides
Surfactants
Residual Lignin
Specific Binding
Dissolved Lignin
M+
N-
P
M

Figure 3

Table 1

Lignin	Non-productive binding effects	ef.
SDL from acidic sulfite pretreatment of eucalyptus	(1) Addition of SDL in filter paper cellulose hydrolysis mixture to 0.4 g/L, cellulose conversion was decreased from 97% to 72%. (2) SDL inhibited cellulase activity more strongly than ODL or KDL.	3
SDL from sulfite pulping of poplar, fractionated by ultrafiltration to SDL1 (Mn~8100), SDL2 (~3800) and SDL3 (~800)	(1) SDL1 (5 g/L) decreased the glucose yield after 72-h hydrolysis of Whatman filter paper by CTEC II from 30% for control to 24%. Equivalent SDL2 and SDL3 improved the glucose yield to 35% and 46% respectively. (2) SDL2 and SL3 offered significantly promoted bioconversion efficiency of dilute acid-aspen, acidic sulfite-aspen, kraft pulped lodgepole pine and acidic sulfite-lodgepole pine. SDL1 offered less beneficial effect.	0
Phenolic compounds simulating LDPMs from biomass pretreatment	The FPUase of cellulasewas enhanced by 28%, 15% and 10%in presence of ferulic acid (0.83 mg/ml), *p*-coumaric acid (0.83 mg/ml), and salicylic acid (0.67 mg/ml)respectively,.	3
Phenolic compounds simulating LDPMs fromsteam explosion of corn stover	(1) Phenolic aldehyde inhibited the activity of *Trichodermaviride*Cellulasestowards Whatman filter paperat 0.05−8 g/L. (2) phenolic acids caused significantly inhibition at 0.05 g/L but slight stimulation at 2−4 g/L. (3) LDPMs mixture had a concentration-dependent effect on cellulase activity	2
Phenolics in aqueous filtrates from liquid hot water pretreatment of bagasse or from acetone extracts	(1) Phenolics from acetoneextraction mainly deactivatedthe β-glucosidase or β-xylosidase components. (2) Pehnolics from hot-water pretreatment liquor (6.2 mg phenolics/mg protein) lowered the cellulose conversion by 45% after 72 h hydrolysis with CTEC II.	7
Isolated ODLs from hardwood poplar and softwood lodgepole pine	(1) Glucose yield from pure cellulose (~60%) was decreased to ~10% by addition of unmodified ODL after 48-h hydrolysis with Celluclast and Novozyme 188. (2) Carboxylation and sulfonation of lignin promoted its	4

	hydrophicilityby 22–30% and thereby reduced the lignin inhibition by 76–96%.	
ODL and ERL from Organosolv pretreatment of Douglasfir	ODL (100 wt% towards substrate) decreased the activity of Spezyme CP (75%) and Celluclast 1.5L (63%) with microcrystalline cellulose as substrate. The addition of ERL led to less inhibition (53% and 37%). Both lignins exerted similar level inhibition to Novozym 188 activity (8-11%)	5
ERL from SO_2-asissted steam explodedDouglas fir	ERL decreased MCC conversion by 49%. The higher pretreatment temperature (e.g. from 190ºC to 210ºC), the lower ERL inhibitory effects	6
ERLs from dilute acid-corn stover, steam explosion-corn stover and steam explosion-rice straw	All ERLs (4 mg/mL) could bind withcellulasein hydrolysis of MCC (6mg/mL),showing lower affinity to enzyme than MCC. At a low enzyme loading (3 FPU/$g_{Cellulose}$), MCC conversion was decreased by 9.5%-11.8% by the three ERLs. Even at a higher loading (15 FPU/$g_{Cellulose}$), ERLs caused considerable decrease in cellulase efficiency.	7
Wood milled lignin (MWL) from original and autohydrolysis pretreated eucalyptus, maple or pine	(1) MWLsisolated from pretreated woods adsorbed 2~6 times more cellulase than those from original woods. (2) Cellulase binding rose with the increase in condensation degree and content of phenolic hydroxyl groups, but declined with the content of total hydroxyl groups. (3) Residual lignin in pretreated biomass (21.5~26.5%) exhibited stronger inhibitory effects than MWL on enzymatic conversion, e.g. about 27% lower cellulose conversion after 96-h hydrolysis by commercial cellulases and xylanases.	8

Note: SDL- Dissolved lignin from sulfite pretreatment; ODL-Dissolved lignin from Organosolv pretreatment; KDL- Dissolved lignin from Kraft process; LDPMs- Lignin-derived phenolic molecules; ERL-enzyme residual lignin;MWL- Wood milled lignin

Table 2

Additives	Substrate	Results	ef.

