ARTICLE

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Effect of Enzyme Supplementation at Moderate Cellulase Loadings on Initial Glucose and Xylose Release From Corn Stover Solids Pretreated by Leading Technologies

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ABSTRACT: Moderate loadings of cellulase enzyme supplemented with β-glucosidase were applied to solids produced by ammonia fiber expansion (AFEX), ammonia recycle (ARP), controlled pH, dilute sulfuric acid, lime, and sulfur dioxide pretreatments to better understand factors that control glucose and xylose release following 24, 48, and 72 h of hydrolysis and define promising routes to reducing enzyme demands. Glucose removal was higher from all pretreatments than from Avicel cellulose at lower enzyme loadings, but sugar release was a bit lower for solids prepared by dilute sulfuric acid in the Sunds system and by controlled pH pretreatment than from Avicel at higher protein loadings. Inhibition by cellobiose was observed to depend on the type of substrate and pretreatment and hydrolysis times, with a corresponding impact of β-glucosidase supplementation. Furthermore, for the first time, xylobiose and higher xylooligomers were shown to inhibit enzymatic hydrolysis of pure glucan, pure xylan, and pretreated corn stover, and xylose, xylobiose, and xylotriose were shown to have progressively greater effects on hydrolysis rates. Consistent with this, addition of xylanase and β-xylosidase improved performance significantly. For a combined mass loading of cellulase and β-glucosidase of 16.1 mg/g original glucan (about 7.5 FPU/g), glucose release from pretreated solids ranged from 50% to75% of the theoretical maximum and was greater for all pretreatments at all protein loadings compared to pure Avicel cellulose except for solids from controlled pH pretreatment and from dilute acid pretreatment by the Sunds pilot unit. The fraction of xylose released from pretreated solids was always less than for glucose, with the upper limit being about 60% of the maximum for ARP and the Sunds dilute acid pretreatments at a very high protein mass loading of 116 mg/g glucan (about 60 FPU).

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KEYWORDS: corn stover; pretreatment; enzymatic hydrolysis; cellulase; β-xylosidase; celloligomers; xylooligomers; inhibition

Introduction

For corn stover, pretreatments by ammonia fiber expansion (AFEX), ammonia recycled percolation (ARP), controlled pH, dilute acid (DA), and lime were previously shown to achieve similarly high glucose and xylose yields for the combined operations of pretreatment and enzymatic hydrolysis at high cellulase mass loadings of about 55 and 220 mg of protein/g glucan in the original corn stover (corresponding to about 15 FPU and 60 FPU/g glucan, respectively; the Spezyme CP used in these studies had a protein content of about 100 mg/mL and activity of 30 FPU/mL) (Mosier et al., 2005; Wyman et al., 2005). Furthermore, the majority of results available in the literature for pretreated solids report high saccharification yields using uneconomically high enzyme loadings. However, because lower enzyme loadings that still achieve high sugar yields are essential to economic success, better knowledge is needed of factors that govern sugar release at more moderate enzyme loadings (Merino Sandra and Cherry, 2007; Saha and Bothast, 1997; Wingren et al., 2005; Wyman, 2007). Nonproductive binding of cellulase and other enzymes with lignin and other portions of the solid (Excoffier et al., 1991; Yang and Wyman, 2006; Yue et al., 2004; Zheng et al., 2007), inhibition by sugar and their

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degradation compounds (Garcia-Aparicio et al., 2006; Kumar and Wyman, 2008a; Kumar and Wyman, 2008c), and their inactivation over time (Desai and Converse, 1997; Vlasenko et al., 1997; Wang et al., 2006) are thought to be at least partly responsible for high enzyme loading requirements, but the complexity of the substrate and enzymes has confounded developing a clear picture of the mechanism. Furthermore, it has not been determined whether carbohydrate oligomers other than cellobiose inhibit cellulase action because these higher oligomers are almost nonexistent at the higher enzyme loadings typically used.

In this study, we sought to develop a new perspective on short term sugar release patterns for corn stover solids prepared by different pretreatments when subjected to moderate enzyme loadings. Thus, glucose and xylose release from corn stover solids pretreated by leading technologies of ammonia fiber expansion (AFEX), ammonia recycle (ARP), controlled pH, dilute sulfuric acid (DA), lime, and sulfur dioxide (SO_2) as well as pure cellulose and xylan were followed for up to 72 h of hydrolysis over a range of cellulase enzyme loadings. In addition, the effect of corn stover deacetylation on 24 h release of glucose and xylose was determined. Data is also presented on the impact of xylooligomers on sugar release and the benefits of supplementation with β-glucosidase, xylanase, and β-xylosidase.

Materials and Methods

Materials

Pure cellulose, Avicel PH-101, was purchased from FMC Corporation, Philadelphia, PA (Cat 11365, Lot 1094627), and birch wood and beech wood xylans were purchased from Sigma Chemicals (St. Louis, MO). Amorphous cellulose was prepared from Avicel PH 101 cellulose using concentrated phosphoric acid as per the method reported by Zhang and Lynd (2005). Xylobiose and xylotriose were purchased from Megazyme International Ireland, Limited (Bray Business Park, Bray, Co. Wicklow, Republic of Ireland). Unpretreated Kramer corn stover was generously provided by the National Renewable Energy Laboratory (NREL) in Golden, CO. Solids resulting from corn stover pretreatment by various 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 pretreatment with the 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. Corn stover was also pretreated in our laboratories using dilute sulfuric acid in a one liter Parr reactor following the method described by Lloyd and Wyman (2005). Reaction conditions and solids compositions as measured according to NREL Laboratory Analytical Procedure 02 (NREL, 2004) are reported in Table I for all of the pretreatments. Corn stover was deacetylated with potassium hydroxide (KOH) at room temperature using the approach of Chang and Holtzapple (2000) and Kong et al. (1992) but for a total solids concentration of 5% (w/w), to facilitate mixing, on a dry basis instead of the 10% in the reported methods.

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), β-glucosidase (31 ± 5 mg protein/mL), and β-xylosidase (75 ± 5 mg protein/mL) and measurements of their protein content/activities were generously provided by Genencor Division of Danisco US, Inc. (Rochester, NY). β-glucosidase (Novozyme188, 140 ± 5 mg protein/mL; 665 CBU/mL) used in some experiments was purchased from Sigma Chemicals. 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).

Table I. Pretreatment methods, conditions, percent of glucan and xylan left in solids, and solids compositions for solids prepared by leading technologies.

		Percent of original left in pretreated solids (%)		Composition of pretreated solids (%)		
Pretreatment	Pretreatment conditions	Glucan	Xylan	Glucan	Xylan	Lignin
AFEX	90°C, 220 psi, 1:1 NH_3 to Biomass, 5 min—NW	100.0	100.0	34.4	22.8	18.0
ARP	170°C, 325 psi, 3.33:1 NH3:Dry Wt, 20 min, and 3.3 mL/g of corn stover—W	98.6	48.1	61.9	17.9	8.8
Dilute acid	Sunds System: 180°C, 0.03H_2SO_4:Dry wt, 90 s, 25% solids-HW	93.4	27.2	59.3	9.3	22.5
	Parr Reactor: 160°C, 0.005 H_2SO_4:Dry wt, 20 min, 5% solids—NW	94.0	NA	64.4	2.9	26.4
Lime	55°C, 0.5:1 $Ca(OH)_2$ to Biomass (dry wt), 4 weeks, water loading—10 g/g dry biomass—W1	97.1	NA	56.7	26.4	14.6
Controlled pH	190°C, 15 min (+5 min heat up)—NW	94.1	NA	52.7	16.2	25.2
SO_2	190°C, 5 min, 3% SO_2—steam explosion-W	96.9	NA	56.9	11.6	23.8
Deacetylation[a]	25°C, 48 h, 1.5 Mmol KOH/gm 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; NA, not available.

[a]Complete deacetylated corn stover. The untreated corn stover contained 38.3 ± 2.2% glucan, 21.7 ± 1.2% xylan, and 20.5 ± 1.1% lignin.

