ARTICLE

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Cellulase Adsorption and Relationship to Features of Corn Stover Solids Produced by Leading Pretreatments

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ABSTRACT: Although essential to enzymatic hydrolysis of cellulosic biomass to sugars for fermentation to ethanol or other products, enzyme adsorption and its relationship to substrate features has received limited attention, and little data and insight have been developed on cellulase adsorption for promising pretreatment options, with almost no data available to facilitate comparisons. Therefore, adsorption of cellulase on Avicel, and of cellulase and xylanase on corn stover solids resulting from ammonia fiber expansion (AFEX), ammonia recycled percolation (ARP), controlled pH, dilute acid, lime, and sulfur dioxide (SO_2) pretreatments were measured at 4°C. Langmuir adsorption parameters were then estimated by non-linear regression using Polymath software, and cellulase accessibility to cellulose was estimated based on adsorption data for pretreated solids and lignin left after carbohydrate digestion. To determine the impact of delignification and deacetylation on cellulose accessibility, purified CBHI (Cel7A) adsorption at 4°C and hydrolysis with whole cellulase were followed for untreated (UT) corn stover. In all cases, cellulase attained equilibrium in less than 2 h, and upon dilution, solids pretreated by controlled pH technology showed the greatest desorption followed by solids from dilute acid and SO_2 pretreatments. Surprisingly, the lowest desorption was measured for Avicel glucan followed by solids from AFEX pretreatment. The higher cellulose accessibility for AFEX and lime pretreated solids could account for the good digestion reported in the literature for these approaches. Lime pretreated solids had the greatest xylanase capacity and AFEX solids the least, showing pretreatment pH did not seem to be controlling. The 24 h glucan hydrolysis rate data had a strong relationship to cellulase adsorption capacities, while 24 h xylan hydrolysis rate data showed no relationship to xylanase adsorption capacities. Furthermore, delignification greatly enhanced enzyme effectiveness but had a limited effect on cellulose accessibility. And because delignification enhanced release of xylose more than glucose, it appears that lignin did not directly control cellulose accessibility but restricted xylan accessibility which in turn controlled access to cellulose. Reducing the acetyl content in corn stover solids significantly improved both cellulose accessibility and enzyme effectiveness.

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KEYWORDS: adsorption; hydrolysis; adsorption capacity; lignin; acetyl content; accessibility; effectiveness

Introduction

According to the billion ton biomass report, corn stover alone, with the current tillage practices and technologies, could provide about 7.5% (75 million ton per year) of the total cellulosic biomass projected to be available in the USA and is the single largest source (38.6% of total 194 million tons) of cellulosic biomass available from agricultural lands (18.0%) (Perlack et al., 2005). In addition, the relatively low recalcitrance of corn stover makes it a favorable feedstock for biological processing (Chandra et al., 2007; Gupta et al., 2008). Consequently, several studies have evaluated corn stover pretreatment and enzymatic digestion (Kaar and Holtzapple, 2000; Liu and Wyman, 2003, 2004a,b; Lloyd and Wyman, 2005; Yang and Wyman, 2004). However, although cellulase adsorption onto solids is the primary step for enzymatic

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saccharification of pretreated corn stover (Kumar and Wyman, 2008; Lynd et al., 2002; Wyman et al., 2005b; Zhang and Lynd, 2004) and the hydrolysis rate and/or yield is often claimed to be directly related to the amount of adsorbed enzymes (Jeoh et al., 2007; Karlsson et al., 1999; Kotiranta et al., 1999; Kumar and Wyman, 2009b), little information is available on substrate–enzyme interactions, including cellulase adsorption kinetics and the accessibility of enzyme to lignin and cellulose in pretreated biomass. Similarly, although hemicellulolytic enzymes must adsorb on the solid surface prior to enzymatic hydrolysis (Kumar and Wyman, 2009e; Sun et al., 1998; Zilliox and Debeire, 1998), the complexity of the hemicellulose structure and the array of enzymes involved have resulted in limited studies of hemicellulolytic enzymes–substrate interactions for real biomass.

Among the primary substrate features impacting enzymatic conversion of biomass, lignin is believed to impede enzyme access to glucan chains by its protective sheathing and also reduce cellulase effectiveness as a result of unproductive binding and steric hindrance (Chang and Holtzapple, 2000; Mansfield et al., 1999). Lignin removal has often been shown to improve cellulose digestibility, with mechanisms postulated to be improved cellulose accessibility, enhancement of cellulase effectiveness by removal of cross linkages to carbohydrates, or both, (Chang and Holtzapple, 2000; Ohgren et al., 2007; Pan et al., 2005; Taniguchi et al., 2005; Yang and Wyman, 2004), but only a few studies claim lignin removal offers little benefit (Grohmann et al., 1986). Moreover, although xylan removal is often reported to enhance glucan digestibility (Allen et al., 2001; Ishizawa et al., 2007; Yang and Wyman, 2004; Zhu et al., 2005), a first of a kind of study showed that xylan removal enhanced biomass digestibility by increasing cellulose accessibility (Jeoh et al., 2007). Furthermore, removal of acetyl content has been reported to enhance digestibility (Chang and Holtzapple, 2000; Grohmann et al., 1989; Wood and McCrae, 1986), but its impact on cellulose accessibility has not been reported. No studies have compared enzyme adsorption for solids resulting from pretreatment by different technologies or tried to determine what features could account for different behavior.

In this study, cellulase and xylanase adsorption and cellulase desorption upon dilution were determined at 4°C for pure Avicel glucan and solids resulting from ammonia fiber expansion (AFEX), ammonia recycled percolation (ARP), controlled pH, dilute acid (DA), lime, and SO_2 pretreatments at near optimal conditions. In addition, cellulase and xylanase adsorption capacities of pretreated solids and cellulase adsorption capacities of the lignin left after enzymatic digestion of the pretreated solids were estimated from adsorption data. The effect of selective delignification and acetyl removal on glucan accessibility and cellulase effectiveness for untreated corn stover was then determined.

Materials and Methods

Materials

Pure cellulose, Avicel PH-101, was purchased from FMC Corporation (Philadelphia, PA) (Cat 11365, Lot 1094627). Bovine serum albumin (BSA, Cat A9056) was from Sigma Chemicals, St. Louis, MO. Unpretreated corn stover was generously provided by the National Renewable Energy Laboratory (NREL) in Golden, CO from a source they had at the Kramer farm in Wray, CO. Solids resulting from pretreatment of corn stover by the following technologies were generously provided by our partners in the Biomass Refining Consortium for Applied Fundamentals and Innovation (CAFI): ARP by Auburn University, AFEX by Michigan State University, dilute acid in a Sunds pilot reactor by NREL, controlled pH by Purdue University, lime by Texas A&M University, and sulfur dioxide by the University of British Columbia. Untreated corn stover was selectively deacetylated with potassium hydroxide (KOH) at room temperature using the approach of Chang and Holtzapple (2000) and Kong et al. (1992) but at a total solids concentration of 5% (w/w), to facilitate mixing, on a dry basis instead of the 10% in the reported method. Reaction conditions and solids compositions as measured according to NREL Laboratory Analytical Procedure 002 (NREL, 2004) by our CAFI partners or in our laboratory are reported in Table I for all of the pretreatments.

Selective Delignification

Untreated corn stover was selectively delignified with peracetic acid (0.1 g to 5.0 g/g dry solids) at room temperature according to the approach of Chang and Holtzapple (Chang and Holtzapple, 2000) at a total solid concentration of 5% (w/w). The delignified solids were neutralized using sodium hydroxide and washed repeatedly unless the filtrate pH was close to neutral. The compositions of delignified solids as measured according to NREL LAP 002 (NREL, 2004) are reported in Table II.

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, Inc. (Rochester, NY). The β-glucosidase used in some experiments (Novozyme188, 140 ± 5 mg protein/mL; 665 CBU/mL) was purchased from Sigma Chemicals. 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 (Smith et al.,

Table I. Pretreatment conditions and compositions for corn stover solids prepared by leading technologies.

