

Compounds inhibiting the bioconversion of hydrothermally pretreated lignocellulose

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Abstract Hydrothermal pretreatment using liquid hot water, steam explosion, or dilute acids enhances the enzymatic digestibility of cellulose by altering the chemical and/or physical structures of lignocellulosic biomass. However, compounds that inhibit both enzymes and microbial activity, including lignin-derived phenolics, soluble sugars, furan aldehydes, and weak acids, are also generated during pretreatment. Insoluble lignin, which predominantly remains within the pretreated solids, also acts as a significant inhibitor of cellulases during hydrolysis of cellulose. Exposed lignin, which is modified to be more recalcitrant to enzymes during pretreatment, adsorbs cellulase nonproductively and reduces the availability of active cellulase for hydrolysis of cellulose. Similarly, lignin-derived phenolics inhibit or deactivate cellulase and β -glucosidase via irreversible binding or precipitation. Meanwhile, the performance of fermenting microorganisms is negatively affected by phenolics, sugar degradation products, and weak acids. This review describes the current knowledge regarding the contributions of inhibitors present in whole pretreatment slurries to the enzymatic hydrolysis of cellulose and fermentation. Furthermore, we discuss various biological

strategies to mitigate the effects of these inhibitors on enzymatic and microbial activity to improve the lignocellulose-to-biofuel process robustness. While the inhibitory effect of lignin on enzymes can be relieved through the use of lignin blockers and by genetically engineering the structure of lignin or of cellulase itself, soluble inhibitors, including phenolics, furan aldehydes, and weak acids, can be detoxified by microorganisms or laccase.

Keywords Lignocellulose · Hydrothermal pretreatment · Inhibitor · Lignin · Phenolics · Detoxification

Introduction

Hydrothermal pretreatment significantly enhances the efficiency of enzymatic hydrolysis by disrupting the recalcitrant structure of lignocellulosic biomass. However, there are several barriers associated with this pretreatment that prevent the development of an efficient bioconversion process. One of the most significant barriers is the generation of inhibitory compounds within the pretreated biomass slurries that interfere with the enzymatic hydrolysis and subsequent fermentation processes. As the high cost of cellulolytic enzymes is still the primary financial hurdle for achieving economical production of bioethanol from lignocellulosic biomass (Klein-Marcuschamer et al. 2012), various efforts have focused on reducing the amount of enzyme required for this process by recognizing the effects of inhibitors on cellulases. Therefore, in this review, we highlight the results of recent studies on the effects of inhibitors, including soluble phenolics, oligosaccharides, sugar degradation products, and insoluble lignins, which are present in the whole slurries of hydrothermally pretreated lignocellulose, on enzymatic hydrolysis as well as on microbial fermentation. Since multiple previous reviews

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have focused on the soluble inhibitors present in the hydrolysates and their effects on microorganisms (Almeida et al. 2007; Jönsson et al. 2013; Mussatto and Roberto 2004; Palmqvist and Hahn-Hägerdal 2000), the recent studies on the impact of inhibitors on enzymes are provided in this work with more details than that on microorganisms. The biological strategies to overcome the negative effects of these inhibitors on cellulolytic enzymes and on fermentative microorganisms are also discussed.

Hydrothermal pretreatment

Lignocellulose has a recalcitrant and complex structure, which is composed of cellulose surrounded by hemicellulose, lignin, and acetyl groups (Kumar et al. 2009). For the efficient conversion of biomass into biofuel, pretreatment is necessary to reduce the recalcitrance of the lignocellulose through the induction of physical or chemical changes in the complex cell wall structures (Mosier et al. 2005). Hydrothermal pretreatment, including liquid hot water, steam explosion, and dilute acid pretreatments, has been widely employed to achieve high conversion yields of lignocellulose. While liquid hot water pretreatment is performed by pretreating biomass at temperatures between 140 and 220 °C under high pressure, to maintain water in the liquid state (Kim et al. 2009b, 2013b, 2015), steam explosion pretreatment employs explosive decompression resulting in the separation of biomass structures (Alvira et al. 2010; Avellar and Glasser 1998; Brownell et al. 1986). During steam explosion pretreatment, biomass is exposed to pressurized steam for several seconds or minutes followed by a sudden decompression. Many steam explosion pretreatments also include acid catalysts (e.g., SO₂ explosion) to enhance hemicellulose solubilization (Brownell et al. 1986; Tengborg et al. 1998). Dilute acid-catalyzed hydrothermal pretreatment has also been accepted as one of the most promising biomass pretreatment strategies (Jung et al. 2013, 2014; Wyman et al. 2009). Although steam or liquid hot water pretreatment is milder than dilute acid-catalyzed hydrothermal pretreatment, the chemical and physical changes that occur in the structure of lignocellulose due to steam or liquid hot water pretreatment are similar to those observed after dilute acid-catalyzed hydrothermal pretreatment (Mosier et al. 2005; Pu et al. 2013). Each of these pretreatment strategies increases the accessibility of cellulase enzymes to cellulose, primarily by solubilizing the hemicellulose fraction while the majority of the lignin and cellulose remain in the solid fraction.

