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Hydrolysis of different chain length xylooliogmers by cellulase and hemicellulase

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ABSTRACT

Commercial cellulase complexes produced by cellulolytic fungi contain enzyme activities that are capable of hydrolyzing non-cellulosic polysaccharides in biomass, primarily hemicellulose and pectins, in addition to cellulose. However, xylanase activities detected in most commercial enzyme preparations have been shown to be insufficient to completely hydrolyze xylan, resulting in high xylooligomer concentrations remaining in the hydrolysis broth. Our recent research showed that these xylooligomers are stronger inhibitors of cellulase activity than others have previously established for glucose and cellobiose, making their removal of great importance. In this study, a HPLC system that can measure xylooligomers with degrees of polymerization (DP) up to 30 was applied to assess how Spezyme CP cellulase, Novozyme 188 β-glucosidase, Multifect xylanase, and non-commercial β-xylosidase enzymes hydrolyze different chain length xylooligomers derived from birchwood xylan. Spezyme CP cellulase and Multifect xylanase partially hydrolyzed high DP xylooligomers to lower DP species and monomeric xylose, while β-xylosidase showed the strongest ability to degrade both high and low DP xylooligomers. However, about 10–30% of the higher DP xylooligomers were difficult to be breakdown by cellulase or xylanase and about 5% of low DP xylooligomers (mainly xylobiose) proved resistant to hydrolysis by cellulase or β-glucosidase, possibly due to low β-xylosidase activity in these enzymes and/or the precipitation of high DP xylooligomers.

1. Introduction

Conversion of lignocellulosic materials into liquid fuels such as ethanol deserves more attention to meet a critical need for environmentally friendly and sustainable transportation fuels that can reduce our heavy dependence on vulnerable supplies of petroleum. Most lignocellulosic materials including woods, grasses, and agricultural residues are primarily composed of cellulose, hemicelluloses and lignin, and high yields from both cellulose and hemicellulose are important to overall economics (Wyman, 2003). The cellulose fraction consists of β-1,4-linked glucose units aligned in long crystalline chains that form fibers (Nishiyama et al., 2003; Wada et al., 2008). The hemicellulose content is somewhat less than for cellulose in cellulosic biomass and is mainly made up of hetero-1,4-β-D-xylan (Poutanen et al., 1986). Unlike cellulose, hemicellulose, primarily xylan, is amorphous and highly branched and much easier to thermochemically depolymerize to monomeric sugars with high yields at the low pH of dilute acid pretreatments (Jacobsen and Wyman, 2000; Saha, 2003). However, as the pH is raised toward neutral, such as in autohydrolysis or controlled pH pretreatments, a large portion of the sugars are released as oligomers with a wide range of chain lengths (Wyman et al., 2005). Many researchers have attributed hemicellulose removal to facilitate hydrolysis of the cellulose remaining in the solids from pretreatment by improving cellulase accessibility to cellulose (Allen et al., 2001; Grohmann et al., 1989; Ishizawa et al., 2007; Kabel et al., 2007; Palonen et al., 2004; Yang and Wyman, 2004; Zhu et al., 2005). However, higher pH pretreatments by ARP (Ammonia Recycle Percolation), lime, and AFEX (Ammonia Fiber Expansion) can also be highly effective even though most of the hemicellulose is left in the pretreated solids (Kim and Holtzapple, 2005; Kim and Lee, 2005; Teymouri et al., 2004). The result is that the xylan content of pretreated solids can range from as low as 5% for dilute acid pretreatment to as high as 25% or more for AFEX pretreated biomass (Wyman et al., 2005).

Because many common fermentative organisms cannot utilize oligomers, cellulose and hemicellulose must be broken down fully to monomers for fermentation to ethanol with high yields (Balat et al., 2008; Sarrouh et al., 2004). A complex of secreted enzymes derived from filamentous fungi, particularly *Trichoderma*, is usually applied to hydrolyze lignocellulosic biomass to fermentable sugars (Berlin et al., 2007), and commercial enzyme preparations such as Spezyme CP that are widely used for enzymatic hydrolysis of cellulose contain high levels of endoglucanase and

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cellobiohydrolase activities. However, supplementation with β-glucosidase is frequently needed to obtain high cellulose conversion (Berlin et al., 2007; Spindler et al., 1989; Sternberg et al., 1977). These enzyme preparations also contain lower amounts of such non-cellulolytic enzyme activities as hemicellulase and pectinase that help remove hemicellulose and pectin that are believed to physically block access of cellulase to cellulose (Jeoh et al., 2007).

The deficiency in xylanase activities in most commercial enzyme preparations (Berlin et al., 2007; Dien et al., 2008; Wood and Mccrae, 1986) can lead to the release of high concentrations of xylooligomers in the hydrolysis broth. Recently, new evidence emerged that xylooligomers are powerful inhibitors of cellulase activity (Qing et al., 2010), suggesting that partial hydrolysis of xylan or insoluble xylooligomers left in pretreated biomass to soluble oligomers could dramatically slow cellulose hydrolysis by enzymes, particularly for the lower enzyme loadings critical to low costs. Although more severe pretreatment conditions could remove more xylan from biomass, this would result in greater degradation of sugars, resulting in lower yields and greater inhibition (Wyman et al., 2005). Therefore, it would be useful to formulate a cocktail of endoglucanases, cellobiohydrolases, β-glucosidases, xylanases, β-xylosidases, and other enzymes that can completely hydrolyze cellulose and hemicellulose to monomers and reduce inhibition of cellulase by xylooligomers.

In this study, the effects of different enzyme preparations on degrading different chain length xylooligomers was evaluated to help us better understand the fate of these oligomers that are so inhibitory to cellulase action during enzymatic hydrolysis. Commercial Spezyme CP cellulase, Novozyme 188 β-glucosidase, Multifect xylanase, and β-xylosidase provided by Genencor, a Danisco Division (Beloit, WI) were applied to xylooligomers made from water-only hydrolysis of birchwood xylan. Changes in the degree of polymerization (DP) of the xylooligomers were then followed using a new HPLC method we devised to follow concentrations of DPs up to 30. In addition to providing insight into the effect enzymes could have in reducing inhibition by xylooligomers, investigation of the biological degradation of xylooligomers by different enzyme preparations could also provide useful insight to industrial xylooligomers production for applications in pharmaceutical, food, and other industries (Akpinar et al., 2009).

