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Eliminating inhibition of enzymatic hydrolysis by lignosulfonate in unwashed sulfite-pretreated aspen using metal salts ☆

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ABSTRACT

This study demonstrated the efficiency of Ca(II) and Mg(II) in removing inhibition of enzymatic hydrolysis by lignosulfonate through non-productive adsorption of enzymes. Adding 1 mmol/g cellulose of either metal salt restores approximately 65% of the activity lost when a pure cellulose/cellulase solution is spiked with lignosulfonate. Addition of either Ca(II) or Mg(II) is also effective in counteracting soluble inhibitors of cellulase present in unwashed aspen solid substrate produced by SPORL (sulfite pretreatment to overcome recalcitrance of lignocelluloses). Soluble inhibitors are often removed by thoroughly washing the lignocellulosic solid substrate following pretreatment. It was determined that adding 1 mmol of $MgSO_4$/g substrate (oven dry) to the unwashed aspen substrate gave enzymatic substrate digestibility (SED) equivalent to that of washing for a range of enzyme loadings. These results demonstrate that applying divalent metal salts eliminates the need for washing, thereby saving considerable process water and cost for production of chemicals and biofuels from lignocellulose.

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1. Introduction

It is well known that lignin can inhibit enzymatic hydrolysis of cellulose through non-productive adsorption (Mansfield et al., 1999; Sewalt et al., 1997; Tengborg et al., 2001). Delignification of lignocellulosic substrate proved to be effective to enhance enzymatic saccharification (Pan et al., 2004). However, it is expensive. The removal of inhibitions by unbound lignin (which here refers to lignin in pretreatment hydrolysate free from wood) and other impurities in pretreatment hydrolysate/spent liquor were commonly achieved by washing the lignocellulosic substrate before enzymatic hydrolysis (Nagle et al., 2002; Sinitsyn et al., 1982; Tengborg et al., 2001). Because the unbound lignin after an acidic-type pretreatment tends to condense and precipitate, a pressurized hot washing process was also developed for further removal of lignin and hemicelluloses (Nagle et al., 2002). However, substrate washing can consume a significant amount of water for commercial cellulosic ethanol production with a typical capacity of 2000 ton biomass/day (a typical pulp mill with similar capacity uses about 10,000 m^3 water/day for pulp washing), which would be a great environmental concern. Furthermore, high-temperature/pressure washing is energy intensive. The significant dilution incurred from washing also requires an energy-intensive concentration step to reclaim the hemicellulose sugars in the washing filtrate. Moreover, the inhibition of enzymatic cellulose hydrolysis by the unbound lignin dissolved in the pretreatment hydrolysate can prevent practicing simultaneous saccharification of lignocellulose and combined fermentation (SSCombF) of the cellulosic fraction with the hemicellulosic sugar stream. The benefits of SSCombF, which include simplifying the production process and alleviating end-product inhibition of enzymes at high carbohydrate loadings, may be outweighed by the non-productive adsorption of enzymes by unbound lignin. Therefore, addressing the inhibition of enzymatic hydrolysis of lignocelluloses by unbound lignin has significant importance for practical applications.

There are several possible alternatives to washing to improve enzymatic hydrolysis of lignocelluloses. Excess cellulase loading in hydrolysis is one solution but at the expense of increasing enzyme cost. Using additives, such as bovine serum albumin (BSA) and surfactants, to "block" lignin from interacting with cellulase has proved effective (Borjesson et al., 2007; Eriksson et al., 2002; Pan et al., 2005; Tu et al., 2009; Yang and Wyman, 2006; Zheng et al., 2008). However, BSA is too expensive to be economically feasible for commercial biorefineries. Surfactant application at the dosage of about 0.2%, as commonly used in the literature, may negatively affect downstream processing and can also be expensive.

☆ This work was conducted on official US government time by Zhu, while Liu was a visiting scientist at the University of Wisconsin-Madison, and USDA Forest Service, Forest Products Laboratory (FPL). The work is in the public domain in the United States.

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We reported in a previous study (Liu et al., 2010) the application of certain metal compounds, namely Ca(II) and Mg(II), to enhance enzymatic hydrolysis of pure cellulose spiked with lignin, i.e., lignosulfonate, kraft lignin, and Organosolv lignin. It is known that certain metal compounds can associate with lignin (Torre et al., 1992). We hypothesized that the formation of lignin–metal complexes (overliming of pretreatment hydrolysate should produce similar phenomena of lignin–Ca(II) complexation) may play a role in reducing affinity of lignin to cellulase enzymes. This study is an extension of our previous work to apply metal salts to unwashed pretreated lignocellulosic substrate containing unbound (dissolved) lignosulfonate. Different metal salts were first applied to a pure cellulose system spiked with purified lignosulfonate from SPORL (sulfite pretreatment to overcome recalcitrance of lignocelluloses) of eucalyptus. Then metal salts alone were applied to unwashed and washed aspen substrate from SPORL. BSA and Tween80 were also used for comparison. The objective of the study is to demonstrate the application of metal salts for enhancing enzymatic hydrolysis of unwashed pretreated lignocellulose. The goal is to eliminate substrate washing to save water or reduce enzyme loading while achieving efficient enzymatic saccharification using inexpensive metal salts.

