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Biological Pretreatment of Corn Stover by *Irpex lacteus* for Enzymatic Hydrolysis

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The feasibility of biological pretreatment for subsequent saccharification largely depends upon an effective pretreatment system. A significant enhancement of saccharification was discovered with corn stover pretreated by white rot fungus *Irpex lacteus* CD2. The highest saccharification ratio reached 66.4%, which was significantly higher than what was reported. Hemicellulose was first destroyed in the process and then lignin. Lignin and hemicellulose were selectively degraded over cellulose, respectively, resulting in increased crystallinity. Enhanced saccharification and the fluctuation in crystallinity together indicated the destruction of the cellulose crystalline structure. Additionally, further studies revealed the disruption of the cell wall and the vital increase of large pores in the pretreated samples, which might be caused by the selective degradation of amorphous components and fungal penetration. Results suggest that *I. lacteus* has a more efficient degradation system than other reported white rot fungi and can be further explored as an alternative to the existing thermochemical processes.

KEYWORDS: *Irpex lacteus*; lignin; hemicellulose; XRD; SEM; pore size distribution

INTRODUCTION

Enzymatic hydrolysis of lignocellulosic materials to sugars that can be fermented into ethanol and other fuels and chemicals has been extensively studied. Similarly, various attempts have been made to improve the process efficiency (*1*, *2*). It is acknowledged that the characteristics of lignocellulosic substrates play a critical role in efficient enzymatic hydrolysis (*3*). Natural lignocellulosic biomass is highly recalcitrant to enzymatic hydrolysis because of its inaccessibility to cellulolytic enzymes (*3*, *4*). Pretreatment is the procedure to reduce the recalcitrance by breaking down the complex structure of the lignin and hemicellulose in the cell wall of the biomass, so that the enzyme can attach to the cellulose biopolymer. Among various technologies, biological pretreatment is desirable because it requires relatively low energy and mild environmental conditions (*1*). The progressive effect of biological pretreatment on enzymatic hydrolysis has been demonstrated (*2*, *5*−*7*). Several white rot fungi, such as *Phanerochaete chrysosporium*, *Pleurotus ostreatus*, *Ceriporiopsis subvermispora*, and *Coriolus versicolor* had been used for biological pretreatment of biomass; however, only low saccharification ratios (35−40%) were obtained when compared to the saccharification ratios by physical and chemical pretreatments (*5*−*7*). Unfortunately, the saccharification ratio of biological pretreatment is too low for industrial use (*8*). Moreover, some important components of biomass are consumed through the degradation of hemicellulose and cellulose by the microorganisms. The promising fungus, *Irpex lacteus*, well-known as an eco-friendly agent in textile industry wastewaters and 2,4,6-trinitrotoluene (TNT) biodegradation (*9*, *10*), has the potential of promoting enzymatic hydrolysis but is rarely studied for use in the biological pretreatment of biomass.

In this work, the effect of biological pretreatment using *I. lacteus* CD2 on enzymatic hydrolysis along pretreatment time was first evaluated over a period of 150 days. Subsequently, the composition and porosity of all of the corn stover samples were determined. Fourier transform infrared (FTIR) spectroscopy, X-ray diffraction (XRD) analysis, and scanning electron microscopy (SEM) observation were conducted to investigate the key factors that led to the enhanced enzymatic hydrolysis. The overall experimental flowchart is summarized in **Figure 1**.

MATERIALS AND METHODS

Microorganism. The white rot fungus *I. lacteus* CD2 was collected from the Shennongjia Nature Reserve of Hubei Province, People's Republic of China. It was identified by rDNA internal transcribed spacer (ITS) sequence analysis, and its GenBank accession number was FJ744594. It was maintained on potato dextrose agar (PDA) slants at 4 °C and was cultured for 1 week before use.

