



Effect of xylanase supplementation of cellulase on digestion of corn stover solids prepared by leading pretreatment technologies

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ABSTRACT

Solids resulting from pretreatment of corn stover by ammonia fiber expansion (AFEX), ammonia recycled percolation (ARP), controlled pH, dilute acid, lime, and sulfur dioxide (SO₂) technologies were hydrolyzed by enzyme cocktails based on cellulase supplemented with β -glucosidase at an activity ratio of 1:2, respectively, and augmented with up to 11.0 g xylanase protein/g cellulase protein for combined cellulase and β -glucosidase mass loadings of 14.5 and 29.0 mg protein (about 7.5 and 15 FPU, respectively)/g of original potential glucose. It was found that glucose release increased nearly linearly with residual xylose removal by enzymes for all pretreatments despite substantial differences in their relative yields. The ratio of the fraction of glucan removed by enzymes to that for xylose was defined as leverage and correlated statistically at two combined cellulase and β -glucosidase mass loadings with pretreatment type. However, no direct relationship was found between leverage and solid features following different pretreatments such as residual xylan or acetyl content. However, acetyl content not only affected how xylanase impacted cellulase action but also enhanced accessibility of cellulose and/or cellulase effectiveness, as determined by hydrolysis with purified CBHI (Cel7A). Statistical modeling showed that cellulose crystallinity, among the main substrate features, played a vital role in cellulase–xylanase interactions, and a mechanism is suggested to explain the incremental increase in glucose release with xylanase supplementation.

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1. Introduction

Highly efficient conversion of carbohydrates in biomass to fermentable sugars is essential to commercially competitive biological processes for making cellulosic ethanol (Wyman, 2007; Yang and Wyman, 2008), and although enzymes realize the high yields required, the corresponding high doses are very expensive (Merino Sandra and Cherry, 2007; Tu et al., 2007; Wingren et al., 2005). Furthermore, current commercial enzymes are mainly intended for pulp and paper and food industries and still lack the proportions of different enzymes and their components required for effective biological production of fermentable sugars from cellulosic biomass (Hespell et al., 1997; Merino Sandra and Cherry, 2007). Typically cellulase, β -glucosidase, xylanase, β -xylosidase, and some accessory activities are required to hydrolyze sugar polymers effectively (Bisaria and Ghose, 1981; Kuhad et al., 1997; Saha and Bothast, 1997), with the proportions of each depending on the type of biomass and pretreatment used. Among leading thermochemi-

cal pretreatments, those at low pH hydrolyze xylan to xylose and xylooligomers (Allen et al., 2001; Kabel et al., 2007a; Vazquez et al., 2002) but can also degrade both of these (Kumar and Wyman, 2008d; Lloyd and Wyman, 2005; Saeman, 1945). Furthermore, because the resulting degradation products are strong inhibitors to cellulase and fermenting microorganisms (Kumar and Wyman, 2008e; Larsson et al., 1999; Palmqvist et al., 1996), the pretreatment liquor must be detoxified prior to fermentation (Lynd et al., 2008; Palmqvist and Hahn-Hagerdal, 2000; Weil et al., 2002). On the other hand, high pH alkaline pretreatments leave most of the xylan in the solids, with the result that xylanase as well as possibly other accessory enzymes are needed in addition to cellulase to realize high sugar yields (Chandra et al., 2007; Kumar and Wyman, in press; Mosier et al., 2005; Yang and Wyman, 2008). It has been reported that thermochemical or enzymatic removal of xylan enhances cellulose digestion by reducing the xylan coating and linkages to cellulose (Allen et al., 2001; Ishizawa et al., 2007), but the mechanism of why xylan impacts cellulose digestion is still not entirely clear. Furthermore, data has not been developed to compare the effect of supplementing cellulase with xylanase on sugar release from solids prepared by different promising pretreatments.

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In this study, baseline sugar release data was developed for a cellulase plus β -glucosidase mass loading of 29.0 mg/g glucan in unpretreated² corn stover for solids produced by the leading pretreatment technologies of ammonia fiber expansion (AFEX), ammonia recycled percolation (ARP), dilute sulfuric acid, lime, controlled pH, and sulfur dioxide (SO₂). Then, xylanase and β -xylosidase supplementation were employed to determine whether these two activities enhanced performance at the same cellulase loading plus a lower cellulase loading of 14.5 mg/g glucan in unpretreated corn stover. In addition, the influence of acetyl content on cellulase–xylanase interactions was studied, and factors and possible mechanisms for enhancement of glucan digestion by xylanase supplementation were also determined.

2. Methods

2.1. Materials

Pure cellulose, Avicel PH-101, was purchased from FMC Corporation, Philadelphia, PA (Cat 11365, Lot 1094627). Birchwood xylan was purchased from Sigma Chemicals, St. Louis, MO. Corn stover was generously provided by the National Renewable Energy Laboratory (NREL, Golden, CO) from a lot that they had collected at the Kramer farm in nearby Wray, CO. Solids prepared by corn stover pretreatment were generously given to us by our CAFI partners from Auburn University, Michigan State University, NREL, Purdue University, Texas A&M University, and the University of British Columbia. Various degrees of corn stover deacetylation were achieved using potassium hydroxide (KOH) at room temperature, as reported elsewhere (Chang and Holtzapfel, 2000; Kong et al., 1992), but for a total solids concentration of 5% (w/w) on a dry basis instead of the 10% in the reported methods. Pretreatment conditions and the composition of the resulting solids as determined by NREL Laboratory Analytical Procedure 002 (NREL, 2004) are reported in Table 1.

2.2. Enzymes

Multifect[®] Xylanase (Lot 301-04021-015; 42 ± 5 mg protein/ml), β -xylosidase (75 ± 5 mg protein/ml), Spezyme[®] CP cellulase (Lot 301-04075-034; 59 ± 5 FPU/ml, 123 ± 10 mg protein/ml), and β -glucosidase (31 ± 5 mg protein/ml) were generously provided by the Genencor Division of Danisco US Inc. (Rochester, NY, USA). Another β -glucosidase used in some experiments (Novozyme188, 180 ± 5 mg protein/ml; 665 CBU/ml) was purchased from Sigma Chemicals, St. Louis, MO. Purified CBHI (18.5 mg/ml) from Spezyme[®] CP cellulase was prepared by Protein Labs (San Diego, CA). Enzyme protein contents were determined by the standard BCA method (Smith et al., 1985), and the activity of Novozyme188 was based on published data (Dien et al., 2008).

