Comparative Evaluation of Magnesium Bisulfite Pretreatment under Different pH Values for Enzymatic Hydrolysis of Corn Stover

Jiwei Ren,[a] Lei Liu,[b] Qianqian Xu,[b] Xin Li,[b] Qiang Yong,[c] and Jia Ouyang [b,c,]*

During pretreatment, the pretreatment pH often plays an important role in removing hemicelluloses and lignin for improving the conversion of biomass to sugars. In this study, corn stover was subjected to magnesium bisulfite pretreatment (MBSP) under various pH conditions. The obtained data showed that the hemicelluloses and lignin were solubilized by MBSP, which led to changes in the structural and chemical properties of the pretreated material. The pretreatment pH could alter the existing forms of SO_2, and magnesium bisulfite was the most effective reagent for removing lignin. A relatively neutral MBSP (pH 5.13) not only considerably improved the enzymatic hydrolysis yield (80.18%), but also produced a large amount of high-value xylo-oligosaccharides in the spent liquor. Furthermore, only the hemicellulose removal showed a linear relationship with the enzymatic hydrolysis yield. These results suggest that removal of all the lignin might not be necessary to improve the hydrolysis efficiency.

Keywords: pH; Corn stover; Magnesium bisulfite pretreatment; Enzymatic hydrolysis

Contact information: a: College of Forestry, Nanjing Forestry University, Nanjing 210037, China;
b: College of Chemical Engineering, Nanjing Forestry University, Nanjing 210037, China;
*c: Key Laboratory of Forest Genetics and Biotechnology of the Ministry of Education, Nanjing Forestry University, Nanjing 210037, China; *Corresponding author: hgouyj@njfu.edu.cn*

INTRODUCTION

Lignocellulosic biomass is the most abundant renewable resource in nature. Because of the rapid depletion of fossil fuels and global climate changes, the efficient utilization of lignocellulosic biomass is gaining great interest for fuel and chemical production (Zhu *et al*. 2010). During this process, the cellulose and hemicelluloses in the biomass are deconstructed first into their corresponding monosaccharides *via* enzymatic hydrolysis, and subsequently fermented into bio-fuels and bulk bio-chemicals (Fougere *et al*. 2015; Vargas *et al*. 2015). However, the recalcitrant nature of native lignocelluloses is a major barrier to the economical production of bio-fuels and bio-chemicals (Zhu *et al*. 2010; Zhang *et al*. 2013a). The lignin present in biomass not only impedes the access of enzymes to cellulose by forming a physical barrier, but also leads to the unproductive adsorption of cellulase, which reduces cellulose hydrolysis (Ohgren *et al*. 2007; Mussatto *et al*. 2008). Hemicelluloses also have been shown to induce a physical and chemical barrier to hydrolysis (Ohgren *et al*. 2007). Thus, the removal of lignin and hemicelluloses can effectively improve the efficiency of the enzymatic hydrolysis of cellulose by increasing the internal surface area and reducing unproductive enzyme binding.

To remove the hemicellulose and lignin components of biomass, pretreatment is needed before enzymatic hydrolysis. Currently, numerous chemical pretreatments have been developed and applied for improving the enzymatic hydrolysis, such as dilute acid

(DA), lime, green liquor (GL), and liquid hot water (LHW) pretreatments. However, only a few pretreatments can effectively remove hemicellulose and lignin at the same time. The DA pretreatment can solubilize a larger amount of the hemicellulose fraction (Ballesteros *et al*. 2008), but its low pH not only impairs the removal of lignin, but also generates serious equipment corrosion problems (Zhao *et al*. 2008). Compared with DA pretreatment, lime and GL (the mixture of sodium carbonate and sodium sulfide) pretreatments are more effective for delignification (Kaar and Holtzapple 2000; Gu *et al*. 2012). However, because of the higher pH, they remove less hemicellulose than the DA process. The hemicelluloses remaining in pretreated biomass can have a negative impact on enzymatic hydrolysis by hindering the access of cellulose and enzymes. As for LHW, it might be attractive and cost-saving because its pH environment is neutral and there is no requirement for purchased chemical reagents. However, LHW pretreatment has not yet been developed at the commercial scale because of its higher water demand and energy requirements (Taherzadeh and Karimi 2008; Alvira *et al*. 2010). On the other hand, LHW can dissolve most hemicelluloses, but it has a weak effect on the removal of lignin in most cases (Sreenath *et al*. 1999).

Recently, our laboratory has developed a novel magnesium bisulfite pretreatment (MBSP) derived from sulfite pulping (Yu *et al*. 2016). However, the influences of different pretreatment pH values during the MBSP process on enzymatic hydrolysis have not been studied. Thus, in the present work, the impacts of pretreatment pH on the properties and enzymatic hydrolysis of pretreated corn stover biomass were investigated in-depth and evaluated. Moreover, the relationship between the removal of hemicellulose and lignin and enzymatic hydrolysis yield was also investigated at different pH values.

EXPERIMENTAL

Materials

Corn stover was harvested from Lianyungang in Jiangsu province, China. Before pretreatment, it was air-dried, crushed, and sieved to achieve the fraction between 20 and 80 mesh size. The prepared samples were stored at room temperature until use.

Two commercial enzyme solutions, Celluclast 1.5L (Cat# C2730) and Novozyme188 (Cat# C6105) from Sigma–Aldrich (Beijing, China), were used for all enzymatic hydrolysis experiments. Sulfur dioxide solution (ACS reagent, ⩾6%) was purchased from Sigma-Aldrich (Shanghai, China). Anhydrous sodium sulfite and magnesium chloride were purchased from Nanjing Chemical Reagent Co., Ltd. Magnesium bisulfate ($MgSO_3$) was prepared as described by Yu *et al*. (2016).

