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# Ball Milling Pretreatment of Corn Stover for Enhancing the Efficiency of Enzymatic Hydrolysis

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**Abstract** Ethanol can be produced from lignocellulosic biomass with the usage of ball milling pretreatment followed by enzymatic hydrolysis and fermentation. The sugar yields from lignocellulosic feed stocks are critical parameters for ethanol production process. The research results from this paper indicated that the yields of glucose and xylose were improved by adding any of the following dilute chemical reagents: $H_2SO_4$, HCl, $HNO_3$, $CH_3COOH$, HCOOH, $H_3PO_4$, and NaOH, KOH, $Ca(OH)_2$, $NH_3{\cdot}H_2O$ in the ball milling pretreatment of corn stover. The optimal enzymatic hydrolysis efficiencies were obtained under the conditions of ball milling in the alkali medium that was due to delignification. The data also demonstrated that ball milling pretreatment was a robust process. From the microscope image of ball milling-pretreated corn stover, it could be observed that the particle size of material was decreased and the fiber structure was more loosely organized. Meanwhile, the results indicate that the treatment effect of wet milling is better than that of dry milling. The optimum parameters for the milling process were ball speed of 350 r/min, solid/liquid ratio of 1:10, raw material particle size with 0.5 mm, and number of balls of 20 (steel ball, $\Phi=10$ mm), grinding for 30 min. In comparison with water milling process, alkaline milling treatment could increase the enzymatic hydrolysis efficiency of corn stover by 110%; and through the digestion process with the combination of xylanase and cellulase mixture, the hydrolysis efficiency could increase by 160%.

**Keywords** Lignocellulosics · Ball milling · Pretreatment · Enzymatic hydrolysis · Corn stover

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## Introduction

Biomass material is the most abundant renewable resources on earth. Recently, biotechnology to produce commercially useful products such as food, fuel, chemical products, and so on, has been employed [1–3].

Corn stover growing in large quantities worldwide consists of three main components: cellulose, hemicellulose, and lignin. The complexity of these components leads to the stability of its biomass and results in the difficulty of its enzymatic hydrolysis. Pretreatment techniques to overcome this difficulty are essential for saccharification from lignocelluloses [4–6] in future biorefineries [3, 7, 8].

Mechanical disruption of cellulose is a candidate method for the preparation of highly accessible surface area cellulose without the loss of low molar mass components. It is an environmentally friendly pretreatment of cellulose biomasses. A conventional ball milling process is one of the techniques widely used to produce powdered cellulose by mechanical shear stress and impaction. This method causes a reduction in the crystallinity of the cellulose [9–11]. The degree of structural disruption depends on the conditions applied, for example, the size of the balls, rotation speed of milling, and time [12–15]. Teramoto and Inoue investigated ball milling combined with hot-compressed water treatment of eucalyptus, which can save energy for pretreatment and enzyme loading for enzymatic hydrolysis [13, 16].

We investigated dilute chemical wet milling of corn stover and compared it with the conventional methods of dry ball milling and water milling pretreatment. To develop a novel, simple technique for environmentally friendly, highly efficient pretreatment for bioethanol production, and to increase the options for combined pretreatments, the optimal conditions of ball milling as pretreatment were investigated as preparation for the enzymatic hydrolysis of corn stover using cellulase and xylanase. Pretreated samples were analyzed by SEM to observe the structure changes of pretreated materials.

## Methods

### Enzyme and Substrate Preparations

The corn stover used in this study was purchased from the Jiangsu province in China. After collection, the stover was chopped, air-dried, milled, and sized to pass through 0.5-, 1.0-, and 1.5-mm screens. It was stored in plastic Ziploc bags and kept at room temperature. The carbohydrates and lignin of the raw material were determined according to the NREL standard methods of Laboratory Analytical Procedure (LAP). The composition of the corn stover is presented in Table 1.

### Enzyme Activity Assays

The cellulase, Accellerase 1000, is provided by Genencor International Inc., the concentration of protein is 63 mg/mL, and the activities of cellulase are listed in Table 2. One unit of cellulose and xylanase activities is defined as the release of 1 μmol of either glucose