2.3. Enzymatic hydrolysis

Consistent with NREL Laboratory Analytical Procedure (LAP 009) (NREL, 1996), enzymatic hydrolysis was performed in at least replicates at 1% (w/v) glucan concentrations in 0.05 M citrate buffer containing antibiotics (400 μ l/100 ml of 10 mg/ml tetracycline in 70% ethanol and 300 μ l/100 ml of 10 mg/ml cyclohexamide in DI water) using 125 ml Erlenmeyer flasks controlled at 48 ± 3 °C and ~200 rpm by a thermostated shaker water bath unit. Substrate blanks without enzyme and enzyme blanks without substrate

were run in parallel. Digestibility was determined at total cellulase (Spezyme CP) and β -glucosidase (CTB) loadings of 29.0 mg of protein per g glucan in the raw biomass (corresponding to about 15 FPU³/g original glucan) supplemented with β -glucosidase at a CBU to FPU activity ratio of ~2 or a protein mass ratio of 0.034. Xylanase was added at ratios of 0.2, 0.5, 1, 2, and 5 mass units per mass of cellulase. In addition, xylanase was also used with a lower CTB loading of 14.5 mg protein/g original glucan (about 7.5 FPU/g original glucan), with the same xylanase to cellulase protein ratios used as for the higher cellulase loading plus an additional ratio of 11. These xylanase loadings were designated as MX_{0.2}, MX_{0.5}, MX₁, MX₂, MX₅, and MX₁₁ for xylanase to cellulase protein mass ratios of 0.2, 0.5, 1, 2, 5, and 11, respectively, unless otherwise noted. The total amounts of protein for each cellulase and xylanase loading are summarized in Table 2.

For hydrolysis of corn stover with purified CBHI, the solids containing 1% (w/v) glucan were hydrolyzed for two hours at 50 °C for a CBHI loading of 15 mg/g glucan. The samples were analyzed for cellobiose and glucose. The equivalent glucose yield was defined as the ratio of total glucan equivalent hydrolyzed (0.9*(glucose + 1.053*cellobiose)) to total potential glucan available in the pretreated solids. Similarly, the xylose yield was the ratio of total xylan hydrolyzed (0.89* xylose) to total potential xylan available in the pretreated solids.

2.4. Sugar analysis

To determine the amount of sugars generated by hydrolysis, liquid samples of about 700 μ l were drawn at 24, 48, and 72 h and then immediately filtered through 0.2 μ m nylon filter vials (Alltech Associates Inc., Deerfield, IL), pipetted into 500 μ l polyethylene HPLC vials (Alltech Associates Inc., Deerfield, IL), and kept refrigerated at 4 °C (frozen at –20 °C for longer) until analyzed. The hydrolysis samples and 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, life science research, Hercules, CA). HPX-87H can analyze acids and sugars while HPX-87P measures only monomeric sugars and cellobiose.

2.5. Determination of acetyl content

The acetyl content of corn stover solids was determined per the NREL LAP 002 method using glacial acetic acid as a calibration standard (NREL, 2004).

3. Results and discussions

3.1. Effect of xylanase supplementation on glucose and xylose release

Pretreated corn stover solids were enzymatically hydrolyzed at fixed CTB mass loadings of 14.5 and 29.0 mg/g glucan in unpretreated corn stover with xylanase supplementation to various degrees for a total of 72 h to establish trends in enzyme effectiveness. However, longer hydrolysis times (e.g., 7 days) would likely be employed commercially to capitalize on the additional sugar release expected.

3.1.1. AFEX

As AFEX pretreatment retains virtually all the carbohydrates intact in biomass (Alizadeh et al., 2005; Murnen et al., 2007; Teymouri et al., 2005), higher sugar yields can be realized with lower

² These pretreatments span a wide pH range and remove different amounts of hemicelluloses and lignin. Therefore, to facilitate comparisons, enzyme loadings were based on the mass of glucan in biomass before pretreatment.

³ Cellulase mixture's 15 FPU was without including the exogenous beta-g supplementation.

Table 1

Pretreatment conditions, compositions, and yields of glucan for solids prepared by leading technologies.

Pretreatment	Pretreatment conditions	Yield of component in pretreated solids (%)		Composition of pretreated solids (%)		
		Glucan	Xylan	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	100	100	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	98.61	48.1	61.9	17.9	8.8
Dilute acid (Sunds System)	180 °C, 0.03H ₂ SO ₄ :Dry wt, 90 s, 25% solids-HW	93.4	27.2	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	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	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-D- 100% deacetylation.

NA- not available.

Table 2Total combined amounts of cellulase, β -glucosidase, and xylanase protein per g glucan in unpretreated corn stover for various xylanase to cellulase protein ratios.

Xylanase to cellulase protein mass ratio [designation]	Total amount of protein, mg/g original glucan	
	14.5 mg cellulase (~7.5 FPU)	29.0 mg cellulase (~15 FPU)
0.0:1 [MX ₀]	14.5	29.0
0.2:1 [MX _{0.2}]	16.8	33.7
0.5:1 [MX _{0.5}]	21.1	42.1
1:1 [MX ₁]	28.1	56.1
2:1 [MX ₂]	42.1	84.2
5:1 [MX ₅]	84.2	168.4
11:1 [MX ₁₁]	168.4	NA

protein demands by reconstituting the enzyme cocktail to include other enzymes than just cellulase (Hespell et al., 1997). As shown in Fig. 1, xylanase supplementation of just 1/5th (MX_{0.2}) of the cellulase mass loading increased⁴ glucose yields by about 9% and 8.5% at 14.5 and 29.0 mg of cellulase plus β -glucosidase mass loading, respectively, and xylose yields by 30% and 15% at the corresponding loadings. Furthermore, 72 h glucose and xylose release continued to increase with further xylanase supplementation. The increase in glucose and xylose yields (40.5% and 101%, respectively) at a xylanase supplementation mass ratio of 5 (MX₅) was much higher at the lower cellulase plus β -glucosidase mass loading of 14.5 mg than the increase of 19.4% and 49.1%, respectively, at 29.0 mg cellulase. Furthermore, glucan digestibility at a cellulase plus β -glucosidase mass loading of 14.5 mg and a xylanase supplementation mass ratio of 1 (MX₁) for a total of 29 mg of protein was ~10% higher than digestibility with cellulase alone with the same total mass of protein (15 FPU) and almost equal to the digestibility for a cellulase plus β -glucosidase mass loading of 120 mg (60 FPU). Thus, adding more xylanase was more effective than adding more cellulase.

Xylose release at a cellulase plus β -glucosidase loading of 14.5 mg and xylanase supplementation mass ratio of 1 was almost equal and a bit lower than at the cellulase mass loading of 29 and 120 mg, respectively. Yet, it was also observed that >90% glucan digestion was obtained at a cellulase plus β -glucosidase loading of just 14.5 mg for a xylanase supplementation mass ratio of 5 (MX₅; total protein ~85 mg), but xylan digestibility was still <80% for both cellulase mass loadings for a xylanase supplementation mass ratio of 5 (total protein 84.0 and 168 mg, respectively). Thus, because soluble xylooligomers might inhibit enzyme action (Kumar and Wyman, 2009-b), 14 mg β -xylosidase was added

per g of glucan for a cellulase plus β -glucosidase mass loading of 14.5 mg at a xylanase supplementation mass ratio of 5 (MX₅) (total protein ~88.0 mg/g glucan in unpretreated biomass). In this case, xylose release was enhanced by about 9%, even though glucose release was virtually unaffected. Thus, even though xylose yield increased to ~90% for supplementation of cellulase with both xylanase and β -xylosidase, small amounts of other xylan debranching enzymes (such as feruloyl esterase) may be beneficial to attain complete hydrolysis of xylan in AFEX pretreated corn stover because xylan branching inhibits xylanase and probably cellulase activity as well (Anand and Vithayathil, 1996; Kormelink and Voragen, 1992; Kumar and Wyman, 2009b; Suh and Choi, 1996).