2. Methods

2.1. Materials

Birchwood xylan with a xylan content measured at ~85% was purchased from Sigma (Lot # is 038K0751) and used in water-only hydrolysis to generate xylooligomer rich solutions. Xylobiose and xylotriose standards of over 95% purity were purchased from Megazyme International Ireland, Ltd. (Bray, Co. Wicklow, Ireland, Cat. No. O-XBI, O-XTR) to calibrate xylooligomer concentrations in solution. Genencor, a Danisco Division (2600 Kennedy Drive, Beloit, WI, 53511) kindly supplied Spezyme CP cellulase (Lot # 301-05330-205, 59 ± 5 FPU/ml, 123 ± 10 mg protein/ml), Multifect xylanase (Lot 301-04021-015; 42 ± 5 mg protein/ml), and β-xylosidase (Lot # 20050881-0882, 75 ± 5 mg protein/ml) enzymes, while Novozyme 188 (066K0676, 665 CBU/ml, 140 ± 5 mg protein/ml) purchased from Sigma was used for β-glucosidase supplementation. In the non-commercial β-xylosidase preparation, β-xylosidase was the most predominant protein expressed in a *Trichoderma* strain in which the major cellulase genes were deleted, specifically CBH1, CBH2, EG1, and EG2. The microbial source and enzyme activities for each enzyme preparation are indicated in Table 1.

2.2. Procedure for production of xylooligomer mixtures

Xylooligomer rich solutions were produced by water-only hydrolysis of birchwood xylan at 200 °C for 15 min in a 1 l stainless steel batch reactor from Parr instruments (Moline, IL) with a solids loading of 10% (w/v). The reactor vessel was sealed and placed in a sand bath set at 320 °C for fast heat up and transferred to a 200 °C sand bath to maintain a constant temperature. After 15 min, the reactor was quenched quickly in ice water to room temperature. The solution was poured out and centrifuged to separate the solid from the liquid hydrolyzate (Yang and Wyman, 2004). The liquid was collected and stored at 50 °C for future use.

2.3. Sugar analysis

Liquid samples were filtered through 0.2 μm nylon filter vials (Alltech Associates Inc., Deerfield, IL). Then, a 20 μl sample was diluted about 50–100 times with HPLC grade water to concentrations desired for Dionex HPLC measurements. The rest of the liquid was pipetted into 500 μl polyethylene HPLC vials (Alltech Associates Inc., Deerfield, IL) and kept refrigerated at 4 °C until analyzed on the Waters HPLC to quantify monomeric xylose concentrations. Liquid samples for measurements of the DP distribution on the Dionex HPLC were diluted immediately after collection and kept at room temperature to prevent precipitation of high DP xylooligomers. Liquid samples together with sugar calibration standards were run on a Waters Alliance HPLC system (Model 2695, Waters Corporation, Milford, MA) employing an Aminex HPX-87P column (Bio-Rad Laboratories, Hercules, CA) and a refractive index detector (Waters 2414). Quantification of xylooligomer chain lengths over a degree of polymerization (DP) range from 1 to 30 was performed with a Dionex DX-600 Ion Chromatograph system. The IC system consisted of a BioLC® GP50, ED50 with cell Au electrode and equipped with a CarboPac PA100 (4 × 250 mm) anion exchange column and guard cartridge (Dionex Corporation, Sunnyvale, CA). The column was run at 30 °C with an eluent flow rate of 1 ml/min. The mobile phases were operated in the gradient mode with 150 mM of NaOH in 1 M of sodium acetate and 150 mM of NaOH according to Curve 6 based on the detection waveform from Dionex Technical Note 21 (Dionex, Sunnyvale, CA). Because the lack of xylooligomer standards with DP greater than 5, the concentration of each higher DP species from DP 4 to 30 was calculated by taking the ratio of their peak height to the peak height for xylobiose and multiplying this ratio by the measured concentration of the latter according to a procedure developed previously (Li et al., 2003; Yang and Wyman, 2008), with xylobiose and xylotriose (purity > 95%, purchased from Megazyme Inc., Wicklow, Ireland) used as calibration standards. The ratio of the peak height of xylotriose to that of xylobiose was used to adjust for possible errors in

Table 1
Microbial sources and key enzyme activities of enzyme preparations used in this study.

Enzyme preparation	Activities (units/mL)			
	Spezyme CP	Novozyme 188	Multifect xylanase	β-Xylosidase
Microbial source[a]	*Trichoderma* sp.	*Aspergillus* sp.	*Trichoderma* sp.	N/A
Total protein (mg protein/ml)	123 ± 10	140 ± 5	42 ± 5	75 ± 5
Cellulase (FPU)	58.2	8.5	0.77	N/A
β-Glucosidase[b]	128	665	35.9	N/A
Xylanase (OSX)[b]	2622	123	25,203	N/A
β-Xylosidase[b]	7.3	16.6	22.6	N/A

[a] Reference: (Berlin et al., 2006a,b).
[b] Reference: (Dien et al., 2008).

Table 2
Distribution of xylooligomer DP and xylose concentrations resulting from water-only pretreatment of birchwood xylan.

Group	DP	Concentration (mg/ml)	Percentage (%)
Monomer	1	0.597	2.53
Low DP xylooligomers	2	0.522	2.21
	3	0.359	1.52
	4	0.297	1.26
	5	0.245	1.04
	6	0.215	0.91
	7	0.163	0.69
	8	0.156	0.66
	9	0.106	0.45
	10	0.092	0.39
	11–30	0.301	1.27
High DP xylooligomers	30 and above	20.55	87.1
	Total	23.6	100

this method. Xylooligomers over the ranges from DP 2 to 30 together are termed as low DP xylooligomers in this paper. The concentrations of each DP group and xylose are showed in Table 2.

