Improved Enzymatic Hydrolysis of Corn Stover by Green Liquor Pretreatment and a Specialized Enzyme Cocktail

Qiulu Chu,[a] Yang Huang,[a] Xin Li,[a] Yimin Fan,[a] Yongcan Jin,[b] Shiyuan Yu,[a] and Qiang Yong [a,*]

An effective strategy for sugar production from corn stover was established through a combination of green liquor pretreatment (8% total titratable alkali charge, 40% sulfidity, 140 °C, and 1 h) and enzymatic hydrolysis with a specialized enzyme cocktail. Green liquor pretreatment was demonstrated as an effective first step for sugar production due to the selective removal of lignin (39.70%), high carbohydrate recovery yield (81.53%), and obvious enhancement of enzymatic hydrolysis after pretreatment. When a specialized enzyme cocktail (cellulase, β-glucosidase, and xylanase at a ratio of 1:1.88:6.61, supplemented with 0.05 g of PEG 6000 *per* g of glucan) was applied, near-theoretical hydrolysis yield was achieved (glucan hydrolysis yield of 93.53% and xylan hydrolysis yield of 86.00%). A total fermentable sugar production of 50.14 g was obtained per 100 g of dry corn stover, including 36.08 g of glucose and 14.06 g of xylose.

Key words: Green liquor pretreatment; Enzymatic hydrolysis; Specialized enzyme cocktail; Sugar production

*Contact information: a: College of Chemical Engineering, Nanjing Forestry University, Nanjing 210037, China; b: College of Light Industry Science and Engineering, Nanjing Forestry University, Nanjing 210037, China; *Corresponding author: swhx@njfu.com.cn*

INTRODUCTION

The concept of producing lignocellulosic biofuel, bioproducts, and chemicals using biomass residues from agricultural and industrial activities has been established for 70 years or more. The key of this concept is to obtain efficient sugar production, through effective pretreatment and enzymatic hydrolysis technology. The heterogeneous complexity and recalcitrance of lignocellulosic materials, which primarily results from lignin as well as hemicellulose, causes difficulty in the degradation of lignocellulosic materials (Van Dyk and Pletschke 2012).

Pretreatment is required to break the lignin seal and/or partially remove the hemicellulose coating to increase the accessibility of enzymes (Saha and Cotta 2006). Among various thermochemical pretreatments, alkaline pretreatments (pH > 7.0) can leave most of the xylan in the solid portion (Kumar and Wyman 2009a), with decreased lignin content in the lignocellulosic materials. An alkaline pretreatment process based on the green liquor in a kraft pulp mill has been developed for wood chips (Jin *et al*. 2010; Wu *et al*. 2010; Meng *et al*. 2014).

Green liquor consists of sodium carbonate and sodium sulfide. Relatively mild conditions are applied in green liquor pretreatment, which can selectively remove lignin (Kim and Lee 2007), and achieve high and competitive polysaccharide recovery in softwood, hardwood, and agricultural residues (Jin *et al*. 2010; Wu *et al*. 2010; Gu *et al*. 2012).

The green liquor pretreatment process was developed with the concept of repurposing an old kraft pulp mill for ethanol production, using existing equipment and technology (Gonzalez *et al*. 2011); such repurposing was considered to lower total cost of green liquor pretreatment, together with spent liquor of green liquor pretreatment and enzymatic hydrolysis residue could be burnt in the recovery boiler to recover inorganic chemicals and energy.

By building on the mature kraft pulping and disk refining technologies already practiced in the pulp and paper industry, the green liquor pretreatment appears to face very few technological barriers and risks for commercialization (Gu *et al*. 2012). Thus, green liquor pretreatment is considered an attractive first step toward efficient sugar production from lignocellulosic materials.

Presently, the focus of efficient sugar production from lignocelluloses has changed from the conversion of cellulose into glucose to the production of both hexoses and pentoses, as it increases the theoretical yield and can substantially improve the economics of the whole process (Merino and Cherry 2007). This change has had an impact on certain aspects of the sugar production process, including enzymes required for efficient hydrolysis.

Xylan, which is retained in the solid from the pretreatment process, is also considered one of the hindrances for cellulose digestion, and it has been reported that enzymatic removal of xylan enhances cellulose digestion (Allen *et al*. 2001; Ishizawa *et al*. 2009). As a result, efficient sugar production from lignocelluloses could be anticipated through a combination of thermochemical removal of lignin by pretreatment and enzymatic removal of xylan prior to or concomitant with cellulose digestion.

Moreover, for any individual biomass and pretreatment regime, it is necessary to tailor the enzyme cocktail (enzyme mixture and additives) to achieve an efficient saccharification process, with the proportions and ratio of each depending on the type of biomass and pretreatment method used. Although the application of enzymes has been investigated for a long time (Gusakov *et al*. 2007; Jorgensen *et al*. 2007), the optimization of the enzymes mixture by altering components and their ratios has not received enough attention.

There is still no enzyme preparation specialized for enzymatic hydrolysis of the material resulting from various specified pretreatments. In order to achieve a near-theoretical sugar yield, typical cellulase, β-glucosidase, and xylanase are required (Van Dyk and Pletschke, 2012). In terms of obtaining the fastest and highest conversions, some surfactants, which are well documented to change the nature of the substrate and enzyme and to positively affect enzyme–substrate interactions (Eriksson *et al*. 2002), should also be taken into consideration.

