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Adsorption of Cellulase on Cellulolytic Enzyme Lignin from Lodgepole Pine

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Enzymatic hydrolysis of lignocellulosic materials is significantly affected by cellulase adsorption onto the lignocellulosic substrates and lignin. The presence of lignin plays an important role in lignocellulosic hydrolysis and enzyme recycling. Three cellulase preparations (Celluclast, Spezyme CP, and MSUBC) were evaluated to determine their adsorption onto cellulolytic enzyme lignin (CEL) from steam-exploded Lodgepole pine (SELP) and ethanol (organosolv)-pretreated Lodgepole pine (EPLP). The adsorption affinity of cellulase (Celluclast) onto isolated lignin (CEL-EPLP and CEL-SELP) was slightly higher than that from corresponding EPLP and SELP substrates on the basis of the Langmuir constants. Effects of temperature, ionic strength, and surfactant on cellulase adsorption onto isolated lignin were also explored in this study. Thermodynamic analysis of enzyme adsorption onto isolated lignin (Gibbs free energy change $\Delta G^0 \approx -30$ kJ/mol) indicated this adsorption was a spontaneous process. The addition of surfactant (0.2% w/v) could reduce the adsorption of cellulase onto CEL-SELP by 60%. Two types of adsorption isotherm were compared for cellulase adsorption onto isolated lignin. A Langmuir adsorption isotherm showed better fit for the experimental data than a Freundlich adsorption isotherm.

KEYWORDS: Cellulase adsorption; cellulolytic enzymatic lignin; surfactant; Langmuir adsorption isotherm; lignocellulosic hydrolysis

INTRODUCTION

Forestry lignocellulosic biomass is a potentially renewable and abundant resource for fuel ethanol production (*1*, *2*). However, the recalcitrant structure of cellulose, hemicellulose, and lignin in woody biomass presents a significant challenge and hindrance to effective enzymatic hydrolysis (*3*, *4*). Therefore, lignocellulosic materials must be pretreated to improve cellulose accessibility to enzymes for hydrolysis (*4*).

Steam explosion, dilute acid, ammonia fiber explosion, and organosolv pretreatments are currently being investigated for their ability to improve the hydrolyzability of lignocellulosic biomass (*3*, *5*−*12*). Of these pretreatment processes, steam explosion is one of the most thoroughly explored processes for bioconversion of hardwood and agricultural residues to ethanol due to its simplicity and effectiveness (*5*, *13*−*15*). Steam explosion typically results in a condensation and enrichment of lignin in the pretreated substrate (*16*), and the high lignin content of steam explosion pretreated substrates has been shown to affect both cellulose hydrolysis and enzyme recycling (*17*, *18*). Organosolv is another pretreatment process that produces cellulose substrate with high hydrolyzability and low lignin content (*3*, *19*, *20*). The precipitated lignin from this process can be used to produce potential value-added byproduct (strand binder and polyurethane foams) (*3*, *20*, *21*). However, commercialization of cellulosic ethanol is still hindered by the high cost of pretreatment processes and the high cost of cellulase enzymes required for lignocellulose hydrolysis (*4*, *22*), even after significant achievement for reduction of the cost of enzymes by Genencor International and Novozymes (*23*). Therefore, cost-effective pretreatment processes need to be developed, and the enzyme cost associated with effective hydrolysis should be reduced significantly.

The recycling of cellulase enzymes during hydrolysis is one of the major strategies being suggested for its potential to decrease the cost of enzymatic hydrolysis for lignocellulosic substrates (*19*, *24*−*27*). Previously, we found that the amount and nature of the residual lignin within the pretreated substrates could be responsible for the inability of cellulase to be recycled after hydrolysis (*17*, *19*, *26*). It was reported that cellulase enzymes were effectively recycled on ethanol-pretreated Lodgepole pine (EPLP), but not on steam-exploded Lodgepole pine (SELP). The apparent difference between SELP (45% of lignin) and EPLP (14% of lignin) was lignin content (*19*), but if the lignin content in both SELP and EPLP substrates is the same, what is the difference for their effects on enzyme recycling? Hence, it is of interest to compare the adsorption of cellulase onto lignin preparations isolated from these two substrates at an equal dosage to determine if the two pretreatment methods would result in differences in cellulase adsorption.

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Table 1. Characteristics of CEL-SELP and CEL-EPLP by Klason Analysis[a]

lignin	arabinose (%)	galactose (%)	glucose (%)	xylose (%)	mannose (%)	nitrogen content (%)	Klason lignin (%)
CEL-SELP	0.074	0.090	3.31	0.09	0.32	0.14	85.76
CEL-EPLP	0.053	0.139	1.72	0.058	0.21	0.29	80.23

[a] Enzymatic lignin was prepared by hydrolyzing the ethanol-pretreated Lodgepole pine (EPLP) and steam-exploded Lodgepole pine (SELP). ~2% of substrate in 100 mL acetate buffer was hydrolyzed with 20 FPU Celluclast/g of cellulose, 40 IU/β-glucosidase/g of cellulose, and 0.2% Tween 80 for 48 h. The hydrolysate was filtered by glass microfiber (Whatman GF/A). The collected residues were hydrolyzed again with the same amount of cellulase, β-glucosidase, and Tween 80 for another 48 h. The residues were recovered by filtration and combined with 0.2% Tween 80 for 2 h of incubation at 45 °C. The collected lignin was washed with 0.5 L of distilled water (~50 °C). The final lignin was put into a vacuum oven to air-dry at room temperature. The lignin samples were ground and screened to a 60 mesh particle size. The percentage of chemical composition is based on the isolated lignin.