2.4. Determination of total xylooligomers and high DP xylooligomers

The amount of all xylooligomers in solution was determined by autoclaving liquid samples in 4% sulfuric acid for 1 h at 121 °C to breakdown oligomers into monomeric sugars as described in NREL laboratory methods (Sluiter et al., 2008). Sugar recovery standards containing known sugar concentrations were also autoclaved in parallel to estimate sugar losses during this post hydrolysis operation. Monomers were then quantified using a Waters HPLC equipped with a refractive index (RI) detector and Bio-rad HPX-87P column (Bio-Rad Laboratories, Hercules, CA). Then the total mass of oligomers was calculated as (Yang and Wyman, 2008):

$$\text{Total oligomers (g)} = \text{Total xylose(g)in the hydrolysate corrected for degradation following post hydrolysis} - \text{Monomeric xylose(g)in the hydrolysate before autoclaving} \quad (1)$$

Xylooligomers with DP higher than 30 that remained in solution at room temperature are termed high DP xylooligomers in this paper. The mass of high DP xylooligomers was calculated as:

$$\text{High DP xylooligomers(g)} = \text{Total xylooligomers following post hydrolysis(g)} - \text{Monomeric xylose in hydrolyzate before post hydrolysis(g)} - \text{Sum of all low DP xylooligomers measured on the Dionex(g)} \quad (2)$$

2.5. Enzymatic hydrolysis

Enzymatic hydrolysis of xylooligomer rich solutions with different enzymes was performed in 25 ml Erlenmeyer flasks to which was added 0.05 M acetate buffer (pH 4.8) according to NREL Laboratory Analytical Procedure LAP 009 (NREL, 1996). Xylooligomers rich solutions were loaded at a concentration of 11.8 mg of total equivalent xylose/ml of solution. To prevent infection by microorganisms, 400 μg of 10 mg/ml tetracycline antibiotic in 70% ethanol and 300 μg of 10 mg/ml cyclohexamide in DI water were added to the hydrolysis broth before adding enzymes. Cellulase, β-glucosidase, xylanase, and β-xylosidase enzymes were added separately at enzyme loadings of 5.37 or 10.73 mg protein/g equivalent xylose. Flasks containing oligomers and enzymes were placed in a thermostated shaker at 48 ± 3 °C with a rotating speed of 150 rpm (NREL, 1996), and samples were taken after 4, 24, 48, 72 h of hydrolysis. Substrate blanks without enzyme and enzyme blanks without substrate were tested in parallel with other samples, and all samples were run in triplicate unless otherwise stated. Samples taken were analyzed with an HPLC to follow the reaction course and determine final yields.

3. Results and discussion

As summarized in Table 1, the three commercial enzyme preparations used in this study all contain some xylanase and β-xylosidase activity. Endo-1,4-β-xylanase primarily randomly hydrolyzes xylan to xylooligomers, while β-xylosidase enzymes release monomeric xylose from the non-reducing ends of xylooligomers (Chen et al., 1997). The ability of these four enzymes in hydrolyzing xylooligomers to xylose not only depends on their endo-1,4-β-xylanase and β-xylosidase activities but also on synergistic action of these two enzymes with esterases to remove acetyl substituents from the β-1,4-linked D xylose backbone of xylan (Coughlan and Hazlewood, 1993; Gubitz et al., 1998). In this study, xylooligomers with a DP range from 2 to 30 were grouped together as low DP xylooligomers, and those with a DP range over 30 were termed as high DP xylooligomers. The commercial enzyme preparations Spezyme CP, Novozyme 188, and Multifect xylanase and the non-commercial enzyme β-xylosidase from Genencor, a Danisco Division were applied based on equal protein content per gram of equivalent xylose to test their effects in degrading different DP range xylooligomers. Enzyme loadings of 5.37 and 10.73 mg protein/g equivalent xylose were applied.

3.1. Spezyme CP cellulase

Fig. 1 shows the concentrations of different groups of xylooligomers over time for addition of 10.73 mg of total Spezyme CP cellulase protein/g equivalent xylose and also for the substrate blank control. High DP xylooligomers were hydrolyzed rapidly in the first hour to lower DP xylooligomers and monomeric xylose, and then the hydrolysis rate gradually slowed down. As a result, a large quantity of lower DP xylooligomers accumulated in hydrolysis broth in the first 24 h, with the highest concentration of 24%

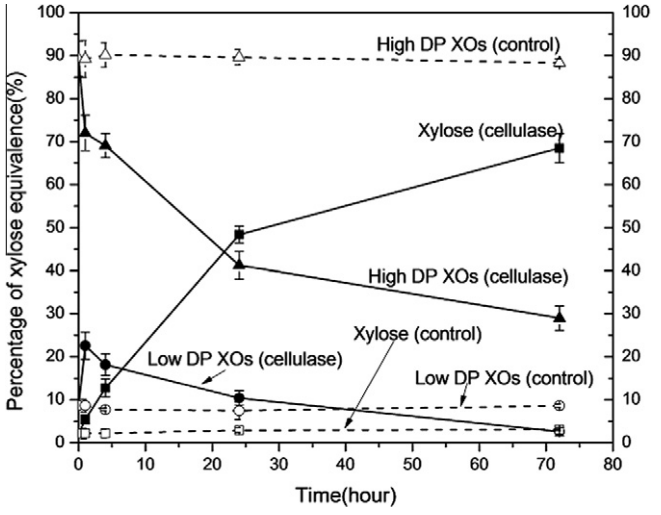

Fig. 1. Xylose and xylooligomer DP group profiles over a 72 h hydrolysis period for mixed DP xylooligomers with and without adding Spezyme CP cellulase at a loading of 10.73 mg protein/g equivalent xylose.

equivalent xylose detected in the first hour. Thus, Spezyme CP cellulase enzymes have limited ability to hydrolyze lower DP xylooligomers. As a result of hydrolysis of both higher and lower DP xylooligomers, the fraction present as total monomeric xylose was 68.1% after 72 h of hydrolysis, with about 62.3% of that total resulting from xylooligomer hydrolysis by Spezyme CP cellulase and the remaining 5.8% present in the xylooligomer solution initially. Although Spezyme CP hydrolyzed a high fraction of the oligomers to xylose, about 28% of the high DP xylooligomers proved more difficult to break down after 72 h of hydrolysis. On the other hand, the shorter chain xylooligomers proved much more easily hydrolyzed by Spezyme CP cellulase enzyme preparation with just small amounts of xylobiose and xylotriose remaining after 72 h, apparently as a result of the low β-xylosidase activities in this cellulase preparation.

