Biological Pretreatment under Non-sterile Conditions for Enzymatic Hydrolysis of Corn Stover

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Pretreatment with white-rot fungi can effectively remove lignin and decompose the structure of biomass to enhance subsequent enzymatic hydrolysis. This study developed a novel fungal pretreatment of biomass, which was operated under non-sterile conditions. The white-rot fungus *Irpex lacteus* colonized stably on the non-sterile substrates and effectively degraded lignin. After non-sterile fungal pretreatment for 42 days, lignin was degraded by 43.8%. The maximum saccharification efficiency was 7-fold higher after enzymatic hydrolysis compared to that of raw corn stover. Furthermore, the production of ethanol from corn stover improved. During non-sterile biological pretreatment, several microorganisms coexisted with *Irpex lacteus*, and the microbial community generated abundant by-products that greatly improved the efficiency of enzymatic hydrolysis. Non-sterile fungal pretreatment presents a feasible and promising technology for the production of biofuels by integrating on-farm wet storage systems. Moreover, it provides a low-cost bioconversion process and a stable, secure, and environmentally friendly energy supply.

Keywords: White-rot fungi; Biological pretreatment; Non-sterile; Phospholipid fatty acid; By-products

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INTRODUCTION

As an abundant source of hexose and pentose, lignocellulosic biomass is a potentially sustainable material for the production of biofuels and biochemicals (Kaparaju *et al.* 2009). However, its complex structural and chemical mechanisms make lignocellulosic biomass resistant to enzymatic hydrolysis (Himmel *et al.* 2007). Pretreatment is a key step in the production of biofuels from lignocelluloses since it can remove lignin, increase the surface area of lignocelluloses, and decrease the crystallinity of cellulose. Pretreatment reduces the resistance to hydrolysis and improves the release of fermentable sugars (Sun and Cheng 2002). Several thermochemical and biological pretreatment technologies have been used to produce biofuel from lignocellulosic biomass. Thermochemical pretreatment methods (acid hydrolysis, alkali hydrolysis, steam explosion, AFEX pretreatment, *etc*.) usually require high energy consumption and special chemicals. Additionally, various harmful by-products (5-hydroxymethylfurfural, levulinic acid, formic acid, *etc*.) are generated, which could inhibit fermentation (Carvalheiro *et al*. 2005; Chen *et al*. 2010).

Currently, white-rot fungi have shown remarkable potential for the pretreatment of biomass because of the mechanism of delignification and the multiple extracellular enzymes (Lee 1997). Biological pretreatment with white-rot fungi, a costless and environmentally friendly method, has been considered an alternative to thermochemical

pretreatment for biomass conversion (Shi *et al*. 2009). White-rot fungi have been reported to selectively degrade lignin, but leave cellulose for the production of biofuels (Ferraz *et al*. 2003; Wolfaardt *et al*. 2004). The white-rot fungus *Irpex lacteus* has promising potential in biological pretreatment, as it effectively decomposes the structure of feedstocks and enhances enzymatic hydrolysis (Novotný *et al.* 2009; Yang *et al.* 2010; Yu *et al.* 2009). This fungus has been reported to degrade wheat straw during the production of ethanol (López-Abelairas *et al*. 2013), combine with alkali pretreatment to enhance saccharification (Zhong *et al*. 2011), and purify manganese peroxidase from fungus to degrade polycyclic aromatic hydrocarbons (Baborová *et al*. 2006).

However, almost all studies on bio-pretreatment with *Irpex lacteus* have been performed under strict sterile conditions. Lignocellulosic biomass generally contains a large amount of microorganisms. Therefore, it is necessary to keep the fermentation axenic and to suppress the indigenous microflora in the substrate before inoculation (Lee 1997). In nature, the biodegradation of lignocellulosic materials occurs in a heterogeneous system, such that the lignocellulolytic microbes coexistent and compete with other non-lignocellulolytic microbes on the natural substrates (Tejirian and Xu 2010). Currently, most research on white-rot fungi in a non-sterile environment is focused on bioremediation in pollutants and dye degradation of wastewater (Lu *et al*. 2009; Sack and Fritsche 1997). However, a few studies have reported on the biological pretreatment of lignocellulosic materials for the production of biofuels under non-sterile conditions. The main challenge in these studies was to keep the functional fungus in a dominant and stable state in non-sterilized biomass (Lu *et al*. 2009). Non-sterile biological pretreatment should also effectively improve the downstream hydrolysis and fermentation. Since biological pretreatment under non-sterile conditions does not require sterilization and special bioreactors, it is novel and will further reduce the cost of pretreatment. Therefore, the study of non-sterile fungal pretreatment will have significant value for biorefineries (Gao *et al*. 2008).

In this study, *Irpex lacteus* was incubated on non-sterile substrates as well as sterilized substrates, for comparison. The composition and efficacy of enzymatic hydrolysis of pretreated corn stover under non-sterile conditions was compared with those under sterile conditions. Moreover, the activities of extracellular enzymes were also investigated. The microbial community during the non-sterile pretreatment process was measured by analysis of phospholipid fatty acids.