Pretreatment	Pretreatment conditions	Composition of pretreated solids (%)		
		Glucan	Xylan	Lignin
Unpretreated	—	38.3 ± 2.2	21.7 ± 1.2	20.5 ± 1.1
AFEX	90°C, 220 psi, 1:1 NH_3 to Biomass, 5 min—NW	34.4	22.8	18.0
ARP	170°C, 325 psi, 3.33:1 NH_3/Dry Wt, 20 min, and 3.3 mL/g of corn stover—W	61.9	17.9	8.8
Controlled pH	190°C, 15 min (+5 min heat up)—NW	52.7	16.2	25.2
Dilute acid (Sunds System)	180°C, 0.03H_2SO_4/Dry wt, 90 s, 25% solids—HW	59.3	9.3	22.5
Lime	55°C, 0.5:1 $Ca(OH)_2$ to Biomass (dry wt), 4 weeks, water loading—10 g/g dry biomass—W1	56.7	26.4	14.6
SO_2	190°C, 5 min, 3% SO_2—steam explosion—W	56.9	11.6	23.8
Deacetylation				
De-A	25°C, 24 h, 0.15 Mmol KOH/g corn stover (dry wt.)—W	43.3	24.5	18.7
De-B	25°C, 24 h, 0.55 Mmol KOH/g corn stover (dry wt.)—W	43.0	26.4	19.7
De-C	25°C, 24 h, 0.75 Mmol KOH/g corn stover (dry wt.)—W	45.9	24.1	18.5
De-D	25°C, 48 h, 1.5 Mmol KOH/g corn stover (dry wt.)—W	42.1	23.4	19.5

NW, not washed; W, water washed; HW, hot water washed; W1, neutralized and washed; De-A, 48.6% deacetylation; De-B, 54.2% deacetylation; De-C, 85.0% deacetylation; De-4, 100% deacetylation.

1985), and the activity for Novozyme188 was based on that by Dien et al. (2008).

Enzymatic Hydrolysis

Enzymatic hydrolysis was performed according to NREL Laboratory Analytical Procedure LAP 009 in at least duplicates at 1% (w/v) glucan concentrations in 0.05 M citrate buffer (pH ~4.8) containing sodium azide (1%) as an antibiotic. These ingredients were mixed in 125 mL Erlenmeyer flasks and controlled at 48 ± 3°C using a thermostated shaker water bath set at ~200 rpm (NREL, 1996). Substrate blanks without enzyme and enzyme blanks without substrate were run in parallel. Digestibility of untreated, delignified, and deacetylated corn stover was determined at a cellulase plus β-glucosidase loading of 32.2 mg of protein/g glucan (corresponding to about 15 FPU/g glucan) supplemented with β-glucosidase at a CBU to FPU activity ratio of ~2, unless otherwise stated. For delignified corn stover digestibility, two separate sets of experiments with a solids loading corresponding to a glucan loading of 1%(w/v) or lignin loading 0.5% (w/v) were run in parallel.

Sugar Analysis

To determine the amount of sugars generated during hydrolysis, liquid samples of about 700 μL were drawn at 24, 48, and 72 h and then immediately filtered through 0.2 μm nylon filter vials (Alltech Associates, Inc., Deerfield, IL), pipetted into 500 μL polyethylene HPLC vials (Alltech Associates, Inc.), and kept refrigerated at 4°C or frozen at −20°C for longer times until analyzed. Hydrolysis samples along with calibration standards were run on a Waters Alliance HPLC system (Model 2695, Waters Corporation, Milford, MA) employing HPX-87P columns (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 Corporation).

Lignin Preparations

Enzyme lignin (EnzL) was prepared by nearly complete hydrolysis of the carbohydrates in pretreated solids with a mixture of cellulase, β-glucosidase, and xylanase at a very high loading (about 150 FPU/g glucan and a xylanase to cellulase protein mass ratio of about 2), as described elsewhere (Willies, 2007). Acid insoluble lignin (AcL) was prepared using 72% acid as per NREL LAP002 (NREL, 2004). Enzyme lignin residues were further cleaned of residual protein left from enzymatic hydrolysis using Pronase K (Sigma Chemicals) according to a method reported elsewhere (Willies, 2007; Yang et al., 2006). Pronase was always used in excess (at least 4 times of minimum) to maximize removal of adsorbed proteins from the lignin. After overnight protease treatment in a phosphate buffer

Table II. Treatment conditions, approximate percentage lignin removal, and composition of resulting delignified corn stover solids (%).

Substrate	Treatment conditions	App. percentage lignin removal	Glucan	Xylan	Lignin
DeL-A	25°C, 24 h, 0.1 g peracetic acid/g corn stover (dry wt.)—W1	28.7	39.5 ± 3.9	23.9 ± 1.2	19.4 ± 0.2
DeL-B	25°C, 24 h, 0.3 g peracetic acid/g corn stover (dry wt.)—W1	52.6	46.1 ± 0.6	25.9 ± 0.9	15.3 ± 0.6
DeL-C	25°C, 24 h, 1 g peracetic acid/g corn stover (dry wt.)—W1	68.4	47.9 ± 0.9	28.1 ± 1.0	14.1 ± 1.2
DeL-D	25°C, 48 h, 5 g peracetic acid/g corn stover (dry wt.)—W1	80.0	46.7 ± 0.9	26.8 ± 0.1	8.2 ± 0.5

DeL- [x], corn stover solids delignified to various degrees; W1, neutralized with sodium hydroxide and washed.

(pH 7.4) at 37°C, the lignin solids were heated at 80°C for 15 min to deactivate the protease followed by washing twice with 1M sodium chloride solution and 3–4 times washing with DI water (Yang et al., 2006). The lignin solids were kept in a refrigerator at 4°C until needed. The composition of EnzL was determined by NREL LAP 002 (NREL, 2004), and the nitrogen content of pretreated corn stover solids and protease treated EnzL was determined with an Elantech nitrogen analyzer (Kumar and Wyman, 2008; Willies, 2007), with the results reported in Table III.

Enzymes Adsorption

To determine cellulase adsorption-desorption kinetics, adsorption on Avicel and pretreated solids was performed in 5 mL 0.05 M citrate buffer at 4°C to avoid hydrolysis, by equilibrating pretreated solids containing 1%(w/w) glucan with cellulase at a loading of 400 mg/g glucan in 15 mL centrifuge tubes turning end-over-end on a rotator driven by a variable speed motor at 60 rpm (Glass-Col, Terre Haute, IN) (Willies, 2007). Tubes in triplicates were periodically removed over 2 h (10, 20, 30, 60, 90, and 120 min) and centrifuged repeatedly for 15 min in a centrifuge at 4,000 rpm. Then, the liquid containing unadsorbed protein was decanted before drying the tubes for 24 h at 105°C for nitrogen analysis of the solids by the Elantech nitrogen analyzer, described elsewhere (Kumar and Wyman, 2008), and the amount of protein adsorbed onto the pretreated solids was estimated by multiplying the percentage nitrogen by a nitrogen factor (NF$\sim = 8.40 \pm 0.3$, 8.27 ± 0.4, and 3.25 ± 0.2 as determined for Spezyme® CP, Multifect® Xylanase, and Novozyme 188, respectively). Because protein in the biomass solids and pretreatments using nitrogen reagents may contribute to the nitrogen content of solids, substrate blanks without enzymes were run in parallel to determine the nitrogen that only came from enzyme protein (Kumar and Wyman, 2008). The remaining tubes, which had equilibrated for 2 h, were used to determine cellulase desorption kinetics. Samples were diluted with equal amounts (5.0 mL) of fresh citrate buffer and equilibrated at 4°C while turning end-over-end on the rotator described above. As before, triplicates of these tubes were periodically removed over 2 h (10, 20, 30, 60, 90, and 120 min) and centrifuged repeatedly followed by decanting the liquid containing unadsorbed protein and drying the tubes at 105°C before nitrogen analysis by a nitrogen analyzer The average amounts of cellulase adsorbed onto pretreated solids and desorbed from the solids upon dilution were then calculated from the nitrogen data as follows:

$$\text{Cellulase desorption}(\%) = 100\left(\frac{C-D}{C}\right)$$

in which C is the average amount of cellulase adsorbed on pretreated solids mg/g solids over 2 h and D is the average amount of cellulase protein left on the pretreated solids after desorption over 2 h-mg/g solids.

For enzyme adsorption capacity, solids were equilibrated overnight with several enzyme loadings (20 mg to 1,600 mg/g glucan or g lignin) using 15 mL test tubes at a biomass loading corresponding to 1% (w/v) glucan or 1% (w/v) EnzL. The amount of protein on solids was then determined as discussed above.

Adsorption of Purified CBHI

To investigate the impact of deacetylation and delignification on cellulose accessibility, CBHI (Cel7A) adsorption was performed at 4°C in a 0.05 M citrate buffer solution in 2 mL centrifuge vials with a loading of 75 mg of CBHI protein/g glucan. Substrate blanks without CBHI and CBHI blanks without substrate were run in parallel. After 4 h of equilibration by rotating the vials end-over-end on the rotator, the vials were centrifuged at 13 K rotor speed for 5 min. Then the amount of free protein in the solid free solution was determined using the BCA method (Smith et al., 1985) with BSA as the protein standard. The amount of CBHI adsorbed was indirectly estimated by subtracting the amount of free protein from the total amount of protein initially added.