Cellulase and Raw Material. Enzyme preparation from *Trichoderma reesei* was provided by Henan Tianguan Enterprise Group Co., Ltd. Corn stover was obtained from Enshi City, Hubei Province in central China. The material (lignin, 20.9%; cellulose, 36.7%; hemicellulose, 26.7%; ash, 3.1%; and ethanol/toluene extractive, 3.1%) was air-dried, ground to 0.425−0.85 mm in size, and subjected to treatment with *I. lacteus* CD2.

Biological Pretreatment. Prior to fungal inoculation, 15 g of air-dried corn stover with 30 mL of distilled water was sterilized in the autoclave for 20 min at 121 °C. After *I. lacteus* CD2 was incubated in potato dextrose

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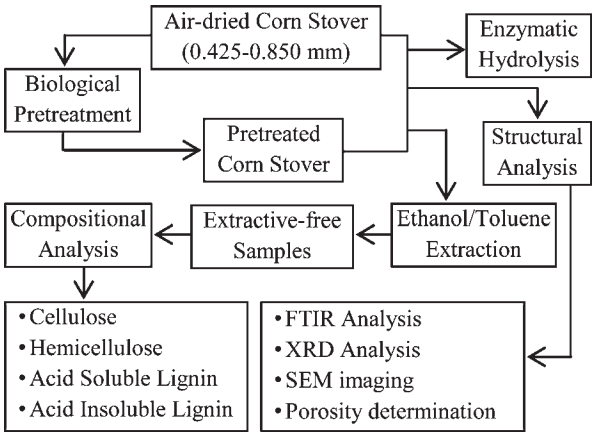

Figure 1. Flowchart of the overall experiment and analyses.

broth (PDB) medium at 28 °C and 150 rpm for 7 days, the inoculums were washed, homogenized, and resuspended with distilled water. A total of 15 mL of mycelium suspension was inoculated to the 15 g of sterilized corn stover. Biological pretreatment of corn stover was carried out statically at 28 °C and atmospheric pressure for varying durations in 250 mL Erlenmeyer flasks. After pretreatment, the mycelium was removed from the biomass as completely as possible and all of the samples were oven-dried at 105 °C and weighed. The corn stover treated in the same condition without fungal inoculation was taken as the control sample. Weight loss was estimated as the difference between the weight of the corn stover at the beginning and at the end of the pretreatment according to the following formula: weight loss (%) = $(W_1 - W_2)/W_1 \times 100$, where W_1 represented the weight of the control sample and W_2 represented the weight of samples after pretreatment. All experiments were performed in triplicate. The mean values are reported subsequently.

Enzymatic Hydrolysis. It was commonly observed that, with an excess of cellulose substrate, the relation of reducing sugar products versus the cellulase concentration followed a hyperbolic curve (*11*). Our preliminary study demonstrated that enzyme loading higher than 20 FPU/g had a little benefit on enzymatic conversion. Therefore, the enzyme loading of 20 FPU/g of sample was taken as the proper concentration for enzymatic hydrolysis. Enzymatic hydrolysis was conducted in 50 mM acetic acid−sodium acetate buffer (pH 4.8) in 10 mL test tubes maintained at 48 °C on a rotary shaker at 200 rpm, with the solids concentration of all samples being 20 g/L. After enzymatic hydrolysis for 48 h, the hydrolyzed corn stover was filtered and the filtrate was used to determine reducing sugars according to the dinitrosalicyclic (DNS) method (*12*). All experiments were performed in triplicate. Upon evaluation of the effects of pretreatment, the saccharification ratio was defined as the percentage of holocellulose (cellulose and hemicellulose) in the raw material converted to reducing sugars, taking into account the weight lost during pretreatment.

Analysis Methods. *Chemical Component Analysis*. Cellulose and hemicellulose contents of corn stover were determined according to the procedures described by Goering and Van Soest (*13*). Insoluble fiber fractions, neutral detergent fiber (NDF) and acid detergent fiber (ADF), were measured in neutral and acid detergent, respectively. The cellulose content was the portion of ADF that was hydrolyzed by 72% (v/v) sulfuric acid. The hemicellulose content was analyzed as the difference between NDF and ADF. Acid-insoluble lignin (AIL) and acid-soluble lignin (ASL) were determined with a standardized method from the National Renewable Energy Laboratory (NREL) (*14*). The average degradation rate of the components was calculated for a given stage over the corresponding time period. Lignocellulose degradation ratios were calculated on the basis of the total lignocellulose weight loss after pretreatment.

