Access of Cellulase to Cellulose and Lignin for Poplar Solids Produced by Leading Pretreatment Technologies

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Adsorption of cellulase on solids resulting from pretreatment of poplar wood by ammonia fiber expansion (AFEX), ammonia recycled percolation (ARP), controlled pH, dilute acid (DA), flowthrough (FT), lime, and sulfur dioxide (SO_2) and pure Avicel glucan was measured at 4°C, as were adsorption and desorption of cellulase and adsorption of β-glucosidase for lignin left after enzymatic digestion of the solids from these pretreatments. From this, Langmuir adsorption parameters, cellulose accessibility to cellulase, and the effectiveness of cellulase adsorbed on poplar solids were estimated, and the effect of delignification on cellulase effectiveness was determined. Furthermore, Avicel hydrolysis inhibition by enzymatic and acid lignin of poplar solids was studied. Flowthrough pretreated solids showed the highest maximum cellulase adsorption capacity (σ_{solids} = 195 mg/g solid) followed by dilute acid (σ_{solids} = 170.0 mg/g solid) and lime pretreated solids (σ_{solids} = 150.8 mg/g solid), whereas controlled pH pretreated solids had the lowest (σ_{solids} = 56 mg/g solid). Lime pretreated solids also had the highest cellulose accessibility ($\sigma_{cellulose}$ = 241 mg/g cellulose) followed by FT and DA. AFEX lignin had the lowest cellulase adsorption capacity (σ_{lignin} = 57 mg/g lignin) followed by dilute acid lignin (σ_{lignin} = 74 mg/g lignin). AFEX lignin also had the lowest β-glucosidase capacity (σ_{lignin} = 66.6 mg/g lignin), while lignin from SO_2 (σ_{lignin} = 320 mg/g lignin) followed by dilute acid had the highest (301 mg/g lignin). Furthermore, SO_2 followed by dilute acid pretreated solids gave the highest cellulase effectiveness, but delignification enhanced cellulase effectiveness more for high pH than low pH pretreatments, suggesting that lignin impedes access of enzymes to xylan more than to glucan, which in turn affects glucan accessibility. In addition, lignin from enzymatic digestion of AFEX and dilute acid pretreated solids inhibited Avicel hydrolysis less than ARP and flowthrough lignin, whereas acid lignin from unpretreated poplar inhibited enzymes the most. Irreversible binding of cellulase to lignin varied with pretreatment type and desorption method. © 2009 American Institute of Chemical Engineers *Biotechnol. Prog.*, 25: 807–819, 2009
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Introduction

Enzymes can break down cellulose and hemicellulose in lignocellulosic biomass to release sugars which in turn can be fermented to fuels and chemicals. Being a heterogeneous reaction, enzymatic saccharification of carbohydrates requires adsorption of multiple enzymes on their surface,[1–4] and several literature studies reported a strong correlation between rates/extent of hydrolysis and enzyme adsorption (Kumar et al., submitted for publication),[5–20] with a few exceptions.[21–23] For example, Karlsson et al.[8,24] observed a linear relation between CBHI adsorption and hydrolysis rates for steam-pretreated willow, and similarly, Ding et al.[18] reported a strong relationship between hydrolysis and adsorption parameters for EGI and CBHI. In another study, Kumar and Wyman (Kumar et al., submitted for publication) showed a linear relationship between cellulase adsorption capacity and 24 h hydrolysis rates for Avicel glucan and corn stover solids prepared by leading pretreatment technologies. However, most of the studies have used pure or nearly pure cellulose, whereas lignocellulosic biomass contains lignin, hemicellulose, and other ingredients in addition to cellulose, and unproductive binding of enzymes on lignin may affect enzymatic saccharification negatively (Kumar et al., submitted for publication).[25,105] Enzyme adsorption on cellulose and lignin most likely takes place concurrently, and the nature and amount of lignin are believed to be largely

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Table 1. Pretreatment Methods, Conditions, Percent of Glucan and Xylan Left in Solids, and Compositions for Solids Prepared by Leading Technologies

		Yield of Component Left in Pretreated Solids (%)		Composition of Pretreated Solids (%)		
Pretreatment	Pretreatment Conditions	Glucan	Xylan	Glucan*	Xylan*	Lignin
AFEX	180°C,700 psi, 2:1 NH_3 to Biomass, 30 min, and 233% moisture content (dwb); NW	100	100	46.6	15	ND
ARP	185°C,400 psi, 3.66:1 NH_3 to Biomass (dry wt), 27.5 min and 23.49 % solid; W	93.2	68.2	57.5	13.5	24.8
Dilute Acid	190°C, 0.02H_2SO_4: Dry wt, 70 s, 30% solids; NW	87.9	8.3	57.3	2.1	46.1
Flowthrough	190°C, 0.05% H_2SO_4, 20ml/min, 15 min	80.2	5.7	65.6	1.6	33.7
Neutral pH	200°C, 10 min (+5 min heat up); NW	98.0	42.1	58.8	7.0	46.1
Lime	65°C, 0.5:1 $Ca(OH)_2$ to Biomass (dry wt); W1	98.1	96.2	53.1	16.8	18.0
SO_2	200, 5 min, 3% SO_2, steam explosion; NW	96.9	9.3	55.1	2.5	ND

W, washed; NW, non washed; W1, washed and neutralized; ND, not determined.
* Glucan defined in terms of $C_6H_{10}O_5$, xylan defined in terms of $C_5H_8O_4$.

responsible for the negative effects of lignin on hydrolysis.[26–28] Although the literature on the effect of lignin on enzymatic hydrolysis is substantial,[29–40] the role of lignin in enzymatic hydrolysis of heterogeneous cellulosic biomass is still not well understood.

Most forms of lignocellulosic biomass must be pretreated to realize high yields of sugars by enzymatic hydrolysis. In the last decade or so, a few pretreatments have been shown to be promising in rendering biomass digestible,[41–44] but their performance appears to vary with the type of lignocellulosic feedstock.[45–51] Among several factors affecting enzymatic hydrolysis, unproductive binding of enzymes to lignin is often claimed to be responsible for the need for high enzyme loadings.[52–56] However, comparative data has not been developed on cellulase adsorption on lignin and cellulose prepared by leading pretreatment options to determine how the balance may shift, and the impact of lignin on enzymatic hydrolysis of solids prepared by these pretreatments has never been collectively reported. Thus, in this study, we sought to develop cellulase adsorption capacity data for pretreated solids, lignin, and cellulose prepared by leading pretreatment options that span a range of pH values including ammonia fiber expansion (AFEX), ammonia recycle percolation, (ARP), dilute sulfuric acid (DA), flowthrough (FT), lime, controlled pH, and sulfur dioxide. Cellulase adsorption data for pretreated solids and cellulase and β-glucosidase adsorption data for enzymatically extracted lignin were developed by equilibrating several concentrations of enzymes with solids at 4°C overnight. Accessibility of cellulose in poplar solids to cellulase, as determined by cellulase adsorption capacity, was indirectly estimated based on the compositional data of solids. In addition, the effectiveness of cellulase adsorbed on solids prepared by all pretreatments was investigated, and further the effect of delignification on cellulase effectiveness was determined. Finally, inhibition of pure cellulose hydrolysis by enzymatic and acid lignin was measured.

