

Enhancing the Enzymatic Hydrolysis of Corn Stover by an Integrated Wet-milling and Alkali Pretreatment

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Abstract An integrated wet-milling and alkali pretreatment was applied to corn stover prior to enzymatic hydrolysis. The effects of NaOH concentration in the pretreatment on crystalline structure, chemical composition, and reducing-sugar yield of corn stover were investigated, and the mechanism of increasing reducing-sugar yield by the pretreatment was discussed. The experimental results showed that the crystalline structure of corn stover was disrupted, and lignin was removed, while cellulose and hemicellulose were retained in corn stover by the pretreatment with 1% NaOH in 1 h. The reducing-sugar yield from the pretreated corn stovers increased from 20.2% to 46.7% when the NaOH concentration increased from 0% to 1%. The 1% NaOH pretreated corn stover had a holocellulose conversion of 55.1%. The increase in reducing-sugar yield was related to the crystalline structure disruption and delignification of corn stover. It was clarified that the pretreatment significantly enhanced the conversion of cellulose and hemicellulose in the corn stover to sugars.

Keywords Lignocellulosic biomass · Corn stover · Pretreatment · Enzymatic hydrolysis · Sugar

Introduction

Lignocellulosic biomass, due to its abundant supply and low cost, has been recognized as a potential resource to decrease our excessive dependence on petroleum for liquid fuels and organic materials, and also to reduce the build-up of greenhouse gasses that is causing global climate change [1–3]. Biological conversion of lignocellulosic biomass involves two main processes: enzymatic hydrolysis of the lignocellulosic biomass to produce reducing sugars and fermentation of the sugars to produce ethanol or other chemicals. However, the sugar yield is very low in the enzymatic hydrolysis of lignocellulosic biomass due to (1)

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cellulose, hemicellulose, and lignin intercross spatially with each other; (2) cellulose and hemicellulose are densely packed within layers of lignin; and (3) cellulose has a crystalline structure, which protects lignocellulosic biomass from enzymatic hydrolysis [4–6]. In order to enhance the hydrolysis rate and the sugar yield, pretreatment of lignocellulosic biomass is necessary.

A variety of physical (comminution, hydrothermolysis), chemical (acid, alkali, ozone), physico-chemical (steam explosion, ammonia fiber explosion), and biological techniques have been developed for the pretreatment of lignocellulosic biomass [7, 8]. An effective pretreatment is characterized by several criteria. It disrupts the barriers to enzymatic hydrolysis of cellulose and hemicellulose, preserves the pentose (hemicellulose) fractions, limits the formation of degradation products that inhibit the growth of fermentative microorganism, minimizes energy and chemicals demands, and reduces the cost [9, 10]. Although many pretreatment methods have been tested over the years, only a few are considered attractive for industrial application. Fundamental understandings of the chemical and physical changes that occur during pretreatment, as well as a clearer understanding of the effects of the chemical composition and physico-chemical structure of lignocellulosic biomass on its enzymatic hydrolysis, are still in demand.

Integrated wet-milling and alkali pretreatment is a new technique developed to enhance the enzymatic hydrolysis of lignocellulosic biomass [11]. In the present research work, the integrated wet-milling and alkali pretreatment was applied to corn stover prior to enzymatic hydrolysis. Dilute sodium hydroxide (NaOH) solution was used as the wet-milling medium. The effects of NaOH concentration on chemical composition, crystalline structure, and reducing-sugar yield of corn stover were investigated, and the mechanism of increasing reducing-sugar yield by the pretreatment was discussed.

Materials and Methods

Corn Stover

The corn stover used in the present study was obtained from the suburb of Nanjing City in October 2007 after the corn (variety, Denghai No.10) was harvested. It was sun-dried to an equilibrium moisture content of 10%, ground to pass through a 40-mesh sieve by a Wiley Laboratory Mill (FZ102, Beijing Yongguangming Medical Instrument Co., China), and then stored until use for pretreatment.