Calculation of Maximum Adsorption Capacity

Adsorption parameters (maximum adsorption capacity [σ] and equilibrium constant [K_d]) were determined by non-

Table III. Carbohydrate content of enzyme lignin (EnzL) solids and percentage nitrogen of pretreated corn stover solids, enzyme lignin, and acid lignin (AcL).

	Carbohydrate content of enzyme lignin (%)		Nitrogen (%)		
Pretreatment	Glucan	Xylan	Corn stover solids	Enzyme lignin	Acid lignin
Untreated	—	—	0.87		1.56
AFEX	7.48	2.90	2.13	2.90	2.27
ARP	4.96	1.30	0.78	2.26	2.53
Controlled pH	5.20	1.48	0.72	0.78	1.47
DA	12.90	4.30	0.82	1.03	1.45
Lime	1.70	0.31	0.46	0.80	—
SO_2	4.84	1.91	0.64	1.14	1.44

linear regression of the adsorption data to the following Langmuir expression using Polymath software (Kumar and Wyman, 2008; Lynd et al., 2002):

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

in which $[CE]$ 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, $[S_t]$ the substrate concentration in mg/mL, and K_d the equilibrium constant $= [C][E]/[CE]$ in mg of enzyme/mL, where $[C]$ is the concentration of free binding sites on the substrates in mg/mL and $[E]$ and $[E_f]$ are the enzyme concentrations not adsorbed on the substrate in mg/mL.

Results

Cellulase Adsorption-Desorption Kinetics

Cellulase adsorption was very rapid for Avicel glucan and pretreated corn stover solids, with the maximum occurring in the first 10 min, as shown in Figure 1. However, for all solids and Avicel glucan, adsorption continued, but very slowly, with time and reached equilibrium in less than 2 h. This observation is consistent with literature findings that about 90 min was needed to reach equilibrium for many similar systems (Lynd et al., 2002). Dilution of solids containing adsorbed cellulase with liquid containing no protein resulted in very rapid release of protein in the first 10 min, with little change over the rest of the equilibration time, as shown in Figure 1.

The amount of protein adsorbed and desorbed varied substantially with the substrate and pretreatment type, as summarized in Figure 2. Lime and dilute acid pretreated solids had the highest adsorption followed by ARP and SO_2, while Avicel solids followed by AFEX and controlled pH had the least. Although pure Avicel cellulose contains no lignin, it desorbed the least amount of protein, while the greatest percentage release was from controlled pH pretreated solids (30%). No direct relationship was observed between the amount of lignin in the solids and enzyme desorption (data not shown).

Modeling Cellulase Adsorption

The adsorption parameters shown in Table IV were estimated by nonlinear regression of adsorption data for Avicel glucan and biomass solids pretreated by the different pretreatments using the Langmuir equation, and cellulase adsorption data followed the Langmuir relationship well, as illustrated in Figure 3 for controlled pH and lime pretreatments. In addition, values of the strength of binding defined as the product of the affinity constant and maximum adsorption capacity are shown in Table IV. Lime pretreated solids had the highest cellulase adsorption capacity of 133 mg/g solids followed by SO_2 pretreated solids (124 mg/g solids). On the other hand, solids produced by dilute acid pretreatment showed the lowest adsorption capacity (90 mg/g solids) followed by solids resulting from AFEX pretreatment (99 mg/g solids). Cellulase affinity for ARP solids was by far the highest at about 40 times the average affinity constant for solids prepared by other pretreatments. Although it is difficult to ascertain the cause of such a high cellulase affinity for ARP solids, the high affinity of lignin for cellulase could be a possible reason, as evidenced by a previous observation that additives that are believed to reduce cellulase adsorption on lignin had a major impact on hydrolysis of ARP pretreated solids (Kumar and Wyman, 2009d,f).

Xylanase Adsorption

Xylanase adsorption at 4°C followed the estimates obtained using the Langmuir equation well, as evident by statistical correlation coefficients $R^2 > 0.95$. Representative predicted and experimental adsorption data are shown in Figure 4a and b for ARP and SO_2 pretreated solids, respectively, and adsorption parameters resulting from fitting the data to the Langmuir equation, are shown in Table V. Lime pretreated solids had the highest xylanase adsorption capacity (148.6 mg/g solids), and AFEX pretreated solids the lowest (60.8 mg/g solids), even though solids from both pretreatments had roughly equal xylan contents. Furthermore, adsorption capacity increased with residual xylan content in the solids, as shown in Figure 5, for all pretreatments except AFEX. However, xylanase adsorption not only takes place on xylan but on glucan and lignin in biomass as well (Boussaid and Saddler, 1999; Gerber et al., 1999; Ryu and Kim, 1998; Tenkanen et al., 1995), suggesting that either AFEX pretreated lignin had a low xylanase capacity or that xylan in AFEX solids was not as accessible as for other pretreatments. By comparing Tables IV and V, we can see that xylanase had a lower affinity for pretreated solids than cellulase, and among the leading pretreatments, AFEX solids had the greatest xylanase affinity (0.96 g/L) followed by dilute acid (0.43 g/L), ARP (0.21 g/L), and SO_2 (0.21 g/L), while lime solids followed by controlled pH had the least.

Cellulase Adsorption on Lignin

Cellulase adsorption was determined for enzymatically extracted lignin solids at 4°C to be consistent with conditions employed for the pretreated solids. Treatment of the lignin solids with a high amount of protease appeared to remove almost all of the residual protein from enzymatic hydrolysis, as shown by the similarities in nitrogen content for enzyme lignin and acid lignin solids in Table III even though acid treatment is expected to remove more biomass protein than enzyme treatment. The high amount of