During hydrothermal pretreatment, water is utilized as the only reaction medium (no additional chemicals are included) for the heating of biomass. This is advantageous in that it lowers the constructing costs of pretreatment reactors and is less hazardous to the environment (Alvira et al. 2010; Avellar and Glasser 1998). During the pretreatment process, the

reaction medium becomes acidic due to the release of acetic acids and other weak acids derived from hemicellulose and to the auto-dissociation of water at elevated temperatures. The release of acetic acid from biomass facilitates the solubilization of hemicellulose and the formation of monomeric sugars and sugar degradation products, such as 5-(hydroxymethyl)furfural (HMF), furfural, formic acid, and levulinic acid (Cantarella et al. 2004; Kim et al. 2009b, 2011; Gurram et al. 2011).

Technical barriers to hydrothermal pretreatment

While hydrothermal pretreatment enhances the efficiency of enzymatic hydrolysis by solubilizing xylan and by increasing the porosity and reducing the particle size of biomass (Kim et al. 2015), several technical barriers still hinder the biomass-to-ethanol conversion process. Pretreatment leads to the formation of compounds that are toxic to enzymes or microorganisms (Kim et al. 2013c, 2015), including soluble sugars (glucose, xylose, cellobioses, and xylobioses), furan derivatives (HMF and furfural), weak acids (acetic, formic, and levulinic acids), and phenolic compounds (vanillin, ferulic acid, etc.) (Gurram et al. 2011; Kim et al. 2011; Klinker et al. 2004; Palmqvist and Hahn-Hägerdal 2000) (Table 1). Depending on the type and severity of the pretreatment, the concentration and distribution of these toxic and inhibitory compounds can vary. The generation of inhibitors also depends on the type and solid loading of lignocellulosic biomass in the pretreatment media (Cantarella et al. 2004; Dekker 1988; Excoffier et al. 1991). When maple woods at 230 g/L were pretreated with liquid hot water at 200 °C for 20 min, the concentrations of xylobioses and xylose in the pretreatment liquid were greater than 11.2 and 9.2 g/L, respectively. Meanwhile, sugar degradation products (HMF and furfural) were formed at levels as high as 4.1 g/L, and the concentration of phenolics was 1.3 g/L (Kim et al. 2011) (Table 1). When the purified and processed cellulose Solka-Floc, a mixed form of crystalline and amorphous cellulose, was incubated with pretreatment liquid containing inhibitors, the initial hydrolysis rate and glucose yield decreased by 50 % compared to that of the Solka-Floc incubated with buffer only (Kim et al. 2011). Inhibitors generated from steam-explosion-pretreated barley straw, such as furfural (0.7 g/L), HMF (0.2 g/L), acetic acid (2.1 g/L), and phenolics (0.2 g/L), also strongly affected the enzymatic hydrolysis step (García-Aparicio et al. 2006). These findings demonstrate that the soluble inhibitors generated from lignocellulose significantly hamper the enzymatic hydrolysis of cellulose.

While pretreated solids may be separated from pretreatment slurries and washed to remove soluble inhibitors (Kim et al. 2013c), the remaining lignin in pretreated solids still provides a significant inhibitory effect on the enzyme hydrolysis of cellulose. Indeed, the pretreated solids retain the

Table 1 Soluble inhibitors present in the prehydrolysates of hydrothermally pretreated lignocellulose

Biomass	Pretreatment conditions	Soluble inhibitors in prehydrolysates							Reference
		Xylooligomer, g/L	Monomeric sugar, g/L	Furan aldehyde, g/L	Phenolics, g/L	Acetic acid, g/L	Others, g/L		
Hardwood (maple)	Liquid hot water (200 °C and 20 min) 230 g/L ^a	11.2	Glucose, 0.6 Xylose, 9.2	4.1	1.3 ^b	13.1	Glycerol, butanediol, 1.0	Kim et al. (2011)	
Agricultural residue (corn stover)	Dilute acid (150 °C and 15 min) 100 g/L ^a	0.2	Glucose, 3.3 Xylose, 17.4 Arabinose, 2.6	HMF, 0.1 Furfural, 0.8	–	3.2	NA	Cao et al. (2013)	
Agricultural residue (corn stover)	Liquid hot water (190 °C and 15 min) 100 g/L ^a	10.7	Glucose, 0.15 Xylose, 1.3 Arabinose, 0.6	HMF, 0.1 Furfural, 0.7	–	2.2	NA	Cao et al. (2013)	
Agricultural residue (barley straw)	Steam explosion (210 °C and 5 min) 100 g/L ^a	NA	Glucose, 4.6 Xylose, 17.4 Arabinose, 1.9	HMF, 0.2 Furfural, 0.7	0.2 ^c	2.1	Formic acid, 0.8	García-Aparicio et al. (2006)	
Hardwood (poplar)	Steam explosion (210 °C and 4 min)	NA	NA	HMF, 0.08 Furfural, 0.5	0.2 ^c	2.1	Formic acid, 0.4	Oliva et al. (2003)	
Softwood (pine)	Dilute acid (160 °C and 30 min) 100 g/L ^a	NA	Glucose, 9.7 Xylose, 9.3 Arabinose, 1.4	HMF, 1.1 Furfural, 2.2	NA	7.3	NA	Gurram et al. (2011)	