Methods

Pretreatment

Pretreatment was carried out in a rotary lab-scale cooking system with an electrically heated oil bath (YRG2-10×1.25, Nanjing Jiezheng, China). The 1.25-L stainless steel bomb reactors with screw caps were contained in the cooking systems. Corn stover samples were subjected to pretreatment in duplicate at 170 °C for 60 min using a pretreatment solution with various initial pH values. The pretreatment liquid to solid ratio was kept at 6 (v/w) for all conditions. The pretreatment solution was prepared by mixing sulfur dioxide with 6% (w/v) magnesium sulfite. The molar ratios of magnesium sulfite to sulfur dioxide (S/A ratio) were 1:0, 1:0.5, 1:1, 1:1.5, and 1:2, corresponding to initial pH values of 9.27, 5.82, 5.13, 2.68, and 2.08, respectively.

After pretreatment, the reactors were placed in cold water immediately to terminate the reaction. The pretreated spent liquors were collected by solid-liquid separation and the liquid portions were stored at -20 °C for high-performance liquid chromatography (HPLC) analysis; the solid was washed 10 times with distilled water and then kept at 4 °C. All experiments were conducted in duplicate.

Enzymatic hydrolysis

Enzymatic hydrolysis was performed at 3% cellulose (w/v) concentration in 30 mL of citrate buffer (pH 4.8, 50 mM) at 50 °C and 150 rpm on a shaker incubator (Innova$^{®}$44, New Brunswick, China) for 48 h. A mixture of Celluclast 1.5 L at 15 FPU/g cellulose and Novozyme 188 at 30 CBU/g cellulose was used for the enzymatic hydrolysis. After 48 h of enzymatic hydrolysis, the samples were withdrawn and centrifuged to remove the insoluble residues. The supernatant liquids were subsequently filtered through a 0.22-μm syringe filter (Millipore, Co., Ltd., USA) and used for subsequent sugar analysis. All hydrolysis experiments were carried out in in duplicate, and each data point was the average of two replicates.

Compositional analysis

The chemical compositions of the original and pretreated corn stover were determined according to the National Renewable Energy Laboratory (NREL, Golden, CO) analytical methods for biomass analysis (Sluiter *et al*. 2008).

Structural analysis

All samples used for structural analysis were first dried in a vacuum refrigerating machine (DZF 6050, Shanghai Hengyi, Co., Ltd., China) at -55 °C for at least 24 h.

Fourier transform infrared (FTIR) spectra of the pretreated sample were obtained using a Thermo-Nicolet VERTEX 80 FTIR (Bruker, Germany) with OMNIC software. Spectra for each sample ranging from 4000 to 400 cm^{-1} were recorded at a spectral resolution of 2 cm^{-1}. Disks were prepared by mixing dried material with KBr at the ratio about 1:50 in an agate mortar.

The microstructure of pretreated materials was evaluated by scanning electron microscope (SEM). All samples were coated with gold using a SC7640 Suto-/Manual high-resolution sputter coater (Quorum Technologies, Co., Ltd., UK) and then mounted on a stub (d = 10 mm) using double-sided tape. The SEM images were acquired using a JEOL JSM 6700F (FEG SEM, Japan) microscope operated at 5 to 15 kV.

HPLC analysis

The fermentable sugars (glucose, cellobiose, and xylose) and several known inhibitors (formic acid, acetic acid, levulinic acid, furfural, and 5-hydroxymethylfurfural (HMF)) in the spent liquors were quantified using an HPLC system (1200 series, Agilent, USA) equipped with a Bio-Rad AminexHPX-87H column (300 mm× 7.8 mm). The mobile phase used was 5 mM H_2SO_4 controlled at a flow rate of 0.6 mL/min at 55 °C. Analysis signals were detected by a refractive index detector (Ouyang *et al*. 2013).

For the analysis of xylo-oligosaccharides (XOS), a 1-mL sample was first mixed with 1 mL of 5 N H_2SO_4 and hydrolyzed at 121 °C for 45 min to convert XOS to xylose, then analyzed by HPLC to quantify the total amount of XOS (Nabarlatz *et al.* 2007). The components of the XOS were analyzed by high performance anion-exchange chromatography (Dionex ICS-3000 system, Thermo Scientific, USA) coupled with pulsed amperometric detection (HPAEC-PAD). The determination method was described in a previous report (Xu *et al*. 2013).

Calculations

The composition (%) of each sample was calculated according to the following equation,

$$\text{Removal ratio of composition (\%)} = (m_{raw} - m_{pre}) \times 100 / m_{raw} \qquad (1)$$

where m_{raw} is the mass of original chemical components (cellulose, hemicellulose, or lignin) in the raw material, and m_{pre} is the mass of components (cellulose, hemicellulose, or lignin) in the pretreated material. The unit of measurement was grams.

The enzymatic hydrolysis yield (EHY) was calculated according to the following equation,

$$\text{Enzymatic hydrolysis yield (\%)} = (C_{glu} + 1.053 \times C_{biose}) \times 0.9 \times 100 / C_{cel} \qquad (2)$$

where C_{glu} is the concentration of glucose in the enzymatic hydrolysate; C_{biose} is the concentration of cellobiose in the enzymatic hydrolysate; and C_{Cel} is the initial concentration of cellulose. The unit of measurement was grams/liter.

