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Does change in accessibility with conversion depend on both the substrate and pretreatment technology?

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ABSTRACT

The accessibility of cellulase and xylanase enzymes to glucan and xylan, respectively, and its change with conversion were measured for pure Avicel glucan and poplar solids that had been pretreated by ammonia fiber expansion (AFEX), ammonia recycled percolation (ARP), dilute acid, and lime. Avicel and pretreated solids were digested to various degrees by cellulase together with β-glucosidase enzymes and then cleaned of residual protein via a biological method using Protease. Glucan accessibility was determined by purified CBHI (Cel7A) adsorption at 4 °C, and 4 and 24 h hydrolysis yields were determined for solids loading containing equal amounts of glucan (1.0% w/v) and lignin (1.0% w/v), in two separate sets of experiments. Consistent with our previous study and in contrast to some in the literature, little change in glucan accessibility was observed with conversion for Avicel, but glucan and xylan accessibility for real biomass varied with the type of pretreatment. For example, AFEX pretreated solids showed a negligible change in glucan accessibility for conversion up to 90%, although xylan accessibility seemed to decline first and then remained constant. On the other hand, a substantial decline in glucan and xylan accessibility with conversion was observed for lime pretreated poplar solids, as shown by initial hydrolysis rates. Yet, an increase in CBHI adsorption with conversion for lime pretreated poplar solids suggested the opposite trend, possibly due to increased lignin exposure and/or reduced effectiveness of adsorbed enzyme.

1. Introduction

The decline in hydrolysis rates with conversion is a well known phenomenon and a major impediment to biological production of sugars from cellulosic biomass (Eriksson et al., 2002b; Lynd et al., 2002; Yang et al., 2006). Although enzyme inactivation (Gunjikar et al., 2001; Kumar and Wyman, 2009a), end-product inhibition (Kumar and Wyman, 2008), and unproductive binding to substrate and/or lignin (Kumar and Wyman, in press-b) are some of causes attributed to hydrolysis rates slowing down with conversion, the heterogeneous nature of biomass, which affects glucan accessibility to cellulase, is believed to be more important (Desai and Converse, 1997; Ma et al., 2008; Wang et al., 2006; Zhang et al., 1999). However, the role of glucan accessibility and its change with conversion has been debatable, with a few studies showing that glucan accessibility becomes limiting with conversion (Ghose and Bisaria, 1979; Hong et al., 2007; Kadam et al., 2004; Wang et al., 2006), and a few others showing little change (Desai and Converse, 1997; Eremeeva et al., 2001; Vlasenko et al., 1997; Yang et al., 2006), as summarized in Table 1. In the majority of these studies, protein removal from spent substrate is either not confirmed/reported or results from using harsh chemicals/reagents, which in turn may change the substrate structure and, if not removed carefully, can adversely affect hydrolysis. For example, Hong et al. (2007) in their study used 10 M sodium hydroxide solution to stop the enzymatic action and then incubated the spent substrate with 1.1%(w/w) sodium dodecyl sulfate (SDS) at 80 °C for 10 min followed by washing three times with 75% ethanol to desorb protein. However, ethanol is reported to be inhibitory to enzymes (Chen and Jin, 2006; Holtzapple et al., 1990), and SDS is known to significantly denature and deactivate enzymes (Eriksson et al., 2002a; Stoner et al., 2006; Xiang et al., 2006). In a similar fashion, Wang et al. (2006) incubated spent cotton fibers with Triton X-100 (0.01%) and glycerol (10%) in NaAc buffer pH 4.8 at 40 °C for 10 min to dislodge adsorbed proteins, again raising questions about the effect on the substrate. In addition, almost no information is available in the literature on the change in glucan and especially xylan accessibility for real biomass, and whether accessibility is a function of pretreatment and substrate type has never been reported.

In this study, we sought to investigate the change in glucan and xylan accessibility with conversion for pure Avicel glucan and

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Table 1
Literature summary on cellulose accessibility/reactivity for pure cellulose and cellulose in real biomass.

Substrate	Pretreatment	Method of enzyme removal from solids	Extent of conversion	Changes with conversion		Ref.
				Rate/reactivity	Cellulase adsorption	
Mixed hardwood	Dilute acid	Water washing	Up to 80%	Not significant	Almost constant	Desai and Converse (1997)
Corn stover	Dilute acid	Water washing	Up to 80%	A linear decline	N.D.	Kadam et al. (2004)
Avicel cellulose	–	Protease treatment	Up to 82%	No change	Almost constant	Yang et al. (2006)
Avicel PH105	–	Chemical method[a]	>80%	A huge decline	Decreased with conversion	Hong et al. (2007)
Rice straw	Dilute acid, steam explosion, AFEX	Washing with buffer[b]	33–38%	No change	N.D.	Vlasenko et al. (1997)
Ball milled cellulose	–	0.2 N NaOH at 80 °C for 10 min	50%	No change	No change in Congo red binding	Fields et al. (2000)
Kraft hardwood pulp	–	NA	NA	The ratio of two fractions does not change.		Eremeeva et al. (2001)
Cotton fibers	–	Chemical method[c]	Up to 80%	A substantial decline	Decrease in CBH I ads.	Wang et al. (2006)

[a] Hydrolysis was stopped by 10 M NaOH then solids were incubated in 1.1% SDS solution at 80 °C for 15 min and then washed by 75% (v/v) ethanol three times and distilled water twice.
[b] Solids were washed with 0.1 M sodium phosphate buffer (pH 7.0), distilled water, and 0.1 M sodium acetate buffer (pH 5.0) to remove enzyme.
[c] Washing with Triton X-100 (0.01%) and glycerol (10%) in sodium acetate buffer pH 4.8 at 40 °C for 10 min.

poplar solids resulting from pretreatment by AFEX (ammonia fiber expansion), ARP (ammonia recycled percolation), dilute acid, and lime. Avicel PH101 glucan and pretreated poplar solids were hydrolyzed to various degrees using cellulase together with β-glucosidase, and the spent solids were cleaned of residual protein via a biological method with measurement of the nitrogen content of fresh and spent solids employed to confirm protein removal. The change in accessibility with conversion was estimated based on 4 and 24 h hydrolysis yields for solids hydrolyzed to various degrees and by measurement of adsorption of purified CBHI at 4 °C.

2. Methods

2.1. Substrates

Avicel PH-101 cellulose was purchased from FMC Corporation, Philadelphia, PA (Cat 11365, Lot 1094627). Solids resulting from the following pretreatments 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 with a pilot scale steam explosion by NREL, and lime by Texas A&M University. Reaction conditions and solids compositions are measured according to NREL Laboratory Analytical Procedure 002 (NREL, 2004) by our CAFI partners or in our laboratory are reported in Table 2 for all of the pretreatments.

