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Effects of Lignin−Metal Complexation on Enzymatic Hydrolysis of Cellulose

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This study investigated the inhibition of enzymatic hydrolysis by unbound lignin (soluble and insoluble) with or without the addition of metal compounds. Sulfonated, Organosolv, and Kraft lignin were added in aqueous enzyme−cellulose systems at different concentrations before hydrolysis. The measured substrate enzymatic digestibility (SED) of cellulose was decreased by 15% when SL was added to a concentration of 0.1 g/L due to nonproductive adsorption of enzymes onto lignin. Cu(II) and Fe(III) were found to inhibit enzymatic cellulose hydrolysis in the presence of lignin. Ca(II) and Mg(II) were found to reduce or eliminate nonproductive enzyme adsorption by the formation of lignin−metal complex. The addition of Ca(II) or Mg(II) to a concentration of 10 mM can almost completely eliminate the reduction in SED caused by the nonproductive enzyme adsorption onto the lignins studied (SL, OL, or KL at concentration of 0.1 g/L). Ca(II) was also found to reduce the inhibitive effect of bound lignin in pretreated wood substrate, suggesting that Ca(II) can also form complex with bound lignin on pretreated solid lignocelluloses. Significant improvement in SED of about over 27% of a eucalyptus substrate produced by sulfite pretreatment to overcome recalcitrance of lignocellulose (SPORL) was achieved with the application of Ca(II).

KEYWORDS: Enzyme adsorption; inhibition; cellulose hydrolysis/saccharificatio; calcium; magnesium; lignin−metal complex

INTRODUCTION

Nonproductive adsorption of enzymes by compounds other than cellulose in enzyme and lignocellulose systems has been recognized as the major cause of loss of cellulase activity that results in reduced efficiency of cellulose hydrolysis (*1*−*5*). Lignin accounts for 20−30% of plant biomass and is one of the key nonproductive enzyme adsorption media (*2*). Research efforts have been made to remove lignin content of lignocellulose substrate through delignification to reduce its inhibition (*6*). However, delignification, such as alkaline oxidation (*6*), is expensive and usually applied to remove the lignin on the solid lignocellulose substrate (bound lignin). Exogenous protein (bovine serum albumin (BSA)) has been used to cover lignin to reduce adsorption of cellulase, resulting in enhanced enzymatic cellulose saccharification (*3*, *4*, *7*). Surfactants have also been used as lignin-blocking agents to improve enzymatic hydrolysis (*1*, *8*−*10*). These two techniques have proven to be efficient at reducing nonproductive adsorption of enzymes by bound lignin on solid substrates. However, the effectiveness of both BSA and surfactants were demonstrated using well-washed substrates that did not containing unbound lignin. Their effectiveness at reducing inhibition by unbound lignin, such as lignin in the pretreatment hydrolysate, has not been well studied (*5*). The differences in molecular weight and chemical structure between the residue lignin on solid substrate (bound) and the unbound lignin (removed by pretreatment) may cause differences in their affinity to BSA or surfactants. Furthermore, both of these techniques potentially cause problems for commercial application. For example, proteins are too expensive to be economically feasible for applications in practical bioconversion of biomass, and surfactants may cause problems in downstream processing and can be expensive.

It is well-known that lignin can form complexes with divalent or multivalent metal ions (*11*−*14*). This mechanism has been used for heavy metal removal (*15*−*17*). It has also been used to remove nonprocess elements in pulp production (*18*). However, the adsorption properties of lignin−metal complex for enzymes have not been examined and reported. We hypothesize that certain metal compounds, such as Ca(II) and Mg(II), when they form complexes with lignin can deactivate adsorption sites for enzymes. Furthermore, these metal compounds are not toxic to enzymes within certain concentration ranges. Therefore, it is possible to reduce or eliminate nonproductive enzyme adsorption in cellulase−lignocellulose systems through this type of lignin−metal complexation and thereby enhance enzymatic hydrolysis of lignocelluloses substrate.