Enzymatic Hydrolysis

Enzymatic hydrolysis was performed according to NREL Laboratory Analytical Procedure LAP 009 in at least duplicates at 1% (w/w) glucan concentrations in 0.05 M citrate buffer (pH = ~4.8) containing antibiotics (400 μL/100 mL of 10 mg/mL tetracycline in 70% ethanol and 300 μL/100 mL of 10 mg/mL cyclohexamide in DI water) in 125 mL Erlenmeyer flasks operated at 48 ± 3°C using a thermostated water bath shaker at ~200 rpm (NREL, 1996). Substrate blanks without enzyme and enzyme blanks without substrate were run in parallel. Unless otherwise stated, digestibility was determined for Spezyme CP cellulase loadings of 6.5, 16.1, 32.2, and 129.0 mg of protein/g glucan in the raw biomass (corresponding to about 3.0, 7.5, 15, and 60 FPU/g of original glucan) supplemented with β-glucosidase at a protein mass ratio of 0.034, resulting in a CBU to FPU activity ratio of ~2. Sugar release from Avicel cellulose and birch wood and beech wood xylan were determined at the same protein loadings for comparison.

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 analysis. 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).

Determination of Oligomers

After 72 h of enzymatic hydrolysis, broths were centrifuged to separate undigested solids and insoluble lignin from the liquid. Then, the solid free liquid was incubated for 1 h with 4% sulfuric acid at 121°C in an autoclave along with sugar recovery standards, as described elsewhere (Yang and Wyman, 2008). Thereafter, liquid was neutralized with $CaCO_3$, and the total amount of glucose and xylose analyzed by HPLC.

The percent yield of celloligomers with a degree of polymerization > cellobiose, G_{3+}, was calculated at follows:

$$G_{3+} = \frac{\begin{array}{c}100^{*}(\text{glucose after post hydrolysis}\\ -\text{glucose before post hydrolysis}\\ -1.053^{*} \times \text{cellobiose before post hydrolysis})\end{array}}{\text{total potential glucose in pretreated solids}}$$

X_{2+}, the percent yield of xylooligomers containing 2 or more xylose units, was calculated as:

$$X_{2+} = \frac{\begin{array}{c}100^{*}(\text{xylose after post hydrolysis}\\ -\text{xylose before post hydrolysis})\end{array}}{\text{total potential xylose in pretreated solids}}$$

In which the yields after post hydrolysis were corrected for sugar degradation using the sugar recovery standard (Yang and Wyman, 2008) and the factor 1.053 accounts for the mass gain due to hydration of cellobiose to glucose.

Effect of β-Glucosidase and β-Xylosidase Supplementation

The effect of β-glucosidase supplementation on the digestibility of Avicel cellulose, amorphous cellulose, corn stover solids following pretreatment with dilute sulfuric acid in the Parr reactor, and corn stover solids following pretreatment with SO_2 was also studied. A combined loading of 16.1 mg of cellulase and β-glucosidase protein/g original glucan was employed for Avicel cellulose and dilute sulfuric acid and SO_2 pretreated corn stover solids, and 10 mg (about 5 FPU) of cellulase and β-glucosidase together/g glucan was added to amorphous cellulose. For all, β-glucosidase was added at activity ratios (CBU to FPU) of 0, 1, 2,5,10, and 15, unless otherwise stated, in addition to the CBU to FPU ratio of ~2.2 for Spezyme CP itself (Dien et al., 2008). The concentration of oligomers in the liquid after 4 h of hydrolysis for amorphous cellulose and 24 h of hydrolysis for Avicel and corn stover solids was determined as described above.

Hydrolysis of birchwood xylan was conducted at two different Multifect xylanase loadings of 7.0 and 14.0 mg/g xylan augmented with up to 10.0 mg of β-xylosidase protein per mg of xylanase protein. Furthermore, hydrolysis of AFEX pretreated corn stover was conducted at a combined mass loading of cellulase and β-glucosidase of 16.1 mg/g original glucan augmented with up to 10.0 mg of β-xylosidase protein per mg of cellulase protein. The impact of β-xylosidase supplementation on sugar yields for SO_2 pretreated corn stover was only determined with a β-xylosidase to cellulase protein ratio of about 0.25. The release of sugar monomers and oligomers into the liquid was measured after 72 h of hydrolysis, as described earlier.

Impact of Xylooligomers on Cellulase Activity

Hydrolysis of 1% (w/w) Avicel cellulose with an equal mass of birch wood xylan [~86% xylan (Gray et al., 2007)] was performed in triplicate at a combined cellulase and β-glucosidase loading of 16.1 mg/glucan. Hydrolysis was also performed at a similar cellulase loading but supplemented with β-xylosidase at a loading of 14 mg/g glucan. To evaluate the effect of xylose alone on cellulose hydrolysis, cellulose containing an equivalent amount of xylose (1.053*× amount of xylan) was enzymatically hydrolyzed in parallel. Controlled experiments for cellulose hydrolysis without xylan, cellulose with β-xylosidase only, and a substrate blank containing cellulose to which xylan was added were run in parallel. Samples were collected 4, 24, 48, and 72 h after initiation of hydrolysis and analyzed for sugars. Liquids produced by 72 h of hydrolysis of samples containing xylan were post hydrolyzed to determine the amount of xylooligomers (see Materials and Methods).

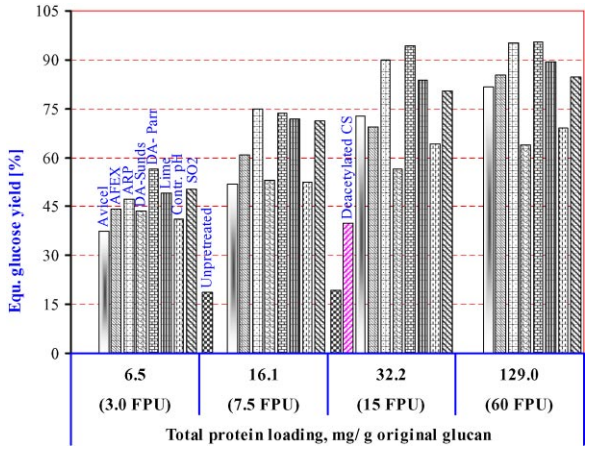

Figure 1. Equivalent glucose yields versus mass protein loadings after 72 h of digestion for Avicel PH 101, unpretreated and deacetylated corn stover, and corn stover solids following pretreatment by leading technologies. Equivalent glucose yield = g of glucose + 1.053 × cellobiose/g glucan in the solids before hydrolysis. [Color figure can be seen in the online version of this article, available at www.interscience.wiley.com.]

Results

Glucose and Xylose Yields Versus Cellulase Loadings

Figures 1 and 2 show the effects of combined cellulase and β-glucosidase protein mass on glucose and xylose yields, respectively, after 72 h of enzymatic hydrolysis expressed as a percent of the glucan and xylan in the pretreated solids. Glucose release was very low for unpretreated corn stover (<20%) at a protein loading of cellulase together with β-glucosidase of 32.2 mg of /g original glucan, and generally, all pretreatments resulted in higher glucose removal than possible from Avicel cellulose at lower enzyme loadings. However, at higher protein loadings, sugar release was a bit lower for solids prepared by dilute sulfuric acid in the Sunds system and by controlled pH pretreatment than from Avicel. On the other hand, solids resulting from dilute sulfuric acid pretreatment in the Parr reactor (severity $[\log R0] = 3.06 = \log\ (t\ \exp(T-100)/14.75)$ in which t is time in minutes and T is temperature in °C) showed much higher digestibility than solids from the Sunds system (severity $[\log R0] = 2.53$) at all protein loadings. The fractional release of xylose (Fig. 2) was much lower from pretreated solids than for glucose (Fig. 1) for all pretreatments. Yet, xylose yields at a high protein loading of 129 mg/g original glucan ranged from 60% to nearly complete saccharification.

