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ORIGINAL PAPER

Xylanase supplementation on enzymatic saccharification of dilute acid pretreated poplars at different severities

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Abstract Three pairs of solid substrates from dilute acid pretreatment of two poplar wood samples were enzymatically hydrolyzed by cellulase preparations supplemented with xylanase. Supplementation of xylanase improved cellulose saccharification perhaps due to improved cellulose accessibility by xylan hydrolysis. Total xylan removal directly affected enzymatic cellulose saccharification. Furthermore, xylan removal by pretreatment and xylanase are indifferent to enzymatic cellulose saccharification. However, more enzymatic xylose and glucose yields were obtained for a substrate with lower xylan content after a severer pretreatment at the same xylanase dosage. The effectiveness of xylanase at increased dosages depended on the substrates structure or accessibility. High xylanase dosages were more effective on well pretreated substrates than on under-pretreated substrates with high xylan content. The application sequence of xylanase and cellulase affected cellulose saccharification. This effect varied with substrate accessibility, perhaps due to competition between xylanase and cellulase binding to the substrate.

Keywords Xylanase supplementation · Enzymatic hydrolysis/saccharification · Poplar/hardwood · Pretreatment severity · Substrate accessibility

This work was conducted while Zhang, Zhuang, and Wang were visiting scientists at the USDA Forest Products Laboratory and on official government time of Zhu, Matt, and St. John.

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Introduction

Lignocellulosic biomass can be a sustainable source for biofuel production (Goldemberg 2007; Scharlemann and Laurance 2008). While the cost-effective production of an adequate supply of biomass continues to be a key to develop a natural resource based economy, developing efficient bioconversion processes and technologies remains a challenge (Humbird et al. 2011; Zhu and Zhuang 2012). A major part of this challenge is the natural recalcitrance of plants to microbial and chemical deconstruction (Himmel et al. 2007; Zhu et al. 2010a; Zhu et al. 2011). The plant cell

wall of lignocellulosic biomass contains three major biopolymers: cellulose, lignin, and hemicelluloses. Hemicelluloses are a broad class of polysaccharide that includes several branched heteropolymers composed of a variety of 5- and 6-carbon sugars. Hemicelluloses are considered potential sites for covalent cross-linking between carbohydrates and ligin, known as lignin-carbohydrate complexes (Fengel and Wegener 1984). The polymer matrix structure of ligin, hemicelluloses, and cellulose makes biological deconstruction of the plant cell wall difficult and costly (Balakshin et al. 2011).

A pretreatment step can overcome the natural recalcitrance of lignocellulosic biomass to liberate the sugars through widening the lignocellulosic complex and making high sugar yields possible (Yang and Wyman 2008; Zhu and Pan 2010). Mild pretreatment conditions can be used to minimize sugar loss and the formation of fermentation inhibitors (Kuhnel et al. 2011; Zhu et al. 2012). However, mild pretreatments result in a significant amount of hemicelluloses, mostly xylan (e.g., for substrates such as hardwoods, agricultural, and herbaceous biomass), remaining associated with the cellulosic-rich water insoluble fraction. It is known that hemicelluloses act as a physical barrier that limits the accessibility of cellulase enzymes to cellulose and affect cellulose enzymatic saccharification (Leu and Zhu 2013; Zhao et al. 2012). In acid-catalyzed pretreatments in which delignification was insignificant, xylan removal from the plant cell wall was critical to facilitate enzymatic cellulose saccharification (Leu and Zhu 2013). Furthermore, the amount of xylan removal was directly correlated to cellulose hydrolysis efficiency (Moxley et al. 2012; Zhang et al. 2013; Zhu et al. 2012). As a result, mild pretreatments result in low yields of total sugars due to reduced cellulose saccharification efficiency.