Materials and Methods

Substrates

Avicel PH-101 was purchased from FMC, Philadelphia, PA (Cat 11365, Lot 1094627). Our partners in the Biomass Refining Consortium for Applied Fundamentals and Innovation (CAFI) generously provided solids resulting from poplar pretreatment by leading technologies: ARP by Auburn University, AFEX by Michigan State University, dilute acid pretreatment with a pilot scale steam explosion by NREL, controlled pH by Purdue University, lime by Texas A&M University, and sulfur dioxide by the University of British Columbia. The pretreatment conditions and solids compositions as determined by NREL Laboratory Analytical Procedure 002[57] by our CAFI partners or in our laboratory are reported in Table 1.

Table 2. Glucan, Xylan, and Lignin Content of Delignified Poplar Solids (%) Prepared by Various Pretreatments

Pretreatment	Glucan	Xylan	Lignin
AFEX	75.8 ± 1.6	23.3 ± 0.14	2.4 ± 0.72
ARP	72.9 ± 4.1	20.9 ± 0.9	3.0 ± 0.8
DA	91.7 ± 2.4	5.9 ± 0.0	3.2 ± 1.3
Lime	72.6 ± 2.0	23.4 ± 1.0	2.2 ± 0.6
Controlled pH	88.4 ± 0.6	11.5 ± 0.1	1.9 ± 0.4
SO_2	92.7 ± 0.8	9.7 ± 0.0	1.5 ± 0.2

Enzymes

Spezyme® CP cellulase (lot 301-04075-034; 59 ± 5 FPU/mL, 123 ± 10 mg protein/mL), Multifect® Xylanase (lot 301-04021-015; 42 ± 5 mg protein/mL), and β-glucosidase (31 ± 5 mg protein/mL) and measurements of their protein content/activities were generously provided by the Genencor Division of Danisco US (Rochester, NY). The β-glucosidase used in some experiments (Novozyme188, 140 ± 5 mg protein/mL; 665 CBU/mL) was purchased from Sigma Chemicals, St. Louis, MO. Purified CBHI (18.5 mg/mL) from Spezyme® CP cellulase was prepared by Protein Labs (San Diego, CA). The enzyme protein contents were determined by the standard BCA method,[58] and the activity for Novozyme188 was based on that by Dien et al.[59]

Delignification

Delignification of pretreated poplar solids was performed in a fume hood using sodium chlorite and acetic acid at 70°C in a water bath.[60] To 80 mL of hot DI water, ~2.5 g dry weight of pretreated poplar solids, 0.5 mL of acetic acid, and 1 g of sodium chlorite were added, and then after every succeeding hour, fresh 0.5 mL of acetic acid and 1 g of sodium chlorite were introduced six times. Samples were left overnight in a water bath and then washed extensively until the filtrate pH was neutral. The solids were dried at 50°C for

Table 3. Carbohydrate Content of Enzyme Lignin Solids, and Percentage Nitrogen of Poplar Solids, Enzyme Lignin (EnzL), and Acid Lignin (AcL)

	Carbohydrate Content of Enzyme Lignin, (%)		Nitrogen, (%)		
Pretreatment	Glucan	Xylan	Poplar Solids	Enzyme Lignin	Acid lignin
Untreated	–	–	0.93	–	1.05
AFEX	6.7	3.0	2.09	2.90	2.93
ARP	5.2	0.0	1.10	2.26	2.09
DA	2.5	0.0	0.75	1.03	0.76
FT	8.8	0.0	ND	ND	ND
Lime	4.7	0.0	0.67	0.80	0.77
SO2	4.5	0.0	0.64	1.14	0.78

ND, not determined.

determining their composition according to NREL LAP 002,[57] as reported in Table 2.

Lignin preparations

Enzyme lignin (EnzL) and acid insoluble lignin (AcL) were prepared by nearly complete hydrolysis of the carbohydrates in pretreated solids with a enzyme mixture at a very high loading (about 150 FPU/g glucan and a xylanase to cellulase protein mass ratio of about 2)[61] and 72% acid as per NREL LAP 002 for carbohydrate and lignin analysis,[57] respectively. Enzyme lignin residues were further cleaned of residual protein left from enzymatic hydrolysis using Pronase K (Sigma Chemicals, St. Louis, MO.) according to a method reported elsewhere.[16,61] The approximate amount of Pronase (mL) in a 10 mg/mL solution required to digest a mg of protein was calculated as follows:

$$\text{Volume of Pronase (mL/mg of protein) of 10 mg/mL solution} = (1/(D \times A \times C \times t))$$

where D is the digestion activity of Pronase (mg protein per Unit per min), A is the activity of Pronase (Units/mg of protease), C is the desired concentration of Pronase (as 10 mg/mL used here), and t is time (taking minimum 60 min). However, Pronase was always used in excess (at least four times minimum) to ensure maximum removal of adsorbed proteins from the lignin. After overnight protease treatment in a phosphate buffer (pH 7.4) at 37°C, the lignin solids were heated at 80°C for 15 min to deactivate the protease followed by washing two times with 1 M sodium chloride solution followed by three to four times with DI water. The lignin solids were kept in a refrigerator at 4°C until needed. AcL solids collected after acid hydrolysis were washed with hot water at least twice, dried at 105°C, and kept refrigerated at 4°C until needed. The compositional data for EnzL as determined according to NREL LAP 002[57] and the percent nitrogen data for pretreated poplar solids, EnzL, and AcL, determined with an Elantech nitrogen analyzer[3,61] are reported in Table 3. The effectiveness of the protease method in removing protein from the lignin can be seen in Figure 1 in which the percentage nitrogen in EnzL and AcL were almost identical for all pretreatments. About 70% of the biomass protein was lost during AcL preparation, as shown in Figure 1, and due to less harsh conditions, less removal is expected for biological isolation of lignin from pretreated biomass.

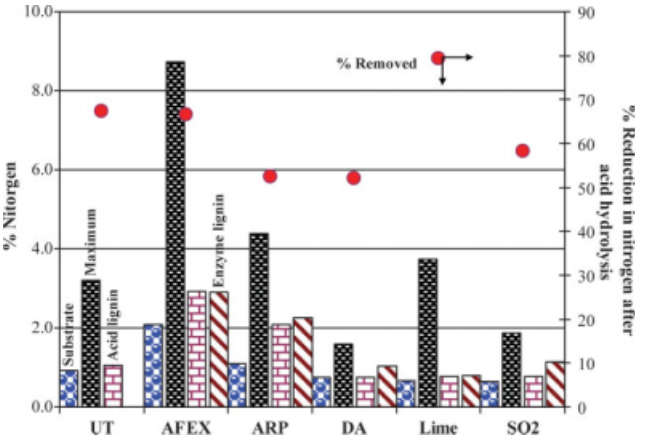

Figure 1. Effectiveness of the protease method as determined by nitrogen analysis of poplar solids produced by enzymatic digestion, EnzL, and acid hydrolysis, AcL, of the solids resulting from leading pretreatments.

*Maximum = % nitrogen of pretreated substrate/fraction of lignin in pretreated solids. EnzL, enzyme lignin; AcL, acid lignin.