2. Methods

2.1. Materials

Two types of cellulosic substrates were used in this study. A pure cellulosic substrate was obtained by disintegrating commercial filter papers (Adventec No. 1, ADVANTEC, Japan) using a disintegrator (TMI, Ronkonkoma, New York) for 8000 revolutions. A lignocellulosic substrate was produced from aspen wood chips using sulfite pretreatment to overcome recalcitrance of lignocellulose (SPORL) (Wang et al., 2009; Zhu et al., 2009). The SPORL solution was made of dilute sodium bisulfite and sulfuric acid. The pretreatment was conducted in a 1-L wood pulping digester using 150 g oven dry (od) wood at 170 °C for 25 min with a liquor-to-wood (L/W) ratio of 3 and acid and bisulfite charge on od wood of 1.1% and 3%, respectively, as described elsewhere (Zhu et al., 2010). At the completion of SPORL, the remaining solids remained as wood chips and were separated from spent liquor (pretreatment hydrolysate) using filter paper (Whatman No. 1, Whatman, England). The separated wood chips were fed into a disk mill (Andritz Sprout-Bauer Atmospheric Refiner with disk-plate pattern D2-B505, Springfield, Ohio) without washing to produce lignocellulosic substrate.

Cellulase enzymes Multifect CL and Novozyme 188 (β-glucosidase) were purchased from Genencor (San Francisco, California) and Sigma–Aldrich (St. Louis, Missouri), respectively. The enzyme activities were 73 FPU/mL and 413 CBU/mL for Multifect CL and Novozyme 188, respectively, determined following the International Union of Pure and Applied Chemistry (IUPAC) recommended method (Ghose, 1987). Soluble lignosulfonates were purified from in-house-produced SPORL hydrolysate using an Amicon ultrafiltration unit (Amicon, Beverly, Massachusetts) with a cut-off from 3 to 10 kDa. The pretreatment hydrolysate was produced from eucalyptus using SPORL at 180 °C with a liquid-to-wood ratio of 3 and sulfuric acid and sodium bisulfite charge on wood (od) 1.1% and 4%, respectively. No carbohydrate was detected in the purified lignosulfonates samples by anion exchange chromatography described in Section 2.5. Standard grade BSA was purchased from Sera Care (Milford, Massachusetts). All other chemicals were from commercial sources and of analytical grade.

2.2. Washing of SPORL aspen solid substrate

Washing experiments were conducted by mixing 2.0 g od SPORL substrate with different volumes (20–500 mL) of deionized (DI) water in several corresponding Erlenmeyer flasks. This is to obtain the maximal amount of removable unbound (dissolved) lignosulfonate (MARUL) from washing at several washing water temperatures—25, 50, 65, 80, 98 °C—based on dilution and extraction. DI water was first heated to a desired temperature and then poured into a flask. The flask was then set on a temperature-controlled rotary incubator (Excella E25, New Brunswick Scientific Co., Edison, New Jersey) at 250 rpm after adding 2.0 g od pretreated substrate. After mixing for 15 min (predetermined through experiments to achieve equilibrium), a sample of about 0.6 mL was taken and centrifuged at 10,000 g for 5 min to separate the solids from liquid. The supernatant was analyzed for lignosulfonate using UV/VIS absorption as described in Section 2.5.

Two thoroughly washed SPORL aspen substrates were produced at temperatures 25 and 98 °C for enzymatic hydrolysis study. The washings were carried out in five stages using 2 g od unwashed pretreated aspen substrate and 500 mL DI water in each stage to ensure achieving maximal removal of unbound lignosulfonate, MARUL, through washing. Again the substrate was thoroughly mixed with 500 mL washing water as described above for 15 min. The substrate was then separated by filtration using Whatman No.1 filter paper. The filter pad was collected and used for the next stage of washing. The residual lignosulfonate concentration, n_w, in the filtrate stream of each stage was monitored using spectrophotometry as described in Section 2.5 and was found undetectable at the fifth stage for both washing experiments. The chemical compositions of the unwashed and two washed substrates are listed in Table 1. Washing enriched bound lignin (here refers to lignin in wood but not necessarily chemically bonded) and carbohydrate content by removing unbound lignin and impurities. The differences in chemical compositions between the two washed substrates at different temperatures are within the measurement uncertainties.

2.3. Applications of additives

Metal salts, Tween80, and bovine serum albumin (BSA) were separately applied to the cellulosic substrates/buffer suspension to study the effect of these additives on enzymatic hydrolysis. Metal salts were used rather than metal alkalis, such as calcium oxide or hydroxide, to avoid the changes of pH of substrate/buffer system. Good mixing of additives with substrate suspension was achieved by setting the Erlenmeyer flask that held the suspension on the temperature-controlled rotary incubator (Excella E25, New

Table 1
Chemical compositions of the unwashed and washed SPORL aspen solid substrates (% w/w on oven dry basis).