3.2. Novozyme 188 β-glucosidase

As shown in Fig. 2, Novozyme 188 β-glucosidase hydrolyzed higher DP xylooligomers much more slowly than Spezyme CP cellulase, with only 11% of the high DP xylooligomer degraded into lower DP species in the first 24 h. Consequently, even though the β-xylosidase enzyme activity of Novozyme 188 is very low, no obvious accumulation of lower DP xylooligomers was found in the hydrolysis broth over the entire hydrolysis time. Overall, about 32.1% of the high DP xylooligomers and 5.3% of the lower DP xylooligomers (based on the equivalent amount mass of monomeric xylose) were hydrolyzed within 72 h, and more xylooligomers were hydrolyzed by commercial β-glucosidase enzymes than for the control substrate blank.

3.3. Multifect xylanase

Fig. 3 shows that addition of Multifect xylanase results in similar xylooligomer profiles to those with Spezyme CP cellulase except that the hydrolysis rate is much greater due to the higher xylanase activities shown in Table 1. A rapid drop in higher DP xylooligomers was observed in the first 24 h, with about 64.2% (based on total equivalent xylose amount) of the high DP xylooligomers hydrolyzed into lower DP species or monomeric xylose during this time. However, after that, the hydrolysis rate dropped off sharply, with just 4% of the total oligomers degraded over the remaining 48 h. The rapid hydrolysis of high DP xylooligomers in the first 24 h not only led to a dramatic increase in the monomeric xylose concentration from 8% to 76% equivalent xylose mass but also resulted in the rapid accumulation of lower DP xylooligomers. Even after just 1 h of hydrolysis, the amount of lower DP xylooligomers increased from 8% to 25% of the total xylose mass. Similar to Spezyme CP, Multifect xylanase could not totally hydrolyze all xylooligomers into monomers even at a higher loading of 10.73 g protein/g equivalent xylose loading, with about 17% of the high DP xylooligomers still in solution after 72 h of hydrolysis.

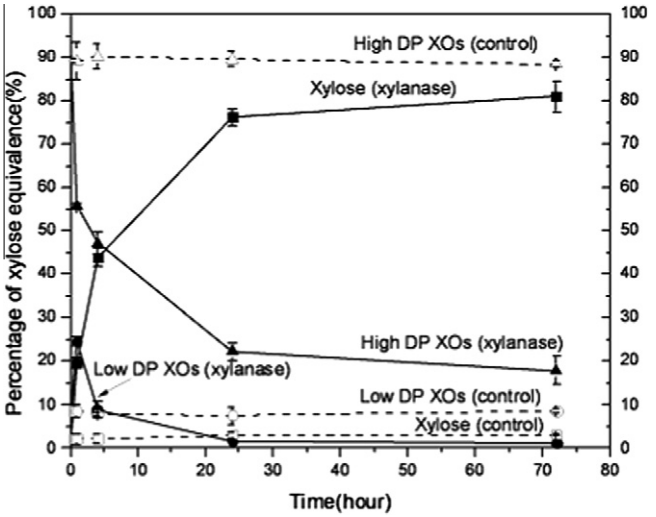

Fig. 3. Xylose and xylooligomer DP group profiles over a 72 h hydrolysis period for mixed DP xylooligomers with and without adding Multifect xylanase at a loading of 10.73 mg protein/g equivalent xylose.

3.4. Genencor β-xylosidase

Unlike the other three enzymes, the β-xylosidase used was a non-commercial enzyme that was kindly provided by Genencor, a Danisco Division. β-Xylosidase dominates protein expression by a *Trichoderma* strain in which the major cellulase genes were deleted, specifically CBH1, CBH2, EG1 and EG2. The data in Fig. 4 clearly indicates that over 76% of the high DP xylooligomers could

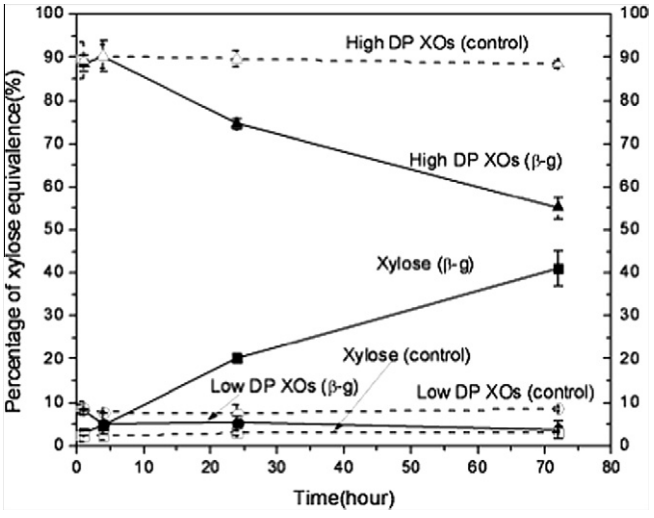

Fig. 2. Xylose and xylooligomer DP group profiles over a 72 h hydrolysis period for mixed DP xylooligomers with and without adding Novozyme 188 β-glucosidase at a loading of 10.73 mg protein/g equivalent xylose.

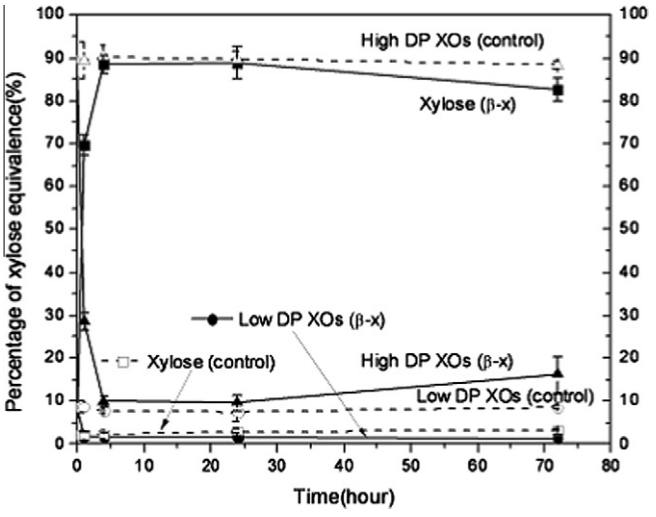

Fig. 4. Xylose and xylooligomer DP group profiles over a 72 h hydrolysis period for mixed DP xylooligomers with and without adding β-xylosidase at a loading of 10.73 mg protein/g equivalent xylose.

be converted into xylose in the first 4 h of hydrolysis, and there was no obvious accumulation of low DP xylooligomers over the reaction time. Furthermore, in contrast with Spezyme CP and Multifect xylanase, this β-xylosidase preparation could totally hydrolyze most of the xylobiose or xylotriose into monomeric xylose in a short reaction time. Also, although around 12% of the high DP xylooligomers still remained after 72 h, this is still much less than the 30% and 17% remaining for Spezyme CP cellulase and Multifect xylanase, respectively. Thus, β-xylosidase removes higher DP xylooligomers more quickly than the other enzymes.