Cellulase adsorption

Replicate adsorption experiments were performed in 15 mL test tubes containing 0.05 M citrate buffer (pH 4.8) at solids loading containing 1% (w/v) glucan or 1% (w/v) EnzL with different enzyme loadings (20 mg–1,600 mg/g glucan or g lignin) at 4°C to minimize hydrolysis. The amount of protein adsorbed on pretreated solids or EnzL was determined by a nitrogen analyzer, as discussed elsewhere.[3] In brief, after overnight equilibration of solids with enzymes in tubes turning end-over-end on a rotator driven by a variable speed motor (Glass-Col, Terre Haute, IN),[61] the tubes were repeatedly centrifuged, the liquid decanted, and the tubes dried for 24 h at 105°C before further nitrogen analysis using an Elantech nitrogen analyzer. The amount of protein adsorbed on the solids was estimated by multiplying the measured nitrogen percent by a nitrogen factor (NF $\cong$ 8.40 $\pm$ 0.3 as determined for Spezyme® CP).[3]

β-glucosidase adsorption

Adsorption experiments for β-glucosidase were performed at 4°C in 2.5 mL vials using a 0.05 M citrate buffer (pH 4.8). The vials containing 1% (w/v) lignin solids and various loadings of β-glucosidase (30 mg–1,500 mg/g lignin) were allowed to equilibrate overnight turning end-over-end on a rotator driven by a variable speed motor (Glass-Col, Terre Haute, IN).[61] Then the liquid and solids were separated by using a low protein binding filters with a pore size of 0.22 μm. The reason for using filters for Novozyme188 was its viscosity and its tendency to stick to tubes wall more prominently than Spezyme CP. The solids were further dried at 105°C, and the solids nitrogen content determined using a Nitrogen analyzer. The amount of protein was estimated by multiplying the measured nitrogen levels by a nitrogen factor (NF $\cong$ 3.25, as determined for Novozyme188).

Calculation of maximum adsorption capacity

Adsorption parameters (maximum adsorption capacity [σ]) and equilibrium constants [K_d]) were determined by nonlinear regression of the adsorption data using Polymath software to the following Langmuir expression:[3,52]

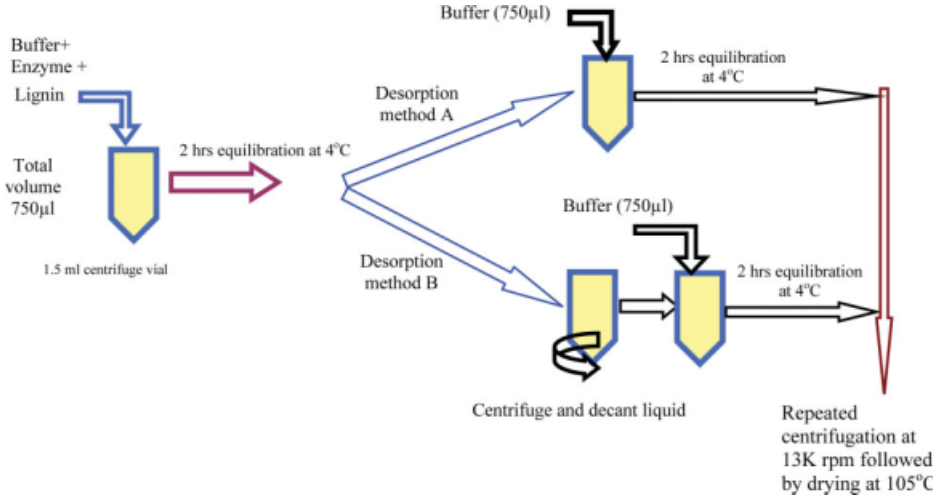

Figure 2. Schematic of cellulase adsorption-desorption approach for lignin solids.

$$[\mathrm{CE}] = \frac{\sigma[S_t][E_f]}{K_d + [E_f]}$$

where [CE] is the amount of adsorbed enzyme in mg/ml, $[E_f]$ the free enzyme concentration in mg/mL, σ the maximum adsorption capacity in mg/mg substrate, [St] the substrate concentration in mg/mL, and K_d the equilibrium constant = $[C]$ $[E]/[CE]$ in mg of enzyme/mL.

Cellulase adsorption-desorption for EnzL

Cellulase adsorption on lignin solids was performed in a citrate buffer at 4°C by equilibrating cellulase at a loading of 100 mg/g lignin with 20 mg of lignin in 1.5 mL centrifuge vials with a total volume of 0.750 mL. After 2 h of equilibration, tubes in triplicate were removed, centrifuged repeatedly, and dried at 105°C. These tubes were taken as a control for further analysis. For remaining vials, cellulase desorption experiments were performed via two methods, as described schematically in Figure 2. Under desorption method A, an equal amount (750 μL) of fresh 50 mM citrate buffer was added to the vials to make a total volume to 1.5 mL and then equilibrated at 4°C for 2 h. Following desorption method B, the vials were repeatedly centrifuged and buffer containing unadsorbed protein was decanted and then replenished with fresh 50 mM citrate buffer followed by equilibration for 2 h. Next, the vials were centrifuged, the liquid decanted, and the tubes dried at 105°C for further nitrogen analysis. The percentage enzyme desorption was calculated as:

$$\%\ \text{Desorption} = 100 \times (C - D)/C$$

where C is the amount of cellulase adsorbed on lignin solids for control and D is the amount of cellulase protein left on lignin solids after desorption.

Inhibition of Avicel hydrolysis by lignin

Inhibition of Avicel hydrolysis by lignin preparations was determined at a cellulase loading of 15 FPU/g glucan supplemented with Novozyme188 at a CBU to FPU activity ratio of ~2. Hydrolysis was performed in triplicates at 1% (w/v) glucan concentrations and 0.5% (w/v) of lignin preparations in a 0.05 M citrate buffer (pH = ~4.8) containing 1% sodium azide in a total volume of 10 mL. These ingredients were mixed in 50 mL Erlenmeyer flasks and controlled at 50°C ± 1°C using an air shaker (Multitron Incubator Shaker, Model no AJ125B, ATR Biotech, Laurel, MD) set at ~150 rpm.[62] Enzymes were added to the flasks after 2 h of incubation of Avicel and lignin preparations at 50°C. Avicel without lignin, controls, substrate blanks without enzyme, and enzyme blanks without substrate were run in parallel. To determine sugar release from residual carbohydrates left in the lignin solids, blanks containing lignin solids and enzymes were also run separately. Samples of about 700 μL in volume were drawn at 4, 24, 48, and 72 h and filtered through a 0.2-μm-nylon filter vials (Alltech Associates, Deerfield, IL). Then samples were pipetted into 500 μL polyethylene HPLC vials (Alltech Associates, Deerfield, IL), and kept refrigerated at 4°C or frozen at −20°C for longer times until analysis. Hydrolysis samples along with calibration standards were run on a Waters Alliance HPLC system (Model 2695, Waters, Milford, MA) employing a Aminex HPX-87P column (Bio-Rad Laboratories, Hercules, CA). Samples were processed at an eluent flow rate of 0.60 mL/min using a refractive index (RI) detector (model 2414, Waters, Milford, MA).