FTIR Analysis. FTIR spectra of all of the samples were measured by direct transmittance using the KBr pellet technique via a VERTEX 70 FTIR spectrometer. All of the spectra were measured at a resolution of 4 cm^{-1}. A total of 100 scans per sample were taken. The peak area was measured using OMNIC software (*15*). Bands were assigned to structural components in the results.

XRD Analysis. The crystallinity measurement of corn stover samples (< 100 μm) was carried out using X'Pert PRO X-ray diffractometry. All

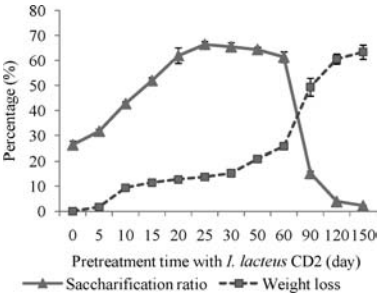

Figure 2. Saccharification ratio (▲) and weight loss (■) of the control (0 day) and pretreated corn stover with *I. lacteus* CD2 for varying durations.

samples were scanned from 2θ = 10° to 50°, with a step size of 0.017°, at 40 kV and 40 mA. The crystallinity index (CrI) was defined as follows:

$$\text{CrI}\ (\%) = (I_{002} - I_{\text{am}})/I_{002} \times 100$$

where I_{002} was the crystalline peak of the maximum intensity at 2θ between 22° and 23° and I_{am} was the minimum intensity at 2θ between 18° and 19° (*16*).

Porosity Determination. The specific surface area and pore volume of corn stover samples were estimated by the Brunauer−Emmett−Teller (BET) method with nitrogen gas adsorption at 77 K (Micromeritics ASAP2100). The analysis condition was as follows: sample mass, 0.3 g; the equilibration interval, 10 s; warm free space, 8.9521 cm^3; and cold free space, 21.8007 cm^3. The total volume was estimated on the basis of the nitrogen gas volume adsorbed at a relative pressure of 0.99.

SEM Imaging. To analyze the microstructural changes and surface characteristics of control and pretreated corn stover samples, prior to imaging with a Sirion 200 scanning electron microscope, SEM was conducted by attaching the samples on a specimen stub using double-coated tape and sputter coating them with AuPd in the presence of argon gas. The accelerating voltage was 20 kV.

RESULTS AND DISCUSSION

Effect of Pretreatment Duration on the Weight Loss and Saccharification Ratio. Figure 2 shows the effect of biological pretreatment duration on the weight loss and saccharification ratio of corn stover. It can be easily seen that the weight loss of corn stover increased with an increasing pretreatment time from 5 to 150 days. The weight loss during the duration shorter than 60 days increased slowly, with the value of the 60 day pretreated sample being 25.7%. However, the weight loss increased sharply after 60 days, and it reached 63.3% after biological pretreatment for 150 days, indicating that the fungus consumed more than half of the biomass. As previously stated, the saccharification ratio after enzymatic hydrolysis was used to evaluate the effect of pretreatment by taking the weight loss into consideration. The changes on the saccharification ratio along pretreatment duration could be divided into three stages. In the first stage, the enhancement of hydrolysis occurred rapidly and the saccharification ratio reached 66.4% after pretreatment for 25 days, taking the weight loss caused by the fungus into consideration. During the second stage, there was a slow decrease in the saccharification ratio. The third stage showed a steep decrease in the saccharification ratio, which might be caused by the long time culture of the fungus. The results demonstrated that the biological pretreatment of corn stover for 5−60 days did improve enzymatic hydrolysis; however, prolonged duration of the treatment had the adverse effect.