This work had three distinctive phases: (1) determining effective green liquor pretreatment conditions for selective removal of lignin, while providing high polysaccharides retention in the solid and enhancement of enzymatic hydrolysis; (2) enzymatic removal of xylan prior to or concomitant with enzymatic hydrolysis of cellulose, to obtain a high yield of both hexoses and pentoses; (3) optimizing an enzyme cocktail that could lead to increasing hydrolysis efficiency with specificity toward the saccharification of green liquor-pretreated corn stover. To the authors' knowledge, no specialized enzyme cocktail has been proposed for the enzymatic hydrolysis of green liquor-pretreated corn stover.

EXPERIMENTAL

Corn Stover and Enzymes

Corn stover was kindly provided by Huhehaote, Neimenggu municipality, China and was cut to 3 to 5 cm in length. Air-dried corn stover was stored in sealed plastic bags at room temperature. The corn stover consisted of 38.14% glucan, 22.68% xylan, 2.78% arabinan, and 23.34% total lignin. A commercial *Trichoderma reesei* cellulase preparation (C2730) and β-glucosidase from *Aspergillus niger* (NZ188) were purchased from Sigma-Aldrich, USA. The filter paper, β-glucosidase, and xylanase activities of the cellulase preparation (C2730) were 96.79 FPU/g, 22.32 U/g, and 238.65 U/g, respectively. The β-glucosidase preparation (NZ188) had β-glucosidase activity of 349.46 U/g and very little filter paper activity or xylanase activity. The filter paper, β-glucosidase, and xylanase activities of xylanase (95595, Sigma-Aldrich; USA; 10 mg/mL) were 0.34 FPU/g, 0.82 U/g, and 16.57 U/g, respectively.

Green Liquor Pretreatment and Enzymatic Hydrolysis of Pretreated Corn Stover

According to our previous studies, pretreatment combinations derived from varying parameters such as total titratable alkali (TTA) charge (2, 4, 6, and 8%) and temperature (100, 120, and 140 ºC), were tested in duplicate. Corn stover was cooked using simulated green liquor (sodium carbonate and sodium sulfide) in the laboratory with a TTA charge of 2, 4, 6, or 8% (w/w) as Na_2O on dry corn stover with a sulfidity of 40% (Jin *et al*. 2010). The ratio of pretreatment liquor to corn stover (oven dried) was 6 (v/w). Corn stover was first impregnated with the pretreatment liquor at 60 ºC for 30 min, and then the temperature was raised to the target temperature at a rate of 2 ºC per min. The pretreatment was maintained at the target temperature (100, 120, or 140 ºC) for 60 min and terminated immediately by cooling the bombs to room temperature in cold water. The pretreated solid was collected and washed with deionized water (with a dry mass-to-water ratio of 1:15) to remove the residual chemicals and dissolved compounds. The washed solid was refined by a laboratory disk refiner (KRK; Japan) at 3000 r/min for the defibration.

Changes in the components of green liquor-pretreated corn stover were determined, while delignification and polysaccharides (glucan and xylan) yield were calculated. Enzymatic hydrolysis of green liquor-pretreated corn stover at a substrate loading of 5% (w/v) was performed in a 250-mL shaker flask at 50 °C, 150 rpm for 48 h in 50 mM citrate buffer (pH 4.8). A cellulase dosage of 50 FPU/g was applied, with complementary β-glucosidase supplementation of 3 U/g, which was considered appropriate to convert cellobiose to glucose, according to previous experiments. Samples (1 mL) were taken at various time points and were immediately added to 0.4 mL of H_2SO_4 to deactivate the enzymes, followed by centrifugation at 5000 rpm for 10 min and filtration for sugar analysis. Enzymatic hydrolysis yield was calculated by averaging values for sample duplicates. The optimal pretreatment condition was chosen for future experiments, according to the delignification, carbohydrate recovery, and enhancement of enzymatic hydrolysis.

$$\text{Solid recovery (\%)} = \text{pretreated solid residue (g)} \times 100 / \text{raw material (g)} \quad (1)$$

Carbohydrate recovery (%) = (glucan + xylan in pretreated solid) (g) × 100/(glucan + xylan in raw material) (g) (2)

Delignification (%) = (lignin in raw material – lignin in pretreated solid)(g) × 100 / lignin in raw material (g) (3)

Glucan hydrolysis yield (%) = glucose in enzymatic hydrolysate (g) × 0.9 × 100 / initial glucan in substrate of enzymatic hydrolysis (g) (4)

Xylan hydrolysis yield (%) = xylose in enzymatic hydrolysate × 0.88 (g) × 100 / initial xylan in substrate (g) (5)

Sugar production (g) = glucose + xylose released from 100 g dry corn stover subject to pretreatment and enzymatic hydrolysis (g) (6)

Specialized Enzyme Cocktail for Enzymatic Hydrolysis of Green Liquor-Pretreated Corn Stover

The effects on the enzymatic hydrolysis yield and sugar production of the enzymatic removal of xylan with the addition of xylanase was evaluated initially. Experiments were carried out at a substrate loading of 5% (w/v) with corn stover pretreated at 140 °C, with a 8% TTA charge for 1 h. Cellulase dosages of 10, 15, 20, 25, 30, or 35 FPU/g, blended with xylanase dosages of 0, 40, 80, 120, or 160 U/g and a β-glucosidase dosage of 3 U/g, were examined. All of the enzyme dosages were *per* gram of glucan.

In addition, the effect of β-glucosidase (3, 5, 10, 15, 20, 25, 37.5, or 50 IU/g) on enzymatic hydrolysis was tested. After that, a series of batch enzymatic hydrolysis experiments were conducted to test the effect of blending β-glucosidase (20, 30, or 40 IU/g) and PEG 6000 on enzymatic hydrolysis, with PEG 6000 dosages of 0.01, 0.02, 0.03, 0.04, 0.05, 0.06, or 0.07 g/g. An enzyme cocktail with a cellulase dosage of 25 FPU/g and xylanase dosage of 120 U/g was used for enzymatic hydrolysis. All these experiments were conducted with a substrate loading of 5% (w/v) at pH 4.8 and 50 ºC in 250-mL Erlenmeyer flasks containing a 50-mL mixture for 48 h. Aliquots of 1 mL were sampled after 48 h for analysis of soluble sugars.

