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Xylooligomers are strong inhibitors of cellulose hydrolysis by enzymes

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ABSTRACT

Typically, the enzymatic hydrolysis rate of lignocellulosic biomass is fast initially but then slows down more rapidly than can be explained by just consumption of substrate. Although several factors including enzyme inhibition, enzyme deactivation, a drop in substrate reactivity, or nonproductive binding of enzyme to lignin could be responsible for this loss of effectiveness, we recently reported evidence that xylose, xylan, and xylooligomers dramatically decrease conversion rates and yields, but clarification was still needed fors the magnitude of their effect. Therefore, in this study, xylan and various xylooligomers were added to Avicel hydrolysis at low enzyme loadings and found to have a greater effect than adding equal amounts of xylose derived from these materials or when added separately. Furthermore, xylooligomers were more inhibitory than xylan or xylose in terms of a decreased initial hydrolysis rate and a lower final glucose yield even for a low concentration of 1.67 mg/ml. At a higher concentration of 12.5 mg/ml, xylooligomers lowered initial hydrolysis rates of Avicel by 82% and the final hydrolysis yield by 38%. Mixed DP xylooligomers showed strong inhibition on cellulase enzymes but not on β-glucosidase enzymes. By tracking the profile change of xylooligomers, a large portion of the xylooligomers was found to be hydrolyzed by Spezyme CP enzyme preparations, indicating competitive inhibition by mixed xylooligomers. A comparison among glucose sugars and xylose sugars also showed that xylooligomers were more powerful inhibitors than well-established glucose and cellobiose.

1. Introduction

Cellulosic biomass is uniquely suited for sustainable production of liquid transportation fuels, and the power of modern biotechnology promises competitive advantages (Lynd et al., 2008). However, the cost of cellulase enzymes coupled with the large amounts required to realize commercially viable yields is by far the dominant economic barrier to large scale implementation. A significant contributor to the high dose demands is that hydrolysis rates slow down as reaction proceeds much faster than can be explained by substrate consumption alone for a typical enzymatic hydrolysis (Ramos et al., 1993; Yang et al., 2006). Many reasons have been offered for the loss in enzyme effectiveness with time and the consequent high doses required for good yields including end-product inhibition, enzyme deactivation with time and temperature, drop in substrate reactivity with conversion due to initial removal of more easily hydrolyzed material, and nonproductive binding of enzyme to lignin (Converse et al., 1988; Eriksson et al., 2002; Holtzapple et al., 1990; Scheiding et al., 1984). Although the exact cause is still uncertain and multiple factors are likely responsible, substrate and end-product inhibition are believed to be very important (Tengborg et al., 2001; Xiao et al., 2004), with glucose and cellobiose identified as the principle cellulase inhibitors that bind to cellulase active sites regardless of the inhibition pattern (Holtzapple et al., 1990). However, other hemicellulose sugars, such as mannose, xylose, and galactose, have been also shown to inhibit cellulase (Xiao et al., 2004), and liquid from Ammonia Recycled Percolation (ARP) pretreatment of corn stover that is rich in xylooligomers, soluble lignin, sugar and lignin degradation products, significantly inhibited cellulase and microbial activities (Kim et al., 2006; Zhu et al., 2006).

Enzymatic hydrolysis of cellulose is a multi-step heterogeneous reaction in which insoluble cellulose is initially broken down at the solid–liquid interface via the synergistic action of endo-glucanases (Xiao et al., 2004) (EC 3.2.1.4) and exo-glucanases/cellobiohydrolases (CBH) (EC 3.2.1.91). This initial reaction is accompanied by further liquid-phase hydrolysis of soluble intermediates, i.e., short cellulooligosaccharides and cellobiose, that are catalytically cleaved to produce glucose by the action of β-glucosidase (BG) (EC 3.2.1.21) (Messner et al., 1991). Enzyme accessibility to cellulose is postulated to be impeded by lignin and hemicellulose coating cellulose and restricting access by enzymes. In this vein, several studies have

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shown that removing a high percentage of hemicellulose can facilitate cellulose conversion by enzymatic hydrolysis (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., 2004). Similarly, addition of purified endoxylanase and hemicellulolytic esterase activities enhanced cellulose conversion by cellobiohydrolase I (Cel7A), and a direct relationship was shown between xylan removal by enzymes and enhancement of cellulose hydrolysis (Selig et al., 2008). Such studies attribute the benefits of hemicellulose removal to improving accessibility of cellulase enzymes to hydrolysis substrates, but additional impacts that hemicelluloses and their hydrolysis products could have on enzyme action have received little attention.

A recent paper determined for the first time that xylobiose and higher DP xylooligomers inhibit enzymatic hydrolysis of pure glucan, pure xylan, and pretreated corn stover, with xylose, xylobiose, and xylotriose having progressively greater impacts on hydrolysis rates (Kumar and Wyman, 2009). This interpretation was supported through showing that addition of xylanase and β-xylosidase improved enzymatic hydrolysis of xylan and pure cellulose. Addition of beta-xylosidase and xylanase also enhanced performance for enzymatic hydrolysis of solids resulting from sulfur dioxide pretreatment and particularly from ammonia fiber expansion (AFEX) pretreatment that leaves virtually all of the xylose in the pretreated solids. These results strongly suggest that xylooligomers inhibit cellulase action and that both glucose and xylose release could be significantly enhanced by supplementation with β-xylosidase and xylanase (Kumar and Wyman, 2009). However, the benefits will likely vary with the amounts of xylose and other hemicellulose oligomers present in the liquid, and the choice of enzyme and enzyme loadings are also expected to vary with substrate composition.

