DOI: 10.1002/cssc.201200859

pH-Induced Lignin Surface Modification to Reduce Nonspecific Cellulase Binding and Enhance Enzymatic Saccharification of Lignocelluloses

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We studied the mechanism of the significant enhancement in the enzymatic saccharification of lignocelluloses at an elevated pH of 5.5–6.0. Four lignin residues with different sulfonic acid contents were isolated from enzymatic hydrolysis of lodgepole pine pretreated by either dilute acid (DA) or sulfite pretreatment to overcome recalcitrance of lignocelluloses (SPORL). The adsorption isotherms of a commercial *Trichoderma reesi* cellulase cocktail (CTec2) produced by these lignin residues at 50 °C were measured in the pH range of 4.5–6.0. The zeta potentials of these lignin samples were also measured. We discovered that an elevated pH significantly increased the lignin surface charge (negative), which causes lignin to become more hydrophilic and reduces its coordination affinity to cellulase and, consequently, the nonspecific binding of cellulase. The decreased nonspecific cellulase binding to lignin is also attributed to enhanced electrostatic interactions at elevated pH through the increased negative charges of cellulase enzymes with low pI. The results validate the hypothesis that the increases in enzymatic saccharification efficiencies at elevated pH for different pretreated lignocelluloses are solely the result of decreased nonspecific cellulase binding to lignin. This study contradicts the well-established concept that the optimal pH is 4.8–5.0 for enzymatic hydrolysis using *Trichoderma reesi* cellulose, which is widely accepted and exclusively practiced in numerous laboratories throughout the world. Because an elevated pH can be easily implemented commercially without capital cost and with minimal operating cost, this study has both scientific importance and practical significance.

Introduction

The conversion of lignocellulosic biomass to biofuel can be a sustainable method to mitigate climate change and reduce global dependence on petroleum fuel.[1–3] The sugar platform for biomass conversion through the enzymatic saccharification of lignocelluloses is a preferred pathway because of the flexibility of sugars as key building blocks to produce fuel and other chemicals.[4] Lignocelluloses consist mainly of lignin and the two polysaccharides cellulose and hemicelluloses, which can be hydrolyzed into sugars. Lignin, as a phenolic polymer, can inhibit enzymatic cellulose saccharification through two mechanisms: physical blockage, which limits cellulose accessibility to cellulase,[5,6] and nonspecific adsorption or binding of cellulase enzymes.[7–9] Chemical pretreatments of lignocelluloses, such as organosolv[10] or sulfite pretreatment to overcome recalcitrance of lignocelluloses (SPORL),[11] are able to partially remove the physical blockage by lignin; however, the lignin content in the pretreated substrates is often enriched because of the simultaneous removal of hemicelluloses during chemical pretreatment. The ratio of lignin to cellulose is often unchanged or slightly increased after chemical pretreatment. As a result, the nonspecific adsorption (binding) of cellulase enzymes to lignin is unavoidable. Consequently, enzyme dosages required to achieve the desired saccharification efficiency are too high to be economical for commercial applications that use current technologies.[1,12]

An effective strategy to reduce enzyme loading is to address the nonspecific cellulase binding to lignin without further delignification during chemical pretreatment as high levels of delignification are very expensive. Passive approaches have been undertaken, such as washing the pretreated solid materials to remove free lignin (i.e., lignin separated from lignocellulosic solids through chemical pretreatment)[13] and the application of a surfactant or metal compound to block bound lignin (i.e., lignin retained in the lignocellulosic solids after pretreatment) from binding to cellulase.[14–18] Washing consumes a significant amount of water, in the order of $10\ m^3_{water}\ ton_{lignocelluloses}^{-1}$,[15] and is an environmental concern both in terms of use and waste of this resource. The application of a surfactant and

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other additives are expensive at the levels required to be effective.

An alternative is to modify the surface properties of lignin. As a hydrophobic interaction is the primary driving force for protein adsorption, a hydrophilic lignin surface has a low affinity for cellulase.[19–21] Increasing the number of surface acid groups, such as sulfonic or carboxylic acid, on the lignin structure through either a separate post-pretreatment step[22,23] or during pretreatment,[11] is very effective to enhance the enzymatic hydrolysis of lignocelluloses by increasing the lignin hydrophilicity. Because a separate post-pretreatment step is economically undesirable, new approaches to reduce the nonspecific lignin binding of cellulase are needed.

This study revealed that by using an elevated pH of 5.5 or higher during enzymatic hydrolysis, which is higher than pH 4.8–5.0 predominantly used in the published literature, a significant increase in the saccharification of lignocelluloses can be achieved. The results are corroborated by our previous studies[24,25] that used lignocellulosic substrates produced from a softwood and a hardwood pretreated by different processes (i.e., dilute acid, alkaline, and SPORL). These results contradict the well-established concept that the optimal pH is 4.8–5.0 for the enzymatic hydrolysis of lignocelluloses using *Trichoderma reesi* cellulase based on optimization with pure cellulose. For pure cellulosic substrates, this optimal pH of 4.8–5.0 is supported by a well-known fact that protein adsorption (binding to cellulose in this case) is maximized in the vicinity of the isoelectric point (pI) of the protein,[21,26] which is around 4.5–5.0 for most cellulase enzymes.[27] However, lignocelluloses are different from pure cellulose, which does not contain lignin. Maximal cellulase binding to a lignocellulosic substrates does not necessarily translate to maximal binding to the cellulose fraction of the lignocellulose because lignin can bind to cellulase nonproductively. Unfortunately, the optimal pH of 4.8–5.0 for the enzymatic hydrolysis of pure cellulose has been widely accepted and exclusively practiced for the saccharification of lignocelluloses in numerous laboratories throughout the world. As a result of the broad applicability of this elevated pH concept in the biochemical conversion of lignocelluloses and because it is a simple pH optimization problem that has been overlooked by the scientific community, this problem is both scientifically important and interesting. From an industrial implementation standpoint, the use of an elevated pH is very simple to practice with only minimal capital and operating costs. An elevated pH of 5.5–6.5 is also beneficial for yeast fermentation compared with pH 5 commonly used in simultaneous enzymatic saccharification and fermentation (SSF). Therefore, it has practical significance in terms of economic benefit for commercial applications.