The concentration of XOS (g/L) was calculated according to the following equation,

$$\text{XOS (g/L)} = (X_{raw} - X_{hyr}) \times 0.88 \qquad (3)$$

where X_{raw} and X_{hyr} are the concentration of xylose in the spent liquor before and after hydrolysis, respectively; 0.88 is the conversion coefficient between xylose before and after hydrolysis. The unit of measurement was grams.

RESULTS AND DISCUSSION

Chemical Compositional Analysis of Pretreated Corn Stover

The original corn stover contained 37.5% cellulose, 18.5% hemicellulose, and 22.2% lignin (3.6% acid-soluble lignin and 18.6% acid-insoluble lignin) according to the NREL analysis. After pretreatment at the various *S*/*A* ratios or initial pH values, the removal ratios for pretreated corn stover are shown in Fig. 1. The effect of pretreatment pH on the removal of hemicellulose and lignin was noticeable. When the S/A ratio increased from 1:0 to 1:1 (corresponding to a pretreatment pH change from 9.27 to 5.13), the hemicellulose and lignin removal ratios increased linearly, from 56.59% to 81.98% and 62.26% to 85.85%, respectively. However, a continuous increase in the *S*/*A* ratio to 1:2 (pH 2.08) did not contribute more lignin removal. The lignin removal decreased to 69.24% when the pretreatment pH was 2.08. Meanwhile, only a slight increase in hemicellulose removal was observed (from 81.97% to 90.01%). As for the loss of cellulose after pretreatment, greater than 90% of the cellulose remained in the pretreated samples, under both acid and alkaline conditions. These results suggest that the pretreatment pH did not have an obvious impact on the cellulose.

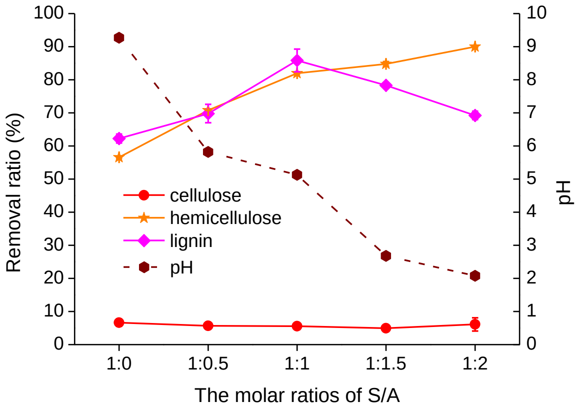

Fig. 1. Effect of pretreatment pH on the removal of cellulose, hemicellulose, and lignin from corn stover (pretreatment conditions: 170 °C for 60 min with magnesium bisulfite at 6% (w/v))

As Fig. 1 shows, the removal of most lignin was found at pH 5.13, which corresponded to the highest cellulose composition (79.03%, data not shown). This result was different from several previous studies (Zhao *et al*. 2008; McIntosh and Vancov 2011) that lignin only could be removed efficiently at an alkaline environment in most case. A possible interpretation is depicted in Fig. 2.

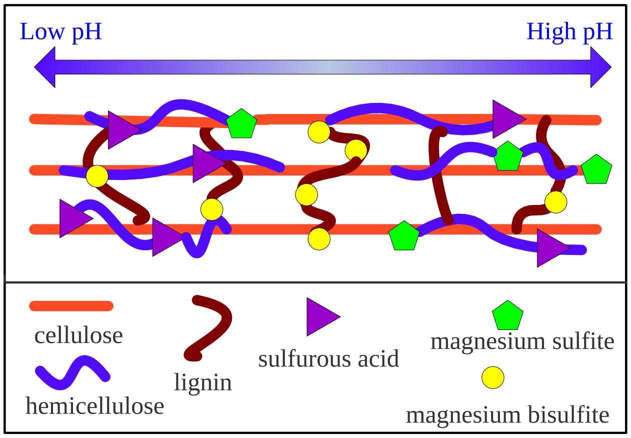

Fig. 2. Scheme of reactions of lignocellulose as affected by pretreatment pH

In MBSP, a molecular form of SO_2 and two kinds of anions, *i.e.*, sulfite (SO_3^{2-}) and bisulfate (HSO_3^-), could exist in the pretreatment liquor. Different initial pH values or *S*/*A* ratios would alter the existing forms of SO_2 and affect the generation of Mg $(HSO_3)_2$. Based on the *S*/*A* ratio, HSO_3^- would occupy the main position at pH 5.13, producing a larger amount of bisulfonating agent. When the pretreatment pH is alkaline, because of the lack of sulfur dioxide, more and more SO_3^{2-} exists in the pretreatment solution. The actual active reagent becomes magnesium sulfite, which is only slightly soluble. Thereby, the effect of delignification became worse at pH values other than 5.13. Regarding the lower pH (< 5), an acid environment might promote lignin condensation and influence the solubilization of lignin (Tao *et al*. 2011). Thus, our results suggest that magnesium bisulfite is the most effective agent for removing lignin during the pretreatment process, and a pH of 5.13 might be suitable for MBSP.

FTIR and Microstructure Analysis of Pretreated Corn Stover

To further investigate the influence of the pH on the chemical structure of pretreated corn stover, FTIR analysis was performed (Fig. 3). The absorbance band at 1731 cm^{-1} assigned to C=O stretching was most prominent at pH 5.13, while it was diminished at pH 2.08 and 9.27. This revealed that the cleavage of acetyl groups linked to hemicellulose was more effective at pH 2.08 and 9.27 than at pH 5.13 .The peak at 1512 cm^{-1} relates to the aromatic skeletal vibrations in lignin (Timung *et al*. 2015). The peak at 1460 cm^{-1} represents the methoxyl group in lignin (Zhang *et al*. 2013b). Both of them were only found at pH 2.08 and 9.27 and disappeared at pH 5.13. These results are consistent with the results of Fig. 1, in which 85.85% of the lignin was removed at pH 5.13. Furthermore, strong absorbance at 1058, 1162, and 897 cm^{-1} was observed at pH 5.13, indicating the exposure of the cellulose fraction in the course of the pretreatment (Thulluri *et al*. 2013).