Samples	Ash	K. lignin	Arabinan	Galactan	Glucan	Xylan	Mannan	Total carbohydrate
Unwashed	0.1	20.4	0.2	0.3	53.3	11.3	1.3	66.5
Washed at 25 °C	Nd	22.5	Nd	Nd	72.7	2.8	0.3	75.8
Washed at 98 °C	Nd	23.6	Nd	Nd	71.6	2.8	0.6	75.0
Relative standard deviations (%)		1.1	5.8	5.7	1.5	4.6	4.2	

Brunswick Scientific Co., Edison, New Jersey) at 200 rpm at 50 °C for 10 min. The application dosages were varied from 15 to 150 and 2.5 to 20 mg/g substrate for Tween80 and BSA, respectively, similar to those reported in the literature (Eriksson et al., 2002; Tu et al., 2009; Yang and Wyman, 2006; Zheng et al., 2008). The dosages of metal salts $MgSO_4$, $CaSO_4$, $CaCl_2$, and KCl ranged from 0.5 to 4 mmol/g substrate. The pH shift caused by these additives was minimal at less than ±0.06. A 5-g/L standard glucose solution was spiked by different additives to examine whether or not the additives affect glucose measurements. The differences in measured glucose concentrations were 0.5%, 0.3%, 0.9%, −0.4%, and −0.6% by adding K(I) at 20 mM, Mg(II) at 40 mM, Ca(II) at 25 mM, Tween80 at 150 mg/L, and BSA at 20 mg/L, respectively. The results suggest additives did not affect glucose measurements.

2.4. Enzymatic hydrolysis

Enzymatic hydrolysis was performed in batch at 50 °C and pH 4.8 in an Erlenmeyer flask on a rotator incubator (Excella E25, New Brunswick Scientific Co., Edison, New Jersey) at 200 rpm. Unless specified, the loading of Multifect CL was 5.0 and 10 FPU/g od substrate for the SPORL aspen and filter paper cellulose, respectively. The loading ratio of Multifect CL to Novozyme 188 was kept at 1 for all studies. The hydrolysis was conducted at about 1% solids consistency (w/v), i.e., 1 od g substrate in 100 mL enzyme/buffer solution, for all substrates of pure cellulose, washed and unwashed aspen substrates from SPORL. Enzymatic hydrolysate was sampled periodically to obtain time-dependent glucose concentration. For each sampling point, a duplicate 0.4 mL suspension was taken for analysis. Duplicate hydrolysis experiments were conducted and the standard deviations were used as measurement errors. Error bars were omitted in some plots for clarity.

2.5. Analytical methods

The chemical compositions of the unwashed and washed (at 25 and 98 °C) solid aspen lignocellulosic substrates were determined by the Analytical Chemistry and Microscopy Laboratory (USDA Forest Products Laboratory, Madison, Wisconsin). The substrates were first 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 as described elsewhere (Zhu et al., 2010). The carbohydrates in the hydrolysate were analyzed using improved high-performance anion exchange chromatography with pulsed amperometric detection (HPAEC-PAD) (Davis, 1998). For fast analysis, the glucose in the enzymatic hydrolysates was measured using a commercial glucose analyzer (YSI-2700, Yellow Springs Instrument Co., Ohio).

The concentrations of soluble lignosulfonate in the washing filtrate streams were determined using a differential UV–VIS spectroscopic method (Lozovik and Kaflyuk, 2005). The absorption spectra of lignosulfonate in alkaline and neutral alcohol–water solution were recorded by a UV–VIS spectrophotometer (Model 3010, Hitachi, Japan). The absorbance at 257 nm was used for quantification. The purified lignosulfonate (Ligno Tech-USA, Rothchild, Wisconsin) was used as standard for calibration. The concentrations of metal ions in substrate residuals after enzymatic hydrolysis were measured by the Analytical Chemistry and Microscopy Laboratory (USDA Forest Products Laboratory). The collected solids were air dried. Very small amounts (~1 mg) were digested in a microwave oven (MDS-2000, CEM Corp., Matthews, North Carolina, USA) with 5 mL of HNO_3 and 5 mL of 30% H_2O_2. ICP-AES (Ultima model, Horiba Jobin–Yvon, Edison, New Jersey, USA) was then used for elemental determinations.

3. Results and discussions

3.1. Effect of unbound lignosulfonate on cellulase hydrolysis of pure cellulose and the abilities of metal salts, BSA, and Tween80 to reverse this effect

The inhibition of enzymatic cellulose hydrolysis by unbound lignosulfonate was first evaluated by spiking purified lignosulfonates into a pure cellulose suspension. Previously, we reported that commercial lignosulfonate can inhibit enzymatic hydrolysis of pure cellulose (Liu et al., 2010). We demonstrated that Ca(II) can eliminate this inhibition through the formation of lignin–Ca(II) complex. By using purified lignosulfonate derived from SPORL, we can demonstrate the potential of various metal salts for enhancing enzymatic hydrolysis of unwashed lignocelluloses from SPORL. The pure cellulosic substrate enzymatic digestibility (SED), determined as the percentage of glucan enzymatically hydrolyzed to glucose in 48 h at a 1% w/w substrate solid loading, decreased from 84% to 73% in the presence of 50 mg of purified lignosulfonate per gram cellulose (or 5% w/w) (Fig. 1). This is in agreement with prior research using milled wood lignin or hydroxypropylated lignin (Sewalt et al., 1997). In quantitative terms, this corresponds to a reduction of 0.22% g cellulose/mg purified lignosulfonate at a cellulase loading of 10 FPU/g cellulose. We call this value the unbound lignin inhibition potential (LIP), defined as

$$\mathrm{LIP} = \lim_{\substack{\text{lignin}=0 \\ \text{enzyme}\to 0}} \frac{d(\mathrm{SED})}{d(\text{lignin})} \tag{1}$$