Xylanase supplementation during biomass saccharification has the potential to improve cellulose saccharification efficiency to address the conundrum discussed above (Hu et al. 2011). Studies reported the synergistic effects between cellulase and non-cellulase enzymes, including primarily xylanase (Murashima et al. 2003; Selig et al. 2008). Much of this synergism between cellulase and xylanase is believed to arise from the ability of xylanase to expose the cellulose microfibril core, by either removing the hemicelluloses or the hemicellulosic side-chains (Yu et al. 2003). However, the experimentally observed synergism varied depending on the pretreatment methods used and was explained by the varied effects on the disruption of xylan association with cellulose (Kumar and Wyman 2009a; Qing and Wyman 2011). An early study on xylanase supplementation for dilute acid pretreated poplar wood used high pretreatment severities that resulted in substrates with low xylan contents between 0.7 and 2.1% or xylan removal between 91.7 and 97.1% (Kumar and Wyman 2009b). Because different pretreatment methods have very different mechanisms in modifying and opening cell wall structure, further studies with more carefully designed experiments are needed to provide improved understanding for effective xylanase applications. This study examines the effectiveness of xylanase supplementation on cellulose saccharification using the same dilute acid pretreatment but under different pretreatment severities, especially at low pretreatment severities that were not studied previously, using two poplar wood samples. The goals are to improve pretreatment energy efficiency and significantly reduce the degradation of xylose into furfural to increase overall xylose yield and to maximize bioconversion efficiency. This practice has now been recognized as an effective strategy to address sugar yield and fermentation difficulties using dilute acid pretreatments as demonstrated by the US National Renewable Energy Laboratory at their pilot scale trails (Schell 2012).

Materials and methods

Materials

Fourteen-year-old trees of poplar clones of NE222 (abbreviated NE2 in sample labels), and NM6 were harvested from the US Forest Service, Hugo Sauer Nursery in Rhinelander, WI. NE222 and NM6, used in this study, were from two different genotypes: *Populus (P.) deltoides* Bartr.ex Marsh × *P. nigra L.* 'NE222' and P. *nigra* × *P. maximowiczii* A. Henry 'NM6', with contrasting yield potential, growth phenologies, and recalcitrance levels. All logs were hand debarked and then chipped at the USDA Forest Service, Forest Products Laboratory, Madison, Wisconsin, using a laboratory chipper. The wood chips were then screened to remove all particles >30 mm

and <6 mm in length. The thickness of the accepted chips ranged from 1 to 5 mm. The chips were kept frozen at a temperature of −16°C until used.

Celluclast 1.5 L and ß-glucosidase Novozyme 188 were generously provided by Novozymes of North America (Franklinton, NC). Accessory xylanase Accellerase® XY was donated by Genencor (Rochester, NY). The protein content and specific activities of these enzymes were determined using the Bradford method (Bradford 1976) and a literature method (Wood and Bhat 1988), respectively, as described previously (Lou et al. 2013). The results are listed in Table 1. Sodium acetate and sulfuric acid were acquired from Sigma-Aldrich (St. Louis, MO). All chemicals were ACS reagent grade.

Substrate production

Wood chips were directly pretreated using dilute sulfuric acid in three 1 L pressure vessels as described previously (Luo et al. 2010; Tian et al. 2011). The pressure vessels were mounted inside a 23 L rotating wood pulping digester heated externally using a steam jacket. Each pressure vessel was loaded with 150 g of wood chips in oven dry (od) weight and dilute acid solution with wood to solution ratio of 1:3 (w/v). Pretreatment temperature, residence time, and acid dosage were varied to vary pretreatment severity and to produce substrates with different xylan contents. Pretreatments of the two poplar samples of NE222 and NM6 under the same condition were conducted in the same batch run using the three pressure vessels. Central composite design of 17 total pretreatments including 3 replicates at the center point was conducted for each poplar sample. The pretreatment conditions of the selected substrates used in this study are listed in Table 2. After pretreatment, the solid substrates were separated from the pretreatment spent liquor by an 8-mesh screen. The pretreatment spent liquors were saved for future analysis and use. The solids were milled by a disk refiner with plates pattern D2-B505 (Andritz Sprout-Bauer Atmospheric Refiner, Springfield, OH) and disk plate gap of 1.02 mm under atmospheric conditions (Zhu et al. 2009; Zhu et al. 2010b). Water was added to facilitate milling to result in a solid discharge consistency was 10%. The milled solids were directly dewatered by pressing using a canvas bag to a solids content of approximately 30% without separate washing.