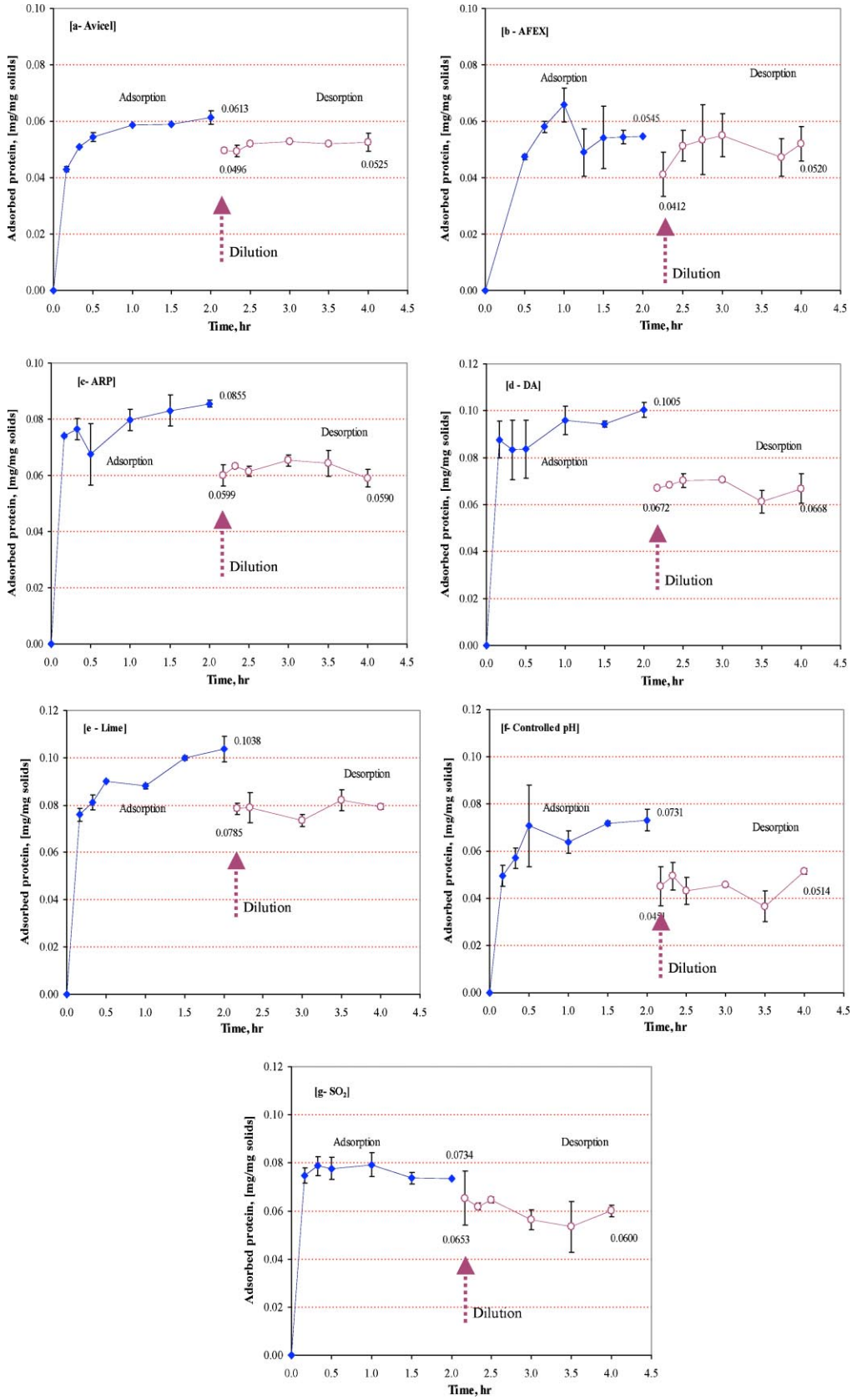

Figure 1. Cellulase adsorption and desorption kinetics upon dilution with an equal amount of fresh buffer at 4°C and at a loading of 400 mg cellulase/g solids for (**a**) Avicel glucan and for corn stover solids pretreated by (**b**) AFEX, (**c**) ARP, (**d**) DA, (**e**) lime, (**f**) controlled pH, and (**g**) SO_2. [Color figure can be seen in the online version of this article, available at www.interscience.wiley.com.]

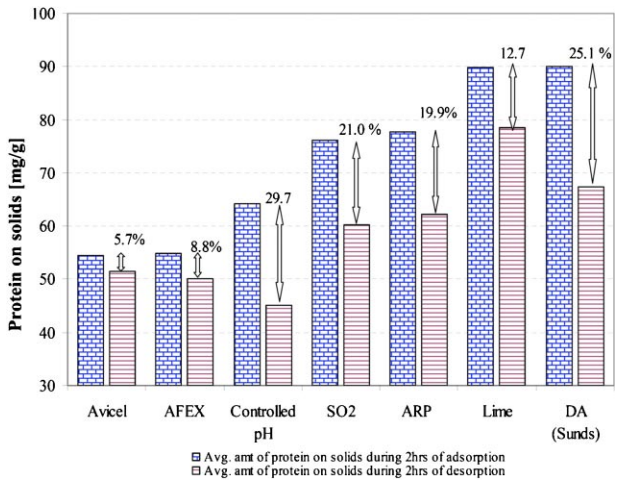

Figure 2. Average amount of cellulase adsorption and desorption after 2 h of equilibration at 4°C for Avicel glucan and pretreated corn stover solids. The numbers on the graph represent the average percentage amount of desorption for each pretreatment. [Color figure can be seen in the online version of this article, available at www.interscience.wiley.com.]

nitrogen in the solids from AFEX and ARP pretreatment probably came from reaction of ammonia to ammoniated lignin derivatives (Chundawat et al., 2007; Weimer et al., 1986). Cellulase adsorption on EnzL followed the Langmuir isotherm estimations reasonably well, as illustrated by results for lignin from ARP and lime pretreatments in Figure 6, but the statistical coefficients R^2 were <0.90. Adsorption parameters fit to the Langmuir equation are shown in Table VI. These results show that SO_2 lignin had the highest adsorption capacity (67 mg/g lignin) followed by lime (65 mg/g lignin) and controlled pH (64 mg/g lignin), while AFEX lignin adsorbed about 45% less protein (38 mg/g lignin) than SO_2 lignin. Although ARP lignin had the second lowest adsorption capacity (42 mg/g lignin) after AFEX, it had the highest affinity for cellulase (11 L/g protein), whereas controlled pH lignin had the lowest affinity (0.60 L/g protein) for cellulase followed by dilute acid lignin (0.68 L/g protein).

Calculation of Cellulose Accessibility

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

$$\sigma_{[\text{cellulose}]} = \frac{\sigma_{[\text{solids}]} - \sigma_{[\text{lignin}]} Lw}{Cw}$$

in which $\sigma_{[X]}$ is the maximum adsorption capacity of solids, cellulose, or lignin, respectively, in the pretreated solids; *Lw* is the fraction of lignin in the pretreated solids, and *Cw* the fraction of cellulose in the pretreated solids. Cellulase adsorption capacities calculated in this way for cellulose in solids prepared by the range of pretreatments are shown in Table VI. Cellulose in AFEX pretreated solids had the highest adsorption capacity (270 mg/g cellulose) followed by lime (220 mg/g cellulose) and SO_2 (191 mg/g cellulose). Cellulose in dilute acid pretreated solids prepared with the Sunds system had the lowest capacity for cellulase (131 mg/g cellulose).

Impact of Lignin on Cellulose Accessibility

Several mechanisms have been hypothesized to account for the effect of lignin on enzymatic saccharification. According to one, lignin slows hydrolysis by impeding cellulase accessibility to cellulose, but it has never been shown whether lignin removal improves cellulose accessibility or enzyme effectiveness or both. Thus, we sought to determine the effect of selective delignification by peracetic acid (Chang and Holtzapple, 2000) on adsorption of purified CBHI at 4°C and on enzymatic digestion at a cellulase plus β-glucosidase mass loading of 32.2 mg/g glucan (~15 FPU).

As shown in Figure 7, delignification increased glucose and xylose release, and at 72 h glucan and xylan hydrolysis was >85% and >55%, respectively, for solids containing 20% of the original lignin content. Similar results were found for ½% lignin solids (data not shown). However, delignification increased initial glucose release (198% in 4 h) by a lower percentage than initial xylose

Table IV. Maximum cellulase adsorption capacities, affinity constants, and strengths of adsorption for Avicel glucan and corn stover solids produced by leading pretreatments.

Pretreatment	Max. cellulase adsorption capacity, σ (mg/g substrate)	Affinity constants, A (L/g)	Strength of binding, $R = \sigma \times A$ (mL/g substrate)	R^2
Avicel	84.0	1.84	154.6	0.98
AFEX	99.7	1.86	185.4	0.75
ARP	113.8	46.20	5257.6	0.75
Controlled pH	101.7	0.77	78.3	0.98
DA	90.7	2.49	225.8	0.90
Lime	133.6	0.88	100.0	0.91
SO_2	124.8	0.90	112.3	0.96

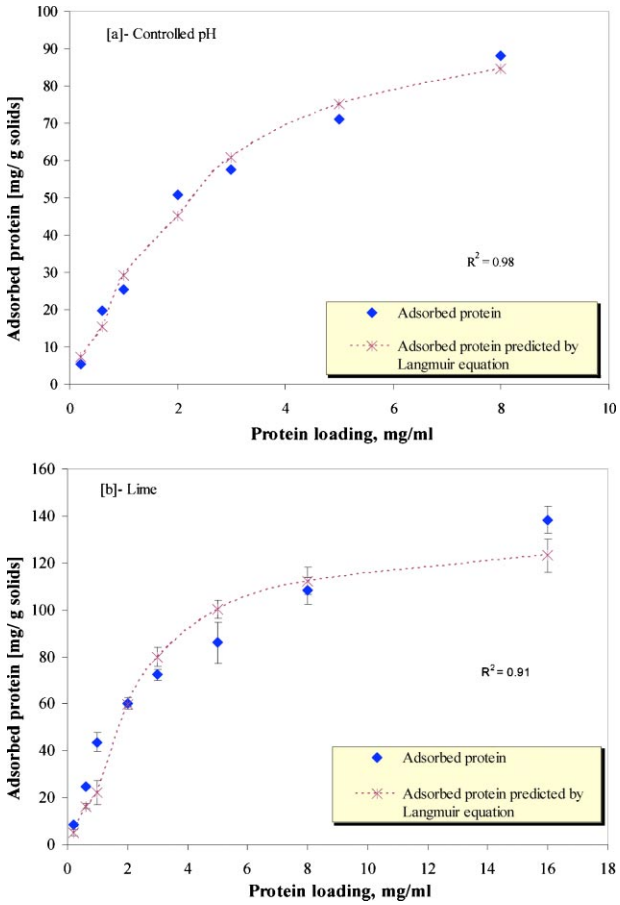

Figure 3. Adsorption of Spezyme CP (mg/g substrate) versus loading (mg/mL) and Langmuir predictions of adsorbed cellulase for (**a**) Controlled pH and (**b**) Lime pretreated solids. [Color figure can be seen in the online version of this article, available at www.interscience.wiley.com.]