A considerable amount of work has been done on cellulase adsorption on different lignocellulosic substrates, such as microcrystalline cellulose (*28*, *29*), lignacious residues (*30*), corn stover (*31*), steam-exploded Douglas fir (*17*), pretreated hardwood (*32*), and isolated lignin from softwood (*33*). However, there is limited information about how to prevent the adsorption of cellulase enzymes onto lignin, especially for nonproductive binding between enzymes and lignin (*31*, *34*).

In this study, adsorption of cellulase onto two kinds of isolated enzyme lignin from EPLP and SELP substrates was investigated in detail. Adsorption kinetics and adsorption isotherm of cellulase onto lignin were determined by using Langmuir and Freundlich isotherms. Effects of temperature, ionic strength, and surfactant on cellulase adsorption onto isolated lignin were also investigated.

MATERIALS AND METHODS

Cellulase and Chemicals. Cellulase preparations derived from *Trichoderma reesei* were used in this study: Celluclast 1.5 L (Novozymes, Franklinton, NC), filter paper activity = 71.7 FPU/mL, protein content = 129.8 mg/mL; Spezyme CP (Genencor International, San Francisco, CA), filter paper activity = 83.5 FPU/mL, protein content = 119.1 mg/mL. In addition to the commercial preparations, another cellulase preparation produced in the Forest Products Biotechnology laboratory (University of British Columbia, Vancouver) derived from *Penicillium* sp. was also used, which will be referred to as MSUBC (filter paper activity = 39.9 FPU/mL, protein content = 65.3 mg/mL). The β-glucosidase used was Novozym 188 (Novozymes), cellobiase activity = 350 IU/mL. Tween 80 and other chemicals were obtained from Sigma.

Steam Explosion and Organosolv Pretreatments. Lodgepole pine (*Pinus contorta*) softwood (cellulose, 47.6%; hemicellulose, 22.9%; lignin, 26.3%; and extractives, 4.7%) was used for the experiments. Oven-dried (OD) 4 × 4 cm chip samples (50 g) were impregnated with 4.0% anhydrous SO_2 (w/w) on OD wood chips in a plastic bag. The samples were loaded in 50 g batches into a preheated 2 L Stake Tech III (Stake Technologies, Norvall ON, Canada) steam gun. The conditions used for Lodgepole pine were 200 °C and 4.0% SO_2 at a residence time of 5 min. After the pretreatment, the pretreated substrates (glucose, 53.4%; lignin, 45.6%) were washed with water and stored at 4 °C for subsequent experiments.

EPLP substrate was prepared by ethanol pulping wood chips (4 × 4 cm) into four vessels (2 L each) by loading 200 g of OD (liquor/wood = 7:1) into a rotating digester manufactured by Aurora Products Ltd. (Savona, BC, Canada). The cooking conditions were 170 °C and 1.1% H_2SO_4 (w/w) in 65% ethanol (v/v) for 60 min. The pretreated substrate (glucose, 90.5%; lignin, 14.5%) was further washed with aqueous ethanol and water as described previously (*35*).

Chemical Analysis of Pretreated Substrates. The lignin content was determined using the Klason technique (TAPPI method T249 cm-85). The carbohydrate composition of acid hydrolysates was quantified using a Dionex DX 2500 high-performance liquid chromatograph (HPLC) on a CarboPac PA-1 column.

Protein Assay. A protein assay utilizing ninhydrin was applied in the present study (*36*), and the protein samples (0.1 mL) were pipetted into glass tubes and placed in an oven at 105 °C for 2 h. After the tubes were completely dry, 0.15 mL of 13.5 N NaOH was added to each tube. The tubes were then autoclaved at 121 °C for 20 min. After cooling, the alkali was neutralized by adding 0.25 mL of glacial acetic acid. A 0.5 mL amount of a 2% solution of ninhydrin reagent (Sigma, St. Louis, MO) was added to each tube and then heated for 20 min in a boiling water bath. After the reaction, the tubes were cooled in an ice water for 10 min, and 2.5 mL of 50% ethanol was further added. The tubes were then shaken vigorously on a vortex. Finally, the solution was read at 570 nm on a UV−vis spectrometer (Perkin-Elmer). Bovine serum albumin (BSA) was used as the protein standard.

Enzymatic Hydrolysis of Lignocellulosic Substrates. Unless otherwise stated, all enzymatic hydrolysis experiments were performed in 50 mL of 50 mM acetate buffer (pH 4.8) at a 2% consistency (based on cellulose). The hydrolysis reaction was incubated at 45 °C, with shaking at 150 rpm for 48 h. The cellulase loading was 20 FPU/g of cellulose, and the β-glucosidase (Novozym 188) loading was 40 IU/g cellulose. Samples were removed from the reaction at different times and centrifuged to remove the insoluble materials. The sugar content was measured by HPLC as described previously (*35*). The hydrolysis yield of the substrate was calculated from the measured monomeric sugars content, as a percentage of the theoretical sugars available in each substrate. The protein content in the supernatant was measured using the ninhydrin assay with BSA as the protein standard.