Although this limited evidence suggested that xylooligomers reduce enzyme activity and therefore effectiveness, the mechanism for inhibition of cellulase and other hydrolysis enzymes by xylooligomers was still uncertain, and more information was needed to clarify the degree to which sugars and oligomers released from hemicellulose affect enzymatic hydrolysis of cellulose. Therefore, in this study, pure cellulose was hydrolyzed in the presence of xylose, xylan, and xylooligomers to determine the degree to which these compounds impact cellulose conversion. In addition, the influence of xylooligomer concentration on cellulose hydrolysis was measured, and the effects of these components on the action of cellulase and β-glucosidase were determined. The results showed for the first time that xylooligomers can play a powerful role in slowing enzymatic hydrolysis, thereby potentially explaining to some extent why hydrolysis rates slow with conversion and why removal of hemicellulose improves cellulose conversion yields.

2. Methods

2.1. Materials

Avicel PH-101 cellulose (cellulose content > 97%, Lot & filling code: 1300045 32806P01) and xylose (xylose purity > 99%, Lot & filling code: 1403673 33308088) were purchased from Sigma (St. Louis, MO). Birchwood xylan with a xylan content measured to be ~85% was also purchased from Sigma (Lot # is 038K0751). A xylobiose standard of over 95% purity was obtained from Megazyme International Ireland Ltd. (Bray, Co. Wicklow, Ireland, Cat No. O-XBI). Genencor International (2600 Kennedy Drive, Beloit, WI, 53511) kindly supplied Spezyme CP cellulase (Lot # 301-05330-205, 59 ± 5 FPU/ml, 123 ± 10 mg protein/ml), while Novozyme 188 (Batch # 066K0676, 665 CBU/ml, 140 ± 5 mg protein/ml) from Sigma was employed for β-glucosidase supplementation.

2.2. Sugar analysis

Liquid samples were filtered through 0.2 μm nylon filter vials (Alltech Associates Inc., Deerfield, IL), pipetted into 500 μl polyethylene HPLC vials (Alltech Associates Inc., Deerfield, IL), and kept refrigerated at 4 °C until analyzed. Liquid samples together with calibration sugar 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). Concentrations of monomeric glucose and xylose were calculated based on calibration sugar standards.

2.3. Enzymatic hydrolysis

Enzymatic hydrolysis of pure cellulose was performed according to NREL Laboratory Analytical Procedure LAP 009 at 2% (w/v) solids loading in 25 ml Erlenmeyer flasks to which was added 0.05 M citrate buffer (pH = 4.8). To prevent possible microbial contamination, 100 μg of 10 mg/ml tetracycline antibiotic in 70% ethanol and 75 μg of 10 mg/ml cyclohexamide in DI water were added to the hydrolysis broth before adding enzymes. The flasks were placed in a thermostated shaker at 48 ± 3 °C with a rotating speed of ~150 rpm (NREL, 1996). The enzyme loading was 5 FPU/g of glucan in Avicel solids (corresponding to about 10.75 mg protein/g of glucan) supplemented with β-glucosidase at a loading of 10 CBU/g of glucan (CBU to FPU activity ratio of 2:1). Substrate blanks without enzyme and enzyme blanks without substrate were tested in parallel with other samples. Samples taken after 1, 4, 24, 48, 72, and 96 h of hydrolysis were analyzed with an HPLC to follow the reaction course and determine final yields.

2.4. Identification of oligomer sugars on Dionex HPLC

Liquid samples were filtered through 0.2 μm nylon filter vials (Alltech Associates Inc., Deerfield, IL) and diluted about 50 times to desired concentrations with HPLC grade water. Then samples were analyzed with a Dionex DX-600 Ion Chromatograph system to quantify xylooligomer chain lengths over a degree of polymerization (DP) range from 1 to 30. The Dionex HPLC was equipped with an electrochemical detector, a CarboPac PA100 (4 × 250 mm) anion exchange column, and guard cartridge (Dionex Corp., Sunnyvale, CA). The mobile phases were operated in gradient mode with 150 mM NaOH in 1 M NaAc and 150 mM NaOH (Dionex application note 67). Xylobiose (purity > 95%, purchased from Megazyme Inc. Wicklow, Ireland) was used as a standard to calibrate and calculate the concentration of xylooligomers (xylooligosaccharides) (DP 2–30). Because the lack of xylooligomer standards with DP higher than five, the concentration of each higher DP species was calculated by taking the ratio of each 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).