3.1.2. ARP

Although ARP pretreatment typically removes about 50% of the xylan and 80% of the lignin from corn stover (Kim and Lee, 2005; Kim et al., 2006; Wu and Lee, 1997), the xylan content in pretreated solids was still about 18% due to high lignin removal. As shown in Fig. 2, xylanase supplementation enhanced digestion less for ARP pretreated corn stover than for AFEX. In fact, a xylanase supplementation mass ratio of 5 (MX₅) increased glucose yields by about 30.0% and 9.0% at cellulase mass loadings of 14.5 and 29.0 mg, respectively, and xylose yields by about 60.5% and 31.7% in the same order of cellulase loadings. Furthermore, glucan and xylan digestion was much higher for the lower cellulase mass loading of 14.5 mg. However, glucan and xylan digestion for both cellulase loadings were still lower than for a cellulase plus β -glucosidase mass loading of 120 mg corresponding to 60 FPU/g original glucan (95.2% and 98% for glucan and xylan, respectively). Although experiments were not run, a low level of β -xylosidase supplementation could possibly improve xylose and glucose release further.

⁴ Based on the total mass of just cellulase and β -glucosidase.

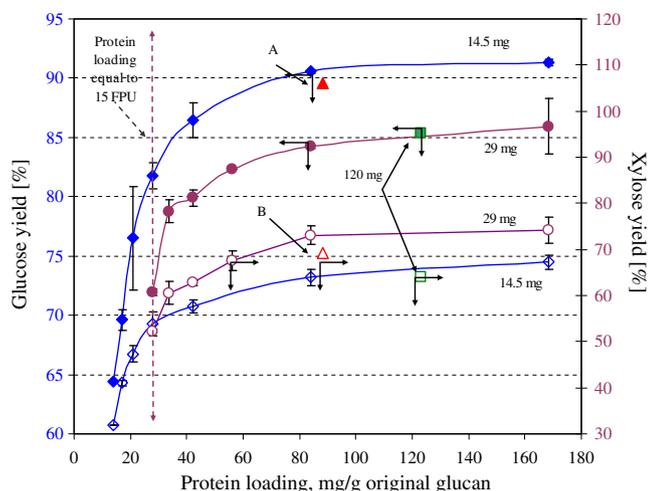


Fig. 1. Glucose and xylose yields after 72 h of hydrolysis vs. total protein loadings for xylanase supplementation of constant cellulase loadings corresponding to about 14.5 and 29 mg protein/g original glucan for AFEX pretreated corn stover solids. Closed symbols represent glucose yields and open symbols designate xylose yields. A and B are glucose and xylose yields at 14.5 mg cellulase + MX_5 + β -xylosidase. Error bars represent the standard deviation.

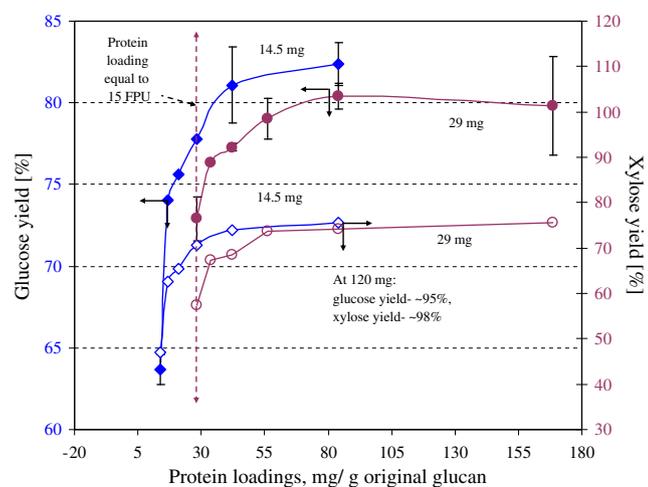


Fig. 2. Glucose and xylose yields after 72 h of hydrolysis vs. total protein loadings for xylanase supplementation of constant cellulase loadings corresponding to about 14.5 and 29 mg protein/g original glucan for ARP pretreated corn stover solids. Closed symbols represent glucose yields and open symbols designate xylose yields. Error bars represent the standard deviation.

3.1.3. Dilute acid

To evaluate how lower pretreatment severity impacted enzyme effectiveness, corn stover was pretreated with dilute acid at a low severity level of $\log R_0 = 2.53$ using the Sunds hydrolyzer (Metso Paper USA, Inc., Norcross, GA, USA) at NREL for comparison to results for a high severity of about 3.06 reported elsewhere (Lloyd, 2005; Lloyd and Wyman, 2005). As is typical, severity is defined as $R_0 = t \cdot \exp((T-100)/14.73)$ with t the time in minutes and T the temperature in $^{\circ}C$ (Lloyd and Wyman, 2005; Overend and Chornet, 1987). In this case, about 94% and 27% of the original glucan and xylan, respectively, were retained in the solids from the Sunds reactor. As shown in Fig. 3, digestion of these solids was much lower at a cellulase mass loading of 14.5 mg/g original glucan than reported in other studies with solids resulting from dilute acid pretreatment of corn stover with a Parr reactor (Lloyd and Wyman, 2005). Furthermore, although xylanase supplementation did not increase glucose release much (3–8%) for the two fixed

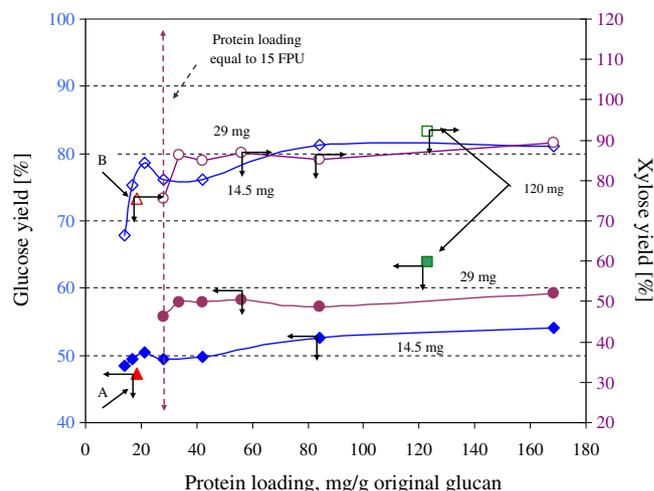


Fig. 3. Glucose and xylose yields after 72 h of hydrolysis vs. total protein loadings for xylanase supplementation of constant cellulase loadings corresponding to about 14.5 and 29 mg protein/g original glucan for dilute acid pretreated corn stover solids. Closed symbols represent glucose yields and open symbols designate xylose yields. A and B are glucose and xylose yields at 14.5 mg cellulase + β -xylosidase.

cellulase mass loadings, xylose release increased by 33.6% and 12.9% for cellulase plus β -glucosidase mass loadings of 14.5 and 29.0 mg, respectively, for a xylanase supplementation mass ratio of 5 (MX_5). At equal protein levels, sugar yields were higher for a mass loading of 120 mg of just cellulase plus beta-glucosidase/g glucan in unpretreated corn stover than possible with xylanase supplementation at cellulase loadings of 14.5 and 29.0 mg. β -xylosidase supplementation increased xylose yield by about 13.6% and had a negligible effect on glucose release.