2.2. Enzymes

Spezyme® CP cellulase (Lot 301-04075-034; 59 ± 5 FPU/ml, 123 ± 10 mg protein/ml) was generously provided by the Genencor Division of Danisco US, Inc. (Rochester, NY, USA). β-Glucosidase (Novozyme188, 140 ± 5 mg protein/ml; 665 CBU/ml) was purchased from Sigma Chemicals, St. Louis, MO, and 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., 1985), and the activity for Novozyme188 was based on that by Dien et al. (2008).

2.3. Preparation of solids digested to various degrees

Solids digested to various degrees were produced by digestion of pure Avicel glucan and pretreated poplar solids by using Spezyme CP supplemented with Novozyme188 at a CBU to FPU activity ratio of ~2. The solids resulting from hydrolysis were washed extensively to remove sugars and solubilized lignin followed by overnight incubation with protease (Pronase, Sigma Chemicals, St. Louis, MO) in a phosphate buffer (pH 7.4) at 37 °C (Willies, 2007; Yang et al., 2006). To deactivate protease completely, the solution containing solids and protease was held at 80 °C for 15 min (Sigma), followed by washing two times with 1 M sodium chloride solution and four times with deionized water (Yang et al., 2006). Then the solids moisture content and composition were determined according to NREL LAP002 (NREL, 2004). Nitrogen contents of unhydrolyzed solids and prehydrolyzed and protease treated solids were determined using a Flash EATM 112 N/Protein plus CHNS/O Analyzer (CE Elantech, Lakewood, NJ) with aspartic acid/BBOT (2,5-Bis-(5-tert-butyl-benzoxazol-2-yl)-thiophen) as a standard, as described elsewhere (Kumar and Wyman, in press-b, 2008; Willies, 2007). The nitrogen contents of solids are shown in Fig. 1, and prehydrolyzed solids compositions as measured according to NREL Laboratory Analytical Procedure 002

Table 2
Pretreatment methods, conditions, and solids compositions for solids prepared by leading technologies.

Pretreatment	Pretreatment conditions	Composition of pretreated solids (%)		
		Glucan[a]	Xylan[a]	Lignin
Avicel	None	~100	~0.0	~0.0
AFEX	180 °C,700 psi, 2:1 NH_3 to biomass, 30 min, and 233% moisture content (dwb) – NW	46.6	15	N.D.
ARP	185 °C, 400 psi, 3.66:1 NH_3 to biomass (dry wt.), 27.5 min and 23.49% solid – W	57.5	13.5	24.8
Dilute acid	190 °C, 0.02 H_2SO_4:dry wt., 70 s, 30% solids – NW	57.3	2.1	46.1
Lime	65 °C, 0.5:1 $Ca(OH)_2$ to Biomass (dry wt.) – W1	53.1	16.8	18.0

W – washed, NW – non washed, W1 – washed and neutralized, N.D. – not determined.
[a] Glucan defined in terms of $C_6H_{10}O_5$, xylan defined in terms of $C_5H_8O_4$.

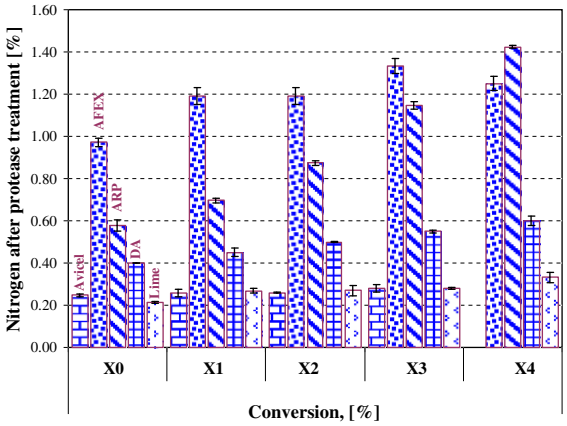

Fig. 1. Percentage nitrogen content of solids digested to various degrees for Avicel glucan and poplar solids prepared by leading pretreatments of AFEX, ARP, dilute acid, and lime. X_0, X_1, X_2, X_3, and X_4 represent different conversion levels as presented in Table 3 for Avicel glucan and poplar solids. Note: the fresh samples (X_0) were not treated with protease.

(NREL, 2004) with degrees of glucan and xylan digestion are reported in Table 3 for the pretreatments studied. In Table 3, X_0, X_1, X_2, X_3, and X_4 represent five different glucan/xylan conversion levels solids that resulted from digestion, with glucan/xylan conversion levels shown in Table 3 for Avicel and pretreated poplar solids.

2.4. Enzymatic hydrolysis

Enzymatic hydrolysis was performed in triplicates in a 0.05 M citrate buffer (pH ~ 4.8) containing sodium azide (1%) as an antibiotic according to NREL Laboratory Analytical Procedure LAP 009. Two separate sets of hydrolysis experiments were run in parallel for fresh and spent solids: (1) with solids loading based on equal concentrations of glucan (1% (w/v)) and (2) with solids loading based on equal concentrations of lignin (1% (w/v)). 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. Solids digestion was performed at a combined cellulase and β-glucosidase mass loading of 33.6 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.

To determine the quantity of sugars generated by enzymatic hydrolysis, about 700 μl of liquid was drawn at 4 and 24 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., Deerfield, IL), 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 Aminex HPX-87H and 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, Milford, MA).

2.5. Adsorption of purified CBHI

Adsorption of purified CBHI (Cel7A) was performed at a mass loading of 75 mg/g glucan in 2 ml centrifuge vials with solids loading containing 1%(w/v) glucan at 4 °C in a 0.05 M citrate buffer. 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 a rotator, the vials were centrifuged at a 13 K rotor speed for 5 min. Then the amount of free protein in solid free solution was determined using the BCA method (Smith et al., 1985) using 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.

Table 3
Percentage conversion of glucan and xylan, compositions, and xylan to glucan (X/G) and lignin to glucan ratios (L/G) of resulting solids for Avicel glucan and poplar solids prepared by AFEX, ARP, dilute acid, and lime pretreatments.