The value of 0.22% g cellulose/mg lignin is an average value and is much smaller than those obtained in our previous study using a commercial lignosulfonate (Liu et al., 2010). The LIP values obtained in the previous study were >1% g cellulose/mg lignin at lignosulfonate dosage less than 40 mg/g cellulose and a lower enzyme loading of 7.5 FPU/g cellulose. The differences in lignin itself may be a factor as well as the lower lignin dosages and enzyme loading used in the previous study.

Metal salts $CaCl_2$ and $MgSO_4$ were separately added to pure cellulose suspensions spiked with purified lignosulfonate to verify the preliminary results obtained in the prior study (Liu et al., 2010). In this study, we demonstrated the complete elimination of the inhibition of enzymatic hydrolysis by lignin including lignosulfonate (0.1 g/L or 10 mg/g cellulose) spiked into a pure cellulose suspension by the application of metal salts, $CaCl_2$ and $MgSO_4$, at a concentration of 10 mM, or 1 mmol/g cellulose. We increased the purified lignosulfonate dosage to 50 mg/g cellulose in this study.

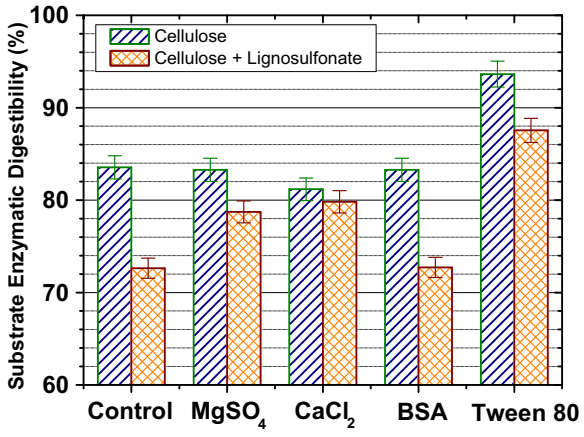

Fig. 1. Effects of the application of purified lignosulfonate and different additives on enzymatic digestibility of pure cellulose. Cellulase loading = 10 FPU/g cellulose.

It should be pointed out that the previous study found that the cellulose inhibition by the spiked lignin reached an asymptotic value using a dosage of about 20 mg/g cellulose with a commercial lignosulfonate. The present results indicate that the applications of $CaCl_2$ or $MgSO_4$ have no effect on enzymatic hydrolysis of pure cellulose (without lignosulfonate). The measured substrate enzymatic digestibilities (SEDs) were all about 83%, the same as for pure cellulose or the control run without the added metal salts (Fig. 1). When $CaCl_2$ and $MgSO_4$ were separately applied to the cellulose suspension spiked with 50 mg/g cellulose purified lignosulfonate, SED was increased from 73% to about 80%. Quantitatively, the application of $CaCl_2$ and $MgSO_4$ at a dosage of 1 mmol/g cellulose eliminated about 65% of the inhibition of enzymatic cellulose hydrolysis by the spiked lignosulfonate.

Tween80 and BSA were also separately applied to the same pure cellulose suspension treated at the same enzyme dosage of 10 FPU/g cellulose, for comparison. It was found that applying BSA at 10 mg/g cellulose was ineffective, demonstrating that had no effect on unbound lignosulfonate (spiked). The application of Tween80 at 15 mg/g cellulose improved SED from 83% to 94% and 73% to 88% (Fig. 1), respectively, for the pure cellulose and lignosulfonate-spiked cellulose suspensions. It is known that surfactants can improve enzymatic hydrolysis of pure cellulose and lignocellulose (Eriksson et al., 2002; Helle et al., 1993; Mizutani et al., 2002; Sewalt et al., 1997). These earlier studies suggested that surfactant enhances cellulose hydrolysis through reducing non-productive attachment/adsorption of endoglucanase to cellulose and lignin, and also by disrupting cellulose structure and increasing enzyme accessibility to cellulose. In comparison, metal salts are only expected to reduce non-productive adsorption of enzymes by complexing with the lignin. As a result, Tween80 is more effective in enhancing SED for the two cellulosic suspensions studied.