Based on these results and data reported elsewhere for dilute acid pretreatment (Lloyd and Wyman, 2005; Yang and Wyman, 2004), digestion is poor for less severe pretreatment, suggesting that disruption of the biomass complex network appears more important than removing/retaining xylan, lignin, or both. For example, the acetyl content in pretreated corn stover solids decreased with severity for dilute acid pretreatment from about 1.1% for the Sunds reactor (180 $^{\circ}C$, 1.5 min, 3.0% acid, $\log R_0 = 2.5$) to 0.2% (160 $^{\circ}C$, 20 min, 0.5% acid, $\log R_0 = 3.1$) and 0.3% (140 $^{\circ}C$, 40 min, 1.0% acid, $\log R_0 = 2.8$). Presumably other internal linkages are disrupted to a greater extent at elevated severity. Gupta and Lee reported that the digestibility of solids from ammonia recycle pretreatment increased with pretreatment temperature, even though the compositions of pretreated solids prepared at different temperature were almost the same (Gupta et al., 2008).

3.1.4. Lime

Similar to other alkaline methods, lime pretreatment leaves a major portion of the initial xylan in the solids (Chang et al., 1997; Kaar and Holtzapple, 2000), and consequently, enzymes must release xylose and other sugars in hemicellulose from the solids in addition to glucose to realize high total sugar yields. However, Kim and Holtzapple reported a xylan digestibility of only about 50% for lime pretreated corn stover solids at a cellulase loading of 15 FPU (Kim and Holtzapple, 2005), and higher mass loadings of cellulase with just β -glucosidase resulted in xylose yields below 75% (Kumar and Wyman, 2009b). The impact of supplementing cellulase with xylanase on xylose and glucose release is shown in Fig. 4 for lime pretreated corn stover at the same two fixed cellulase plus β -glucosidase mass loadings as employed previously. Now xylanase supplementation at a mass ratio of 5 (MX_5) resulted in modest 8% and 3.8% increases in glucose yields for cellulase plus β -glucosidase mass loadings of 14.5 and 29.0 mg,

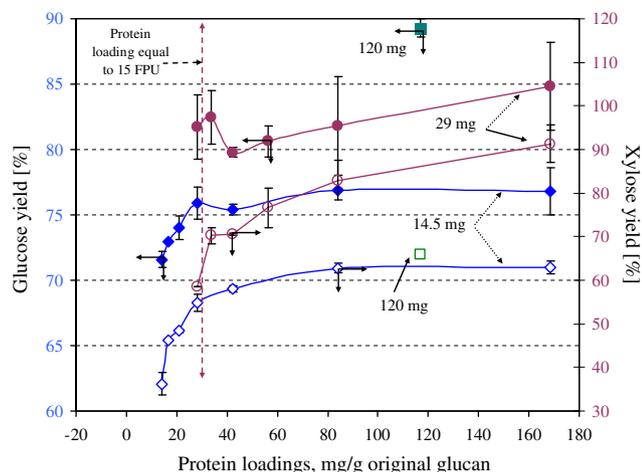


Fig. 4. Glucose and xylose yields after 72 h of hydrolysis vs. total protein loadings for xylanase supplementation of constant cellulase loadings corresponding to about 14.5 and 29 mg protein/g original glucan for lime pretreated corn stover solids. Closed symbols represent glucose yields and open symbols designate xylose yields. Error bars represent the standard deviation.

respectively but much higher increases in xylose yields of 74% and 58%, respectively. Furthermore, although glucan digestibility at a cellulase plus β -glucosidase mass loading of 120 mg was much higher than for both of the lower cellulase mass loadings of 14.5 and 29.0 mg with xylanase supplementation, xylose release at 120 mg cellulase plus β -glucosidase mass loading was about equal to that for a cellulase mass loading of 14.5 mg with xylanase supplementation and much lower than for a cellulase mass loading of 29.0 mg with xylanase supplementation. On the other hand, adding β -xylosidase to 14.5 mg of cellulase plus β -glucosidase supplemented with xylanase at a mass ratio of 5 (MX_5) had a negligible impact on glucose and xylose release (1% and 2.5%, respectively) (data not shown). Overall, based on these results and those reported elsewhere (Kumar and Wyman, *in press*) for poplar substrate, enzymatic removal of xylan resulted in a negligible increase in glucan digestion for lime pretreated corn stover solids, making xylanase supplementation of cellulase appear less promising than for AFEX and APR pretreated solids. However, as reported elsewhere (Kumar and Wyman, *in press-a*), additives had a greater impact on glucose and xylose release when added to just cellulase and β -glucosidase than the enhancement observed here with xylanase supplementation. Thus, use of surfactants may be more effective with lime pretreatment than supplementing with xylanase or β -xylosidase.

3.1.5. Controlled pH

As shown in Fig. 5, xylanase supplementation at a mass ratio of 5 (MX_5) resulted in modest 10% and 15% increases in glucose yields for cellulase plus β -glucosidase mass loadings of 14.5 and 29.0 mg, respectively, but much higher increases in xylose yields of 110% and 39%, respectively. Thus, these solids displayed limited enhancement in glucose release with xylan removal, similar to results for lime pretreatment and in contrast to those for AEX and ARP. Both lime and controlled pH left significant amounts of xylan in the pretreated solids, as shown in Table 1, but the fact that virtually all of the xylan is left in solids following AFEX pretreatment would appear to contradict a firm conclusion. Adding β -xylosidase (14.0 mg/g glucan) with a cellulase mass loading of 14.5 mg and a xylanase mass supplementation ratio of 5 resulted in an additional 6% improvement in xylose release, and a negligible increase in glucose release (data not shown). Overall, cellulase with just β -glucosidase at a total protein loading of 29.0 mg/g glucan in

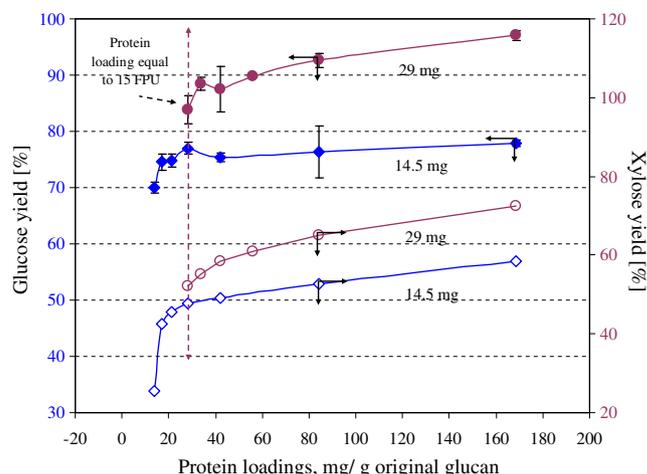


Fig. 5. Glucose and xylose yields after 72 h of hydrolysis vs. total protein loadings for xylanase supplementation of constant cellulase loadings corresponding to about 14.5 and 29 mg protein/g original glucan for controlled pH pretreated corn stover solids. Closed symbols represent glucose yields and open symbols designate xylose yields. Error bars represent the standard deviation.

unpretreated corn stover released more glucose and xylose than adding xylanase to cellulase for the same total mass of protein.