**Table 1** Composition of corn stover expressed as percent of dry matter (%).

| Glucan | Xylan | Ligin | Ash |
|---|---|---|---|
| 36.8 | 21.5 | 17.5 | 9.3 |

**Table 2** Activities of cellulase.

| Filter paper activity (FPIU/mL) | CMC activity (IU/mL) | $\beta$- Glucosidase activity (IU/mL) |
|---|---|---|
| 93 | 7.38 | 1632.9 |

or xylose equivalents per minute. Measurements of three types of enzyme activities were accomplished. Firstly, the filter paper activity of the cellulase preparations was determined according to standard methods of the NREL LAP. Secondly, the endoglucanase activity assay was performed in sodium citrate buffer (50 mmol/L, pH 4.8), which consisted of carboxymethy cellulose-Na (CMC-Na, Sigma, 1.0%, w/v). The activity was measured by assaying reducing sugars released after 30 min of enzyme reaction with 1.0% CMC-Na. Thirdly, *p*-Nitrophenyl-glucose (*p*-NPG) was a substrate for the assays of $\beta$-1,4-glucan cellobiohydrolase activity, and one unit of activity was defined as 1 μmol of *p*-nitrophenol released per minute. The released *p*-nitrophenol was measured at 400 nm with a spectrophotometer after adding $Na_2CO_3$ to a final concentration of 60 mmol/L. All of the experiments were performed in triplicate. Xylanase (1,4-$\beta$-D-xylanase) activity was determined under similar conditions as described above, except that 1% xylan solution (birch wood xylan, Sigma) was used as the substrate [17]. Xylanase (964 IU/mL) is provided by the Zesheng Company (China).

## Enzymatic Hydrolysis

The solid concentration was 6%. Enzyme loading concentrations were: cellulases, 15 FPU/g glucan or 10.2 mg cellulase/g glucan; xylanase 200 IU/g dry biomass. Samples were hydrolyzed in shake flasks with 50 mmol/L sodium citrate buffer, pH 4.8, with agitation of 130 r/min, which were placed in a laboratory shaking incubator at 50°C.

## Sugar Measurement

Glucose and xylose in the liquid were analyzed by HPLC (Summit P680 HPLC, Dionex; Shodex RI-101 Refractive Index Detector; Aminex HPX-87H Ion Exclusion Column 300 mm×7.8 mm, Bio-Rad). The mobile phase was 5 mmol/L $H_2SO_4$ at a flow rate of 0.6 mL/min, and column temperature used for HPLC analysis was 85°C [1].

## Screening of Ball Milling Parameters

The corn stover samples were milled with a planetary ball mill (XQM, Chishun China), as previously described [18]. The 3.00 g of each mass sample was milled in 60.0 mL steel milling-cup with stainless 10-mm-diameter steel ball (slurry volume 40 mL). The main factors important to ball milling are rotational speed, ratio of solid to liquid, particle size of raw materials, and the number of balls per volume. The optimum process parameters were evaluated based on the sugar content in the hydrolysis. The design factors were presented in Table 3, with application of the Minitab 15.1 software for statistical analysis.

## Chemical Augmentation of Milling

The optimal chemical additive to the milling process was selected with a concentration of 1%, with the use of the following: $H_2SO_4$, HCl, $HNO_3$, $CH_3COOH$, HCOOH, $H_3PO_4$, and

**Table 3** Factors and levels of orthogonal experiment design.

| Factors | A | B | C | D |
|---|---|---|---|---|
| Name | Solid/liquid ratio | Speed (rpm) | Particle size (mm) | Balls |
| Level 1 | 1: 20 | 250 | 0.5 | 10 |
| Level 2 | 1: 15 | 300 | 1.0 | 15 |
| Level 3 | 1: 10 | 350 | 1.5 | 20 |

alkali, NaOH, KOH, $Ca(OH)_2$, $NH_3{\cdot}H_2O$. The results of adding each of these reagents to the milling process are shown in Fig. 2.

### Morphology Observation

The morphology of corn stover cellulose powder of different milling treatment was observed under a Hitachi S-3400 scanning electron microscope imaging system. The samples were selected as unmilled, milled in water, and milled in 1% NaOH.

### Definitions of Yields for Glucose and Xylose

The final glucose and xylose yields are defined as follows:

$$\text{Glucose yield}\,(\%) = \frac{[\text{Glucose}]}{1.111f[\text{Biomass}]} \times 100\%$$

$$\text{Xylose yield}\,(\%) = \frac{[\text{Xylose}]}{1.136f[\text{Biomass}]} \times 100\%$$

[Glucose]: glucose concentration (g/L)
[Xylose]: xylose concentration (g/L)
[Biomass]: dry biomass concentration at the beginning of the hydrolysis (g/L)
*f*: cellulose or hemicellulose fraction in dry biomass (g/g)

## Results and Discussion

### Screening of Ball Milling Parameters

The experiments are designed to optimize the main factors in the ball milling process. Crushing of raw materials increases the accessibility of cellulose surface area, so the hydrolysis rate increases (Table 4). In the theory of ball mill [19], water results in softening the cellulose, which binds the microfibrils; it still needs some shear forces in order to break the bonds. Size reduction is a process in which particles are broken into smaller pieces. Generally, there are three processes involved: (1) cutting, (2) abrasion, (3) crushing.