The objective of this study is to investigate the effects of various lignin−metal complexes on enzymatic hydrolysis of cellulose to validate the above proposed hypothesis. Specifically, the study will find the "right" metal compounds that are not only nontoxic to enzymes but can also deactivate the adsorption sites of lignin for enzymes to reduce or eliminate nonproductive enzyme adsorption.

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Table 1. Pretreatment Conditions and Chemical Analysis Results of the Eucalyptus Substrates

	agents[a]		composition of substrates (%)					acid groups	
pretreatment	sulfuric acid (%)	sodium bisulfite (%)	glucan	xylan	galactan	lignin	substrate solids yield (%)	$-SO_3H$ μmol/g	$-COOH$ μmol/g
untreated wood			41.8	10.4	1.8	28.5	100.0		
hot-water	0	0	53.5	2.3	0.2	37.2	76.0	0	112
dilute-acid	1.1	0	55.6	0.6	ND	40.6	71.2	0	123
SPORL high pH	0	4	56.7	1.5	ND	33.8	69.8	24	113
SPORL low pH	1.1	4	56.3	0.3	ND	36.6	67.6	11	136
relative standard deviation (%)			1.3	4.6	5.7	0.7		2.5	6.0

[a] On oven dry (od) wood base in w/w.

The significance of this study lies in its potential to solve three practical problems: (1) achieving simultaneous saccharifcation and combined fermentation (SSCombF) of the cellulose fraction (lignocellulose solid substrate) with the hemicelluloses sugar stream (pretreatment spent liquor or hydrolysate). The unbound lignin in the hemicellulose sugar stream can inhibit enzymatic saccharification of cellulose, (2) reducing extensive water washing of the cellulose fraction (pretreated lignocellulose solids), which is required currently before enzymatic saccharification to reduce the amount of unbound lignin from cellulose solid substrate, (3) improving the low enzymatic digestibility of biomass cellulose (especially woody biomass) substrate produced by most existing pretreatment methods due to the inhibition of bound lignin on the substrate using low enzyme dosage.

MATERIALS AND METHODS

Substrates. Two types of solid substrates were used in this study: pure cellulose and lignocelluloses from pretreated eucalyptus wood. Whatman Quantitative Filter Paper (grade no. 41, Whatman, England; ash content <0.01) was defibrated for 8000 revolutions using a disintegrator (TMI, Ronkonkoma, NY) to produce pure cellulosic substrate. Eucalyptus wood chips were screened to remove particles smaller than 6 mm and larger than 38 mm. The accepted wood chips have thickness ranging from 3 to 8 mm and were subjected to hot-water, dilute-acid, and two sulfite pretreatment to overcome recalcitrance of lignocellulose (SPORL) pretreatments and then disk-milled to produce lignocellulosic solid substrates as described in our previous studies (*19*, *20*). All pretreatments were conducted at 180 °C with a liquid to wood ratio (L/W) of 3 for a fixed pretreatment duration of 30 min. An extensive washing using deionized water was applied to milled fibrous substrates after disk milling to remove dissolved (unbound) lignin adsorbed to the substrates. The major chemical components of the substrates were analyzed (**Table 1**). The lignin data includes both bound and the remaining (after washing) unbound lignin.

Enzymes. The cellulase complex used was a mixture of Cytolase CL preparation (Genencor, Menlo Park, CA) and β-glucosidase (Novozyme 188) from commercial source (Sigma-Aldrich, St. Louis, MO). The enzyme activities of 43 FPU/mL for Cytolase CL and 415 CBU/mL for Novozyme 188 were calibrated using a method from the literature (*21*).