3.5. Xylobiose profile with different enzyme preparations

To facilitate comparisons of the effect of the different enzymes on xylooligomers, the fraction of xylobiose during xylooligomer hydrolysis by the different enzyme preparations is plotted in Fig. 5A over the 72 h time period. Due to the comparatively low β-xylosidase activity in Spezyme CP and Multifect preparations and quick hydrolysis of higher DP ones into xylobiose, a large fraction of xylobiose accumulated during the first 24 h when xylooligomers were hydrolyzed with these two enzymes, although xylobiose levels dropped more slowly with Spezyme CP than xylanase. However, as the decomposition rate of the of higher DP xylooligomers gradually dropped over time, the amount of xylobiose in the hydrolysis broth dropped, and the maximum fraction of xylobiose was 23.4% and 13.2% of the total equivalent xylose mass for Multifect xylanase and Spezyme CP cellulase, respectively. Xylobiose concentrations were virtually constant at low levels for addition of β-glucosidase and the control, which might be due to slow depolymerization of higher DP oligomers to form xylobiose. Most striking is that β-xylosidase hydrolyzed most of xylobiose into xylose immediately after formation, in contrast to limited hydrolysis by the control and Novozyme 188 β-glucosidase.

3.6. The effect of different enzymes on xylobiose and xylotriose

As stated above, xylobiose was noticed to be an important fraction in lower DP xylooligomer groups that was relatively resistant to rapid hydrolysis by Spezyme CP cellulase and Multifect xylanase. Thus, the effect of these four different enzyme preparations on degrading xylobiose and xylotriose was studied, with the results summarized in Fig. 5B. Purified xylobiose and xylotriose were used as hydrolysis substrates at a solids loading of 1% to which an enzyme loading of 5.37 mg protein/g equivalent xylose was applied. For both xylobiose and xylotriose, monomer xylose was detected for all of the four enzymes in the first 24 h of hydrolysis. However, β-xylosidase hydrolyzed 71.0% and 70.2% of the xylobiose and xylotriose into monomers in the first 24 h, respectively. β-Glucosidase, on the other hand, only hydrolyzed 19.1% and 21.8% of xylobiose and xylotriose, respectively, in this time span. Although, Multifect xylanase and β-xylosidase showed comparable capacities in degrading xylobiose and xylotriose at longer times, β-xylosidase had a much greater hydrolysis rate than Multifect xylanase as indicated by the conversions measured after 4 and 24 h. In addition, Fig. 5B shows that Spezyme CP cellulase could hydrolyze more xylobiose and xylotriose in a shorter time than Novozyme 188 β-glucosidase, although its β-xylosidase activity is lower than for Novozyme 188. As expected, xylanase displayed a much greater ability to degrade xylobiose and xylotriose than cellulase. The accumulation of high amounts of xylobiose during hydrolysis of mixed DP xylooligomers was due to fast hydrolysis of high DP oligomers into lower DP fragments and xylobiose.

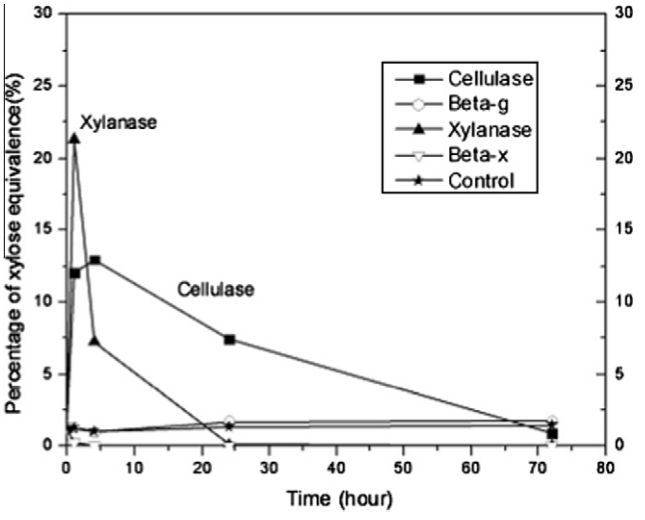

Fig. 5A. Xylobiose profiles over a 72 h hydrolysis period for mixed DP xylooligomers with and without adding various enzymes at a loading of 10.73 mg protein/g equivalent xylose.

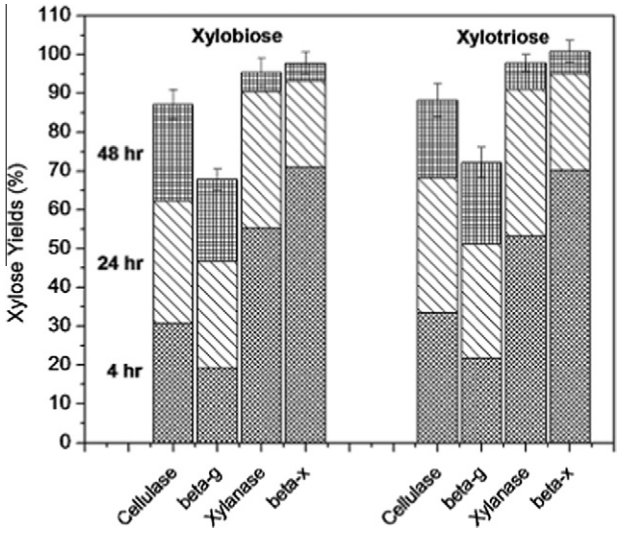

Fig. 5B. Xylose yields for enzymatic hydrolysis of xylobiose and xylotriose with different enzyme preparations at an enzyme loading of 5.37 mg protein/g equivalent xylose.