Pretreatment

Corn stover (40 g) at 10% (w/v) solid concentration was wet-milled at room temperature for 1 h, using a planet-type ball mill (XQM-4 L, Nanjing Kexi Institute of Experimental Instruments Co., China) with a rotational speed of 500 rpm. Sodium hydroxide (NaOH) solution with a concentration of 0% (i.e., water), 0.2%, 0.5%, 1%, and 2%, respectively, was used as the wet-milling medium. After the wet-milling, the corn stover suspension was centrifuged at 7,000 rpm for 30 min. The solid fraction was collected and stored at 4 °C before the composition determination and the enzymatic hydrolysis tests. No washing treatment was performed for the solid fraction. Solid recovery, R_S (%), was calculated as follows:

$$R_S = \frac{M_p}{M} \times 100\% \quad (1)$$

Where, M_p is the mass of dry matter in pretreated corn stover (g); M is the mass of dry matter in corn stover before wet-milling (g). The dry matter content of the untreated and the pretreated corn stovers was determined by drying a 5-g corn stover sample at 105 °C for 24 h.

The content of water extractives, cellulose, hemicellulose, lignin, and ash in the untreated and the pretreated corn stovers was determined with Van Soest's method [12, 13], using a Fiber Analysis System (Fibertec System M6, FOSS, Denmark).

The particle size distributions of the pretreated corn stovers were measured by a particle size analyzer (2000, Malvern, UK).

X-ray diffractometry was performed for the untreated and the pretreated corn stovers, using an X-ray diffractometer (ARL X'TRA, Thermo Electron Corporation, USA). The pretreated corn stovers were dried at 60 °C for 48 h before the analysis. Each corn stover sample was packed tightly into a square plastic cell (20×20 mm), and exposed to the X-ray beam from an X-ray generator running at 45 kV and 35 mA. The diffraction angle (2θ) was from 5° to 40°.

Enzymatic Hydrolysis

The untreated and the pretreated corn stovers were enzymatically hydrolyzed by a commercial cellulolytic complex (Cellulase, Zhaodong Richeng Enzymes Co., China). The cellulolytic complex was produced from *Trichoderma reesei*. It had a cellulase activity of 89 FPU per gram and inherent activities of xylanase and beta-glucosidase. The cellulase activity was determined according to the method reported by Ghose [14]. The unit of FPU was defined as the amount of enzyme which produces 2.0 mg reducing sugar (as glucose) from 50 mg filter paper in 1 h under optimum conditions: temperature of 50 °C, and pH of 4.8. In the hydrolysis process, the cellulase loading was controlled at 7 FPU/g-substrate. A corn stover sample containing 4 g dry matter was used as the substrate in each enzymatic hydrolysis test. It was mixed with 0.05 M sodium citrate buffer (pH 4.8) at 1% (w/v) solid concentration in a 500-mL Erlenmeyer flask. The enzymatic hydrolysis was performed at 50°C on a thermostated water bath.

During hydrolysis, samples of the hydrolysis liquid were taken and centrifuged at 4,500 rpm for 15 min. Reducing sugars contained in the supernatant were analyzed with the 3,5-dinitrosalicylic acid method [15] and expressed by dextrose equivalent. Reducing-sugar yield, Y_r (%), was defined as follows:

$$Y_r = \frac{C_r \times V}{M_s} \times 100\% \quad (2)$$

Where C_r is the reducing-sugar concentration of hydrolysis liquid (g/L); V is the volume of hydrolysis liquid (L); M_s is the mass of dry substrate corn stover used in the enzymatic hydrolysis (g).

Holocellulose was defined as the sum of cellulose and hemicellulose in lignocellulosic biomass. Holocellulose conversion, η_h (%), was calculated with the equation below.

$$\eta_h = \frac{Y_r}{m_h} \times 0.9 \quad (3)$$

Where, m_h is the holocellulose content of dried substrate used in the enzymatic hydrolysis (–); 0.9 is the mass ratio of anhydroglucose to its free glucose.