The objective of this study was to develop a mechanistic understanding of the observed enhancement in the saccharification of lignocelluloses at elevated pH. We hypothesize that an elevated pH decreases the nonspecific binding of cellulase to lignin. Specifically, an elevated pH can induce a lignin surface charge through the lignin surface functional groups, which alters the lignin surface hydrophilicity (pH-induced lignin surface modification) and leads to a reduced lignin affinity to cellulase. Furthermore, the induced surface charge may also produce favorable electrostatic interactions between lignin and cellulase through Coulombic repulsion. Although global electrostatic interactions between a protein and a surface do not dominant protein adsorption,[21,26] it has been suggested that Coulombic repulsion is a dominant opposition force to protein adsorption (cellulase binding to lignin in the present study) at a pH far from the pI of cellulase (pH 5.5 or higher in this case).[26]

We prepared four lignin residues derived from the enzymatic hydrolysis of four different lodgepole pine solid substrates pretreated by using dilute acid (DA) or SPORL with three variations in the sulfite loading to produce lignin with varied contents of surface sulfonic acid groups. The zeta potentials of the lignin and the cellulase adsorption by lignin at different pH values were measured. Enzymatic hydrolysis of the four different substrates was also conducted at various pH values. Our goal was to develop effective strategies to reduce enzyme loading in the saccharification of lignocelluloses through lignin surface modification to limit the binding interactions between lignin and cellulase.

Results and Discussion

Effect of pH on the enzymatic saccharification of lignocelluloses

The four lignocellulosic substrates of lodgepole pine pretreated by using DA and SPORL with three sulfite loadings (Tables 1 and 2) were enzymatically saccharified in a pH range of 4.5–6.0. This was to verify the enhancement of the enzymatic saccharification of lignocelluloses at elevated pH as observed in our previous studies that used different substrates.[24,25] The results clearly indicate that the pH value required to achieve maximum substrate enzymatic digestibility (SED), defined as the percentage of glucan in the substrate that was enzymatically hydrolyzed to glucose, was between 5.5 and 6.0 for all four lignocellulosic substrates (Figure 1), and not at 5.0, which is used almost exclusively in the published literature and suggested by the manufacturer (Novozymes) of the cellulase cocktail Cellic CTec2. The SED values were low as a low

Table 1. List of pretreated lignocellulosic substrates studied along with pretreatment conditions.

Pretreated substrate label[a]	Pretreatment	Chemical dosage on wood [wt %]	*T* [°C]	*t* min]
DA	DA	H_2SO_4: 2.5	175	25
SP-B2	SPORL	H_2SO_4: 2.5 $NaHSO_3$: 2.0	175	25
SP-B4	SPORL	H_2SO_4: 2.5 $NaHSO_3$: 4.0	175	25
SP-B6	SPORL	H_2SO_4: 2.5 $NaHSO_3$: 6.0	175	25

[a] B# represents the sodium bisulfite loading [%] on the oven-dry wood for SPORL pretreatment. All samples were washed separately.

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Table 2. Chemical compositions of the untreated and pretreated lodgepole pine substrates listed in Table 1 and the corresponding hydrolysis lignin residues.

Sample	Klason lignin [%]	Main carbohydrates [%] glucan	xylan	mannan	sum	Sulfonic acid groups [mg g_{lignin}^{-1}]
untreated lodgepole pine						
	28.6	41.9	5.5	11.7	87.7	0
pretreated lodgepole pine						
DA	47.4	45.9	0.3	0.7	94.3	0
SP-B2	47.1	43.1	0.2	0.7	91.2	4.12
SP-B4	44.8	44.9	0.5	0.9	91.1	6.22
SP-B6	42.0	45.5	0.8	0.9	89.3	10.85
hydrolysis lignin residues						
L-DA	85.8	8.2	0.4	1.0	95.4	0
L-SP-B2	91.0	3.8	0.3	0.9	95.9	5.46
L-SP-B4	91.4	3.6	0.3	1.0	96.3	8.07
L-SP-B6	89.3	4.6	0.4	1.0	95.3	11.84

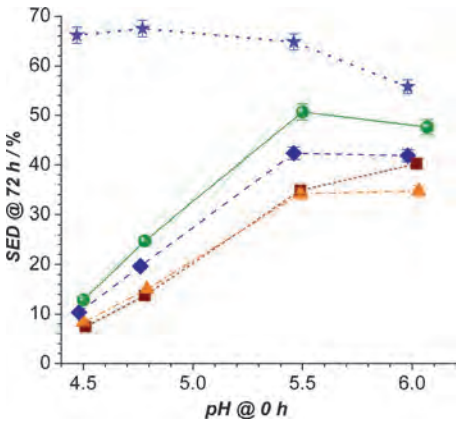

Figure 1. Effects of suspension pH on the SED of four lignocelluloses and Whatman paper. ■ DA; ▲ SP-B2; ◆ SP-B4; ● SP-B6; ★ Whatman filter paper.

