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Combined Mechanical Destruction and Alkaline Pretreatment of Wheat Straw for Enhanced Enzymatic Saccharification

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Wheat straw was pretreated by combined mechanical destruction and alkaline pretreatments to enhance enzymatic saccharification. Four strategies were employed to evaluate the potential of wheat straw as a feedstock for fermentable sugar production. The effects of the pretreatments on the substrate morphology, size distribution, chemical composition, and cellulose crystallinity, along with the subsequent enzymatic digestibility, were investigated. Optical microscope images showed that mechanical pretreatment alone resulted in poor fiber defibrillation, wherein samples mostly consisted of rigid fiber bundles, while integrated mechanical destruction and alkaline pretreatment led to relatively good fiber defibrillation. Low temperature NaOH/urea pretreatment can fibrillate the rigid fiber bundles into a relatively loose network and alter the structure of the treated substrate to make cellulose more accessible. The glucan conversion rates were 77% and 95% for integrated mechanical destruction and alkaline pretreatments and mechanical destruction followed by low temperature NaOH/urea and ammonium/urea pretreatments, respectively, after 72 h of enzymatic hydrolysis with enzyme loadings of 10 FPU cellulase *per* g of oven-dry substrate.

INTRODUCTION

The shortage of fossil fuels and environmental pollution caused by combustion of fossil fuels have prompted the development of biofuel as an alternative energy source (Ragauskas *et al.* 2006). Bioethanol is one of the most promising renewable biofuels. Second-generation bioethanol based on non-food lignocellulose substrates offers the possibility for large-scale production of renewable fuels (Somerville *et al.* 2010). Agricultural residues such as wheat straw and corn stover are the largest and most important renewable feedstock in China (Chen *et al.* 2009).

The efficient saccharification of lignocellulose is a prerequisite step for advanced biofuels and chemicals (Zhang and Lynd 2004). Many substrate-related factors, such as chemical components and structure, surface area and porosity, cellulose content, and accessibility to cellulose, can affect enzymatic saccharification (Leu and Zhu 2012; Wang *et al.* 2012). Pretreatment plays a critical role in efficient conversion of lignocellulose to fermentable sugars. The purpose of pretreatment is therefore to (1) increase the reactivity

of polysaccharides, (2) avoid the degradation of carbohydrate and formation of inhibitors, and (3) be cost-effective (Kumar *et al.* 2009; Talebnia *et al.* 2010; Wyman 2013). Many physical, mechano-chemical, chemical, and biological pretreatments have been developed over the years. Physical size reduction, such as milling, grinding, or chipping, can improve the efficiency of downstream processing in most pretreatment processes. Unfortunately, mechanical pretreatment requires high energy consumption, especially for wood feedstock (Zhu *et al.* 2011). Mechano-chemical pretreatment can effectively increase the specific surface area. The mechano-chemical pretreatment of lignocellulose with alkaline media has been studied extensively to improve enzymatic hydrolysis by removing lignin. Nevertheless, traditional alkaline treatment is generally conducted at a high temperature, *i.e.*, 100 to 160 °C (Kaar and Holtzapple 2000; Karp *et al.* 2014). Chemical pretreatment of lignocellulose has been conducted by adding chemicals such as acids, alkalines, organic solvents, or ionic liquids as catalysts (Lee *et al.* 2009; Wyman 2013). Specific pretreatment processes often have different effects on lignocellulosic substrates. Alkaline pretreatments are more efficient for lignin removal, while dilute acid pretreatments are more effective for hemicellulose dissolution (Talebnia *et al.* 2010). Organosolv pretreatment and ionic liquid pretreatment provide a clean process for fractionation of biomass. The biggest challenge for both organosolv pretreatment and ionic liquid pretreatment is the efficient recovery of the organic solvents or ionic liquids. Biological pretreatment uses microorganisms such as white-, brown-, and soft-rot fungi to degrade hemicellulose and lignin in mild conditions (Sun and Cheng 2002). Biological pretreatment requires less energy consumption than the other methods. Unfortunately, the rate of lignocellulosic substrate degradation is usually very low, so a long residence time is one of the major drawbacks for biological pretreatment (Wyman 2013).