Metal Compounds and Lignins. $CaCl_2$, $CuCl_2$, $MgSO_4$, and $FeCl_3$ were all of analytical grade from a commercial source (Sigma-Aldrich, St. Louis, MO). High purity sulfonated lignin (SL) D748 from softwood sulfite pulping was donated by LignoTech USA (Rothschild, WI). Organosolv lignin (OL) was purchased from Sigma-Aldrich (St. Louis, MO). Kraft lignin (KL) was kindly provided by S. Ralph of the U.S. Forest Service, Forest Products Laboratory (Madison, WI). The SL and OL resemble the dissolved lignin in the pretreatment hydrolysates by SPORL and Organosolv pretreatments, respectively. These two pretreatments are the only two methods proven to produce excellent cellulose digestibility from woody biomass, especially softwood species. SL is water-soluble and was directly added into cellulose suspension during experiments. OL and KL were dissolved in dilute NaOH solutions (pH 12) separately to make lignin solutions with concentration of 10 g/L for hydrolysis experiments. This is to better simulate enzymatic hydrolysis of unwashed lignocelluloses with unbound lignin. No pH changes were observed when OL and KL solution were added up to concentration of 0.1 g/L. However, when OL or KL lignin concentration was increased to 0.2 and 0.4 g/L, the measured pH was increased slightly by 0.04 and 0.09, respectively. About 20 and 50 μL of HCl (1M) was added, respectively, to adjust the pH back to 4.8 ± 0.01.

Enzymatic Hydrolysis of Cellulosic Substrate. Enzymatic hydrolysis of the cellulosic substrate was conducted at 1% solids concentration (w/v) in 50 mM acetate buffer (pH 4.8). After the addition of lignin or/and metal compound, the reaction flasks were incubated at 50 °C on the rotary shaker at 200 rpm for 15 min, then the enzymes were added to start hydrolysis. The enzyme loadings were 15 and 7.5 FPU/g substrate for pure cellulose and pretreated wood substrates, respectively. The ratio of cellulase and β-glucosidase loading was 1:1. The pretreated substrates have low cellulose contents from 42 to 57% (**Table 1**), therefore enzyme loading on a cellulose base is in the range of 13−18 FPU/g cellulose, similar to that for the pure cellulose substrate and those in the literature (*22*, *23*). It was found that the pH of the aqueous cellulose−enzyme suspension was not affected by the addition of either SL or/and metal compounds. Aliquots of 300 μL were taken at different time points (1, 2, 4, 6, 12, 24, 36, and 48 h), and immediately heated in boiling water bath to stop enzymatic hydrolysis. The samples were then centrifuged at 4000*g* for 5 min before glucose analysis. Control experiments were carried out without the addition of lignin and metal compound. Replicating hydrolysis experiments were conducted. The relative standard deviation of 2.5% determined from replicate measurements was used as error bars.

Analytical Methods. The chemical composition of the original and pretreated solid substrates was measured by the Analytical and Microscopy Laboratory (USDA Forest Products Laboratory). The solid biomass substrates were air-dried and then Wiley milled to a size passing a 20 mesh (~1 mm) screen. The resulting materials were hydrolyzed using sulfuric acid in two stages. The hydrolysis conditions were acid concentration of 72% (v/v) at 30 °C and 3.6% (v/v) at 120 °C for the first and second stage, respectively. The hydrolysis duration time was 1 h for both stages. The hydrolysate was analyzed for carbohydrates using an improved high-performance anion exchange chromatography with pulsed amperometric detection (HPAEC-PAD) method (*24*). The Klason lignin content was measured gravimetrically after washing and drying the solid residue from the acid hydrolysis. For fast analysis, glucose in the hydrolysate supernatant was measured using a commercial glucose analyzer (YSI 2700S, YSI Inc., Yellow Springs, OH). A phosphate buffer YSI 2357 stabilized by1.5 mM EDTA and 6.9 mM sodium benzoate was used (*25*). The error caused by the addition of Ca(II) (10 mM), Cu(II) (1 mM), Mg(II) (10 mM), Fe(III) (2.5 mM), and SL (0.4 g/L) on the analysis of glucose using the YSI glucose analyzer was determined to be 0.3, −0.8, 0.9, −2.1, and 0.6%, respectively. Therefore, the effects of metal ions and SL on the accuracy of the YSI system for glucose measurements can be ignored. All sugar measurements were performed at least twice. The averaged data were reported. The sulfonic and carboxylic group contents of the pretreated substrates were measured by a conductometric titration method (*26*, *27*)

RESULTS AND DISCUSSION

Inhibition of the Enzymatic Hydrolysis of Pure Cellulose by Unbound Lignin. A few studies reported the inhibition of cellulase by unbound lignin (*5*, *28*) which can be simulated by the purified lignin spiked into cellulose suspension systems. When lignin (SL) was added into pure cellulose (Whatman filter paper) suspensions