When the rotational speed is slow, the resulting centrifugal force is insufficient to effectively crush the stover fragment. With the increase of speed, the crushing speed increases. When the rotation speed is too high, centrifugal force keeps the balls in place which minimizes crushing and grinding. When the speed reaches a certain value, the speed of the

**Table 4** Results of orthogonal experiment design.

| No. | A | B | C | D | Glucose yield (%) |
|---|---|---|---|---|---|
| 1 | 0.050 | 250 | 0.5 | 10 | 29.76 |
| 2 | 0.050 | 300 | 1 | 15 | 37.68 |
| 3 | 0.050 | 350 | 1.5 | 20 | 43.72 |
| 4 | 0.067 | 250 | 1 | 20 | 59.68 |
| 5 | 0.067 | 300 | 1.5 | 10 | 56.24 |
| 6 | 0.067 | 350 | 0.5 | 15 | 61.92 |
| 7 | 0.100 | 250 | 1.5 | 15 | 71.12 |
| 8 | 0.100 | 300 | 0.5 | 20 | 87.68 |
| 9 | 0.100 | 350 | 1 | 10 | 78.24 |
| Delta | 5.368 | 0.878 | 0.465 | 0.995 | |
| Order | 1 | 3 | 4 | 2 | |

crushing effects will reach the largest, but beyond the set value cannot increase the crushing effects. Thus, it can be seen that the speed plays an important role in the grinding mill.

The ratio of material to liquid in the milling process is one of the key factors. If there is too much moisture, the ball mill slurry will be too thin and reduce the effective use of the energy required to turn the mill, less water will lead to a high viscous slurry and restrict ball movement which would reduce the grinding crushing and abrasion of materials.

Hydrolysis rate is decreased with the increase of particle size because smaller particles have a greater surface area to volume ratio. Large particles size of raw materials need greater energy and shear stress to crush them.

From Fig. 1, it could be observed that the effects of factors on the orthogonal design were speed, solid/liquid ratio, and number of balls, which increased with the parameter

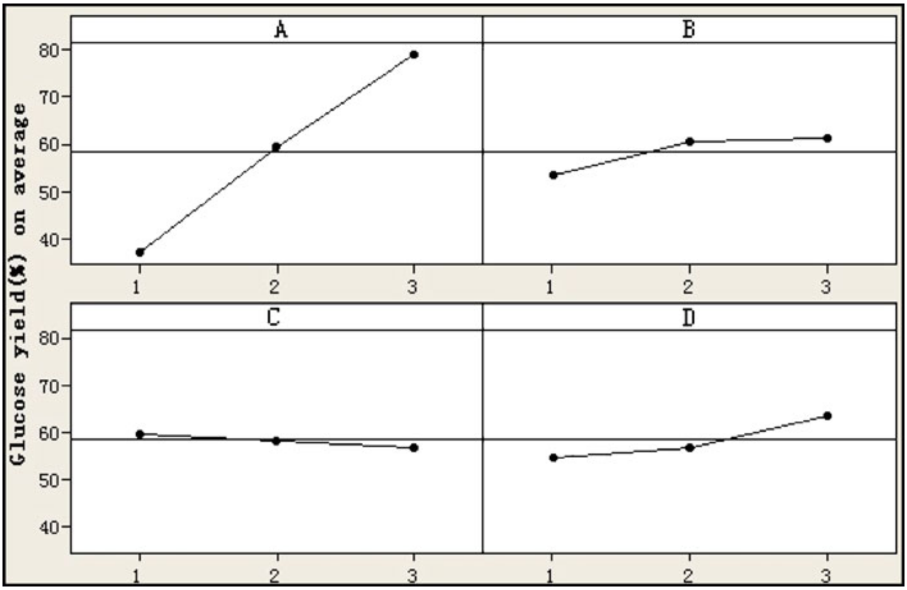


**Fig. 1** Analysis of orthogonal experiment factors

values. After crushing of raw materials, special surface area increased, so that the hydrolysis rate increased. The particle size of raw material exhibited the negative correlation effect, with increasing particle size of raw materials, after smashing decreased the rate of hydrolysis. The reason is that with large particle size of raw materials, the milling process needs a greater energy and shear stress to crush them, thereby reducing the crushing effect.