No single pretreatment can meet all of the requirements mentioned above. As an alternative, a combination of different pretreatment strategies can be applied to achieve a better result. Therefore, the preliminary aim of the present work is to develop a combined mechanical destruction and alkaline pretreatment process and to evaluate the feasibility of wheat straw as a feedstock for bioethanol production. For this purpose, four pretreatment strategies, including simple mechanical pretreatment (WS-M), integrated mechanical destruction and alkaline pretreatment (WS-MA), and mechanical destruction followed by low temperature NaOH/urea and ammonium/urea pretreatments (WS-M-A1 and WS-M-A2, respectively), were conducted. The structural and chemical changes involved in the process were characterized by optical microscopy, laser particle size analysis, and X-ray diffraction. The enzymatic saccharification efficiencies of substrates prepared using the four types of pretreatment were also compared.

EXPERIMENTAL

Materials

Substrates and chemicals

Wheat straw, obtained from a local farmer, was air-dried and manually chopped into 1- to 2-cm lengths and stored in a sealed plastic bag at room temperature. A commercial cellulase complex, Cellic CTec2, was obtained from Novozymes North America (Franklinton, NC). Sodium hydroxide, urea, and other chemicals were all purchased from a commercial source.

Methods

Pretreatment

Pretreatments of wheat straw were conducted according to the schematic flow diagram shown in Fig. 1. First, 9.98 g (9.85% moisture content) of wheat straw and 600 mL of water (25 °C) or preheated NaOH solution (0.5%, 80 °C) were placed in a 1-L kitchen blender (JYL-C051, Joyoung Co., Ltd., China). The suspension was violently agitated at 22,000 rpm for 10 min and then separated by filtration using a Büchner funnel. The mechanically treated substrate was denoted WS-M, and the mechano-chemically pretreated substrate was denoted WS-MA. The WS-M substrate was further treated by NaOH/urea or ammonium/urea solution at a low temperature. To accomplish this, 8 g of WS-M substrate was soaked in 100 g of a precooled mixture of alkaline/urea in distilled water (7:12:81 by weight) at -20 °C for 6 h. The mixture was vigorously stirred at 200 rpm for 2 min, and the substrate was washed and filtered. These two substrates were denoted WS-M-A1 and WS-M-A2, respectively.
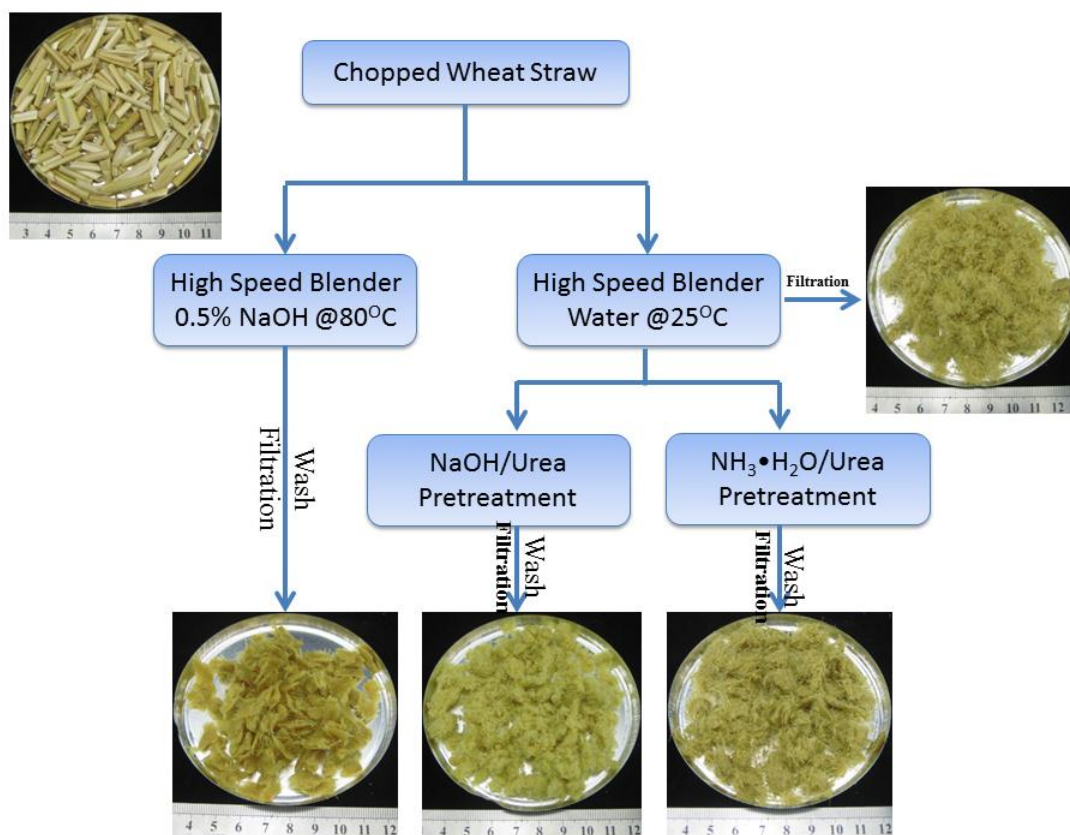