Cellulase effectiveness

Cellulase effectiveness was defined as the ratio of 24 h hydrolysis rate of poplar solids to the maximum cellulase adsorption capacity of the cellulose in the poplar solids. The maximum capacity of cellulose for cellulase was estimated as described earlier. Hydrolysis data for poplar solids pretreated with leading technologies were collected at a cellulase combined with β-glucosidase mass loading of 32.2 mg/g glucan in the unpretreated poplar solids (corresponding to about 15 FPU), as reported elsewhere.[25] Cellulase was supplemented with β-glucosidase at a CBU to FPU activity ratio of ~2. Similarly, hydrolysis data for delignified poplar solids were collected at the same enzyme loadings as poplar solids. All hydrolysis experiments were performed according to NREL LAP 009 with solids containing 1% (w/v) glucan in a

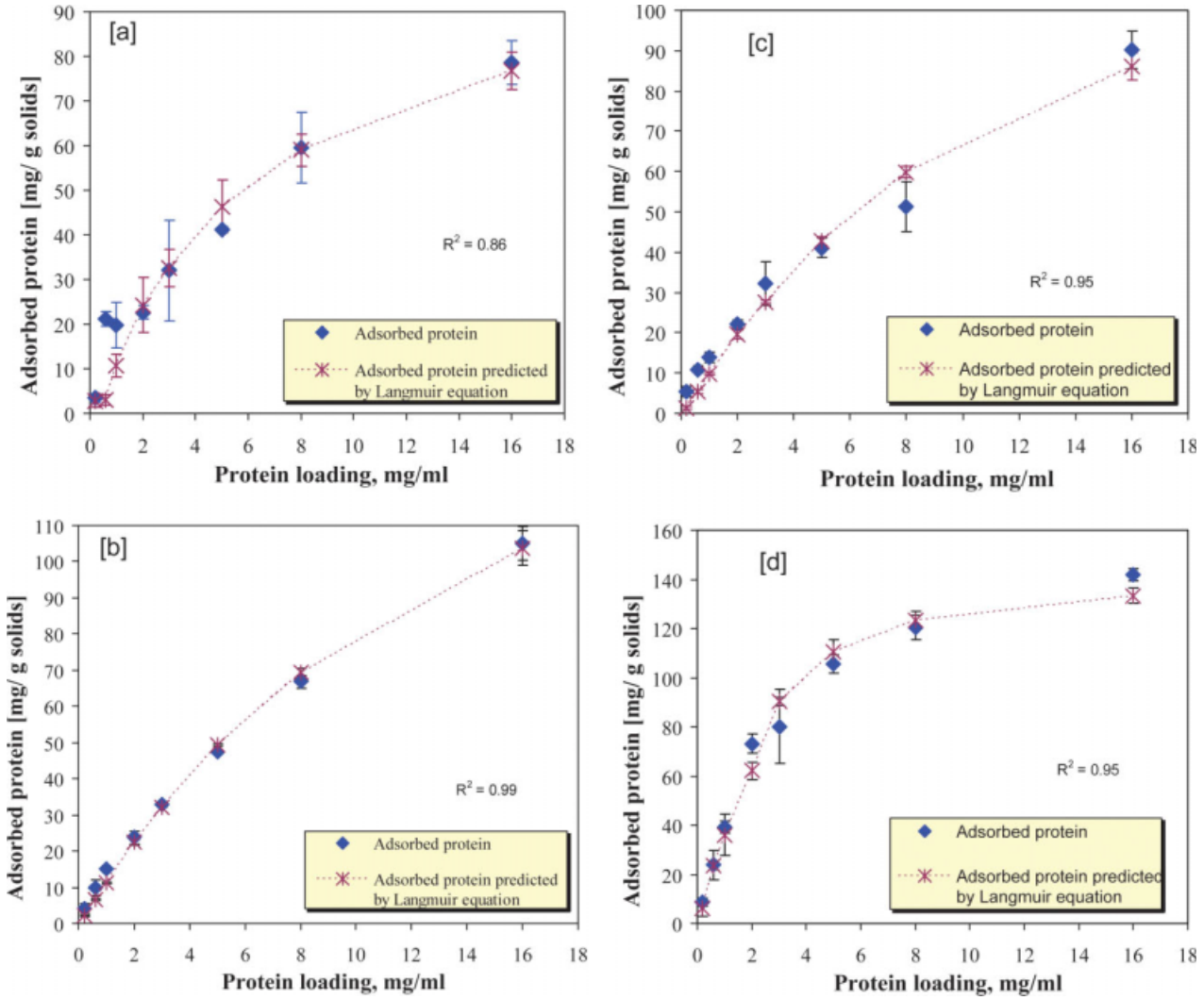

Figure 3. Amount of Spezyme CP adsorbed (mg/g substrate) vs. cellulase loading (mg/mL) and predicted amounts of adsorbed cellulase by Langmuir equation for (a) AFEX, (b) FT, (c) Lime, and (d) SO2 pretreated solids.

Table 4. Maximum Cellulase Adsorption Capacity, Affinity Constants, and Strength of Adsorption for Avicel Glucan and Solids Produced by Leading Pretreatments

Pretreatment	Max. Cellulase Adsorption Capacity, σ (mg/g substrate)	Affinity Constants, A, (L/g)	Affinity by Gibbs Free Energy, ΔGa, KJ/mol	Strength of Binding, $S = \sigma \times A$ (mL/g substrate)	R^2
Avicel	84.5	1.84	−26.30	154.5	0.98
AFEX	107.4	0.21	−21.28	23.0	0.86
ARP	113.5	0.11	−21.49	13.1	0.98
Controlled pH	56.2	0.43	−22.92	23.9	0.91
DA	170.9	0.94	−24.73	159.0	0.98
FT	195.2	0.08	−19.05	15.6	0.99
Lime	150.8	0.09	−19.47	14.5	0.95
SO_2	142.2	1.14	−25.19	161.0	0.95

50 mM citrate buffer.[62] Sugars in samples were analyzed as described previously.

Results

Cellulase adsorption on poplar solids

As per the literature, adsorption is affected by temperature and other physical parameters.[3,63–67] For example, some studies reported increasing temperature enhanced enzyme adsorption,[61,68,69] others observed a negative effect,[70–72] and a few others found a negligible impact of temperature on cellulase adsorption for a range of temperatures.[69,73] In this study, cellulase adsorption on solids was performed at 4°C to avoid carbohydrate hydrolysis during the measurements. Adsorption isotherms of cellulase on poplar solids were found to follow the Langmuir equation well with $R^2 > 0.86$, as shown in Figure 3 for AFEX, FT, lime, and SO_2 pretreated solids. By fitting the adsorption data to the Langmuir equation, adsorption capacities (σ), equilibrium constants (K_d), affinity constants ($1/K_d$), and strength of binding ($R = \sigma \times$ affinity constant) were estimated, as shown in Table 4.

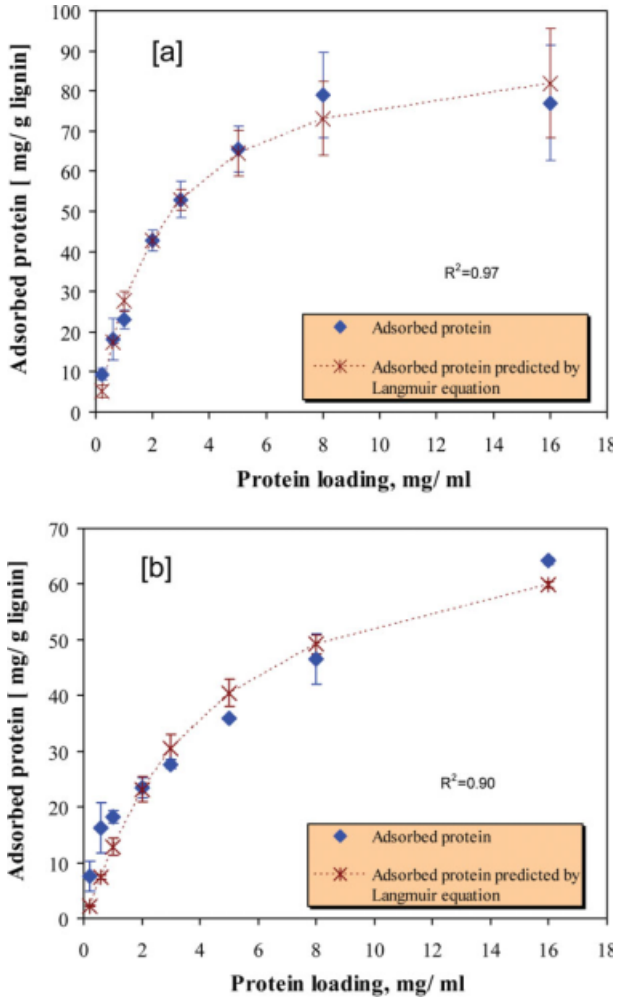

Figure 4. Amount of Spezyme CP adsorbed (mg/g lignin) vs. cellulase loading (mg/mL) and predicted amounts of adsorbed cellulase by Langmuir equation for (a) ARP and (b) DA pretreated lignin.