NA not available

^a Solid loading (gram solid per liter)

^b Total phenolics were measured using the Folin-Ciocalteu method (Kim et al. 2011)

^c Concentrations of phenolics, including 4-hydroxybenzaldehyde, 4-hydroxybenzoic acid, catechol, syringaldehyde, syringic acid, vanillin, vanillic acid, ferulic acid, and coumaric acid, were measured by high-performance liquid chromatography (HPLC) (García-Aparicio et al. 2006)

majority of the initial lignin after the hydrothermal pretreatment, including liquid hot water, steam explosion, and dilute acid treatments (Carvalho et al. 2009; Hu et al. 2012; Sannigrahi et al. 2008). As the pretreatment solubilizes hemicellulose and opens up macropores within the biomass, the amount of lignin exposed to cellulase also increases. Lignin adsorbs cellulases nonproductively, inhibiting the activity of these enzymes on cellulosic substrates (Kim et al. 2015; Ko et al. 2015a, b).

Pretreatment-derived inhibitors for enzymatic hydrolysis and fermentation

The inhibitory compounds present in whole slurries of pretreated lignocellulose fall into five categories: lignin, soluble phenolics, sugars, furan aldehydes, and weak acids (Fig. 1). The production of each of these inhibitors during pretreatment and their impact on the enzymatic hydrolysis of cellulose are reviewed here.

Lignin

Lignin is one of the most abundant macromolecules in nature, comprising 15–40 % of plant biomass (Gellerstedt and Henriksson 2008). Lignin is widely accepted as the major inhibitory factor generated during enzymatic hydrolysis of cellulose, inhibiting hydrolysis through the formation of physical barriers and the nonproductive binding of cellulase enzymes (Chang and Holtzapple 2000). Therefore, the presence of lignin restricts both the access of enzymes to cellulose and reduces the amount of active enzymes for cellulose hydrolysis (Nakagame et al. 2011b). After hydrothermal pretreatment using liquid hot water, steam explosion, or dilute acids, most of the initial lignin remains within the solid fraction, while hemicellulose is removed (Carvalho et al. 2009; Ko et al. 2015a, b; Sannigrahi et al. 2008). Indeed, in a recent study, the lignin recovery in the solid fraction was 80–90 % when hardwoods were pretreated with liquid hot water at temperatures of 180–220 °C (Ko et al. 2015a). As the pretreatment severity increases, the lignin content in the biomass composition of the pretreated solids also increases. The increase in lignin content during hydrothermal pretreatment accompanies the changes in lignin structure due to the simultaneous de- and re-polymerization reactions of lignin (Chua and Wayman 1979a, b; Ko et al. 2015a, b; Li et al. 2007). Recent findings suggest that liquid hot water pretreatment modifies the lignin structure to a more condensed and heterogeneous form that is more inhibitory to cellulase than that of lignin prior to pretreatment (Ko et al. 2015a).

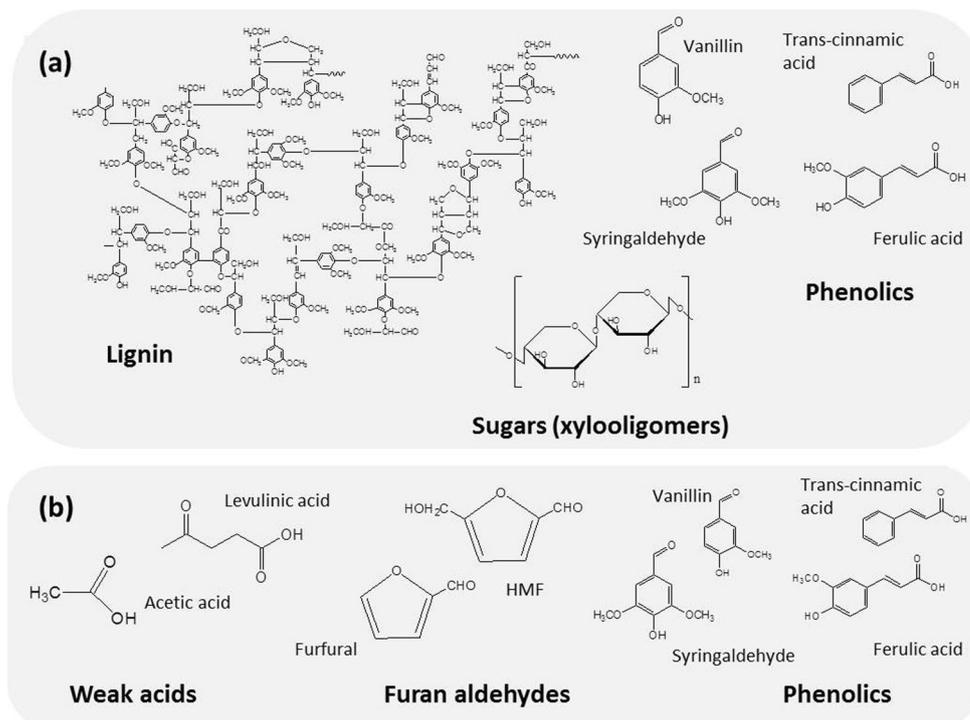
The mechanism by which lignin inhibits hydrolysis via nonproductive adsorption has been widely reported (Bernardez et al. 1993; Tu et al. 2009). Many previous studies