2.5. Determination of total oligomers

To determine the total oligomers in solution, liquid samples were autoclaved 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 post-hydrolysis losses. The 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). From this procedure, the total oligomers were calculated as (Yang and Wyman, 2008):

Total oligomers (g) = Total xylose (g) in the hydrolyzate corrected for degradation − Monomeric xylose (g) in the hydrolyzate before autoclaving.

2.6. Procedure for production of xylooligomer mixtures

Xylooligomer mixtures 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 5% (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 keep at the target temperature. After 15 min, the reactor was quenched quickly in ice water to room temperature (Yang and Wyman, 2004). The solution was cooled down to room temperature and centrifuged to separate the solid from the liquid hydrolyzate. The Dionex HPLC was then applied to determine the xylooligomer distribution, as shown in Fig. 1, and their concentration in the liquid portion. The Waters HPLC was employed to determine the xylose and degradation product (mainly furfural) concentrations, following the procedures described above. Post-hydrolysis was employed to quantify the total oligomer concentration in the liquid hydrolyzate. The relative weight percentage of each fraction is shown in Table 1.

2.7. Production of xylose from birchwood xylan

Birchwood xylan was first pretreated as described above, and the liquid hydrolysate was separated from the remaining solids by filtration. Then, 0.1 M citrate buffer was added to the xylooligomer rich solution to adjust the pH to 4.8 and make the final concentration 0.05 M. Multifect xylanase enzymes were added next at a high loading to ensure total conversion of xylooligomers to xylose. To prevent possible microbial contamination, 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. The flask was then placed in a thermostated shaker at 48 ± 3 °C with a rotating speed of ~150 rpm (NREL, 1996). After 72 h of hydrolysis, hydrolysate samples were taken every 12 h and tested on the Waters HPLC. Hydrolysis was stopped once most of the xylooligomers were converted to xylose. Comparing the effects of adding this solution to cellulose hydrolysis to that for addition of purchased pure xylose provided insight into whether other components released into the xylooligomer solution during its preparation were responsible for inhibiting cellulose hydrolysis.

Table 1
Xylooligomer concentrations in xylan hydrolyzate as determined by a Dionex IC system equipped with a CarboPac PA100 column and by post-hydrolysis.

Composition[a]	Weight[b],%
DP1 (xylose)	8.21
DP2 (xylobiose)	6.91
DP3 (xylotriose)	5.73
DP4 (xylotetraose)	4.89
DP5 (xylopentaose)	4.07
DP6	3.81
DP7	2.89
DP8	2.54
DP9	2.17
DP10	1.82
DP11–30	4.98
High DP (31 and above)	44.9
Degradation products	2.26
Total	95.78

[a] Xylooligomers with DP 2–30 are quantified by Dionex IC equipped with a CarboPac PA100 column and higher DP xylooligomers are quantified by post-hydrolysis.
[b] All xylooligomer weight percentages are based on equivalent xylose mass.

3. Results and discussion

3.1. Effects of xylan derivatives on enzymatic hydrolysis of pure cellulose

Xylooligomers are important intermediates in hemicellulose hydrolysis, and we sought to better quantify how inhibitory they are to cellulose hydrolysis by enzymes. Because pure xylooligomers with DPs greater than five are not commercially available and even those available are extremely expensive, a mixture of xylooligomers was produced by water-only hydrolysis of Birchwood xylan, with the distribution of different DP xylooligomers shown in Fig. 1. Then, xylan, xylose, and xylooligomers derived from xylan were added to pure cellulose hydrolysis on equivalent xylose mass basis (adjusted for waters of hydrolysis) in all cases. Furthermore, to better understand their role in hydrolysis of real substrates, these compounds were added based on similar glucan to xylan ratios as in pretreated corn stover. At one extreme, a ratio of 12:1 (Avicel: xylose compounds) was applied because this ratio is similar to the ratio of these compounds in solids produced by dilute acid pretreatment of corn stover. At the other end of the spectrum of possibilities, a ratio of 8:5 was employed to simulate the glucan to xylan ratio for solids produced by ammonia fiber expansion (AFEX) pretreatment as well as that in raw biomass without

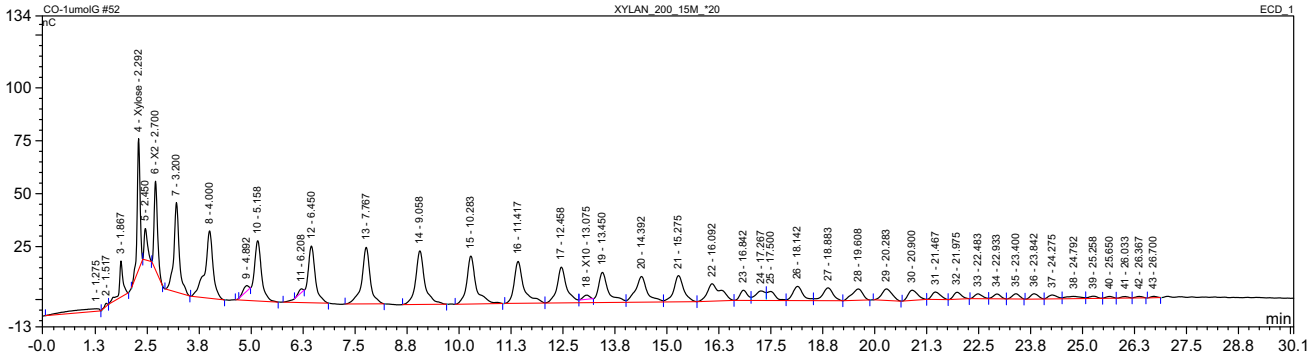

Fig. 1. Distribution of the degree of polymerization of soluble xylooligomers produced from xylan as determined by a Dionex IC system equipped with a CarboPac PA100 column.

pretreatment as the two are virtually the same (Elander et al., 2009; Wyman et al., 2005; Wyman et al., 2009).