Mono- and disaccharides in the supernatant of the hydrolysis liquid were analyzed using a high-performance liquid chromatograph (Ultimate 3000, Dionex, USA) equipped with a refractive index detector and a Bio-Rad Aminex HPX-87H column (300 mm×7.8 mm).

The samples were diluted with deionized water, filtered through Sep Pak C₁₈ filters (Millipore) and thus injected into the chromatograph under the conditions: column temperature of 55 °C, 5 mM H₂SO₄ as mobile phase at a flow rate of 0.6 ml/min, and an injection volume of 20 µl.

The pretreatment and enzymatic hydrolysis tests under each condition were conducted in duplicate, and the data presented here are the average results.

Results and Discussion

Changes in the Crystal Structure of Corn Stover

Figure 1 shows two examples of particle size distributions of the pretreated corn stovers. The NaOH concentration in the wet-milling was 0% and 1%, respectively. Under the same solid loading and wet-milling time, NaOH concentration had no significant effect on the particle size distribution. For all pretreated corn stovers, the particle size followed a normal distribution in a range of 0.5 to 500 µm, with a median diameter of about 25 µm.

X-ray diffraction patterns of the untreated and the pretreated corn stovers are shown in Fig. 2. In the untreated corn stover, there was a diffraction peak at $2\theta=22^\circ$ and a diffraction bottom at $2\theta=18^\circ$ [16, 17]. The diffraction peak and bottom stood for the intensity of crystalline and amorphous regions, respectively. In the crystalline region, molecules were packed densely so that the enzymatic digestibility of cellulose and hemicellulose is very low. On the other hand, the molecules in the amorphous region were packed loosely, which was propitious for enzymatic hydrolysis. The diffraction peak and bottom of pretreated corn stovers decreased gradually with increasing NaOH concentration. They disappeared at the NaOH concentration of 1%, indicating that the crystal structure was destroyed completely.

Dry-milling had less effect on the crystalline structure of lignocellulosic biomass, in contrast to wet-milling. For example, it took 108 h to destroy the crystalline structure of Masson Pine wood (under an 80-mesh sieve) by dry-milling in a ball mill [18]. In addition, a microcalorimetric study indicated that the concentration of NaOH solution in alkali

Fig. 1 Particle size distributions of the pretreated corn stovers

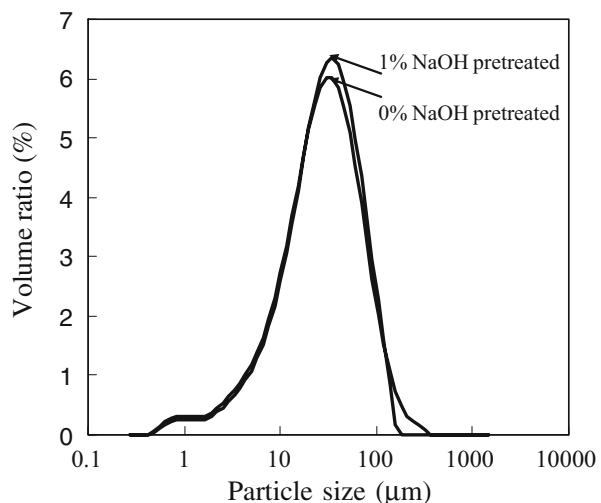
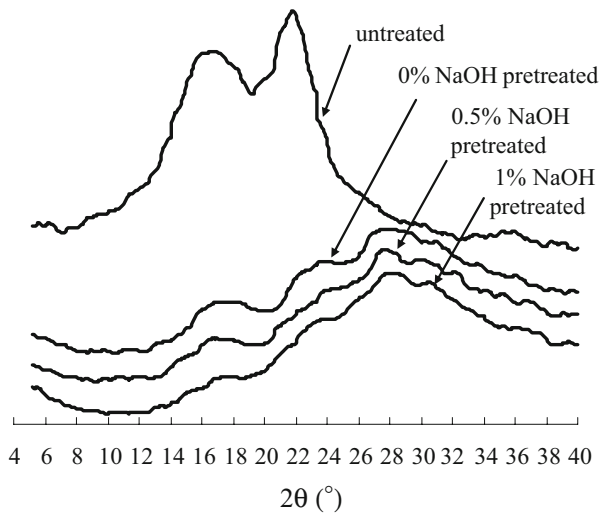


