Effect of Combined Wet Alkaline Mechanical Pretreatment on Enzymatic Hydrolysis of Corn Stover and Its Mechanism

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Research Article

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Abstract

**Background:** To further optimize the mechnochemical pretreatment process, a combined wet alkaline mechanical pretreatment of corn stover at room temperature was proposed.

**Results:** The combined alkaline mechanical pretreatment significantly enhanced enzymatic hydrolysis resulting in a glucose yield (Y_G) of 491.9 mg/g (91.9%) with 3% NaOH and ball milling for 10 min. The liquid fraction after treatment contained by-products such as monosaccharides, oligosaccharides, acetic acid, and lignin but no furfural and 5-hydroxymethylfurfural. Quantitative correlation analysis showed that Y_G = 41.41 + 4.09’ball milling time (min) + 112.7’NaOH concentration (%) (R^2 = 0.87), Y_G = 37.79’cellulose content - 1421.55 (R^2 = 0.88), and Y_G = -72.47’lignin content + 1482.77 (R^2 = 0.95).

**Conclusion:** The impact of the physical and chemical properties of corn stover pretreated with different ball milling times and/or different NaOH concentrations on the subsequent enzymatic hydrolysis was investigated, which would be beneficial to illustrate the effective mechanism of mechnochemical pretreatment method.

**Background**

With the extensive development and application of fuel ethanol worldwide, lignocellulosic biomass, such as straw, has become a raw material source for fuel ethanol production owing to its advantages of large output and availability[1–3]. However, lignocellulosic biomass has a complex microstructure and chemical composition. A dense macromolecular network composed of cellulose, hemicellulose, and lignin in the cell wall of lignocellulosic biomass improves its recalcitrance of cellulose to chemical reagents and enzymes, seriously hindering the ethanol conversion efficiency[4]. Therefore, pretreatment of lignocellulosic biomass is generally required to reduce its biological resistance and increase the ethanol conversion efficiency[5].

Alkaline pretreatment using sodium hydroxide (NaOH) solution is a common and efficient chemical pretreatment method for lignocellulose. This process improves the accessibility of cellulose to enzymes by cleaving ester and ether bonds in lignin and hemicellulose through degreasing and saponification, and removing lignin and some hemicellulose in plant cell walls[6–9]. The alkaline pretreatment process doesn't require expensive and complicated equipment, but long reaction times or high reaction temperatures are often needed to achieve desired efficiency in the subsequent enzymatic hydrolysis[10]. For example, Li et al. mixed corn stover with 7 wt% and 10 wt% NaOH solutions at a mass ratio of 1:10, followed by grinding for 30 min at 140 and 160°C, obtaining the maximum glucose yield from enzymatic hydrolysis after pretreatment using 10 wt% NaOH at 160 °C[11].

Ball milling pretreatment is an environmentally friendly physical pretreatment method that can disrupt the dense and complex physical structure of plant cell walls, reduce the size of biomass particles, destroy the crystalline structure of cellulose, and increase the degree of exposure to cellulose through mechanical force, which result in increased enzymatic hydrolysis efficiency[12–14]. Ji et al. reported that the glucose yield from enzymatic hydrolysis of rice straw increased after ball milling for 20 min owing to the average particle size being at the cellular scale (<30–50 µm), destruction of the straw cell wall structure, and reduced crystallinity[15]. Although the ball milling process is environmentally friendly, pollution-free, and cannot change the original chemical composition of biomass, its energy consumption is high[14].

Recently, to utilize the advantages of alkaline and mechanical pretreatments while avoiding the problems of single pretreatment, mechnochemical pretreatment has attracted research attention[16–18]. Barakat et al. mixed wheat straw with NaOH solution and ammonia (5% w/w) in a 5:1 (w/v) ratio at room temperature for 5 h and then the mixtures were ball-milled after drying in an oven at 105°C[19]. Chuetor et al. mixed bagasse with NaOH (5% w/w) at a ratio of 1:2 or 1:5 (w/v) for 3 h, followed by centrifugal grinding treatment after drying (maintaining moisture at 8-10%) in 60°C oven[20]. The above combined mechnochemical treatments can enhance enzymatic hydrolysis efficiency, but the long treatment time and drying step are not favorable for industrial process.