3.2. Determination of the maximal amount of removable unbound lignosulfonate by washing at different temperatures

The washing of SPORL lignocellulosic substrate to remove unbound lignin follows a dilution and extraction mechanism (Crotogino et al., 1987). The maximal amount of removable unbound lignosulfonate (MARUL) from the initial unwashed substrate, m_0, can be determined by measuring the unbound lignosulfonate concentration in the diluted substrate suspension after adding washing water, n_w, based on mass balance as

$$m_0 = n_w(V_0 + V_w) \quad (2)$$

where V_0 is the unknown volume of the water (moisture) in the initial unwashed wet substrate and V_w is the volume of dilution water used in washing. Eq. (2) can be rewritten as

$$\frac{1}{n_w} = \frac{V_0}{m_0} + \frac{V_w}{m_0} \quad (3)$$

The MARUL through washing, m_0, can be determined through linear regression of the measured n_w and V_w, i.e., m_0 is the inverse of the slope of Eq. (3). Values of n_w and V_w can be obtained by conducting a set of washing experiments using different amounts of washing water (dilutions) at a given washing temperature or pressure. The results showed excellent linear relationships between $1/n_w$ and V_w for the five sets of washing experiment conducted at five different temperatures (Fig. 2a).

It is known that washing efficiency can be improved by heating wash water and by increasing the solubility of impurities. Results indicate that MARUL, m_0, determined from the data in Fig. 2a was linearly proportional to washing water temperature (Fig. 2b) in the temperature range studied. At 25 °C, m_0 was about 45.4 mg from washing 1 od g substrate (85.1 mg/g cellulose based on glucan content of unwashed substrate). When washing water temperature was increased to 98 °C, m_0 was almost doubled to 89.6 mg/g od substrate (or 168.1 mg/g cellulose), or about 9% of the lignocellulosic substrate solids. Because both lignin sulfonation and condensation reactions could occur during SPORL (acid sulfite pretreatment), a high washing water temperature increased the solubility of the re-precipitated lignin (Adler, 1977; Nagle et al., 2002).

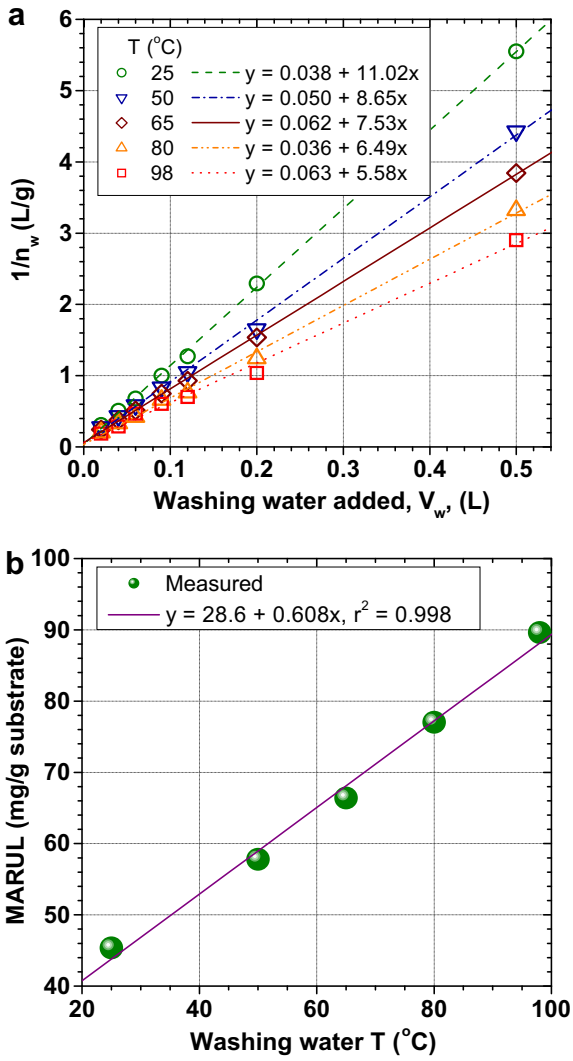

Fig. 2. (a) Correlations between the amount of washing water application and lignin concentration in the washing filtrate stream. (b) Effect of washing water temperature on the maximal amount of removable unbound lignin (MARUL).

3.3. Quantification of enzymatic hydrolysis inhibition by soluble components in an unwashed SPORL aspen substrate

Substrate enzymatic digestibilities (SEDs) of unwashed and washed SPORL aspen substrates were compared to verify the inhibitory effect of soluble components in the pretreatment hydrolysate, including that of unbound lignosulfonate. The comparative experiments were conducted at several enzyme loadings using the unwashed and two thoroughly washed substrates at 25 and 98 °C, respectively. The results clearly show that washing improved SED at 48 h for all the enzyme loadings studied (Fig. 3). Washing at 98 °C removed more unbound lignosulfonate than at 25 °C (Fig. 2b) and, further, improved SED for all the enzyme loadings studied (Fig. 3). The improvements are most significant at low en-

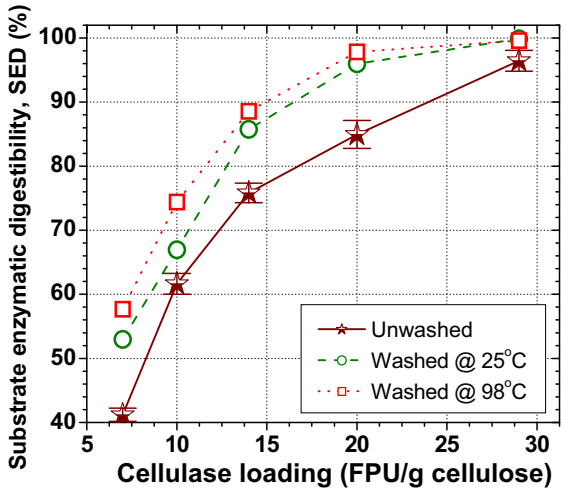

Fig. 3. Effects of washing on substrate enzymatic digestibility (SED) of a SPORL aspen substrate at various enzyme loadings. Averages were plotted for all curves.