Up to now, the pretreatment of biomass with white rot fungi has been reported on rice straw, wheat straw, and bamboo residues. Generally, a saccharification ratio of 35−40% can be expected after pretreatment with white rot fungi (*6*, *7*), which is far from satisfying requirements of fuel and chemical production from lignocellulosic biomass. In this study, a much higher

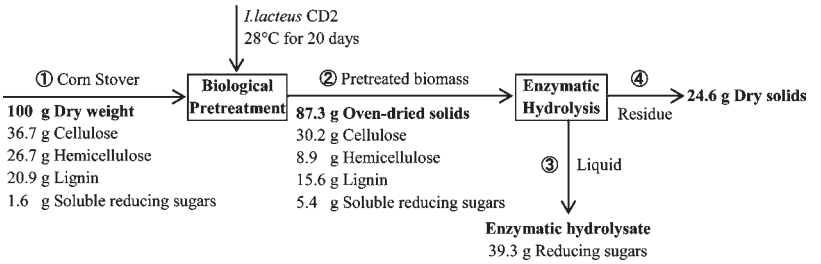

Figure 3. Mass balance flow diagram for pretreatment and enzymatic hydrolysis (the sample pretreated by *I. lacteus* CD2 for 20 days).

saccharification ratio of 66.4% was obtained after pretreatment with the white rot fungus *I. lacteus* CD2, which was about 152.5% higher than the control. Therefore, *I. lacteus* CD2 was a more efficient white rot fungus for biological pretreatment of corn stover compared to other white rot fungi. This is a significant improvement for the biological pretreatment of biomass, because the low saccharification ratio of the biological process has restricted its industrial application (*8*).

To be comparable, mass balance information during the pretreatment and enzymatic hydrolysis is provided. **Figure 3** presents the mass balance for biological pretreatment (20 days) and enzymatic hydrolysis based on 100 g of dry corn stover. After biological pretreatment, 6.5 g of cellulose and 17.8 g of hemicellulose were removed before enzymatic hydrolysis, with 3.8 g of soluble reducing sugars accumulated in the biomass. Further saccharification released 39.3 g of reducing sugars.

Component Contents and Degradation. The sequence of polymer degradation suggested that, for the amorphous components, hemicellulose degradation occurred first, followed by lignin degradation (**Figure 4**). Here, a significant difference in the composition content between the control value and that of pretreated samples was determined with a two-sided t test, and a p value of < 0.05 was indicative of statistical significance.

Significant decreases in the hemicellulose content could be seen at the very early stages of pretreatment, and by 20 days, only approximately 10% of hemicellulose remained. Little decrease in the cellulose content occurred during the first 30 days ($p < 0.05$), while the cellulose contents of the 40−60 day pretreated samples were almost the same as that of the untreated material ($p > 0.05$), suggesting that biological pretreatment did not cause a distinct change in the cellulose content in the first 60 days. However, the cellulose content decreased remarkably after 90−150 days. The AIL content increased slightly after 5 days because of the quick degradation of hemicellulose and then gradually decreased to 10.8% at 30 days of pretreatment. The ASL content increased gradually from 1.6 to 4.6% with increasing pretreatment time until 150 days (**Figure 4a**). ASL, with less carbon content than AIL, is a small fraction of lignin, and its structure remains largely unknown (*17*, *18*). It is probably composed of two components: lignin degradation products, such as low-molecular-weight lignin, and secondarily formed hydrophilic materials (*17*). An important relation has been found between ASL and syringyl lignin by comparing different materials with varied methoxyl group content (*17*, *18*). Therefore, the increase of the ASL content might reveal the lignin modification during biological pretreatment.