Cellulolytic Enzyme Lignin (CEL) Isolation and Elemental Analysis. Enzymatic lignin was prepared by hydrolyzing the ethanol-pretreated Lodgepole pine and steam-exploded Lodgepole pine substrates. The substrates were suspended at a 2% consistency (based on cellulose) in 100 mL of acetate buffer and hydrolyzed with 20 FPU Celluclast/g of cellulose, 40 IU of β-glucosidase/g of cellulose, and 0.2% Tween 80 for 48 h. The hydrolysate was filtered using glass microfiber (Whatman GF/A). After the reaction, the residue was collected and further hydrolyzed for another 48 h using identical amounts of cellulase, β-glucosidase, and Tween 80 for 48 h. After the second hydrolysis stage, the residues were recovered by filtration, resuspended in 0.2% Tween 80, and incubated for 2 h at 45 °C. The resulting lignin preparation was washed with 500 mL of distilled water (~50 °C). The final lignin was put into vacuum oven to air-dry at room temperature. The lignin samples were ground and screened through 60 mesh. A summary of the characteristics of cellulolytic enzyme lignin from steam-exploded Lodgepole pine (CEL-SELP) and cellulolytic enzyme lignin from ethanol-pretreated Lodgepole pine (CEL-EPLP) is shown in **Table 1**.

An elemental analysis of CEL-SELP and CEL-EPLP was performed using a Perkin-Elmer series II CHNS/O 2400 analyzer (Norwalk, CT). The protein content of isolated lignin was calculated by multiplying the nitrogen content value by 6.25. All samples were measured in triplicate.

Cellulase Adsorption Kinetics and Isotherm on Cellulolytic Enzyme Lignin. To measure adsorption kinetics, 30 mg of lignin (CEL-SELP or CEL-EPLP) sample was suspended in 5 mL of acetate buffer (50 mM, pH 4.8) with protein content at ~0.15 mg/mL of three cellulase preparations (Celluclast, Spezyme, and MSUBC). The reactions were incubated with shaking for 6 h at 25 °C. Aliquots (0.15 mL) were taken at 0, 15, 30, 60, 120, 180, and 360 min during the incubation. The protein content was determined using the ninhydrin assay (*19*).

To determine the adsorption isotherm, different concentrations (0.05−0.4 mg/mL) of cellulase were incubated with ~20 mg of lignin in 1 mL of 50 mM acetate buffer at 25 °C for 3 h to reach equilibrium. The protein content in the supernatant was determined for the nonadsorbed cellulase. The adsorbed cellulase was calculated from the difference between the initial cellulase dosage and the nonadsorbed cellulase. Cellulase

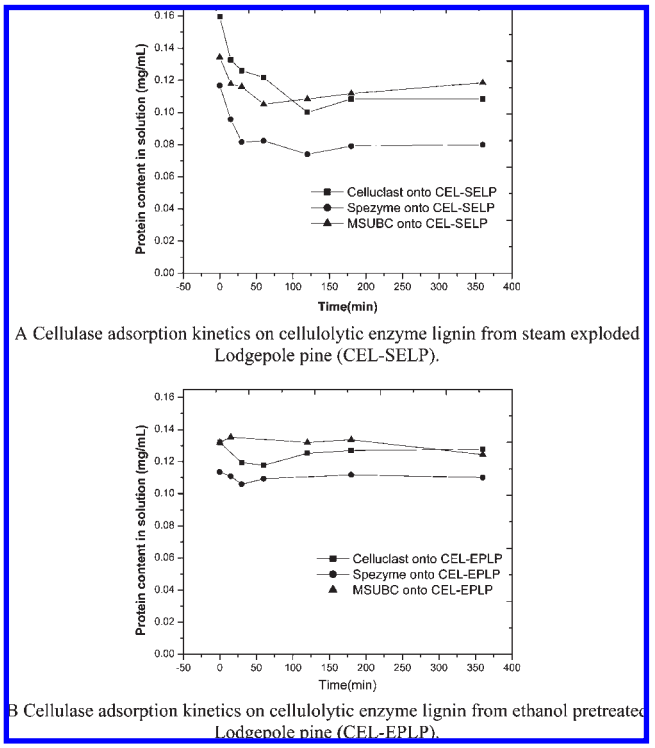

Figure 1. Cellulase adsorption kinetics on isolated CEL-SELP and CEL-EPLP at 25 °C. For adsorption kinetics, 30 mg lignin samples were placed into Falcon tubes and incubated in 5 mL of acetate buffer with 0.10−0.15 mg/mL of commercial cellulases for 6 h at 25 °C on a rotary shaker. Aliquots of 0.15 mL were taken for each time point during the incubation.

adsorption on lignin samples was characterized by Langmuir adsorption isotherm (*32*).