The affinity of cellulase to poplar solids was determined by the Gibb's free energy equation as well, $\Delta Ga = -R \times T \times \ln(K_d)$,[74] where the larger negative values of free energy indicate more spontaneous cellulase adsorption on solids. However, although the glucan content in the pretreated poplar solids from all pretreatments was only about 50%, all but those from controlled pH pretreatment showed higher adsorption capacity than the nearly pure glucan in Avicel. Solids prepared by flowthrough pretreatment had the highest adsorption capacity (σ) of 195 mg/g substrate followed by dilute acid (170 mg/g substrate) and then lime (151 mg/g substrate) pretreatments. Controlled pH pretreated solids had the least adsorption capacity, with the second least being for AFEX pretreatment.

As Table 4 shows, dilute acid and SO_2 pretreated solids had the highest binding strength for cellulase. However, not much data is available for comparison in the literature on cellulase adsorption for poplar solids. An adsorption capacity of 210 mg/g solids was reported in a study for dilute acid pretreated corn stover solids.[61] Using SO_2 catalyzed steam exploded Douglas fir, Lu et al. reported an adsorption capacity of about 171 mg/g substrate.[75] However, Tu et al.[76] reported an adsorption capacity of 101 mg/g substrate for SO_2 impregnated steam exploded Lodgepole pine containing 53.4% glucan, 0.4% xylan, and 45% lignin. In contrast, Zheng and coworkers[77] reported a very low cellulase adsorption capacity (42.5 mg/g solids) for dilute acid pretreated Creeping Wild Ryegrass, whereas Galbe et al. measured a much higher adsorption capacity (473 m/g solids) for steam pretreated willows.[78]

Cellulase adsorption on lignin

For adsorption experiments, EnzL was used because this biological method of lignin extraction should introduce less chemical changes in lignin than acid or other chemical methods,[79] and cellulase adsorption was performed at 4°C as before. Cellulase adsorption on EnzL followed the Langmuir equation well, as shown in Figure 4 for examples of ARP and dilute acid pretreatments, and the adsorption parameters are summarized in Table 5 for cellulase adsorption on lignin prepared by all of the leading pretreatments. Table 5 shows that lignin prepared by lime pretreatment had the highest capacity ($\sigma_{[lignin]} = 127$), while AFEX pretreatment had the least ($\sigma_{[lignin]} = 56.2$) followed by second lowest capacity for dilute acid pretreated lignin ($\sigma_{[lignin]} = 74.2$). Consistent with this, although adsorption experiments were performed at 40°C, Ooshima at al.[27] estimated a capacity of about 66 mg/g lignin residue and an affinity of 0.43 g/L for mixed hardwood lignin pretreated at somewhat different pretreatment conditions (200°C, 1% sulfuric acid, and 8.7 s; severity factor* $[R_0] = 2.10$ compared to severity factor of 2.70 used in this study). Performing adsorption experiments at 50°C in a recent study, Zheng et al.[77] showed an adsorption capacity of 86.1 mg/g lignin for dilute acid pretreated Creeping Wild Ryegrass (severity factor $[R_0] = 2.81$). In contrast, another study reported a very high cellulase adsorption capacity of 590 mg/g lignin for dilute acid pretreated corn stover.[61] Furthermore, the affinity constants in Table 5 suggest that cellulase strongly adsorbs onto lignin. Lime pretreated lignin, however, had the highest adsorption capacity but the lowest affinity for cellulase followed by dilute acid and SO_2 pretreated lignin.

Calculation of cellulose accessibility

The cellulase adsorption capacity for the cellulose fraction in pretreated solids was estimated from adsorption data for pretreated solids and EnzL by assuming complete accessibility of cellulase to cellulose and lignin and negligible cellulase adsorption on hemicellulose, resulting in the following formula:

$$\sigma_{[cellulose]} = (\sigma_{[solids]} - \sigma_{[lignin]} \times L_w)/C_w$$

where $\sigma_{[X]}$ is the maximum adsorption capacity of solids, cellulose, or lignin in pretreated solids; L_w is the fraction of lignin in the pretreated solids, and C_w the fraction of cellulose in the pretreated solids. Cellulase adsorption capacities calculated in this way for cellulose produced by the range of pretreatments are shown in Table 5. Lime and FT pretreated solids ($\sim\sigma_{[cellulose]} = 240$ mg/g) followed by dilute acid ($\sigma_{[cellulose]} = 236$ mg/g) had the highest cellulose accessibility, while ARP had the least. Again, there is not much information available in literature for comparison, but Lu et al.

*Severity factor, defined as Log R_0 = log ($t \cdot \exp[T_H - T_R]/14.75$]) includes only time and temperature.

Table 5. Cellulase Adsorption Parameters for Lignin and Maximum Adsorption Capacity of Cellulose Prepared by Leading Pretreatments

Pretreatment	Max. Adsorption Capacity of Lignin, $\sigma_{[lignin]}$ (mg/g lignin)	Affinity, 1/Kd (L/g protein)	Adsorption Strength, $S = \sigma \times$ Affinity (mL/g lignin)	Max. Adsorption Capacity of Cellulose, $\sigma_{[cellulose]}$ (mg/g cellulose)
AFEX	56.8	2.14	121.8	184.1
ARP	92.1	0.59	54.8	157.6
DA	74.0	0.29	21.2	235.8
FT	112.8	0.67	75.9	239.4
Lime	126.9	0.11	14.3	241.0
SO_2	83.7	0.25	21.0	179.8