Pretreatment	% Conversion level			Composition of fresh and spent solids (%)			Xylan to glucan ratio (X/G)	Lignin to glucan ratio (L/G)
	Symbol	Glucan	Xylan	Glucan	Xylan	Lignin		
Avicel	X_0	0.0	0.0	100.0	0.0	0.0	0.00	0.00
	X_1	22.0	–	100.0	0.0	0.0	0.00	0.00
	X_2	48.7	–	100.0	0.0	0.0	0.00	0.00
	X_3	70.0	–	100.0	0.0	0.0	0.00	0.00
AFEX	X_0	0.0	0.0	46.6	15.0	29.0	0.32	0.62
	X_1	35.2	55.0	43.5	9.1	27.1	0.21	0.62
	X_2	45.0	70.0	40.6	8.8	31.0	0.22	0.76
	X_3	65.0	80.0	34.7	7.8	36.5	0.22	1.05
	X_4	85.0	85.0	29.0	6.9	46.2	0.24	1.59
ARP	X_0	0.0	0.0	57.5	13.5	24.8	0.23	0.43
	X_1	25.4	44.8	53.6	6.8	31.3	0.13	0.58
	X_2	47.3	69.5	42.0	5.6	41.7	0.13	0.99
	X_3	60.5	81.6	32.5	4.2	51.4	0.13	1.58
	X_4	90.3	93.1	18.2	2.0	66.9	0.11	3.68
Dilute acid	X_0	0.0	0.0	57.3	2.1	46.1	0.04	0.80
	X_1	16.2	23.9	52.6	3.6	44.6	0.07	0.85
	X_2	32.2	31	43.4	3.3	53.6	0.08	1.24
	X_3	63.2	56.4	11.1	1.6	80.3	0.14	7.23
	X_4	90	95	7.72	1.1	80.5	0.14	10.43
Lime	X_0	0.0	0.0	53.1	16.8	18.0	0.32	0.34
	X_1	27.8	38.4	53.2	17.1	33.0	0.32	0.62
	X_2	40.2	46.5	48.6	16.2	36.7	0.33	0.76
	X_3	60.2	60.4	38.2	14.3	46.9	0.37	1.23
	X_4	72.0	72.0	29.9	12.5	54.8	0.42	1.83

3. Results

The 4 h hydrolysis rates, 24 h sugar yields, and CBHI adsorption data were determined for Avicel glucan and poplar solids prepared by AFEX, ARP, dilute acid, and lime pretreatments hydrolyzed to various degrees. Because these solids had different lignin to glucan ratios, as shown in Table 3, hydrolysis was performed with solids loadings containing 1% (w/v) glucan and with solids loading containing 1% (w/v) lignin in parallel.

3.1. Avicel

Nitrogen contents of the solids left after various degrees of conversion and treatment with protease were determined using a nitrogen analyzer, and the results in Fig. 1 confirmed that the spent solids nitrogen contents were not much different than for fresh material, suggesting that protease was effective in removing most of the proteins from the solid surface. Although initial[2] rates declined a little after 70% cellulose conversion, the 24 h glucose yield was apparently the same for all solids prehydrolyzed to various degrees, as shown in Fig. 2a and b, respectively. These results are consistent with findings reported by Yang and Wyman with Avicel previously that specific reactivity of Avicel glucan (rate per mg protein adsorbed) did not change with conversion (Yang et al., 2006). However, in contrast, Hong et al. recently applied harsher chemicals to remove proteins from spent solids to show a drastic decline in initial rate (>85%) after 80% glucan conversion (Hong et al., 2007). Similarly, Wang and coworkers showed about a 20–30% decline in hydrolysis rates for long term hydrolysis of cotton fiber (6 days) but did not reportedly clean residual protein from the spent substrate prior to restart experiments. In other studies, Gusakov et al. (1985) and Nidetzky and Steiner (1993) showed that the rate of glucose production for prehydrolyzed (50%) cotton stalks and microcrystalline cellulose, respectively, was much lower than for fresh material.

CBHI adsorption revealed that cellulose accessibility did not change much with conversion, as shown in Fig. 2a, consistent with the hydrolysis data. Other results not included here showed that the cellulase adsorption capacity (σ_{max}) of the solids, as determined by complete Spezyme adsorption at 4 °C at various concentrations and then estimating adsorption parameters via fitting the data to the Langmuir equation, did not change with conversion. Consistent with these findings, Yang et al. (2006) showed that the cellulase adsorption capacity for Avicel cellulose remained constant over a wide range of conversions and then increased a little at high conversion. In addition, for a shorter run hydrolysis, Ma et al. (2008) recently reported a negligible change in the amount of reducing ends over the course of hydrolysis for microcrystalline cellulose. In contrast, Hong et al. and Wang et al. showed that the decline in rate is more profoundly affected by limited glucan accessibility during the course of cellulose conversion, as determined by the adsorption of a fusion protein containing a cellulose-binding module (CBM) and a green fluorescent protein (GFP) and purified CBHI, respectively (Hong et al., 2007; Wang et al., 2006). Hong et al. showed that accessibility is limited by a loss of reducing ends as conversion proceeds, but the BCA reagent method employed is very sensitive to interference by sugars, chemicals, and proteins (Kumar and Wyman, 2008; Zhang and Lynd, 2005). Ooshima et al. (1991)) also showed a decline in cellulase adsorption as Avicel was hydrolyzed to various degrees, but found that the substrate specific reactivity (rate per mg of enzymes adsorbed) did not change much with conversion. Thus, it appears that proteins not completely removed from the solid may hinder binding

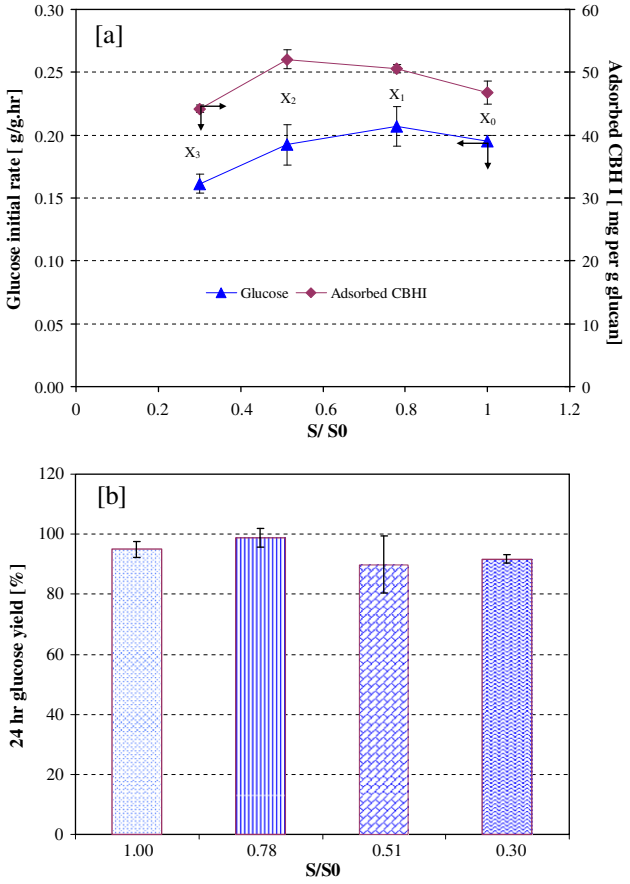

Fig. 2. (a) Initial glucose release rates and CBHI adsorption and (b) 24 h glucose yields vs. glucan conversion for Avicel.

of enzymes, reducing hydrolysis rates and cellulase adsorption and possibly interfering strongly with highly sensitive assays such as the BCA method used in some studies (Hong et al., 2007; Zhang and Lynd, 2005). Furthermore, using harsh chemicals for protein removal may also strongly inhibit enzymes. For example, the drop in cellulase adsorption capacity (CAC) with conversion for the Hong et al. (2007) study did not seem to be in good agreement with the drop in hydrolysis rates (substrate reactivity).