Although release of sugars was lower with removal of acetyl groups in Figures 1 and 2 than for any of the pretreatments, there was a nearly linear relationship between glucose yields and acetyl removal as shown in Figure 3a for a protein mass loading of 32.2 mg/g glucan. Furthermore, glucose yields

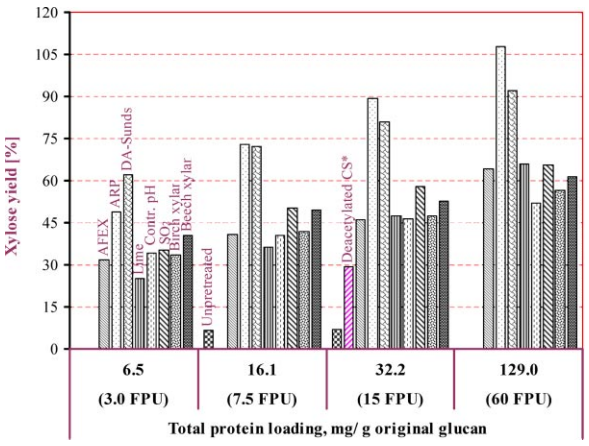

Figure 2. Xylose yields versus mass protein loadings after 72 h of digestion for pure xylan, unpretreated and deacetylated corn stover, and corn stover solids prepared by leading pretreatment technologies. [Color figure can be seen in the online version of this article, available at www.interscience.wiley.com.]

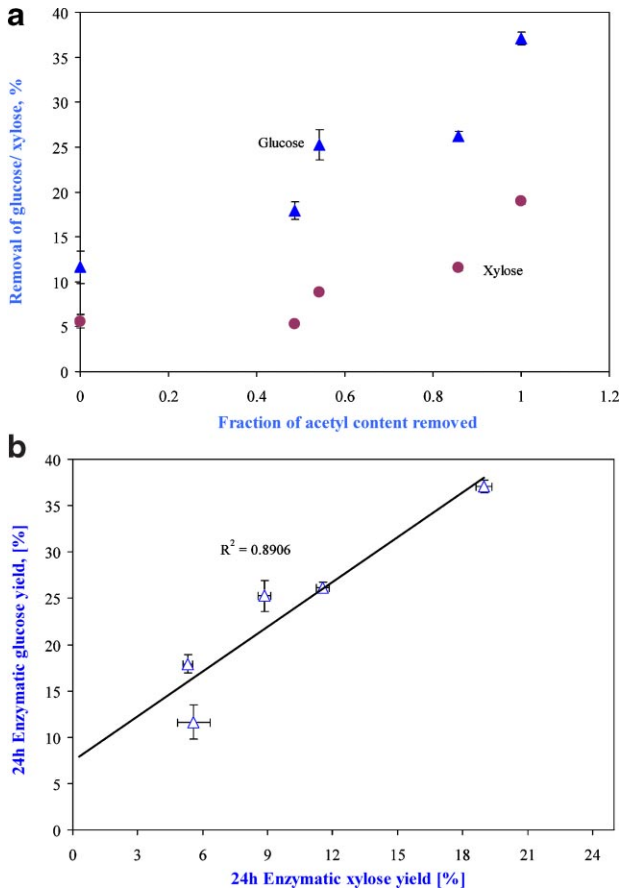

Figure 3. **a**: Effect of deacetylation on glucan and xylan removal after 24 h of enzymatic hydrolysis for an enzyme loading of 32.2 mg/g glucan. **b**: Twenty four hours enzymatic glucose yields versus xylose yields for untreated and deacetylated corn stover. [Color figure can be seen in the online version of this article, available at www.interscience.wiley.com.]

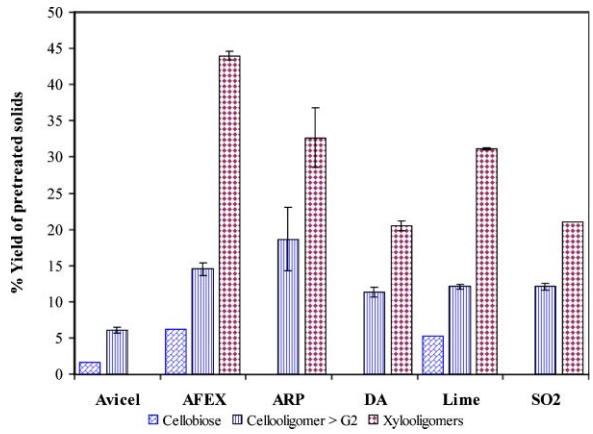

Figure 4. Yields of cellobiose, cellooligomers with degrees of polymerization greater than cellobiose, and xylooligomers as determined by post hydrolysis following enzymatic hydrolysis for 72 h at a combined cellulase and β-glucosidase protein loading of 16.1 mg/g original glucan. [Color figure can be seen in the online version of this article, available at www.interscience.wiley.com.]

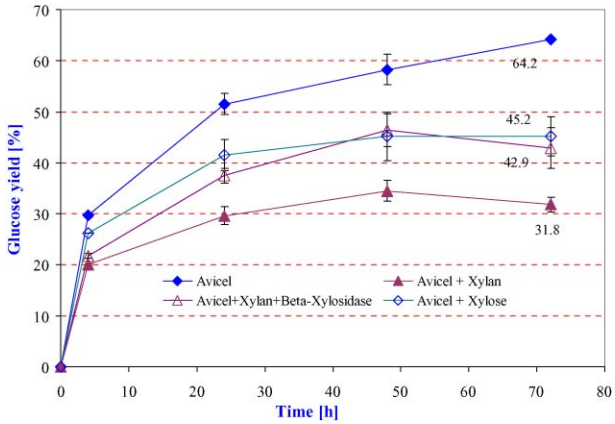

Figure 5. Effect of adding xylan (10 g/L), xylose (12 g/L), and β-xylosidase on hydrolysis of Avicel glucan for a combined cellulase and β-glucosidase protein loading of 16.1 mg/g glucan. [Color figure can be seen in the online version of this article, available at www.interscience.wiley.com.]

approximately doubled over the range of modest acetyl removal achieved, and xylose removal was significantly enhanced as well. In addition, a linear relation was observed between xylose and glucose removal for untreated and deacetylated corn stover (Fig. 3b).

Using only Spezyme CP cellulase that is low in xylanase activity is expected to result in the incomplete removal of xylan, as confirmed by Figures 1 and 2. The low xylanase activity of cellulase could also explain why xylooligomer yields as determined by post hydrolysis were as high as 45% following 72 h of hydrolysis, as shown in Figure 4 for a protein loading of 16.1 mg/g original glucan. However, to our surprise, substantial amounts of cellobiose and particularly higher degree of polymerization cellodextrins (>cellobiose) were also found in the hydrolyzates, with yields of up to 20% after 72 h of hydrolysis, depending upon the substrate and pretreatment. The cause of this substantial cellooligomers accumulation could be adsorption/inactivation of cellulase components and/or β-glucosidase on lignin or lower crystallinity. Although several studies have reported the beneficial effect of β-glucosidase supplementation on glucan digestibility, no universally effective loading of β-glucosidase has been defined, perhaps due to variations in β-glucosidase activity in cellulase and the effects of the substrates used. Therefore, more detailed investigations appear warranted on whether the effectiveness of β-glucosidase supplementation is influenced by pretreatment choice and the type of substrate as well as enzymatic hydrolysis conditions such as the duration of hydrolysis, temperature, and substrate loading.