Table 1 List of protein content and specific activities of different enzymes

Enzyme preparation	Protein content (mg/mL)	FPU (FPU/mL)	ß-Glucosidase (IU/mL)	Xylanase (u/mL)
Celluclast 1.5 L	129.2	70	17	438.8
Novozyme 188	233.4	n/a	239	32.63
Accellerase® XY	126.25	0.7	n/a	15267.18

Determination of pretreatment severity

The combined hydrolysis factor *(CHF)* developed previously was used to determine pretreatment severity (Zhu et al. 2012). Unlike the commonly used combined severity factor *(CSF)* (Chum et al. 1990) that cannot provide good predictions of xylan dissolution, *CHF* combined slow and fast xylan fractions and their respective reaction rates and is capable of accurately determining xylan dissolution, important to reflect the pretreatment severity relevant to substrate xylan content. *CHF* is also applicable to pretreatment with more than one catalyst, i.e., acid and sulfite.

$$CHF = e^{\left(\alpha - \frac{E}{RT} + \beta C_A + \gamma C_B\right)}(C_A + C_B)t \quad (1a)$$

For the present study with only one catalyst (acid), Eq. (1a) can be simplified as

$$CHF = e^{\left(\alpha - \frac{E}{RT} + \beta C_A\right)} C_A \cdot t \quad (1b)$$

Where α and β are adjustable parameters, E is the apparent activation energy, R is universal gas constant = 8.314 (J/mole/K), C_A is acid molar concentration. The symbols α, E, β along with θ and f in Eq. (2) were determined by fitting the measured xylan contents of the pretreated substrates with *CHF* using Eq. (2). Equation (2) was developed based on xylan dissolution kinetics that takes slow and fast reacting xylan into consideration (Zhu et al. 2012). The slow xylan represents a small fraction of xylan intimately associated with cellulose that is hard to be hydrolyzed (Harris et al. 1963). The fitting results are listed in Table 3 for the two wood samples studied.

Table 2 Chemical compositions of the untreated and pretreated substrates along with pretreatment conditions and seventies

Sample label	T (°C)	Time (min)	Acid (% v/v)	*CHF*	Solid Yield (%)	Chemical composition (wt% of dry matter)			
						Glucan	Xylan	Mannan	Lignin
Untreated wood									
U-NE222	–	–	–			40.75	16.42	3.79	23.5
U-NM6	–	–	–			39.39	15.81	3.70	25.2
Pretreated substrate									
NE2-6	150	50	0.3	4.97	74.6	54.29	4.62	1.54	30.2
NE2-8	165	30	0.15	2.94	78.4	52.89	6.36	1.73	27.7
NE2-7	120	50	0.3	0.50	94.7	45.93	12.19	2.67	24.9
NM6-5	165	70	0.15	7.17	66.3	60.50	4.71	1.11	32.2
NM6-6	150	50	0.3	5.99	75.6	56.76	6.43	1.95	31.3
NM6-7	120	70	0.3	0.67	93.1	45.30	12.59	3.20	26.2

$$X_R = (1-\theta)e^{-CHF} + \theta e^{-fCHF} \quad (2)$$

Where X_R is the fraction of xylan remained in the solid substrate after pretreatment, θ is the fraction of slow reacting xylan and f is the ratio of the rate of constant between the slow and fast xylan.

Different pretreatment conditions were applied to two poplar wood chip samples of NE2 and NM6 to produce solid lignocellulosic substrates with different xylan contents. Three pairs of substrates with xylan contents of approximately 4.6, 6.4, 12.5%, respectively, were selected for the enzymatic hydrolysis study. The pretreatment conditions and severities *(CHF)* along with chemical compositions of the 6 selected substrates are listed in Table 2.