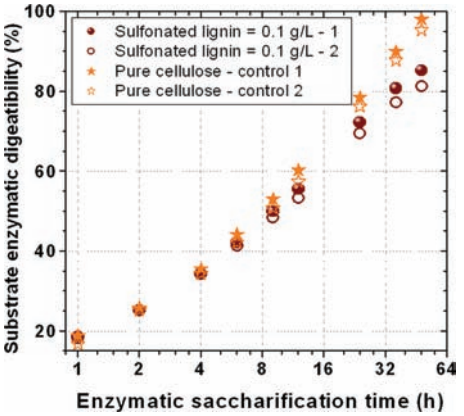

Figure 1. Time-dependent substrate enzymatic digestibilities of pure cellulose with and without the addition of sulfonated lignin.

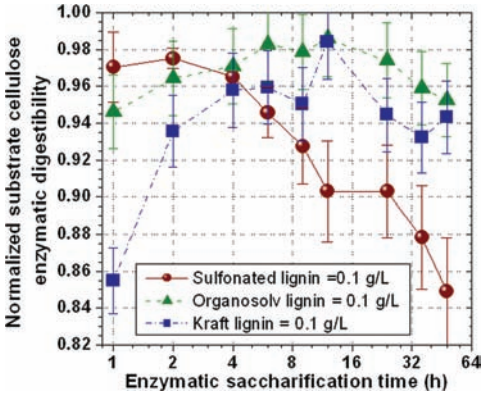

Figure 2. Effects of different types of lignin at 0.1 g/L on normalized time-dependent enzymatic digestibility of pure cellulose.

at a concentration of 0.1 g/L, the substrate cellulose saccharification efficiency was decreased as reflected by the substrate cellulose digestibility (SED) (**Figure 1**). SED is defined as the percentage of glucan in substrate converted to glucose by the enzymes. Time-dependent data from duplicate hydrolysis experiments of the control (no lignin addition) and with LS addition are shown in **Figure 1**, which demonstrates good experimental repeatability was obtained with only a small systematic error (about 4%) at each sampling point. The duplicate experiments were conducted two weeks apart. So, the systematic errors were most likely from glucose analysis using different calibrations. The reduction in SED by SL after 48 h hydrolysis was about 14%. SED always increases as hydrolysis proceeds, and enzyme adsorption by lignin is also time-dependent. As a result, the dynamic information about the inhibition of enzymatic hydrolysis by the spiked lignin was obscured by the rapid increase in SED as hydrolysis proceeds. The data in **Figure 1** can only tell the reduction in SED at a particular time. To illustrate the dynamic behavior of nonproductive adsorption of enzymes by lignin, we normalized each SED data point from a lignin-spiked experiment by the corresponding SED at the same hydrolysis time of the pure cellulose (control) experiment. The normalized SED after 48 h hydrolysis was decreased by 15% when SL was added (**Figure 2**). When the same amount of OL or KL was added, the SED was reduced by about 6% (**Figure 2**). This could be due to the differences in the affinity of different lignin with enzymes. The hydrophilic nature of SL may result in a stronger affinity to enzymes. The lignin solubility may also play a role. SL is soluble at pH of 4.8, whereas OL and KL are not. Adsorption study is needed to elucidate this behavior in the future. The reduced SED is a result of

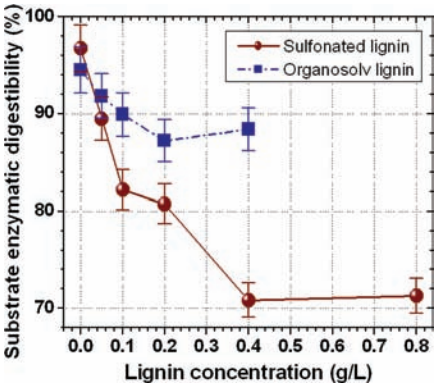

Figure 3. Effects of the amount of sulfonated and Kraft lignin added on enzymatic digestibility of pure cellulose measured at 48 h.

nonproductive enzyme adsorption onto lignin as reported in the literature (*1*−*5*).