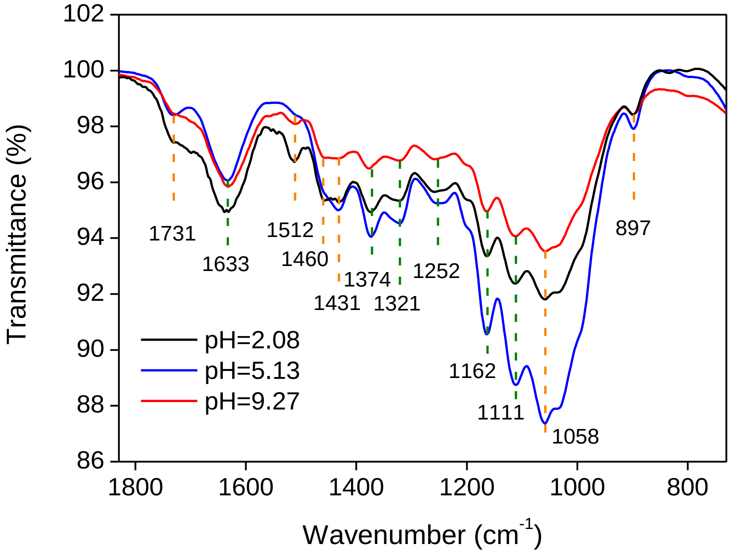

Fig. 3. Effects of pretreatment pH on FT-IR spectra of pretreated corn stover

The surface morphology of pretreated substrates at different pH values is shown in the SEM micrograph in Fig. 4. Compared with the raw corn stover (Fig. 4a), alkaline pretreatment led to the appearance of a crumpled surface, while a corrugated and porous surface was found for material treated by MBSP at pH 2.08 (Fig. 4c). This unconsolidated structure would be beneficial to access by enzymes and materials. In addition, we also found some lignin droplets on the fiber surface at either pH 9.27 (Fig. 4b) or 2.08, which indicated that lignin was still not removed completely. It has been reported that lignin droplets can inhibit enzymatic hydrolysis by preventing the adsorption of enzymes (Koo *et al*. 2012).

For the pretreatment at pH 5.13 (Fig. 4d), the pretreated corn stover revealed a spongy surface and no lignin droplets were found on the surface. We also found many vascular bundles on the surface of the cellulose fibers. These results account for the access of enzymes and cellulose under this pH condition.

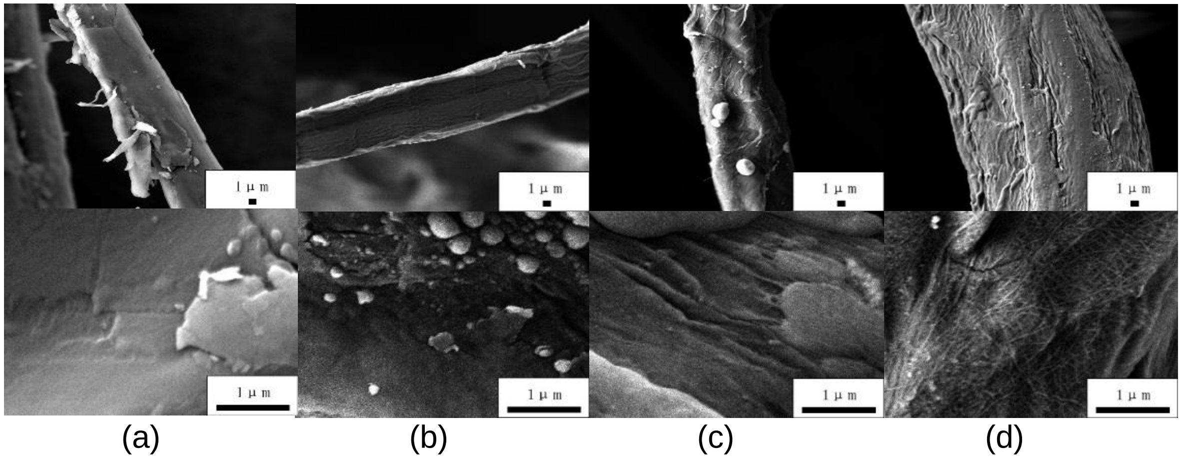

Fig. 4. SEM images of the raw material and pretreated samples: (a) untreated (3000×), (15000×); (b) pretreated at pH = 9.27 (3000×), (15000×); (c) pretreated at pH = 2.08 (3000×), (15000×); and (d) pretreated at pH = 5.13 (3000×), (15000×).