CTec2 loading of 10 FPU g_{glucan}^{-1} was used to better reflect any differences produced by pH variation (Figure 1). Furthermore, the SED response to the pH was very steep and linear from pH 4.5–5.5. The SED values of the four substrates more than doubled if the pH was increased from 4.8 to 5.5. The pH shown in Figure 1 is the pH of the substrate suspension measured at 0 h of enzymatic hydrolysis. If a pure cellulosic substrate (Whatman paper) was used, the optimal pH at maximum saccharification was pH 4.8 and the SED profile was very flat from pH 4.5–5.5. By comparing the SED profiles of the four lignocellulosic substrates with that of Whatman paper, the shift in the optimal pH to an elevated value for maximal cellulose saccharification is clear (Figure 1) and in agreement with our previous data from lignocellulosic substrates pretreated differently.[24]

Effect of pH on the nonspecific binding of CTec2 to lignin

Cellulase adsorption by the four hydrolysis lignin residues, derived after two enzymatic hydrolysis steps from the four pretreated lodgepole pine substrates (Table 2), were measured at different pH values to verify that the decrease in nonspecific cellulase binding to lignin at elevated pH is the mechanism of the enhanced saccharification observed. The time-dependent adsorption of CTec2 by the four different hydrolysis lignin residues at pH 4.8 and 50 °C with a CTec2 loading of 400 $mg_{protein}$ L^{-1} reached an asymptotic value at approximately 30 min, which suggests that the adsorption had reached an equilibrium. Therefore, all cellulase adsorption data reported here were measured at a fixed time of 30 min. The isotherms of CTec2 adsorption at pH 4.8 and 5.5 and 50 °C by the hydrolysis lignin residues L-DA and L-SP-B6, derived from substrates DA and SP-B6, respectively (Tables 1 and 2), are shown in Figure 2. The results clearly show the reduced CTec2 binding

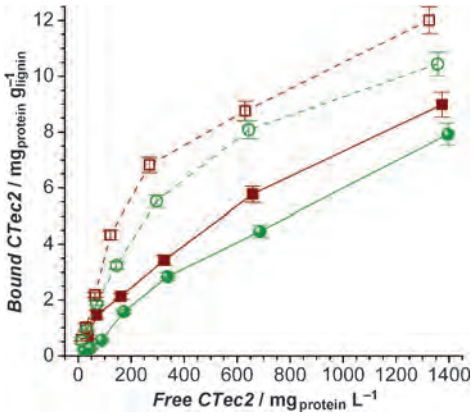

Figure 2. Isotherms of CTec2 binding to L-DA and L-SP-B6 at 50 °C at pH 4.8 and 5.5. ■ L-DA; ● L-SP-B6. Solid symbols represent data obtained at pH 5.5 and open symbols at pH 4.8.

(adsorption) to lignin at pH 5.5 compared with the corresponding values at pH 4.8 over a range of CTec2 loadings with a concentration of free CTec2 in the suspension ranging from 0–1400 $mg_{protein}$ L^{-1}. Similar results were observed for the other two lignin residues (data not shown). The ratio of CTec2 binding to lignin at pH 5.5 compared to that at pH 4.8 for the corresponding substrates was found to depend on the content of sulfonic acid groups in the lignin (Figure 3). The CTec2 binding

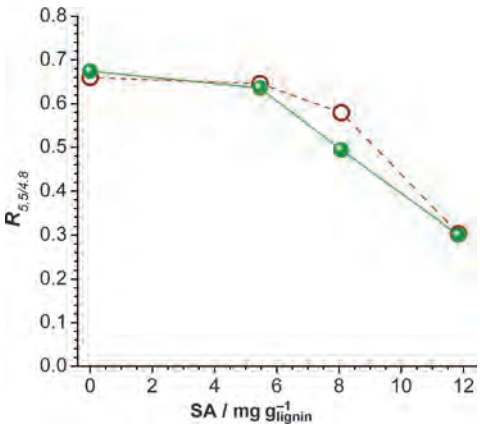

Figure 3. Effects of lignin sulfonic acid group content on the ratio $R_{5.5/4.8}$ of CTec2 binding to hydrolysis lignin residues at pH 5.5 compared to that at pH 4.8 at two CTec2 loadings: ○ = 50 $mg_{protein}$ L^{-1}; ● = 100 $mg_{protein}$ L^{-1}.

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to lignin at pH 5.5 was approximately 70% of that at pH 4.8 for L-DA. This ratio decreased as the content of sulfonic acid groups in the lignin increased and decreased to 30% for L-SP-B6. Furthermore, the ratios obtained at CTec2 loadings of 50 and 100 $mg_{protein}\,L^{-1}$ were almost identical for each of the four hydrolysis lignin residues (Figure 3).

The decrease in nonspecific cellulase binding to lignin was observed at all elevated pH values over the pH range studied. The decrease in the nonspecific binding of CTec2 to L-DA and L-SP-B6 is linearly proportional to pH (Figure 4a) and was near zero for L-SP-B6 at pH 6.0. Similar linear decreases occurred for L-SP-B2 and L-SP-B4 (data not shown). The slopes (absolute