3.2. AFEX

The results in Fig. 1 reveal that the nitrogen content of hydrolyzed AFEX solids increased by about 0.2–0.35% with conversion, which is roughly a 2.0–2.5% increase in residual protein on the solids (% protein = nitrogen factor [NF] × % nitrogen; NF = 8.4 for Spezyme CP (Kumar and Wyman, 2008)). However, as discussed elsewhere (Chundawat et al., 2007; Kumar and Wyman, in press-a), ammonia used for AFEX and ARP pretreatments strongly reacts with biomass lignin and presumably increases the nitrogen content slightly as the fraction lignin increases with conversion (L/G increased from 0.60 to 1.60), as shown in Table 3. Although, it is difficult to confirm experimentally that the increase in nitrogen was associated with the lignin and not left over enzyme, the hydrolyzed solids were assumed to be pretty much free of residual enzyme proteins.

Hydrolysis data for both sets of experiments showed that there was a negligible change in glucose initial rate with conversion, shown in Fig. 3a and b, whereas the xylose initial rate first dropped and then remained constant. However, although the 24 h glucose yield dropped first and then increased slightly with conversion

[2] The initial rate is the release of glucose/xylose determined after 4 h of hydrolysis.

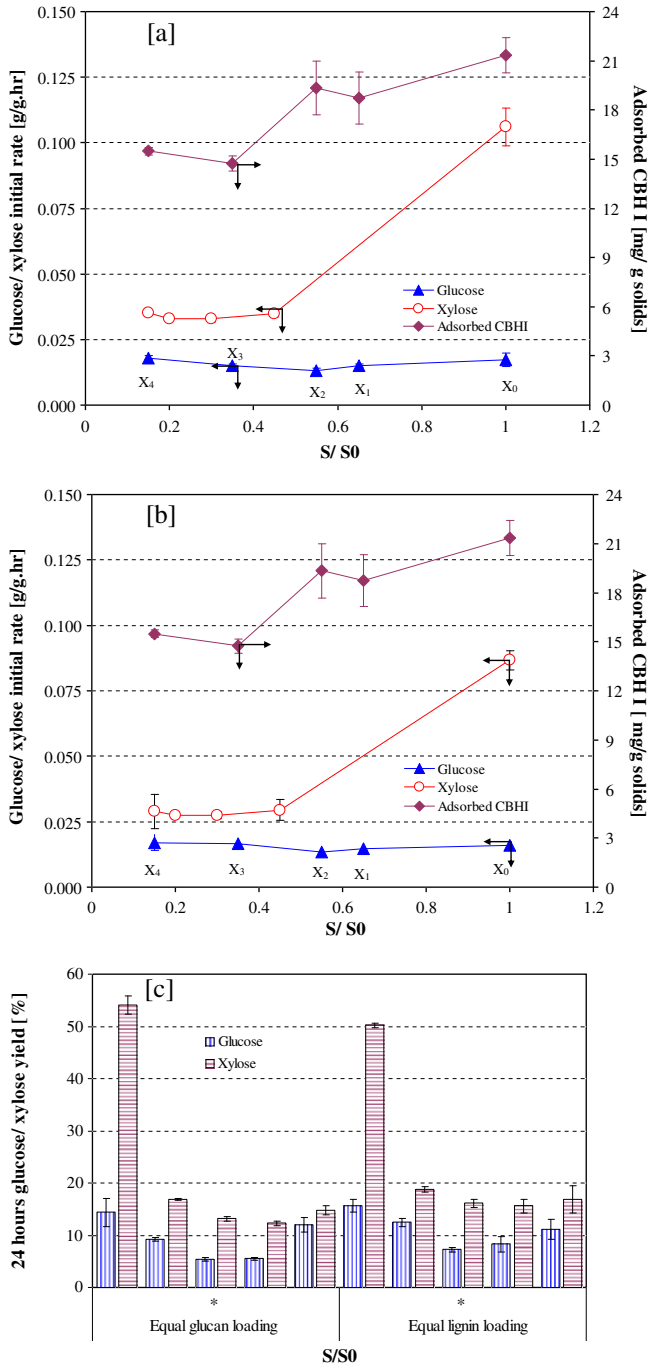

Fig. 3. (a) Glucose and xylose initial rates and CBHI adsorption vs. conversion for substrate loadings of AFEX poplar solids containing (a) equal glucan (1% (w/v)) and (b) equal lignin (1% (w/v)) and (c) 24 h glucose and xylose yield vs. conversion for solids containing equal masses of glucan or lignin. S/S_0 is the fraction of glucan/xylan left, and X_0, X_1, X_2, X_3, and X_4 are the glucan/xylan conversion levels. In figure (c) S/S_0 is 1 on the left side.

for solids loadings containing equal amounts of glucan, the drop in 24 h glucose yields was less prominent when solids loadings were based on equal amounts of lignin, shown in Fig. 3c. In addition, the 24 h xylose yield data seemed consistent with the initial xylose release rates, as yield first dropped and then remained unchanged. In addition, though the cause for a drop in 24 h glucose yields is not known, the xylan and lignin contents increased as the solids were digested, possibly exposing more lignin as conversion proceeds. However, the fact that solids hydrolyzed to various degrees apparently had the same specific CBHI adsorption (at least until the conversion level X_2), as shown in Fig. 3a, refutes the possibility that lignin exposure increased with conversion. The negligible changes in initial glucose release rates and CBHI adsorption with conversion suggests that glucan accessibility for AFEX poplar solids does not change with conversion. Although, it is expected that xylan removal should enhance cellulose accessibility (Jeoh et al., 2007; Kumar and Wyman, in press-d), this was not the case here. Furthermore, although endoxylanase adsorption was not performed, it appears that xylan[3] accessibility dropped by about 67% with conversion and then remained constant (after X_1).