3.1.6. SO_2

Xylanase supplementation significantly increased glucose and xylose release from SO_2 pretreated corn stover solids that contained about 11% xylan. As shown in Fig. 6, xylanase supplementation at a mass ratio of 5 (MX_5) resulted in 15% and 9% increases in 72 h glucose yields for cellulase plus β -glucosidase mass loadings of 14.5 and 29.0 mg, respectively, and higher increases in xylose yields of 39% and 22%, respectively. However, for a cellulase mass plus β -glucosidase loading of 14.5 mg, glucose and xylose release continued to increase beyond the xylanase mass supplementation ratio of 5, as shown in Fig. 6. At an equal amount of total protein loading of 29.0 mg, as shown by the vertical dashed line, the 72 h release of glucose and xylose was higher when xylanase was added to a lower cellulase mass loading of 14.5 mg/g glucan in unpretreated corn stover than for a higher cellulase plus β -glucosidase loading without xylanase supplementation. At an equal total

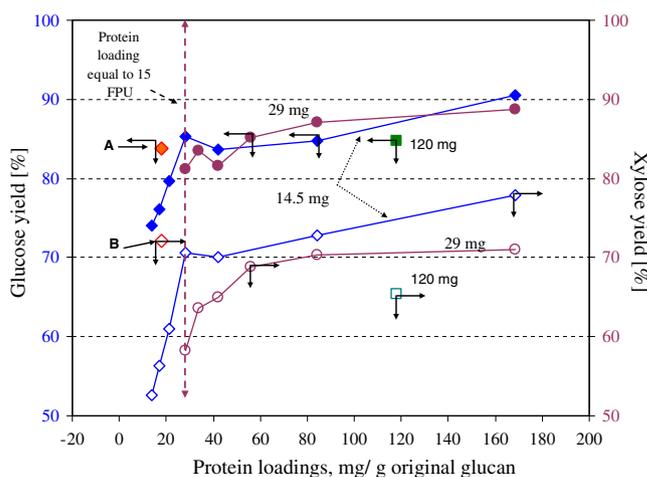


Fig. 6. Glucose and xylose yields after 72 h of hydrolysis vs. total protein loadings for xylanase supplementation of constant cellulase loadings corresponding to about 14.5 and 29 mg protein/g original glucan for SO_2 pretreated corn stover solids. Closed symbols represent glucose yields and open symbols designate xylose yields. A and B are glucose and xylose yields at 14.5 mg cellulase + β -xylosidase.

protein mass loading, glucose and particularly xylose release were higher for a cellulase mass loading of 14.5 and 29.0 mg with xylanase supplementation than for using 120 mg of just cellulase and β -glucosidase.

In additional experiments, using β -xylosidase at a loading of 14.0 mg/g glucan with cellulase at a mass loading of 14.5 mg increased glucose and xylose release by 12% and 35%, respectively, the same as gained by a xylanase mass supplementation ratio of 1. Furthermore, adding β -xylosidase to cellulase plus β -glucosidase at a mass loading of 14.5 mg supplemented with xylanase at a mass ratio of 1 increased glucose and xylose release by 15% and 58%, respectively (data not shown in the Figure). Thus, a significant amount of soluble xylooligomers apparently formed during enzymatic hydrolysis of SO_2 pretreated corn stover that β -xylosidase effectively removed.

3.2. Cellulase–xylanase interactions

Synergism is often defined as the ratio of the rate or yield of a single product released by the simultaneous action of enzymes to the sum of rate or yield of these products when produced by the action of individual enzymes when used separately in the same amounts as in the mixture. However, because this study followed release of two products, glucose and xylose, over a range of loadings of xylanase, a new term xylanase leverage was defined as the ratio of the percent increase in glucose release to the percent increase in xylose release to illustrate how releasing additional xylan enhanced glucan hydrolysis, as discussed elsewhere (Kumar and Wyman, *in press*). As summarized in Table 3, the degree of xylanase leverage on cellulase effectiveness varied with the type of pretreatment.

As shown in Fig. 7a and b for AFEX and ARP pretreated corn stover solids, respectively, a strong linear relation ($R^2 = 0.97$ and 0.85 for AFEX and 0.97 and 0.89 for ARP corn stover solids for 14.5 and 29 mg cellulase plus β -glucosidase mass loading, respectively) was found between the increase in glucose release and the increase in xylose release. However, it is also interesting to note that the impact of xylanase was more sensitive to enzyme loadings for ARP than AFEX, which means that with increased cellulase loading the leverage factors for ARP decreased more than for AFEX. In any event, linear relationships of this nature were observed for enzymatic digestion with xylanase supplementation of solids prepared by the other pretreatments, inferring that enhancing xylan removal with xylanase made glucan more accessible to cellulase.

3.2.1. Factors affecting cellulase–xylanase interaction

As shown in Table 3, xylanase leverage varied with the type of pretreatment. Furthermore, while ARP pretreated solids showed the highest xylanase leverage for a cellulase mass loading of 14.5 mg, controlled pH pretreated solids had the highest glucose release for a given increase in xylose release at a cellulase mass loading of 29.0 mg. On the other hand, lime pretreated solids con-

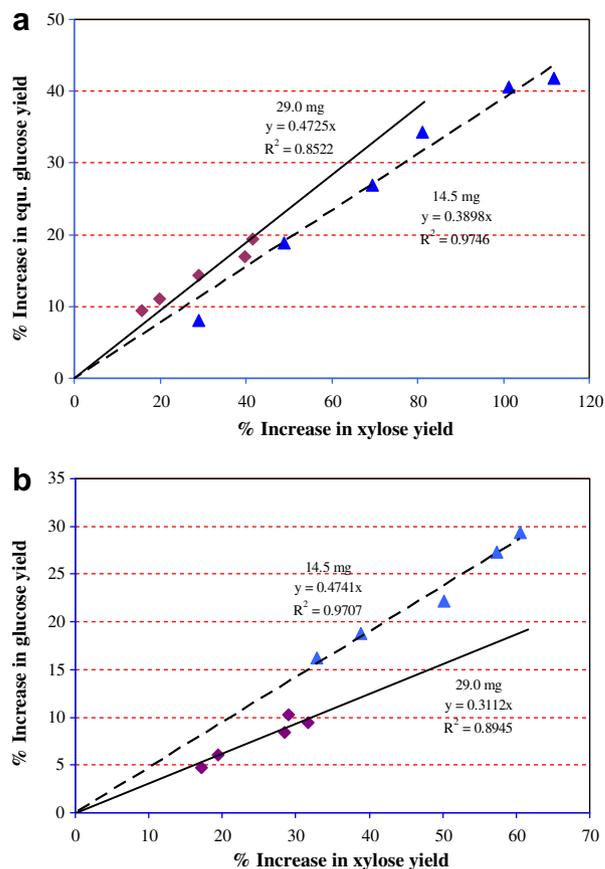


Fig. 7. Relationship between release of glucose and removal of xylose as a result of enzymatic hydrolysis with fixed cellulase loadings of 14.5 and 29.0 mg/g original glucan and different levels of xylanase supplementation for corn stover solids produced by (a) AFEX and (b) ARP pretreatments.

taining residual xylan in solids comparable to other alkaline pretreatments showed a very low leverage for both cellulase mass loadings. Overall, the data in Table 3 make it difficult to conclude that removal of xylan always enhances glucan conversion, as apparently there was no direct relationship between xylanase leverage and the amount of residual xylan in solids. Consistent with this observation, García-Aparicio et al. (2007) reported that xylanase leverage on cellulase action was not dependent on the amount of residual xylan in pretreated biomass. Furthermore, as observed in this study, there was no evident relationship between the amount of acetyl groups and xylanase leverage for both cellulase mass loadings. The residual amount of acetyl groups, xylan, and lignin in pretreated solids; the degree of cellulose crystallinity; enzyme loadings; and length of hydrolysis time have also been thought to affect xylanase leverage (Kumar and Wyman, *in press*;

Table 3

The degree of leverage between cellulase and xylanase for two fixed cellulase plus β -glucosidase mass loadings with multiple xylanase supplementations.