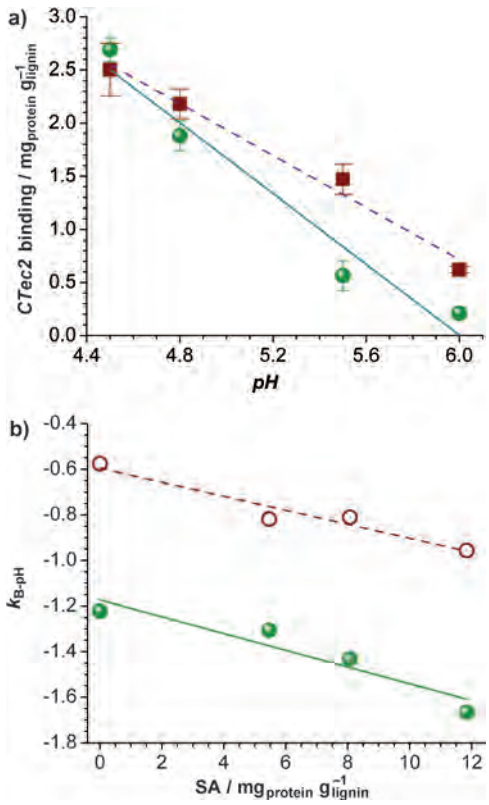

Figure 4. a) Effects of pH on CTec2 binding to L-DA and L-SP-B6 at 50 °C with a CTec2 loading of 100 $mg_{protein}\,L^{-1}$. ■ L-DA, ----- $y=-1.22x+8.1$, $r^2=0.997$; ● L-SP-B6, —— $y=-1.67x+10.0$, $r^2=0.937$. b) Effects of lignin sulfonic acid group content (SA) on the slope (k_{B-pH}) of the correlation between CTec2 nonspecific binding (i.e., the slope of the lines in Figure 4a) and pH at two CTec2 loadings. CTec2 loading: ○ = 50 $mg_{protein}\,L^{-1}$, ----- $y=-0.031x-0.60$, $r^2=0.91$; ● = 100 $mg_{protein}\,L^{-1}$, —— $y=-0.037x-1.17$, $r^2=0.84$.

values) of the linear correlations between the amount of nonspecifically bound CTec2 and the pH for the four hydrolysis lignin residues increase linearly with the content of sulfonic acid groups in the lignin (Figure 4b), which suggests that the decrease in the nonspecific binding of CTec2 to lignin at elevated pH is more pronounced for sulfonated lignin with a high content of sulfonic acid groups. This is in agreement with the results shown in Figure 3.

To further quantify the nonspecific binding of cellulase to lignin at elevated pH, the cellulase adsorption isotherms were fitted to the Langmuir model [Eq. (1)]. Good fits were obtained for all experimental data collected. The CTec2-binding parameters, that is, the maximum binding capacity σ, affinity constant A, and binding strength S ($S=A\sigma$), and the linear correlation coefficient r^2 are listed in Table 3. The values of σ, A, and S for the four lignin residues at pH 4.8 are all higher than those at pH 5.5. The ratio of σ at pH 5.5 to that at pH 4.8, $\sigma_{pH\,5.5}/\sigma_{pH\,4.8}$,

Table 3. Results from linear regressions of CTec2 adsorption isotherms by the four hydrolysis lignin residues by using the Langmuir model [Eq. (1)].

Hydrolysis lignin residue	pH	σ [$mg\,g_{lignin}^{-1}$]	A [$L\,g_{protein}^{-1}$]	S [$mL\,g_{lignin}^{-1}$]	r2	$\sigma_{pH\,5.5}/\sigma_{pH\,4.8}$
L-DA	4.8	10.53	1.10	11.63	0.986	0.982
	5.5	10.34	0.48	4.99	0.979	
L-SP-B2	4.8	15.61	0.72	11.31	1.000	0.671
	5.5	10.48	0.53	5.54	0.992	
L-SP-B4	4.8	8.12	1.74	14.16	0.999	0.503
	5.5	4.09	1.59	6.51	0.987	
L-SP-B6	4.8	7.71	1.27	9.82	0.983	0.417
	5.5	3.22	0.80	2.56	0.889	

decreased rapidly as the content of sulfonic acid groups in the lignin increased. This is in agreement with the experimental data shown in Figures 3 and 4b and indicates that the sulfonic acid groups in lignin are directly related to and play a significant role in the observed pH-induced decrease in the nonspecific binding of cellulase to lignin.

pH-induced lignin surface charge and correlation with nonspecific cellulase binding

The zeta potentials of the four hydrolysis lignin residues at different pH values were measured to validate the pH-induced lignin surface charge. The results clearly show that the zeta potentials of L-DA and L-SP-B6 increased (absolute values) linearly with increasing pH from 4.5–6.0 (Figure 5). Therefore, lignin becomes more negatively charged as the pH increases. A negatively charged surface is less hydrophobic, which is not favorable for binding cellulase through hydrophobic interac-

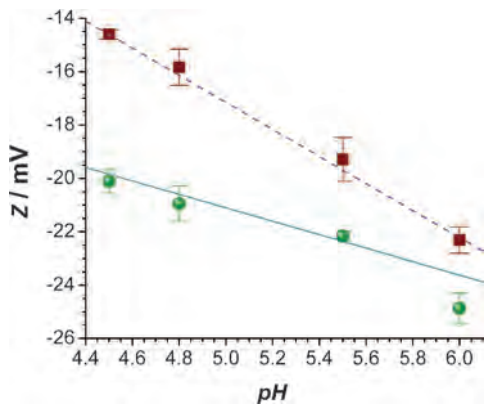

Figure 5. Effects of pH on pH-induced surface charge (Zeta potential, *Z*) of L-DA and L-SP-B6. ■ L-DA, ----- $y=-5.07x+8.2$, $r^2=0.99$; ● L-SP-B6, —— $y=-2.52x-8.5$, $r^2=0.79$.