3.7. The effect of enzyme loadings

In addition to an enzyme loading of 10.73 mg protein/g equivalent xylose, a lower loading at 5.37 mg protein/g equivalent xylose was applied to understand how enzyme loadings impact xylooligomer degradation for the four different preparations, as shown in Fig. 6 for both loadings. For Spezyme CP (Fig. 6A), reducing the total protein loading reduced the rate of conversion of high DP xylooligomers to lower DP species and xylose, but the profiles of low DP xylooligomers were similar for both loadings. For β-glucosidase enzymes (Fig. 6B), lower enzyme loadings also resulted in a comparatively lower xylose yields and high DP xylooligomer conversion, but the benefits of increasing enzyme loading were more modest in that just 7% more xylooligomers were hydrolyzed. Dropping the xylanase loading more strongly influenced hydrolysis during the first 24 h than later (Fig. 6C), and more high DP xylooligomers were converted into lower DP species and then hydrolyzed to monomeric xylose for the higher total protein loading. However, higher enzyme loadings did not reduce xylooligomer amounts at

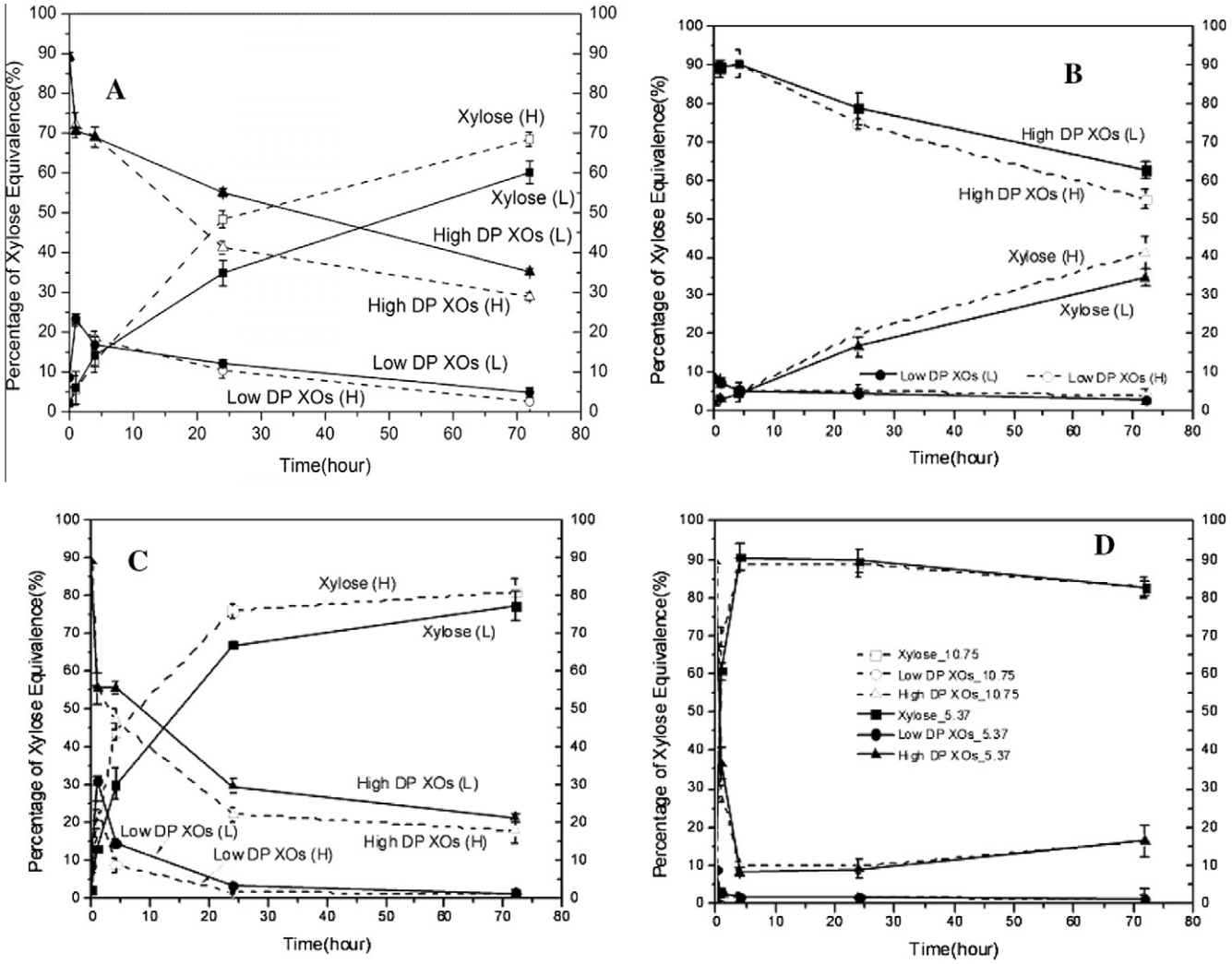

Fig. 6. Comparison of xylose and xylooligomer profiles during enzymatic hydrolysis of mixed DP xylooligomers for 72 h with 5.37 and 10.73 mg protein/g of equivalent xylose. The four enzyme preparations used are Spezyme CP cellulase (A), Novozyme 188 β-glucosidase (B), Multifect xylanase (C), and Genecor β-xylosidase (D).

the end of 72 h (Fig. 6C). A possible explanation is precipitation of longer chain xylooligomers at the enzymatic hydrolysis temperature due to the drop in solubility of high DP xylooligomers with decreasing temperature (Gray et al., 2007). Finally, we can see that cutting β-xylosidase loadings in half had almost no effect on the xylobiose yields in Fig. 6D, showing that this enzyme is very effective in rapidly hydrolyzing xylobiose.

3.8. Kinetic implications

Enzymatic hydrolysis of xylan or xylooligomers is a heterogeneous reaction that benefits from synergistic action of different enzyme activities, and the presence of a wide range of chain length xylooligomers further complicates the kinetic view of the reactions. On top of that, inhibition of enzymes by the different DP xylooligomers and xylose must be factored into the analysis (Kumar and Wyman, 2009a). Moreover, even if enzymes are quite specific and the enzyme–substrate complex follows a "lock and key" mechanism, xylan or xylooligomers could bind with cellulase active sites with an even higher km value than for cellulose (Kanda et al., 1976). Our recent research suggests that mixed DP xylooligomers competitively inhibit cellulase and that inhibition by higher DP xylooligomers is particularly strong. The result is that the capacity of different enzyme preparations to decompose different DP xylooligomers cannot be easily predicted by comparing xylanase or β-xylosidase activities alone.