Therefore, to further reduce treatment times and avoid energy consumption in the drying process, a combined wet alkaline mechanical pretreatment at room temperature was proposed in this study. The relationships of the microstructure and chemical composition of corn stover treated with different ball milling times and/or different concentrations of NaOH with the corresponding glucose yield from enzymatic hydrolysis were analyzed. The composition change of liquid fraction was explored with a view to providing experimental data to elucidate the effective mechanism of mechnochemical pretreatment method.

**Results And Discussion**

**Effects of different pretreatments on yield from enzymatic hydrolysis of corn stover**

Figure 1 shows the glucose yields and Figure 2 shows the xylose yields (b) from treated corn stover. The sugar yields of control group were 65.5 mg/g (SY =14.5%) of glucose yield and 11.8 mg/g (SY=4.7%) of xylose yield. For samples treated only by ball milling (WBMx-NaOH0%), the yield of glucose and xylose increased with increasing ball milling time and reached maximum of 137.9 mg/g (SY =31.2%) glucose yield and 30.9 mg/g (SY =12.3%) xylose yield at 20 min. The relationship between glucose yield (Y_G) and ball milling time (x) can be fitted as the following exponential function, Y_G=164.19–99.34´0.94^x (R^2 = 0.98) (Fig. 3). For corn stover only treated with NaOH (WBM0-NaOHy), both the glucose yield and xylose yield significantly increased with increased NaOH concentration. At a NaOH concentration of 3%, the glucose yield was 307.1 mg/g (SY =60.8%), and the xylose yield was 110.8 mg/g (SY =42.6%). The quantitative relationship between glucose yield and NaOH concentration (y) was represented by the following equation: Y_G=WBM0 = 74.17exp (0.48y) (R^2 = 0.98) (Fig. 4). By comparison, the effect of NaOH treatment on the enhancement in enzymatic hydrolysis was higher than that of ball milling.

For the samples treated with combined wet alkaline mechanical, the glucose yield clear increased and the xylose yield showed a trend of increasing first and then decreasing with increasing NaOH concentration at the same ball milling time. However, when the ball milling time exceeded 10 min, the glucose yield
showed no clear change. The relationship between NaOH concentration and glucose yield could be correlated using the following exponential function: $Y = 156.056x^0.39y$ (R² = 0.90) (Fig. 4). The highest glucose yield of 491.4 mg/g (SY=91.9%) could be obtained from WBM10-NaOH3%, and the highest xylose of 157.9 mg/g (SY=62.1%) could be obtained from WBM10-NaOH2%. Therefore, compared with a single mechanical or chemical pretreatment method, combined wet alkaline mechanical pretreatment had a multiplicative effect on the glucose yield efficiency, which greatly enhanced the enzymatic hydrolysis from corn stover. Further binary analysis (Fig. 5) showed that the relationship of ball milling time and NaOH concentration with the glucose yield could be fitted with the following plane function: $Y = 41.41 + 4.09x + 112.78y$ (R² = 0.87).

**Mechanistic analysis of the enhancement of enzymatic hydrolysis efficiency by mechaenochemical pretreatment based on physical and chemical characterization**

According to scanning electron micrographs of corn stover from different pretreatments (see Fig.6), the particle size of samples was reduced after ball milling for 10 min, but the morphology and size of the particles did not change much with increased milling time.

The particle size distribution curves for corn stover from different pretreatments were shown in Figure 7. Furthermore, Table 1 shows the changes of microstructure parameters and main chemical compositions of corn stover with different pretreatments. Table 2 showed the Pearson correlation analysis results.