Enzymatic hydrolysis

Enzymatic hydrolysis was carried out at 2% substrate solids loading (w/v) in 50 mL of sodium acetate buffer of pH 5.0 and 50 mM on a shaker (Thermo Fisher Scientific, Model 4450, Waltham, MA) at 50 °C and 200 rpm. Cellulase loading was Celluclast 1.5 L at 7.5 FPU/g glucan and Novozyme 188 (ß-glucosidase) at 11.25 CBU/g glucan. This relatively low enzyme dosage was chosen to better differentiate sugar release among substrates with different xylan contents. The Accellerase XY (xylanase) loading was varied from 0 to 0.7 mL/g glucan to investigate the effect of xylanase supplementation on enzymatic hydrolysis of pretreated substrates.

Table 3 Fitting parameters of xylan dissolution data using **Eq.** (2)

	NM6	NE2	Unit
E	99,777	105,118	J/mole
α	28.2	29.5	None
β	15.0	15.2	L/mole
θ	0.398	0.246	None
f	0.060	0.054	None

Analytical methods

The solid biomass substrates were ground using a Wiley mill (model #2, Arthur Thomas Co, Philadelphia, PA) to pass a 40 mesh screen. The resulting materials were hydrolyzed using sulfuric acid in two stages as described previously (Luo et al. 2010). The carbohydrates in the hydrolysate were analyzed using a HPLC (Dionex IC-3000, Dionex Corporation, Sunnyvale, CA) with pulsed amperometric detection (HPAEC-PAD). The system was equipped with a second pump to deliver a post column stream of 200 mM sodium hydroxide at a flowate of 0.3 mL/min. to increase the eluent pH to approximately 12.4. A Dionex Carbo Pac PA-1 Analytical column (4 × 250 mm) with a 4 × 20 mm PA-1 guard column was conditioned using aqueous 220 mM Sodium Acetate and 380 mM Sodium Hydroxide for a period of 2 min followed by an 8 min equilibration period with 100% water prior to injection of 10 uL sample. The sample was then eluted with 100% water. The column compartment was maintained at 22° C throughout the run sequence. The Klason lignin

content was measured gravimetrically after washing and drying the solid residues from acid hydrolysis (Dence 1992). The same HPLC system was used to analyze sugars in the enzymatic hydrolysates.

Results and discussion

Effect of pretreated substrate xylan content on enzymatic xylan saccharification

Several studies reported a strong relationship between xylan removal and the extent of enzymatic cellulose saccharification (Moxley et al. 2012; Yang and Wyman 2004; Zhang et al. 2013; Zhu et al. 2012). Previous study indicated that the effectiveness of xylanase supplementation is indirectly related to xylan content (Kumar and Wyman 2009a). We therefore evaluated the effectiveness of xylanase supplementation on xylan saccharification of three pairs of substrates with very different xylan contents produced by dilute acid pretreatment at different severities (Table 2). The amounts of xylan removal by the pretreatments were approximately 80, 70, and 30% for NE2-6 and NM6-5, NE2-8 and NM6-6, and NE2-7 and NM6-7, respectively. It was found that enzymatic hydrolysis xylose yield (EHXY) (wt% of wood xylan) varied with initial xylan content and pretreatment severity (Fig. 1a) as expressed by the combined hydrolysis factor *(CHF)* (Zhu et al. 2012). The data indicate that xylan hydrolysis to xylose without account for xylooligomers by xylananse was most significant for the substrate with lowest initial xylan content (NM6-5) that was pretreated with the highest severity (*CHF* = 7.2) and least significant for the least pretreated substrate (NM6-7) with the lowest severity (*CHF* = 0.7).