Figure 4b demonstrated how the degradation of corn stover components proceeded from the beginning of pretreatment to 150 days. The course of biodegradation was divided into five stages according to the different characteristics of component degradation. On the basis of these observations, it is apparent that, during the early stage (0−5 days), CD2 preferentially degraded the hemicellulose over cellulose and lignin and the average degradation rate was 7.4%/day. No lignin degradation was measured during

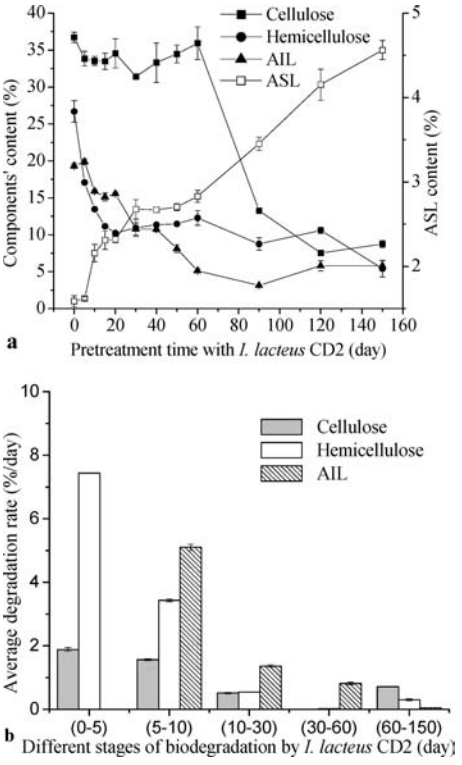

Figure 4. Component degradation by *I. lacteus* CD2 for varying durations. (a) Cellulose (■), hemicellulose (●), AIL (▲), and ASL (□) contents of the control and pretreated samples. (b) Average degradation rate of components at different stages of biodegradation. The gray, blank, and diagonal bars represent average degradation rates of cellulose, hemicellulose, and AIL, respectively.

this stage. During the second stage (5−10 days), *I. lacteus* CD2 selectively degraded lignin and hemicellulose at the average degradation rate of 5.1 and 3.4%/day, respectively. During the following two stages, selective degradation of lignin and hemicellulose proceeded with lower speed. At the last stage, *I. lacteus* CD2 selectively degraded cellulose over hemicellulose and lignin. In summary, *I. lacteus* CD2 selectively degraded lignin during the pretreatment duration of 5−60 days, hemicellulose during the pretreatment duration of 0−10 days, and cellulose during the pretreatment duration of 60−150 days. The AIL decrease and ASL increase jointly suggest the degradation and modification of lignin.

Lignin has been demonstrated to be largely responsible for recalcitrance to enzyme hydrolysis, as reported in the literature (*19*, *20*). The removal and modification of lignin (*20*, *21*) undoubtedly impart substantial modifications to the structure and accessibility of the lignocellulosic substrate. It is well-recognized that lignin content and distribution have an important impact on enzymatic hydrolysis, via two distinct mechanisms: (1) forming a physical barrier that prevents attack by cellulases and (2) binding cellulases nonproductively (*19*, *20*). The degradation

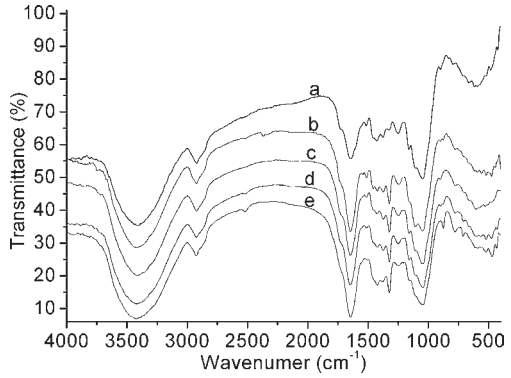

Figure 5. FTIR spectra of corn stover samples with differing degrees of pretreatment with *I. lacteus* CD2: (a) control and pretreated samples for (b) 5 days, (c) 20 days, (d) 30 days, and (e) 60 days.