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tions.[19–21] Similar results were observed for the other two lignin residues (data not shown). The slopes and intercepts obtained from linear regression of the zeta potential data with pH, $Z = k\,\mathrm{pH} + Z_0$, for the four hydrolysis lignin residues are listed in Table 4. These results indicate that pH has a stronger effect on the surface charge of L-DA than on L-SP-B2, L-SP-B4, and especially L-SP-B6. This is evidenced from the larger slope of the data for L-DA than that of the SPORL-derived samples (Table 4). However, L-DA still has a smaller negative charge than L-SP-B2, L-SP-B4, and L-SP-B6, especially in the pH range of 4.5–6.0.

Table 4. Linear regression results of zeta potentials at different pH for four hydrolysis lignin residues: $Z = k\,\mathrm{pH} + Z_0$.

Hydrolysis lignin residue	k	Z_0 [mV]	r^2
L-DA	−5.07 ± 0.16	8.2 ± 0.74	0.997
L-SP-B2	−3.51 ± 0.70	0.6 ± 3.39	0.888
L-SP-B4	−2.07 ± 1.24	−9.1 ± 6.63	0.372
L-SP-B6	−2.52 ± 0.72	−8.5 ± 3.86	0.791

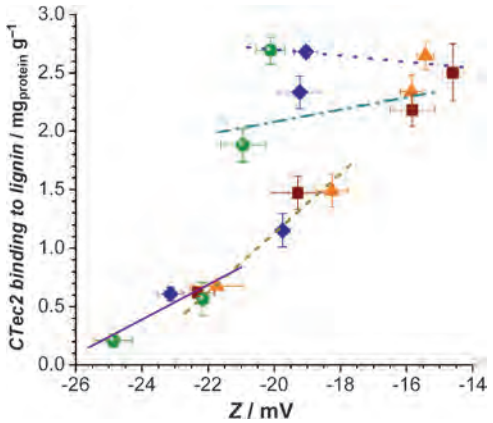

Figure 6. Correlations between lignin surface charge (Zeta potential, Z) and nonspecific CTec2 binding to the four hydrolysis lignin residues at different pH. ■ DA; ▲ SP-B2; ◆ SP-B4; ● SP-B6; pH 4.5 ····· $y = -0.026x + 2.18$, $r^2 = 0.65$; pH 4.8 –·–· $y = 0.053x + 3.14$, $r^2 = 0.40$; pH 5.5 ----- $y = 0.253x + 6.19$, $r^2 = 0.94$; pH 6.0 —— $y = 0.150x + 3.99$, $r^2 = 0.90$.

Electrostatic interactions can also contribute to cellulase binding to lignocelluloses through Coulombic repulsion and protein structural rearrangement.[21,26,28] CTec2 is a mixture that contains endoglucanase, exoglucananse, and β-glucosidase. Different cellulases have different pI values,[27,29–32] and most cellulases have pI values ≤5.0, for example, EGI (Cel7B, pI 3.9, 4.5, 4.7), EGII (Cel5A, pI 4.2, 5.5), CBHI (Cel7A, pI 3.6–3.9), and β-glucosidase (Aspergillus sp., pI 4.0). These enzymes will have negative surface charges at an elevated pH of 5.5 (>pI) and will, therefore, have less affinity to lignin (also negatively charged as discussed above) owing to Coulombic repulsion to reduce their nonspecific binding to lignin. Only β-glucosidase I (pI 8.5) and EGIII (Cel12A, pI 6.8–7.4) have very high pI values >6.0 and will have a positive charge even at an elevated pH of 5.5–6.0. This suggests that future enzyme formulation should only overdose cellulase enzymes with pI values >6.0. This is because only these enzymes, which are likely to have a relatively high affinity to lignin owing to a lack of Coulombic repulsion, will result in lost activities through nonspecific binding to lignin if the enzymatic hydrolysis of lignocelluloses is conducted at an elevated pH of 5.5–6.0.

The zeta potentials of the four hydrolysis lignin residues at different pH values and the amounts of CTec2 bound to the lignin residues were plotted to examine the effects of pH-induced lignin surface charge on nonspecific cellulase binding. The data for each lignin at different pH values were plotted with the same symbol, and each cellulase-binding data set for the four lignin residues measured at the same pH was fitted by a linear equation (Figure 6). The results indicate that the effect of lignin sulfonation on the relationship between the zeta potential and nonspecific binding varies with pH. At low pH values of 4.5 and 4.8, the nonspecific binding is not significantly affected by lignin sulfonation as evidenced by the near-zero slopes of the regression lines shown in Figure 6. This suggests that the variations in surface hydrophobicity among the four lignin samples are not sufficiently large to alter the cellulase binding. It also suggests the lack of Coulombic repulsion between the lignin and cellulase enzymes that have low negative, zero, or slightly positive surface charges, because pH values of 4.5 and 4.8 are in the vicinity of the pI values of most cellulase enzymes (discussed above). However, at elevated pH (5.5 and 6.0), the nonspecific binding of CTec2 decreased rapidly with the zeta potential as shown by the slopes of the lines displayed in Figure 6. This can be explained by the increased lignin surface hydrophilicity as evidenced by the increased negative charges. Furthermore, the increased negative charges of most cellulase enzymes at elevated pH values of 5.5–6.0 (>pI) also increased the Coulombic repulsion against the negatively charged lignin. Although hydrophobic interactions are the primary driving force in protein adsorption,[21,26] Coulombic forces have been identified as the dominant opposition to protein adsorption through lateral interactions at pH values far from the pI of the protein (if negatively charged),[26] that is, elevated pH of 5.5 or higher in this study. Elevated pH-induced protein charge can also result in a relatively low native-state stability of the protein to affect adsorption.[28] The data set for pH 5.5 and 6.0 can be combined and fit by the same equation ($y = 0.207x + 5.28$, $r^2 = 0.936$). The difference in the slopes of the fitted lines between these two data sets is approximately equal to the sum of the fitting errors in the slopes from separate regressions.