| Sample | $D_{50}$ (μm) | Crystallinity (%) | solid | liquid |
|--------|---------------|-------------------|-------|--------|
|        |               |                   | Cellulose (%) | Hemicellulose (%) | Lignin (%) | Glucose (mg/g) | Xylose (mg/g) | Arabinose (mg/g) | Acetic acid (mg/g) |
| WBM10-NaOH0% | 182.8±8.2 | 43.8±3.0 | 40.5±0.3 | 26.8±0.2 | 19.4±0.4 | 46.5±0.1 | 15.9±0.2 | 2.1±0.0 | 1.1±0.0 |
| WBM10-NaOH1% | 226.6±10.9 | 42.9±1.1 | 42.4±0.1 | 28.2±0.3 | 17.8±0.4 | 49.6±0.2 | 19.6±0.1 | 3.4±0.0 | 25.4±0.2 |
| WBM20-NaOH2% | 250.2±13.1 | 47.1±0.2 | 44.2±0.3 | 28.6±0.2 | 16.7±0.6 | 51.8±0.0 | 25.1±0.1 | 5.8±0.0 | 33.3±0.0 |
| WBM20-NaOH3% | 283.8±7.6 | 43.6±1.8 | 45.5±1.2 | 28.4±0.6 | 14.4±0.6 | 54.7±0.2 | 36.2±0.3 | 8.1±0.1 | 34.1±0.1 |
| WBM10-NaOH0% | 81.5±1.2 | 41.5±0.7 | 39.5±1.3 | 26.5±0.6 | 18.1±0.7 | 46.7±0.1 | 21.9±0.1 | 3.4±0.0 | 1.7±0.0 |
| WBM10-NaOH1% | 86.5±0.7 | 40.5±0.9 | 42.7±0.2 | 29.0±0.2 | 16.5±0.9 | 41.9±0.1 | 20.5±0.0 | 4.8±0.0 | 29.6±0.2 |
| WBM10-NaOH2% | 94.0±1.0 | 41.2±0.4 | 46.6±0.8 | 28.3±0.5 | 14.8±1.0 | 47.0±0.0 | 52.4±0.9 | 16.7±0.0 | 31.4±0.0 |
| WBM10-NaOH3% | 96.1±0.6 | 42.7±1.4 | 48.1±0.2 | 26.6±0.3 | 14.3±0.1 | 60.2±0.1 | 98.8±0.1 | 24.1±0.0 | 35.7±0.0 |
| WBM20-NaOH0% | 37.3±0.2 | 36.6±0.6 | 39.9±1.2 | 27.0±0.8 | 17.0±0.4 | 56.5±0.2 | 24.2±0.2 | 3.7±0.0 | 2.2±0.1 |
| WBM20-NaOH1% | 50.3±0.2 | 38.4±1.0 | 44.7±0.6 | 30.1±0.4 | 17.4±0.4 | 46.2±0.0 | 22.6±0.1 | 5.0±0.0 | 28.1±0.1 |
| WBM20-NaOH2% | 65.3±0.3 | 37.4±1.5 | 47.7±0.9 | 28.9±0.4 | 14.8±0.5 | 49.8±0.1 | 56.0±0.0 | 17.7±0.0 | 31.0±0.1 |
| WBM20-NaOH3% | 77.2±0.3 | 35.2±0.7 | 50.1±0.2 | 27.0±0.7 | 14.5±0.3 | 58.5±0.0 | 102.2±0.3 | 24.1±0.0 | 33.7±0.2 |
| WBM30-NaOH0% | 27.1±0.2 | 33.6±0.4 | 41.6±0.9 | 28.5±0.4 | 17.7±0.0 | 40.4±0.1 | 25.6±0.2 | 3.9±0.0 | 1.3±0.0 |
| WBM30-NaOH1% | 37.7±0.1 | 36.1±1.1 | 43.2±0.6 | 29.3±0.4 | 16.5±0.9 | 37.6±0.2 | 19.8±0.0 | 4.5±0.0 | 23.6±0.2 |
| WBM30-NaOH2% | 56.8±0.1 | 34.0±0.1 | 49.6±0.7 | 29.3±0.5 | 14.4±0.3 | 54.0±0.0 | 56.1±0.3 | 17.7±0.1 | 34.3±0.1 |
| WBM30-NaOH3% | 67.4±0.4 | 35.4±0.5 | 50.7±0.6 | 26.2±0.2 | 14.3±0.0 | 61.7±0.2 | 103.3±1.3 | 25.3±0.2 | 35.6±0.1 |