Table 1. Intensity of the Lignin-Associated Band with Carbohydrate Bands for Corn Stover Samples

pretreatment time (day)	I_{1515}/I_{898}	I_{1515}/I_{1161}	I_{1515}/I_{1324}	I_{1515}/I_{1377}	I_{1515}/I_{2921}
0	0.700	0.389	0.900	0.984	0.063
5	1.420	0.930	0.235	1.112	0.059
20	0.973	0.326	0.124	0.411	0.033
30	0.336	0.217	0.085	0.327	0.023
60	0.074	0.162	0.029	0.198	0.008

and modification of pretreated lignin by *I. lacteus* CD2 revealed the deconstruction of the physical barrier and the decrease of nonspecific adsorption of cellulase to lignin, which consequently enhanced enzymatic hydrolysis.

On the other hand, within the plant cell wall architecture, hemicellulose is thought to coat cellulose fibrils, also resulting in reduced accessibility, and is considered to be connected with lignin by covalent bonds and with cellulose by hydrogen bonding (*21*). In comparison to studies on the lignin content, the effect of the hemicellulose content on enzymatic hydrolysis of lignocellulose has been considered less frequently because of its sensitivity to pretreatment conditions (*3*). The positive effect of hemicellulose removal on cellulose conversion has been addressed by adding xyloglucanases into cellulase (*22*) or chemical pretreatment (*23*). During the early stage within 5 days of pretreatment with *I. lacteus* CD2, enhanced saccharification and selective degradation of hemicellulose, other than lignin, were detected. The result suggested that hemicellulose degradation did enhance enzymatic hydrolysis when pretreated with *I. lacteus* CD2. Thus, the cleavage of hemicellulose might also play a key role in recalcitrance reduction by *I. lacteus* CD2.

Moreover, not all of the soluble reducing sugars generated during pretreatment were used by *I. lacteus* CD2 or converted to CO_2 and H_2O (*24*). The retained soluble reducing sugars in the residue, mostly generated from hemicellulose, might also contribute to a high saccharification ratio. Hemicellulose, consisting of heterogeneous and branched polymers of pentoses, hexoses, and acetylated sugars (*25*), was also extensively studied as a potential source of valuable products (*26*). Therefore, the soluble sugar accumulation caused by hemicellulose degradation also brought about the efficient biological process. Hemicellulose degradation indeed contributes significantly to the promotion of enzymatic hydrolysis during the early stage of pretreatment with *I. lacteus* CD2.

FTIR Analysis. FTIR analysis was adopted to qualitatively observe the changes of functional groups and further evaluate component modification. Significant changes in FTIR spectra

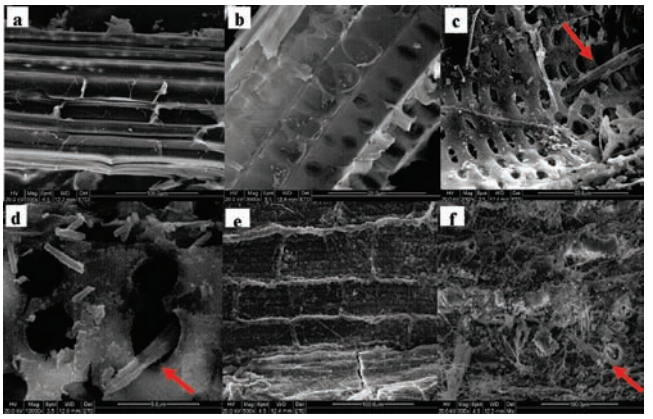

Figure 6. SEM micrographs of control and pretreated corn stover samples with *I. lacteus* CD2: (a and b) control sample, 500×; (c) pretreated for 10 days, 10000×; (d) pretreated for 20 days, 10000×; (e) pretreated for 30 days, 500×; and (f) pretreated for 60 days, 500×.