RESULTS AND DISCUSSION

Cellulase Adsorption Kinetics. Cellulase adsorption kinetics onto isolated lignin were determined in a similar approach for characterizing the adsorption of cellulase onto cellulosic substrates (*19*, *37*). The enzymatic lignin preparations (CEL-EPLP and CEL-SELP) were suspended in acetate buffer (pH 4.8, 50 mM). To each suspension of lignin samples, various cellulase preparations from *T. reesei* (Celluclast and Spezyme CP) and *Penicillium* sp. (MSUBC) were assessed for adsorption kinetics. The adsorption of cellulases onto CEL-EPLP and CEL-SELP required approximately 3 h to reach equilibrium (**Figure 1**), which is considerably longer than the time (30−60 min) required for cellulase adsorption on EPLP and SELP substrates (*19*). Previously, Kyriacou reported that the equilibrium time for cellulase components (CBH I, EG I, EG II, and EG III) on Solka Floc (pure cellulose) was ∼60 min (*38*). This indicated that cellulase adsorption kinetics onto cellulose was considerably different from those onto lignin. It might provide us a new angle to explain why the lignocellulosic substrates even with high lignin content were hydrolyzed quickly at the initial stage (*39*), because cellulase adsorption onto lignin is slower than that onto cellulose. Also from adsorption kinetics of cellulase onto CEL-SELP (**Figure 1A**) and CEL-EPLP (**Figure 1B**), less cellulase enzyme adsorbed onto CEL-EPLP than onto CEL-SELP. This suggested that during the initial hydrolysis stage, less cellulase enzyme adsorbed onto the lignin portion of EPLP substrate than onto the lignin portion of SELP substrate. As a result, the majority of cellulase enzymes adsorbed onto the cellulose portion of the EPLP substrate, whereas considerable cellulase enzymes adsorbed onto the lignin portion for SELP substrate. This could be further applied to elucidate why the organosolv-pretreated biomass had great hydrolyzability (*3*). Further information from adsorption kinetics should also be noted: the concentration of MSUBC increased after 60 min (**Figure 1A**), probably caused by the enzymatic hydrolysis of glucan (3−4%) in lignin samples, because partially adsorbed MSUBC on glucan was released during the hydrolysis. Typically, *Penicillium*-derived cellulases (MSUBC) could hydrolyze lignocellulosic substrates without the addition of β-glucosidase, whereas Celluclast and Spezyme do not have sufficient β-glucosidase activity to hydrolyze lignocelluloses (*40*). Subsequently, to further quantify cellulase adsorption onto isolated lignin, an adsorption isotherm was used to characterize the cellulase adsorption capacity and adsorption affinity on cellulolytic enzyme lignin.

Cellulase Adsorption Isotherms on Cellulolytic Enzyme Lignin. The Langmuir and Freundlich isotherms have been utilized extensively to study protein adsorption onto various particle surfaces (*33*, *41*, *42*). The Langmuir adsorption isotherm is a nonlinear model that assumes a reversible monolayer adsorption, no interaction among the dilute adsorbate (cellulase enzymes), and uniform binding sites on the adsorbent (lignin).

$$\Gamma = \frac{\Gamma_{max} KC}{1+KC} \quad (1)$$

C is the concentration of unadsorbed protein in bulk solution (mg/mL), Γ is the adsorbed protein (mg/g of lignin), Γ_{max} is the maximal adsorbed protein (mg/g of lignin), and K is the Langmuir constant (mL/mg of protein)

Alternatively, the adsorption process can also be monitored using the Freundlich model. The Freundlich isotherm is also a nonlinear model, which assumes a heterogeneous surface on the adsorbent with various affinities of the binding sites for the adsorbate, and it accounts for interactions among the adsorbed molecules (*43*).

$$\Gamma = K_F C^n \quad (2)$$

Γ is the adsorbed protein (mg/g of lignin), C is the concentration of unadsorbed protein in bulk solution (mg/mL), K_F is the Freundlich constant, and n is the heterogeneity factor, which has a lower value for most heterogeneous surfaces.

The linearized form of the Freundlich isotherm is

$$\ln \Gamma = \ln K_F + n \ln C \quad (3)$$

K_F and n are determined by data fit.

To establish the adsorption isotherm, various concentrations of cellulase enzymes were incubated with a ∼2% suspension of lignin in an acetate buffer at 25 °C for 3 h to reach equilibrium. The protein content in the supernatant was then determined. The Langmuir adsorption isotherms were compared under different cellulase preparations (Celluclast and MSUBC) onto CEL-SELP and CEL-EPLP (**Figure 2** and **Table 2**). The experimental data fit well into Langmuir adsorption isotherm models ($R^2 >$ 0.85). Langmuir adsorption isotherms revealed significant differences between Celluclast and MSUBC adsorption onto CEL-SELP (**Figure 2**). The adsorption capacity of Celluclast onto CEL-SELP was $\Gamma_{max} = 10.20 \pm 5.93$ mg/g of lignin, which was twice higher than the adsorption capacity of MSUBC onto CEL-SELP ($\Gamma_{max} = 4.87 \pm 1.99$ mg/g of lignin). A similar cellulase adsorption capacity (15 mg/g of lignin) on isolated enzymatic lignin from steam-exploded spruce (CEL-SPS) was calculated from a previous paper (*33*). Ooshima et al. also reported the maximum adsorbed cellulase (*Trichoderma*) was 12.3 mg/g of