Solid/liquid ratio indicated the greatest effect. The number of balls, rotation speed, and particle size had only a minor effect on the recovery of sugar. The optimal milling conditions were selected as follows: solid/liquid ratio of 1:10, ball speed of 350 r/min, raw material particle size with 0.5 mm, adding the amount of grinding media and number of balls of 20 (steel ball, $\Phi$=10 mm), 30 min after grinding.

## Screening of Ball Milling Condition

According to the results in Fig. 2, the hydrolysis results of corn stover with milling in dilute alkali medium were better than that of in dilute acid medium. The overall glucose and xylose yields increased significantly due to the delignification effect of alkali chemicals. Alkali pretreatment technologies are rather similar to the Kraft paper pulping technology. The major effect of the alkaline pretreatment is the removal of lignin from the biomass, which can improve the reactivity of the remaining polysaccharides [20]. On the basis of the analyses of ball milling corn stover, it was shown that about 38% of initial lignin were solubilized with alkaline treatment; thus, the enzyme digestibilities of pretreated corn stover were enhanced.

Enzymatic hydrolysis was carried out to evaluate the pretreatment conditions. Figure 3 showed the results of enzymatic hydrolysis under the conditions of untreated raw materials, dry milling, water ball milling, ball milling with alkali and alkali-milling cellulose hydrolysis processing with addition of xylanase. It could be observed that dry milling and unmilling pretreatment resulted in lower amounts of sugars with the enzymatic hydrolysis of the solid fraction, while the dilute alkali milling-pretreated materials exhibited the best

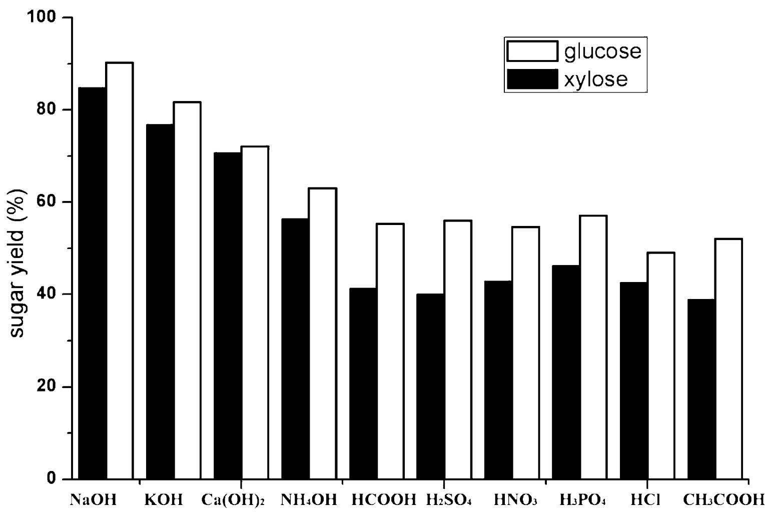


**Fig. 2** Results of hydrolysis with diluted acid and alkali ball milling

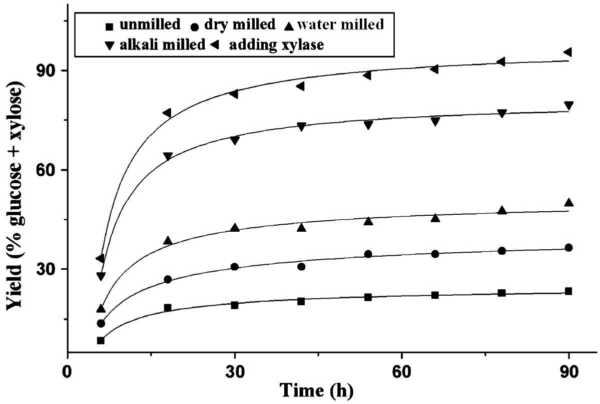


**Fig. 3** Results of corn stover hydrolysis under different milling conditions

enzymatic hydrolysis efficiency. In comparison with water milling process, alkaline milling treatment could increase the enzymatic hydrolysis efficiency of corn stover by 110%; and through the digestion process with the combination of xylanase and cellulase mixture, the hydrolysis efficiency could increase by 160%.

Morphology Observation

The structure of corn stover cellulose after mechanical treatment by ball mill could be analyzed by microscopic observation. Figure 4a showed that the cellulose fiber bundles were tight together and well organized. From Fig. 4b, the fiber bundles became looser and less organized after milling in water medium, and from Fig. 4c, we could get an intuitive view of the disruption of cellulose fiber bundles milled with NaOH medium (the fiber bundles were broken totally). The NaOH solution could cause cellulose swelling and lignin removal, so the loose structure of fiber bundle could provide more surface binding for the hydrolysis of cellulose.