Effect of Xylooligomers on Cellulase Activity

Although several studies reported inhibition of cellulase activity by monomeric sugars and cellobiose (Holtzapple et al., 1990; Todorovic et al., 1987; Xiao et al., 2004), limited information is available on the effect of higher cellooligomers and xylooligomers. For example, Kim et al. (2006) reported that effluent exiting from low liquid ammonia recycled percolation (LLARP) pretreatment strongly inhibited cellulase and microbial activities, but it was unclear whether high amounts of xylooligomers or solubilized lignin in the liquid were responsible (Kim and Lee, 2005; Wu and Lee, 1997). Therefore, we added Spezyme CP supplemented with β-glucosidase at a 1:2 ratio, respectively, to equal concentrations of 10 g/L for both xylan and Avicel and found that the 72 h glucose yield was about half that of the control, as shown in Figure 5. We also measured the concentration of xylose in the hydrolyzate to be 5.5 g/L. However, supplementing cellulase with β-xylosidase at a protein mass loading of 14 mg/g glucan improved yields by ~34%, resulting in about the same performance as realized when an amount of xylose equivalent to the xylan concentration originally employed, 12.5 mg/mL, was added initially. Yet, only about 6.7 mg/mL of xylose was released by hydrolysis of the mixture with β-xylosidase supplementation at a protein loading of 14 mg/g glucan. Furthermore, in the initial period of cellulose hydrolysis in the presence of xylan, cellobiose appeared in higher amounts than for the control, suggesting inhibition of β-glucosidase activity by xylooligomers and/or xylan. However, cellobiose virtually disappeared after 24 h of hydrolysis, and β-xylosidase had very low cellulase activity (data not shown). Thus, these observations strongly suggest that xylooligomers inhibit cellulase action, and glucose and xylose release could be significantly enhanced by β-xylosidase supplementation. However, the benefits of adding β-xylosidase are expected to vary with the amount of xylan left in the solids which is in turn affected by the type of pretreatment and substrate. In addition, the choice of enzyme and enzyme loadings are also expected to be important.

Effect of β-Glucosidase Loading

Cellobiose produced by the action of cellobiohydrolases on cellulose is well recognized to strongly inhibit cellulase action (Ghosh et al., 1982; Gusakov et al., 1985; Oh et al., 2000), and several studies reported the effects of adding β-glucosidase on cellulose hydrolysis (Breuil et al., 1992; Ghosh et al., 1982; Spindler et al., 1989; Stockton et al., 1991; Sun and Cheng, 2004).

However, the impacts of β-glucosidase supplementation on cellodextrin release after enzymatic hydrolysis and inhibition by cellodextrins with degrees of polymerization greater than for cellobiose have seldom been studied. For example, despite adding amounts of β-glucosidase consistent with literature recommendations (Lloyd and Wyman, 2005; Murnen et al., 2007; Yang et al., 2006; Yang and Wyman, 2006), yields of higher DP cellooligomers were high for pure cellulose and lignin containing substrates, as shown in Figure 4, even though the yield of cellobiose was lower. This is consistent with reports that the effectiveness of β-glucosidase drops with increasing chain lengths of soluble celloligomers (Wilson et al., 1994). Furthermore, β-glucosidase is well known to lose activity by exposure to heat and agitation (Aguado et al., 1995; Gunjikar et al., 2001; Mukataka et al., 1983), nonproductive binding with lignin (Shawky et al., 1984; Sutcliffe and Saddler, 1986; Tatsumoto et al., 1988; Xu et al., 2008; Yang and Wyman, 2006), and the effect of lignin degradation compounds (Garcia-Aparicio et al., 2006; Kaya et al., 1999; Vohra et al., 1980). In addition, the effectiveness of a given β-glucosidase loading may vary with the type of substrate, pretreatment method, substrate loading, and time of hydrolysis (Breuil et al., 1992; Gruno et al., 2004; Stockton et al., 1991; Tengborg et al., 2001).

Figure 6 shows the effect of β-glucosidase supplementation at a fixed cellulase mass loading on glucose release from amorphous cellulose, highly crystalline cellulose, and cellulose in corn stover solids left after SO_2 and dilute sulfuric acid pretreatment in the Parr reactor. Contradictory to expectations, increasing β-glucosidase activity did not significantly enhance cellulose hydrolysis to glucose and cellobiose for amorphous cellulose, even though it did increase cellobiose conversion to glucose up to a CBU to FPU activity ratio of 2, as shown in Figure 6a. Furthermore, β-glucosidase supplementation beyond that ratio reduced cellobiose

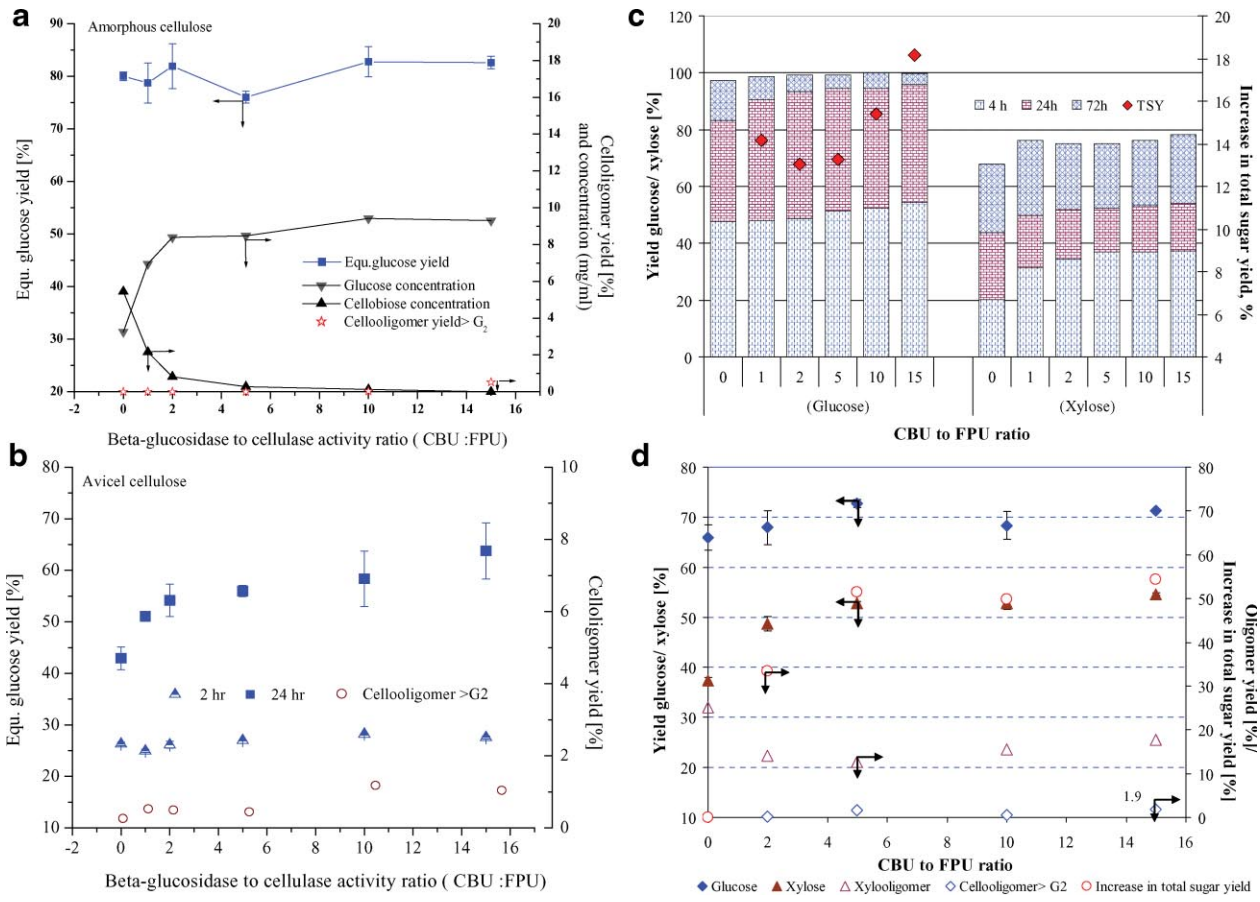

Figure 6. Effect of β-glucosidase supplementation on sugar release and oligomer yields at a combined cellulase and β-glucosidase mass loading of 10.0 mg/g glucan for (**a**) amorphous cellulose after 4 h hydrolysis with cellulase and β-glucosidase combined at a mass loading of 16.1 mg/g glucan; (**b**) Avicel PH101 after 2 and 24 h; (**c**) dilute acid pretreated corn stover after 72 h; and (**d**) SO_2 pretreated corn stover after 24 h. [Color figure can be seen in the online version of this article, available at www.interscience.wiley.com.]