Table 2. CrI Values of Corn Stover Samples with Differing Degrees of Pretreatment with *I. lacteus* CD2

pretreatment time (day)	0	5	10	15	20	25	30	50	60
CrI (%)	63.7	67.3	69.5	71.1	69.7	67.1	69.3	72.5	71.5

could be seen in the early stage of pretreatment. The peak at 2921 cm^{-1} was attributed to the aliphatic C−H stretching vibration (*15*). The lignin-associated peaks at 1515 and 1324 cm^{-1} were assigned to aromatic ring stretch vibrations and syringyl ring breathing in lignin, respectively (*27*). The four carbohydrate-related peaks at 1729, 1377, 1161, and 898 cm^{-1} were assigned to the C=O unconjugated bond for the acetyl and uronic ester groups in xylan or the ester linkage of the carboxylic group of ferulic acid, C−H deformation in carbohydrate, C−O−C vibration in cellulose and hemicellulose, and C−H deformation in cellulose, respectively (*15*). As shown in **Figure 5**, the intensities of carbohydrate bands decreased rapidly with the pretreatment time. The reduced intensity of C=O stretching in hemicellulose indicated cleavage of lignin side chains (*28*).

The intensities of the lignin bands at 1515 cm^{-1} increased slightly in the initial 5 days because of the degradation of carbohydrates and then quickly decreased in later stages of pretreatment. The peaks at 1515 cm^{-1} were almost absent after 60 days of pretreatment. The only "pure" band at 1515 cm^{-1} was related to the aromatic moieties in lignin and was used as a reference band. The relative intensity of aromatic skeletal vibration (I_{1515}) against typical bands for carbohydrates is presented in **Table 1**. Values of I_{1515}/I_{898}, I_{1515}/I_{1161}, and I_{1515}/I_{1377} increased at the early stages of pretreatment and then decreased quickly. Values of I_{1515}/I_{1324} and I_{1515}/I_{2921} kept decreasing during pretreatment. These changes also confirmed the preference for hemicellulose degradation at the beginning of pretreatment and the selective degradation of lignin with pretreatment time, which coincides with the results based on the component contents and average degradation rate.

XRD Analysis. Lignocellulosic biomass is mainly composed of cellulose (amorphous and crystalline), hemicellulose, and lignin, with cellulose protectively surrounded by lignin and hemicellulose (*1*, *3*, *4*). Among several effecting factors, crystallinity is believed to significantly affect enzymatic saccharification of cellulose (*29*). However, because of the impossibility of completely separating cellulose from other components of the fibers, the direct measurements of cellulose crystallinity in biomass are

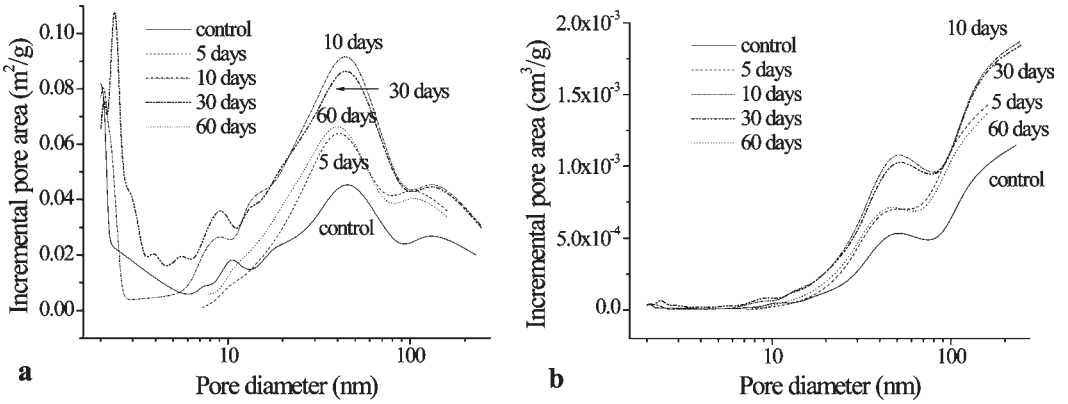

Figure 7. Distribution profiles of the control and pretreated corn stover with *I. lacteus* CD2: (a) specific area distribution and (b) pore volume distribution.

hindered (*30*). Anyway, X-ray measurement of CrI is still the best option to estimate the impacts of pretreatment on biomass crystallinity (*31*). In the present study, the CrI values of corn stover samples were determined by measuring the relative amount of crystalline cellulose in the total solid. The CrI values of different samples were calculated from corresponding XRD patterns, and the result was shown in **Table 2**.