The time-dependent SED data suggest that there are competing physical and chemical processes are taking place in the lignin-containing aqueous cellulose−enzyme suspensions. Because of the soluble nature of SL, SED decreased with hydrolysis time continuously when SL was added (**Figure 2**). As described in the Materials and Methods section, the KL or OL solution (pH = 12) was added and preincubated on the shaking bed for 15 min first before enzymes were added to beginning hydrolysis. However, KL and OL are not soluble in the aqueous cellulose−enzyme suspension of pH about 4.8. KL and OL precipitation occurred. The precipitation process of the dissolved KL and OL in the alkaline solution onto the cellulose substrate is similar to that occurred during washing of Organosolv and Kraft pulps. Washed Kraft and Organosolv fiber surfaces were covered by an adsorbed layer of reprecipitated lignin (*29*, *30*). This suggests that a similar layer of reprecipitated lignin covered the cellulose surface after KL or OL lignin solution was added, which resulted in a significant reduction (15%) in SED at the beginning of the hydrolysis (**Figure 2**). As hydrolysis proceeds, the cellulose fibers were broken down by the enzymes, which increased the cellulose accessibility to the enzymes and effectively reduced the cellulose covering area by KL. As a result, SED mostly recovered by 4 h (**Figure 2**). The desorbed lignin can still bind with enzymes, which continues to suppress enzyme activities and therefore SED (**Figure 2**). Similar explanations can be applied to the data set for OL.

The results (**Figure 2**) have significant implications to all pretreatment technologies that remove a fraction of biomass lignin, especially to the two most robust biomass pretreatment processes for sugar and ethanol production, Organosolv (*23*) and SPORL (*22*, *31*). Organosolv pretreatment dissolves as much as 70% of biomass lignin (*23*) into the pretreatment hydrolysate. SPORL can remove about 20−40% of biomass lignin (*19*, *32*) in the form of lignosulfonate. The results from the KL addition experiment suggest that certain alkaline pretreatments will suffer from the same consequence of nonproductive enzyme adsorption by unbound lignin. A thorough washing of lignocellulose solid substrate has been applied as a common practice to reduce nonproductive adsorption before enzymatic hydrolysis.

To further evaluate the effect of unbound lignin on enzymatic hydrolysis of solid lignocellulosic substrate, different amounts of OL and SL were added to the pure cellulose substrate suspension before enzymatic hydrolysis. The increases in lignin (OL or SL) concentration in the aqueous substrate suspension resulted in further inhibition of cellulose saccharification as observed from the reduced normalized SED after 48 h hydrolysis (**Figure 3**).

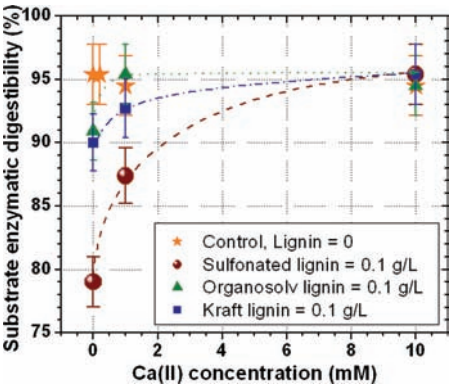

Figure 4. Recoveries of pure cellulose enzymatic digestibility measured at 48 h by the addition of $CaCl_2$ in a cellulose−enzyme system containing different types of lignin at 0.1 g/L.

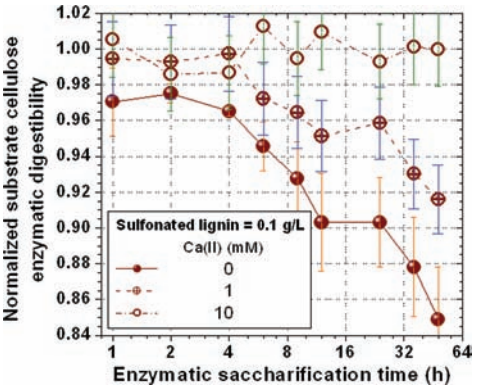

Figure 5. Recoveries of the time-dependent enzymatic digestibility of pure cellulose by the addition of $CaCl_2$ in a cellulose−enzyme system containing sulfonated lignin at 0.1 g/L.