concentrations to virtually zero, but the yield of higher celloligomers remained at about 0.5%, even at a very high ratio of β-glucosidase to cellulase activity. Although β-glucosidase supplementation did not have much effect on the initial rate of hydrolysis for Avicel PH101, as shown in Figure 6b, it had a major impact on the 24 h glucose yield, and surprisingly, the yield of higher celloligomers ($>G_2$) was around 1–2% even with very high β-glucosidase supplementations (CBU to FPU ratio ~15). β-glucosidase supplementation had a small effect on the initial rate of glucose release and a major effect on initial xylose release for dilute sulfuric acid pretreated corn stover, as shown in Figure 6c, but had a significant impact on 24 h yields of both glucose and xylose (increase in total sugar yield ~39%). However, β-glucosidase supplementation had a noticeable effect on total sugar yields in the first 24 h but only increased glucose and xylose yields by 2.8% and 15%, respectively, after 72 h. Therefore, for dilute acid pretreated corn stover prepared with the Parr reactor, a CBU to FPU activity ratio of 1 appears adequate for achieving nearly theoretical glucose yields even at a low cellulase loading of 16.1 mg/g glucan. Supplemental β-glucosidase had little effect on glucose release at a cellulase loading of 32.2 mg/g glucan (15 FPU) with a substrate loading of 1% (w/v) glucan (data not shown). For SO_2 pretreated corn stover, 24 h glucose yields increased with increasing β-glucosidase supplementation up to a ratio of 5 (by 10%) and then remained constant, as shown in Figure 6d, and the overall increase in total glucose plus xylose yields was about 50% at a CBU to FPU activity ratio of 5. It is interesting to note that β-glucosidase supplementation resulted in a 30% decrease in yield of xylooligomers to about 25% of the total xylan available in the pretreated solids, and the yield of celloligomers was around 2%, even with a very high β-glucosidase supplementation. Therefore, β-glucosidase does not hydrolyze all of the higher cellodextrins, and cellulose crystallinity, type of pretreatment, and hydrolysis duration may influence the effectiveness of β-glucosidase supplementation, as suggested in the literature (Tengborg et al., 2001; Todorovic et al., 1987). The increase in xylose yields with β-glucosidase supplementation may result from xylanase activity in Novozyme188 (Dien et al., 2008).

Effect of β-Xylosidase Supplementation

Enzymatic hydrolysis requires synergy between endo-xylanase and β-xylosidase for unsubstituted xylan (Kumar and Wyman, 2008a; Robert, 1983; Suh and Choi, 1996; Tenkanen et al., 1996) and among xylanase components and accessory enzymes for substituted xylan (de Vries et al., 2000; Kormelink and Voragen, 1992). However, although the role of β-xylosidase has some similarities to that for β-glucosidase, it is more effective in hydrolyzing higher DP soluble xylans than β-glucosidase is in hydrolyzing higher DP celloligomers (Anand and Vithayathil, 1996; Basaran and Ozcan, 2008; Belkacemi and Hamoudi, 2003; Ghose and Bisaria, 1987; Silveira et al., 1999). As shown previously in Figure 4, the yield of xylooligomers after enzymatic hydrolysis with just cellulase and β-glucosidase can be as high as 45%, as observed for solids containing 1% (w/w) glucan and depend on type of pretreatment, possibly due to limited β-xylosidase activity in Spezyme CP. Therefore, birch wood xylan was hydrolyzed at two xylanase mass protein loadings of 7 mg and 14 mg/g xylan with β-xylosidase supplementation up to 10 mg/mg of xylanase protein, and xylose monomer yields are summarized

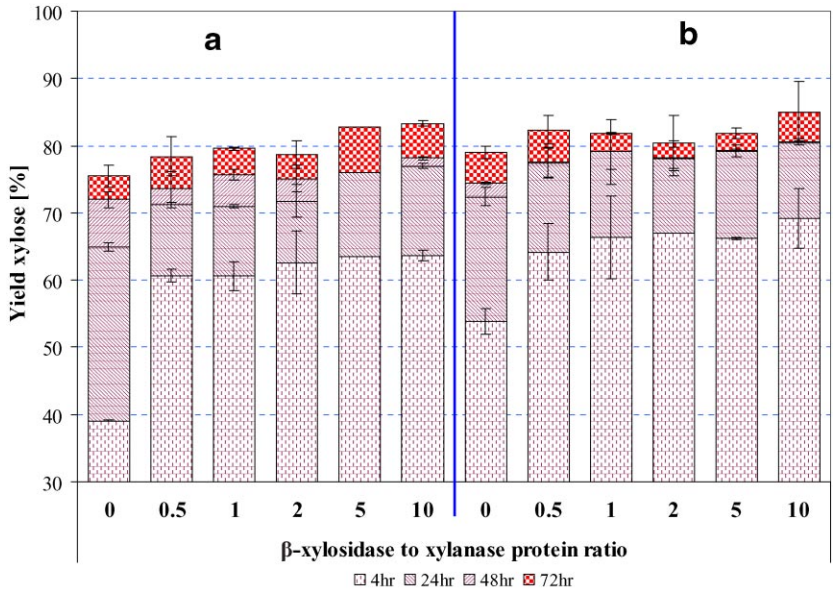

Figure 7. Effect of β-xylosidase supplementation on xylose release from birchwood xylan at fixed xylanase mass loadings of (**a**) 7 mg protein/g xylan and (**b**) 14 mg/g xylan. [Color figure can be seen in the online version of this article, available at www.interscience.wiley.com.]

in Figure 7 for samples collected after 4, 24, 48, and 72 h. This data showed that β-xylosidase supplementation did not increase longer time xylose yields significantly beyond a protein mass ratio of 1, possibly due to the high activity of β-xylosidase in Multifect xylanase. However, β-xylosidase supplementation had a substantial effect on the initial rate of xylose release for each xylanase mass loading, with about a 55% and 20% increase for 7 mg and 14 mg of xylanase protein, respectively, in the first 4 h. Furthermore, β-xylosidase supplementation dropped xylooligomers yields to essentially 0% (data not shown) from 5% with a xylanase mass loading of 7 and to 0% from 1.7% with a xylanase mass loading of 14 mg/g xylan. Although higher xylanase loadings and β-xylosidase supplementation both increased initial xylose yields significantly, 72 h yields were quite similar at both xylanase mass loadings with β-xylosidase supplementation.

To better understand how β-xylosidase supplementation impacts glucan and xylan hydrolysis for substrates containing appreciable amounts of xylan and lignin, AFEX pretreated corn stover was hydrolyzed at a cellulase mass loading of 16.1 mg including β-glucosidase/g original glucan with up to 10 mg of β-xylosidase supplementation per mg of cellulase. Similarly, SO_2 pretreated corn stover was hydrolyzed at a combined cellulase and β-glucosidase loading of 16.1 mg/g with 0.25 mg of β-xylosidase/mg of cellulase and with an equal amount of xylanase protein as the combined amount of