Analysis Methods

Material was analyzed using the National Renewable Energy Laboratory (NREL) standard methods or the determination of structural carbohydrates and lignin in biomass (NREL 2012). Enzyme activity was measured using an assay based on the Ghose methodology (Ghose 1987). The concentrations of glucose and xylose were determined using a high-performance liquid chromatography system with a refractive index (RI) detector (HPLC, Agilent technology 1100 series, Palo Alto, CA). Samples were filtered through a 0.22-μm filter. The analysis was performed on a Bio-rad minex HPX-87H ion exclusion column (300×7.8 mm; USA) with 5 mM H_2SO_4 as the eluent at a flow rate of 0.6 mL/min at 55 ºC (Tengborg *et al*. 2001).

RESULTS AND DISCUSSION

Effect of Green Liquor Pretreatment on Corn Stover

Green liquor pretreatment, which has been demonstrated to enhance the enzymatic hydrolysis efficiency of hardwood polysaccharides (Jin *et al*. 2010) and softwood polysaccharides (Wu *et al*. 2010), was utilized for corn stover. The components of green liquor pretreated corn stover under different pretreatment conditions were determined, and the results are illustrated in Table 1. As seen, glucan was the major component of green liquor pretreated corn stover (~49.93 %), followed by xylan (~23.74 %) and lignin (~24.74 %). The glucan content increased with TTA charge and temperature, while the lignin content decreased. Xylan was the main component of hemicellulose in the solids, for there was only a small amount of arabinan (~3.98 %), and trace amounts of mannan and galactan (details not shown). Results suggest that the green liquor pretreatment, acting as a kind of alkaline pretreatment, selectively decreased the lignin content of lignocellulosic material, resulting in cellulose-enriched material (Silverstein *et al*. 2007; Wang *et al*. 2012).

Table 1. Main Components of Corn Stover, Recovery Yield, and Delignification under Different Pretreatment Conditions

Temp. (°C)	TTA charge (%)	Glucan content (%)	Xylan content (%)	Total lignin (%)	Solid recovery yield (%)	Glucan recovery yield (%)	Xylan recovery yield (%)	Carbohydrate recovery yield (%)	Lignin removal (%)
100	2	40.84	22.88	24.35	88.92	95.21	89.69	92.43	7.25
	4	42.53	23.33	23.62	85.37	95.20	87.83	92.74	13.68
	6	43.06	23.57	22.80	83.31	94.06	86.59	90.69	18.64
	8	44.15	23.74	21.50	81.09	93.87	84.90	90.01	25.40
120	2	41.51	23.02	24.74	86.01	93.61	87.31	90.66	8.85
	4	43.92	23.11	23.48	80.94	93.21	82.46	88.78	18.61
	6	45.45	23.16	22.18	78.47	93.51	80.12	87.66	25.49
	8	47.84	22.60	20.66	74.52	93.47	74.25	84.96	34.06
140	2	42.41	23.02	24.78	83.55	92.90	84.81	89.18	11.34
	4	47.03	22.96	23.60	75.21	92.74	76.14	85.71	23.98
	6	48.67	22.42	21.70	71.89	91.74	71.07	83.69	33.25
	8	49.93	21.76	20.19	69.71	91.26	66.90	81.53	39.70

Component percentages are on dry-weight basis for all data.

The solid recovery yield under listed conditions was in the range of 69.71% (under relatively severe conditions, 140 ºC, 8% TTA charge) to 88.92% (w/w) (under mild pretreatment conditions, 100 ºC, 2% TTA charge), on the basis of raw materials. Yield dropped with ascending TTA charge and temperature, indicating that both TTA

charge and temperature had an impact on solid recovery yield, consistent with the work of McIntosh and Vancov (2010). In addition, the glucan recovery yield decreased slightly with the ascending pretreatment severity (95.21 to 91.26%), suggesting that cellulose was basically stable under the green liquor pretreatment conditions (Jin *et al*. 2010). However, the xylan recovery yield remained lower than that of glucan, which is consistent with the fact that the chemical and thermal stability of hemicellulose are lower than that of cellulose, most likely owning to its lack of crystallinity and lower degree of polymerization (Wang *et al*. 2012). A major portion of xylan was retained in the solid (66.90 to 89.69%), and this may have been due to the 4-o-methylglucoronic acid group attached as side chain at the C-2 position of xylan slowing down the primary peeling reaction in favor of the stopping reaction in the green liquor pretreatment (Ibrahim *et al*. 2011; Jin *et al*. 2010). As a result, the carbohydrate recovery yield varied from 92.74% to 81.53%, suggesting a major part of carbohydrate remained in the solid, which was considered an important factor to obtain an improved yield of total fermentable sugars.

Green liquor pretreatment also caused delignification, which varied with pretreatment severity, and peaked at 140 ºC and a TTA charge of 8% (lignin removal of 39.70%). Previous reports suggest that hydrosulfide ions (HS^-), when released from the Na_2S of green liquor, cause the cleavage of phenolic baryl ether bonds of the lignin, leading to the selective degradation and removal of lignin macromolecules (Lee *et al*. 2009). It is also reported that delignification of about 30% is sufficient for effective enzymatic hydrolysis (Jin *et al*. 2010) and complete delignification is not essential to achieve efficient hydrolysis (Varnai *et al*. 2010). Ishizawa *et al*. (2009) also demonstrated that severe delignification can decrease cellulose digestibility.