Pretreatment	% Residual xylan in pretreated solid	% Acetyl content	Degree of leverage ^a	
			@ 7.5 FPU	@ 15 FPU
Untreated	22.8	2.51	–	–
AFEX	22.8	1.69	0.390	0.473
ARP	17.9	0.30	0.474	0.311
Dilute acid (Sunds)	9.5	1.13	0.221	0.286
Lime	26.4	0.20	0.099	0.001
Controlled pH	16.2	1.14	0.089	0.660
SO_2	11.6	1.15	0.427	0.349

^a Leverage = % increase in glucan digestion/% increase in xylan conversion.

Selig et al., 2008). For example, because xylan digestion is enhanced by removal of acetyl or any other xylan branching groups, Grohmann et al. (1989) suggested that such groups affect xylanase leverage and their removal should enhance cellulose accessibility (Fernandes et al., 1999; Kormelink and Voragen, 1992; Suh and Choi, 1996; Wood and McCrae, 1986). But, experimental verification is lacking of whether removal of acetyl group results into increased xylanase leverage or cellulose accessibility or both.

To determine the role of acetyl groups in xylanase leverage and whether their removal enhanced cellulose accessibility, two separate set of hydrolysis experiments were run for corn stover solids, which were deacetylated to various degrees, with cellulase plus β -glucosidase supplemented with xylanase and with just purified CBH-I (Cel7A). As shown in Fig. 8a for a cellulase plus β -glucosidase mass loading of 29.0 mg with xylanase supplementation mass ratios of 2 and 5 (MX₂ and MX₅), xylanase supplementation increased glucose and xylose release much more for deacetylated corn stover (De-B and De-D) compared to untreated corn stover (UT CS). In addition, the linear relationships between increases in glucose and xylose release for the limited data available in Fig. 8b suggest that leverage increased with removal of acetyl groups. For example, glucose release increased almost twice as much with xylose removal for completely deacetylated corn stover compared to untreated corn stover and about 1.3 times as much when 50% of the acetyl content was removed. As shown in Fig. 8c, deacetylation of corn stover enhanced cellobiose production by purified CBH-I at a loading of 15 mg/g glucan, with generation increasing by about 530% for complete removal. Hence, removal of acetyl groups not only increased xylanase leverage but enhanced cellulose accessibility and/or CBHI effectiveness as well. Yet, the fact that lime pretreated solids with the lowest residual acetyl groups did not display the highest xylanase leverage suggests that acetyl content is not the only factor controlling xylanase leverage. Furthermore, no clear correlation was found between acetyl content and the degree of xylanase leverage in this study.

To unravel this complex situation, xylanase leverage data were correlated with substrate features and enzyme loadings using the statistical software Minitab based on the xylanase leverage data; cellulase mass loadings; acetyl, xylan, and lignin content; and cellulose crystallinity index (CrI) summarized in Table 4. The multiple variables model used for linear regression is as follows:

$$Y = A_0 + A_1 * [\text{CrI}] + A_2 * [\% \text{acetyl content}] + A_3 * [\% \text{glucan}] + A_4 * [\% \text{xylan}] + A_5 * [\% \text{lignin}] + A_6 * [\text{cellulase loading}]$$

in which Y = xylanase leverage, as defined by the ratio of percentage increase in glucan conversion to percentage increase in xylan conversion, A₀ to A₆ = constants, and cellulase loading = mg protein/g original glucan.

The parameters for substrate features and cellulase loadings were linearly regressed to give the coefficients shown in Table 5 for the best fit as determined in terms of the statistical coefficient R² (0.537). The low value of the statistical coefficient R² indicates that leverage is more complex than can be explained by this model, and further refinement is needed in the parameters included and their relationships to leverage. Nonetheless, although the correlation was not great, acetyl content appeared to have the biggest impact on xylanase leverage, followed by crystallinity of biomass and lignin content. However, the importance of the parameters may also be interpreted based on their p values (set value 0.1 at 90% confidence interval) shown in Table 5, from which crystallinity index would be concluded to have the greatest effect on xylanase leverage. Experimental and predicted values of xylanase leverage factors (LF) are shown in Table 4 and Fig. 9.