Fig. 1. Experimental schematic flow diagram illustrating different pretreatment methods for wheat straw and visual images of pretreated substrates. In the images, the scale between each set of numbers is 10 mm.

Enzymatic hydrolysis of pretreated wheat straw substrates

The pretreated wheat straw substrates were hydrolyzed by cellulase at a dosage of 10 FPU/g substrate at 2% substrate solids (w/v) in acetate buffer (50 mM, pH 4.8) with 50 ppm tetracycline as an antibiotic. The reaction medium was incubated on a shaker (KYC 100B; Shanghai Fuma Test Equipment Co., Ltd., China) at 50 °C and 200 rpm. Samples were taken periodically, and glucose concentration was measured in replicate using a

commercial glucose analyzer (SBA-40D; Institute of Biology of Shandong Province Academy of Sciences, China). The glucan conversion (%) was described as follows,

$$\text{Glucan conversion(\%)} = \frac{\rho_g \times 50}{1.1 \times 1 \times C_g} \times 100\% \qquad (1)$$

where ρ_g (g/mL) is the glucose concentration in the supernatant and 50 is total volume of hydrolysate (mL). 1.1 is the factor that converts glucan to an equivalent glucose and 1 (g) is the pretreated substrate added. C_g is the glucan content (%) in pretreated substrate.

Chemical composition determination of pretreated substrate

The chemical compositions of pretreated wheat straw substrates were determined according to the standard NREL protocol (Sluiter *et al.* 2008). Monomeric sugars were measured by a Shimadzu high performance liquid chromatograph (HPLC; Japan) equipped with a Shodex SP-1011 sugar column (Showa Denko) with a refractive index detector (RID10A). The column was operated with 0.01 N H_2SO_4 at a flow rate of 0.5 mL/min, with a column and detector temperature of 50 °C.

Substrate morphology and size distribution

A Keyence VHX-1000 digital microscope (Japan) equipped with a CCD camera was employed to collect the morphology images of pretreated wheat straw substrates. Size distributions of pretreated substrates were measured by a laser particle size analyzer (BT-2003; Dandong Bettersize Instruments, China). Three measurements were conducted for each sample, and the average is reported.