Enzymatic hydrolysis was also performed on the green liquor-pretreated corn stover, to determine the influence of pretreatment severity on hydrolysis efficiency. In addition, sugar production was tested, which was defined as the weight of the sugar recovered from 100 g dry corn stover after pretreatment and enzymatic hydrolysis. This procedure incorporated the effect on the sugar yield during green liquor pretreatment and also during enzymatic hydrolysis. Sugar production was a very useful statistic because it helped determine which combination of green liquor pretreatment and enzymatic hydrolysis conditions gave the best fermentable sugar yield.

As observed in Fig. 1, elevated pretreatment severity (TTA charge and temperature) improved enzymatic hydrolysis yield. For instance, the glucan and xylan hydrolysis yields were 28.87% and 18.92%, respectively, under mild pretreatment conditions (100 °C, 2% TTA charge), and were enhanced to 78.16% and 55.70%, respectively, at 140 °C, 8% TTA charge. However, these treatments showed relatively less influence on sugar production efficiency, because elevated pretreatment severity caused lower carbohydrate recovery yield in solids. Correspondingly, sugar production from 100 g dry mass was enhanced from 15.90 g (11.53 g glucose and 4.37 g xylose) to 39.53 g (29.93 g glucose and 9.60 g xylose). The green liquor pretreatment at 8% TTA and 140 °C resulted in the most efficient sugar production. Elevated pretreatment severity caused increased enzymatic hydrolysis efficiency and sugar production, which was considered largely due to the delignification of the lignocellulosic biomass (Hu and Wen 2008; Jeoh *et al*. 2007), because lignin directly acted as a physical barrier, restricting cellulase access to cellulose, and inactivating cellulase through non-productive binding. The removal of lignin exposes more accessible cellulose, and reduces the strong surface interaction between lignin and enzyme (Yu *et al*. 2011).

It has been reported that a successful pretreatment process must be based on various considerations, including the extent to which lignin is removed, the extent to which polysaccharides are retained within the pretreated biomass, and significant enhancement of enzymatic hydrolysis after pretreatment (Merino and Cherry 2007; Romani *et al*. 2010; Van Dyk and Pletschke 2012). According to the above results, it could be concluded that green liquor pretreated (140 °C, 8% TTA charge) corn stover retained a high amount of carbohydrates in the solid (81.53%), selectively removed lignin (39.70%), while enabling obvious enhancement of sugar production, suggesting green liquor based pretreatment is an ideal step for the production of fermentable sugars from corn stover.

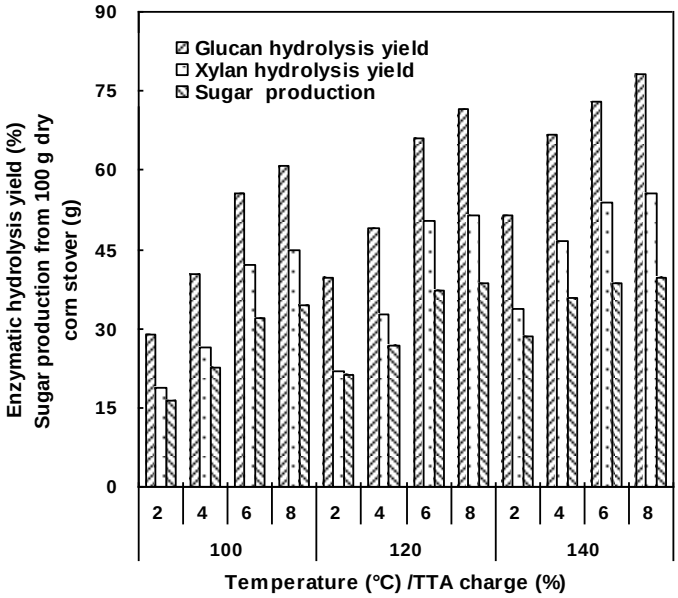

Fig. 1. Effect of green liquor pretreatment conditions on enzymatic hydrolysis, the relationship between delignification and sugar production

Effect of Enzymatic Removal of Xylan on Hydrolysis Efficiency and Sugar Production

Similar to other alkaline methods, green liquor pretreatment leaves a major portion of the initial xylan in the solids (Kumar and Wyman 2009a), and therefore, enzymes must release xylose in addition to glucose from the solids to realize high total sugar yields. The impact of supplementing xylanase with cellulase on enzymatic hydrolysis of green liquor pretreated corn stover is shown in Fig. 2. The general finding is that xylanase supplementation could increase both glucan and xylan hydrolysis yields, especially at a low cellulase dosage. For example, very low polysaccharides hydrolysis yield was obtained at a cellulase dosage of 10 FPU/g, including a glucan hydrolysis yield of 44.67% and xylan hydrolysis yield of 41.43%. They increased to 58.50% and 57.78%, respectively, at a cellulase loading of 10 FPU/g supplemented with xylanase at 160 U/g. Reasons for these results might be that hemicellulose (xylan) acts as a physical barrier around cellulose, ultimately restricting cellulase access and hydrolysis efficiency (Jeoh *et al*. 2007). The enzymatic removal of xylan enhances cellulose digestion by reducing the xylan coating and linkages to cellulose (Allen *et al*. 2001; Murashima *et al*. 2003; Teleman *et al*. 2001). However, increased enzyme dosage did lead to increased hydrolysis yield, but only up to a certain point, after which hydrolysis slows down due to

various factors (Van Dyk and Pletschke 2012). As observed in Fig. 2a, increasing cellulase dosage from 10 FPU/g to 25 FPU/g improved glucan hydrolysis yield, whereas glucan hydrolysis yield changed less with further increase of cellulase dosage from 25 FPU/g to 35 FPU/g. A similar tendency was also observed in xylan hydrolysis yield (Fig. 2b). A cellulase dosage of 25 FPU/g with a xylanase dosage over 120 U/g failed to promote or deliver greater sugar gains. Thereby, a cocktail with a cellulase dosage of 25 FPU/g and xylanase dosage of 120 U/g was considered to be adequate for effective saccharification of green liquor pretreated corn stover. The hydrolysis yield of glucan was 74.34%, xylan was 70.56%, and the sugar production from 100 g dry corn stover was 40.21 g, including 28.68 g glucose and 11.53 g xylose.