First, we set out to determine how these compounds impact enzymatic hydrolysis of pure cellulose at low commercially relevant enzyme loadings of about 5 FPU/g glucan, and Fig. 2A shows enzymatic hydrolysis of 2% Avicel with addition of 1.67 mg/ml (based on a 12:1 Avicel: xylose compounds ratio) of xylose, xylooligomers, or xylan and a control sample for comparison. Thus, addition of even such low concentrations of xylose and xylan derivatives lowered enzymatic hydrolysis rates and yields substantially from those with pure cellulose even at the low solids loadings used. As shown in Fig. 2A, hydrolysis yields after 96 h were 76% for addition of xylose, 71% for addition of xylan, and 68% for addition of mixed xylooligomers, about 5–13% lower than the yield from the control. It was also noticed that xylose and xylan derivatives inhibited enzymatic hydrolysis of pure cellulose most strongly within the first 48 h, at which point conversions dropped to just 44% and 47% for samples with addition of xylooligomer mixtures and xylan, respectively, while the control reached around 75% glucose conversion in that time. However, at 96 h, Fig. 2A shows that the differences between the yields for addition of xylose compounds and those for the control sample became much smaller, possibly due to the depolymerization of the xylose polymers by cellulase and β-glucosidase.

As shown in Fig. 2B, higher concentrations of xylose compounds had a much more obvious effect on final yields and initial hydrolysis rates. For example, addition of xylose, xylan, and xylooligomers lowered the final hydrolysis yields by 20%, 29%, and 38%, respectively, while the yield with the control was 81%. Both Fig. 2A and B clearly show that addition of xylooligomer mixtures inhibited cellulose hydrolysis the most followed by xylan and xylose, as confirmed by others (Ximenes et al., 2010). Furthermore, inhibition by these compounds was much more obvious at higher concentrations than at lower concentrations. In addition, although enzymatic hydrolysis of cellulose in the presence of xylan and xylooligomers had similar hydrolysis patterns, the final hydrolysis yields in the presence of xylooligomers were lower than with xylan. Because soluble xylooligomers are released from xylan, these results suggested that xylooligomers over some range of DPs could have stronger inhibition effects on enzymes than others.

The effects of xylose and xylan derivatives on the initial hydrolysis rates of enzymatic hydrolysis were evaluated by comparing yields in the first hour of enzymatic hydrolysis of cellulose in the presence of different concentrations of these compounds. As indicated in Fig. 2C, addition of 1.67 mg/ml xylose, xylan, or xylooligomers reduced initial hydrolysis rates by 9.7%, 34.5%, and 23.8%, respectively, compared to the control. For comparison, a higher concentration (12.5 mg/ml) of xylose, xylan and xylooligomers reduced initial rates of enzymatic hydrolysis by 37.8%, 53.2% and 81.9%, respectively. Therefore, addition of these compounds dramatically reduced the initial rate of enzymatic hydrolysis, and similar to the results described above, xylooligomers had a greater effect on initial rates than xylan or xylose at similar concentrations. In addition, higher concentrations of all these xylose compounds resulted in lower initial hydrolysis rates.

3.2. Effects of xylose, mixed xylooligomers, and xylan on β-glucosidase performance

The effect of xylose and its oligomers on β-glucosidase activity was evaluated by comparing glucose yields from cellobiose hydrolysis with or without addition of 12.5 mg/ml of xylose, xylooligomers, or xylan at a β-glucosidase loading of 10 CBU/g cellobiose. As shown in Fig. 3, no obvious differences were observed in glucose release among the control and samples with xylose or xylan derivatives although strong inhibitory effects on cellulases were observed with same concentration of these compounds. Thus, although xylose, xylooligomers, and xylan strongly inhibited cellulase enzymes, they had little if any effect on β-glucosidase, consistent with previous report on effects of xylose alone (Xiao et al., 2004).