X-ray diffraction (XRD) analysis

X-ray diffraction of pretreated wheat straw substrates was carried out on an X-ray diffractometer (D8 ADVANCE, Bruker; Germany) at 30 kV and 15 mA from 10° to 35° with a Cu $K_{\acute{a}1}$ radiation source. The cellulose crystallinity index (CrI) in the pretreated substrate was determined (Segal *et al.* 1959) from Eq. 2,

$$\text{CrI} = (I_{002} - I_{am})/I_{002} \qquad (2)$$

where I_{002} and I_{am} are the maximum and minimum intensity of diffraction at approximately $2\theta=21.0-22.0°$ and $2\theta=18.0-19.0°$, respectively. I_{002} represents the peak intensity of the crystalline and amorphous material, and I_{am} represents the amorphous region only.

RESULTS AND DISCUSSION

Chemical Composition of Pretreated Substrates

The chemical compositions of wheat straw after simple mechanical pretreatment (WS-M), integrated mechanical destruction and alkaline pretreatment (WS-MA), and mechanical destruction followed by low temperature NaOH/urea and ammonium/urea pretreatments (WS-M-A1 and WS-M-A2, respectively) are given in Table 1. The glucan, xylan, and lignin contents of untreated wheat straw were 39.4%, 16.0%, and 19.1%, respectively. As expected, substrates after WS-M pretreatment had chemical components similar to untreated wheat straw. The biggest difference was in the ash content. Table 1

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shows that the solid recoveries of wheat straw ranged from 53% to 92% after pretreatment. The WS-MA and WS-M-A1 pretreatments increased glucan content in retained solids by partially removing lignin, waxes, ash, and other components. Because of the relatively mild pretreatment conditions, more than 90% glucan was retained in recovered wheat straw solids for all pretreatments. With WS-MA, WS-M-A1, and WS-M-A2 pretreatments, the total amount of lignin in substrates was reduced from the original 17.7% to 6.4%, 8.4%, and 6.3%, respectively. Less lignin in pretreated substrates allows cellulase to access cellulose more efficiently. The WS-M-A1 substrate had an approximate 3.2% reduction in xylan content compared with untreated wheat straw, while other pretreatments had limited effect in xylan removal.

Table 1. Chemical Compositions of Untreated and Pretreated Wheat Straw Substrates

Pretreatments	Glucan (%)	Xylan (%)	Total lignin (%)	Ash (%)	Solid recovery (%)
Untreated WS	39.4	16.0	19.1	7.1	100
WS-M	39.5	15.8	20.0	2.4	92.2
WS-MA	54.0	17.1	11.1	1.6	74.5
WS-M-A1	69.1	13.8	10.0	1.7	53.2
WS-M-A2	44.7	16.0	15.8	1.0	85.5