Fig. 2 X-ray diffraction patterns of the untreated and the pretreated corn stovers



pretreatment affected the enzymatic hydrolysis of pretreated wheat straws. The maximum reaction heat of enzymatic hydrolysis was observed when the wheat straw was pretreated by 4% NaOH at the room temperature [19]. It is clear that the integrated wet-milling and alkali pretreatment significantly reduces both the milling time and the NaOH concentration required to disrupt the crystalline structure of lignocellulosic biomass. The main reason for this might be that the pretreatment incorporates both physical and chemical effects: (1) NaOH solution dissolves lignin, thus increasing the porosity of biomass [8, 20, 21]; (2) comminution reduces particle size and disrupts the crystalline structure [18, 22], which facilitates the chemical reaction between NaOH and lignin.

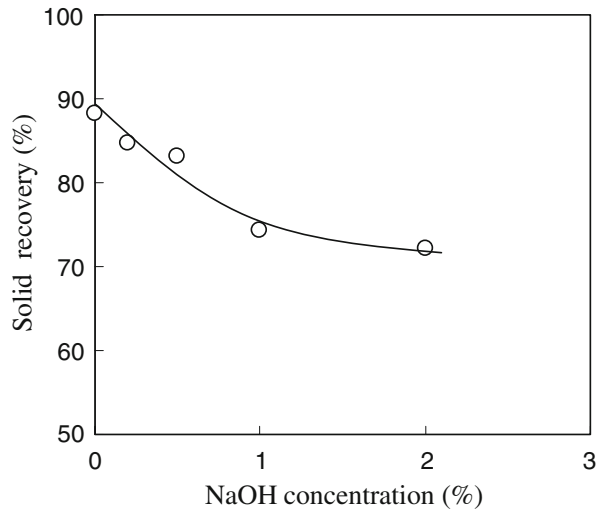
Changes in the Chemical Composition of Corn Stover

Solid recovery changed with NaOH concentration in the pretreatment process. As shown in Fig. 3, the solid recovery was 90% at the NaOH concentration of 0%, and 72% at the NaOH concentration of 2%. The decrease of solid recovery in the NaOH concentration range of 0–1% was much larger than that in the NaOH concentration range of 1–2%.

Table 1 shows the chemical composition of the untreated and the pretreated corn stovers on dry basis. The untreated corn stover consisted of 8.6% water extractives, 34.9% cellulose, 31.5% hemicellulose, 19.1% lignin, and 5.9% ash. The content of cellulose, hemicellulose and ash increased, while the content of water extractives and lignin decreased by the pretreatment with NaOH solution in the concentration range of 0–1%, since a part of water extractives and lignin was removed from the corn stover. The 1% NaOH pretreated corn stover had 4.5% water extractives, 41.3% cellulose, 32.2% hemicellulose, 15.0% lignin, and 7.9% ash. Increasing of NaOH concentration from 1% to 2% did not significantly improve delignification.

In the steam explosion pretreatment of lignocellulosic biomass, 87.6% of hemicellulose together with 25% of lignin in the raw material was removed, so that the solid recovery was only 40–60% [23–25]. In comparison with steam explosion pretreatment, the integrated wet-milling and alkali pretreatment in the present study was more effective in both removing lignin from lignocellulosic biomass and retaining its cellulose and hemicellulose composition.