lignin on enzymatic lignin from steam-exploded hardwood at 220 °C. The lower adsorption of *Penicillium* enzymes (MSUBC) onto lignin probably was due to their structural differences of *Penicillium*-derived cellulase enzymes (*44*). The two significant cellulase components EG a and EG b1, which comprise 25% of the total protein from *Penicillium*, do not have a cellulose binding domain (*44*). Similar results have been reported by Berlin et al., who found that *Penicillium*-derived enzymes had lower adsorption on cellulolytic enzyme lignin from organosolv-pretreated Douglas fir than *Trichoderma*-derived enzymes (*45*).

As for the different isolated lignin samples, the adsorption capacity of Celluclast onto CEL-SELP was nearly 3 times higher than that onto CEL-EPLP (Γ_{max} = 2.73 ± 0.82 mg/g of lignin). One probable reason for this is that the CEL-SELP from steam explosion had more binding sites on the lignin particle surface due to the preservation of functional groups (phenolic hydroxyl and benzyl) and lignin branches during the pretreatment (*16*, *46*). The CEL-EPLP obtained from organosolv pretreatment possessed fewer binding sites and fewer branches because a larger proportion of the lignin is solubilized during the pretreatment process (*3*, *40*). This indicated that different pretreatment processes significantly affected the adsorption of cellulase enzymes on lignin. The MSUBC showed no detectable enzyme adsorption on CEL-EPLP; therefore, no adsorption isotherm was presented in **Figure 2**. The data of the Spezyme CP adsorption isotherm also was not shown here, because the results were similar to those obtained from Celluclast.

Langmuir constants from adsorption isotherm represent equilibrium affinity constants of cellulase onto cellulose or lignin, which have often been used to evaluate the affinity of cellulase

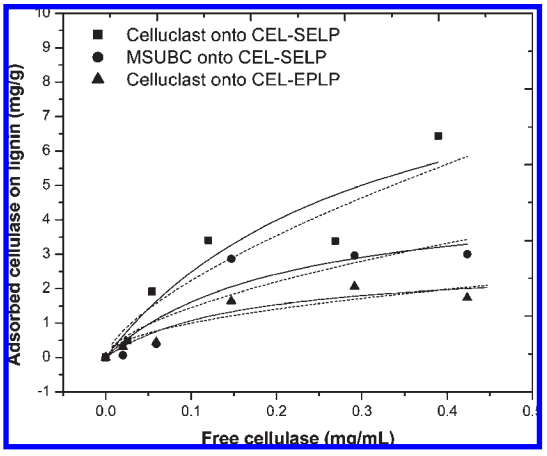

Figure 2. Cellulase adsorption isotherms on CEL-SELP and CEL-EPLP at 25 °C (solid line from Langmuir model, dotted line from Freundlich model). For the adsorption isotherm, different concentrations of cellulases were incubated with 2% of lignin in 50 mM acetate buffer for 3 h to reach equilibrium. The protein content in the supernatant was determined as the nonadsorbed cellulase. The adsorbed cellulase was calculated from the difference between initial cellulase content and nonadsorbed cellulase content in the supernatant.

enzymes on different cellulose substrates (*47*, *48*). On the basis of Langmuir constants of Celluclast onto CEL-SELP and CEL-EPLP (**Table 2**), it is of interest to note that the Celluclast (*Trichoderma*-derived enzymes) showed a higher affinity (higher *K* value) to CEL-EPLP than to CEL-SELP. This interestingly coincided with the higher protein content (residual enzyme) in CEL-EPLP (0.29% of nitrogen), whereas CEL-SELP had 0.14% of nitrogen after the same protocol for isolating the lignin samples from pretreated Lodgepole pine. This suggested that cellulase had a higher affinity for the CEL-EPLP lignin. Because of the higher solubilization of lignin branches during the pretreatment, the potentially hydrophobic nature of the resulting CEL-EPLP lignin may show a greater affinity for cellulase due to hydrophobic interactions between enzymes and lignin (*18*, *45*).

When cellulase adsorption onto lignin is compared to cellulase adsorption onto lignocellulosic substrate (EPLP) (Γ_{max} = 87.69 mg/g, *K* = 3.48 mL/mg) and SELP substrate (Γ_{max} = 101.05 mg/g, *K* = 1.48 mL/mg) (*19*), cellulase adsorption onto corresponding isolated lignin showed a much lower adsorption capacity (Γ_{max} = 2.73−10.20 mg/g) and a slightly higher affinity (*K* = 3.21−6.44 mL/mg). This most likely explained why the lignin in the lignocellulosic substrates had a negative effect on the enzymatic hydrolysis because of the higher affinity of cellulase to lignin. Comparable results have been reported before: the Langmuir affinity constant of *Clostridium* cellulase enzymes onto lignin was 17.9 mL/mg (*32*); the variation probably was caused by the unique scaffolding structure with multiple cohesion domains for *Clostridium* cellulase enzymes (*49*, *50*).