Morphology Observation and Size Distribution

The visual appearance of pretreated wheat straw substrates is shown in Fig. 1. Pretreated WS-M and WS-M-A2 substrates have rigid fiber bundle structures. Fibers of the WS-MA substrate are intertwined together and formed into fiber blocks, while fibers from the WS-M-A1 substrate form a loose network. Optical microscopy was used to study the actual morphology of pretreated substrates in the wet state, and avoid the deformation that occurs during sample drying. As shown in Fig. 2, optical images reveal information similar to that discussed previously based on visual examination. Chopped wheat straw was subjected to intense shear forces, producing long wheat straw pulp that retained its stem tissue structure and small fragments with ripped cell walls. Mechanical pretreatment by the high speed blender can also result in a nanosize fiber structure in some regions (Uetani and Yano 2011). The WS-MA substrate consists of single fibers or relatively small fiber bundles, forming a complex network. The presence of sodium hydroxide during mechanical fibrillation may play a critical role in the occurrence of fibrillation. Lignin was partially removed by alkaline attack during pretreatment, producing more weak points for mechanical fibrillation. The sodium hydroxide extended the fibrillation of the substrate to a deeper level. The WS-M-A1 substrate has more loose and distorted bundles, exhibiting more internal surfaces. These significant changes can be ascribed to the dissolution and regeneration of cellulose in the low-temperature NaOH/urea solution (Zhao *et al.* 2008). The changes of chemical composition and morphology of the treated sample suggested that the sodium hydroxide/urea pretreatment could effectively disrupt the complex lignocellulosic structure, increasing the substrate accessibility and reactivity. The morphology of WS-M-A2 is very similar to that of the WS-M substrate obtained using only mechanical destruction.
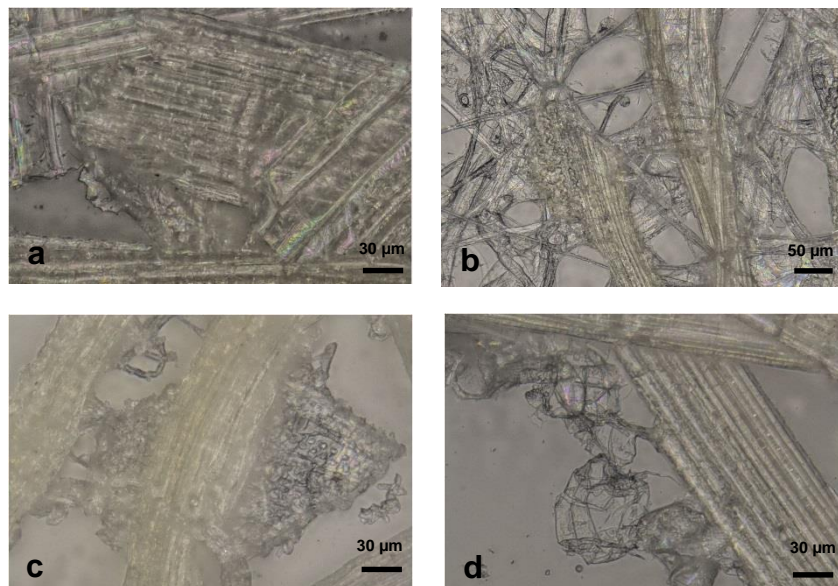
Fig. 2. Digital microscopic images of pretreated wheat straw substrates after various pretreatments. (a) WS-M, (b) WS-MA, (c) WS-M-A1, and (d) WS-M-A2

A laser particle size analyzer was used to determine the particle size distributions of pretreated substrates. The size frequency distribution of the tested wheat straw substrates was very wide, ranging from 1 to 1000 μm (Fig. 3). There were no obvious differences between the WS-M, WS-M-A1, and WS-M-A2 substrates, while the WS-MA substrate exhibited a slightly shorter particle size, on average. Particle frequency between 10 and 100 micrometer of WS-MA substrate was higher as compared to others.
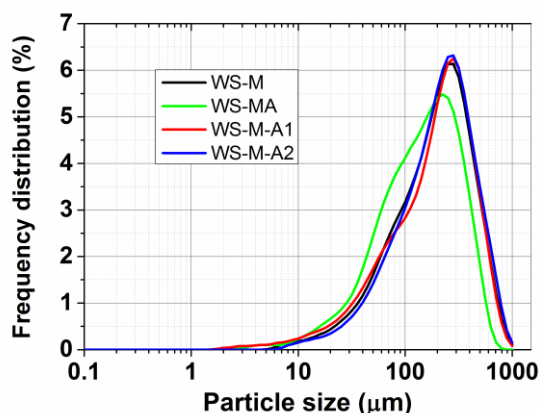

Fig. 3. Wheat straw particle size distribution after various pretreatments

X-Ray Diffraction (XRD) Analysis of Pretreated Wheat Straw Substrates

The crystallinity index (CrI) of cellulose in pretreated lignocellulosic substrates is a key factor affecting the efficiency of enzymatic hydrolysis. The X-ray diffraction spectra of pretreated wheat straw substrates were examined and compared, as shown in Fig. 4. The CrI for all samples was calculated from the XRD data. The CrI of WS-M cellulose was 29.1%, and after integrated mechanical destruction and alkaline pretreatment, there was an observed increase in the CrI (33.3%). This increase in CrI after mechano-chemical pretreatment is probably due to the increase in the relative cellulose content in the