Table 1. Effects of Pretreatment pH on Degradation Products of Pretreated Corn Stover in Spent Liquor

Pretreatment pH	Formic acid (g/L)	Acetic acid (g/L)	Furfural (g/L)	HMF (g/L)	Glucose (g/L)	Xylose (g/L)	XOS (g/L)
2.08	ND	3.99±0.05	1.92±0.09	0.41±0.18	1.78±0.19	5.32±0.23	6.51±0.22
2.68	ND	3.48±0.09	1.39±0.20	0.39±0.04	1.26±0.04	3.44±0.11	9.11±0.35
5.13	ND	3.23±0.10	1.54±0.31	0.32±0.03	0.94±0.05	2.04±0.15	11.04±0.13
5.82	ND	3.92±0.01	0.36±0.05	ND	0.62±0.02	0.81±0.03	10.32±0.46
9.27	0.69±0.21	4.20±0.17	0.26±0.24	0.20±0.29	0.64±0.11	0.46±0.02	11.76±0.35

ND: Not determined

Degradation Compounds of Lignocelluloses after Pretreatment

In most cases, pretreatment inevitably led to the degradation of partial lignocellulosic biomass, and various other small compounds were formed (such as acetic acid, furfural, formic acid, and HMF) (Cantarella *et al*. 2004; Jönsson *et al*. 2013). Thus, after MBSP, the composition of the spent liquor was determined to evaluate the effect of the pretreatment pH on the formation of degradation compounds.

Table 1 shows the concentration profile of the formed degradation compounds in pretreatment spent liquor. During a typical pretreatment process, the cellulose generally is depolymerized to glucose and subsequently converted to HMF, formic acid, and levulinic acid (Girisuta *et al*. 2013). In this work, because of the slight loss of cellulose after MBSP, the concentrations of these degradation compounds from glucose remained at low levels at various pH values. Meanwhile, various decomposition compounds from the hemicelluloses fraction were found in spent liquor after MBSP. Interestingly, pH 5.13 seemed to be a turning point for the amount of those decomposition compounds. Acetic acid, generated from the cleavage of acetyl groups directly linked to hemicellulose, is an important inhibitor of the activity of enzymes and fermentation (Zhang *et al*. 2011; Matsushika and Sawayama 2012). Along with the deacetylation of corn stover at lower or higher pH environments and the solubilization of hemicelluloses, MBSP inevitably led to the release of acetic acid. The lowest concentration of acetic acid, 3.23 g/L, was discovered at a pretreatment pH of 5.13. Furfural is a major xylose dehydration product that also inhibits fermentation (Pfeifer *et al*. 1984). In MBSP, increasing the pH could considerably reduce the formation of furfural. When the pretreatment pH was increased to 5.82, the concentration of furfural dropped to less than 1 g/L.

In addition to the presence of the above inhibitors, XOS and xylose were the main compounds in pretreatment spent liquor. An inverse relationship was observed between the concentrations of XOS and xylose with increasing pH. When the pH increased from 2.08 to 5.82, the amount of xylose dropped from 5.32 to 0.81 g/L, while the amount of XOS noticeably increased, from 6.51 to 10.32 g/L. Further increasing the pH did not obviously affect the concentrations of XOS and xylose. The XOS is a novel sweetener that can improve health as a prebiotic (Garrote *et al*. 1999). Many reports have shown that it can be prepared by auto-hydrolysis of biomass and partial acid or enzymatic hydrolysis of hemicellulose (Akpinar *et al*. 2009). Using MBSP at pH 5.13, the amount of XOS reached 11.04 g/L, which is considerably higher than that in liquid hot water pretreatment (7.50 g/L) (Yu *et al*. 2014). In addition, the different preparation methods of XOS resulted in different molecular weight distributions of XOS (Akpinar *et al*. 2009). Figure 5 shows at least five different XOS molecular weight distributions that were identified in the spent liquor after pretreatment at pH 5.13. Among them, X2 and X3 were the main components.

In-Depth Investigation of Enzymatic Hydrolysis of Pretreated Corn Stover

After pretreatment, enzymatic hydrolysis of pretreated corn stover was conducted to assess the effects of the pretreatment pH on the EHY (Fig. 5a). When the pretreatment pH value was below 5.13, not much difference was observed for the EHY. The 48-h enzymatic hydrolysis yield of the pretreated corn stover was approximately 78.61% to 80.71%. However, a turning point occurred at pH 5.13.