3.3. ARP

Although the initial nitrogen content of ARP pretreated poplar solids (~0.80%) was lower than that for AFEX pretreated solids (~1.0%), the incremental change in nitrogen content with conversion was much greater than for other pretreatments, as shown in Fig. 1. However, the lignin to glucan ratio (L/G) increased by about a factor of 6 for conversion X_4 for ARP vs. about 2.5 times for AFEX pretreated solids at the same conversion level. Furthermore, nitrogen analysis of acid insoluble lignin for ARP solids (Kumar and Wyman, in press-a,b) showed that ammonium hydroxide seemed to strongly react with lignin. As a result, much of the increase in nitrogen content with conversion could be attributed to the increase in the concentration of nitrogen rich lignin as hemicellulose and cellulose are removed and not to residual enzyme proteins.

Digestion data for fresh ARP solids and solids hydrolyzed to different degrees showed that the initial glucose reaction rate first increased by a significant amount (by 210%[4] from conversion level X_0 to X_1) and then dropped with conversion to a level of only about 42% (after 90% [X_4] conversion), lower than for fresh substrate, as shown in Fig. 4a. The cause for this sharp increase in glucose initial rate may be the increased exposure of glucan chains due to xylan removal (Jeoh et al., 2007; Kumar and Wyman, 2009, in press-d) and reduced enzymes inhibition by xylooligomers (Kumar and Wyman, 2009, submitted for publication-b; Suh and Choi, 1996), as the xylan content in the hydrolyzed solids for a 1% glucan loading dropped from 1.1 g/l for fresh substrate to 0.78 g/l at the X_1 conversion level. Furthermore, when hydrolysis was conducted with solids containing 1% (w/v) lignin, the drop in the glucose rate shown in Fig. 4b was somewhat sharper (57% compared to 42% observed for 1%(w/v) glucan based hydrolysis). The initial xylose release followed a similar trend as glucose, although the initial increase in xylose release was nowhere as great as for glucose (52% than 210% for glucose). Although not shown, a linear relationship was observed between the initial rates of glucose and xylose release. Furthermore, 24 h glucose and xylose yields were roughly in agreement with initial rates. Glucose yields increased first and then dropped with conversion, whereas xylose yields decreased with conversion, and the drop in xylose yields with conversion was much higher than the decline in glucose yields (82% vs. 68% for X_4 solids), as shown in Fig. 4c.

Similar to glucose initial rates, 4 °C CBHI adsorption on fresh and hydrolyzed ARP solids revealed that glucan accessibility first increased with conversion (about 165% increase after conversion level of X_1), and then dropped to a level roughly equal to the fresh substrate, as shown in Fig. 4a. The increased CBHI adsorption and glucose initial rates indicated enhanced glucan accessibility, possibly due to xylan removal as suggested by Jeoh et al. (2007). Thus, it could be concluded that glucan accessibility for ARP pretreated solids was limited by xylan and that xylan removal beyond a

[3] The basis for change in xylan accessibility was the initial xylose release over 4 h.

[4] Percent change in rate/yield was calculated as follows: % change in rate/yield = 100 * (rate/yield of spent solids − rate/yield of fresh solids)/rate/yield of fresh solids.

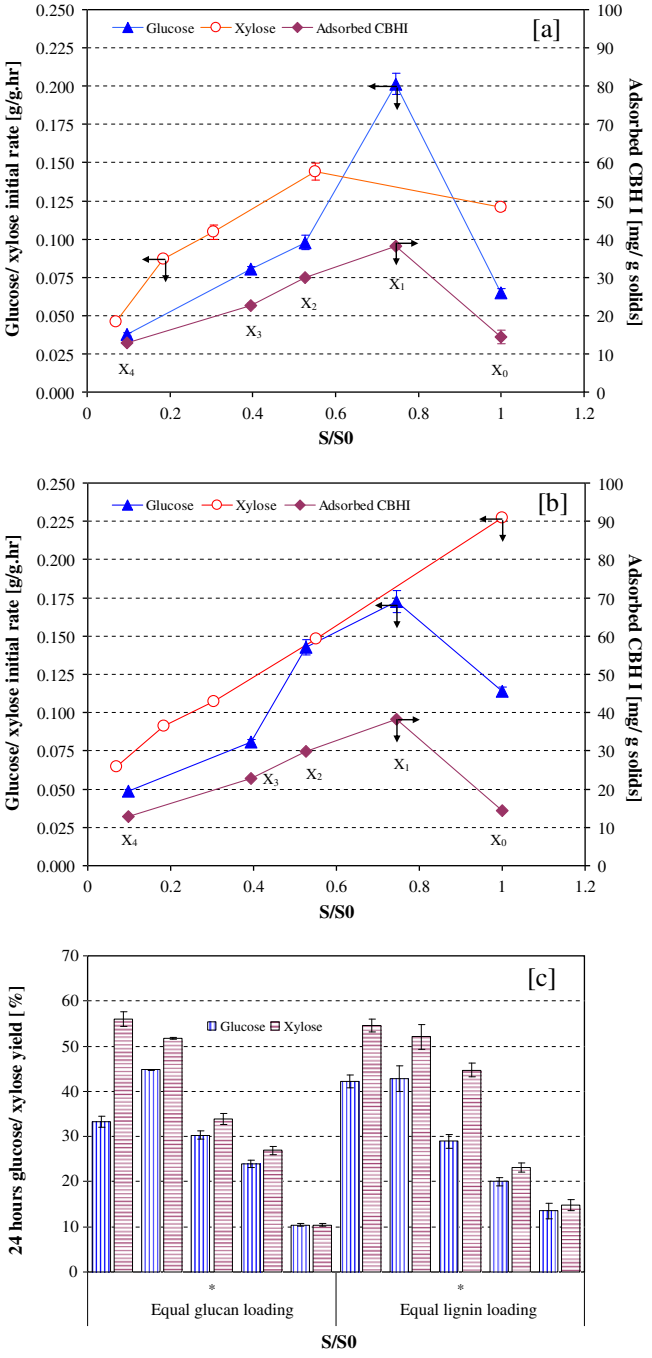

Fig. 4. (a) Glucose and xylose initial rates and CBHI adsorption vs. conversion for substrate loadings with ARP pretreated poplar solids containing (a) equal glucan (1% (w/v)) and (b) equal lignin (1% (w/v)), and (c) 24 h glucose and xylose yields vs. conversion for equal glucan or lignin levels for ARP pretreated poplar solids. S/S_0 is the fraction of glucan/xylan left and X_0, X_1, X_2, X_3, and X_4 are the glucan/xylan conversion levels. In figure (c) S/S_0 is 1 on the left side.

certain level (45%) resulted in little additional enhancement; however, the drop in initial glucose rates after a sudden initial increase suggested that glucan accessibility declined modestly, possibly due to increased lignin content and/or increased lignin exposure as hydrolysis proceeded. In addition, xylan accessibility also initially increased with conversion and then seemed to drop, possibly affecting glucan accessibility as suggested in the literature that coating of glucan by xylan limits glucan accessibility (Chambat et al., 2005; Whitney et al., 1995; Yang and Wyman, 2004).