Note: (1) mg/g corn stover before pretreatment. (2) Different lowercase letters in the same column indicate significant differences (P<0.01).
According to the results in Figure 7 and Table 1, the average particle size (D_{50}) of corn stover without mechanochemical treatment (WBM0-NaOH0) was 182.8±8.2 mm, which was at the tissue scale (100-500mm) [21]. For corn stover subjected to wet ball milling treatment, the D_{50} was reduced from 81.5±1.2 mm at 10 min to 27.1±0.2 mm at 30 min, which was at cellular scale. The plant tissue structure and hydrogen bonds between crystalline cellulose were destroyed by mechanical forces, resulting in decreased crystallinity[14, 21]. Although cellulose macromolecules in ball-milled samples were hardly depolymerized, the cellulose exposure was increased owing to the increase in specific surface area, which was beneficial to improving the enzymatic hydrolysis efficiency to some extent [22-24]. For NaOH pretreatment, as shown in Table 1, the crystallinity of corn stover did not change much with increased NaOH concentration, which was due to the overall NaOH loading being low [23, 24]. However, the D_{50} value increased (p<0.01) owing to the swelling effect of NaOH solution on corn stover [20]. The removal of lignin and hemicellulose from the solids by NaOH resulted in a significant increase in the proportion of cellulose and a significant decrease in the lignin content (p<0.01), while the hemicellulose content decreased only slightly. Compared with the contents of liquid fraction, it could be seen that the xylose content increased 127.8% from 15.9 mg/g (WBM0-NaOH 0%) to 36.2 mg/g (WBM0-NaOH 3%). The arabinoxylan and acetic acid contents also showed a similar pattern of change, which indicated that the increase in NaOH concentration made more hemicellulose decompose into monosaccharides or oligosaccharides, resulting in no discernable increase of content in the solid[7]. According to previous research, lignin could cause non-productive binding with cellulase, and limit the accessibility of xylan to enzymes[25-27]. Therefore, the glucose and xylose yield increased with the lignin content decreasing by NaOH pretreatment, which was consistent with the correlation analysis results in Table 2.

There was no furfural or HFM formation during the combined wet alkaline mechanical pretreatment. With the same ball milling time, the particle size of treated samples increased (Fig. 7), and the cellulose characteristic diffraction peak intensity weakened with increased NaOH solution concentration (Fig. 8). Furthermore, the data in Table 2 shows that samples subjected to mechanochemical pretreatment had decreased size and crystallinity, with the trend similar to that observed for samples subjected to single ball milling pretreatment. The changes in chemical characteristics caused by single NaOH treatment, including the increased cellulose content and lignin removal, were further enhanced by mechanochemical pretreatment. For example, the cellulose content of WBM0-NaOH0% was 40.5%, which was increased to 45.5% after treatment with 3% NaOH and 50.7% after treatment with 3% NaOH and ball milling for 30 min. Furthermore, the decrease in crystallinity and increase in average particle size caused by the swelling effect of NaOH contributed to promoting the diffusion of cellulase molecules and the accessibility of cellulose to the enzyme, which resulted in a much greater effect on the glucose yield, as shown in Fig. 4 [23].

### Table 2

Pearson correlation analysis

|                  | NaOH concentration (%) | WBM (min) | D_{50} (µm) | Crystallinity (%) | Cellulose (%) | Hemicellulose (%) | Lignin (%) | Glucose yield (mg/g) | Xylose yield (mg/g) |
|------------------|------------------------|-----------|-------------|------------------|---------------|------------------|------------|---------------------|-------------------|
| NaOH concentration (%) | 1                      | 0         | 0.260       | 0.43             | 0.893**       | -0.406          | -0.897**   | 0.884**             | 0.787**           |
| WBM (min)        | 1                      | -0.837**  | -0.945**    | 0.338            | -0.403        | -0.282          | 0.321      | 0.206               |
| D_{50} (µm)      | 1                      | 0.807**   | 0.090       | 0.099            | 0.057         | -0.169          | -0.060     |                     |
| Crystallinity (%)| 1                      | -0.304    | 0.089       | 0.258            | -0.270        | -0.124          |           |                     |
| Cellulose (%)    | 1                      | -0.404    | -0.884**    | 0.944**          | 0.821**       |                 |           |                     |
| Hemicellulose (%)| 1                      | 0.333     | -0.370      | -0.242           |               |                 |           |                     |
| Lignin (%)       | 1                      | -0.939**  | -0.882**    |                 |               |                 |           |                     |
| Glucose yield (mg/g) | 1                     |           |             | 0.886**          |               |                 |           |                     |
| Xylose yield (mg/g) | 1                     |           |             |                 |               |                 |           |                     |