EXPERIMENTAL

Microorganism and Inoculum

The microorganism *Irpex lacteus* was isolated from the Shennongjia Scenic Area in Hubei, China. The isolate was identified by rDNA internal transcribed spacer sequence analysis. The GenBank accession number was FJ744594. The isolate was cultured on potato dextrose agar (PDA) slants for 7 days and transferred to potato dextrose broth (PDB) medium for 5 days at 28 °C before the inoculum was prepared. The inoculum medium, consisting of corn stover with 70% moisture content, was sterilized for 30 min at 121 °C. The inoculum was prepared as follows: 2 mL fungal inocula was added into the 2 g inoculum medium and cultured for 6 days.

Non-sterile Biological Pretreatment

Corn stover from Macheng (Hubei, China) was ground to pass through a 40-mesh screen. The materials were air-dried and stored at room temperature. Before inoculation, corn stover was disinfected in 0.1% (w/v) hydrated lime solution for 24 h at room temperature, then washed with distilled water until the pH was neutral. Non-sterile biological pretreatment was carried out in 250 mL Erlenmeyer flasks with solid inocula and 8 g disinfected corn stover. The autoclaved corn stover with 70% moisture content was prepared for biological pretreatment under sterile conditions (2 g solid inocula and 8 g raw corn stover). Cultures were incubated statically at 28 °C for 0, 14, 28, 42, and 56 days.

Components Determination of Corn Stover

The contents of acid-soluble lignin (ASL), acid-insoluble lignin (AIL), and structural carbohydrates in pretreated and raw corn stover were determined according to the National Renewable Energy Laboratory (NREL) (Sluiter *et al*. 2008). Structural carbohydrates were reported as percentages of glucan and xylan, which were determined using high-performance liquid chromatography (HPLC). Xylan and glucan contents were calculated based on xylose and glucose, using anhydrocorrections of 0.88 and 0.9, respectively. Lignin, the major noncarbohydrate component, was calculated as the sum of ASL and AIL. Acid-insoluble lignin was measured by gravimetric analysis and acid-soluble lignin was measured by UV-spectrometry at 320 nm.

The dry weight loss was calculated as the percentage of total solids lost after pretreatment. Lignin degradation, glucan loss, and xylan loss were defined as the percentages of lignin, glucan, and xylan reduction.

Fourier Transform Infrared (FTIR) Spectroscopy of Corn Stover

Discs were prepared by homogenizing dried samples (untreated and bio-pretreated corn stover) with KBr in a mortar and then pressing at 10 MPa for 3-4 min. The FTIR spectra were recorded with a NEXUS 670 spectrometer (Thermo Nicolet Corporation, Madison, WI) between 2000 and 700 cm^{-1} (Yang *et al*. 2010).

Enzymatic Hydrolysis of Corn Stover

After biological pretreatment, one part of the pretreated corn stover was subjected to subsequent enzymatic hydrolysis and another part was first dried at 60 °C for 72 h and then subjected to enzymatic hydrolysis. Enzymatic hydrolysis was performed at 2% substrate concentration (w/v) in 50 mM sodium acetate buffer (pH 4.8) with cellulase (30 total filter paper units/g substrate) in a rotary shaker (150 rpm and 48 ± 2 °C) for 72 h. The glucose content after enzymatic hydrolysis was determined by a high performance liquid chromatography (HPLC) system (Agilent 1200, USA). The saccharification efficiency was calculated as a percentage of theoretical glucose yield in corn stover with the following equation:

$$\text{Saccharification efficiency (\%)} = (\text{g of glucose after enzymatic hydrolysis} \times 100) / (\text{g of glucan in biopretreated corn stover} \times 1.1) \quad (1)$$

Simultaneous Saccharification and Fermentation (SSF)

The SSF experiment was performed using yeast (*Saccharomyces cerevisiae*) obtained from Anqi Co. (Hubei, China). Raw and pretreated corn stover were added to a

250 mL flask containing sodium acetate buffer (50 mM, pH 4.8) with 2% substrate concentration (w/v). Ethanol production was carried out with cellulase (30 total filter paper units/g substrate) and activated yeast (~7×10^7 cells/ml) at 40 ± 2 °C for 48 h. Ethanol was determined by a high performance liquid chromatography (HPLC) system (Agilent 1200, USA). The yield of ethanol was calculated from the following equations:

Ethanol yield (mg/g) = (mg of ethanol after fermentation) / (g of raw corn stover) (2)

Ethanol conversion (%) = (g of ethanol after fermentation × 100) / (g of glucan × 1.1 × 0.511) (3)

Enzyme Activity Assay

The pretreated corn stover was extracted with distilled water at 2.0% (w/v) substrate concentration. Water extraction was carried out with shaking (150 rpm) at 28 ± 2 °C for 4 h, and then the sample was analyzed for enzyme activities after filtration through filter paper.