demonstrated that the nonproductive adsorption of enzymes to lignin is a significant hurdle for improving the efficiency of enzymatic hydrolysis of cellulose at low enzyme loadings (Bernardez et al. 1993; Kim et al. 2015; Ko et al. 2015a, b; Nakagame et al. 2011a; Rahikainen et al. 2011; Tu et al. 2009). When Avicel, the microcrystalline cellulose derived from wood pulp, was hydrolyzed in the presence of lignin isolated from hardwoods, the glucose yield decreased from 62.5 to 51 % (Ko et al. 2015a, b). Nakagame et al. (2011a) also showed that the hydrolysis yield of Avicel decreased from 70 to 40–45 % in the presence of lignin isolated from steam-pretreated softwood. The inhibitory role of lignin was also indirectly evaluated in multiple studies using lignin surface blockers such as surfactants and noncatalytic proteins (Eriksson et al. 2002; Kumar et al. 2012; Yang and Wyman 2006). To better understand lignin-enzyme interactions, the adsorption behaviors of enzymes in the presence of steam-, liquid hot water-, or dilute-acid-pretreated lignocellulose or isolated lignin were investigated (Ko et al. 2015b; Nakagame et al. 2011a; Tu et al. 2009). The Langmuir isotherm has been widely applied to model the adsorption process by correlating the amount of adsorbed molecules with that of free (unadsorbed) molecules in equilibrium (Langmuir 1918). The values of Langmuir adsorption constants of cellulases, including the maximum adsorption capacity and the adsorption affinity, indicated that enzyme-lignin interactions are dependent on the biomass type, the pretreatment type, and the severity of pretreatment. With respect to biomass type, softwood lignin adsorbed cellulase more strongly than lignin from hardwoods or agricultural residues and thereby inhibited the enzymatic hydrolysis of cellulose to a higher degree in these samples (Nakagame et al. 2010, 2011a). The strong inhibition of softwood lignin on enzymatic hydrolysis might be due to its more condensed and hydrophobic structure resulting in the enhanced hydrophobic interaction between lignin and enzymes (Ko et al. 2015b; Nakagame et al. 2011b). In addition, the strength of the interactions between lignin and cellulase increased as the pretreatment severity increased (Ko et al. 2015b). When 10 mg of cellulase was incubated with lignin isolated from liquid-hot-water-pretreated hardwoods, 50–60 % of the total cellulase was lost due to the adsorption to lignin. Furthermore, among the enzymatic components of the commercial cellulase preparation Cellic CTec2, the majority of the β -glucosidase activity (>90 %) was lost due to adsorption to lignin (Ko et al. 2015b). Recently, the importance of recognizing and mitigating the inhibitory effects of lignin on enzymatic activities to achieve high glucose yields at low enzyme loadings was brought into focus (Kim et al. 2015).

Lignin-derived phenolics

Phenolics derived from lignocellulose, when present at micromolar to millimolar concentrations, can impart a significant

Fig. 1 Compounds present in the whole slurries of hydrothermally pretreated lignocellulose that are inhibitory to **a** cellulase or **b** fermenting microorganisms



inhibitory effect on enzymes during cellulose conversion (Tejirian and Xu 2011; Ximenes et al. 2010). When high solid pretreatment is performed, soluble phenolics can be generated at levels that are inhibitory to enzymatic hydrolysis (Ximenes et al. 2011). Furthermore, the inhibition and/or deactivation of enzymes was reportedly caused by phenolics extracted from pretreated slurries or by model phenolic compounds (Kim et al. 2011; Tejirian and Xu 2011; Ximenes et al. 2010, 2011). Simple phenolic compounds within pretreatment slurries originate from lignin degradation products, including syringic acid, vanillin, ferulic acid, *p*-hydroxybenzoic acid, syringaldehyde, vanillic acid, *trans*-cinnamic acid, and coumaric acid (Jönsson et al. 1998; Kim et al. 2013c; Ximenes et al. 2010).

Among the soluble toxic compounds, including soluble sugars, furan derivatives, weak acids, and phenolics, originating from liquid hot water-pretreated hardwood slurries, phenolics were the strongest inhibitors of cellulase (Kim et al. 2011). At 1 and 25-mg enzyme loadings per gram of glucan, the presence of phenolics at 1.3 g/L decreased both the rate and extent of cellulose hydrolysis by half (Kim et al. 2011). In a recent study, a 10-mM (=1.8 g/L) concentration of a phenolic compound, syringyl aldehyde, reduced the yield of enzymatic hydrolysis of cellulose by approximately 70 % (Tejirian and Xu 2011). Meanwhile, oligomeric phenolics, such as tannic acid, were found to exhibit even greater inhibitory activity toward cellulase than simple phenolics (Tejirian and Xu 2011; Ximenes et al. 2011). The inhibitory effect of tannic acid on cellulase has also been reported (Mandels and Reese 1965). In

particular, a 1-mM concentration of tannic acid inhibited cellulose hydrolysis by 70–80 %, and the I_{50} of tannic acid was found to be 0.27 mM (Tejirian and Xu 2011).