Fig. 3 Change of solid recovery with NaOH concentration in the pretreatment process



Increase in Reducing-sugar Yield

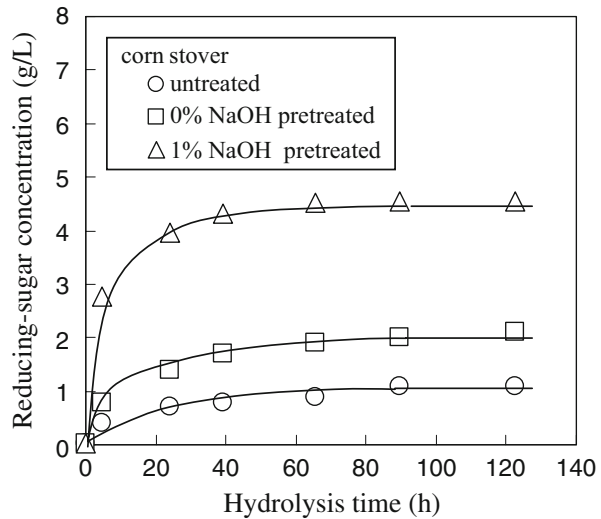
Figure 4 shows the variation of the reducing-sugar concentration of hydrolysis liquid during the enzymatic hydrolysis of corn stovers. For all corn stovers, the hydrolysis liquid did not contain sugars at the beginning. The reducing-sugar concentration increased due to the degradation of cellulose and hemicellulose. It had little change after 90 h, suggesting that the hydrolysis had almost finished. Therefore, the reducing-sugar concentration at 120 h was used to calculate the reducing-sugar yield and the holocellulose recovery in the present study. The reducing-sugar concentration at 120 h was 1.1%, 2.1%, and 4.6% for the untreated corn stover, 0% NaOH pretreated corn stover, and 1% NaOH pretreated corn stover, respectively.

The untreated corn stover only had a reducing-sugar yield of 11.0% in the enzymatic hydrolysis process. The reducing-sugar yield was improved by the pretreatment. Figure 5 shows the change of reducing-sugar yield with the NaOH concentration of pretreatment. The reducing-sugar yield increased from 20.2% to 46.7% when the NaOH concentration increased from 0% to 1%, and it was almost constant at 46.7% in the NaOH concentration range of 1% to 2%. From Eq. 3, it could be found that the untreated and the 1% NaOH pretreated corn stovers had a holocellulose conversion of 16.5% and 55.1%, respectively. The increase in reducing-sugar yield was related to the crystalline structure disruption (Fig. 2) and delignification (Table 1) of the corn stover in the pretreatment process. It was

Table 1 Chemical composition of the untreated and the pretreated corn stovers (% , db).

Corn stover	Water extractives	Cellulose	Hemicellulose	Lignin	ash
Untreated	8.6	34.9	31.5	19.1	5.9
0% NaOH pretreated	5.3	39.2	31.2	18.1	6.2
0.2% NaOH pretreated	5.2	39.4	31.3	17.7	6.4
0.5% NaOH pretreated	5.1	39.7	31.4	17.2	6.6
1% NaOH pretreated	4.5	41.3	32.2	15.0	7.9
2% NaOH pretreated	3.4	41.3	32.1	14.5	8.7

Fig. 4 Variation of the reducing-sugar concentration of hydrolysis liquid during the enzymatic hydrolysis of corn stovers