pretreated wheat straw substrate. The CrI values were 14.8% and 33.2% for WS-M-A1, and WS-M-A2 substrates, respectively, suggesting that low-temperature NaOH/urea treatment can break down the crystalline cellulose to amorphous cellulose by the dissolution and regeneration process. During the dissolution of cellulose in low-temperature NaOH/urea solution, decrystallization was first observed, and then crystal type changed (from cellulose I to cellulose II) (Wang and Deng 2009). Low-temperature NH_3/urea treatment is unable to break apart the hydrogen bonds in cellulose.
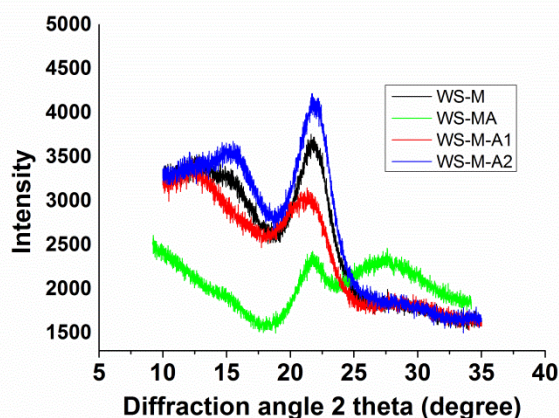

Fig. 4. XRD spectra of pretreated wheat straw substrate

Cellulose Enzymatic Saccharification

To compare the effects of pretreatments on sugar release, enzymatic glucan hydrolysis profiles of wheat straw substrates were examined at a loading of 10 FPU/g of substrate, as shown in Fig. 5. The glucan conversion of the WS-M substrate was only 34.1% after 72 h of enzymatic hydrolysis. Combined mechanical destruction and alkaline pretreatment increased the substrate enzymatic digestibility of glucan in wheat straw, as can be seen from the time courses of glucan conversion during enzymatic hydrolysis. The saccharification yield of the WS-MA substrate increased steadily to approximately 80% after 72 h. Based on the trend of the curve, complete saccharification was not achieved in 72 h. The enzymatic digestibility of substrate pretreated by low temperature NaOH/urea solution increased rapidly for 48 h and then plateaued at approximately 95.6%, compared to only 41.3% for low-temperature NH_3/urea pretreatment.
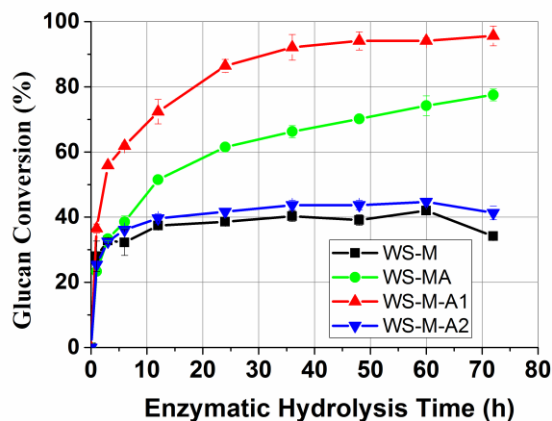

Fig. 5. Time course of enzymatic hydrolysis of glucan in wheat straw

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Some previous investigations have attributed the improvement of enzymatic saccharification of pretreated lignocellulose to reduced substrate particle size (Dasari and Berson 2007; Yeh *et al.* 2010; Agger and Meyer 2012). In this study, no obvious difference in size distribution was detected in the pretreated wheat straw substrates, other than the WS-MA samples, which indicated that particle size is not the key factor affecting the enzymatic digestibility in this study. However, the loose structure and increased surface area of pretreated wheat straw substrate enable the enzyme to more easily penetrate, absorb, and hydrolyze the cellulose; thus, the glucan conversion was greatly improved. This great enhancement of enzymatic hydrolysis of pretreated wheat straw can also be ascribed to the removal of lignin, as well as to the reduction in cellulose CrI. WS-M-A2 pretreatment had little effect on the CrI reduction and lignin removal. The WS-MA pretreatment had a limited effect on cellulose crystallinity, but a considerable amount of lignin was removed, which led to a noticeable increase in the efficiency of enzymatic hydrolysis. Lignin was reduced to half of the initial content and the CrI was markedly reduced by low-temperature NaOH/urea pretreatment, which greatly enhanced the efficiency of enzymatic saccharification. The relatively low CrI value implied that there was a relatively higher proportion of amorphous cellulose in the samples, which was ascribed to the dissolution and regeneration of cellulose in the low-temperature NaOH/urea solution. More extensive studies related to the mechanism of interaction between alkali and the components of lignocellulosic biomass during pretreatment will be performed in the future.