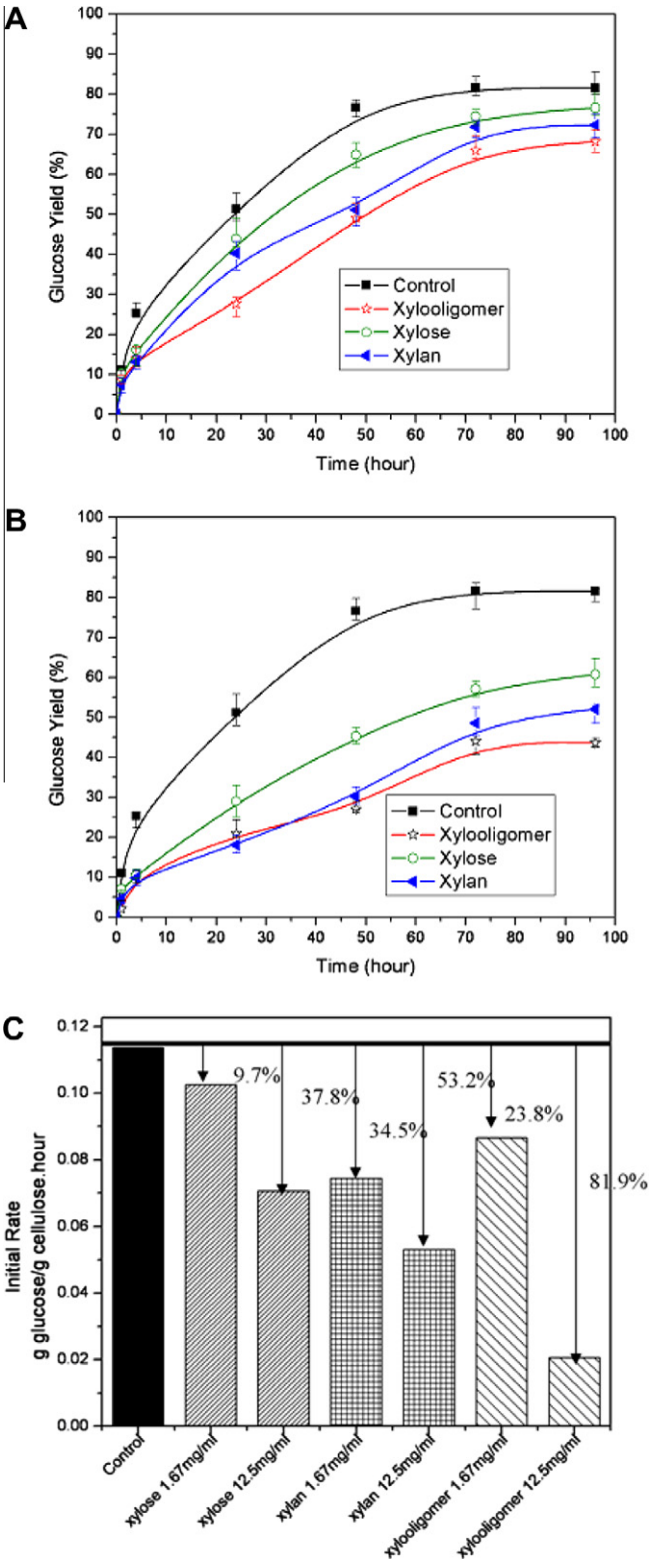

Fig. 2. Enzymatic hydrolysis of 2% Avicel alone (the control) and following initial addition of xylose, mixed xylooligomers, or xylan at a mass ratio of 12:1 corresponding to a concentration of 1.67 mg/ml (A) and 12.5 mg/ml (B) of monomeric xylose for an enzyme loading of 5 FPU/g glucan plus 10 CBU/g glucan. Comparison of initial rates of 2% Avicel enzymatic hydrolysis with addition of 1.67 mg/ml and 12.5 mg/ml xylose, mixed xylooligomers, and xylan (C).

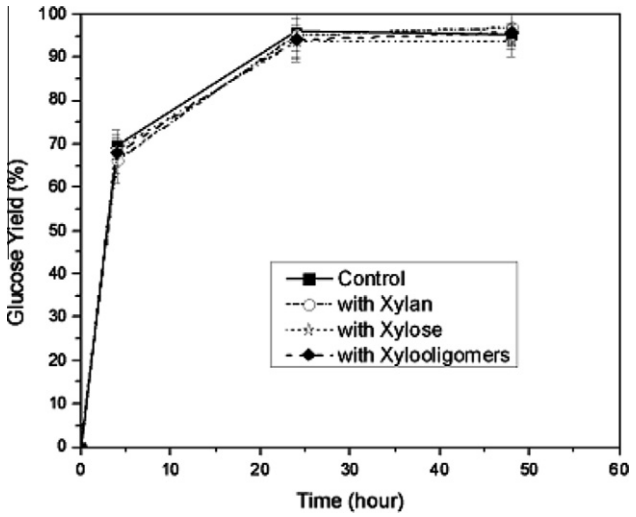

Fig. 3. Enzymatic hydrolysis of 2% cellobiose alone (the control) and with addition of 12.5 mg/ml xylan, mixed xylooligomers, or xylose at a beta-glucosidase loading of 10 CBU/g cellobiose.