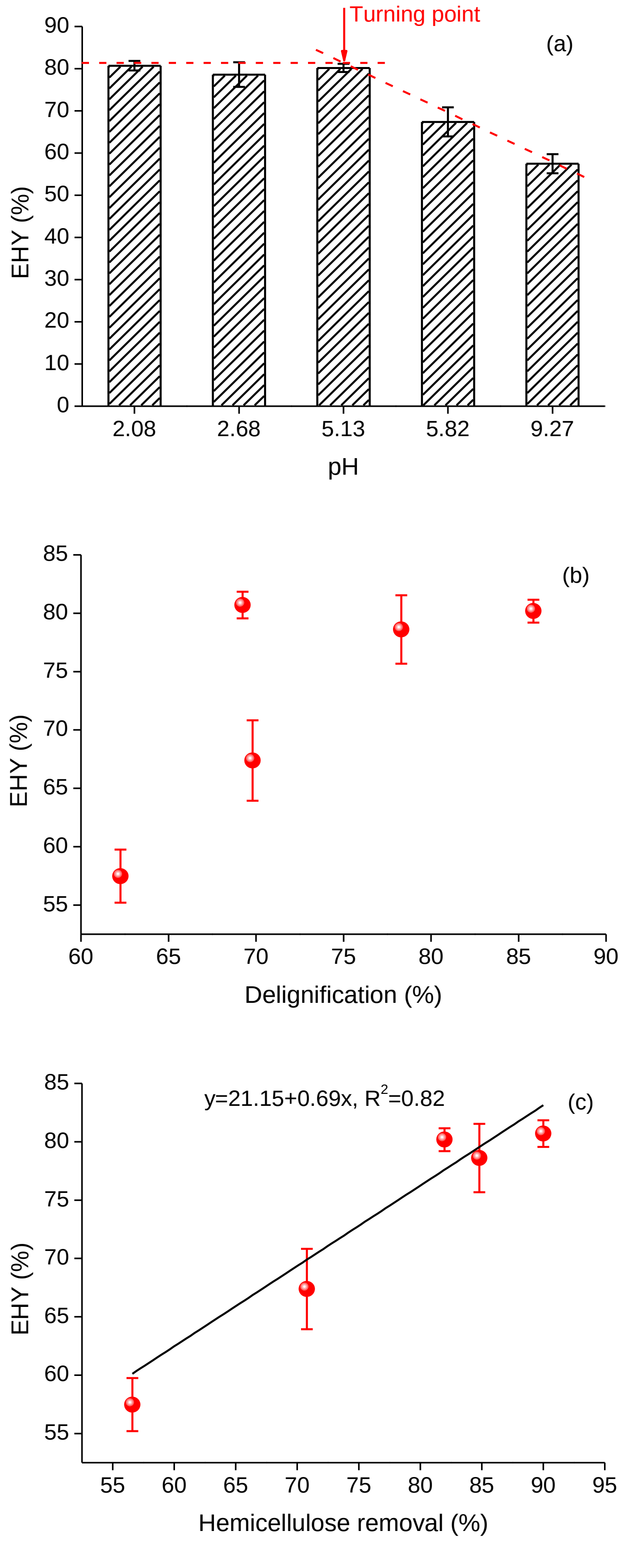

Fig. 5. Relationships between enzymatic hydrolysis yield (EHY) and (a) pretreatment pH; (b) delignification; and (c) hemicellulose removal

When the pH increased from 5.13 to 9.27, the EHY decreased linearly by 22.71% (from 80.18% to 57.47%). In the past few years, high-efficiency enzymatic hydrolysis of lignocellulosic materials has been achieved with various pretreatment methods (Ballesteros *et al*. 2008; McIntosh and Vancov 2011). However, the pH of these pretreatment methods was partially acidic or alkaline (Yang and Wyman 2008). In addition, although magnesium bisulfite had a long history for pulping, the pH ranges were below 5 (Zhu and Pan 2010; Yuliani *et al*. 2012), However, it was encouraging that the present work resulted in a pretreatment method that could obtain a high EHY at pH 5.13.

As shown in previous research reports, the presence of lignin can reduce the efficiency of the enzymatic hydrolysis of lignocellulosic materials (Mussatto *et al*. 2008). To explore the relationship between the lignin content and the enzymatic digestibility at various pH values, the correlation of EHY with the delignification is shown in Fig. 5b. When the delignification level increased by 6.98% (from 62.26% to 69.24%), the 48-h EHY increased sharply, by 23.24% (from 57.47% to 80.71%). This demonstrated that enzymatic hydrolysis could be enhanced by delignification and there existed a positive correlation between delignification and EHY. However, more delignification (from 69.24% to 85.85%) did not have any effect on the EHY (80.71% to 80.18%). Therefore, there was not a linear relationship between delignification and EHY, which contradicts other researchers' results (Koullas *et al*. 1992; Zhang *et al*. 2013b). Our results suggest that it was not necessary to have more delignification, and an efficient enzymatic hydrolysis could be obtained when 70% lignin was removed. In addition, different EHYs (80.71% and 67.38%) were obtained even at the same delignification (about 69.24%) level. This may have occurred because of the lower EHY that was obtained at the pretreatment pH of 5.82, which had a lower hemicellulose removal than at pH 2.68. Figure 5c shows the relationship between EHY and hemicellulose removal. A linear relationship was observed after fitting the experimental results. The linear equation was described as:

$$\text{EHY (\%)} = 21.15 + 0.69 \times \text{Hemicellulose removal (\%)},\ R^2=0.82. \qquad (4)$$

Hence, hemicellulose removal can be regarded as a key factor in evaluating the efficiency of pretreatment for enzymatic hydrolysis. It is important to remove all the hemicellulose and part of the lignin during pretreatment to enhance enzymatic hydrolysis.

CONCLUSIONS

1. During MBSP, controlling the pretreatment pH can alter existing forms of SO_2, and magnesium bisulfite is the most effective agent for removing lignin during the pretreatment.

2. Removing all the hemicellulose and part of the lignin from corn stover is regarded as a key factor in enhancing the enzymatic hydrolysis. At pretreatment pH 5.13, MBSP removed 81.98% lignin and 85.85% hemicelluloses from corn stover, which considerably improved the enzymatic hydrolysis yield (80.18%).

3. A great advantage of the relatively neutral MBSP is that it can produce a large amount of high-value xylo-oligosaccharides in the spent liquor.

ACKNOWLEDGMENTS

This study was supported by Funds for International Cooperation and Exchange of the National Natural Science Foundation of China (51561145015). The authors are also grateful to the Priority Academic Program Development of Jiangsu Higher Education Institutions (PAPD) for partial funding of this study.