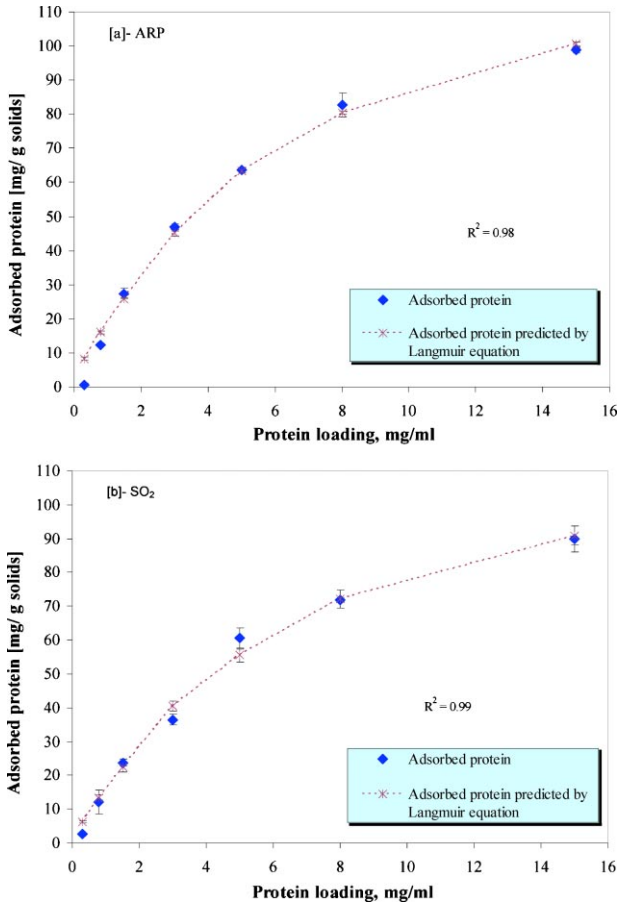

Figure 4. Adsorption of xylanase (mg/g substrate) versus xylanase loading (mg/mL) and Langmuir predictions of adsorbed protein for (**a**) ARP and (**b**) SO_2 pretreated solids. [Color figure can be seen in the online version of this article, available at www.interscience.wiley.com.]

release (780%). Furthermore, a nearly linear relationship was evident between glucan and xylan removal for untreated corn stover solids and solids delignified to various degrees, as shown in Figure 8. As shown in Figure 9, delignification had a negligible effect on adsorption of purified CBHI on untreated and delignified solids, inferring little change in cellulose accessibility. Yet, hydrolysis data shows that delignification enhanced cellulase and probably xylanase (present in Spezyme CP) effectiveness significantly.

Consistent with the findings here, another study (Kumar and Wyman, 2009b) showed that delignification of solids with a xylan content of <3% resulting from pretreatments at lower pH with dilute acid and SO_2 had a negligible impact on glucose release. On the other hand, delignification of solids produced by high pH pretreatments, such as AFEX and ARP, that contained a significant amount of xylan enhanced glucose release. However, in the latter case, delignification increased xylose release much more than glucose release.

Table V. Maximum xylanase adsorption capacity, affinity constants, and strength of adsorption for corn stover solids produced by leading pretreatments.

Pretreatment	%Xylan	Maximum adsorption capacity, σ(mg/g solids)	Affinity, A (L/g)	Binding strength, $R = A \times \sigma$ (mL/g solids)	R^2
AFEX	22.8	60.8	0.96	58.4	0.80
ARP	17.9	133.6	0.23	30.7	0.98
Controlled pH	16.2	122.9	0.21	25.8	0.98
Dilute acid	9.3	95.50	0.43	41.1	0.97
Lime	26.4	148.6	0.17	25.3	0.99
SO_2	11.6	117.8	0.23	26.6	0.99

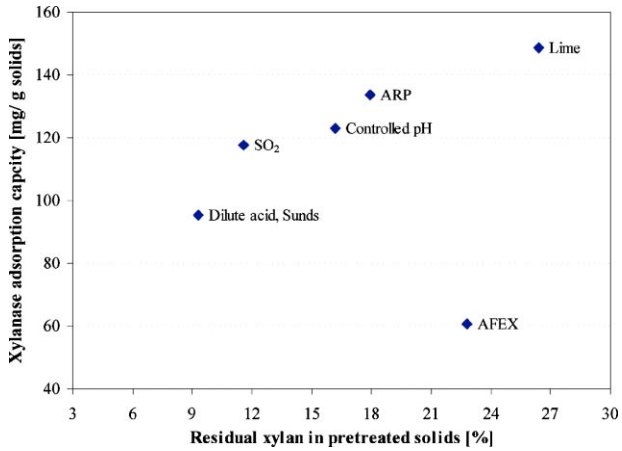

Figure 5. Relationship between residual xylan in pretreated solids and xylanase adsorption capacity for pretreated corn stover solids produced by leading pretreatments. [Color figure can be seen in the online version of this article, available at www.interscience.wiley.com.]

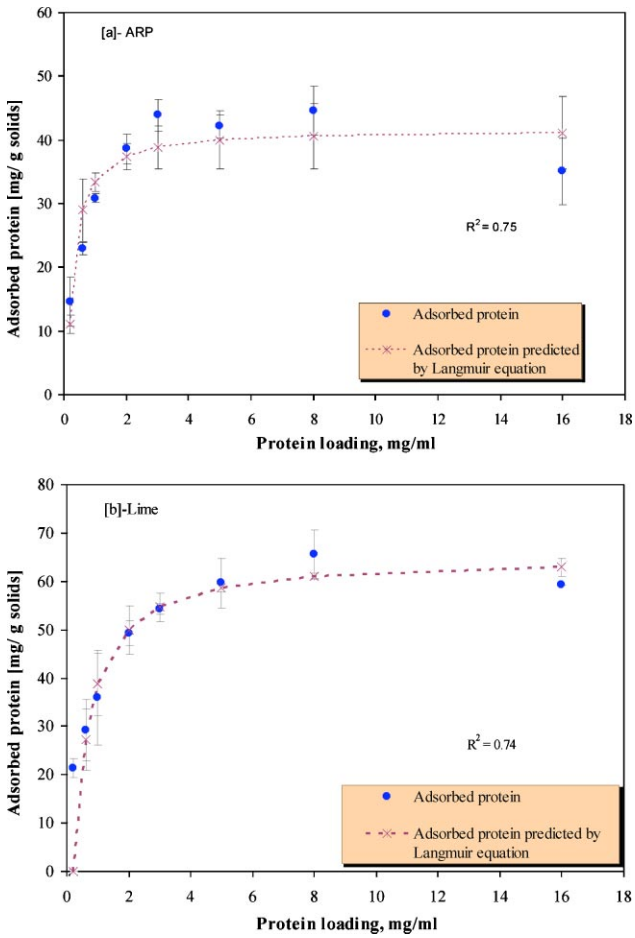

Figure 6. Amount of cellulase adsorbed (mg/g lignin) versus cellulase loading (mg/mL) and predicted amounts of adsorbed protein by Langmuir equation for (**a**) ARP and (**b**) lime pretreated lignin. [Color figure can be seen in the online version of this article, available at www.interscience.wiley.com.]

Effect of Acetyl Content on Cellulose Accessibility

To determine the impact of deacetylation on cellulose accessibility, CBHI adsorption was performed on untreated corn stover solids and untreated corn stover solids deacetylated to various degrees. It appears that deacetylation had a higher impact on cellulose accessibility than delignification, as shown in Figure 10, but had a smaller impact on cellulase effectiveness probably due to lignin networking with carbohydrates. Interestingly, complete deacetylation increased initial glucose and xylose release by 65% and 74%, as shown elsewhere (Kumar and Wyman, 2009a), compared to an initial increase of 198% and 780% in glucose and xylose release observed for delignification at a similar cellulase plus β-glucosidase mass loading of 32.2 mg/g glucan, respectively.