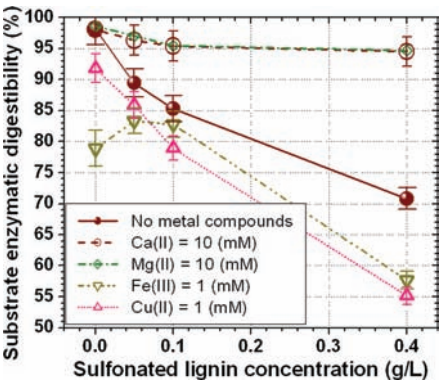

Figure 6. Effects of the additions of different metal compounds on enzymatic digestibility of pure cellulose measured at 48 h in a cellulose−enzyme system containing sulfonated lignin at 0.1 g/L.

Fortunately, the reduction in SED reached an asymptotic value at OL and SL concentrations of 0.2 and 0.4 g/L, respectively. These two critical lignin concentrations may be related to the amount of enzyme applied.

Calcium(II) to Reduce Inhibition of Enzymatic Cellulose Hydrolysis by Unbound Lignin. We hypothesized that lignin−metal complexes can reduce nonproductive enzyme adsorption in aqueous cellulose−enzyme systems. Different amounts of $CaCl_2$ were added into the pure cellulose suspensions that contained 0.1 g/L lignin (OL, KL, or SL) before enzymatic hydrolysis. It was found that $CaCl_2$ has no effect on enzymatic hydrolysis of pure cellulose without the addition of lignin (**Figure 4**). The slight reduction of about 1% in saccharification efficiency at Ca(II) concentration of 10 mM is within the measurement uncertainty. When $CaCl_2$ was added into lignin containing cellulose suspensions, the recoveries of SEDs are obvious (**Figure 4**). With the addition of Ca(II) to a concentration of just 1 mM, 50% or more of the reduction in cellulose saccharification caused by lignin addition was recovered for all the lignin-containing cellulose suspensions. The reduction in SED due to lignin addition was almost completely eliminated at Ca(II) concentration of 10 mM.

The effectiveness of Ca(II) for reducing nonproductive enzyme adsorption can also be observed from the time-dependent enzymatic saccharification efficiency of pure cellulose with the addition of lignin (**Figure 5**). When SL was added into the pure cellulose suspension to a concentration of 0.1 g/L, the normalized SED by the corresponding SED of the pure cellulose system at the same hydrolysis time was reduced by 3% after the first hour of hydrolysis and further decreased by 15% after 48 h. When $CaCl_2$ was added into the suspension to a Ca(II) concentration of 1 mM, the reduction in SED by SL at 48 h recovered by 50% (**Figure 5**). When Ca(II) concentration was increased to 10 mM, the inhibition of enzymatic hydrolysis by the added SL was almost completely eliminated throughout the entire hydrolysis process. It was reported that calcium ion can bind to lignisulfonate-sodium plasticizer (*33*) and calcium ion can also exchange sodium ion on SL in neutral solutions in room temperature (*34*). These suggest that the sodium based SL used in this study serves as a chelating agent to bind calcium ions to form complex. This complex has low affinity to cellulase enzymes which reduced nonproductive enzyme absorption and resulted in the observed enhancement in enzymatic hydrolysis of cellulose.

Effect of Other Metals on Enzymatic Cellulose Hydrolysis in the Presence of Unbound SL. The effectiveness of various metal compounds for reducing lignin inhibition of enzymatic cellulose hydrolysis was evaluated. Pure cellulose suspensions in the presence of SL with a concentration range up to 0.4 g/L were added with different metal compounds before hydrolysis. Cu(II) was known to be toxic to most cellulase enzymes. The addition of $CuCl_2$ to a Cu(II) concentration of just 1 mM produced additional significant reduction in SED of the substrate cellulose (**Figure 6**). The addition of $FeCl_3$ showed the similar results to those obtained using $CuCl_2$. The addition of $CaCl_2$ to a Ca(II) concentration of 10 mM effectively eliminated the inhibition of enzymatic hydrolysis by SL in the concentration range tested up to 0.4 g/L (**Figure 6**). The results clearly show that the addition of $MgSO_4$ to a Mg(II) concentration of 10 mM also effectively eliminated inhibition of enzymatic hydrolysis by SL. It is known that Ca(II) is nontoxic to yeast and Mg(II) has a positive effect on yeast growth in fermentation within certain concentration ranges, therefore the addition of Ca(II) or Mg(II) may improve simultaneous enzymatic saccharification and fermentation using unwashed or less washed solid substrate to reduce water consumption in production.