## Conclusions

Ball milling pretreatment combined with the addition of dilute acid and alkali is an effective processing to enhance the enzymatic hydrolysis efficiency of corn stover. As observed from electron microscope analyses, the structure of cellulose fiber bundles was destroyed totally after ball milling treatment that increased the accessibility of cellulose. The results indicated that the treatment effect of wet milling is better than that of dry milling. The optimum parameters for the milling process were ball speed of 350 r/min, solid/liquid ratio of 1:10, raw material particle size with 0.5 mm, and number of balls of 20 (steel ball, $\Phi$=10 mm), grinding 30 min. In comparison with water milling process, alkaline milling treatment could increase the enzymatic hydrolysis efficiency of corn stover by 110%; and through the digestion process with the combination of xylanase and cellulase mixture, the hydrolysis efficiency could increase by 160%.

Indeed, ball milling pretreatment is a high-energy-consumption processing, though wet mill mode can save energy to some extent. However, ball milling is a definitely effective process for reducing particle size and disrupting the structure of cellulose fiber bundles, and thus reduces the crystallinity of the cellulose to enhance enzymatic efficiency, and it can be

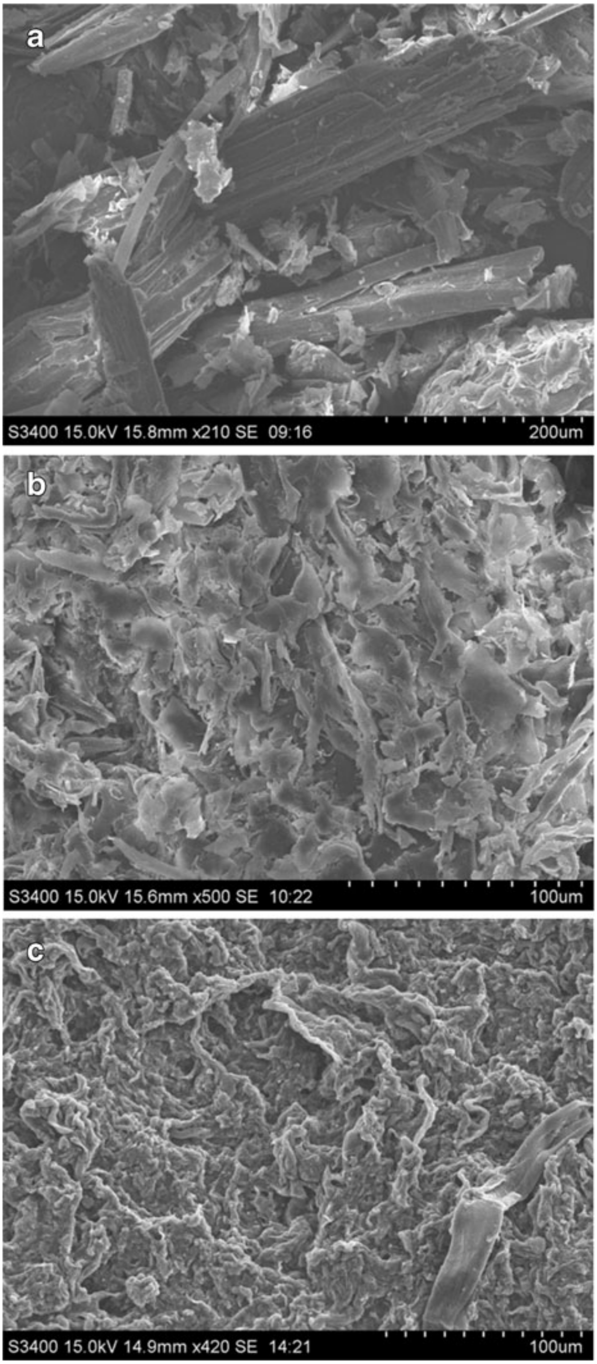


**Fig. 4** Microscope photos of enlarged corn stover cellulose: (**a**) corn stover cellulose unmilled, (**b**) corn stover cellulose milled in water, and (**c**) corn stover cellulose milled in NaOH

carried out at ambient conditions with less fermentation inhibitors formed during the processing. Therefore, the ball milling technology is a useful approach for lignocellulosic biomass pretreatment, and the operation parameters are easy to enlarge to an industrialized scale.

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