However, the hypotheses of removal of xylan coating and disruption of xylan linkages to glucan are difficult to prove experimentally due to the complex nature of biomass. Experiments were carried out to determine the relationship between xylan removal and the increase of sugar production. As shown in Fig. 2c, when only cellulase was added in the enzymatic hydrolysis, 34.70 g sugar was obtained at 48 h (strategy 1); when only xylanase was added in the enzymatic hydrolysis, only 1.71 g sugar was obtained at 48 h (strategy 2). However, the sugar production of strategy 3 was 40.26 g, with cellulase and xylanase added simultaneously at 0 h, while the initial rate of sugar release during the first 4 h was obviously higher than that of strategy 1 and 2. A cellulase-xylanase iteration, defined as xylanase leverage (Kumar and Wyman 2009b), was observed here, which was similar with synergism, but happened to different kinds of enzymes, cellulase and xylanase. In strategy 4, enzymatic hydrolysis was carried out with xylanase added at 0 h, followed by a cellulase addition at 4 h. As seen, little sugar was released during the first 4 h, with no cellulase in hydrolysis; the rate of sugar release increased dramatically, as the cellulase worked cooperatively with xylanase after 4 h. The sugar production at 48 h was 39.39 g. In strategy 5, enzymatic hydrolysis was performed with only cellulase added at 0 h, followed by a xylanase addition at 4 h. As observed, the rate of sugar release in strategy 5 was the same as that of strategy 2 during the first 4 h, but was enhanced obviously after 4 h, owning to the xylanase addition. The sugar production at 48 h was 38.42 g. As detected, xylanase leverage played an important role in enhancing the sugar production. The xylanase leverage could be explained by the xylan and/or xylooligomers aggregation on cellulose and its binding by covalent and hydrogen bonds; xylanase removes redeposited xylan and xylooligomers from the surface, thereby increasing the accessibility of cellulose microfibrils to cellulase. Similarly, xylan accessibility could in turn be limited by the presence of glucan microfibrils (Teleman *et al*. 2001). Thus, xylan could be further hydrolyzed, due to the removal of cellulose. In addition, Qing *et al*. (2010) proposed that xylose, xylan, and xylooligomers dramatically decreased conversion rates and yields of cellulose, while xylan and xylooligomers were even stronger inhibitors than xylose to cellulase. In order to enhance enzymatic hydrolysis efficiency, it is suggested to remove xylan or to convert xylan to xylose, which was considered less inhibitory to cellulase. Furthermore, the results suggested that xylanase addition concomitant with cellulase led to higher sugar production (strategy 3) than that of xylanase addition prior to (strategy 4) or after cellulase addition (strategy 5). So far, a strategy for sugar production from corn stover was established though the combination of thermochemical removal of lignin by green liquor pretreatment and enzymatic removal of xylan concomitant with cellulose digestion.