3.3. Xylooligomer profiles during cellulose hydrolysis

Measuring cellulase inhibition by xylooligomer mixtures is more complicated than that by xylose alone due to changes in the xylooligomer molecular weight distribution during enzymatic hydrolysis of cellulose. Therefore, changes in the degree of polymerization of xylooligomers were monitored during enzymatic hydrolysis of pure cellulose, to which 6.25 mg/ml of the previously mentioned xylooligomer mixture of 8% xylose, 42% DP2–30 xylooligomers, and 45% high DP xylooligomers (total equivalent xylose percentage = 95%) had been added initially. Similar to results above, Fig. 4A points out that the glucose yield in the presence of xylooligomers was 12% lower than that of the control at 96 h, while Fig. 4B shows that the concentration of high DP oligomers (DP > 30) dropped rapidly to near the detectable limit within 24 h. The yield of low DP oligomers (DP = 2–30) increased rapidly over the first 3 h due to breakdown of the high DP oligomers but then dropped rapidly in concentration to about 20% within a day, with the composition shifting to mostly xylobiose before decreasing further to about 8% at 96 h. As a result of the fast breakdown of xylooligomers, the xylose concentration increased rapidly in the first 24 h and then slowed as it approached the maximum yield of 82% at 48 h. These results showed that a significant portion of the high DP xylooligomers first formed low DP oligomers that in turn broke down to xylobiose and xylose. In addition, xylobiose tended to be more difficult to break down by cellulases than higher DP oligomers, possibly due to low β-xylosidase activity in commercial cellulases. For comparison, a mixed xylooligomers sample without enzyme addition was run in parallel. In this case, Fig. 4C reveals that the concentrations of each xylose or xylooligomer group did not change much during the entire time period in the absence of cellulase and β-glucosidase while Fig. 4B shows that commercial cellulase enzymes could actually convert most of the xylooligomers into xylose during this same time span. Although hydrolysis rates did pick up once cellulase rapidly broke down virtually all of the soluble xylooligomers to xylose and some xylobiose, as shown in Fig. 4A and B, the final yields were still considerably lower than observed without addition of the mixed xylooligomers. Considering the comparative low xylanase and β-xylosidase enzyme activities in Spezyme CP cellulase enzymes (Berlin et al., 2006) and the efficient hydrolysis of most high and