As for the Freundlich adsorption isotherm, the heterogeneity factor (*n*) from Celluclast adsorption onto CEL-SELP (0.67 ± 0.20) was higher than that from Celluclast adsorption onto CEL-EPLP (0.49 ± 0.23) (**Table 2**). This perhaps further demonstrated that the CEL-SELP from steam explosion had more binding sites than CEL-EPLP from the organosolv process. The adsorption of cellulases on lignin plays an important role in the enzymatic hydrolysis of lignocellulose because the adsorption influences the efficiency of hydrolysis and the potential to recover cellulase enzymes. Cellulase adsorption onto CEL-SELP and CEL-EPLP preparations basically followed the Langmuir adsorption model. The adsorption capacity of cellulases (Celluclast) onto CEL-SELP was 3 times higher than that obtained onto CEL-EPLP. It is interesting to point out that Freundlich adsorption might be used to characterize the heterogeneity of lignocellulosic substrates. However, in this study, the data fit from the Langmuir adsorption isotherm was better than that from the Freundlich adsorption isotherm for cellulase adsorption onto lignin. Hence, the later experiments were mainly assessed using Langmuir adsorption isotherm.

Effect of Temperature on Cellulase Adsorption onto Lignin. Previously, temperature showed significant effects on cellulase adsorption onto Solka Floc and microcrystalline cellulose (*38*, *47*, *51*). It is of interest to gauge the effects of various temperatures on cellulase adsorption onto isolated lignin. Different concentrations of cellulase enzymes (0.05−0.4 mg/mL) were incubated with ∼20 mg of lignin in 1.0 mL of 50 mM acetate

Table 2. Adsorption Isotherm Parameters of Different Cellulases on CEL-SELP and CEL-EPLP at 25 °C[a]

	Langmuir			Freundlich		
cellulase adsorption	Γ_{max} (mg/g)	*K* (mL/mg)	R^2	K_F	*n*	R^2
Celluclast onto CEL-SELP	10.20 ± 5.93	3.21 ± 3.45	0.847	10.34 ± 2.68	0.67 ± 0.20	0.887
MSUBC onto CEL-SELP	4.87 ± 2.30	4.95 ± 5.31	0.842	5.74 ± 2.25	0.60 ± 0.28	0.775
Celluclast onto CEL-EPLP	2.73 ± 0.95	6.44 ± 5.57	0.819	3.12 ± 1.05	0.49 ± 0.23	0.732

[a] R^2 is the coefficient of determination.

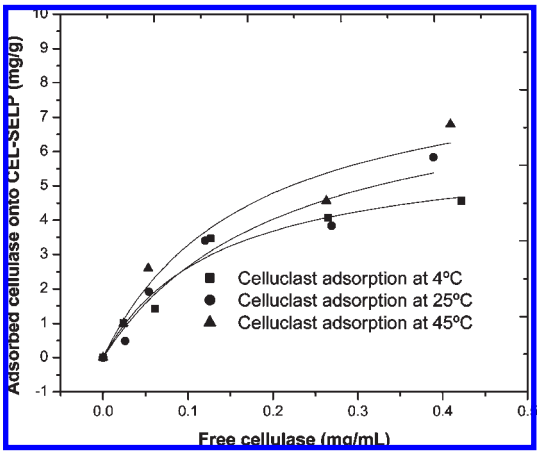

Figure 3. Cellulase adsorption isotherms on CEL-SELP at different temperatures.

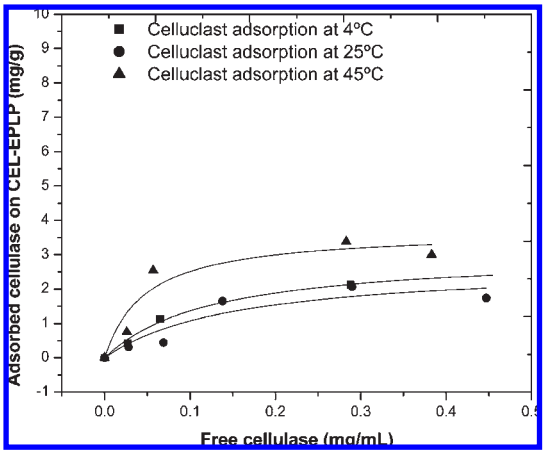

Figure 4. Cellulase adsorption isotherms on CEL-EPLP at different temperatures.

buffer for 3 h. Cellulase adsorption onto both CEL-SELP and CEL-EPLP was measured at 4, 25, and 45 °C (**Figures 3** and **4**). Increase of temperature from 4 to 45 °C resulted in a slight increase in the adsorption onto CEL-SELP and CEL-EPLP, although the temperature showed much less considerable effect on cellulase adsorption onto lignin than onto cellulose (*38*). The cellulase adsorption isotherms were further assessed through thermodynamic analysis.