CONCLUSIONS

1. Combined mechanical destruction and alkaline pretreatments were successfully carried out in this study. Nearly half of the original lignin in wheat straw was removed by the simultaneous mechanical destruction and alkaline pretreatment (WS-MA), which is favorable for subsequent enzymatic hydrolysis. Low-temperature NaOH/urea pretreatment disrupted the linkages among cellulose, hemicelluloses, and lignin, as well as dissolved cellulose fibers, resulting in a more open structure as shown by optical microscopy.

2. Enzymatic cellulose conversion of over 75% and enzymatic hydrolysis glucose yield of approximately 30% wt o.d. wheat straw were achieved with WS-MA pretreatment after 72 h of enzymatic hydrolysis with an enzyme loading of 10 FPU cellulose *per* g of o.d. substrate.

3. The substrates pretreated by a low-temperature NaOH/urea solution were hydrolyzed even faster. The cellulose digestibility was as high as 95.6% in wheat straw pretreated by the low-temperature NaOH/urea solution, compared to only 34% in mechanically treated wheat straw.

ACKNOWLEDGMENTS

REFERENCES CITED

Agger, J., and Meyer, A.S. (2012). "Alteration of biomass composition in response to changing substrate particle size and the consequences for enzymatic hydrolysis of corn bran," *BioResources* 7(3), 3378-3397. DOI: 10.15376/biores.7.3.3378-3397

Chen, L., Xing, L., and Han, L. (2009). "Renewable energy from agro-residues in China: Solid biofuels and biomass briquetting technology," *Renewable and Sustainable Energy Reviews* 13(9), 2689-2695. DOI: 10.1016/j.rser.2009.06.025

Dasari, R.K., and Berson, R.E. (2007). "The effect of particle size on hydrolysis reaction rates and rheological properties in cellulosic slurries," *Applied Biochemistry and Biotechnology* 137, 289-299. DOI: 10.1007/s12010-007-9059-x

Kaar, W. E., and Holtzapple, M. T. (2000). "Using lime pretreatment to facilitate the enzymic hydrolysis of corn stover," *Biomass and Bioenergy* 18(3), 189-199. DOI: 10.1016/S0961-9534(99)00091-4

Karp, E. M., Donohoe, B. S., O'Brien, M. H., Ciesielski, P. N., Mittal, A., Biddy, M. J., and Beckham, G. T. (2014). "Alkaline Pretreatment of corn stover: Bench-scale fractionation and stream characterization," *ACS Sustainable Chemistry & Engineering* 2(6), 1481-1491. DOI: 10.1021/sc500126u

Kumar, P., Barrett, D.M., Delwiche, M.J. and Stroeve, P. (2009). "Methods for pretreatment of lignocellulosic biomass for efficient hydrolysis and biofuel production," *Industrial & Engineering Chemistry Research* 48(8), 3713-3729. DOI: 10.1021/ie801542g

Lee, S. H., Doherty, T. V., Linhardt, R. J., and Dordick, J. S. (2009). "Ionic liquid-mediated selective extraction of lignin from wood leading to enhanced enzymatic cellulose hydrolysis," *Biotechnology and Bioengineering* 102(5), 1368-1376. DOI: 10.1002/bit.22179