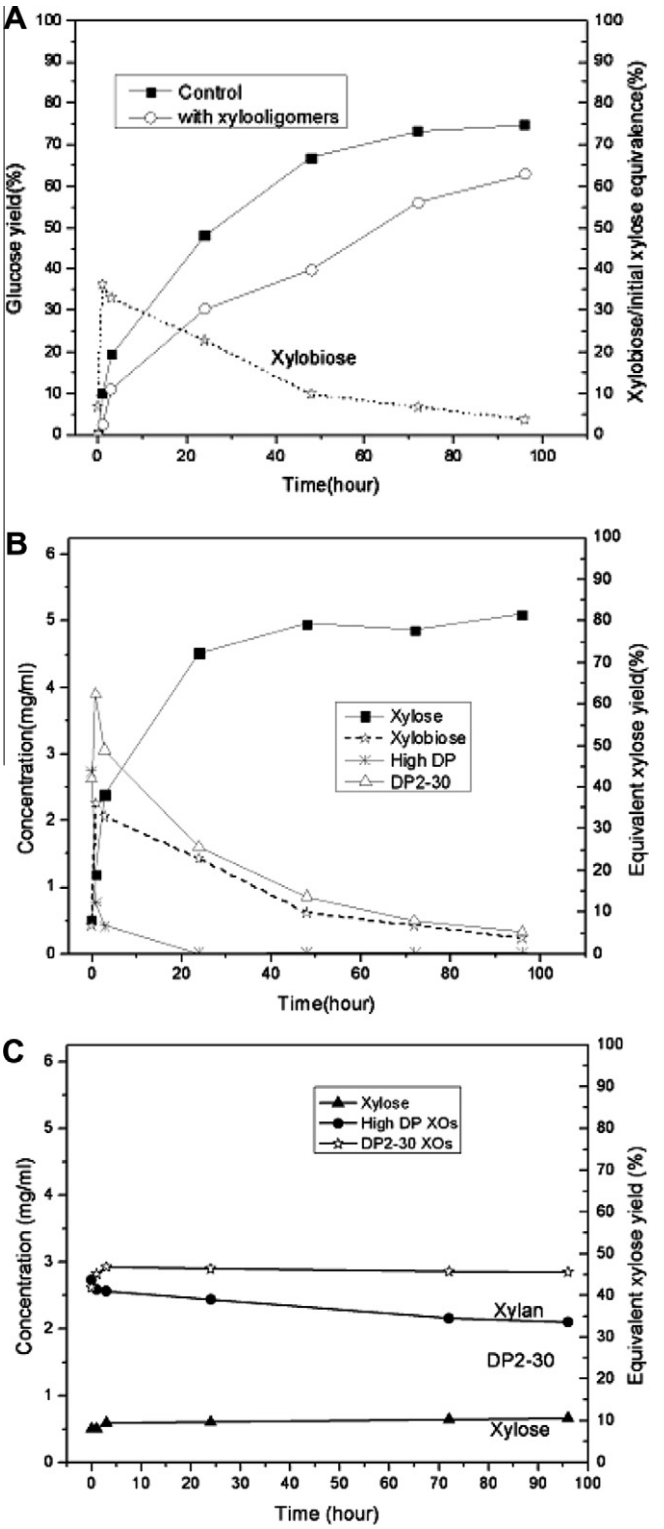

Fig. 4. Glucose yields and xylobiose concentrations for 96 h enzymatic hydrolysis of 2% Avicel cellulose concentrations with and without addition of mixed xylooligomers at a concentration of 6.25 mg/ml (A). Enzymatic hydrolysis was performed at 50 °C and pH = 4.8 with an enzyme loading of 5 FPU/g glucan plus 10 CBU/g glucan. Xylose and xylooligomer profiles during enzymatic hydrolysis of cellulose with addition of mixed xylooligomers for the yield profiles of Fig. 4A. The initial mixed xylooligomer concentration was 6.25 mg/ml, and its initial composition was 8% xylose, 42% DP2–30 oligomers, and 45% higher DP oligomers (B). Xylose and xylooligomer profiles for 96 h hold time at the same conditions, xylose compound concentrations, and hold times as for Fig. 4A and B but without added enzymes (C).

low DP xylooligomers during this 96 h time span, the results suggest competitive inhibition of cellulase by xylooligomers.

3.4. Comparison of inhibition by glucan and xylan derivatives

Glucose, cellobiose and degradation products of cellulose hydrolysis have been shown to be inhibitors in many previous studies (Gusakov and Sinitsyn, 1992; Holtzapple et al., 1990; Xiao et al., 2004). Thus, inhibition by xylose derivatives was compared to that by glucan derivatives to understand the relative importance of xylose and xylooligomers in inhibiting cellulose hydrolysis. As a basis for comparison, 10 mg/ml of glucose and the same concentration of cellobiose were added separately to a 2% cellulose concentration prior to enzymatic hydrolysis using the same low enzyme loading of 5 FPU/g glucan of cellulase supplemented with 10 CBU/g glucan of beta-glucosidase as above. Equal molar concentration of xylose, xylobiose and mixed xylooligomers were added based on the same equivalent xylose mass to compare their inhibition effects with glucan derivatives. As shown in Fig. 5B, glucose and cellobiose decreased initial hydrolysis rates by 96% and 95% respectively, while xylose, xylobiose and xylooligomers lowered initial hydrolysis rates by 38%, 39% and 79% respectively. However, although the well-established inhibitors glucose and cellobiose inhibited cellulose enzymatic hydrolysis much more strongly during the first hour than any of the xylan derivatives, mixed DP xylooligomers were far more inhibitory to cellulase than either glucose or cellobiose in the longer term. As indicated by Fig. 5A, mixed DP xylooligomers exhibited the strongest inhibition of cellulose conversion after 8 h of hydrolysis, dropping cellulose to glucose conversion by 52% compared to the control. Furthermore, inhibition by glucose and cellobiose gradually dropped after the first few hours, with the result that 96 h yields were reduced by 15% and 13% for glucose and cellobiose sugars, respectively. On the other hand, the mixed xylooligomer sugars dropped the 96 h glucose yield by 32%. Fig. 5A shows that xylose and xylobiose had a much smaller effect on cellulase performance than the higher DP xylooligomers, glucose, or cellobiose, with 96 h yields dropping by only 7% and 9%, respectively. These results illustrate that although mixed DP xylooligomers are not the strongest inhibitors initially, they were more powerful in the long term. Thus, more attention should be given to reducing xylooligomers inhibition to achieve a higher cellulose conversion with lower enzyme loadings.