While the inhibition of enzymes occurs instantly upon exposure to phenolics, the enzyme deactivation results in a steady loss of enzymatic activity as the length of exposure to the phenolics increases (Kim et al. 2011; Ximenes et al. 2011). It is because the phenolic compounds present in the pretreatment liquid coprecipitates with cellulase (Kim et al. 2011). The inhibition or deactivation of cellulase is dependent on the microbial source and the type (exo- and endocellulases and β -glucosidase) of enzyme, as well as on the type of phenolic compounds present. While gallic or *p*-coumaric acid deactivated 50–70 % of *Trichoderma reesei* β -glucosidase activity (Spezyme CP) after 24 h of incubation, the β -glucosidase from *Aspergillus niger* was more resistant to phenolics, exhibiting less than a 20 % loss in enzyme activity after a similar exposure to these compounds (Ximenes et al. 2011). Comparison of the deactivation rate constants of the β -glucosidases from *T. reesei* and *A. niger* upon exposure to polymeric and monomeric phenolics demonstrated that binding of phenolic inhibitors at the enzyme active site resulted in time-dependent deactivation of β -glucosidase (Ximenes et al. 2011).

Soluble phenolics are toxic, not only to cellulase but also to fermenting microorganisms via the disruption of microbial membrane integrity (Almeida et al. 2007; Heipieper et al. 1994; Palmqvist and Hahn-Hägerdal 2000). Previous studies demonstrated an association between the concentrations of

phenolics in prehydrolysates and the degree of inhibition of both yeast growth and ethanol production (Adeboye et al. 2014; Clark and Mackie 1984; Delgenes et al. 1996; Larsson et al. 2000). Low-molecular-weight phenolics, including syringaldehyde, syringic acid, hydroxybenzaldehyde, coniferyl aldehyde, and vanillin, are known to be strong inhibitors of fermentation, even at low concentrations (Adeboye et al. 2014; Clark and Mackie 1984; Delgenes et al. 1996; Palmqvist and Hahn-Hägerdal 2000). For example, coniferyl aldehyde at >0.2 g/L significantly inhibited both the growth and ethanol production of *Saccharomyces cerevisiae* (Larsson et al. 2000).

Soluble sugars

Substrate and end product inhibition of enzymatic activity have long been a primary concern for enzymatic hydrolysis of cellulose (Gong et al. 1977; Hong et al. 1981). During enzymatic hydrolysis, the accumulation of glucose and cellobiose inhibits β -glucosidase and cellobiohydrolase, respectively (Philippidis et al. 1993). Furthermore, several studies have shown that the glucose, cellobiose, and celooligomers generated during cellulose hydrolysis inhibit cellulase (Gusakov and Sinitsyn 1992; Holtzaple et al. 1990). As a result, these sugar compounds are regarded as significant inhibitors of cellulases that affect this enzyme by binding to the active site (Qing et al. 2010; Tengborg et al. 2001). Prior to enzymatic hydrolysis, high levels of soluble sugar monomers and oligomers released during pretreatment may result in an inhibitory effect on enzymatic activity.

The solubilization of hemicellulose and the formation of monomeric sugars from hemicellulose occur during hydrothermal pretreatment, whether or not catalysts are utilized (Kim et al. 2009b). As shown in Table 1, for example, dilute-acid-pretreated corn stover generated 17.4 g/L of xylose and 0.2 g/L of xylooligomers (Cao et al. 2013). In comparison, liquid hot water pretreatment of corn stover generated 10.7 g/L of xylooligomers and 1.3 g/L of xylose in the liquid fraction (Cao et al. 2013). Similarly, 11.2 g/L of xylooligomers was present in the liquid fraction of hot-water-pretreated maple (Kim et al. 2011). Among xylose, xylan, and xylooligomers, xylooligomers were the strongest inhibitor of hydrolysis when added to Avicel (Kim et al. 2011; Qing et al. 2010). Specifically, the enzymatic hydrolysis of Avicel decreased from 80 to 40 % in the presence of 12.5 g/L of xylooligomers (Qing et al. 2010). Similarly, in the presence of 8 g/L of xylooligomers, the rate of enzymatic hydrolysis of cellulose decreased by approximately 40 %, and thus, the glucose yield was reduced by 20 %. Therefore, the supplementation of cellulase for pretreated biomass with xylanase which hydrolyzes xylooligomers to less inhibitory xylose has been suggested as an alternative method for enhancing the enzymatic hydrolysis of cellulose (Kumar and Wyman 2009;

Qing et al. 2010). Also, hydrolysis and fermentation can be performed simultaneously (simultaneous saccharification and fermentation, SSF; simultaneous saccharification and cofermentation, SSCF) to avoid inhibition of cellulase by sugars (Jönsson et al. 2013).