In the research reported in this paper, we sought to better understand the capability of different enzyme preparations to hydrolyze different chain length xylooligomers, and the results indicated that commonly used Spezyme CP cellulase and Novozyme 188 β-glucosidase both have some xylanase and β-xylosidase activity, consistent with other findings (Berlin et al., 2006a,b, 2007; Sorensen et al., 2007). However, these two enzymes still left significant amounts of higher DP xylooligomers in the hydrolysis broth (Figs. 1 and 2). Because our research has shown that higher DP xylooligomers are strong inhibitors of enzymatic hydrolysis and partially responsible for gradually slowing down hydrolysis rates, it is important to remove as much as possible of these xylooligomers to achieve higher cellulose conversion and enzyme efficiency during enzymatic hydrolysis.

Supplementation with xylanase has been identified as desirable to enhance cellulase efficiency by increasing cellulase accessibility (Garcia-Aparicio et al., 2007; Gupta et al., 2008; Kumar and Wyman, 2009b; Wyman et al., 2005, 2009). Moreover, removal of xylooligomers from solution could not only help remove physical blocking but also hydrolyze xylooligomers that are highly inhibitory

to cellulase. However, the results of this research indicate that even high loadings of Multifect xylanase did not totally hydrolyze long chain xylooligomers within 72 h (Fig. 3), and the comparatively smaller amount of xylooligomers left could still strongly inhibit cellulase action. On the other hand, the results reported here show that the same mass of β-xylosidase protein nearly eliminated all of the high DP and low DP xylooligomers (Fig. 4).

4. Conclusions

The four enzyme preparations studied in this research all showed the ability to degrade different chain length xylooligomers. However, a considerable amount of high DP xylooligomers could not be hydrolyzed by Spezyme CP cellulase and Novozyme 188 β-glucosidase combined. To reduce inhibition by xylooligomers and achieve a high xylose monomer recovery, cellulase should be supplemented with xylanase and β-xylosidase. Further study is warranted to identify ratios of β-xylosidase to cellulase that maximize total sugar yields while reducing enzyme loadings and to establish the important of removing xylooligomers to enhancing cellulase effectiveness.

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References

Akpinar, O., Erdogan, K., Bostanci, S., 2009. Enzymatic production of xylooligosaccharide from selected agricultural wastes. Food and Bioproducts Processing 87, 145–151.

Allen, S.G., Schulman, D., Lichwa, J., Antal, M.J., Jennings, E., Elander, R., 2001. A comparison of aqueous and dilute-acid single-temperature pretreatment of yellow poplar sawdust. Industrial and Engineering Chemistry Research 40, 2352–2361.

Balat, M., Balat, H., Oz, C., 2008. Progress in bioethanol processing. Progress in Energy and Combustion Science 34, 551–573.

Berlin, A., Balakshin, M., Gilkes, N., Kadla, J., Maximenko, V., Kubo, S., Saddler, J., 2006a. Inhibition of cellulase, xylanase and beta-glucosidase activities by softwood lignin preparations. Journal of Biotechnology 125, 198–209.

Berlin, A., Gilkes, N., Kilburn, D., Maximenko, V., Bura, R., Markov, A., Skomarovsky, A., Gusakov, A., Sinitsyn, A., Okunev, O., Solovieva, I., Saddler, J.N., 2006b. Evaluation of cellulase preparations for hydrolysis of hardwood substrates. Applied Biochemistry and Biotechnology 130, 528–545.

Berlin, A., Maximenko, V., Gilkes, N., Saddler, J., 2007. Optimization of enzyme complexes for lignocellulose hydrolysis. Biotechnology and Bioengineering 97, 287–296.

Chen, C., Chen, J.L., Lin, T.Y., 1997. Purification and characterization of a xylanase from *Trichoderma longibrachiatum* for xylooligosaccharide production. Enzyme and Microbial Technology 21, 91–96.

Coughlan, M.P., Hazlewood, G.P., 1993. Beta-1,4-D-xylan-degrading enzyme-systems – biochemistry, molecular-biology and applications. Biotechnology and Applied Biochemistry 17, 259–289.

Dien, B.S., Ximenes, E.A., O'Bryan, P.J., Moniruzzaman, M., Li, X.L., Balan, V., Dale, B., Cotta, M.A., 2008. Enzyme characterization for hydrolysis of AFEX and liquid hot-water pretreated distillers' grains and their conversion to ethanol. Bioresource Technology 99, 5216–5225.

Garcia-Aparicio, M.P., Ballesteros, M., Manzanares, P., Ballesteros, I., Gonzalez, A., Negro, M.J., 2007. Xylanase contribution to the efficiency of cellulose enzymatic hydrolysis of barley straw. Applied Biochemistry and Biotechnology 137, 353–365.

Gray, M.C., Converse, A.O., Wyman, C.E., 2007. Solubilities of oligomer mixtures produced by the hydrolysis of xylans and corn stover in water at 180 °C. Industrial and Engineering Chemistry Research 46, 2383–2391.

Grohmann, K., Mitchell, D.J., Himmel, M.E., Dale, B.E., Schroeder, H.A., 1989. The role of ester groups in resistance of plant-cell wall polysaccharides to enzymatic-hydrolysis. Applied Biochemistry and Biotechnology 20–21, 45–61.

Gubitz, G.M., Stebbing, D.W., Johansson, C.I., Saddler, J.N., 1998. Lignin–hemicellulose complexes restrict enzymatic solubilization of mannan and xylan from dissolving pulp. Applied Microbiology and Biotechnology 50, 390–395.

Gupta, R., Kim, T.H., Lee, Y.Y., 2008. Substrate dependency and effect of xylanase supplementation on enzymatic hydrolysis of ammonia-treated biomass. Applied Biochemistry and Biotechnology 148, 59–70.

Ishizawa, C.I., Davis, M.F., Schell, D.F., Johnson, D.K., 2007. Porosity and its effect on the digestibility of dilute sulfuric acid pretreated corn stover. Journal of Agricultural and Food Chemistry 55, 2575–2581.

Jacobsen, S.E., Wyman, C.E., 2000. Cellulose and hemicellulose hydrolysis models for application to current and novel pretreatment processes. Applied Biochemistry and Biotechnology 84–86, 81–96.

Jeoh, T., Ishizawa, C.I., Davis, M.F., Himmel, M.E., Adney, W.S., Johnson, D.K., 2007. Cellulase digestibility of pretreated biomass is limited by cellulose accessibility. Biotechnology and Bioengineering 98, 112–122.