Examination of the results based on lignin produced from the same substrate but tested at a different pH shows that lignin residues from different substrates behaved differently, which agrees with the results reported in Figures 4 and 5. The binding data for L-SP-B2, which has a low degree of sulfonation (Table 2), are similar to the data for L-DA (Figure 6). Notably, the variation in the zeta potential of L-SP-B3 at pH 4.5, 4.8, and 5.5 was very small, which suggests that the surface hydrophobicity and charge were not significantly changed; however, CTec2 binding to L-SP-B3 was significantly reduced as the pH

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increased from 4.5 to 5.5. This suggests that lignin surface hydrophobicity and charge cannot fully explain the observed decrease in nonspecific cellulase binding. Variations in pH inherently result in the variation of the surface charge of cellulase enzymes. Consequently, a variation in pH can affect the cellulase structural conformability and stability,[21,26,28] as well as the Coulombic repulsion between lignin and cellulase enzymes, which depends on the pI of the enzymes. Both pH-induced lignin surface hydrophilicity (hydration), cellulase-surface-charge-induced Coulombic repulsion between lignin and enzymes, and enzyme protein structural rearrangement contribute to the observed decrease in the nonspecific binding of cellulase to lignin.[26,28] These processes may produce a synergistic effect to decrease the cellulase binding to lignin.[26]

Reduction in nonspecific cellulase binding and enhancement of enzymatic saccharification

The increase in enzymatic saccharification shown in Figure 1 measured by the increase in SED at an elevated pH, $\Delta(SED)_{pH}$, was plotted for all four pretreated substrates against the decrease in lignin binding of CTec2. Data points for $\Delta(SED)_{5.5}$ are calculated by subtracting either the SED at pH 4.5 or 4.8 from the SED at pH 5.5, as well as the SED at pH 4.5 from the SED at pH 4.8. Likewise, $\Delta(SED)_{6.0}$ is calculated by subtracting the SED at pH 4.5, 4.8, or 5.5 from the SED at pH 6.0. The results indicate that $\Delta(SED)_{pH}$ for all four substrates with different lignin structures fall to a single line for a given pH of either 5.5 or 6.0 (Figure 7). Linear regressions produced almost identical slopes for the two data sets. The difference in the two slopes is within the fitting errors for $\Delta(SED)_{6.0}$ of 3.7 and for $\Delta(SED)_{5.5}$ of 1.0. The regression line for the data set of $\Delta(SED)_{5.5}$ has a zero intercept, that is, there is no enhancement in enzymatic cellulose saccharification at a zero decrease of nonspecific cellulase binding to lignin. The regression line for the data set for $\Delta(SED)_{6.0}$ has a negative intercept at -11.6 ± 2.8, which indicates that increasing the pH beyond 5.5 did not produce a benefit in terms of lignocellulose saccharification efficiency (in agreement with Figure 1), even though nonproductive binding of CTec2 to lignin was further reduced at pH 6.0 (Figures 5 and 6). This is because too high a pH can reduce the cellulase activities to hydrolyze cellulose. This can be clearly seen from the enzymatic hydrolysis of a pure cellulosic substrate, such as Whatman paper, over a wide pH range (Figure 1). The SED of Whatman paper decreased from approximately 65% at pH 5.5 to 55% at pH 6.0. This decrease of 10% is within one standard deviation of the intercept of the regression line for $\Delta(SED)_{6.0}$ of $-11.6 \pm 2.8\%$ (Figure 7). In addition, this 10% decrease on increasing the pH from 5.5 to 6.0 could account for all the difference between the intercepts of the two regression lines for $\Delta(SED)_{5.5}$ and $\Delta(SED)_{6.0}$. The above discussion of the results in Figure 7 indicates that the decrease in the nonspecific binding of cellulase is solely responsible for the observed enhancement of the enzymatic saccharification of lignocelluloses at elevated pH.

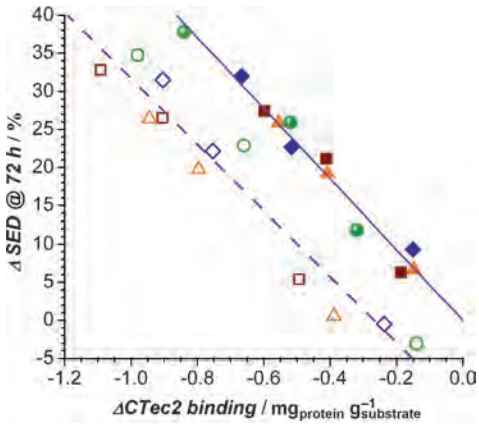

Figure 7. Correlation between the increase $\Delta(SED)$ of four lignocellulosic substrates and the decrease in nonspecific CTec2 binding to lignin derived from the corresponding substrate at 50 °C and elevated pH 5.5 and 6.0. Solid symbols: $\Delta(SED)_{5.5}$, ■ DA; ▲ SP-B2; ◆ SP-B4; ● SP-B6; —— $y = -46.3x$, $r^2 = 0.99$ Open symbols: $\Delta(SED)_{6.0}$, □ DA; △ SP-B2; ◇ SP-B4; ○ SP-B6; ----- $y = -43.3x - 11.6$, $r^2 = 0.93$.