When comparing two substrates with the same or very similar xylan content but from different wood samples, the effectiveness of xylanase on xylan hydrolysis to xylose was different (Fig. 1b). The effectiveness of xylanase not only depends on the substrate xylan content, the pretreatment severity *CHF* (Table 2) and the pretreated substrate structure, but also depends on the wood chemical composition such as lignin and xylan content and the initial cell wall structure. As clearly seen from Fig. 1b, xylanase is more effective on sample NE222 than on NM6 at relatively high xylan content 6.4 and 12%, but the difference decreased at xylan content of 4.6% probably due to the higher pretreatment severity of NM6-5 (*CHF* = 7.2 Table 2) than NE2-6 (*CHF* = 5.0, Table 2). A previous study suggested that NM6 is more recalcitrant than NE222 partially due to its higher wood lignin content (Wang et al. 2012) and a higher slow xylan content of approximately 40% than NE222 of 25% (Table 3). As discussed early, the slow xylan is the fraction closely associated with cellulose that is difficult to be hydrolyzed (Harris et al. 1963). The results from the current study suggest that the effects of NM6 recalcitrant structure can be overcome by a more severe pretreatment that can remove some fraction of slow xylan to improve substrate accessibility to enzymes and therefore xylan saccharification.

Effect of xylanase dosage on xylan and cellulose saccharification

Xylanase supplementation increased xylan saccharification of all NM6 substrates (Fig. 2). However, increasing xylanase dosage beyond 0.1 mL/g glucan did not produce additional xylose from NM6-7 that is very mildly pretreated (*CHF* = 0.7, Table 2). Improving pretreatment severity improved the xylanase effectiveness at high xylanase dosages. This is clearly seen from the EHXY of NM6-5 produced with *CHF* = 7.2 (Table 2). Very similar xylanase dosage effects on enzymatic hydrolysis glucose yield (EHGY) were also observed (Fig. 2). Furthermore, similar results of xylan and cellulose saccharification were also observed from the three substrates of NE222 (data not shown).

The effectiveness of xylan supplementation on cellulose saccharification

The EHGY at 72 h were plotted against EHXY at 72 h for all xylanase loadings, to quantitatively examine the effect of xylan removal on improving cellulose saccharification. The results indicate that EHGY was linearly correlated to EHXY for both the NM6 and NE222 substrates (Fig. 3a). Similar linear correlation was also obtained for a given substrate when saccharification data from different enzymatic hydrolysis times at different dosages were plotted (Fig. 3b). The slope of the correlations, $\frac{d(EHGY)}{d(EHXY)}$, a mathematical

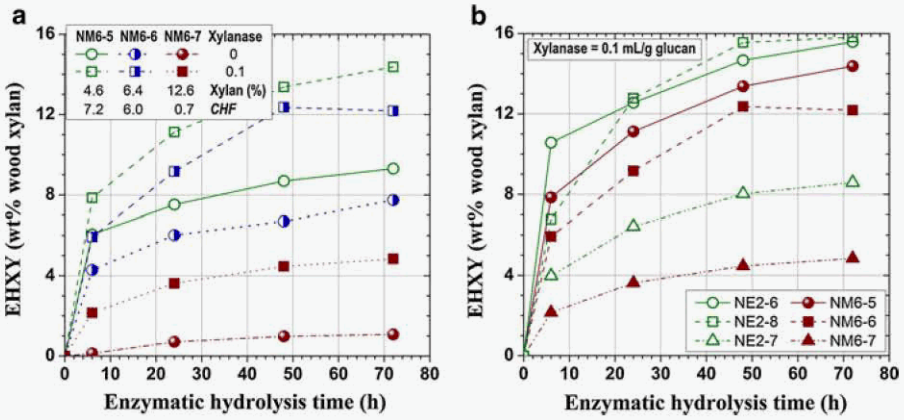

Fig. 1 Time-dependent enzymatic hydrolysis xylose yield (EHXY) in wt% wood xylan of differently pretreated poplars with xylanase supplementation. a Effect of xylanase dosage; b Comparisons between two poplar samples

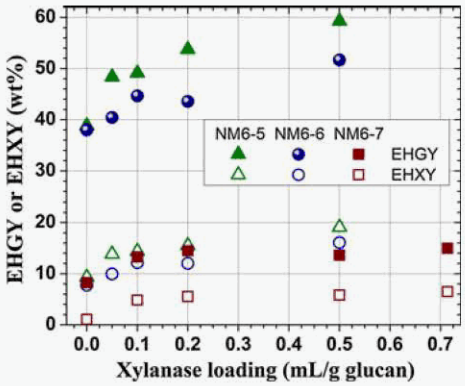

Fig. 2 Effect of xylanase loading on enzymatic hydrolysis yield of glucose (EHGY) or xylose (EHXY) in wt% wood glucan or xylan, respectively

expression of xylanase leverage (Kumar and Wyman 2009a), however, was independent of wood samples, pretreatment severity, and substrate xylan content (Fig. 3a), the difference in the fitted slopes were within error margin.