Note: **indicates extremely significant correlation between parameters (P<0.01), *indicates significant correlation between parameters (P<0.05).

As shown in Table 2, the ball milling time had a negative effect on the D_{50} value and crystallinity (p<0.01). The NaOH concentration had a positive effect on the cellulose content, glucose yield and xylose yield, while it had a negative effect on the lignin content (p<0.01), which was consistent with the above analysis results. It is worth noting that there was no discernable correlation between xylose yield and hemicellulose content. It is possible that the removal of lignin increased the xylose yield with slight changes of hemicellulose content in the solid, leading to irrelevant relationship between xylose yield and hemicellulose content.

The cellulose content had a positive effect on the glucose yield (P<0.01), while there was no relationship between glucose yield with D_{50} and crystallinity. For example, the cellulose content reached maximum after 10 min ball milling in combined alkaline mechanical pretreatment, and further changes in the chemical composition with an extended ball milling time were limited. It was similar to the change trend in glucose yield, as shown in Fig. 1, which may explain the ball milling time and glucose yield showed no clear linear correlation in Table 2. The scatter plot of glucose yield and cellulose content was shown in Fig. 9, in which the relationship was described as the following linear regression equation: \( Y = 37.79 \times \text{cellulose content} - 1421.55 \) (R^2 = 0.88). Li et al. found...
that the glucose yield of different samples treated with ball milling, alkaline hydrogen peroxide and ammonia fiber expansion could be fitted with a univariate linear correlation with their cellulose content[24]. It is consistent with author's results. Also, the cellulose content of the substrate increases regardless of different pretreatments, the glucose yield will increase accordingly within a certain range[24, 28]. The lignin content had a negative effect on the glucose yield (P<0.01). The scatter plot of glucose yield and lignin content was shown in Fig. 10, in which the relationship was described as the following linear regression equations: \( Y_G = -72.47 \times \text{lignin content} + 1482.77 \) (\( R^2 = 0.95 \)). Loustau-Cazela et al. mentioned that the main reason for the enhancement of enzymatic hydrolysis was the removal of lignin and lignin-carbohydrate complexes rather than the change of cellulose crystallinity in a study of NaOH-VBM pretreatment[29]. Previously, Ishiguro et al. found a linear negative correlation between glucose yield and lignin content in obtained eucalyptus treated with hydrothermal-mechanical chemical[30]. Yang and Wyman et al. demonstrated that lignin removal facilitated the degradation of corn stover by cellulase[31]. However, Li et al. discovered an unclear correlation between glucose yield and lignin content when studying corn stover by different pretreatment, and Kumar et al. revealed that the reason why lignin hinders glucose yield may depend on its chemical properties rather than content \([22, 32]\). Combined with the previous data, it can be seen that the lignin content of WBM0-NaOH3% and WBM10-NaOH3% is basically the same (14.4% and 14.3%, respectively), but the glucose yield of the latter is higher (307.1 mg/g and 491.4 mg/g, respectively). It may be caused by changes in physical properties such as loosening of structure, reduction in particle size and crystallinity, and chemical properties such as increased cellulose content of the latter through wet ball milling treatment. The fitted equation between glucose and lignin content has some limitations, which may be caused by the wet ball milling treatment. It reflects the enhanced effect of the wet ball milling on the NaOH treatment in the combined mechanochemical pretreatment, while there are limits to this enhancement, as shown by the fact that extending the ball milling time had little effect.