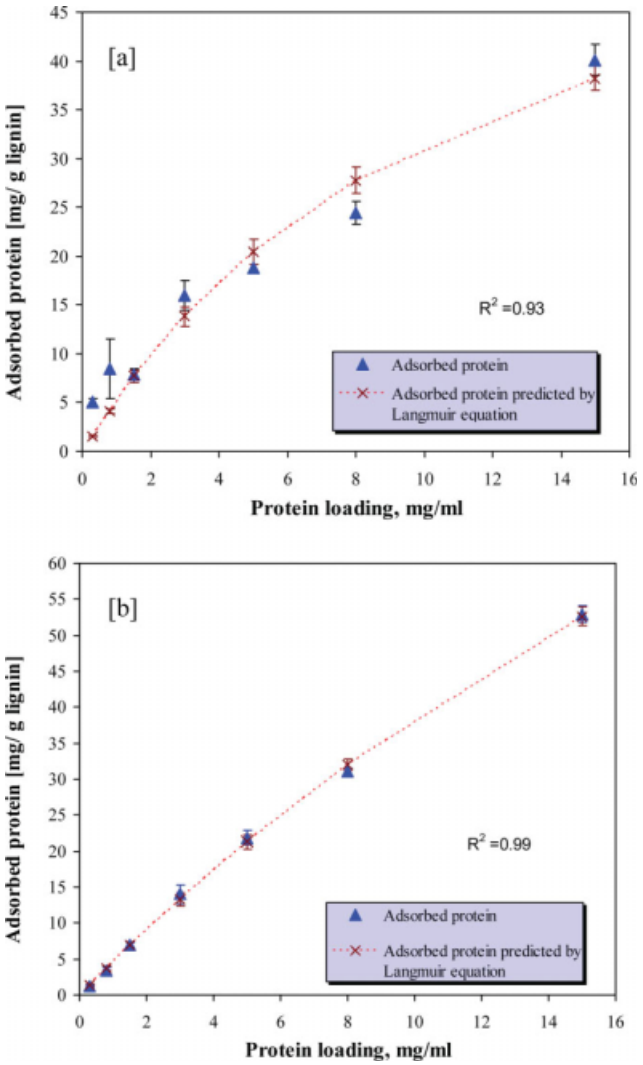

Figure 5. Amount of *β*-glucosidase adsorbed (mg/g lignin) vs. *β*-glucosidase loading (mg/mL) and predicted amounts of adsorbed *β*-glucosidase by Langmuir equation for (a) AFEX and (b) DA pretreated lignin.

reported an adsorption capacity estimated simply by dividing cellulase adsorption capacity of solids by the cellulose fraction in the solids of 340 mg/g cellulose for SO_2 catalyzed steam exploded Douglas fir. Ooshima et al.[27] indicated a much lower accessibility (31 mg/g cellulose) for dilute acid pretreatment at a temperature of 200°C (severity factor [R_0] = 2.10) for mixed hardwood. The probable causes for differences may be the type of substrate used (Ooshima et al. used mixed hard wood), pretreatment equipment (Ooshima et al. used a plug flow reactor), and the method employed for determining protein adsorption (conventional Bradford method[80]). There is no information available on cellulase adsorption for lignin preparations for the other pretreatments reported in this study.

β-glucosidase adsorption on lignin

Similar to cellulase adsorption on poplar solids and EnzL, *β*-glucosidase adsorption followed the Langmuir isotherm reasonably well ($R^2 > 0.93$), as illustrated in Figure 5 for AFEX and lime pretreated lignin, and maximum adsorption capacity, affinity constant, and adsorption strength for *β*-glucosidase are shown in Table 6 for all pretreatments. However, although the *β*-glucosidase adsorption capacity of EnzL was almost double that for cellulase capacity for all pretreatments but AFEX, the *β*-glucosidase affinity for lignin was much lower than for cellulase. Consistent with the estimated affinity constants here, Berlin et al. reported[81] for several enzymes that *β*-glucosidase activity was the least affected by lignin preparations from ethanol-organosolv pretreatment. Furthermore, a lower adsorption of *β*-glucosidase on pretreated substrates has also been reported in some other studies as well.[82–85] In contrast, several other studies reported that *β*-glucosidase adsorbs strongly on lignin and its activity is affected most by lignin.[26,86] Nonetheless, as reported in the literature and shown in Table 6, the capacity and affinity of *β*-glucosidase for lignin is a function of pretreatment and methods of preparation. AFEX EnzL had the least adsorption capacity (66.6 mg/g lignin) followed by lime (210 mg/g lignin), while SO_2 EnzL showed the highest capacity (320 mg/g lignin), and the highest affinity was for AFEX EnzL. Compared to the capacity for dilute acid poplar EnzL (301.3 mg/g glucan) reported here, a lower *β*-glucosidase capacity (173.5 mg/g lignin) but a much higher affinity (0.75 L/g protein) was reported by Zheng et al.[77] for dilute acid pretreated Creeping Wild Ryegrass EnzL, although it was not reported whether residual protein from lignin surface was completely removed. By working with enzymatically extracted and protease treated dilute acid corn stover lignin, an adsorption capacity and affinity of 170 mg/g lignin and 0.39 g/L, respectively, were measured at 4°C.[61]

Cellulase desorption from lignin

Applying the two methods of experimentally measuring the binding strength of cellulase to lignin depicted in Figure 2 revealed that cellulase desorption varied with the lignin type and desorption method employed, as shown in Figure 6. Upon dilution of samples with an equal amount of fresh citrate buffer, the percentage desorption from EnzL ranged from a minimum of 3.2% for that from SO_2 pretreatment to a maximum of 67% for that resulting from dilute acid. However, when the buffer containing unadsorbed proteins was replaced by an equal amount of fresh buffer, the percentage desorption from EnzL was lowest for ARP (26.1%) followed by second lowest for FT (40.9%), and it was highest for

Table 6. β-Glucosidase Adsorption Parameters for Poplar EnzL Prepared by Leading Pretreatments

Pretreatment	Maximum Adsorption Capacity, σ (mg/g lignin)	Affinity Constant, $A = 1/Kd$ (L/g protein)	Adsorption Strength, $S = \sigma \times A$ (mL/g lignin)	R^2
AFEX	66.6	0.092	6.15	0.93
ARP	278.6	0.009	2.73	0.98
DA	301.3	0.013	4.02	0.99
FT	257.7	0.020	5.20	0.99
Lime	209.4	0.026	5.50	0.99
SO_2	319.9	0.009	3.16	0.99

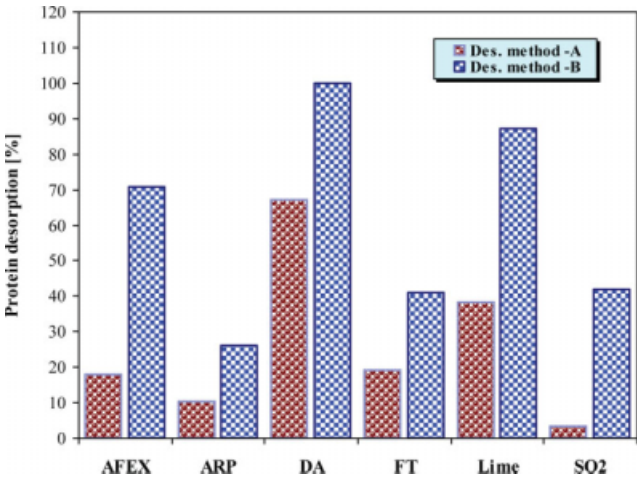

Figure 6. The extent of cellulase desorption upon dilution as determined via two methods in Figure 2 for poplar EnzL resulting from various pretreatments.

dilute acid (100%) followed by lime (87%) and AFEX (70.8%). Furthermore, for method A, the degree of desorption for all lignin solids but SO_2 had a good correlation to lignin binding strength (shown in Table 5), but no clear relationship was found with method B. Thus, although the activity of desorbed cellulase was not determined in this study, the data reported here should help in designing the enzyme recycling strategies.