Conclusions

This study revealed that pH-induced lignin surface modification reduced the nonspecific cellulase binding to lignin and resulted in significantly enhanced enzymatic saccharification of lignocelluloses at an elevated pH of 5.5 or higher. This contradicts the well-established concept that an optimal pH is 4.8–5.0 for enzymatic hydrolysis using *Trichoderma reesi* cellulase, which is based on optimization by using pure cellulose and is widely accepted and exclusively practiced in numerous laboratories throughout the world. The study indicates that there are at least two mechanisms for the observed decrease in nonspecific cellulase binding to lignin at elevated pH: (1) the pH-induced lignin surface charge (negative) increased the lignin surface hydrophilicity (hydration) and (2) the pH-induced change in the negative surface charge of most cellulase enzymes with low pI values produced a Coulombic repulsion between lignin (also negatively charged) and these enzymes. The experimentally observed increases in the enzymatic saccharification efficiencies, represented by SED, of the differently pretreated lignocelluloses by using an elevated pH is solely the result of the decreased nonspecific cellulase binding to lignin. This is validated by the near-perfect correlations between these two measured quantities independent of the chemical structure of lignin derived by using different pretreatment methods.

Experimental Section

Materials

CTec2 was generously provided by Novozymes North America. A Bradford protein assay kit and bovine serum albumin (BSA) were purchased from Bio-Rad Laboratories. BSA was used as standard to calibrate the protein content of CTec2 by the Bradford method.[33] The protein concentration of CTec2 was 73.6 mg mL^{-1}, and its cellulase activity was 147 FPU m^{-1} as cali-

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brated by a literature method.[34] Sodium acetate buffer, sulfuric acid, and sodium bisulfite were used as received from Sigma–Aldrich. All chemicals were ACS reagent grade.

A lodgepole tree (*Pinus contorta*) killed by mountain pine beetle (*Dendroctonus ponderosae*; estimated infestation 4 years; abbreviated BD4) was harvested at Sulphur Ranger District, Arapaho–Roosevelt National Forest, Colorado, as described previously.[35,36] The wood logs were debarked onsite, shipped to the U.S. Forest Service, Forest Products Laboratory, Madison, Wisconsin, USA, and chipped by using a laboratory chipper. The wood chips were then screened to remove all particles greater than 38 mm and less than 6 mm in length. The thickness of the accepted chips ranged from 1–5 mm. The chips were kept frozen at approximately −16 °C until used.

Substrate production

DA and SPORL pretreatments were employed to produce four lignocellulosic solid substrates from the BD4 wood chips by using three bomb reactors placed in an autoclave configuration in a 23 L rotating pulping digester as described previously.[36,37] The pretreatment conditions such as temperature and duration, sulfuric acid and sodium bisulfite dosages are listed in Table 1. The liquid-to-solid ratio (L/W) was fixed at 3:1 for all pretreatments. The pretreated wood chips remained intact and were separated from the pretreatment hydrolysate (hemicellulosic sugar stream) with a screen. The pretreated wood chips were then disk-milled by using a laboratory atmospheric disk refiner (Andritz Sprout-Bauer) with a disk plate pattern of D2B-505 and disk plate gap of 1.00 mm. The size-reduced solids were directly dewatered to a solids content of approximately 30% by vacuum pressing in a canvas bag (as prewashing). Additional thorough washing was conducted. The chemical compositions of both the washed solid substrates and the untreated wood chips were analyzed (Table 2).

Enzymatic hydrolysis

Enzymatic hydrolysis of the pretreated solid substrate was conducted by using CTec2 at 2% solids (*w/v*) in a flask on a shaker/incubator (Thermo Fisher Scientific, Model 4450, Waltham, MA) at 50 °C and 200 rpm. The solid substrate (1 g oven-dry (od) weight) was added to an acetate buffer solution (50 mL, 50 mM) along with CTec2 (10 FPU g_{glucan}^{-1}). The pH values of the buffer solutions were varied between 4.5 and 6.0 by using different ratios of sodium acetate and acetic acid. The amount of glucose in the enzymatic hydrolysate was determined. SED, defined as the percentage of substrate glucan enzymatically saccharified to glucose, was used to represent the enzymatic saccharification efficiency. The experimental error in enzymatic hydrolysis was 2.5% on average based on replicate runs.

Preparation of hydrolysis lignin residues

The enzymatically hydrolysis lignin residues, L-DA, L-SP-B2, L-SP-B4, and L-SP-B6, were prepared from four pretreated lodgepole pine substrates, namely, DA, SP-B2, SP-B4, and SP-B6, respectively. The two-step enzymatic hydrolysis of each pretreated lodgepole pine substrate was conducted with an excess of CTec2 at a loading of 20 FPU $g_{substrate}^{-1}$ in each step. The preparation procedure was as follows: (1) the pretreated and disk-milled substrates were milled again with the same disk refiner by using the same disk plates and disk plate gap described in the Substrate production section; (2) the resultant substrate (ca. 16 g) was enzymatically hydrolyzed by using CTec2 (20 FPU $g_{substrate}^{-1}$) with a solid loading of 2% (*w/v*) at pH 5.5 and 50 °C by using a shaker at 200 rpm for 48 h; (3) the supernatant was decanted after standing overnight; (4) the remaining solids were enzymatically hydrolyzed again by adding fresh CTec2 at the same loading under the same conditions described in step (2); (5) the supernatant was decanted after centrifuging at 10000 rpm for 30 min; (6) the decanted lignin solid residue was washed with distilled water at r.t.; (7) the residual protein from CTec2 on the lignin residue was removed by using an excess amount (ca. 0.18 mg $g_{lignin\ residue}^{-1}$) of Pronase K (6556, 30 units mg^{-1} protein) in a borax/$CaCl_2$ buffer solution at pH 8 at 5% (*w/v*) and 37 °C for 48 h; (8) the supernatant was again decanted after centrifuging at 10000 rpm for 30 min, and the resultant lignin residue was washed with distilled water, 1.0 M NaCl, and distilled water, sequentially; (9) the protease on the lignin residues was deactivated in deionized (DI) water at 100 °C for 2 h and washed twice with DI water; (10) the lignin residue was then dried at 50 °C for 60 h until no further weight loss was observed; (11) the lignin residue was milled by using a Wiley mill (Model #2, Arthur Thomas Co) with a 50 mesh screen. The accepted sample was used for analysis and adsorption experiments.