REFERENCES CITED

Akpinar, O., Erdogan, K., and Bostanci, S. (2009). "Production of xylooligosaccharides by controlled acid hydrolysis of lignocellulosic materials," *Carbohydr. Res.* 344(5), 660-666. DOI: 10.1016/j.carres.2009.01.015

Alvira, P., Tomas-Pejo, E., Ballesteros, M., and Negro, M. J. (2010). "Pretreatment technologies for an efficient bioethanol production process based on enzymatic hydrolysis: A review," *Bioresour. Technol.* 101(13), 4851-61. DOI: 10.1016/j.biortech.2009.11.093

Ballesteros, I., Ballesteros, M., Manzanares, P., Negro, M. J., Oliva, J. M., and Sáez, F. (2008). "Dilute sulfuric acid pretreatment of cardoon for ethanol production," *Biochem. Eng. J.* 42(1), 84-91. DOI: 10.1016/j.bej.2008.06.001

Cantarella, M., Cantarella, L., Gallifuoco, A., Spera, A., and Alfani, F. (2004). "Effect of inhibitors released during steam-explosion treatment of poplar wood on subsequent enzymatic hydrolysis and SSF," *Biotechnol. Prog.* 20(1), 200-206. DOI: 10.1021/bp0257978

Fougere, D., Clarke, K., Zhao, Y., and Li, K. (2015). "Chemical–mechanical pretreatment of wood: Reducing downsizing energy and increasing enzymatic digestibility," *Biomass. Bioenergy.* 80, 17-29. DOI: 10.1016/j.biombioe.2015.04.011

Garrote, G., Dominguez, H., and Parajo, J. C. (1999). "Mild autohydrolysis: An environmentally friendly technology for xylooligosaccharide production from wood," *J. Chem. Technol. Biotechnol.* 74(11), 1101-9. DOI: 10.1002/(sici)1097-4660(199911)74:11<1101::aid-jctb146>3.0.co;2-m

Girisuta, B., Dussan, K., Haverty, D., Leahy, J. J., and Hayes, M. H. B. (2013). "A kinetic study of acid catalysed hydrolysis of sugar cane bagasse to levulinic acid," *Chem. Eng. J.* 217(2), 61-70. DOI: 10.1016/j.cej.2012.11.094

Gu, F., Yang, L., Jin, Y., 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. DOI: 10.1016/j.biortech.2012.08.054

Jönsson, L. J., Alriksson, B., and Nilvebrant, N. -O. (2013). "Bioconversion of lignocellulose: Inhibitors and detoxification," *Biotechnol. Biofuels.* 6(1), 16. DOI: 10.1186/1754-6834-6-16

Kaar, W. E., and Holtzapple, M. T. (2000). "Using lime pretreatment to facilitate the enzymic hydrolysis of corn stover," *Biomass. Bioenergy.* 18(3), 189-199. DOI: 10.1016/s0961-9534(99)00091-4

Koo, B. -W., Min, B. -C., Gwak, K. -S., Lee, S. -M., Choi, J. -W., Yeo, H., and Choi, I. -G. (2012). "Structural changes in lignin during organosolv pretreatment of *Liriodendron tulipifera* and the effect on enzymatic hydrolysis," *Biomass. Bioenergy.* 42, 24-32. DOI: 10.1016/j.biombioe.2012.03.012

Koullas, D. P., Christakopoulos, P., Kekos, D., Macris, B. J., and Koukios, E. G. (1992). "Correlating the effect of pretreatment on the enzymatic hydrolysis of straw," *Biotechnol. Bioeng.* 39(1), 113-6. DOI: 10.1002/bit.260390116

Matsushika, A., and Sawayama, S. (2012). "Characterization of a recombinant flocculent *Saccharomyces cerevisiae* strain that co-ferments glucose and xylose: II. Influence of pH and acetic acid on ethanol production," *Appl. Biochem. Biotechnol.* 168(8), 2094-2104. DOI: 10.1007/s12010-012-9920-4

McIntosh, S., and Vancov, T. (2011). "Optimisation of dilute alkaline pretreatment for enzymatic saccharification of wheat straw," *Biomass. Bioenergy* 35(7), 3094-3103. DOI: 10.1016/j.biombioe.2011.04.018

Mussatto, S .I., Fernandes, M., Milagres, A. M. F., and Roberto, I. C. (2008). "Effect of hemicellulose and lignin on enzymatic hydrolysis of cellulose from brewer's spent grain," *Enzyme Microb. Technol.* 43(2), 124-129. DOI: 10.1016/j.enzmictec.2007.11.006

Nabarlatz, D., Ebringerová, A., and Montané, D. (2007). "Autohydrolysis of agricultural by-products for the production of xylo-oligosaccharides," *Carbohydr. Polym.* 69(1), 20-28. DOI: 10.1016/j.carbpol.2006.08.020

Ohgren, K., Bura, R., Saddler, J., and Zacchi, G. (2007). "Effect of hemicellulose and lignin removal on enzymatic hydrolysis of steam pretreated corn stover," *Bioresour. Technol.* 98(13), 2503-10. DOI: 10.1016/j.biortech.2006.09.003

Ouyang, J., Liu, B., Zhang, M., Zheng, Z., and Yu, H. (2013). "Enzymatic hydrolysis, adsorption, and recycling during hydrolysis of bagasse sulfite pulp," *Bioresour. Technol.* 146, 288-93. DOI: 10.1016/j.biortech.2013.07.019

Pfeifer, P. A., Bonn, G., and Bobleter, O. (1984). "Influence of biomass degradation products on the fermentation of glucose to ethanol by *Saccharomyces carlsbergensis* W 34," *Biotechnol. Lett.* 6(8), 541-546. DOI: 10.1007/bf00139999

Sluiter, A., Hames, B., Ruiz, R., Scarlata, C., Sluiter, J., Templeton, D., and Crocker, D. (2008). "Determination of structural carbohydrates and lignin in biomass," *Technical Report* NREL LAP.