We plotted substrate cellulose and xylan enzymatic digestibilities (SCED and SXED), defined as percentage substrate glucan and xylan enzymatically saccharified to glucose and xylose, respectively, against total xylan removal by pretreatment and enzymatic hydrolysis at different xylanase dosages (Fig. 4a, b). Excellent linear correlations were obtained. This suggests that xylanase removal by pretreatment and xylanase are indifferent to enzymatic cellulose saccharification. The slopes of the corrections are similar for substrate pairs of NE2 and NM6 with the same xylan content, but very different for substrate pairs with very different xylan content. The substrate glucan and xylan content significantly contributed to the differences due to the way SCED and SXED are defined. Substrate xylan content or pretreatment severity, however, is a factor contributing to this difference. This is evidenced by the fact that the relative difference in substrate glucan content (Table 2) is much smaller than the differences in the slopes of the correlations in Fig. 4a. This is also corroborated by the results shown in Fig. 2. This again supports that significant xylan (or hemicellulose) removal is critical to improve enzymatic cellulose saccharification. A severe pretreatment with high xylan removal can improve xylanse supplementation and consequently facilitate cellulose saccharification.

Effect of xylanase and cellulase application sequence on cellulose saccharification

A previous study suggested that application of xylanase before cellulase produced better cellulose saccharification efficiency using Avicel (Qing and Wyman 2011). On the other hand, It was also reported that there was no difference in sugar release from very well pretreated poplar substrate by dilute acid between application xylanase first (24 h prior to application of cellulase) and application of xylanase and cellulase simultaneously (Kumar and Wyman 2009b). The results from Avicel and a well pretreated substrate with very low xylan content may not be applicable to lignocellulosic substrates with substantially higher xylan content. We evaluated three enzyme application sequences in this study using substrates, NM6-5 and NE2-6, both with xylan content of 4.6%. The sequences were: (1) xylanase was applied 24 h prior to the application of cellulase, (2) cellulase was applied 24 h before the application of xylanase,

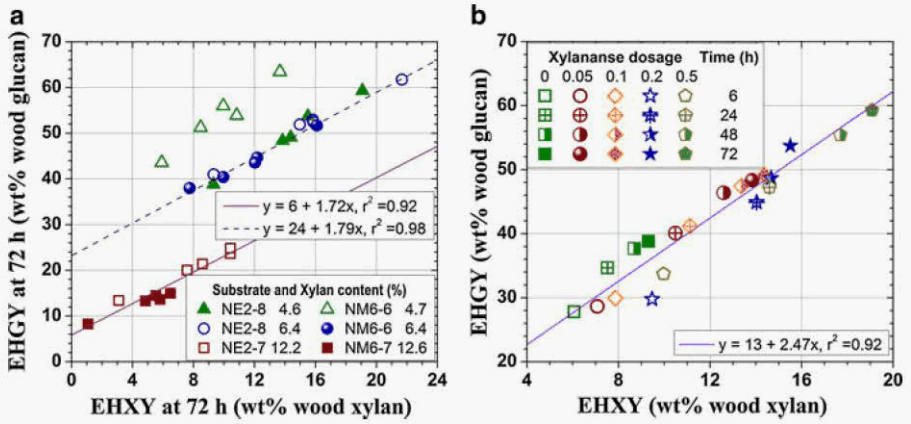

Fig. 3 Correlation between enzymatic hydrolysis glucose yield (EHGY) and xylose yield (EHXY) at different xylanase loadings. **a** All substrates at 72 h; **b** NM6-5 at all times