Furan aldehydes and weak acids

Sugars can be further degraded to furan aldehydes during hydrothermal pretreatment. Furan aldehydes, such as furfural and HMF, are formed from pentose and hexose, respectively (Dunlop 1948; Ulbricht et al. 1984). Moreover, while acetic acid is liberated from hemicellulose during pretreatment or hydrolysis, weak acids such as levulinic acid and formic acid can be generated through degradation of furan aldehydes (Jönsson et al. 2013; Ulbricht et al. 1984). Furan aldehydes and acetic acid are well-known inhibitors of fermenting microorganisms but have little effect on the activity of cellulases (Allen et al. 2010; Kim et al. 2011; Palmqvist et al. 1999). Indeed, the presence of acetic acid (13 g/L) and furfural (4 g/L) had no effect on cellulase activity (Kim et al. 2011). As fermentation inhibitors, furfural and HMF inhibit cell growth and ethanol production of yeast (Ask et al. 2013; Banerjee et al. 1981; Larsson et al. 1999a). Furan aldehydes inhibit yeast fermentation by reducing enzymatic and biological activities and by causing oxidative cellular damages (Allen et al. 2010; Modig et al. 2002; Sanchez and Bautista 1988). Furthermore, yeast can reduce furfural to furfuryl alcohol in fermentation media, resulting in a prolonged lag phase during ethanol fermentation. It was demonstrated that both furfural and HMF decreased the volumetric production of ethanol by yeast, although a decrease in the final ethanol yield was not observed (Larsson et al. 1999a). Notably, however, furfural was found to have a greater inhibitory effect on cell growth than on ethanol production (Palmqvist et al. 1999).

Weak acids, including acetic, levulinic, and formic acid, are known to inhibit microbial cell growth likely due to the influx of undissociated acids into the cytosol (Lück and Jäger 1997). The resulting pH drop within the cytosol of these cells leads to plasma membrane ATPase-mediated efflux of protons from the cells (Pampulha and Loureiro-Dias 1989; Verduyn et al. 1990). At high acid concentrations, however, the cellular energy reserves would be exhausted, resulting in cytoplasmic acidification and eventually cell death (Imai and Ohno 1995). Lignocellulose, such as corn stover and poplar, contains 3.6–5.6 % (w/w) of acetic acid (Lu et al. 2009). For economical production of ethanol, the minimum initial biomass loading should be >20 % (w/v) (Ximenes et al. 2010). Based on this criterion, the concentrations of acetic acid in the hydrolysates of corn stover and poplar are estimated to be 11.2 and 7.2 g/L, respectively (Lu et al. 2009). Previously, when 7.5 or 15 g/L (125 or 250 mM) of acetic acid was present in the fermentation broth at pH 5–6, there was a significant

decrease in yeast cell growth (Casey et al. 2010). These findings indicate that the predicted concentrations of acetic acid within the corn stover and poplar hydrolysates would be sufficient to inhibit fermentation. Additionally, the performance of *S. cerevisiae* was notably reduced in the presence of high concentrations of weak acids (>100 mM) (Larsson et al. 1999a).

Biotechnological strategies to mitigate the effects of inhibitors and future perspectives

There are various strategies to avoid or minimize the effects of the inhibitors generated during hydrothermal pretreatment and to thereby enhance the bioconversion of lignocellulose (Table 2).

Lignin-blocking additives

Various efforts have focused on improving the efficiency of enzymatic hydrolysis by blocking exposed lignin surfaces using noncatalytic proteins or surfactants (Eriksson et al. 2002; Kumar et al. 2012; Sipos et al. 2010; Yang and Wyman 2006). The lignin-blocking additives prevent the nonproductive adsorption of cellulases, thereby reducing the loss of cellulase activity. When the lignin present in liquid hot water-pretreated hardwoods was blocked using bovine serum albumin (BSA), the enzymatic hydrolysis yield with a cellulase loading of 2 filter paper unit (FPU)/g of glucan increased dramatically from <10 to approximately 70 %, and the degree of improvement in hydrolysis yield correlated with the severity of pretreatment (Kim et al. 2015). It was recently suggested, however, that further efforts are required to identify more cost-effective blocking proteins that would provide substitutes for BSA (Kim et al. 2015). Such proteins include soybean and whey proteins (Yang and Wyman 2013), hydrophobic proteins extracted from corn stover (Han and Chen 2010), and bacterial expansin (Kim et al. 2013a). Indeed, a 30 % increase in glucose yield was obtained when the hydrophobic protein from corn stover was supplemented in place of BSA during pretreatment (Han and Chen 2010). Also, the bacterial expansin, which is known to have synergism with cellulase in cellulose hydrolysis (Kim et al. 2009a), was found to preferentially bind to lignin (Kim et al. 2013a). These positive results motivate the search for other promising protein additives that are more cost-effective than BSA.

Genetic engineering of lignin composition

While the nonproductive adsorption of cellulase enzymes is prevented by the use of lignin blockers, an alternative approach involves the genetic manipulation of plants to reduce the content or the recalcitrance of lignin (Bonawitz et al. 2014;

Chapple et al. 2007; Chen and Dixon 2007). Lignin structures vary depending on the plant source and can also be modified by engineering lignin biosynthesis (Chen and Dixon 2007). Softwood lignin, which is rich in guaiacyl monolignol, was shown to inhibit the enzymatic hydrolysis of cellulose to a greater degree than the guaiacyl-syringyl (G-S)-rich hardwood lignin (Nakagame et al. 2010; Obst 1982). Notably, a transgenic plant with syringyl-rich lignin was more susceptible to deconstruction by liquid hot water or maleic acid pretreatment and was enzymatically hydrolyzed more efficiently than plants with guaiacyl-rich lignin or wild-type lignin (Li et al. 2010; Ciesielski et al. 2014). Furthermore, modification of the lignin structures in both switchgrass (Fu et al. 2011) and poplar (Mansfield et al. 2012) by genetic engineering resulted in reduced recalcitrance of lignocellulose. For example, transgenic switchgrass with a reduced lignin content increased the ethanol yield by up to 38 % using the conventional SSF when compared to the wild type. This transgenic crop needed less severe pretreatment with lower cellulase loadings to achieve the equivalent ethanol productivity (Fu et al. 2011).