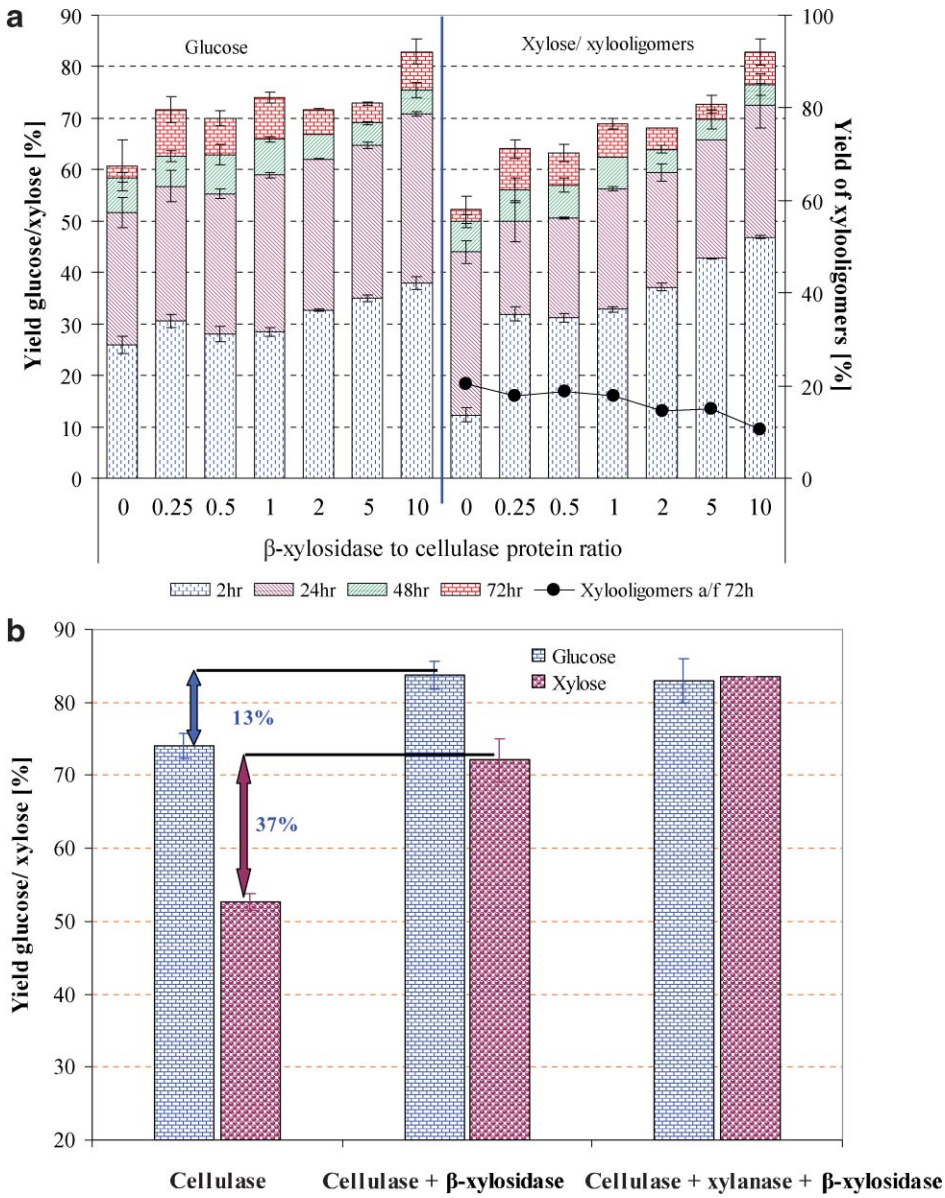

Figure 8. Effect of β-xylosidase supplementation on glucose, xylose, and xylooligomer release at a combined cellulase and β-glucosidase mass loading of 16.1 mg/g original glucan for (**a**) AFEX pretreated corn stover and (**b**) SO_2 pretreated corn stover. [Color figure can be seen in the online version of this article, available at www.interscience.wiley.com.]

cellulase and β-xylosidase. As shown in Figure 8a, β-xylosidase supplementation increased 4 h glucose and xylose releases by up to 46% and 285%, respectively, at high supplementation ratios. These results for glucan hydrolysis support earlier evidence that xylooligomers significantly inhibit cellulase activity. In addition, 72 h glucose and xylose yields at a protein mass ratio of β-xylosidase to cellulase of 0.25 were enhanced by about 18% and 23%, respectively. However, although β-xylosidase supplementation enhanced sugar release, a significant yield of xylooligomers (~10%) was still found after 72 h of hydrolysis even for very high β-xylosidase supplementation, as shown in Figure 8a. For SO_2 pretreated corn stover with β-xylosidase supplementation, glucose and xylose release increased by about 13% and 37%, respectively, as shown in Figure 8b. However, although supplementation of the enzyme mixture containing cellulase, β-glucosidase, and xylanase with β-xylosidase enhanced xylose release by 16%, a negligible increase in glucose release was observed, suggesting that β-xylosidase supplementation may not be beneficial with a high xylanase loading. Thus, β-xylosidase alone does not appear to be sufficient to hydrolyze high DP soluble xylooligomers, and supplementation with both xylanase and β-xylosidase appears desirable to realize high monomeric xylose yields, as found for pure xylan and AFEX and SO_2 pretreated corn stover. Further study is underway on the impact of xylanase supplementation on corn stover hydrolysis.

Effect of Xylose Oligomers Chain Length on Cellulase Activity

As shown in previous sections, significant amounts of xylooligomers were measured in the hydrolyzates after enzymatic hydrolysis. Furthermore, these xylooligomers were also found in the liquor from pretreatment prior to enzymatic hydrolysis, with the amounts depending upon the type of pretreatment, substrate, and substrate loading (Kabel et al., 2007; Kim and Holtzapple, 2005; Kumar and Wyman, 2008b; Liu and Wyman, 2003; Wyman et al., 2005; Yang and Wyman, 2008). As shown above, such xylooligomers can slow glucose and xylose release, but better quantification was desired on whether oligomers concentration and chain length affect this inhibition. Therefore, 12.5 mg/mL (80 mM xylose equivalent) of xylose, 6.6 mg/mL (46 mM xylose equivalent) and 16.6 mg/mL (117 mM xylose equivalent) of xylobiose, and 6.6 mg/mL (48 mM xylose equivalent) of xylotriose were added to 2 mL centrifuge tubes containing 30 mg of Avicel in 1.5 mL of liquid at a cellulase loading of 30 FPU/g glucan, and initial hydrolysis rates were measured. Xylose alone at this concentration inhibited yields by about 10%, but about half as much xylobiose, 46 mM of xylose equivalent, reduced glucose release by 18%, as shown in Figure 9. Furthermore, glucose release dropped by about 32% at a higher xylobiose loading of 117 mM of xylose equivalent. Adding almost half of the xylose concentration and about the same as the lower concentration of xylobiose, xylotriose (48 mM xylose equivalent) reduced glucose release even more, by about

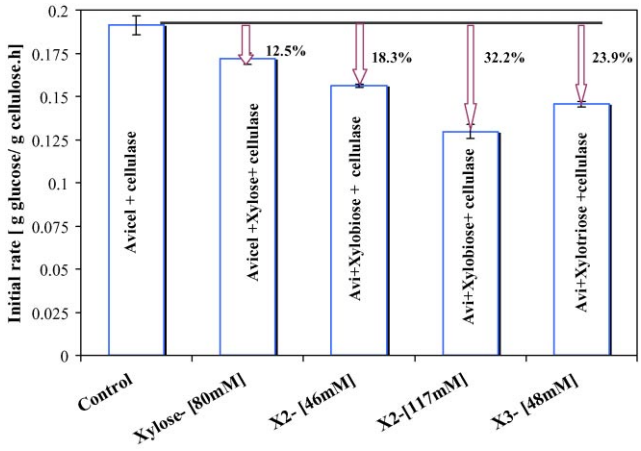

Figure 9. Effect of xylose oligomer chain length on Avicel glucan initial hydrolysis rate at a cellulase loading of 30 FPU/g glucan and CBU to FPU activity ration of 2. [Color figure can be seen in the online version of this article, available at www.interscience.wiley.com.]

24%. These results show that enzyme inhibition increases with chain length and concentration for these three species, but more work is needed to determine whether these trends continue to build for longer xylooligomer chain lengths. Further study with purified cellulase components could clarify which activities xylooligomers particularly affect.