Tween 80 (0.95% w/v)	Alkaline/H_2O_2 pretreated Sugarcane bagasse	29 % improvement in glucose concentration after 120-h hydrolysis with cellulase (4.1 FPU/g solids) and β-glucosidase (18.2 CBU/g)	8
Tween 80 or Triton X-100 (2.5 g/L)	Alkaline-auto claved elephant grass	The yield of reducing sugar was increased from516 mg/g $_{Biomass}$ to 567 mg/g $_{Biomass}$ and 717 mg/g $_{Biomass}$ with Tween 80 and Triton X-100, respectively, within 72-h hydrolysis by *P. echinulatum* cellulase.	9
Tween 80 (0.4%, v/v)	Hot water treated newspaper	10% improvement in sugar conversion after 48-h enzymatic hydrolysis. Mechanical refining weakened the role of surfactant	0
BSA, PEG 6000, Tween 80, and/or xylanase	Ammonia-pret reated bamboo residues	Glucose yield was increased from 21~29% to 32~36% with BSA, 33~35% with PEG 6000, 32~37% with Tween 80, 44~62% with xylanase, or 50~73% with PEG 6000 and xylanase, depending on biomass species	1
PEG 6000	Avicel& steam- exploded corn stover	Conversion rates of corn stover and Avicel PH101 were increased to 54% and 87% with addition of PEG 6000. More cellulase activity (68%) was retained than control (39%).	2
PEG 4000	Steam-mecha nical pretreated softwood	Conversion of biomass to glucose was improved from 27% to 43% after 72-h enzymatic hydrolysis with 0.01 g/g$_{Substrate}$ PEG.	3
BSA and Tween 80	Acidic sulfite-aspen	(1) For unwashed substrate, BSA (10 mg/g $_{Biomass}$) offered 22% increment in cellulose conversion, comparable with the effects by using 70 mg/g $_{Biomass}$Tween 80. (2) For washed substrates, cellulose conversion was increased by 28~37%.	5
BSA, Corn steep liquor, yeast extract, and peptone	Alkaline-pretr eated Rice straw	Glucose concentration after 72 h of hydrolysis was increased by 12.7%, 13.5%, and 13.7% after	4

		addition of corn steep liquor, yeast extract, and peptone, respectively, better than BSA.	
Expansin-like non-catalyticprotein from *Xanthomonascampestris*	Cellulose & pretreated grass	(1) Sugar release from pure cellulose was increased by up to36 %. (2) Preferential binding of BsEXLX1 to lignin than cellulose, with higher affinity compared with CBM.	[5]
Engineered peptides	Heat-pretreated Napier grass	Peptides (40 μg/mL) promoted the production of reducing sugars by 90%.	[6]
Amphiphilic lignin derivatives	Soda-AQ pulp	(1) Sugar yield was promoted from 0.1 to 0.77 g/g. (2) Cellulase could be recycled by many times. (3) Bioethanol yield could be increased by 30%	[7,98]
Amphiphilic lignin derivatives	Steam-exploded corn stover	Glucose yield of corn stover at 20% (w/v) was improved from 32.8% to 63.8%	[9]
Cationic polyacrylamide or polyDADMAC (0.04 $g/g_{Substrate}$)	Avicel, kraft pulp & Recycled paper mill sludge	Digestibilities of glucanwas improved from 50-60% to 58-68% after 120-h hydrolysis with CTEC II (5 FPU/g_{Glucan}) depending on the solid consistency.	[00]
NIPAm-based copolymers (2 g/L)	Avicel& acid-assisted steam exploded *Miscanthus*grass	Celluclastrequirement was reduced by 60% to yield the same level of glucose from*Miscanthus* after 12-h hydrolysis. Much less beneficial effect was found in the case of Avicel.	[01]