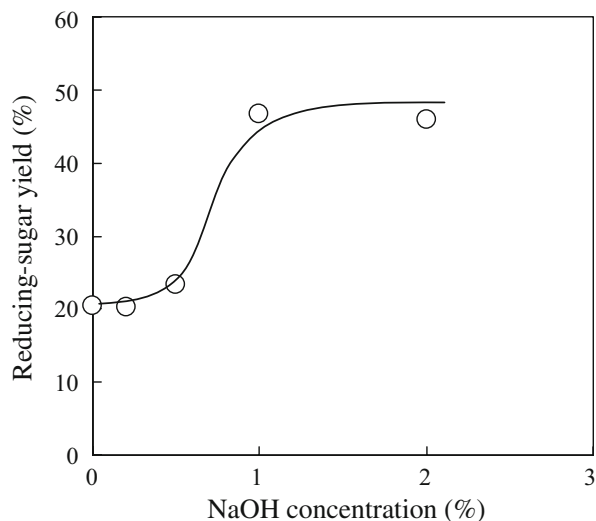


also reported that lignin removal greatly enhanced the ultimate hydrolysis extent of hybrid poplar, while crystallinity reduction tremendously increased the initial hydrolysis rate and reduced the amount of enzyme required to attain high digestibility [26].

Analysis with high-performance liquid chromatography indicated that the reducing sugars, which was produced in the enzymatic hydrolysis of 1% NaOH pretreated corn stover, consisted of 63.9% glucose, 33.6% xylose, 2.4% arabinose, and 0.2% cellobiose. It is suggested that both cellulose and hemicellulose contained in the corn stover were hydrolyzed.

NaOH is the most commonly used base for chemical pretreatment of lignocellulosic biomass [9]. Among the pretreatments with sulfuric acid (H_2SO_4), sodium hydroxide (NaOH), hydrogen peroxide (H_2O_2) and ozone, NaOH pretreatment (2% NaOH, 121 °C, 90 min) resulted in the highest level of delignification (65.6%) for the raw material of cotton stalks and the highest level of glucan conversion (60.8%) for the pretreated cotton

Fig. 5 Change of reducing-sugar yield with the NaOH concentration of pretreatment



stalks [8]. Steam explosion pretreatment at 220 °C improved the glucose yield from pretreated sunflower stalks by four times in comparison with that (18.1%) from untreated sunflower stalks [25]. The NaOH pretreatment and steam explosion pretreatment, however, usually need a temperature higher than 200 °C. Furthermore, few data for xylan conversion are available in the previous reports.

The integrated wet-milling and alkali pretreatment enhanced the enzymatic hydrolysis of lignocellulosic biomass to sugars. The sugar yield expects to be further improved by the optimizing the enzymatic hydrolysis conditions such as enzyme complex and its loading. In addition, the integrated wet-milling and alkali pretreatment also has an advantage of energy-saving, in comparison with the conventional NaOH pretreatment and steam explosion pretreatment, since it is performed at the room temperature. For the industrial application of the new pretreatment technology, the hydrolysis operation with high substrate loading and the hydrolysis reaction rate of pretreated lignocellulosic materials should be discussed to increase the sugar concentration of hydrolysis liquid.

Conclusions

An integrated wet-milling and alkali pretreatment was studied for enhancing of the enzymatic hydrolysis of corn stover. The experimental results could be concluded as follows:

1. The particle size of pretreated corn stovers followed a normal distribution in a range of 0.5 to 500 μm , with a median diameter of about 25 μm . The crystalline structure of corn stover was disrupted completely by the pretreatment with 1% NaOH in 1 hour.
2. Delignification of corn stover increased with increasing NaOH concentration in the range of 0% to 1%. The pretreatment was effective in removing lignin from corn stover, while retaining its cellulose and hemicellulose composition.
3. The reducing-sugar yield of pretreated corn stovers increased from 20.2% to 46.7% when the NaOH concentration increased from 0% to 1%, and it was almost constant at 46.7% in the NaOH concentration range of 1% to 2%. The 1% NaOH pretreated corn stover had a holocellulose conversion of 55.1%.
4. The increase in reducing-sugar yield was related to the crystalline structure disruption and delignification of corn stover. The pretreatment significantly enhanced the conversion of cellulose and hemicellulose in corn stover to sugars.

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