To understand fully how cellulase adsorption onto lignin proceeds, it is also necessary to examine the free energy change ($\Delta G°$), enthalpy change ($\Delta H°$), and entropy change ($\Delta S°$) associated with the adsorption. The value $\Delta G°$ was obtained from equilibrium constants (K), which were estimated from the Langmuir adsorption isotherm of cellulase onto CEL-SELP and CEL-EPLP under various temperatures. The Gibbs free energy change of the adsorption process is shown in the equation (*52*−*54*)

$$\Delta G^{\circ} = -RT \ln K = \Delta H^{\circ} - \mathrm{T}\Delta S^{\circ} \qquad (4)$$

where $\Delta G°$ is the free energy change (J/mol), R is the universal gas constant, 8.314 (J/mol·K), T is the absolute temperature, and K is the Langmuir constant. A negative $\Delta G°$ value indicates an exergonic reaction, whereas a positive $\Delta G°$ value indicates an endergonic reaction.

Equation suggested that the cellulase adsorption process is governed by both entropy and enthalpy of the adsorption

Table 3. Thermodynamic Parameters of Cellulase Adsorption on CEL-SELP and CEL-EPLP[a]

	cellulase on CEL-SELP lignin			cellulase on CEL-EPLP lignin		
temperature (K)	K (mL/mg)	$\Delta G°$ (kJ/mol)	R^2	K (mL/ mg)	$\Delta G°$ (kJ/mol)	R^2
277	7.41 ± 2.46	−29.95	0.98	7.92 ± 1.95	−30.10	0.99
298	4.66 ± 2.51	−31.07	0.95	6.44 ± 4.80	−31.87	0.88
318	5.89 ± 3.38	−33.78	0.96	20.91 ± 12.05	−37.13	0.92

[a] When calculating the Gibbs free energy change from Langmuir constants, the volumetric concentration was converted to molar concentration (*62*). $\Delta G° = RT\ln K'$. Here $K' \approx KM_A$, M_A is the molecular weight of cellulases (assume average M_A = 60000). R^2 is the coefficient of determination.

reaction (*52*). Typically, the $\Delta G°$ for physical adsorption is in the range from 0 to −20 kJ/mol and the $\Delta G°$ for chemical adsorption (adsorption that results from chemical bond formation) in the range from −80 to −400 kJ/mol (*55*). Here, we found that the $\Delta G°$ for cellulase adsorption on lignin was from −29.9 to −37.1 kJ/mol (**Table 3**), which indicated that the binding of cellulases on lignin was the result of physical adsorption (*56*). The negative values of $\Delta G°$ suggest that the process of cellulase adsorption on both CEL-SELP and CEL-EPLP is a spontaneous process (*54*). The different results for the change in free energy indicate that the adsorption of cellulase on lignin differs significantly depending on the pretreatment applied to the lignocellulosic biomass. Similar results have been reported about cellobiohydrolases I and II (CBH I and CBH II) on microcrystalline cellulose (*54*); the Gibbs free energy changes of CBH I and CBH II on Avicel were from −27.9 to −34.1 kJ/mol (*54*). This further suggested that cellulase adsorption on lignocellulosic substrates and isolated lignin likely followed a similar pattern.

Previously, contradicting results have been reported for the effect of temperature on cellulase adsorption process. Kim (*54*) and Ooshima (*57*) suggested that cellulase adsorption on lignocellulose was an exothermic and enthalpy-controlled reaction. They found that the amount of cellulase adsorption decreased as the temperature increased (*28*, *54*, *57*). On the contrary, Hoshino (*47*) and Creagh (*58*) proposed that cellulase adsorption on cellulose was an endothermic and entropy-driven reaction. Our results showed a higher level of cellulase adsorption onto lignin at 45 °C than at 4 and 25 °C, indicating that cellulase adsorption on CEL-SELP and CEL-EPLP probably was an endothermic reaction. This most likely is true for the cellulase adsorption onto lignin, because hydrophobic interaction is the main force between lignin and cellulase enzymes (*18*, *19*), and the entropy could be the predominant driving force in the adsorption process through hydrophobic interactions (*59*). This entropy-driven adsorption process was demonstrated previously by experimental data from titration microcalorimetry (*47*).

Thermodynamic analysis of cellulase adsorption is a potentially useful approach for evaluating the ability of a pretreatment process to produce substrates with a lower tendency to adsorb cellulase. Because the CEL-SELP showed a greater capacity to adsorb cellulase, further work was done to determine if cellulase adsorption onto CEL-SELP could be decreased by varying the ionic strength of the reaction and/or by the addition of a surfactant.

Effect of Ionic Strength on Cellulase Adsorption onto Lignin. The ionic strength (I) is a function of the concentration of all ions present in that solution

$$I = \frac{1}{2}\sum_{i=1}^{n} c_i z_i^2$$

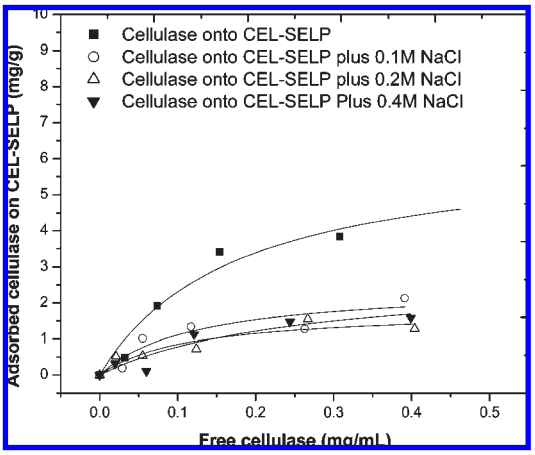

Figure 5. Effect of ionic strength on cellulase (Celluclast) adsorption on CEL-SELP.