Comparison of Effectiveness

To compare the effectiveness of adsorbed cellulase for delignified versus deacetylated corn stover solids, cellulase effectiveness was defined as:

$$\text{Effectiveness} = \frac{\%\text{increase in initial glucose release with complete cellulase}}{\text{increase in CBHI adsorption(mg)}}$$

Based on this definition, delignification enhanced cellulase effectiveness by about five times more than deacetylation (78.5%/mg of CBHI for delignification vs. 14.2%/mg of CBHI for deacetylation), even though accessibility was nearly unchanged for delignification and increased by 70% for deacetylation. Furthermore, the ratio of the increase in glucose release to the amount of xylose release was higher for deacetylation (0.88) compared to delignification (0.3). These results suggest that lignin removal makes xylan more accessible to xylanase and that in turn makes cellulose more accessible to cellulase, consistent with the finding by Jeoh et al. (2007) that xylan removal enhances cellulose accessibility. Thus, data reported here and in the literature support the hypothesis that lignin removal has a greater impact on xylan than glucan removal (Ford, 1983; Morrison, 1983), but further experimental validation is needed.

Adsorption Capacity Versus Hydrolysis

As discussed earlier and suggested in the literature, enzyme adsorption on solids and their effectiveness are the two factors hypothesized to have a major impact on enzymatic hydrolysis (Chang and Holtzapple, 2000; Kumar, 2008; Zhang and Lynd, 2004, 2006). However, these factors are affected by substrate and enzyme features and physical parameters such as the presence of inhibitors, temperature, pH, ionic strength, agitation, and substrate and enzymes loadings (Kim and Hong, 2000; Kumar, 2008; Kumar and Wyman, 2008, 2009a,f; Kyriacou et al., 1988; Ooshima et al., 1983; Peitersen et al., 1977; Reinikainen et al., 1995). Several

Table VI. Cellulase adsorption parameters determined for enzyme lignin (EnzL) and calculated maximum adsorption capacities of cellulose in solids from leading pretreatments.

Pretreatment	Maximum adsorption capacity, σ_{Lignin} (mg/g lignin)	Affinity, A_{Lignin} (L/g protein)	Binding strength, $S_{Lignin} = A \times \sigma$ (mL/g lignin)	Cellulose adsorption capacity, $\sigma_{cellulose}$ (mg/g cellulose)
AFEX	38.7	2.99	116.0	270.55
ARP	41.6	10.70	445.0	178.22
Controlled pH	63.6	0.60	36.2	173.36
DA	53.0	0.68	174.5	131.15
Lime	64.9	2.69	37.8	219.72
SO_2	67.5	6.39	431.5	191.11

studies reported a linear relationship between adsorption capacity and hydrolysis rate/yield (Kotiranta et al., 1999; Lee and Fan, 1981; Ryu and Lee, 1982), while others reported the opposite to be true (Beldman et al., 1987; Mooney et al., 1997). Thus, 24 h glucan hydrolysis rate data[1] collected at various combined cellulase and β-glucosidase mass loadings, as reported elsewhere (Kumar and Wyman, 2009a) and summarized in Table VII, were correlated with cellulase adsorption capacities estimated here, and the relationships between 24 h glucan release for various enzyme mass loadings and cellulase adsorption capacities for Avicel glucan and pretreated corn stover solids are shown in Figure 11. Overall, 24 h glucan release correlated well ($R^2 > 0.75$) with adsorption capacities, consistent with the hypothesis, but 72 h yields did not relate well to capacities (data not shown). This result suggests that enzyme effectiveness dropped over time, possibly due to a combination of substrate (Mansfield et al., 1999; Yang et al., 2006; Zhang et al., 1999) and enzyme features (Desai and Converse, 1997; Mansfield et al., 1999) and physical parameters such as enzymes inhibition by sugars and their oligomers (Holtzapple et al., 1990; Kumar and Wyman, 2009a; Mandels and Reese, 1965).

Xylan released in 24 h from pretreated solids by cellulase combined with β-glucosidase did not correlate well with xylanase adsorption capacity (data not shown). Although xylan hydrolysis is much more complex than glucan hydrolysis, the weak relationship could result from incomplete xylan hydrolysis due to low activity of xylanase, β-xylosidase, and/or debranching enzymes availalable in Spezyme CP (Dien et al., 2008). Findings by Zilliox and Debeire (1998) for the hydrolysis of wheat straw by a thermostable endoxylanase are consistent with these results, in that they found no correlation between adsorbed xylanase and xylan degradation.

Discussion

The focus of this study was to better understand how enzyme–substrate interactions differed for corn stover solids

[1]Hydrolysis was performed in a 0.05 M citrate buffer at 50°C with solids containing 1% (w/v) glucan at a cellulase together with β-glucosidase mass loadings of 6.0, 16.1, 32.2, and 118.0 mg (corresponding to about 3.0, 7.5, 15, and 60.0 FPU respectively)/g glucan in unpretreated corn stover.

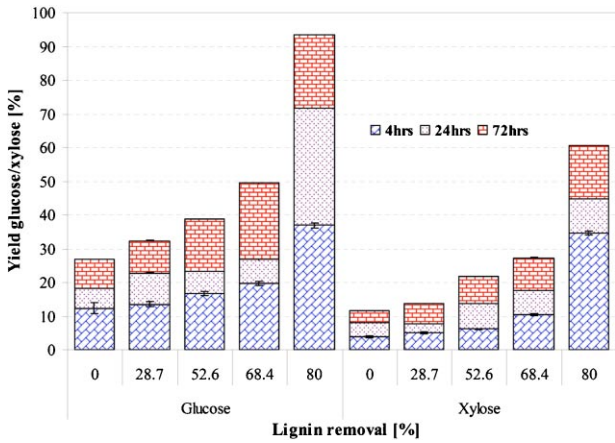

Figure 7. Effect of lignin removal on 72 h sugar release at a cellulase plus β-glucosidase mass loading of 32.2 mg/glucan for solids containing 1% (w/v) glucan. [Color figure can be seen in the online version of this article, available at www.interscience.wiley.com.]

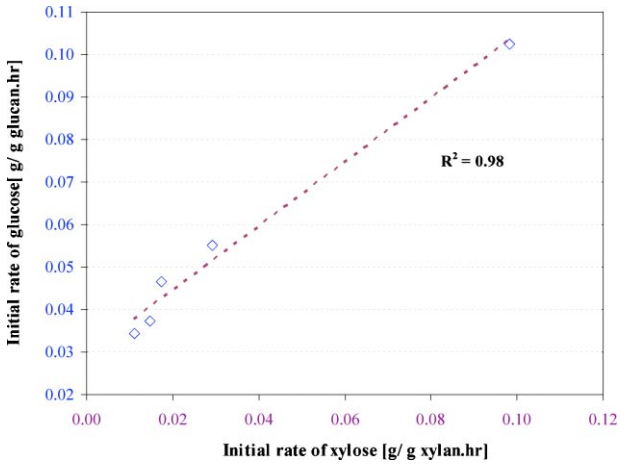

Figure 8. Relationship between initial rate of glucose and xylose release for untreated corn stover solids and solids delignified to various degrees. [Color figure can be seen in the online version of this article, available at www.interscience.wiley.com.]

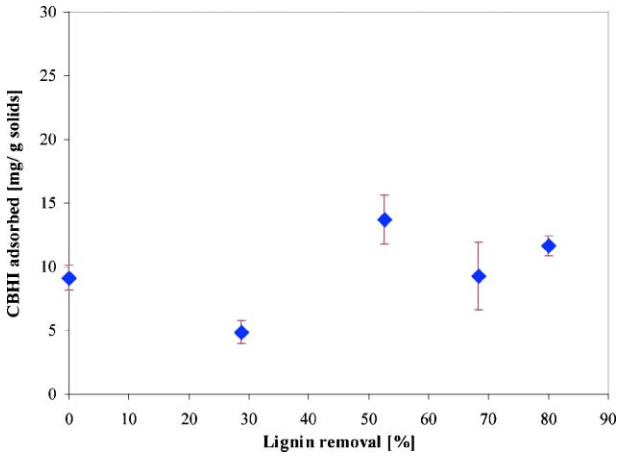

Figure 9. Effect of lignin removal on CBHI adsorption performed at 4°C and an enzyme loading of 75 mg/g glucan. [Color figure can be seen in the online version of this article, available at www.interscience.wiley.com.]

produced by AFEX, ARP, controlled pH, dilute acid, lime, and SO_2 pretreatments that span a range of pretreatment conditions. In line with this goal, cellulase adsorption and desorption kinetics were measured for solids pretreated by these methods as well as for Avicel glucan, and cellulase adsorption parameters were estimated via fitting the adsorption data to the Langmuir relationship. Consistent with the literature, cellulase adsorption on the solids was virtually at equilibrium within one and half hours of incubation, but the amount of enzyme adsorbed varied by a factor of about 2 from a low with AFEX and Avicel to a high with lime and dilute acid pretreated solids. Furthermore, little relationship was found of enzyme adsorption to pretreatment pH or hemicellulose or lignin content.