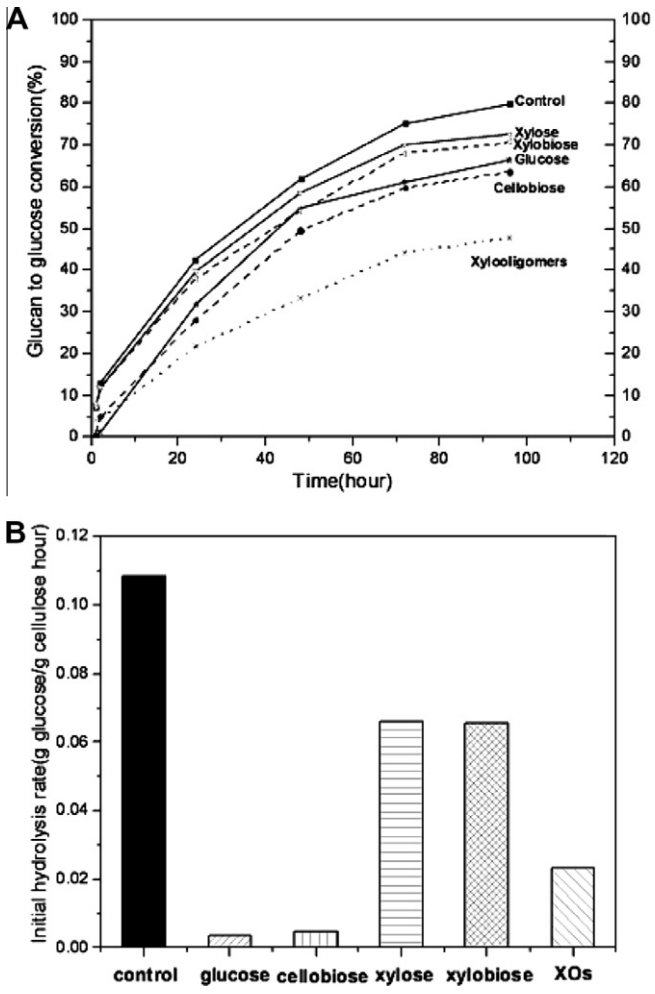

Fig. 5. Enzymatic hydrolysis (A) and initial 1 h hydrolysis rates comparison (B) of 2% Avicel alone (the control) and with addition of xylose, xylobiose, mixed xylooligomers, glucose, or cellobiose, all with an enzyme loading of 5 FPU/g glucan plus 10 CBU/g glucan. Glucose and cellobiose concentrations were 10 mg/ml (0.0556 mol/ml). Xylose, xylobiose, and mixed xylooligomers concentrations were based on equivalent monomeric xylose mass concentrations that were all equal to the molar concentration of glucose (8.33 mg/ml).

3.5. Comparison of pure xylose and xylose made from birchwood xylan

Some glucan, lignin, and other unknown compounds are contained in birchwood xylan, and water-only treatment of birchwood xylan to produce different DP xylooligomers introduces degradation products into the xylooligomer hydrolyzate. Therefore, it is important to be sure that the inhibition observed with the mixed DP xylooligomer solution is really due to the xylooligomers and not any of these other compounds present in the hydrolyzates employed. To address this concern, a xylose solution derived by enzymatic hydrolysis of xylooligomers made from birchwood xylan was added to enzymatic hydrolysis of cellulose for comparison to cellulose hydrolysis in the presence of purchased pure xylose. In addition, mixed DP xylooligomers produced from xylan hydrolysis were added separately at an equivalent xylose concentration for comparison. As shown in Fig. 6, xylose made from birchwood xylan had similar inhibition effects as pure xylose, and both inhibited cellulose conversion far less than the mixed DP xylooligomers. Thus, it is fair to conclude that the various chain length xylooligo-

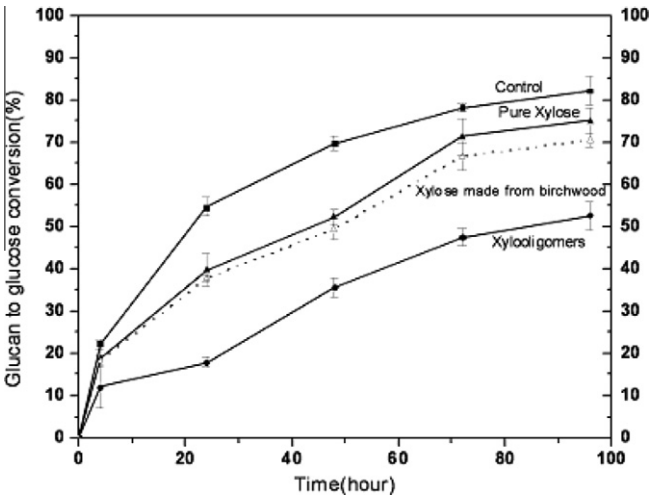

Fig. 6. Enzymatic hydrolysis of 2% Avicel with addition of purchased xylose, monomeric xylose made from birchwood, and xylooligomers made by birchwood hydrolysis at an enzyme loading of 5 FPU/g glucan plus 10 CBU/g glucan. All xylose and xylooligomers were added based on equivalent xylose concentration of 5 mg/ml prior to adding enzymes.

mers in solution and not degradation products and other background compounds were primarily responsible for the strong inhibition of cellulose hydrolysis by xylooligomers.

The strong inhibition of cellulase by xylooligomers and xylooligomer hydrolysis by cellulase reported here suggests that hemicellulose removal from lignocellulosic materials prior to enzymatic hydrolysis may not only increase enzyme accessibility but also reduce cellulase inhibition by xylooligosaccharides released during enzymatic hydrolysis. This possibility is consistent with reports that hemicellulose removal before enzymatic hydrolysis was more beneficial to hydrolysis rates and yields than adding a higher dose of enzyme (Liao et al., 2005).

This result also reinforces the importance of enhancing xylanase and β-xylosidase activities in the widely used cellulase and β-glucosidase enzyme combinations to totally hydrolyze xylooligomers into less inhibitory xylose, thereby reducing enzyme doses needed to achieve higher cellulose and hemicellulose conversions (Elander et al., 2009; Gupta et al., 2008; Kumar and Wyman, 2009).

4. Conclusion

In summary, our results indicated that xylose, xylooligomers, and xylan strongly inhibited cellulase. In addition, inhibition increased with concentration of these compounds, and xylooligomers were more inhibitory to cellulase than either xylan or xylose for an equivalent amount of xylose at the concentrations studied. Xylooligomers were also far more inhibitory to cellulase than equal molar amounts of glucose or cellobiose. However, additional research is needed to determine which xylooligomer chain lengths inhibit cellulose hydrolysis the most, how inhibition by these oligomers changes with enzyme loadings and substrate concentrations, and the mechanism of enzyme inhibition.

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