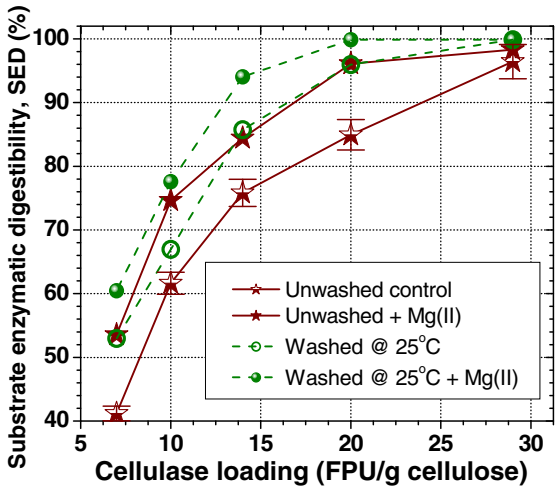

Fig. 4. Effects of $MgSO_4$ application (1 mmol/g substrate) on SED of unwashed and washed aspen substrate at 25 °C at various enzyme loadings.

zyme loadings and diminished at very high enzyme loadings. For example, SED was improved by 30% and 40%, respectively, by washing at 25 and 98 °C at enzyme loading of 7 FPU/g cellulose. The SED improvements were less than 5% at enzyme loading of 29 FPU/g cellulose. The observed effects of washing on SED improvement agree with those reported in the literature (Sinitsyn et al., 1982; Tengborg et al., 2001). We can use the amount of lignosulfonate removed as surrogate of the total soluble inhibitors to estimate this inhibitory effect. The total amount of unbound lignosulfonate removed is assumed to be equal to the MARUL, 168.1 and 85.1 mg/g cellulose, obtained from washing at 98 and 25 °C, respectively. Results (Table 2) clearly show the marginal inhibitory effect decreases with increase in (1) enzyme loading due to the availability of more free cellulase and (2) lignosulfonate content of the substrate. The estimated degrees of enzymatic hydrolysis inhibition (Table 2) of the 25 °C washed substrate are about the same as the 0.22% g cellulose/mg lignosulfonate quantified using pure cellulose spiked with purified lignosulfonate, except at very high enzyme dosages of 20 FPU/g cellulose and greater. It should be pointed out that the adsorbed soluble glucose on the substrate (washed at 50 °C) is less than 9 mg/g unwashed substrate, not considerable enough to cause end-product inhibition (Holtzapple et al., 1990).

3.4. Eliminating inhibition of enzymatic hydrolysis by lignosulfonate in an unwashed SPORL aspen substrate using metal salts, BSA, and Tween80

Metal salts along with BSA and Tween80 were separately applied to enzymatic hydrolysis of the unwashed and two thoroughly washed SPORL aspen substrates to evaluate the potentials of these additives for eliminating substrate washing. When 1 mmol $MgSO_4$/g substrate was applied, the SEDs of the unwashed substrate matched the corresponding SED of the washed substrate without $MgSO_4$ in a wide range of enzyme loadings (Fig. 4). $MgSO_4$ was effective on both unwashed and washed substrate in improving SED (Figs. 4 and 5). This clearly suggests that washing at 25 °C

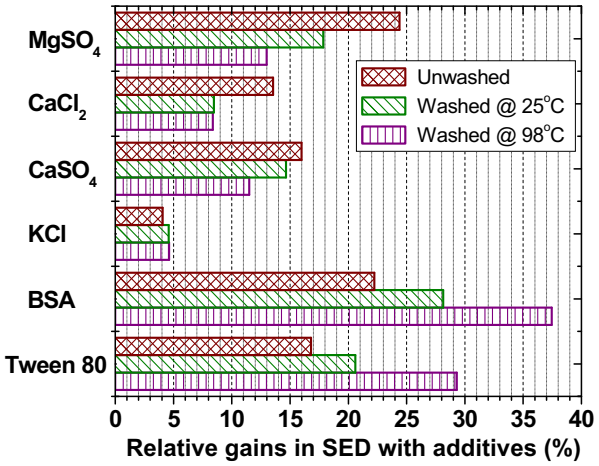

Fig. 5. Relative (to control) gains in SED by the application of various additives for the unwashed and two washed SPORL aspen substrates. Additive dosages: Ca(II) and Mg(II) = 1 mmol/g substrate; K(I) = 2 mmol/g substrate; Tween80 = 15 mg/g substrate; BSA = 10 mg/g substrate; control = no additive; cellulase loading = 5 FPU/g substrate.

Table 2
Effects of washing and increased cellulase loadings on lignin inhibition of enzymatic hydrolysis of the SPORL aspen substrate.