Cellulase effectiveness and effect of delignification

The effectiveness of adsorbed cellulase, as defined by the ratio of the 24 h hydrolysis rate to cellulose adsorption capacity, was determined for pretreated poplar solids and pure glucan. Enzymatic hydrolysis of poplar solids and pure Avicel glucan was conducted at combined cellulase and β-glucosidase mass loadings of 16.1 and 32.2 mg/g glucan in unpretreated poplar (corresponding to about 7.5 and 15 FPU/g, respectively). On the basis of the ratio of the effectiveness for poplar solids compared with Avicel shown in Figure 7a, we can see that at a low combined cellulase and β-glucosidase mass loading, the best effectiveness ratio was about 60% for SO_2 pretreated poplar solids, whereas, the ratio for AFEX pretreated solids was the lowest. Effectiveness increased with cellulase plus β-glucosidase mass loadings for all pretreatments except SO_2 which apparently had reached its peak, and the increase was greatest for DA pretreated solids (about 70% of Avicel). Therefore, effectiveness appears to be a function of enzyme loading, probably due to unproductive binding of enzymes to lignin and substrate. Furthermore, the effect of delignification on effectiveness was determined assuming that the estimated cellulase adsorption capacity of cellulose does not change with delignification

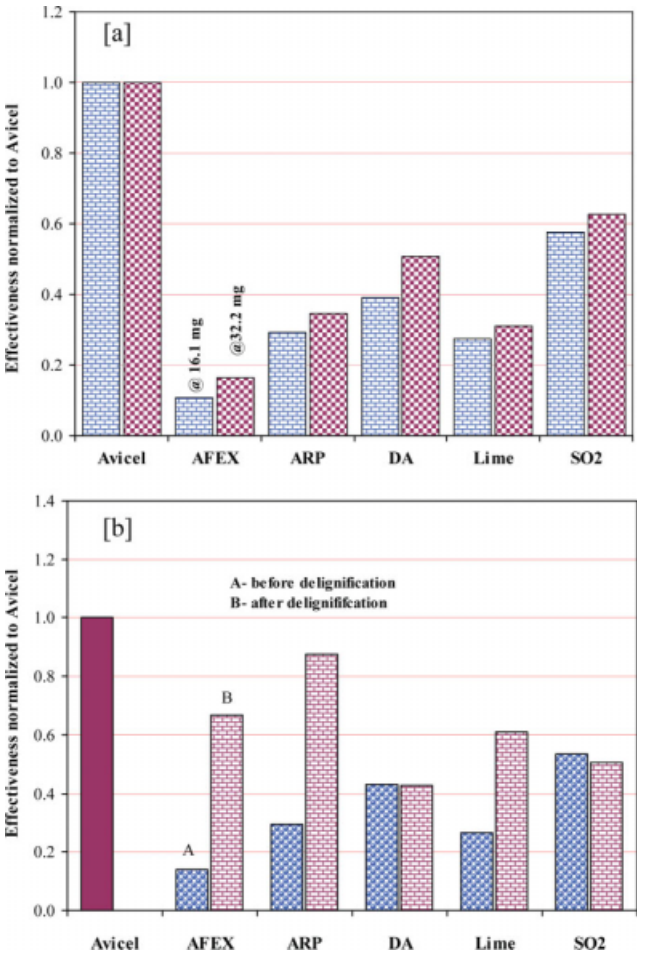

Figure 7. (a) Cellulase effectiveness for poplar solids normalized to Avicel glucan for a cellulase together with β-glucosidase mass loading of 16.1 and 32.2 mg/g glucan in unpretreated solids.

(b) Effect of delignification on cellulase effectiveness for poplar solids normalized to Avicel for a cellulase together with β-glucosidase mass loading of 32.2 mg/g glucan in unpretreated solids.

and drying of delignified solids at 50°C. Nevertheless, it was observed that the chlorite-acetic acid delignification procedure reduced the cellulose degree of polymerization (DP) (data not shown) and enhanced cellulose crystallinity, as discussed elsewhere.[60] However, although numerous studies have shown a negative impact of crystallinity on hydrolysis and cellulase adsorption,[2,70,87–89] no information is available on the effect of cellulose DP on cellulase adsorption and hydrolysis.

The impact of delignification on cellulase effectiveness is shown in Figure 7b for hydrolysis conducted at 32.2 mg/g glucan or glucan in unpretreated poplar. Delignification had a very large impact on cellulase effectiveness for AFEX

(enhanced by 3.75 times) and ARP (enhanced by 1.97 times) pretreated poplar solids followed by a modest impact for lime (enhanced by 1.30 times) and almost a negligible impact for dilute acid and SO_2 pretreated solids. Consistent with this, Schwald et al. found a negligible impact of delignification on glucan hydrolysis for SO_2 catalyzed steam exploded aspen wood.[90] This finding suggests that dilute acid and SO_2 pretreated solids used in this study had the least networking between lignin and carbohydrate components. In addition, delignification increased xylose release (770% for AFEX, 665% for ARP, 1494% for controlled pH, and 756% for lime pretreated solids) much more than glucose release (375% for AFEX, 197% for ARP, 318% for controlled pH, and 113% for lime pretreated solids). Furthermore, there was a liner relation between the release of these sugars, and because solids pretreated at low pH had much less xylan, delignification had a negligible impact on their glucose release. Thus, it appeared that lignin impeded enzyme access to xylan more than to glucan, which in turn affects glucan accessibility (Kumar et al., submitted for publication).[25,106] In another study, we showed a negligible impact of delignification on the accessibility of purified CBHI to glucan in unpretreated corn stover (Kumar et al., submitted for publication), consistent with this finding. However, the increase in effectiveness for AFEX, ARP, and lime pretreated solids may be due to the reduced DP of cellulose during delignification because these high pH pretreatments do not change cellulose DP much during pretreatment. However, the reduction in DP alone cannot be fully responsible for such a major increase in effectiveness and requires more study.

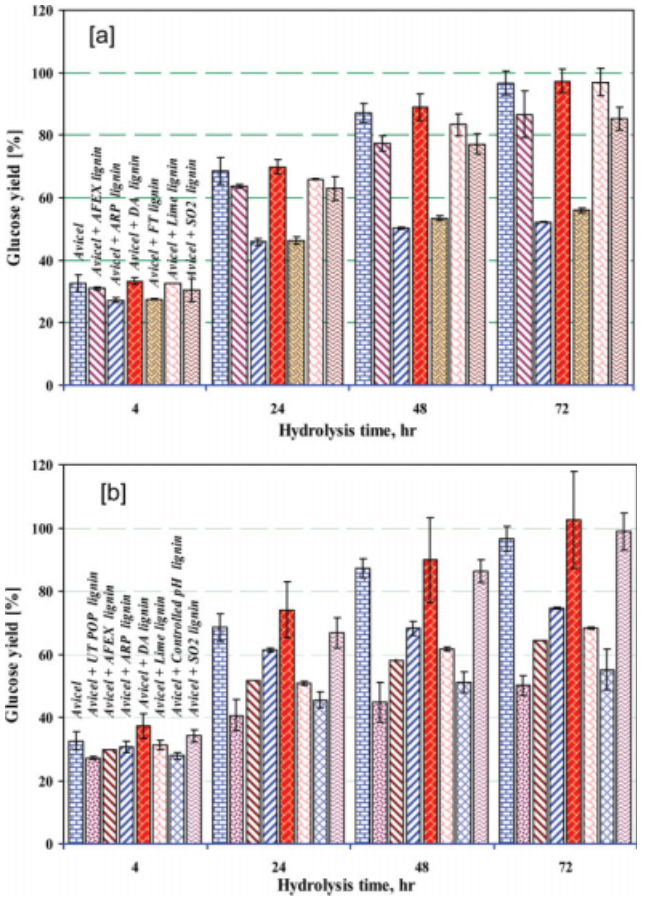

Figure 8. Glucose yield vs. hydrolysis time for Avicel hydrolysis at a combined cellulase and *β*-glucosidase mass loading of 32.2 mg/g glucan alone and with (a) EnzL or (b) AcL extracted from untreated and pretreated poplar solids prepared by leading pretreatments.