**Conclusions**

A combined wet alkaline mechanical pretreatment of corn stover at room temperature was proposed in this study, which significantly enhanced the enzymatic subsequent hydrolysis. The highest glucose yield of 491.4 mg/g (SY=91.9%) was obtained after treatment with 3% NaOH and ball milling for 10 min, and the highest xylose yield of 157.91 mg/g (SY=62.1%) was obtained from corn stover treated with 2% NaOH and ball milling for 10 min. The quantitative relationships between glucose yield with NaOH concentration, ball milling time, cellulose content and lignin content were established. The component of liquid fraction after pretreatment was determined and less harmful inhibitors were generated in this pretreatment process.

**Materials And Methods**

**Corn stover and reagents**

Corn stover collected from the Shangzhuang Experimental Station in Beijing, China, was dried naturally, chopped into small pieces (3–5 cm), and coarsely milled through a 1-mm sieve using RT-34 milling machine (Hongquan Pharmaceutical Machinery Ltd., China), with the resulting milled corn stover denoted as CM. Its main components were 36.3% of cellulose, 22.6% of hemicellulose and 16.9% of lignin.

Sodium hydroxide (purity, ≥96.0%) was purchased from Beijing Chemical Plant and used without further purification.

**Mechanochemical treatments of corn stover**

CM samples and NaOH solutions of different concentrations (0, 1, 2, and 3 wt%) were thoroughly mixed in a 1:6(w/w) ratio (optimized from our pre-experiment) and were allowed to stand for a certain period \( t_1 \). An ultrafine vibration ball mill (CJM-SY-B; Qinhuangdao Taiji Ring Nano Ltd., Hebei, China) was then used to crush the above mixture for different amounts of time \( t_2 \) (0, 10, 20, and 30 min) under the optimized conditions obtained from our preliminary tests, in which ZrO\(_2\) balls and CM were mixed in a volume ratio of 2:1, with the ZrO\(_2\) balls occupying 35% of the ball mill tank volume. A circulating cooling water was passed around the tank to maintain the temperature at around 25°C during the milling process. All samples were in contact with NaOH solutions of different concentrations for 1h \((t_1+t_2=1h)\). The samples obtained were denoted as WBMx-NaOH\(y\), where \( x \) was the milling time and \( y \) was the NaOH concentration. The control treatment was as follows: CM samples were mixed thoroughly with deionized water for 1h, without ball milling.

The pH of treated samples was adjusted to neutral pH by deionized water and dilute hydrochloric acid. The liquid was collected and volume was recorded. A portion of the solid from treated corn stover was stored at 4 °C for subsequent enzymatic hydrolysis, and the remaining solid was dried in a vacuum freeze dryer to determine the lignocellulosic composition and crystallinity. The solid yield was calculated using equation (1)[33]:

\[
\text{Solid yield} (\%) = \left( \frac{m_1}{m_2} \right) \times 100\%
\]

where \( m_1 \) and \( m_2 \) are the masses (g) of dry matter after and before treatment, respectively.

**Particle size distribution measurement**

The particle size distribution of treated samples (diluted to 1 wt% of the original concentration for measurement) was obtained using a MASTERSIZER 3000 laser particle size analyzer (Malvern, UK)[34]. Particle sizes \( D_{10}, D_{50}, \) and \( D_{90}, \) representing 10%, 50%, and 90% of the accumulated volume fraction, respectively, were determined using the obtained particle size distribution curves. Each sample was measured five times.
Cellulose crystallinity (CrI) analysis

The cellulose crystallinity (CrI) of the obtained dried samples was measured using a XD3 series X-ray diffractometer (Puxi, Beijing) with Cu Ka radiation at 36 kV and 20 mA. The diffraction intensity was obtained in the 2q range of 5–40° with a step size of 0.2° at a scanning speed of 2°/min. Each sample was measured in duplicate. The CrI was calculated according to equation (2)[35]:

\[
CrI(\%) = \frac{I_{\text{max}} - I_{\text{am}}}{I_{\text{am}}} \times 100\%
\]

2 where \(I_{\text{max}}\) is the maximum intensity of the diffraction peak at approximately \(2q = 22°\), and \(I_{\text{am}}\) is the intensity of the amorphous background at approximately \(2q = 18°\).