3.4. Dilute acid

Enzymatic digestion of dilute acid pretreated fresh and partially hydrolyzed solids at a combined cellulase and β-glucosidase loading of 33.6 mg/g glucan showed that initial glucose rates first rapidly increased with conversion (by 65% after X_1 conversion) and then dropped to a somewhat lower level (9% after X_4 conversion) than for fresh substrate, as shown in Fig. 5a and b. Although the initial rate dropped with conversion, it was not as linear with time as shown by Kadam et al. (2004) for dilute acid pretreated corn

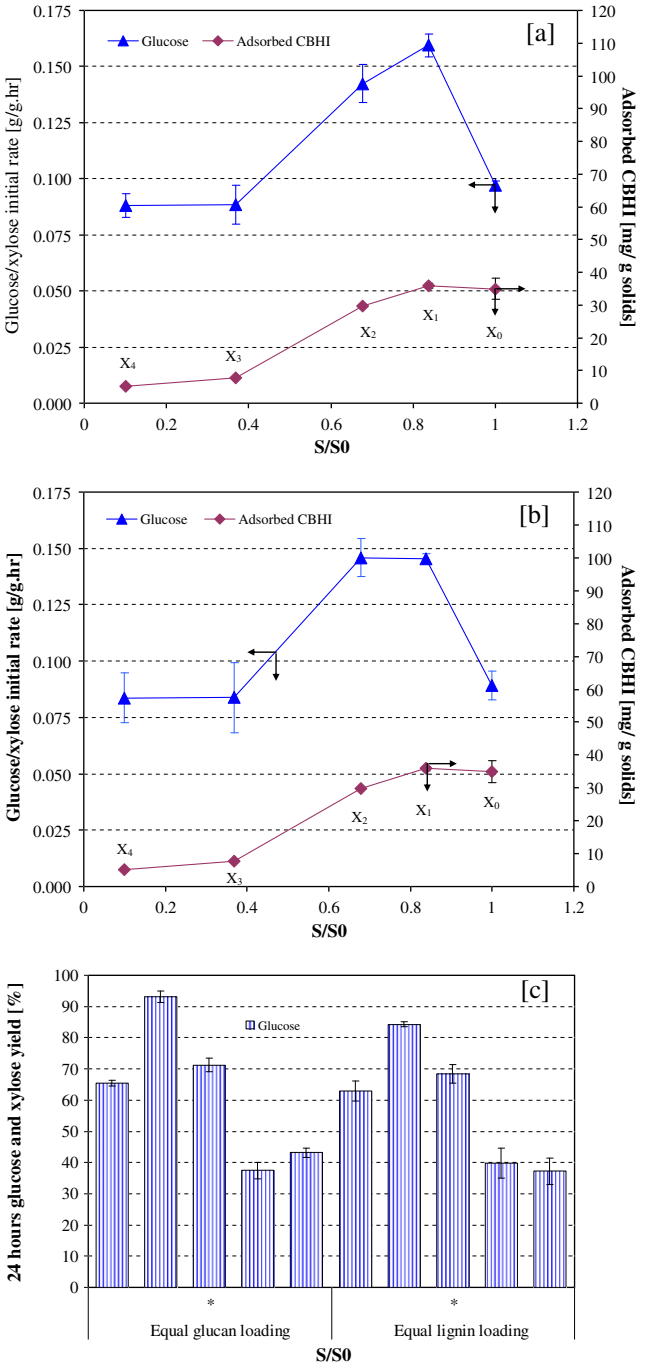

Fig. 5. (a) Glucose and xylose initial rates and CBHI adsorption vs. conversion for substrate loadings with dilute acid pretreated poplar solids containing (a) equal glucan (1% (w/v)) and (b) equal lignin (1% (w/v)), and (c) 24 h glucose and xylose yields vs. conversion for equal glucan or lignin levels for dilute acid pretreated poplar solids. S/S_0 is the fraction of glucan left and X_0, X_1, X_2, X_3, and X_4 are the glucan conversion levels. In figure (c) S/S_0 is 1 on the left side.

stover; however, they did not dislodge adsorbed enzymes prior to re-hydrolysis. Xylose initial rate data was not reported because xylan was almost negligible in the pretreated solids and could not be detected in the hydrolyzate. Although 24 h glucose yields were consistent with initial rates, the drop in yield with conversion was much higher (35–40%) than the drop in initial rates (9%), possibly due to a drastic drop in glucan accessibility with hydrolysis (especially for solids prehydrolyzed to X_3 and X_4). In addition, the fact that hydrolysis rates dropped when conducted with equal amounts of lignin suggests that the greater exposure of lignin as hydrolysis proceeded could adsorb enzymes.

In contrast to glucose rate data, a negligible change was found in CBHI adsorption up to the X_2 conversion level followed by a major drop (about 85%) with further conversion, even though adsorption was always performed with solids containing 1% (w/v) glucan. Therefore, cellulose accessibility does not seem to be responsible for an initial increase in rates with conversion, and the sudden increase in rate after X_1 conversion could be attributed to removing inhibitory compounds such as xylooligomers (Kumar and Wyman, 2009b), other sugars (Garcia-Aparicio et al., 2006; Kumar and Wyman, 2008; Xiao et al., 2004), and other chemical compounds produced by dilute acid pretreatment (Berlin et al., 2006; Weil et al., 2002). In addition, the fresh solids used for hydrolysis were unwashed, and as shown elsewhere (Kumar and Wyman, in press-d), washing can significantly affect cellulase effectiveness. After the X_2 level of conversion, both adsorption and initial glucose rates dropped. Consistent with this finding, Desai and Converse showed that both restart rate and cellulase adsorption declined with conversion by about 83.9% and 86.4%, respectively, for dilute acid pretreated mixed hardwood, when constant cellulase loadings were added to hydrolyzed solids, and a similar finding was reported by Ghose and Bisaria for steam exploded bagasse (Ghose and Bisaria, 1979).