Of the three main components, the amorphous part includes not only amorphous cellulose but also hemicellulose and lignin (*30*, *32*). The X-ray methods measure the crystallinity of the entire material, including the hemicellulose and lignin, in addition to amorphous cellulose (*32*, *33*). Therefore, including the crystallinity of cellulose itself, the CrI is also influenced by the composition of the biomass. As seen from **Table 2**, the CrI values slightly increased after biological pretreatment and there was a little decrease on the 25th day. Increased CrI might be caused by the degradation and modification of amorphous components (*4*), hemicellulose and lignin, as reported in previous studies (*34*, *35*). Therefore, the CrI increase in pretreated samples suggested that cellulose became more exposed after pretreatment. In comparison of **Figures 2** and **4** to **Table 2**, it can be seen that the samples pretreated for 25 days had a higher saccharification ratio but a lower CrI value and more comprehensive degradation of lignin and hemicellulose than that pretreated for other durations. The decrease of the CrI value of the 25 day pretreated sample firmly convinced that the crystalline structure of cellulose was destructed in the pretreated corn stover. Additionally, the evidently increased cellobiohydrolase (CBH) activity during this period in the previous study (*24*) also demonstrated the depolymerization of crystalline cellulose. Therefore, the enhanced enzymatic hydrolysis in pretreated corn stover by *I. lacteus* CD2 was associated with not only the removal of amorphous components but also the destruction of the crystalline structure.

Microstructrual Analysis. During sampling, it was found that pretreated samples were much softer and easier to be ground than the raw material, which revealed the apparent changes on physical characteristics after pretreatment. **Figure 6** showed changes in surface characteristics of the samples. As seen, the dissected control sample showed a smooth and compact section (panels **a** and **b** of **Figure 6**). After pretreatment, the tissue surface and cell wall were altered (panels **c**−**f** of **Figure 6**). Fungal growth and cell wall disruption caused the deposition of unknown materials on the surface of pretreated samples. Cracks and larger holes were also seen in the pretreated samples, leaving the inner parts of the cell wall exposed. In addition, hyphal penetration can also be seen in the pretreated corn stover (arrows in panels **c** and **d** of **Figure 6**). It was reported that fungal penetration of the biomass cell wall by hyphal sheath facilitated the decay process and eventually resulted in the expansion of xylem defense in softwood (*36*). After pretreatment for a long duration, it was observed that the residual hair, which might have come from the tissue of the leaf or bract in the corn stover, was also removed from the tissue (arrow in **Figure 6f**). Clearly, the SEM analysis results demonstrated that the biological pretreatment could cause severe degradation and evident damage to the intact cell structure, thus resulting in enhanced digestibility of pretreated corn stover.

The surface area and pore distribution are generally considered as important roles in the accessibility and adsorption of enzymes. The pore size distribution of the control and pretreated corn stover samples was shown in **Figure 7**. The result indicated that, although there was a similar distribution curve compared to the control sample, the pretreated samples had a totally different pore size distribution. The biological pretreatment enhanced the pore volume and pore size remarkably, especially in the pretreated samples of 10 and 30 days. Small pores contributed more to the specific area than large pores (**Figure 7a**). The pretreatment significantly increased the pore size over 10 nm (**Figure 7b**). Both the selective biodegradation of amorphous components by the fungus and the fungal penetration might contribute to the pore size increase. It was reported that the enzymatic hydrolysis is in close relation to the available surface area (*37*). Therefore, the result on pore size distribution suggested that the increased available pore size and surface area might also contribute to the enhanced efficiency of enzymatic hydrolysis of pretreated samples.

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