Analytical methods

The chemical compositions of the original and pretreated biomass were analyzed at the Analytical and Microscopy Laboratory of the Forest Products Laboratory as described previously.[36] Briefly, a two-stage acid hydrolysis procedure was employed to hydrolyze the milled lignocellulosic substrates. The supernatant after filtration through filter paper was used for carbohydrate analysis by using high-performance anion-exchange chromatography with pulsed amperometric detection (HPAEC-PAD). Klason lignin (acid insoluble) retained on the filter paper was quantified gravimetrically after drying. The typical standard deviation of the chemical composition analysis was approximately 0.3%.[36] Glucose in the enzymatic hydrolysate was measured in duplicate by using a commercial glucose analyzer (YSI 2700S, YSI Inc.).

Cellulase adsorption by hydrolysis lignin residues

Cellulase adsorption experiments were conducted in acetic buffer solutions of pH 4.5, 4.8, 5.5, and 6.0 at 50 °C with hydrol-

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ysis lignin residues at a solids consistency of 2% (*w/v*) (0.100 g lignin and 4.900 g CTec2 solution). The initial concentrations of CTec2 were 25, 50, 100, 200, 400, 800, and 1600 $mg_{protein}L^{-1}$. After incubation for 30 min (kinetic experiments were not conducted), the solution (50 mL) was centrifuged at 12000 rpm for 10 min. The supernatant was again centrifuged at 12000 rpm for 10 min. An aliquot of the final supernatant was placed into a sampling cuvette. The amount of cellulase adsorption by hydrolysis lignin residue was quantified by using a UV/Vis spectrometer (Model 8453, Agilent Technologies) with a dual wavelength method (276 and 400 nm) as described previously.[38] The dual-wavelength method can correct for spectral interference from light scattering of small particles such as any remaining insoluble lignin. The lignin solution without enzyme application was used as a blank to correct for spectral absorption from dissolved lignin present in the lignin–cellulase solution. The amount of CTec2 adsorbed or bound on the hydrolysis lignin residue was calculated by subtracting the amount of free CTec2 in the supernatant from the total amount of CTec2 applied initially.

Determination of adsorption parameters

The Langmuir model [Eq. (1)] was used to fit the adsorption isotherm data. The maximum cellulase adsorption capacity σ and the cellulase adsorption equilibrium constant K_d were determined accordingly:

$$[CE] = \frac{\sigma[S_t][E_f]}{K_d + [E_f]} \quad (1)$$

in which [CE] is the amount of adsorbed CTec2 [$mg_{protein}L^{-1}$], [E_f] is the free CTec2 concentration [$mg_{protein}mL^{-1}$], σ is the maximum adsorption capacity [$mg_{protein}g_{lignin}^{-1}$], [$S_t$] is the substrate concentration, that is, 2 gL^{-1} for this study, and K_d is the adsorption equilibrium constant [$mg_{protein}mL^{-1}$]. The affinity constant ($A = 1/K_d$) and binding strength ($S = A\sigma$) can then be calculated.

Sulfur-content analysis

The sulfur content of the pretreated substrates and hydrolysis lignin residues was analyzed by using inductively coupled plasma MS (ICP-MS; Ultima model, Horiba Jobin–Yvon). The samples were weighed and then transferred to Teflon digestion flasks. All samples were digested at 145 °C for 15 min in a microwave oven (MDS-2000, CEM Corp.) by using 70% HNO_3 (ca. 5 mL) and 30% H_2O_2 (3 mL) before analysis.

Zeta potential measurement

The zeta potentials of the hydrolysis lignin residues at different pH values were measured in buffer solutions (50 mL) by using a shaker/incubator at 50 °C and 200 rpm. Buffer solutions of acetate at pH 4.5, 4.8, 5.5, and 6.0 were used. The lignin concentration in the buffer solutions was 0.033% (*w/w*). The lignin solution was mixed with a magnetic stirrer for 30 min and allowed to stand for 60 min. The supernatant was tested by using a Zeta Potential Analyzer (Zeta Plus). All zeta potential measurements were performed in duplicate with seven readings in each experiment to ensure experimental repeatability. The averages were reported, and the standard deviations were used as error bars in plotting.

Acknowledgements

This work was partially supported by a USDA Small Business Innovative Research (SBIR) Phase II project (Contract Number: 2010-33610-21589) to Biopulping International and the Agriculture and Food Research Initiative Competitive Grant no. 2011-68005-30416 from the USDA National Institute of Food and Agriculture (NIFA) through the Northwest Advanced Renewables Alliance (NARA). The financial support of these projects along with that of the China Excellent Young Scientist Fund (20925622) made the scientific visit of Hongming Lou to the US Forest Service (USFS), Forest Products Laboratory (FPL) possible. We acknowledge Fred Matt and Kolby Hirth (both USFS-FPL) for performing carbohydrate and sulfur-content analyses.

Keywords: adsorption · biomass · biotransformation · enzymes · proteins

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Received: November 9, 2012
Published online on March 28, 2013

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