Enzyme loadings (FPU/g cellulose)	SED of unwashed substrate (%)	SED gains with washing at 25 °C	SED gains with washing at 98 °C	SED reduction in substrate washed at 25 °C (% g cellulose/mg lignin)[a]	SED reduction in unwashed substrate (% g cellulose/mg lignin)[b]
7	41.2	19.2	21.2	0.226	0.126
10	61.6	16.0	17.5	0.188	0.104
14	75.8	18.2	19.6	0.214	0.117
20	85.0	14.9	14.6	0.175	0.087
29	96.4	3.4	3.4	0.040	0.020

[a] Amount of unbound lignosulfonate removed: 85.1 mg/g cellulose.
[b] Amount of unbound lignosulfonate removed: 168.1 mg/g cellulose.

can be completely eliminated with the application of $MgSO_4$ at 1 mmol/g substrate without sacrificing SED. Moreover, the SEDs of the substrate washed at 25 °C with the application of $MgSO_4$ are higher than the corresponding values of the substrate washed at 98 °C at the same enzyme loading (not shown in Fig. 4 for clarity) for the range of enzyme loadings studied. Therefore, $MgSO_4$ can substitute for hot washing when further improvement of SED is desired.

Similar effects were observed using other metal salts at 1 mmol/g substrate with an enzyme loading of 5 FPU/g substrate (Fig. 5). All tested metal salts improved SEDs of the two washed substrates with smaller relative gains in SED over that of the unwashed substrate (Fig. 5). This is, first, because bound lignin contains acidic groups (Liu et al., 2010), although at a much lower fraction than unbound lignosulfonate, which serve as binding sites for metal ions (Torre et al., 1992) to form lignin–metal complexes. Second, some bound lignin was released into the substrate suspension from the lignocellulosic substrates, becoming unbound, due to the release of glucan by enzyme actions as hydrolysis proceeds. This unbound lignin may also have binding sites for cellulase. Because the washed substrates have significantly lower amounts of unbound lignosulfonate, especially the substrate washed at 98 °C, than the unwashed substrate, the relative gains in SED of the washed substrates are lower than that of the unwashed substrate with the application of metals. The relative gain in SED of the substrate washed at 98 °C is the smallest among the three substrates studied. The slight improvement of about 4% in SED with the application of KCl (2 mmol/g substrate) on the three substrates is probably simply due to the increase in ionic strength. It was suggested that high ionic strength can promote enzymatic hydrolysis of cellulose (Reinikainen et al., 1995).

Applications of BSA (10 mg/g substrate) and Tween80 (15 mg/g substrate) are more effective on the two washed substrates, which have relatively lower unbound and higher bound lignin content than the unwashed substrate (Fig. 5), opposite to what was observed when metal salts were applied. This is because BSA cannot overcome inhibition of enzymatic hydrolysis by unbound lignosulfonate based on the study using pure cellulose (Fig. 1). Its effect on enhancing enzymatic hydrolysis is through reducing inhibition by blocking non-productive adsorption of enzyme only onto bound lignin. It should be pointed out that the applied dosages of BSA and Tween80 are high from an economical point of view when compared with calcium salts.

The effects of application dosages of additives on improving SED of unwashed substrate were also studied at an enzyme loading of 5 FPU/g substrate (or 9.4 FPU/g cellulose). SED increases with the increase in $CaCl_2$ and $MgSO_4$ dosage from 60% and then reaches an asymptotic value of about 75% (Fig. 6). The 15% point gain in SED corresponds to total inhibition by the unbound lignosulfonate in the unwashed substrate. This can be seen from the difference in SEDs between the unwashed and washed at 98 °C substrates at the same enzyme loadings (Fig. 3). This suggests that the application of 1 mmol/g substrate $MgSO_4$ completely eliminated the inhibition of enzymatic hydrolysis by unbound lignosulfonate in unwashed substrate. As a result, further improvement in SED is not possible at higher $MgSO_4$ dosages (Fig. 6). The increase in dosage of BSA and Tween80 also enhanced SED of unwashed substrate (Fig. 6). The slope of the BSA curve is about the same as that of the $MgSO_4$ curve (Fig. 6). Although the Tween80 curve has a much steeper slope, the Tween80 dosage of 15 mg/g substrate is regarded as high in practical applications.

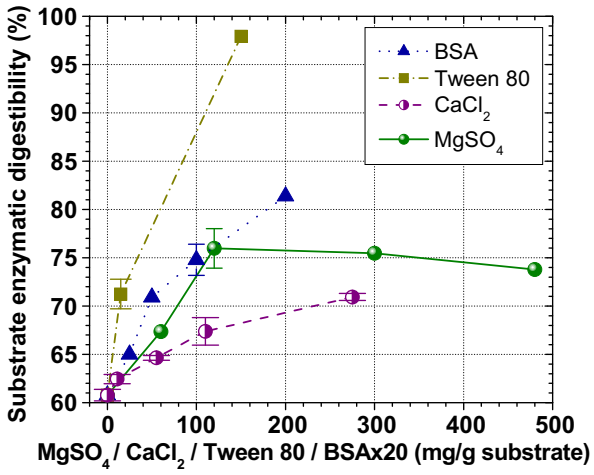

Fig. 6. Effects of additive dosages on SED of unwashed SPORL aspen substrate. Cellulase loading = 5 FPU/g substrate.