Sreenath, H. K., Koegel, R. G., Moldes, A. B., Jeffries, T. W., and Straub, R. J. (1999). "Enzymic saccharification of alfalfa fibre after liquid hot water pretreatment," *Process Biochem.* 35(1), 33-41. DOI: 10.1016/s0032-9592(99)00029-1

Taherzadeh, M. J., and Karimi, K. (2008). "Pretreatment of lignocellulosic wastes to improve ethanol and biogas production: A review," *Int. J. Mol. Sci.* 9(9), 1621-51. DOI: 10.3390/ijms9091621

Tao, L., Aden, A., Elander, R. T., Pallapolu, V. R., Lee, Y., Garlock, R. J., Balan, V., Dale, B. E., Kim, Y., and Mosier, N. S. (2011). "Process and technoeconomic analysis of leading pretreatment technologies for lignocellulosic ethanol production using switchgrass," *Bioresour. Technol.* 102(24), 11105-11114. DOI: 10.1016/j.biortech.2011.07.051

Thulluri, C., Goluguri, B. R., Konakalla, R., Shetty, P. R., and Addepally, U. (2013). "The effect of assorted pretreatments on cellulose of selected vegetable waste and enzymatic hydrolysis," *Biomass. Bioenergy* 49, 205-213. DOI: 10.1016/j.biombioe.2012.12.022

Timung, R., Mohan, M., Chilukoti, B., Sasmal, S., Banerjee, T., and Goud, V. V. (2015). "Optimization of dilute acid and hot water pretreatment of different lignocellulosic biomass: A comparative study," *Biomass. Bioenergy* 81, 9-18. DOI: 10.1016/j.biombioe.2015.05.006

Vargas, F., Dominguez, E., Vila, C., Rodriguez, A., and Garrote, G. (2015). "Agricultural residue valorization using a hydrothermal process for second generation bioethanol and oligosaccharides production," *Bioresour. Technol.* 191, 263-70. DOI: 10.1016/j.biortech.2015.05.036

Xu, Y., Fan, L., Wang, X., Yong, Q., and Yu, S. -Y. (2013). "Simultaneous separation and quantification of linear xylo-and cello-oligosaccharides mixtures in lignocellulosics processing products on high-performance anion-exchange chromatography coupled with pulsed amperometric detection," *BioResources* 8(3), 3247-3259. DOI: 10.15376/biores.8.3.3247-3259

Yang, B., and Wyman, C. E. (2008). "Pretreatment: The key to unlocking low-cost cellulosic ethanol," *Biofuels. Bioprod. Biorefin.* 2(1), 26-40. DOI: 10.1002/bbb.49

Yu, Q., Xu, C., Zhuang, X., Yuan, Z., He, M., and Zhou, G. (2014). "Xylo-oligosaccharides and ethanol production from liquid hot water hydrolysate of sugarcane bagasse," *BioResources* 10(1), 30-40. DOI: 10.15376/biores.10.1.30-40

Yu, H., Ren, J., Liu, L., Zheng, Z., Zhu, J., Yong, Q., and Ouyang, J. (2016). "A new magnesium bisulfite pretreatment (MBSP) development for bio-ethanol production from corn stover," *Bioresour. Technol.* 199, 188-193. DOI: 10.1016/j.biortech.2015.08.090

Yuliani, G, Qi, Y., Hoadley, A. F. A., Chaffee, A. L., and Garnier, G. (2012). "Lignite clean up of magnesium bisulphite pulp mill effluent as a proxy for aqueous discharge from a ligno-cellulosic biorefinery, " *Biomass. Bioenergy* 36, 411-418. DOI: 10.1016/j.biombioe.2011.11.012

Zhang, J. G., Liu, X. Y., He, X. P., Guo, X. N., Lu, Y., and Zhang, B. R. (2011). "Improvement of acetic acid tolerance and fermentation performance of *Saccharomyces cerevisiae* by disruption of the FPS1 aquaglyceroporin gene," *Biotechnol. Lett.* 33(2), 277-84. DOI: 10.1007/s10529-010-0433-3

Zhang, C., Lei, X., Scott, C. T., Zhu, J. Y., and Li, K. (2013a). "Comparison of dilute acid and sulfite pretreatment for enzymatic saccharification of earlywood and latewood of Douglas fir," *BioEnergy Res.* 7(1), 362-370. DOI: 10.1007/s12155-013-9376-6

Zhang, Z., O'Hara, I. M., and Doherty, W. O. S. (2013b). "Effects of pH on pretreatment of sugarcane bagasse using aqueous imidazolium ionic liquids," *Green Chem.* 15(2), 431-438. DOI: 10.1039/c2gc36084e

Zhao, Y., Wang, Y., Zhu, J. Y., Ragauskas, A., and Deng, Y. (2008). "Enhanced enzymatic hydrolysis of spruce by alkaline pretreatment at low temperature," *Biotechnol. Bioeng.* 99(6), 1320-8. DOI: 10.1002/bit.21712

Zhu, J. Y., Pan, X., and Zalesny Jr., R. S. (2010). "Pretreatment of woody biomass for biofuel production: Energy efficiency, technologies, and recalcitrance," *Appl. Microbiol. Biotechnol.* 87(3), 847-57. DOI: 10.1007/s00253-010-2654-8

Zhu, J.Y., and Pan, X.J. (2010). "Woody biomass pretreatment for cellulosic ethanol production: Technology and energy consumption evaluation, " *Bioresour. Technol.* 101(13), 4992-5002. DOI: 10.1016/j.biortech.2009.11.007

Article submitted: March 24, 2016; Peer review completed: May 31, 2016; Revised version received and accepted: June 29, 2016; Published: July 14, 2016.
DOI: 10.15376/biores.11.3.7258-7270