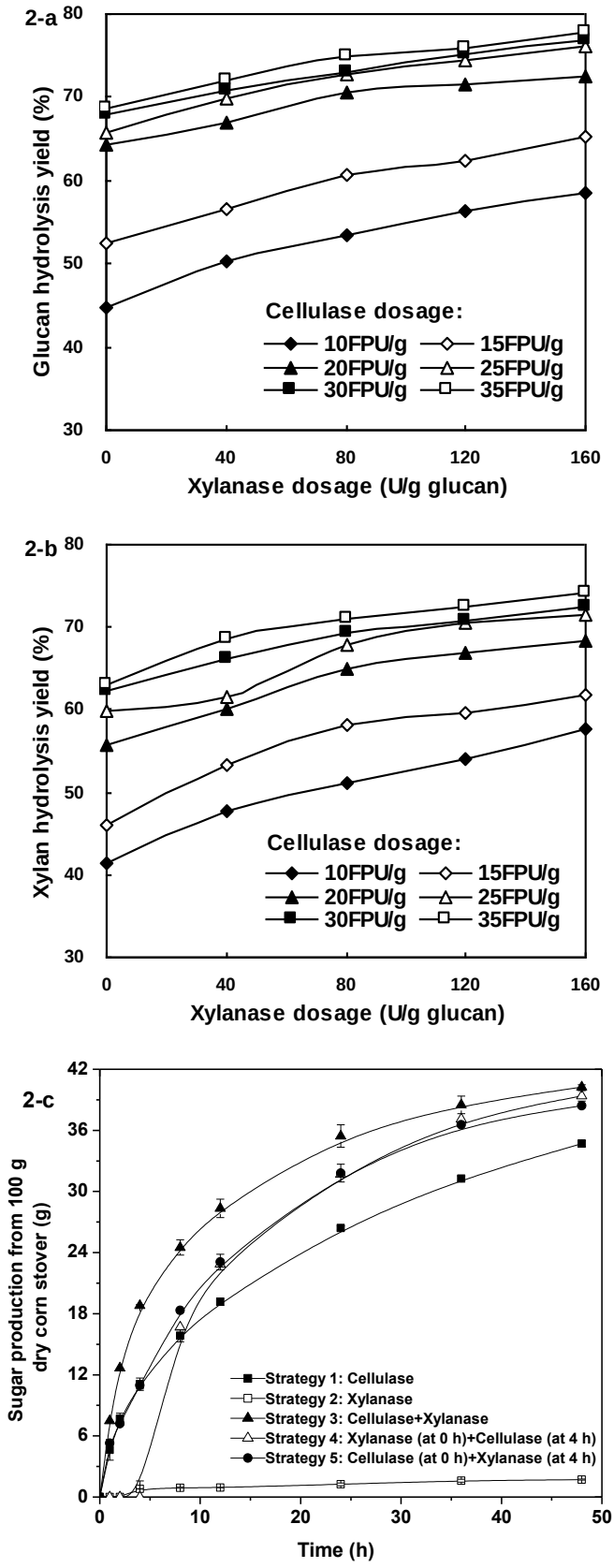

Fig. 2. Effect of enzymatic removal of xylan on enzymatic hydrolysis yield and sugar production from green liquor pretreated corn stover

Enzyme Cocktail Specialized for Green Liquor Pretreated Corn Stover

To reach near-theoretical sugar yields, it is necessary to tailor the saccharification process (enzyme mixture and additives) for any individual biomass and pretreatment regime. The effect of β-glucosidase dosage on enzymatic hydrolysis and sugar production is shown in Fig. 3a. As can be seen, with β-glucosidase dosage increasing from 3 IU/g to 20 IU/g, the glucan hydrolysis yield and xylan hydrolysis yield were enhanced to 84.59% and 77.19%, respectively.

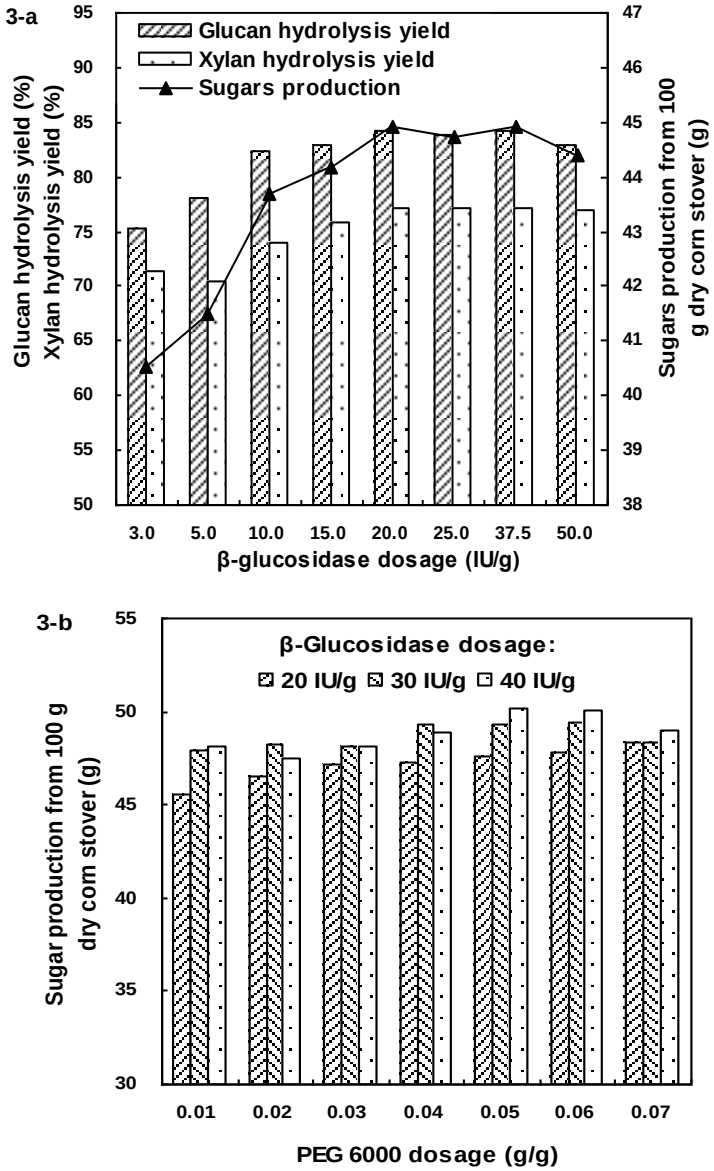

Fig. 3. Effect of β-glucosidase and surfactant addition on enzymatic hydrolysis yield and sugar production from green liquor-pretreated corn stover

A further increase of β-glucosidase dosage failed to promote hydrolysis yield. In addition, the highest sugar production of 45.25 g was obtained at a β-glucosidase dosage of 20 to 37.5 IU/g. Results suggest that β-glucosidase addition is an important parameter

to include in order to achieve high sugar yields during enzymatic hydrolysis, which is in agreement with Pallapolu *et al*. (2011). Reasons might be that some cellulases have low cellobiose cleaving activity, and cellobiose accumulation could occur in the initial stages of hydrolysis, thereby inhibiting the cellulase activity. β-glucosidase has been reported to regulate the cellulolytic process and is the rate-limiting enzyme during enzymatic hydrolysis of cellulose, because both endoglucanase and cellobiohydrolase activities can be inhibited by cellobiose.