Ca(II) to Reduce Inhibition of Enzymatic Cellulose Hydrolysis by Lignin in Pretreated-Wood Substrate. Reducing the inhibition of cellulase activities by lignin in lignocellulosic substrate can improve biomass substrate saccharification efficiency. $CaCl_2$, $CuCl_2$, and $FeCl_3$ were separately applied to enzymatic hydrolysis of each of four eucalyptus substrates produced by hot-water, dilute-acid, and SPORL high pH and SPORL low pH pretreatment. Different pretreatments resulted in very different substrate enzymatic cellulose digestibility (**Figure 7**). The addition of $CuCl_2$ and $FeCl_3$ reduced SED as expected based on the discussions in the previous section. The addition of $CaCl_2$ with Ca(II) concentration of 10 mM in the substrate suspension increased SED for

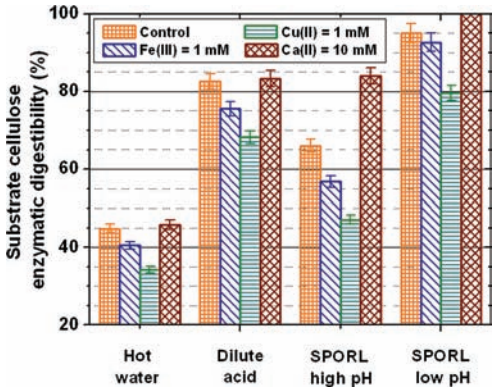

Figure 7. Effects of the additions of different metal compounds on enzymatic digestibilities of washed pretreated-eucalyptus substrates measured at 48 h.

two SPORL pretreated substrates tested (**Figure 7**). The increase was especially significant for the SPORL high pH substrate with an increase in SED of about 20% (or about 27%). Complete enzymatic cellulose saccharification of the SPORL low pH substrate was achieved. However, the increase was not obvious for the hot-water and dilute-acid pretreated substrates. The difference in the effectiveness of $CaCl_2$ addition on enhancement of cellulose enzymatic saccharification efficiency of different substrates may be explained by the difference in lignin chemical structure or functional groups that can interact with metal ions to form a lignin−metal complex. A high degree of sulfonation may promote the formation of a lignin−metal complex. The higher sulfonic acid group content of SPORL high pH substrate than that of the SPORL low pH substrate (**Table 1**) may explain the difference in the enhancement of SED of these two substrates when the same amount of 10 mM Ca(II) was applied. On the other hand, no sulfonation reaction occurred during the dilute-acid and hot-water pretreatment as evidenced by the measured sulfonic acid group content (**Table 1**). Moreover, washing removed almost all of the unbound lignin on the pretreated wood substrates. As a result, no obvious effective enhancement of SED of the dilute-acid pretreated substrates (hot-water is a special case of dilute-acid pretreatment) was observed (**Figure 7**).

Both the bound lignin on solid substrate and the unbound lignin (can be dissolved or insoluble) in the lignocellulosic substrate−enzyme system can inhibit cellulose enzymatic hydrolysis through nonproductive adsorption of enzymes. The application of Ca(II) and Mg(II) were proven to be effective at reducing or eliminating both unbound and bound lignin inhibition likely through the formation of lignin−metal complex. A plausible explanation for this is that the lignin−metal complexation can deactivate the enzyme adsorption sites. This mechanism can be validated by enzyme adsorption measurement in future studies. The time-dependent SED data presented in this study represent the dynamic and competing processes of the formation of lignin−metal complex and the adsorption/desorption of enzymes.

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