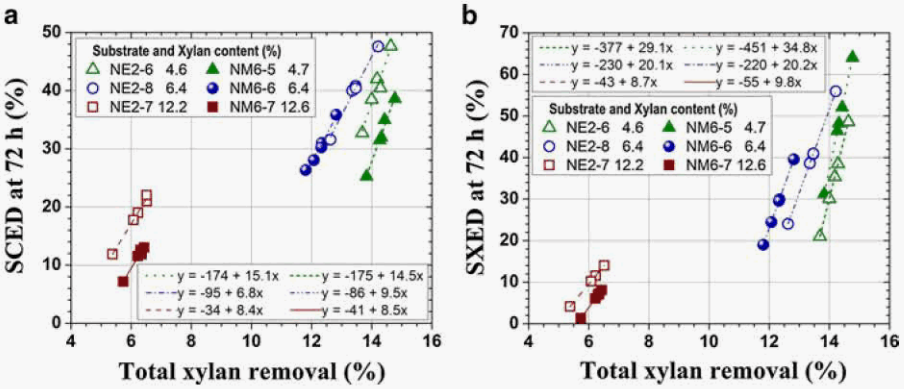

Fig. 4 Substrate enzymatic digestibility as total xylan removal by pretreatment and xylanase **a** substrate cellulose enzymatic digestibility (SCED); **b** substrate xylan enzymatic digestibility (SXED)

(3) xylanase and cellulase were applied at the same time (the baseline runs for most of the present study). The results indicate that application of cellulase 24 h prior to the application of xylanase produced the highest cellulose saccharification efficiency after 72 h for both substrates (Fig. 5a), i.e., SCED = 35 and 48 % for NM6-5 and NE2-6, respectively. The application of xylanase 24 h prior to the application of cellulase produced the lowest cellulose saccharification, i.e., SCED = 27 and 37 % for NM6-5 and NE2-6, respectively. The time-dependent results in Fig. 5a also show that the cellulose saccharification rate was higher in the first 24 h when cellulase and xylanase were applied at the same time than that produced by applying cellulase first; however, it did not produce a higher final cellulose saccharification efficiency (72 h). The xylo-oligomers produced by the xylanase is known to be inhibitive to affect cellulose saccharification (Qing et al. 2010). The 24 h delay in applying cellulase (sequence (3)) may produce significant amount of xylo-oligomers to reduce cellulose conversion substantially and never recovered even after 72 h.

The results also show that when cellulase was applied 24 h prior to xylanase supplementation, which produced the highest cellulose saccharification, xylan saccharification was intermediate for both NM6-5 and NE2-6, i.e., between those when xylanase and cellulase applied at the same time and those when xylanase applied 24 h prior to cellulase application (Fig. 5b). This suggests that the application of cellulase also produces synergistic effect on xylan saccharification. Cellulose saccharification opens the cell wall structure to improve xylanase accessibility to xylan to facilitate xylan saccharification, which in turn improved cellulose saccharification. It appears that there is a competition between cellulase and xylanase binding to the lignocellulosic substrate to hydrolyze cellulose and xylan, respectively, i.e., cellulose and xylan. This can be seen from the large discrepancies in xylan saccharification efficiency between NM6-5 and NE2-6