3.5. Lime

As shown in Fig. 1 for lime pretreated solids, the nitrogen content of the solids changed little (0.15%) with conversion, suggesting that the solids were reasonably free of the protein used for hydrolysis. However, the minor increase in nitrogen content could be attributed to the increased lignin content as hydrolysis proceeded retaining more protein.

Initial rates of glucose and xylose release from hydrolyzed and fresh lime pretreated solids dropped with conversion (from normalized rates of 100% for fresh substrate to about 30% for X_4 conversion), as shown in Fig. 6a. Although the drop in initial xylose rate was much greater than for glucose (18.4% for xylose vs. 3% for glucose) for solids hydrolyzed to lower degrees, they were roughly the same for both sugars for solids prehydrolyzed to > 30%. Unlike ARP pretreated solids, initial rates did not seem to increase with xylose removal, suggesting that xylan for lime pretreated solids does not affect glucan accessibility much, as reported elsewhere (Kumar and Wyman, 2009, in press-d). The 24 h glucose and xylose yields shown in Fig. 6c also dropped with conversion, but the drop was somewhat less than the drop in initial rates (58% vs. 69% for conversion level of X_4).

CBHI adsorption was performed for fresh and hydrolyzed solids containing equal amounts of glucan (1% (w/v)) and a common CBHI mass loading of 75 mg/g glucan. As shown in Fig. 6a, CBHI adsorption first increased (by 140%) with conversion and then dropped to a level almost equal to that for fresh substrate. The initial increase could be attributed to increased lignin exposure with hydrolysis, because unlike ARP pretreated solids, the increase in CBHI adsorption did not result in increased initial glucose release rates. On the other hand, the accessibility of glucan might have increased with conversion, but enzyme effectiveness was limited by lignin networking with carbohydrates. Thus, it is difficult to firmly determine the exact cause for the increase in CBHI adsorption with conversion for lime pretreated solids.

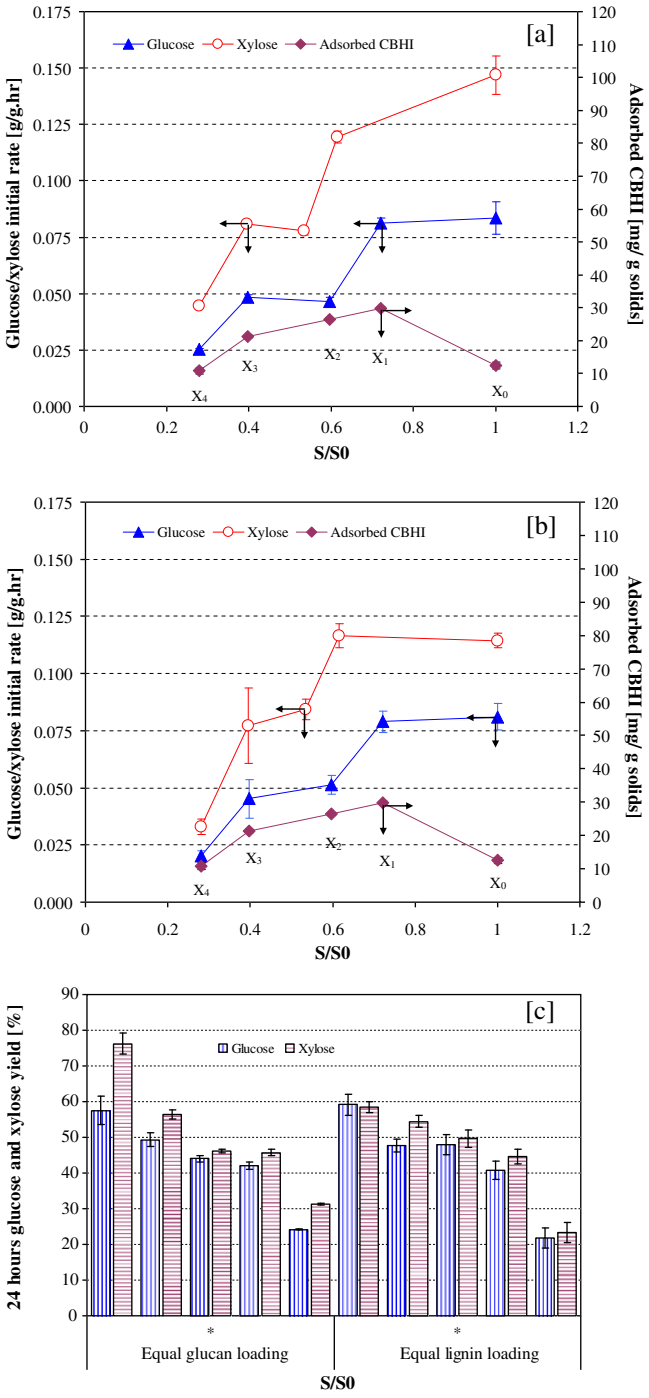

Fig. 6. (a) Glucose and xylose initial rates and CBHI adsorption vs. conversion for substrate loadings with lime pretreated poplar solids containing (a) equal glucan (1% (w/v)) and (b) equal lignin (1% (w/v)), and (c) 24 h glucose and xylose yields vs. conversion for equal glucan or lignin levels for lime pretreated poplar solids. S/S_0 is the fraction of glucan/xylan left and X_0, X_1, X_2, X_3, and X_4 are the glucan/xylan conversion levels. In figure (c) S/S_0 is 1 on the left side.

4. Discussion

The change in glucan and xylan accessibility with conversion was determined for Avicel glucan as a reference and poplar solids prepared by leading pretreatments of AFEX, ARP, dilute acid, and

Table 4
Summary of glucose 4 h rate, CBHI adsorption, and specific activity for Avicel glucan and poplar solids prepared by leading pretreatments.