The application of metal salts may not only affect the fermentability of the resultant hydrolysate, but also impact lignin recovery due to the formation of lignin–metal complex. The metal ions retained on the residual solid substrate at the completion of enzymatic hydrolysis were analyzed. The hydrolysis residual solids yields were estimated by subtracting the measured glucan loss through hydrolysis from the initial substrate mass. The metal ion contents on the residual solids were only about 1–2% of the dosage applied (Table 3). This suggests that the application of metal compounds should not have significant effects on the utilization of lignin from the solid residual. If $MgSO_4$ is applied, the amount of Mg(II) remaining in the hydrolysate (~10 mM) is within the dosage applied in laboratory fermentation and should prove beneficial to fermentation (Rees and Stewart, 1997; Thanonkeo et al., 2007).

Table 3
Metal contents in the solid residues at the completion of enzymatic hydrolysis (48 h) of SPORL-pretreated substrates[a].

Samples	Metal salts	Estimated hydrolysis solids yield (%)	Metal content in solid residue (mg/g)	Retained (%)
Unwashed	KCl	63.1	1.73	1.4
	$MgSO_4$	56.5	0.46	1.07
	$CaCl_2$	60.5	0.90	1.36
	$CaSO_4$	59.7	1.13	1.68
Washed at 25 °C	KCl	56.2	1.93	1.39
	$MgSO_4$	51.3	0.55	1.18
	$CaCl_2$	52.6	0.74	0.97
	$CaSO_4$	51.5	0.95	1.23
Washed at 98 °C	KCl	53.9	1.80	1.25
	$MgSO_4$	51.5	0.57	1.22
	$CaCl_2$	51.4	0.65	0.84
	$CaSO_4$	47.5	0.85	1.01

[a] Hydrolysis was conducted at 1% solids consistency with the addition of metal salts at a dosage of 2 mmol/g substrate for KCl and 1 mmol/g substrate for all other metal salts. Solids yield after enzymatic hydrolysis is estimated by subtracting the measured glucan loss (by hydrolysis) from the initial solids.

Table 4
Estimated enzyme savings with washing or Mg(II) application at substrate (SPORL-pretreated aspen) enzymatic digestibility of 90%.

Samples	Cellulase loading at SED = 90% (FPU/g cellulose)	Lignin removed (mg/g cellulose)/ water used (L)	Savings in cellulase by washing (FPU/g cellulose)	Cellulase savings by washing (FPU/mg lignin removed/FPU/L)	$MgSO_4$ loadings (mg/g cellulose)	Savings in cellulase with $MgSO_4$ (FPU/g cellulose)	Cellulase savings with Mg(II) (FPU/ mg $MgSO_4$)
Unwashed	24.0						
Washed at 25 °C	16.5	85.1/2.5	7.5	0.088/3.0			
Washed at 98 °C	15.0	168.1/2.5	9.0	0.054/3.6			
Unwashed + Mg(II)	17.0				22.51	7.0	0.311
Washed at 25 °C + Mg(II)	13.0				16.51	3.5	0.212
Washed at 98 °C + Mg(II)	12.6				16.76	2.4	0.143

3.5. Quantification of the savings of enzymes and water with the application of $MgSO_4$

The reduction in enzymatic hydrolysis inhibition by unbound lignosulfonate with washing and/or application of metal salts can save enzyme to achieve a desired SED. To quantify the amount of enzyme savings while achieving SED of 90% for the SPORL aspen substrate, the amounts of cellulase required for the unwashed and two washed substrates with and without $MgSO_4$ application are determined based on measured SED data (Table 4). The savings in cellulase by either washing or $MgSO_4$ application can be calculated based on MARUL and the applied $MgSO_4$ dosage on cellulose base. Every milligram of lignosulfonate removal (based on unit gram of cellulose) through washing at 25 °C can save 0.088 FPU cellulase. We can also calculate the cellulase savings in terms of water consumption to be 3.0 FPU/L water (2.5 L water was used in producing the washed substrate). On the other hand, the application of 1 mg $MgSO_4$/g cellulose can save 0.311 FPU cellulase for the unwashed substrate. When washing is substituted by the application of $MgSO_4$, the equivalent substitution ratios are 8.0 and 11.6 mg $MgSO_4$/L water for washing at 25 and 98 °C, respectively. The highly efficient washing at 98 °C requires more $MgSO_4$ to substitute washing to achieve the same SED. Because $CaSO_4$ is much less expensive and readily available, the efficacy of $CaSO_4$ for substitute washing will be evaluated in a future study.

4. Conclusions

This study demonstrated lignin–metal complexation for eliminating inhibition of cellulase by unbound lignosulfonate in unwashed pretreated lignocellulosic substrates. With the application of 1 mmol/g substrate metal salts (e.g., $MgSO_4$), the reduction in substrate enzymatic digestibility (SED) by unbound lignosulfonate in an unwashed SPORL aspen substrate was recovered. The enhancement of SED by lignin–metal complexation can substitute for washing to save water or reduce enzyme loading in biomass conversion to biofuels.

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