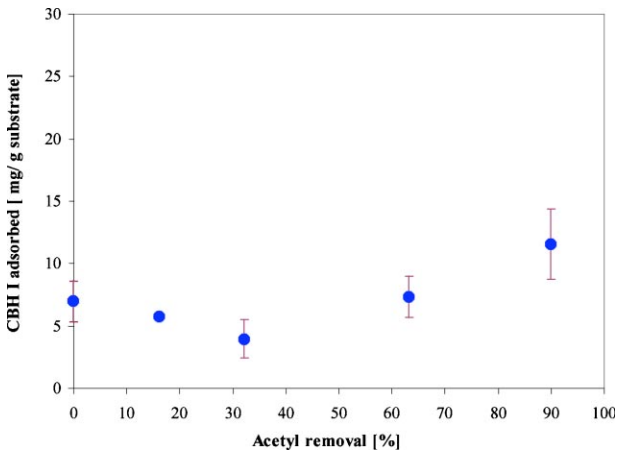

Figure 10. Effect of corn stover deacetylation on CBHI adsorption at 4°C for an enzyme loading of 75 mg/g glucan. [Color figure can be seen in the online version of this article, available at www.interscience.wiley.com.]

Table VII. The 24 h glucan hydrolysis rate (g/g glucan-h) at various cellulase together with β-glucosidase loadings (mg/g glucan) for Avicel glucan and corn stover solids prepared by leading pretreatments.

	Total protein loading, mg/g original glucan			
Pretreatment	6.0	16.1	32.2	118
Avicel	0.0124	0.0182	0.0226	0.0296
AFEX	0.0130	0.0205	0.0244	0.0337
ARP	0.0180	0.0306	0.0413	0.0502
Controlled pH	0.0130	0.0172	0.0229	0.0257
DA	0.0130	0.0188	0.0249	0.0254
Lime	0.0230	0.0386	0.0517	0.0642
SO_2	0.0160	0.0285	0.0333	0.0377

Upon dilution of these solids containing adsorbed cellulase, a new equilibrium was established within 20 min (Lynd et al., 2002; Palonen et al., 1999) based upon the average amount of protein desorbed after equilibration for 2 h. The average amount of protein desorption increased in the following order with the amount of residual lignin and cellulase affinity for solids noted in parentheses: Avicel (0%; 1.84 L/g) < AFEX (18%; 1.86 L/g) < lime (14.6%; 0.88 L/g) < ARP (8.8%; 46.2 L/g) < SO_2 (23.8%, 0.9 L/g) < dilute acid (22.5%; 2.49 L/g) < controlled pH (25.2%; 0.77 L/g). Thus, the amount of cellulase desorbed did not correlate with residual lignin; however, we did find a linear relationship with affinity for all pretreatments except for ARP and dilute acid pretreatment with the Sunds reactor.

Although there is not much information on cellulase desorption from pretreated solids, studies on cellulase desorption from pure cellulose are numerous and inconclusive. For example, some studies reported that cellulase or its components bind irreversibly and do not return to solution upon dilution (Beltrame et al., 1982; Carrard and Linder, 1999; Kyriacou et al., 1989), while others reported the opposite (Linder and Teeri, 1996; Palonen et al., 1999). Our observations with pure Avicel glucan and pretreated solids showed that desorption is a function of substrate and pretreatment type. The low protein desorption from Avicel could be attributed at least partially to its high crystallinity. Consistent with this observation, Rad and Yazdanparast (1998) reported a 30% desorption of cellulase from Avicel when liquid containing buffer and unadsorbed protein was replaced by fresh citrate buffer. These differences in protein adsorption and desorption among pretreatments could have important implications for performance in that enzymes bound irreversibly to substrate may lose activity (Palonen, 2004; Park et al., 2002), but some cellulase components must adsorb strongly on the cellulose surface for effective saccharification (Klyosov, 1990; Klyosov et al., 1986). Thus, although literature data is limited, substrate features are expected to have a significant impact on performance.

Cellulase adsorption parameters for pretreated solids revealed that solids prepared by dilute acid with the Sunds reactor had the lowest adsorption capacity followed by AFEX, an alkaline pretreatment, showing similarities for two

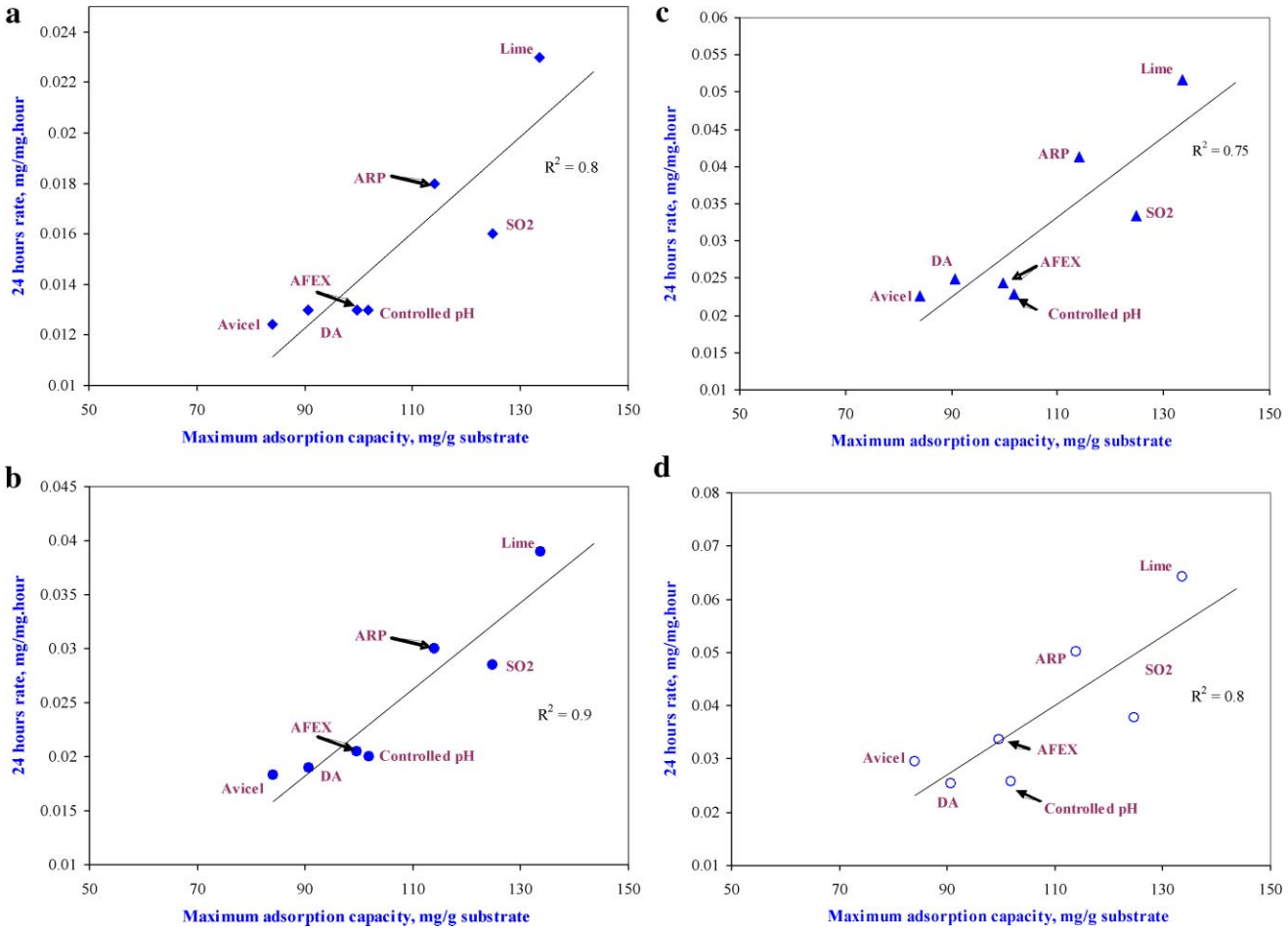

Figure 11. Relationship between maximum cellulase adsorption capacity (mg/g substrate) and the 24 h glucan hydrolysis rate (g/g-h) at cellulase together with β-glucosidase mass loadings of (**a**) 6.0 mg/g original glucan, (**b**) 16.1 mg/g original glucan, (**c**) 32.2 mg/g original glucan, and (**d**) 120 mg/g original glucan for Avicel and pretreated corn stover solids. [Color figure can be seen in the online version of this article, available at www.interscience.wiley.com.]

extremes in pretreatment pH. Although the adsorption parameters estimated in this study for solids from dilute acid pretreatment in the Sunds reactor (σ, 90 mg/g; affinity, 2.8 L/g) were much lower than reported by others for dilute acid pretreated solids (σ, 210 mg/g; affinity, 6.3 L/g) (Willies, 2007), the latter solids were produced in a Parr reactor at 140°C with 1.0% (w/w) acid in 40 min. Another study reported an affinity of 2.1 L/g for steam exploded Birchwood (Lee et al., 1994), consistent with the value estimated here for dilute acid pretreated solids, but the adsorption capacity was much higher (214 mg/g solids). An adsorption capacity of 60 mg/g solids was reported by Kadam et al. (2004) for dilute acid pretreated corn stover solids produced at a similar conditions as used in this study. Unfortunately, comparative data is not available for the other pretreatments.