As can be seen in Fig. 3b, the interaction of β-glucosidase and PEG 6000 enhanced the polysaccharides hydrolysis yield. The reasons for enhanced hydrolysis yield might be that PEG prevented nonproductive adsorption of enzymes to the pretreated corn stover. Thus, more productive enzymes including endoglucanase and cellobiohydrolase were adsorbed to substrate, liberating more cellooligosacchrides (Van Dyk and Pletschke 2012) and cellobiose. To alleviate the cellobiose accumulation and to further enhance hydrolysis efficiency, a higher β-glucosidase dosage was required (Andrić *et al*. 2010). When a specialized enzyme cocktail with cellulase of 25 FPU/g, β-glucosidase of 40 U/g, xylanase of 120 U/g, and PEG 6000 of 0.05 g/g was applied, the highest hydrolysis yield and sugar production were achieved. The glucan hydrolysis yield was 93.53% and xylan hydrolysis yield was 86.00%; the sugar production from 100 g dry corn stover also peaked (50.14 g), including 36.08 g glucose and 14.06 g xylose. Therefore, a specialized enzyme cocktail for green liquor-pretreated corn stover was obtained, with an actual cellulase, β-glucosidase, and xylanase ratio in enzymatic hydrolysis system of 1:1.88:6.61, due to the cross-specificity of enzymes (Van Dyk and Pletschke 2012), supplemented with 0.05 g/g PEG 6000.

CONCLUSIONS

1. The enzymatic hydrolysis efficiency of corn stover could be greatly improved by green liquor pretreatment (8% total titratable alkali charge, 40% sulfidity, 140 °C, and 1 h), followed by enzymatic hydrolysis using a specialized enzyme cocktail. Sugar production of 50.14 g from 100 g dry corn stover was obtained, including 36.08 g of glucose and 14.06 g of xylose.
2. Green liquor pretreatment was demonstrated to be an effective first step for sugar production due to the selective removal of lignin (39.70%), high carbohydrate recovery yield (81.53%), and obvious enhancement of enzymatic hydrolysis after pretreatment.
3. When a specialized enzyme cocktail (cellulase, β-glucosidase, and xylanase at a ratio of 1:1.88:6.61, supplemented with 0.05 g of PEG 6000 *per* g of glucan) was applied in the enzymatic hydrolysis of green liquor-pretreated corn stover, near-theoretical hydrolysis yield was achieved (glucan hydrolysis yield of 93.53% and xylan hydrolysis yield of 86.00%).

ACKNOWLEDGMENTS

This research was supported by the International Advanced Forestry Technology Introduction Project Funding (Grant No. 2012-4-18), National Natural Science Foundation of China (Grant No. 31070523), Research Fund for the Doctoral Program of Higher Education of China (Grant No. 20113204120013), Priority Academic Program Development of Jiangsu Higher Education Institution (PAPD), and Doctorate Fellowship Foundation of Nanjing Forestry University.

REFERENCES CITED

Allen, S. G., Schulman, D., Lichwa, J., Antal, M. J., Jennings, E., and Elander, R. (2001). "A comparison of aqueous and dilute-acid single-temperature pretreatment of yellow poplar sawdust," *Ind. Eng. Chem. Res.* 40(10), 2352-2361.

Andrić, P., Meyer, A. S., Jensen, P. A., and Dam-Johansen, K. (2010). "Reactor design for minimizing product inhibition during enzymatic lignocellulose hydrolysis: II. Quantification of inhibition and suitability of membrane reactors," *Biotechnol. Adv.* 28(3), 407-425.

Eriksson, T., Börjesson, J., and Tjerneld, F. (2002). "Mechanism of surfactant effect in enzymatic hydrolysis of lignocellulose," *Enzyme Microb. Technol.* 31(3), 353-364.

Ghose, T. K. (1987). "Measurement of cellulase activities," *Pure Appl. Chem.* 59(2), 257-268.

Gonzalez, R., Treasure, T., Phillips, R., Jameel, H., and Saloni, D. (2011). "Economics of cellulosic ethanol production: green liquor pretreatment for softwood and hardwood, greenfield and repurpose scenarios," *BioResources* 6(3), 2551-2567.

Gu, F., Yang, L. F., Jin, Y. C., Han, Q., Chang, H. M., Jameel, H., and Phillips, R. (2012). "Green liquor pretreatment for improving enzymatic hydrolysis of corn stover," *Bioresour. Technol.* 124, 299-305.

Gusakov, A. V., Salanovich, T. N., Antonov, A. I., Ustinov, B. B., Okunev, O. N., Burlingame, R., Emalfarb, M., Baze, M., and Sinitsyn, A. (2007). "Design of highly efficient cellulase mixtures for enzymatic hydrolysis of cellulose," *Biotechnol. Bioeng.* 97(5), 1028-1038.

Hu, Z. H., and Wen, Z. Y. (2008). "Enhancing enzymatic digestibility of switchgrass by microwave-assisted alkali pretreatment," *Biochem. Eng. J.* 38(3), 369-378.

Ibrahim, M. M., El-Zawawy, W. K., Abdel-Fattah, Y. R., Soliman, N. A., and Agblevor, F. A. (2011). "Comparison of alkaline pulping with steam explosion for glucose production from rice straw," *Carbohyd. Polym.* 83(2), 720-726.

Ishizawa, C. I., Jeoh, T., Adney, W. S., Himmel, M. E., Johnson, D. K., and Davis, M. F. (2009). "Can delignification decrease cellulose digestibility in acid pretreated corn stover," *Cellulose* 16(4), 677-86.

Jeoh, T., Ishizawa, C., Davis, M., Himmel, M., Adney, W., and Johnson, D. (2007). "Cellulase digestibility of pretreated biomass is limited by cellulose accessibility," *Biotechnol. Bioeng.* 98(1), 112-122.