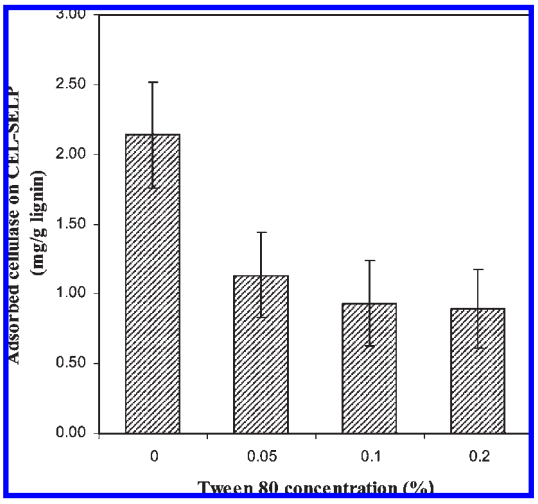

Figure 6. Effect of surfactant concentration on cellulase (Celluclast) adsorption on CEL-SELP. Approximately 0.13 g/mL of cellulase was incubated with ~20 mg of lignin in 1.0 mL of 50 mM acetate buffer at 25 °C for 3 h to reach equilibrium.

where c_i is the molar concentration of ion and z_i is the charge number of that ion. For sodium chloride, the ionic strength is equal to the concentration. The effect of ionic strength on cellulase adsorption onto CEL-SELP lignin was evaluated by increasing the NaCl concentration from 0 to 0.4 M (**Figure 5**). Different concentrations of cellulase were incubated with ~20 mg of CEL-SELP lignin in acetate buffer at 25 °C. The cellulase adsorption onto CEL-SELP lignin decreased with increasing NaCl concentration as the NaCl concentration was raised to 0.1 M. Kim and Hong similarly found that CBH I adsorption onto microcrystalline cellulose decreased with increase of the sodium acetate concentration from 0.01 to 0.4 M (*54*). The salt ions could either compete against the protein for binding sites or change the configuration of the protein, resulting in a decrease in hydrophobic interactions between the protein and the solid surface (*60*). Similarly, the adsorption capacity of β-lactoglobulin on a hydrophobic silicon surface decreased from 1.06 to 0.49 μg/cm^2 as the NaCl concentration was raised from 0.1 to 0.5 M (*41*).

In this study, the decrease in cellulase adsorption onto CEL-SELP occurred at a low concentration of NaCl (< 0.1 M). As the concentration of NaCl increased from 0.1 to 0.4 M, cellulase adsorption did not decrease further. The ineffectiveness of NaCl concentrations (> 0.1 M) could be due to a saturation of the lignin surface at low salt concentrations. As the salt concentration increased past the saturation point, the salt could not further compete with the cellulase protein for binding sites because all of the binding sites are depleted. However, we can conclude that ionic strength had a considerable effect on cellulase adsorption on CEL-SELP lignin, as cellulase adsorption decreased with increasing NaCl concentration from 0 to 0.4 M. Because our previous results showed that Tween 80 addition was highly successful in improving cellulase desorption from substrates (*19*), the next set of experiments examined the effects of Tween 80 on the adsorption of cellulase on CEL-SELP lignin.

Effect of Tween 80 on Cellulase Adsorption onto Isolated Lignin. The effect of Tween 80 on the adsorption of cellulases (Celluclast) onto CEL-SELP was studied by applying Tween 80 at 0−0.2% (w/v) to the adsorption process. The adsorption capacity decreased from 2.5 to 1.0 mg/g of lignin as the Tween 80 concentration was raised (**Figure 6**). The decrease in cellulase adsorption was most likely due to the competitive adsorption of Tween 80 onto the hydrophobic lignin surface. Eriksson et al. also suggested that nonionic surfactants such as Tween 20 and Tween 80 could reduce enzyme adsorption because the hydrophobic portion of the surfactant binds to lignin to prevent the nonproductive binding of cellulase to lignin.

A similar effect of Tween 80 on the adsorption of insulin onto the hydrophobic surface of silane-modified quartz slides has been reported (*61*), as it was hypothesized that Tween 80 displaced adsorbed insulin from the silane-modified quartz (*61*). In this study, the adsorption of cellulase onto CEL-SELP was reduced by 60% with the addition of 0.1−0.2% Tween 80. The results indicated that the addition of Tween 80 to a lignocellulosic hydrolysis system could significantly reduce enzyme adsorption onto lignin and potentially improve the enzymatic hydrolysis and enzyme recycling (*19*). Overall, the results show that each pretreatment method has unique effects on the downstream cellulase−substrate lignin interactions that occur during hydrolysis. The finding of interactions between cellulase enzymes and lignins from different pretreatments will help us design suitable strategies for effective enzymatic hydrolysis and cellulase recovery.

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