Lime pretreated solids had the highest adsorption capacity for both cellulase and xylanase, but their affinity for xylanase was about 1/5th of that for cellulase. ARP solids had the highest affinity constant for cellulase while AFEX solids had the highest affinity but the least capacity for xylanase. If the data point for AFEX solids is excluded, an almost linear relationship was found between xylanase adsorption capacity and xylan content of the solids, implying something different about the effect of ammonia on adsorption.

Although lignin left from enzymatic digestion of ARP pretreated solids had the second lowest adsorption capacity after AFEX, its affinity was much higher than for AFEX, almost by a factor of 3, as shown in Table VI. The high affinity constant estimated for ARP lignin is consistent with the large affinity constant for ARP pretreated solids shown in Table IV. Consistent with the adsorption parameters determined here, additives thought to preferentially adsorb on lignin had a huge impact on sugar release from ARP pretreated solids, as reported elsewhere (Kumar and Wyman, 2009d,f). SO_2 lignin had the highest capacity for cellulase, but its affinity was a lower than for ARP.

The low cellulase adsorption capacity observed for AFEX lignin and xyalanse capacity for AFEX solids compared to

other pretreatments indicated that ammonia probably reduced lignin's hydrophobicity which resulted in reduced unproductive binding (Golovchenko et al., 1992; Kumar et al., 2008; Tilton et al., 1991; van Oss, 1995). Another study reported a much higher cellulase adsorption capacity of 590 mg/g lignin for corn stover lignin resulting from dilute acid pretreatment in a Parr reactor instead of the Sunds unit (Willies, 2007). However, consistent with the values reported here, enzymatic lignin extracted from dilute acid pretreated Creeping Wild Ryegrass had an adsorption capacity of 86 mg/g lignin and cellulase affinity of 0.51 L/g protein (Zheng et al., 2007). Furthermore, Ooshima et al. (1990) reported an adsorption capacity of 66 mg/g lignin and affinity of 0.66 L/g protein for lignin residue extracted from pretreated hardwood, although cellulase adsorption was performed at 40°C instead of 4°C and pretreatment conditions were somewhat different than for this study.

Cellulose accessibility, as estimated based on adsorption data for solids and lignin, was the highest for solids prepared by AFEX (270.5 mg/g cellulose) followed closely by lime pretreatment (219.7 mg/g cellulose). This very high cellulose accessibility for AFEX pretreated corn stover solids could explain to some extent, at least, the somewhat higher digestibility measured for AFEX solids compared to other pretreatments reported elsewhere (Wyman et al., 2005a). Although the cellulose in dilute acid pretreated solids prepared by the Sunds system had the lowest capacity for cellulase, and that probably was the main cause for a very low digestion reported in this study, Figure 11, and shown elsewhere as well (Kumar and Wyman, 2009a), Ooshima et al. estimated a cellulose capacity of 81 mg/g cellulose for dilute acid pretreated mixed hardwood prepared at somewhat different conditions (continuous plug flow reactor operated at 220°C for a residence time of 8.7 s giving a severity parameter[2] $\log R_o = 2.70$) compared to this study (Sunds reactor, 180°C, 1.5 min, $\log R_o = 2.5$). Willies (2007) applied the approach by Ooshima et al. to estimate a capacity of 89 mg/g cellulose for dilute acid pretreated corn stover solids (Parr reactor, 140°C, 40 min, $\log R_o = 2.78$). Furthermore, using SO_2 catalyzed and steam exploded Douglas fir, Lu et al. (2002) reported a much higher cellulose capacity (342 mg/g cellulose) for Douglas fir than Willies reported for dilute acid pretreated corn stover and Osshima et al. for dilute acid pretreated mixed hardwood. Unfortunately, no data is available in the literature for comparison to the other pretreatments employed in this study.

For the first time and contrary to many beliefs, CBHI adsorption and hydrolysis data showed that delignification plays a greater role in enhancing cellulase effectiveness than in improving cellulose accessibility. This result suggests that lignin does not directly limit glucan accessibility but greatly restricts xylan accessibility which in turn limits glucan accessibility, consistent with thoughts by others (Beveridge and Richards, 1975; Dehority et al., 1962; Teixeira et al., 1999). Although several studies indicated negligible impacts of delignification on cellulose accessibility, none have considered its significance (Eriksson et al., 2002; Mes-Hartree et al., 1987; Mooney et al., 1997, 1998). For example, Lu et al. (2002) reported that steam-exploded Douglas-fir (46.1% lignin; capacity, 171.3 mg/g solids) had the same adsorption capacity as steam-exploded-hot-alkali-extracted Douglas fir (8.1% lignin; capacity—162.4 mg/g solids). However, the initial hydrolysis rate for the latter was almost three times that of the former. In another study, Mooney et al. (1998) showed that delignified refiner mechanical pulp (RMP) had the same adsorption capacity as refiner mechanical pulp that was not delignified.

Deacetylation improved cellulose accessibility more than delignification but had a limited impact on effectiveness, as measured by the percentage increase in glucose release per mg of CBHI adsorbed. This result could be explained in terms of the extensive networking of lignin with carbohydrates reducing effectiveness, just as branching and substitution of glucan and xylan chains reduce enzyme effectiveness (Anand and Vithayathil, 1996; Glasser et al., 1995; Kormelink and Voragen, 1992; Samios et al., 1997; Silveira et al., 1999; Suh and Choi, 1996). The 24 h glucan hydrolysis rate data collected at four protein mass loadings of cellulase in combination with a fixed ratio of β-glucosidase related well to cellulase adsorption capacities, but 72 h glucan hydrolysis yields did not. This observation strongly supports the hypothesis (Kumar, 2008) that hydrolysis is mainly controlled by cellulase adsorption and its effectiveness, once adsorbed, which in turn are affected by substrate and enzymes features and physical parameters. Furthermore, the 24 h xylan hydrolysis rate data was not related to xylanase adsorption capacities suggesting that xylan hydrolysis is more complicated than glucan hydrolysis.

Conclusions

Enzyme-substrate interactions showed that enzyme adsorption is much faster than saccharification, and enzyme desorption, carbohydrate accessibilities, and lignin affinity towards enzymes are impacted by the choice of substrate and pretreatment. Cellulase adsorption onto pretreated solids/cellulose, designated as cellulose accessibility, had no clear correlation with xylan/lignin removal, probably because xylan removal is not selective for the leading pretreatment options. The greater cellulose accessibility for AFEX pretreated solids suggests that disruption of lignin-carbohydrates linkages plays a greater role in enhancing accessibility than xylan/lignin removal. However, consistent with another finding (Kumar and Wyman, 2009b), the data here for selective lignin removal supports the hypothesis that lignin controls xylan accessibility, which consequently does not have a major impact on cellulase adsorption. The high cellulose accessibility for AFEX and lime pretreated solids could account for their good glucose release reported in the

[2]Severity factor, defined as $\log R_o = \log(t \cdot \exp[(T_H - T_R)/14.75])$. Includes only time and temperature.

literature at cellulase loadings of 15 and 60 FPU (Wyman et al., 2005a). However, the presence of inhibitors such as xyloligomers also affects enzyme effectiveness significantly, as reported elsewhere (Kumar and Wyman, 2009a,d,f), and the low digestion rate data for AFEX shown in Figure 11 despite its highest cellulose accessibility also supports this idea. Furthermore, the strong relationship of 24 h glucan hydrolysis rate data to cellulase adsorption capacities supports the hypothesis that carbohydrate saccharification is primarily controlled by enzyme adsorption onto carbohydrates and effectiveness, as discussed in more detail elsewhere (Kumar, 2008).

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