Jin, Y. C., Jameel, H., Chang, H. M., and Phillips, R. (2010). "Green liquor pretreatment of mixed hardwood for ethanol production in a repurposed kraft pulp mill," *J. Wood Chem. Technol.* 30(1), 86-104.

Jorgensen, H., Kristensen, J. B., and Felby, C. (2007). "Enzymatic conversion of lignocellulose into fermentable sugars: challenges and opportunities," *Biofuels Bioprod. Bioref.* 1(2), 119-134.

Kim, T. H. and Lee, Y. Y. (2007). "Pretreatment of corn stover by soaking in aqueous ammonia at moderate temperatures," *Appl. Biochem. Biotech.* 136-140, 81-92.

Kumar, R., and Wyman, C. E. (2009a). "Effects of cellulase and xylanase enzymes on the deconstruction of solids from pretreatment of poplar by leading technologies," *Biotechnol. Prog*. 25(2), 302-314.

Kumar, R., and Wyman, C. E. (2009b). "Effect of xylanase supplementation of cellulase on digestion of corn stover solids prepared by leading pretreatment technologies," *Bioresour. Technol.* 100(8), 4203-4213.

Lee, J. M., Shi, J., Venditti, R. A., and Jameel, H. (2009). "Autohydrolysis pretreatment of Coastal Bermuda grass for increased enzyme hydrolysis," *Bioresour. Technol.* 100(24), 6434-6441.

McIntosh, S. and Vancov, T. (2010). "Enhanced enzyme saccharification of *Sorghum bicolor* straw using dilute alkali pretreatment," *Bioresour. Technol.* 101, 6718-6727.

Meng, X., Geng, W., Ren, H., Jin, Y. C., Chang, H. M., and Jameel, H. (2014). "Enhancement of enzymatic saccharification of poplar by green liquor pretreatment," *BioResources* 9(2), 3236-3247.

Merino, S. T., and Cherry, J. (2007). "Progress and challenges in enzyme development for biomass utilization," *Adv. Biochem. Eng. Biotechnol.* 108(6), 95-120.

NREL (National Renewable Energy Laboratories) (2012). *Determination of Structural Carbohydrates and Lignin in Biomass*, <http://www.nrel.gov/biomass/analytical_procedures.html> [consulted 08.03.12]

Pallapolu, V. R., Lee, Y. Y., Garlock, R. J., Balan, V., Dale, B. E., Kimd, Y., Mosier, N. S., Ladisch, M. R., Falls, M., Holtzapple, M. T., Sierra-Ramirez, R., Shi, J., Ebrik, M. A., Redmond, T., Yang, B., Wyman, C. E., Donohoe, B. S., Vinzant, T. B., Elander, R. T., Hames, B., Thomas, S., and Warner, R. E. (2011). "Effects of enzyme loading and β-glucosidase supplementation on enzymatic hydrolysis of switchgrass processed by leading pretreatment technologies," *Bioresour. Technol.* 102, 11115-11120.

Qing, Q., Yan, B., and Wyman, C. E. (2010). "Xylooligomers are strong inhibitors of cellulose hydrolysis by enzymes," *Bioresour. Technol.* 101, 9624-9630.

Romani, A., Garrote, G., Alonso, J. L., and Parajo, J. C. (2010). "Bioethanol production from hydrothermally pretreated *Eucalyptus globulus* wood," *Bioresour. Technol.* 101, 8706-8712.

Saha, B. C., and Cotta, M. A. (2006). "Ethanol production from alkaline peroxide pretreated enzymatically saccharified wheat straw," *Biotechnol. Prog.* 22(2), 449-453.

Silverstein, R. A., Chen, Y., Sharma-Shivappa, R. R., Boyette, M. D., and Osborne, J. (2007). "A comparison of chemical pretreatment methods for improving saccharification of cotton stalks," *Bioresour. Technol.* 98(16), 3000-3011.

Teleman, A., Larsson, P., and Iversen, T. (2001). "On the accessibility and structure of xylan in birch kraft pulp," *Cellulose* 8(3), 209-215.

Tengborg, C., Galbe, M., and Zacchi, G. (2001). "Reduced inhibition of enzymatic hydrolysis of steam-pretreated softwood," *Enzyme Microb. Technol.* 28(9-10), 835-844.

Van Dyk, J. S., and Pletschke, B. I. (2012). "A review of lignocellulose bioconversion using enzymatic hydrolysis and synergistic cooperation between enzymes—Factors affecting enzymes, conversion and synergy," *Biotechnol. Adv.* 30(6), 1458-1480.

Varnai, A., Siika-aho, M., and Viikari, L. (2010). "Restriction of the enzymatic hydrolysis of steam-pretreated spruce by lignin and hemicellulose," *Enzyme. Microb. Technol.* 46(3-4), 185-193.

Wang, Z. Y., Li, R. Y., Xu, J. L., Marita, J. M., Hatfield, R. D., Qu, R. D., and Cheng, J. J. (2012). "Sodium hydroxide pretreatment of genetically modified switchgrass for improved enzymatic release of sugars," *Bioresour. Technol.* 110, 364-370.

Wu, S. F., Chang, H. M., Jameel, H., and Phillips, R. (2010). "Novel green liquor pretreatment of loblolly pine chips to facilitate enzymatic hydrolysis into fermentable sugars for ethanol production," *J. Wood Chem. Technol.* 30(3), 205-218.

Yu, Z. Y., Jameel, H., Chang, H. M., and Park, S. (2011). "The effect of delignification of forest biomass on enzymatic hydrolysis," *Bioresour. Technol.* 102, 9083-9089.

Article submitted: April 2, 2014; Peer review completed: May 16, 2014; Revised version received and accepted: June 2, 2014; Published: June 10, 2014.