Engineering of cellulase enzymes to reduce lignin adsorption

The loss of cellulase activity due to nonproductive adsorption of cellulases to lignin is dependent on the enzyme components and sources. For example, after incubating Cellic CTec2 (derived from *T. reesei*) with lignin, 50–60 % of the initial cellobiohydrolase and endoglucanase remained in the enzyme reaction supernatant, while the majority of β -glucosidase was adsorbed to the lignin (Ko et al. 2015b). Consistent with these findings, previous reports also demonstrated high levels of adsorption of *Trichoderma* β -glucosidase to lignin (Rahikainen et al. 2011). However, the loss of activity of β -glucosidases harvested from other microbial sources, such as *A. niger*, was not as significant as that of the *Trichoderma* β -glucosidase (Berlin et al. 2006; Ko et al. 2015b; Sipos et al. 2010). Therefore, one strategy for alleviating enzymatic inhibition is to engineer enzymes to be more resistant to adsorption to insoluble lignin (Berlin et al. 2006). It was revealed that the adsorption of β -glucosidase to lignin was highly dependent on the pH and the concentration of salt ions, indicating the contribution of electrostatic interactions (Ko et al. 2015b). Therefore, through the engineering of surface charges, the adsorption of this enzyme to lignin could be reduced. Indeed, the chemical modification of reactive functional groups on cellulases increased the ratio of negative-to-positive surface charges (Nordwald et al. 2014). Because lignin is negatively charged, the repulsive interactions between the negatively charged cellulase and lignin increased the cellulose hydrolysis efficiency (Nordwald et al. 2014).

Table 2 Biological strategies to overcome the effects of inhibitors derived during hydrothermal pretreatment

Strategy	Inhibitor	Description	Effect	Limitation	Reference
Lignin blocker	Lignin	-Block lignin surface using hydrophobic proteins (BSA or proteins extracted from corn stover) to reduce the adsorption of cellulase	-Improve the efficiency of cellulose hydrolysis by enzymes	-Still need to find more cost-effective blocking proteins	Han and Chen (2010) Kim et al. (2015)
Lignin engineering	Lignin	-Regulation of syringyl/guaiacyl ratio of lignin	-Improve both enzymatic hydrolysis and microbial fermentation	-Can stunt plant growth	Yang and Wyman (2013) Bonawitz et al. (2014) Chapple et al. (2007)
Enzyme engineering	Lignin, phenolics	-Reduce the lignin content of plants	-Need less severe pretreatment		Chen and Dixon (2007) Fu et al. (2011) Li et al. (2010)
Biological detoxification (bio-abatement)	Phenolics, furan aldehydes, and weak acids	-Reduce nonproductive adsorption of cellulase by modifying the charge of the enzymatic surface -Removal of inhibitors using microorganisms or laccase, which transforms the inhibitors into the inactive forms	-Improve the efficiency of cellulose hydrolysis by enzymes -Improve microbial fermentation	-Loss of fermentable sugars -Need additional detoxification process	Cao et al. (2013) Larsson et al. (1999b) López et al. (2004) Nichols et al. (2008) Palmqvist et al. (1997)
Adaptation or genetic engineering of microorganisms	Phenolics, furan aldehydes, and weak acids	-Engineering of fermenting microorganisms to be more resistant to inhibitors	-Improve microbial fermentation -No additional detoxification process is required	-Development of strains resistant to multiple inhibitors is still in demand	Hasunuma et al. (2011) Heer et al. (2009) Keller et al. (1998) Larsson et al. (2001) Liu et al. (2005)

Removal of inhibitors

While xylooligomers or other oligomeric sugars that act as cellulase inhibitors might be removed through hydrolysis and pentose fermentation (Qing and Wyman 2011; Wei et al. 2013), other inhibitory compounds, including phenolic compounds, weak acids, or furan aldehydes, still inhibit the enzymatic and fermentation processes (Kim et al. 2013c). Various approaches, categorized into chemical, physical, and biological methods, have been proposed for converting inhibitory compounds into inactive forms or for reducing the concentrations of these compounds (Larsson et al. 1999b; Mussatto and Roberto 2004; Palmqvist and Hahn-Hägerdal 2000). Previous studies found that the inhibitory compounds were removed by pH adjustment with alkali (i.e., overliming) (Cao et al. 2013), vacuum evaporation for the removal of volatile inhibitors (Converti et al. 2000; Larsson et al. 1999b), sulfite addition (Larsson et al. 1999b), and treatment with adsorbents (Kim et al. 2011, 2013c). Among the chemical detoxification methods tested, treatment with polymeric resin or activated carbon adsorbents effectively removed phenolics, resulting in relatively higher ethanol yields or rates of productivity than other methods (Grant and King 1990; Kim et al. 2011, 2013c; Larsson et al. 1999b). However, many of these detoxification methods also lead to the loss of fermentable sugars, the increase in the production costs, and the generation of additional waste streams (Cao et al. 2013; Larsson et al. 1999b).