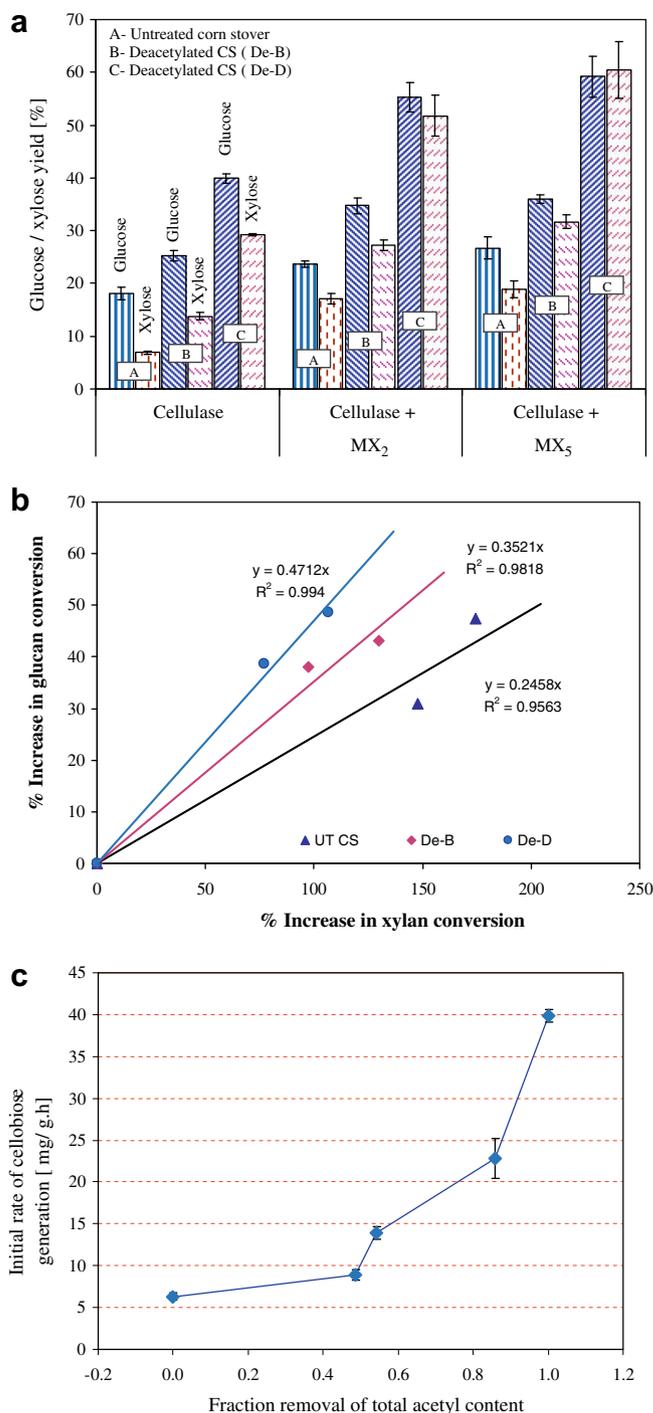


Fig. 8. (a) Glucose and xylose yields after 72 h of hydrolysis for xylanase supplementation of constant cellulase mass loadings of 29 mg/g original glucan for untreated (UT CS) and deacetylated (54%-De-B, 100%-De-D) corn stover solids; (b) changes in glucan conversion with xylan conversion for UT and De CS; and (c) impact of deacetylation of biomass on cellobiose generation for hydrolysis with purified CBH-I. Error bars represent the standard deviation.

3.2.2. Mechanism of xylanase leverage

Several studies reported a strong relationship between xylan removal and the extent of glucan digestion (Allen et al., 2001; Ishizawa et al., 2007; Kumar and Wyman, in press; Yang and Wyman, 2004; Zhu et al., 2005), and two hypothesized mechanisms are removal of xylan that coats glucan chains, making them more accessible to cellulase, and disruption of xylan linkages to glucan (Murashima et al., 2003; Teleman et al., 2001). Unfortunately,

Table 4
Cellulase–xylanase leverage data and substrate features for unpretreated and deacetylated corn stover and corn stover pretreated by leading pretreatment technologies.

Pretreatment	% Glucan	% Xylan	% Lignin	% Acetyl content	CrI ^d	Cellulase loading ^e	Xylanase leverage factor (exp.)	Xylanase leverage factor (model)
UT- CS	38.3	21.7	20.5	2.51	50.3	29.0	0.2458	0.2262
De-B ^a	43.0	26.4	19.7	1.15	50.3 ^c	29.0	0.3521	0.3055
De-D ^b	42.1	23.4	19.5	0.03	50.3 ^c	29.0	0.4712	0.4763
AFEX	34.4	22.8	18.0	1.70	36.3	14.5	0.3898	0.4129
ARP	61.9	17.9	8.8	0.30	25.3	14.5	0.4741	0.3423
DA	59.3	9.3	22.5	1.13	52.5	14.5	0.2210	0.2645
Lime	56.7	26.4	14.6	0.30	56.2	14.5	0.0988	0.0333
Controlled pH	52.7	16.2	25.2	1.70	44.5	14.5	0.0892	0.3609
SO ₂	56.9	11.6	23.8	1.15	53.0	14.5	0.4274	0.2864
AFEX	34.4	22.8	18.0	1.70	36.3	29.0	0.4725	0.4862
ARP	61.9	17.9	8.8	0.30	25.3	29.0	0.3112	0.4156
DA	59.3	9.3	22.5	1.13	52.5	29.0	0.2859	0.3378
Lime	56.7	26.4	14.6	0.30	56.2	29.0	0.0009	0.1066
Controlled pH	52.7	16.2	25.2	1.70	44.5	29.0	0.6600	0.4342
SO ₂	56.9	11.6	23.8	1.15	53.0	29.0	0.3485	0.3597

^a ~54% deacetylated corn stover.

^b ~100% deacetylated corn stover.

^c It was assumed that with deacetylation the CrI of biomass does not change.

^d Crystallinity values were taken from Laureano-Perez et al. (2005).

^e Cellulase loading- mg/g glucan in unpretreated corn stover.

Table 5
Variable coefficients, their standard error, and significance (p value).

Predictor	Coefficient	Standard error of coeff.	p value
Constant [A0]	1.110	0.791	0.198
CrI [A1]	-0.013	0.007	0.087
% Acetyl content [A2]	-0.128	0.097	0.225
% Glucan [A3]	-0.008	0.008	0.350
% Xylan [A4]	-0.008	0.014	0.597
% Lignin [A5]	0.022	0.019	0.275
Cellulase loading [A6]	0.005	0.006	0.416

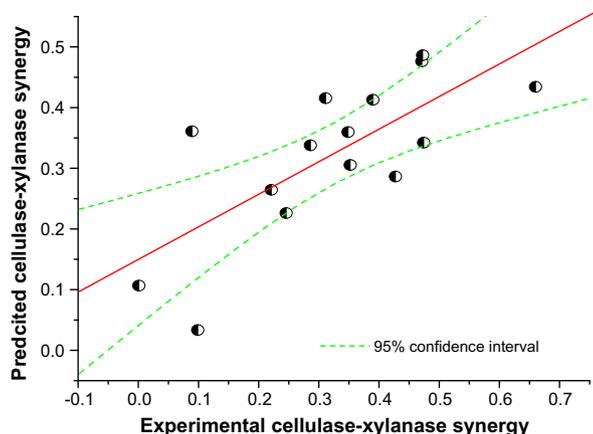


Fig. 9. Predicted vs. experimental xylanase leverage for two CTB loadings for xylanase supplementation mass ratios up to 11.

these hypotheses are difficult to prove experimentally due to the complex nature of biomass. In the case of xylanase leverage, a plausible explanation could be that xylanase removes redeposited xylan and xylooligomers from the surface, thereby increasing the accessibility of cellulose microfibrils to cellulase. However, the extent of xylan and/or xylooligomers aggregation on cellulose and the strength of its binding by covalent and hydrogen bonds are affected by physical features, the type of xylan, cellulose characteristics, and the presence of lignin (Chambat et al., 2005; Gray et al., 2007; Hansson and Hartler, 1969; Kabel et al., 2007b; Kaya et al., 2005; Linder et al., 2003a,b; Mora et al., 1986; Westbye et al.,

2007; Whitney et al., 1995). In addition, xylan accessibility could in turn be limited by the presence of glucan microfibrils (Teleman et al., 2001), as xylan is believed to be intertwined with glucan chains and may play a vital role in xylanase leverage on cellulase action.

Sugars and oligomers released during hydrolysis could also be factors. As reported elsewhere (Kumar and Wyman, 2009a), the low xylanase activity in commercial enzymes produces a significant amount of xylooligomers during enzymatic hydrolysis of biomass solids that contain xylan, and these xylooligomers are strongly inhibitory to cellulase. For example as shown in Fig. 10, the initial rate of glucose release during hydrolysis of Avicel glucan mixed with an equal amount of birchwood xylan at a cellulase loading of 7.5 FPU/g glucan dropped by 55% compared to hydrolysis of Avicel alone. While xylose mixed with Avicel in an amount equivalent of xylan, had a limited inhibition of enzyme action (12%), inhibition by xylose, xylooligomers, and/or xylan was reduced by ~70% when β -xylosidase was added to the cellulase. Thus, removing xylooligomers by β -xylosidase or its activity in xylanase could reduce inhibition of cellulase and speed conversion.