Conclusions

Glucose and xylose release data were developed for solids prepared by leading pretreatment options that span a range of pH values to better understand what factors drive the need for high enzyme doses to realize high yields. It was observed that about 70–80% of the glucan was digestible at a combined cellulase and β-glucosidase mass loading of 16.1 mg/g glucan and that glucose release was better from all pretreatments than from Avicel at these lower enzyme loadings. However, xylose yields were much lower and remained lower even with very high cellulase mass loadings. Analysis of the hydrolyzate following pretreatment and in the liquid after 72 h of enzymatic hydrolysis showed an appreciable amount of oligomers, with the former containing large amounts of xylooligomers. Furthermore, enzymatic hydrolysis was slower when xylooligomers were present, suggesting that they strongly inhibited cellulase activity and possibly endoxylanase activity as well (Suh and Choi, 1996). Supplementation of cellulase with β-xylosidase and xylanase improved sugar release significantly, with their effectiveness depending on the type of substrate and pretreatment and the length of time for hydrolysis. In addition, xylose, xylobiose, and xylotriose were shown to inhibit enzymatic hydrolysis, and the degree of inhibition was found to increase with chain length and concentration for these three compounds. However, further study is needed to determine if inhibition continues to increase with degree of polymerization for longer chained

oligomers and assess how these species impact the activity of individual cellulase components.

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References

Aguado J, Romero MD, Rodriguez L, Calles JA. 1995. Thermal deactivation of free and immobilized b-glucosidase from Penicillium funiculosum. Biotechnol Prog 11(1):104–106.

Anand L, Vithayathil PJ. 1996. Xylan-degrading enzymes from the thermophilic fungus Humicola lanuginosa (Griffon and Maublanc) Bunce: Action pattern of xylanase and [beta]-glucosidase on xylans, xylooligomers and arabinoxylooligomers. J Ferment Bioeng 81(6):511–517.

Basaran P, Ozcan M. 2008. Characterization of [beta]-xylosidase enzyme from a Pichia stipitis mutant. Bioresource Technol 99(1):38–43.

Belkacemi K, Hamoudi S. 2003. Enzymatic hydrolysis of dissolved corn stalk hemicelluloses: Reaction kinetics and modeling. J Chem Technol Biotechnol 78(7):802–808.

Breuil C, Chan M, Gilbert M, Saddler JN. 1992. Influence of beta-glucosidase on the filter paper activity and hydrolysis of lignocellulosic substrates. Bioresource Technol 39(2):139–142.

Chang VS, Holtzapple MT. 2000. Fundamental factors affecting biomass enzymatic reactivity. Appl Biochem Biotechnol 84–86:5–37.

de Vries RP, Kester HCM, Poulsen CH, Benen JAE, Visser J. 2000. Synergy between enzymes from Aspergillus involved in the degradation of plant cell wall polysaccharides. Carbohydr Res 327(4):401–410.

Desai SG, Converse AO. 1997. Substrate reactivity as a function of the extent of reaction in the enzymic hydrolysis of lignocellulose. Biotechnol Bioeng 56(6):650–655.

Dien BS, Ximenes EA, OBryan PJ, Moniruzzaman M, Li X-L, Balan V, Dale B, Cotta MA. 2008. Enzyme characterization for hydrolysis of AFEX and liquid hot-water pretreated distillers grains and their conversion to ethanol. Bioresource Technol 99(12):5216–5225.

Excoffier G, Toussaint B, Vignon MR. 1991. Saccharification of steam-exploded poplar wood. Biotechnol Bioeng 38(11):1308–1317.

Garcia-Aparicio MP, Ballesteros I, Gonzalez A, Oliva JM, Ballesteros M, Negro MJ. 2006. Effect of inhibitors released during steam-explosion pretreatment of barley straw on enzymatic hydrolysis. Appl Biochem Biotechnol 129–132:278–288.

Ghose TK, Bisaria VS. 1987. Measurement of hemicellulase activities. Part 1: Xylanases. Pure Appl Chem 59(12):1739–1752.

Ghosh P, Pamment NB, Martin WRB. 1982. Simultaneous saccharification and fermentation of cellulose: Effect of β-glucosidase activity and ethanol inhibition of cellulases. Enzyme Microb Technol 4(6):425–430.

Gray MC, Converse AO, Wyman CE. 2007. Solubilities of oligomer mixtures produced by the hydrolysis of xylans and corn stover in water at 180°C. Ind Eng Chem Res 46(8):2383–2391.

Gruno M, Valjamae P, Pettersson G, Johansson G. 2004. Inhibition of the Trichoderma reesei cellulases by cellobiose is strongly dependent on the nature of the substrate. Biotechnol Bioeng 86(5):503–511.

Gunjikar TP, Sawant SB, Joshi JB. 2001. Shear deactivation of cellulase, exoglucanase, endoglucanase, and β-glucosidase in a mechanically agitated reactor. Biotechnol Prog 17(6):1166–1168.

Gusakov AV, Sinitsyn AP, Gerasimas VB, Savitskene RY, Steponavichus YY. 1985. A product inhibition study of cellulases from Trichoderma longibrachiatum using dyed cellulose. J Biotechnol 3(3):167–174.

Holtzapple M, Cognata M, Shu Y, Hendrickson C. 1990. Inhibition of Trichoderma reesei cellulase by sugars and solvents. Biotechnol Bioeng 36(3):275–287.

Kabel MA, Bos G, Zeevalking J, Voragen AG, Schols HA. 2007. Effect of pretreatment severity on xylan solubility and enzymatic breakdown of the remaining cellulose from wheat straw. Bioresource Technol 98(10): 2034–2042.

Kaya F, Heitmann JA Jr, Joyce TW. 1999. Effect of dissolved lignin and related compounds on the enzymic hydrolysis of cellulose model compound. Cell Chem Technol 33(3–4):203–213.

Kim S, Holtzapple MT. 2005. Lime pretreatment and enzymatic hydrolysis of corn stover. Bioresource Technol 96(18):1994–2006.

Kim TH, Lee YY. 2005. Pretreatment and fractionation of corn stover by ammonia recycle percolation process. Bioresource Technol 96(18): 2007–2013.

Kim TH, Lee YY, Sunwoo C, Kim JS. 2006. Pretreatment of corn stover by low-liquid ammonia recycle percolation process. Appl Biochem Biotechnol 133(1):41–57.

Kong R, Engler CR, Soltes EJ. 1992. Effects of cell-wall acetate xylan backbone and lignin on enzymatic hydrolysis of aspen wood. Appl Biochem Biotechnol 34:23–35.

Kormelink FJM, Voragen AGJ. 1992. Combined action of xylan-degrading and accessory enzymes on different glucurono-arabino xylans. Progr Biotechnol 7(XylansXylanases): 415–418.

Kumar R, Wyman CE. 2008a. Effects of cellulase and xylanase enzymes on the deconstruction of solids from pretreatment of poplar by leading technologies. Biotechnol Prog (in press).

Kumar R, Wyman CE. 2008b. The impact of dilute sulfuric acid on the selectivity of xylooligomer depolymerization to monomers. Carbohydr Res 343(2):290–300.

Kumar R, Wyman CE. 2008c. An improved method to directly estimate cellulase adsorption on biomass solids. Enzyme Microb Technol 42(5): 426–433.

Liu C, Wyman CE. 2003. The effect of flow rate of compressed hot water on xylan, lignin, and total mass removal from corn stover. Ind Eng Chem Res 42(21):5409–5416.

Lloyd TA, Wyman CE. 2005. Combined sugar yields for dilute sulfuric acid pretreatment of corn stover followed by enzymatic hydrolysis of the remaining solids. Bioresource Technol 96(18):1967–1977.

Merino Sandra T, Cherry J. 2007. Progress and challenges in enzyme development for biomass utilization. Adv Biochem Eng Biotechnol 108:95–120.

Mosier N, Wyman C, Dale B, Elander R, Lee YY, Holtzapple M, Ladisch M. 2005. Features of promising technologies for pretreatment of lignocellulosic biomass. Bioresource Technol 96(6):673–686.