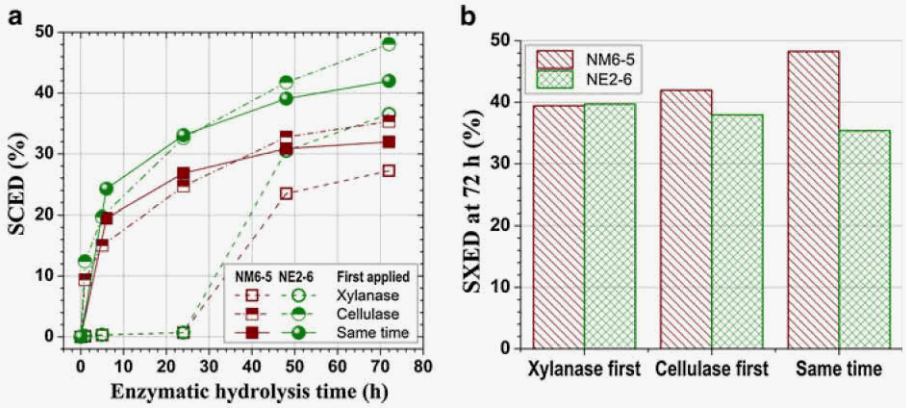

Fig. 5 Effect of xylanase and cellulase application sequence on saccharification. **a** Time-dependent substrate cellulose enzymatic digestibility (SCED): **b** xylan enzymatic digestibility (SXED) at 72 h

when xylanase and cellulase were applied at the same time (Fig. 5b). The xylan saccharification of NM6-5 was the highest when cellulase and xylanase were applied at the same time and lowest when xylan applied first. Opposite trend was observed from NE2-5, i.e., xylan saccharification was the lowest when cellulase and xylanase were applied at the same time and highest when xylanase was applied first. This perhaps can be explained by the difference in the substrate accessibility between NM6-5 and NE2-6. NM6 is more recalcitrant than NE222 as suggested by our previous study (Wang et al. 2012) and this also can be seen from the cellulose saccharification data in Fig. 3a and b. It is expected that the substrate accessibility of NM6-5 is lower than that of NE2-5 with its higher lignin content as confirmed by the WRV data (Table 2). As a result, applying xylanase first did not produce a higher xylan saccharification than that produced when xylanase and cellulase were applied at the same time due to improved xylan accessibility by cellulose saccharification. Although a commercial xylanase Accellerase® XY was used in this study with unknown active glycosyl hydrolase family of endoxylanases in hydrolyzing the xylan fraction, the primary xylanase activity is expected to be represented by one, or both, of the two major endoxylanase families. At least for the case of glycosyl hydrolase family 10 endoxylanases, the molecular weight will be comparable or greater than the typical cellulase catalytic domain. As a result, cellulose saccharification by cellulase may be critical to allowing the xylanase to infiltrate the cell wall and facilitate xylan saccharification. Delayed application of xylanase may reduce xylanase binding in competing with the cellulase binding to the substrate, which could result in an intermediate xylan saccharification (Fig. 5b). The accessibility of NE2-6 is relatively improved over than of NM6-5; therefore, xylan saccharification can proceed fast enough without the aid from cellulose saccharification to improve xylan accessibility to the xylanase. This explains the highest xylan saccharification when xylanase was applied first for NE2-6.

Conclusions

Xylanase supplementation can facilitate cellulose saccharification by improving cellulose accessibility through xylan hydrolysis. The effectiveness of xylanase supplementation in terms of enzymatic glucose and xylose yields depends on substrate structure and accessibility to enzymes determined by pretreatment severity. For a given wood sample, this dependence can be reflected by the variation of xylanase effectiveness with substrate xylan content. Enzymatic cellulose and xylan saccharification efficiencies are linearly proportional to total xylan removal irrespective of the xylan removed by pretreatment or xylanase. The appropriate xylanase dosage and application sequence of xylanase and cellulase to effectively facilitate cellulose saccharification are also dependent on substrate structure and/or pretreatment severity. High xylanase loading can increase xylose and glucose yield only for substrates will low xylan content, which pose a challenge to the

strategy of low pretreatment severity with xylanase supplementation.

Application of cellulase has a synergistic effect on xylan saccharification. Cellulose saccharification opens the cell wall structure to improve xylan accessibility to xylanase to facilitate xylan saccharification, which in turn improved cellulose saccharification. However, there is a competition between cellulase and xylanase binding to the lignocellulosic substrate to hydrolyze their respective component due to limited substrate accessibility, especially for those mild pretreated substrates. Xylo-oliomers produced by xylanase supplementation can also contribute to the effectiveness of a xylanase supplementation for enhancing cellulose saccharification. This may result in different effectiveness of xylanase supplementation on xylan saccharification using a given xylanase supplementation sequence on substrates with different accessibility to enzymes.

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