Avicel hydrolysis inhibition by EnzL and AcL

Avicel hydrolysis with and without (control) adding lignin preparations EnzL and AcL at a loading of 50% [w/w] of glucan was conducted in triplicates at a combined cellulase and *β*-glucosidase mass loading of 32.2 mg/g glucan for 72 h. Samples were then collected at 4, 24, 48, and 72 h of hydrolysis, with data shown in Figures 8a,b and tabulated in Table 7. In all cases, hydrolysis inhibition increased with hydrolysis time. Furthermore, among the EnzL preparations, ARP followed by FT pretreated lignin resulted in the greatest inhibition (46 and 42.1%, respectively after 72 h of hydrolysis), and AFEX and SO_2 pretreated EnzL produced only a modest inhibition of 10 to 11% after 72 h of hydrolysis. Lime pretreated EnzL had a modest inhibition (3–4%) during the early stages of hydrolysis but a negligible inhibition after 72 h. However, dilute acid pretreated EnzL had a negligible impact on Avicel hydrolysis, in contrast to general perceptions but consistent with literature reports of a limited effect of dilute acid pretreated lignin on glucan hydrolysis.[91,92]

Similar to EnzL, inhibition by AcL increased with hydrolysis time, as shown in Table 7. Furthermore, AcL from unpretreated poplar solids had the highest inhibition (48%) followed by AcL from controlled pH pretreated solids (42.8%), with this similarity suggesting that control pH pretreatment at the conditions applied did not change the poplar native lignin much. Comparing inhibition to that by EnzL, inhibition by AcL was higher for AFEX and lime, lower for

Table 7. Percentage Inhibition of Avicel Hydrolysis by EnzL and AcL of Poplar Solids Prepared by Leading Pretreatments at a Cellulase Plus *β*-Glucosidase Mass Loading of 32.2 mg/ g Glucan

	Hydrolysis Time (h)							
	Enzyme Lignin (EnzL)				Acid Lignin (AcL)			
Pretreatment	4	24	48	72	4	24	48	72
Control*	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
UT	–	–	–	–	16.6	40.6	48.6	48.1
AFEX	5.2	7.2	11.2	10.5	8.6	24.5	33.4	33.5
ARP	16.5	33.0	42.4	46.0	5.9	10.5	21.8	22.8
Controlled pH	–	–	–	–	14.3	33.5	41.4	42.9
DA	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
FT	15.7	32.5	38.7	42.1	–	–	–	–
Lime	0.1	3.8	4.4	0.0	4.0	25.8	29.3	29.4
SO2	6.3	8.1	11.4	11.8	0.0	2.3	1.0	0.0

* Control—Avicel plus enzymes only.

ARP and SO_2, and unchanged for dilute acid pretreatment. The negligible inhibition by dilute acid AcL, similar to DA EnzL discussed above, further confirms that poplar lignin prepared by dilute acid pretreatment using steam explosion had the lowest inhibition of Avicel hydrolysis.

As implied in the literature[93–95] and from the nitrogen contents of EnzL and AcL from AFEX in Table 3, it appears that ammonia strongly interacts with lignin and makes it less inhibitory to hydrolysis, consistent with the finding shown earlier that AFEX lignin had a very low adsorption capacity for enzymes. In addition, treatment of AFEX lignin with acid (used for extraction of AcL) seemed to have reduced this effect, as hydrolysis inhibition with AFEX AcL was much higher than observed with AFEX EnzL. The low inhibition observed with SO_2 lignin could result from lignin sulfonation,[90,96–98] and high inhibition by AcL than EnzL could be caused by acid treatment enhancing the hydrophobicity of lignin.

Discussion

Cellulase adsorption was measured at 4°C for poplar solids and lignin left from those solids following pretreatments by leading options that span a range of pH values: dilute acid, SO_2 steam explosion, FT, controlled pH, AFEX, ARP, and lime. Adsorption parameters were estimated by fitting adsorption data to the Langmuir equation via a nonlinear regression using Polymath software. The accessibility of cellulose to cellulase, as determined by maximum cellulase adsorption capacity, was estimated indirectly. Solids produced by FT and dilute acid pretreatment had a higher cellulase adsorption capacity (195 mg/g glucan and 170 mg/g glucan, respectively) than other pretreatments. The affinity or strength of cellulase binding to solids, which is often considered vital among enzyme factors controlling hydrolysis,[71,74,99] was also very high for SO_2 and dilute acid pretreated solids followed by that for pure Avicel glucan. However, although it is difficult to ascertain a single factor impacting glucan hydrolysis results, the normalized effectiveness of cellulase to Avicel for these two pretreatments was found to be higher than for other pretreatments, as shown in Figure 7. EnzL prepared by AFEX lignin had the least cellulase adsorption capacity (56.8 mg/g glucan) and the highest adsorption affinity (2.14 L/g protein), whereas lime EnzL had the highest cellulase adsorption capacity (126 mg/g glucan) but the lowest affinity for cellulase (0.11 L/g protein). Surprisingly, dilute acid EnzL had the second lowest adsorption capacity and third lowest, after lime and SO_2, affinity for cellulase.

Cellulose accessibility was observed to be highest for lime followed by FT and dilute acid pretreated solids. However, estimation of cellulose accessibility depends on underlying assumptions as cellulase may unproductively adsorb on hemicellulose part of biomass,[100] and cellulose and lignin in pretreated biomass may not be as completely accessible to cellulase for some pretreatments[92,101,102] as assumed here. Similar to cellulase, β-glucosidase adsorption parameters were estimated for poplar EnzL. Nevertheless, although lignin solids had a much higher β-glucosidase capacity than for cellulase for all pretreatments but AFEX, the affinity of lignin for β-glucosidase was lower. AFEX EnzL had the least β-glucosidase adsorption capacity (66.6 mg/g glucan) and SO_2 had the highest (320 mg/g glucan). In contrast, Willies[61] estimated a much higher capacity of dilute acid pretreated corn stover EnzL for cellulase (590 mg/g lignin) than for β-glucosidase (170 mg/g glucan). Cellulase desorption from EnzL after 2 h of incubation at 4°C showed that cellulase binds strongly with lignin for some pretreatments whereas for others the binding was partially and completely reversible upon dilution. The normalized effectiveness of adsorbed cellulase showed that cellulase adsorbed on SO_2 and dilute acid pretreated solids was more effective than for other pretreatments, and effectiveness increased with cellulase together with β-glucosidase mass loading for all pretreatments but SO_2. However, delignification had a huge impact on cellulase effectiveness for AFEX, ARP, and lime pretreatments (375, 197, and 130% increase, respectively) and a negligible improvement for dilute acid and SO_2 pretreatments. Based on the increase in xylose and glucose release, lignin impedes enzyme access to xylan which in turn limits access to glucan due to xylan coating/binding to glucan, consistent with a recent study by Joeh et al.[101] and prior reports.[103,104]

In addition, dilute acid pretreated lignin caused negligible inhibition of enzymatic hydrolysis of Avicel, while EnzL resulting from ARP and FT had the highest. AFEX and SO_2 EnzL inhibited hydrolysis by 10–11% after 72 h of hydrolysis. Lime EnzL inhibited hydrolysis to some extent in the early stages, but the effect was negligible after 72 h of hydrolysis. For both AcL and EnzL, the extent of inhibition increased with hydrolysis time.

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