Leu, S.-Y., and Zhu, J. Y. (2012). "Substrate-related factors affecting enzymatic saccharification of lignocelluloses: Our recent understanding," *BioEnergy Research* 6(2), 405-415. DOI: 10.1007/s12155-012-9276-1

Ragauskas, A. J., Williams, C. K., Davison, B. H., Britovsek, G., Cairney, J., Eckert, C. A., Frederick Jr., W. J., Hallett, J. P., Leak, D. J., Liotta, C. L., *et al.* (2006). "The path forward for biofuels and biomaterials," *Science* 311(5760), 484-489. DOI: 10.1126/science.1114736

Segal, L., Creely, J., Martin, A., and Conrad, C. (1959). "An empirical method for estimating the degree of crystallinity of native cellulose using the X-ray diffractometer," *Textile Research Journal* 29(10), 786-794. DOI: 10.1177/004051755902901003

Sluiter, A., Hames, B., Ruiz, R., Scarlata, C., Sluiter, J., Templeton, D., and Crocker, D. (2008). "Determination of structural carbohydrates and lignin in biomass," *Laboratory analytical procedure NREL/TP-510-42618*, National Renewable Energy Laboratory, Golden, CO.

Somerville, C., Youngs, H., Taylor, C., Davis, S. C., and Long, S. P. (2010). "Feedstocks for lignocellulosic biofuels," *Science* 329(5993), 790-792. DOI: 10.1126/science.1189268

Sun, Y., and Cheng, J. (2002). "Hydrolysis of lignocellulosic materials for ethanol production: A review," *Bioresource Technology* 83(1), 1-11. DOI: 10.1016/S0960-8524(01)00212-7

Talebnia, F., Karakashev, D., and Angelidaki, I. (2010). "Production of bioethanol from wheat straw: An overview on pretreatment, hydrolysis and fermentation," *Bioresource Technology* 101(13), 4744-4753. DOI: 10.1016/j.biortech.2009.11.080

Uetani, K., and Yano, H. (2011). "Nanofibrillation of wood pulp using a high-speed blender," *Biomacromolecules* 12(2), 348-353. DOI: 10.1021/bm101103p

Wang, Q. Q., He, Z., Zhu, Z., Zhang, Y. H., Ni, Y., Luo, X. L., and Zhu, J. Y. (2012). "Evaluations of cellulose accessibilities of lignocelluloses by solute exclusion and protein adsorption techniques," *Biotechnology and Bioengineering* 109(2), 381-389. DOI: 10.1002/bit.23330

Wang, Y., and Deng, Y. (2009). "The kinetics of cellulose dissolution in sodium hydroxide solution at low temperatures," *Biotechnology and Bioengineering* 102(5), 1398-1405. DOI: 10.1002/bit.22160

Wyman, C. E. (2013). *Aqueous Pretreatment of Plant Biomass for Biological and Chemical Conversion to Fuels and Chemicals*, John Wiley & Sons, New York. DOI: 10.1002/9780470975831

Yeh, A., Huang, Y., and Chen, S. (2010). "Effect of particle size on the rate of enzymatic hydrolysis of cellulose," *Carbohydrate Polymers* 79(1), 192-199. DOI: 10.1016/j.carbpol.2009.07.049

Zhang, Y.H. and Lynd, L.R. (2004). "Toward an aggregated understanding of enzymatic hydrolysis of cellulose: noncomplexed cellulase systems, " *Biotechnology and Bioengineering* 88(7), 797-824. DOI: 10.1002/bit.20282

Zhao, Y. L., Wang, Y., Zhao, J. Y., Ragauskas, A., and Deng, Y. L. (2008). "Enhanced enzymatic hydrolysis of spruce by alkaline pretreatment at low temperature," *Biotechnology and Bioengineering* 99(6), 1320-1328. DOI: 10.1002/bit.21712

Zhu, J., Zhang, X., and Pan, X. (2011). *Sustainable Production of Fuels, Chemicals, and Fibers from Forest Biomass*, American Chemical Society, Washington, DC. DOI: 10.1021/bk-2011-1067