Kabel, M.A., Bos, G., Zeevalking, J., Voragen, A.G.J., Schols, H.A., 2007. Effect of pretreatment severity on xylan solubility and enzymatic breakdown of the remaining cellulose from wheat straw. Bioresource Technology 98, 2034–2042.

Kanda, T., Wakabayashi, K., Nisizawa, K., 1976. Xylanase activity of an endo-cellulase of carboxymethyl-cellulase type from *Irpex lacteus* (Polyporus-Tulipiferae). Journal of Biochemistry 79, 989–995.

Kim, S., Holtzapple, M.T., 2005. Lime pretreatment and enzymatic hydrolysis of corn stover. Bioresource Technology 96, 1994–2006.

Kim, T.H., Lee, Y.Y., 2005. Pretreatment and fractionation of corn stover by ammonia recycle percolation process. Bioresource Technology 96, 2007–2013.

Kumar, R., Wyman, C.E., 2009a. Effect of xylanase supplementation of cellulase on digestion of corn stover solids prepared by leading pretreatment technologies. Bioresource Technology 100, 4203–4213.

Kumar, R., Wyman, C.E., 2009b. Effects of cellulase and xylanase enzymes on the deconstruction of solids from pretreatment of poplar by leading technologies. Biotechnology Progress 25, 302–314.

Li, X., Converse, A.O., Wyman, C.E., 2003. Characterization of molecular weight distribution of oligomers from autocatalyzed batch hydrolysis of xylan. Applied Biochemistry and Biotechnology 105, 515–522.

Nishiyama, Y., Sugiyama, J., Chanzy, H., Langan, P., 2003. Crystal structure and hydrogen bonding system in cellulose 1(alpha), from synchrotron X-ray and neutron fiber diffraction. Journal of the American Chemical Society 125, 14300–14306.

NREL, 1996. Enzymatic Saccharification of Lignocellulosic Biomass. National Renewable Energy Laboratory, Golden, CO (LAP009).

Palonen, H., Thomsen, A.B., Tenkanen, M., Schmidt, A.S., Viikari, U., 2004. Evaluation of wet oxidation pretreatment for enzymatic hydrolysis of softwood. Applied Biochemistry and Biotechnology 117, 1–17.

Poutanen, K., Puls, J., Linko, M., 1986. The hydrolysis of steamed birchwood hemicellulose by enzymes produced by *Trichoderma reesei* and *Aspergillus awamori*. Applied Microbiology and Biotechnology 23, 487–490.

Qing, Q., Yang, B., Wyman, C.E., 2010. Xylooligomers are strong inhibitors of cellulose hydrolysis by enzymes. Bioresource Technology 101, 9624–9630.

Saha, B.C., 2003. Hemicellulose bioconversion. Journal of Industrial Microbiology and Biotechnology 30, 279–291.

Sarrouh, B.F., Jover, J., Gonzalez, E., 2004. Progress in the production of bioethanol from lignocellulosic biomass. Ingenieria Quimica 3, 6.

Sluiter, A., Hames, B., Ruiz, R., Scarlata, C., Sluiter, J., Templeton, D., 2008. Determination of Sugars, Byproducts, and Degradation Products in Liquid Fraction Process Samples. Laboratory Analytical Procedure (LAP) (NREL/TP-510-42623).

Sorensen, H.R., Pedersen, S., Jorgensen, C.T., Meyer, A.S., 2007. Enzymatic hydrolysis of wheat arabinoxylan by a recombinant "minimal" enzyme cocktail containing beta-xylosidase and novel endo-1,4-beta-xylanase and alpha-(L)-arabinofuranosidase activities. Biotechnology Progress 23, 100–107.

Spindler, D.D., Wyman, C.E., Grohmann, K., Mohagheghi, A., 1989. Simultaneous saccharification and fermentation of pretreated wheat straw to ethanol with selected yeast strains and beta-glucosidase supplementation. Applied Biochemistry and Biotechnology 20–21, 529–540.

Sternberg, D., Vijayakumar, P., Reese, E.T., 1977. Beta-glucosidase – microbial-production and effect on enzymatic-hydrolysis of cellulose. Canadian Journal of Microbiology 23, 139–147.

Teymouri, F., Laureano-Perez, L., Alizadeh, H., Dale, B.E., 2004. Ammonia fiber explosion treatment of corn stover. Applied Biochemistry and Biotechnology 113–116, 951–963.

Wada, M., Nishiyama, Y., Chanzy, H., Forsyth, T., Langan, P., 2008. The structure of celluloses. Powder Diffraction 23, 92–95.

Wood, T.M., Mccrae, S.I., 1986. The effect of acetyl groups on the hydrolysis of ryegrass cell-walls by xylanase and cellulase from *Trichoderma koningii*. Phytochemistry 25, 1053–1055.

Wyman, C.E., 2003. Potential synergies and challenges in refining cellulosic biomass to fuels, chemicals, and power. Biotechnology Progress 19, 254–262.
Wyman, C.E., Dale, B.E., Elander, R.T., Holtzapple, M., Ladisch, M.R., Lee, Y.Y., 2005. Comparative sugar recovery data from laboratory scale application of leading pretreatment technologies to corn stover. Bioresource Technology 96, 2026–2032.
Wyman, C.E., Dale, B.E., Elander, R.T., Holtzapple, M., Ladisch, M.R., Lee, Y.Y., Mitchinson, C., Saddler, J.N., 2009. Comparative sugar recovery and fermentation data following pretreatment of poplar wood by leading technologies. Biotechnology Progress 25, 333–339.
Yang, B., Wyman, C.E., 2004. Effect of xylan and lignin removal by batch and flowthrough pretreatment on the enzymatic digestibility of corn stover cellulose. Biotechnology and Bioengineering 86, 88–95.
Yang, B., Wyman, C.E., 2008. Characterization of the degree of polymerization of xylooligomers produced by flowthrough hydrolysis of pure xylan and corn stover with water. Bioresource Technology 99, 5756–5762.
Zhu, Y.M., Lee, Y.Y., Elander, R.T., 2005. Optimization of dilute-acid pretreatment of corn stover using a high-solids percolation reactor. Applied Biochemistry and Biotechnology 121, 1045–1054.