Scanning electron microscopy (SEM) analysis

The surface morphologies of treated corn stover were observed using a Hitachi SU3500 electron microscope (Hitachi, Japan). Samples with a concentration of 0.1 wt% were dropped onto carbon tape, dried in an oven at 60 °C overnight, and sprayed with gold before subjecting to SEM observation.

Analysis of main chemical components

The cellulose, hemicellulose, and lignin contents in the solid were measured using the method of NREL-TP-510-42618[36]. Each sample was measured in duplicates.

The total sugar content (monosaccharide and oligosaccharide) of glucose, xylose and arabinose, and byproduct contents such as furfural, 5-hydroxymethylfurfural (HFM), acetic acid in liquids were measured according to NREL-TP-510-42623[37]. The acid soluble lignin content was measured according to method NREL-TP-510-42618. Each sample was measured in duplicates.

Enzymatic hydrolysis of corn stover

The enzymatic hydrolysis of pretreated corn stover was performed using cellulase enzyme of CellicCtec2 (Novozymes, Denmark). Pretreated samples (0.5 g dry basis) and citrate buffer (pH 4.8) were mixed in a 1:20 (w/v) ratio and were kept at 200 rpm and 50°C for 72 h in a shaking incubator. Tetracycline hydrochloride (0.08 g/L) was added and the enzyme loading was 20 FPU/g solid, according to the method of NREL/TP-510-42623 [38]. Each sample was measured in duplicates. The monomeric sugar concentration was measured after enzymatic hydrolysis using a HPLC (Hitachi, Japan). The glucose or xylose yield (Y) was calculated using the following equation (3):

\[
Y(\text{mg/g}) = \frac{c \times V}{m}
\]

3 where \(c\) is the monomeric sugar concentration (mg/mL) in the enzymatic hydrolysate, \(V\) is the volume of enzymatic hydrolysate (mL), and \(m\) is the amount of pretreated corn stover added into the enzymatic hydrolysis mixture (g).

The enzymatic digestibility of sugars (SY) was calculated using the following equation (4):

\[
SY(\%) = \frac{m_e}{m_{\text{max}}}
\]

4 Where \(m_e\) is the monomeric sugars weight (mg) of added solid after enzymatic hydrolysis, and \(m_{\text{max}}\) is the maximum weight (mg) of monomeric sugars in the solid.

Statistical analysis

The results of repeated experiments were expressed as means ± standard deviation. One-way analysis of variance was performed using Duncan's test at the 99% level (p < 0.01) using SPSS 20.0 software. Data fitting was performed using Origin 2018 software.

Declarations

Authors’ contributions

Jie Yang designed and performed experiments, analyzed samples, and wrote the manuscript; Chongfeng Gao joined in the design of the experiments; Xueqi Yang and Yanfu Su took part in the performance of experiments; Prof. Suan Shi and Prof. Lujia Han joined in the discussion of experimental plans and edited
the manuscript. All authors read and approved the final manuscript.

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Availability of data and materials

All data generated or analyzed during this study are included in this published article.

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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**Figures**

Figure 1

Glucose yield of corn stover treated with different pretreatments (different lowercase letters indicate significant differences, P<0.01).
Figure 2
Xylose yield of corn stover treated with different pretreatments (different lowercase letters indicate significant differences, P<0.01).

Figure 3
Glucose yield as a function of ball milling time.

Y=164.19-99.34*0.94^x R^2=0.98

Figure 4
Glucose yield as a function of NaOH concentration.
Figure 5
Glucose yield as a function of ball milling time and NaOH concentration.

Figure 6
SEM images of corn stover samples from different pretreatments.

Figure 7
Particle size distribution curve of corn stover from different pretreatments.

Figure 8
X-ray diffraction pattern of corn stover from different pretreatments.

Figure 9
Relationship of glucose yield from corn stover with cellulose content.
Figure 10

Relationship of glucose yield from corn stover with lignin content.

\[ Y_G = -72.47 \times \text{lignin content} + 1482.77 \quad R^2 = 0.95 \]