Pretreatment	% conversion	Glucose initial rate (g/g h) (a)	% change in initial rate[a]	CBHI adsorption (mg/g) solids (b)	Specific activity (a/b)[b]	Normalized specific activity[c]
Avicel	0.0	0.1954	0.0	46.8	0.0042	100.0
	22.0	0.2068	5.8	50.6	0.0041	97.9
	48.7	0.1923	−1.6	52.0	0.0037	88.5
	70.0	0.1613	−17.5	44.2	0.0036	87.4
AFEX	0.0	0.0172	0.0	21.3	0.0008	100.0
	35.2	0.0151	−12.5	18.7	0.0008	99.7
	45.0	0.0130	−24.6	19.3	0.0007	83.3
	65.0	0.0151	−12.6	14.7	0.0010	126.5
	85.0	0.0180	4.3	15.5	0.0012	143.7
ARP	0.0	0.0651	0.0	14.4	0.0045	100.0
	25.4	0.2015	209.7	38.2	0.0053	117.1
	47.3	0.0978	50.3	29.8	0.0033	72.7
	60.5	0.0805	23.8	22.7	0.0036	78.8
	90.3	0.0377	−42.1	12.8	0.0029	65.1
Dilute acid	0	0.0972	0.0	34.9	0.0028	100.0
	16.2	0.1595	64.1	35.9	0.0044	159.8
	32.2	0.1424	46.5	29.7	0.0048	172.3
	63.2	0.0885	−9.0	7.8	0.0114	408.4
	90	0.0881	−9.4	5.1	0.0172	617.6
Lime	0.0	0.0835	0.0	12.5	0.0067	100.0
	27.8	0.0812	−2.8	29.8	0.0027	40.8
	40.2	0.0466	−44.2	26.4	0.0018	26.4
	60.2	0.0483	−42.2	21.2	0.0023	34.1
	72.0	0.0252	−69.9	10.8	0.0023	34.9

[a] Percentage change in rate = 100 * (rate of spent solids − rate of fresh solids)/rate of fresh solids.
[b] Specific activity was defined by the ratio of glucose initial rate and CBHI adsorption per g solids.
[c] Normalized specific activity was determined as the ratio of specific activity for fresh/spent solids and fresh solids.

lime. Overall, these experiments showed that glucan accessibility was a function of substrate and pretreatment type, as summarized in Table 4. Fig. 7 further illustrates how specific activity, as defined by the ratio of glucose initial rate and adsorbed CBHI, changes with conversion.

Consistent with a previous study by our group (Yang et al., 2006) and other studies in literature (Desai and Converse, 1997; Ooshima et al., 1991), initial glucose release rates, CBHI adsorption, and specific activity were almost constant throughout Avicel hydrolysis, as shown in Table 4 and Fig. 7. For AFEX pretreated poplar solids, both initial glucose rate and CBHI adsorption and consequently specific activity remained close to the levels for fresh substrate for carbohydrate conversions up to about 85%, and the xylose initial rate first decreased with conversion and then remained constant. Consistent with these observations, Vlasenko et al. (1997) showed that the reactivity of AFEX pretreated rice straw did not change with conversion when substrate collected after 30% to 35% conversion was restarted with fresh enzyme. However, although it was expected that glucan accessibility should increase with xylan removal from AFEX solids, the results reported here did not support this hypothesis, a finding also consistent with data reported elsewhere for limited xylan removal (Kumar and Wyman, in press-d). Unlike AFEX pretreated poplar solids, initial glucose, and xylose rates for ARP pretreated solids increased with conversion by 210% and 51% after conversion level of X_1, respectively, and then dropped to somewhat lower levels than for the fresh substrate (42% and 62% drop in glucose and xylose initial rates, respectively, after X_4 conversion). CBHI adsorption followed the same trend as initial rate of glucose resulting in apparently constant specific reactivity (Fig. 7). The decrease in specific activity after 90% carbohydrate conversion was about 35%, probably attributable to reduced xylan accessibility with conversion; however, the drop in specific reactivity at the same conversion level was somewhat higher (50%) when hydrolysis was conducted with solids containing an equal concentration of lignin. For dilute acid pretreated solids, the increase in initial glucose rate data could be attributed to washing because, unlike ARP solids, CBHI adsorption was not in agreement with the higher initial glucose rate. Although CBHI adsorption and initial glucose release rates decreased with conversion, the specific activity of enzymes with dilute acid pretreated poplar solids increased by a factor of 4 to 6, as shown in Table 4 and Fig. 7, compared to fresh substrate; however, the specific activity for fresh substrate was seemingly hampered by the presence of sugars/oligomers (Kumar and Wyman, 2009b). This enhancement was much higher than observed with AFEX solids and could not be explained. However, consistent with these findings, Desai and Converse (1997) reported a very slight decline in specific activity with conversion for dilute acid pretreated mixed hardwood. In addition, working with acid catalyzed steam exploded rice straw prehydrolyzed to about 33%, Vlasenko et al. reported a negligible decline in substrate reactivity (Vlasenko et al., 1997). Although solids prepared by lime pretreatment had a negligible change in initial glucose rate up to 27% carbohydrates conver-

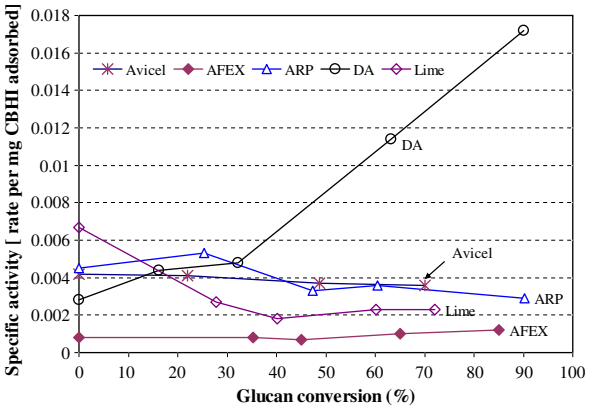

Fig. 7. Relation of specific activity, defined as the 4 h rate per mg of CBHI, to conversion for Avicel glucan and poplar solids prepared by leading pretreatments.

sion, glucan, and xylan hydrolysis rates dropped by approximately 70% after 72% carbohydrate conversion. However, increased CBHI adsorption with conversion suggested that either glucan accessibility or lignin exposure or both increased with conversion. But because the initial glucose rate did not increase with CBHI adsorption, it can be hypothesized that glucan accessibility increased with conversion, while adsorbed CBHI effectiveness dropped. Although specific activity first dropped by about 65% and then remained constant until the carbohydrate conversion reached 72%, the decline in specific reactivity can be misleading because dividing a constant or reduced initial rate by greater CBHI adsorption will result in a decline in specific activity.

In summary, it can be concluded that glucan accessibility for pure Avicel glucan does not drop with conversion, in contrast to hypotheses suggested in other studies that easily digestible glucan is hydrolyzed first (Hong et al., 2007; Park et al., 2007; Sattler et al., 1989; Wald et al., 1984; Wang et al., 2006). However, consistent with our results, a number of other studies concluded that the substrate underwent negligible changes in macro and micro characteristics with conversion (Desai and Converse, 1997; Eremeeva et al., 2001; Ghose and Bisaria, 1979; Lenz et al., 1990). On the other hand, glucan and xylan accessibility was dependent on the pretreatment system applied to poplar solids; however, the specific activity did not decline as drastically as reported in the literature, suggesting that enzyme features and the chemical/physical environment are mainly responsible for the decline in rate with conversion, consistent with studies by Desai and Converse (1997) and Yang et al. (2006).

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