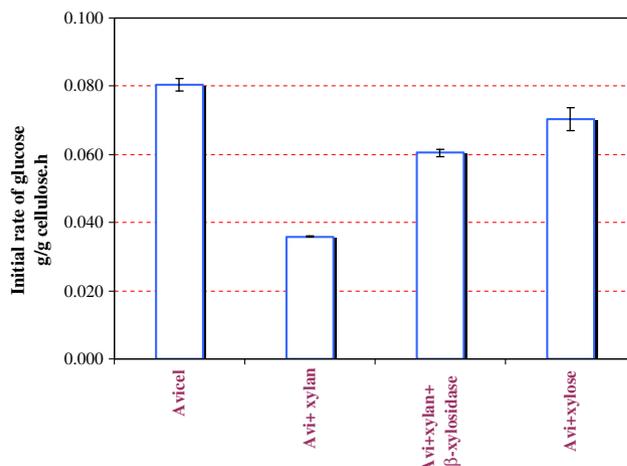


Fig. 10. Effect of xylooligomers on the 4 h rate of 1%(w/v) Avicel glucan digestion at a cellulase loading of 7.5 FPU/g glucan. Avicel + xylan- 1%(w/v) each, Avicel + xylose- xylose equivalent to 1%(w/v) xylan. β -xylosidase loading was 28 mg/g glucan. Error bars represent the standard deviation.

Table 6

Summary of percentage increase in 72 h glucose and xylose release for cellulase β -glucosidase mass loadings of 14.5 and 29.0 mg/g glucan in unpretreated corn stover with xylanase supplementation mass ratios of 1 and 5.

Xylanase to cellulase protein mass ratio	SO ₂		DA		Controlled pH		AFEX		ARP		Lime	
	CE-A	CE-B	CE-A	CE-B	CE-A	CE-B	CE-A	CE-B	CE-A	CE-B	CE-A	CE-B
<i>% Increase in glucose yield</i>												
1 (MX ₁)	15.1	14.8	2.1	4.3	9.9	7.1	26.9	14.4	22.1	8.4	3.3	1.3
5 (MX ₅)	15.2	24.5	8.6	6.2	10	15.7	40.5	19.4	29.4	9.5	7.4	1.6
<i>% Increase in xylose yield</i>												
1 (MX ₁)	34.1	25	20.9	15.3	87.7	17	69.5	29.1	50.1	28.5	33.3	28.2
5 (MX ₅)	38.2	37.6	33.6	18.5	108	39	101.5	41.6	59.9	31.7	59.9	52.7
<i>% Increase in total sugar yield</i>												
1 (MX ₁)	49.2	39.8	23	19.6	97.6	24.1	96.4	43.5	72.2	36.9	36.6	29.5
5 (MX ₅)	53.4	62.1	42.2	24.7	118	54.7	142	61	89.3	41.2	67.3	54.3

CE-A – cellulase plus β -glucosidase mass loading of 14.5 mg/g glucan in unpretreated corn stover.

CE-B – cellulase plus β -glucosidase mass loading of 29.0 mg/g glucan in unpretreated corn stover.

4. Conclusions

Glucan and xylan digestion data for cellulase plus β -glucosidase mass loadings of 14.5 and 29.0 mg/g glucan in raw corn stover with xylanase supplementation ratios of 1 and 5 are summarized in Table 6. Xylanase supplementation substantially increased xylose release from solids resulting from all pretreatments but had less effect on glucose release. Overall, xylanase supplementation enhanced pretreatment performance at a given enzyme loading in the following order of increasing impact with the levels of xylan left in the solids after pretreatment indicated in parentheses: dilute acid (9.3%) < lime (26.4%) < SO₂ (11.6%) < ARP (17.9%) < controlled pH (16.2%) < AFEX (22.8%). Therefore, as expected, xylanase supplementation tended to be more effective for pretreatments that removed less xylose into solution prior to enzymatic hydrolysis, but lime pretreatment proved a substantial exception to this generalization.

Although total sugar release decreased with decreasing cellulase plus β -glucosidase mass loading, as shown in Table 6, xylanase leverage, the ratio of the increase in glucose release to the increase in xylose release, was not directly related to cellulase mass loadings, consistent with the lower enhancement in sugar release at higher cellulase mass loadings reported elsewhere (Kumar and Wyman, in press; Selig et al., 2008). Furthermore, leverage varied with the choice of pretreatment. ARP solids, at the lower CTB loading of 14.5 mg, showed the highest xylanase leverage (0.474) followed by SO₂ (0.427) and AFEX (0.390). However, at the higher CTB loading of 29 mg, controlled pH solids demonstrated the highest xylanase leverage (0.660) followed by AFEX (0.473) and SO₂ (0.349), and leverage for lime solids was almost negligible. Thus, for some pretreatments such as AFEX, dilute acid, and controlled pH, leverage increased with cellulase loading while for others the opposite resulted. Possible reasons for this behavior could be higher acetyl content in pretreated solids for these pretreatments, as shown in Table 4, and inhibition by larger amounts of xylooligomers generated at lower cellulase loadings. In addition, it is expected that acetylated soluble xylooligomers should be more inhibitory to enzymes than simpler soluble xylooligomers (Anand and Vithayathil, 1996; Kabel et al., 2007a; Kumar and Wyman, 2009b; Suh and Choi, 1996).

Linear regression statistical modeling for untreated and pretreated corn solids showed that xylanase leverage did not have a significant dependence on acetyl content ($R^2 = 0.021$, p value = 0.605 at 90% confidence interval), xylan content ($R^2 = 0.03$, p value = 0.536 at 90% confidence interval), or the two together ($R^2 = 0.044$, p value = 0.764 at 90% confidence interval). However, the statistical model applied showed that cellulose crystallinity had the most significant effect, with less significance for acetyl

content and lignin. Contrary to statistical modeling predictions, selective acetyl removal from untreated corn stover enhanced xylanase leverage and had a significant impact on cellulose accessibility or cellulase effectiveness or both, as hydrolysis of solids with purified CBHI showed a significant increase in hydrolysis rate.

Consistent with the statistical modeling, the amount of xylan in biomass can be hypothesized to play an indirect role in xylanase leverage. During cellulose synthesis by plants, hemicellulose can alter crystallinity, the ratio of different crystalline polymorphs ($I\alpha/I\beta$) of cellulose (Hanus and Mazeau, 2006; Heiko Winter, 2005; Tokoh et al., 1998, 2002a,b; Uhlin et al., 1995; Whitney et al., 1995), surface area, and other features which in turn could impact hemicellulose linkages/coating/association with cellulose (Chambat et al., 2005; Linder et al., 2003a; Whitney et al., 1995). Therefore, strong xylan coating/linkages to amorphous cellulose could be left intact for pretreatments that do not remove much xylan or lignin during pretreatment such as AFEX, possibly explaining the strong leverage observed between cellulase and xylanase. Other pretreatments such as dilute acid and lime likely disrupt the strong xylan association/coating on amorphous cellulose and remove some xylan loosely associated with crystalline cellulose, resulting in a low degree of xylanase leverage. However, further research is needed to verify such hypotheses and identify what other factors control xylanase leverage on glucan release.

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