Various biological detoxification methods (bio-abatement) using either microorganisms or microbial enzymes (laccase or peroxidase) have been employed to decrease the inhibitory impacts of phenolics, furan aldehydes, or weak acids (Cao et al. 2013; López et al. 2004; Nichols et al. 2008; Parawira and Tekere 2011). Indeed, a large number of microorganisms, including *Paecilomyces variotii*, *Coniochaeta ligniaria*, *Ureibacillus thermosphaericus*, *T. reesei*, and *S. cerevisiae* mutants, have been utilized for the effective removal of inhibitors present in acid-pretreated lignocellulose hydrolysates prior to fermentation (Cao et al. 2013; Larsson et al. 1999b; López et al. 2004; Nichols et al. 2008; Palmqvist et al. 1997; Pereira et al. 2012). Recently, bio-abatement using *C. ligniaria* NRRL30616 was applied to mitigate the impact of inhibitors, especially phenolics, on the enzymatic hydrolysis of dilute acid or liquid hot water-pretreated corn stover slurries (Cao et al. 2013). In this study, bio-abatement removed >95 % of the acetic acid and >50 % of the HMF, furfural, and phenolic compounds from the slurries, which was accompanied by a 16 % increase in the cellulose conversion yield (Cao et al. 2013). However, biological detoxification also results in the loss of sugars and in the need for prolonged incubation with the detoxifying microorganisms (Parawira and Tekere 2011).

Development of microorganisms that are tolerant of inhibitors

Adaptation or evolutionary engineering of fermenting microorganisms to tolerate inhibitors has been accepted as a promising alternative detoxification approach. This method, based on successive cultivations, is desirable since it requires no additional detoxification treatment processes (Parawira and Tekere 2011). The ethanol-producing microorganism *S. cerevisiae* has an innate tolerance to some inhibitors, such as furan and phenolics, and is capable of converting these inhibitors to less toxic compounds (Almeida et al. 2007; Liu et al. 2005). For example, HMF and furfural are reduced to 2, 5-bis(hydroxymethyl)furan (HMF alcohol) and furfuryl alcohol by *S. cerevisiae*, respectively (Liu et al. 2005). Successive and continuous fermentation in the presence of inhibitors enables yeast strains to become more tolerant to these compounds by gaining enhanced conversion capabilities (Liu et al. 2005; Parawira and Tekere 2011). The adaptation of yeast to hydrolysates was shown to enhance microbial growth and ethanol productivity in several studies (Keller et al. 1998; Liu et al. 2005; Olsson and Hahn-Hägerdal 1996; Palmqvist and Hahn-Hägerdal 2000). Furthermore, genetic or metabolic engineering of yeast strains can be employed to improve microbial performance in the presence of inhibitors. Indeed, *S. cerevisiae* has been genetically modified by the overexpression of genes associated with enzymes resistant to phenolics (Larsson et al. 2001), furan aldehydes (Liu et al. 2005), or weak acids (Hasunuma et al. 2011). For example, laccase-producing *S. cerevisiae* mutants have been developed to detoxify the phenolic compounds from hydrolysates (Larsson et al. 2001). Also, engineered *S. cerevisiae* strains exhibiting tolerance to furan aldehydes have been constructed by overexpressing various oxidoreductases such as alcohol dehydrogenases (Liu et al. 2008). However, different compounds present in hydrolysates may have different inhibitory potentials depending on the pretreatment condition and the biomass type and may exhibit synergistic toxic effects on microorganisms (Klinke et al. 2004). The development of robust microorganisms tolerant to multiple inhibitors will be a challenge for the metabolic engineering approach (Nevoigt 2008).

Conclusions

Understanding the effects of inhibitors derived during hydrothermal pretreatment of natural biomass on the performance of enzymatic or microbial catalysts is invaluable for developing methods for lowering the cost of the production of biofuels from biomass. For example, understanding how the lignin present in pretreated biomass reacts with cellulases could suggest promising technologies to prevent the loss of cellulase activity due to nonproductive adsorption to lignin. The

negative impact of lignin can be mitigated by genetic engineering of lignin composition, blocking lignin surfaces, and enzyme engineering. Meanwhile, bio-abatement and metabolic engineering of microorganisms appear to be the most promising approaches for overcoming the inhibitory effects of soluble phenolics, furan aldehydes, and weak acids on microbial performance and to thereby enhance the efficiency of the conversion of whole pretreatment slurries into biofuels. However, since various types of inhibitors are formed during hydrothermal pretreatment and have synergistic or additive effects on enzymes or on microorganisms, the solutions to minimize the combined effects of these inhibitors need to be developed. For microorganisms, these efforts would be desirable to develop the strains which are tolerant to multiple inhibitors, not only to individual inhibitors.

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