Mukataka S, Tada M, Takahashi J. 1983. Effects of agitation on enzymic hydrolysis of cellulose in a stirred-tank reactor. J Ferment Technol 61(6):615–621.

Murnen HK, Balan V, Chundawat SPS, Bals B, Da Costa Sousa L, Dale BE. 2007. Optimization of ammonia fiber expansion (AFEX) pretreatment and enzymatic hydrolysis of miscanthus x giganteus to fermentable sugars. Biotechnol Prog 23(4):846–850.

NREL. 1996. Enzymatic saccharification of lignocellulosic biomass. Golden, CO: National Renewable Energy Laboratory. LAP009.

NREL. 2004. Determination of structural carbohydrates and lignin in biomass. Golden, CO: National Renewable Energy Laboratory. LAP02.

Oh KK, Kim SW, Jeong YS, Hong SI. 2000. Bioconversion of cellulose into ethanol by nonisothermal simultaneous saccharification and fermentation. Appl Biochem Biotechnol 89(1):15–30.

Robert FHD. 1983. Bioconversion of hemicellulose: Aspects of hemicellulase production by Trichoderma reesei QM 9414 and enzymic saccharification of hemicellulose. Biotechnol Bioeng 25(4):1127–1146.

Saha BC, Bothast RJ. 1997. Enzymes in lignocellulosic biomass conversion. ACS Sym Ser 666(Fuelsand Chemicals from Biomass): 46–56.

Shawky B, Kasulke U, Philipp B, Schulz G, Hirte W. 1984. Sorption of Penicillium cellulase on cellulose and lignin. Acta Biotechnol 4(3): 267–274.

Silveira FQP, Ximenes FA, Cacais AOG, Milagres AMF, Medeiros CL, Puls J, Filho EXF. 1999. Hydrolysis of xylans by enzyme systems from solid cultures of Trichoderma harzianum strains. Braz J Med Biol Res 32(8):947–952.

Smith PK, Krohn RI, Hermanson GT, Mallia AK, Gartner FH, Provenzano MD, Fujimoto EK, Goeke NM, Olson BJ, Klenk DC. 1985. Measurement of protein using bicinchoninic acid. Anal Biochem 150(1):76–85.

Spindler DD, Wyman CE, Grohmann K, Mohagheghi A. 1989. Simultaneous saccharification and fermentation of pretreated wheat straw to ethanol with selected yeast strains and beta -glucosidase supplementation. Appl Biochem Biotechnol 20–21:529–540.

Stockton BC, Mitchell DJ, Grohmann K, Himmel ME. 1991. Optimum β-glucosidase supplementation of cellulase for efficient conversion of cellulose to glucose. Biotechnol Lett 13(1):57–62.

Suh J-H, Choi Y-J. 1996. Synergism among endo-xylanase, β-xylosidase, and acetyl xylan esterase from Bacillus stearothermophilus. J Microbiol Biotechnol 6(3):173–178.

Sun Y, Cheng J. 2004. Enzymatic hydrolysis of rye straw and bermudagrass using cellulases supplemented with β-glucosidase. Trans ASAE 47(1): 343–349.

Sutcliffe R, Saddler JN. 1986. The role of lignin in the adsorption of cellulases during enzymic treatment of lignocellulosic material. Biotechnol Bioeng Symp 17:749–762.

Tatsumoto K, Baker JO, Tucker MP, Oh KK, Mohagheghi A, Grohmann K, Himmel ME. 1988. Digestion of pretreated aspen substrates. Hydrolysis rates and adsorptive loss of cellulase enzymes. Appl Biochem Biotechnol 18:159–174.

Tengborg C, Galbe M, Zacchi G. 2001. Influence of enzyme loading and physical parameters on the enzymatic hydrolysis of steam-pretreated softwood. Biotechnol Prog 17(1):110–117.

Tenkanen M, Siika-aho M, Hausalo T, Puls J, Viikari L. 1996. Synergism of xylanolytic enzymes of Trichoderma reesei in the degradation of acetyl-4-O-methylglucuronoxylan. Biotechnology in the Pulp and Paper Industry: Recent Advances in Applied and Fundamental Research, Proceedings of the International Conference on Biotechnology in the Pulp and Paper Industry, 6th, Vienna, June 11–15, 1995:503–508.

Todorovic R, Grujic S, Matavulj M. 1987. Effect of reaction end-products on the activity of cellulolytic enzymes and xylanase of Trichoderma harzianum. Microbios Lett 36(143–144):113–119.

Vlasenko EY, Ding H, Labavitch JM, Shoemaker SP. 1997. Enzymic hydrolysis of pretreated rice straw. Bioresource Technol 59(2& 3):109–119.

Vohra RM, Shirkot CK, Dhawan S, Gupta KG. 1980. Effect of lignin and some of its components on the production and activity of cellulase(s) by Trichoderma reesei. Biotechnol Bioeng 22(7):1497–1500.

Wang L, Zhang Y, Gao P, Shi D, Liu H, Gao H. 2006. Changes in the structural properties and rate of hydrolysis of cotton fibers during extended enzymatic hydrolysis. Biotechnol Bioeng 93(3):443–456.

Wilson CA, McCrae SI, Wood TM. 1994. Characterisation of a β-glucosidase from the anaerobic rumen fungus Neocallimastix frontalis with particular reference to attack on cello-oligosaccharides. J Biotechnol 37(3):217–227.

Wingren A, Galbe M, Roslander C, Rudolf A, Zacchi G. 2005. Effect of reduction in yeast and enzyme concentrations in a simultaneous-saccharification-and-fermentation-based bioethanol process: Technical and economic evaluation. Appl Biochem Biotechnol 121–124:485–499.

Wu Z, Lee YY. 1997. Ammonia recycled percolation as a complementary pretreatment to the dilute-acid process. Appl Biochem Biotechnol 63–65:21–34.

Wyman CE. 2007. What is (and is not) vital to advancing cellulosic ethanol. Trends Biotechnol 25(4):153–157.

Wyman CE, Dale BE, Elander RT, Holtzapple M, Ladisch MR, Lee YY. 2005. Comparative sugar recovery data from laboratory scale application of leading pretreatment technologies to corn stover. Bioresource Technol 96(18):2026–2032.

Xiao Z, Zhang X, Gregg DJ, Saddler JN. 2004. Effects of sugar inhibition on cellulases and beta-glucosidase during enzymatic hydrolysis of softwood substrates. Appl Biochem Biotechnol 113–116:1115–1126.

Xu F, Ding H, Osborn D, Tejirian A, Brown K, Albano W, Sheehy N, Langston J. 2008. Partition of enzymes between the solvent and insoluble substrate during the hydrolysis of lignocellulose by cellulases. J Mol Catal B Enzym 51(1–2):42–48.

Yang B, Wyman CE. 2006. BSA treatment to enhance enzymatic hydrolysis of cellulose in lignin containing substrates. Biotechnol Bioeng 94(4): 611–617.

Yang B, Wyman CE. 2008. Characterization of the degree of polymerization of xylooligomers produced by flowthrough hydrolysis of pure xylan and corn stover with water. Bioresource Technol 99(13):5756–5762.

Yang B, Willies DM, Wyman CE. 2006. Changes in the enzymatic hydrolysis rate of Avicel cellulose with conversion. Biotechnol Bioeng 94(6): 1122–1128.

Yue Z, Bin W, Baixu Y, Peiji G. 2004. Mechanism of cellobiose inhibition in cellulose hydrolysis by cellobiohydrolase. Sci China C Life Sci 47(1): 18–24.

Zhang YH, Lynd LR. 2005. Determination of the number-average degree of polymerization of cellodextrins and cellulose with application to enzymatic hydrolysis. Biomacromolecules 6(3):1510–1515.

Zheng Y, Pan Z, Zhang R, Labavitch J, Wang D, Teter S. 2007. Adsorption characteristics of cellulase and β-glucosidase to lignin, cellulose and pretreated creeping wild ryegrass. Minnesota: Minneapolis. pp. 1–13.