The activity of total paper cellulase (FPA), endoglucanase (EG), cellobiohydrolase (CBH), and xylanase were determined by monitoring the release of reducing sugar, using the following substrates: filter paper strip, carboxymethyl cellulose-Na (CMC-Na), microcrystalline cellulose (Avicel), and xylan (from beech wood), respectively. β-glucosidase (BG) activity was determined using ρ-Nitrophenyl-β-D-glucopyranoside (ρ-NPG) as the substrate (Cai *et al*. 1999; Machuca and Ferraz 2001). Fe^{3+}-reducing activity (FeRA) was based on the formation of ferrozine-Fe^{2+} complex (Ferraz *et al*. 2001). Laccase activity was determined using 2, 2'-azinobis-3-ethylbenzthiazoline-6-sulphonate (ABTS) as substrate, according to the procedure in the literature (Hakala *et al*. 2005). Lignin peroxidase (Lip) activity was determined using Azure B and H_2O_2 in 50 mM sodium tartrate buffer (Archibald 1992). The manganese peroxidase (MnP) activity was based on $MnSO_4$ and H_2O_2 in 50 mM sodium malonate buffer (Steffen *et al*. 2000).

The activities of enzymes were expressed in international units (IU) per gram of initial corn stover. Fe^{3+}-reducing activity was defined as the increase of absorbance at 562 nm per minute (A/min).

Analysis of the Microbial Community under Non-sterile Conditions

The changes in the microbial community during biological pretreatment under non-sterile conditions were investigated by phospholipid fatty acid (PLFA) analysis (McKinley *et al*. 2005). The samples were freeze-dried before analysis. Total lipids in the microorganisms were extracted with a phosphate buffer-chloroform-methanol mixture (0.8:1:2). Phospholipids were separated from other lipids using a silicic acid column (Zhang *et al*. 2008). Finally, phospholipid fatty acids were methylated to fatty acid methyl esters (FAME) for further analysis by GC-MS (Agilent 7890A/5975C) (Denef *et al*. 2009). Nonadecanoic acid methyl ester was used as an internal standard to determine the total quantity of phospholipid fatty acids.

Statistical Analysis

All of the experiments were prepared in triplicate. The experimental results were validated by statistical analysis using Microsoft Excel and Origin 8.0 software. Mean values were presented with their standard errors. An analysis of variance (ANOVA) and

least-significant difference (LSD) at a 0.05 level were conducted using SPSS software. The correlation coefficient (r) was used to test for significant relationships between variables.

RESULTS AND DISCUSSION

Biodegradation of Corn Stover

During biological pretreatment under non-sterile conditions, the growth of *I. lacteus* was stable and stayed in a dominant state throughout the 56-day pretreatment. The compositional analysis of corn stover pretreated under non-sterile conditions is shown in Fig. 1a, which was compared to the biological pretreatment under pure culture conditions (Fig. 1b). In the initial biodegradation stage (14 days), the loss of lignin was significant in both non-sterile and sterile substrates (18.2% and 20.4%). The maximum rate of delignification appeared in the 28-day pretreatment; more than 40% of the lignin was removed in both types of pretreated corn stover. After prolonged pretreatment, delignification by *I. lacteus* slowed down. Lignin was degraded by 43.8% and 49.3% under the non-sterile and sterile conditions, respectively (42-day pretreatment). Furthermore, *I. lacteus* selectively utilized xylan as the carbon sources. The degradation of xylan by *I. lacteus* was faster than that of glucan, whether the corn stover was sterilized or not. Interestingly, more glucan was conserved under non-sterile conditions, which might be beneficial to saccharification. The losses of glucan were 13.2%, 15.4%, 33.9%, and 36.3% after non-sterile biological pretreatment for 14, 28, 42, and 56 days. The corresponding losses of glucan were 18.7%, 27.4%, 32.8%, and 33.1% under sterile conditions. Rapid degradation of glucan by *I. lacteus* was obtained after 28-day pretreatment. However, because of the coexistence of indigenous microflora in corn stover, the degradation rate of glucan was slower in the early stages of non-sterile bio-pretreatment. Especially after 28-day pretreatment, the loss of glucan decreased by 31.0%, compared to that of corn stover pretreated under sterile conditions.

Delignification was inhibited slightly, accompanied by greater glucan conservation during the process of non-sterile biological pretreatment. It seemed that the degradation patterns of corn stover were different between the sterile and non-sterile biological pretreatments. Lignin was the main barrier to the hydrolysis of corn stover. Greater losses of lignin meant that the recalcitrance of biomass was further reduced. Glucan was the only substrate to produce glucose. More glucan meant there was potentially more glucose. There must be a balance between the amounts of lignin and glucan, however, because it effects enzymatic hydrolysis. Because the substrates were not sterilized, the indigenous microflora in raw corn stover might have competed with *I. lacteus* and sought more nutrients and energy for colonization. The degradation of lignin and glucan seemed to be slightly inhibited compared with that under sterile conditions. However, in this study, the delignification of *I. lacteus* under non-sterile conditions was still more extensive than other fungi, such as *Pleurotus ostreatus* (Taniguchi *et al.* 2005) and *Phanerochaete chrysosporium* (Shi *et al.* 2008). Overall, biological pretreatment was successfully carried out in the substrates without sterilization, and there was an efficient delignification of corn stover by *I. lacteus*.

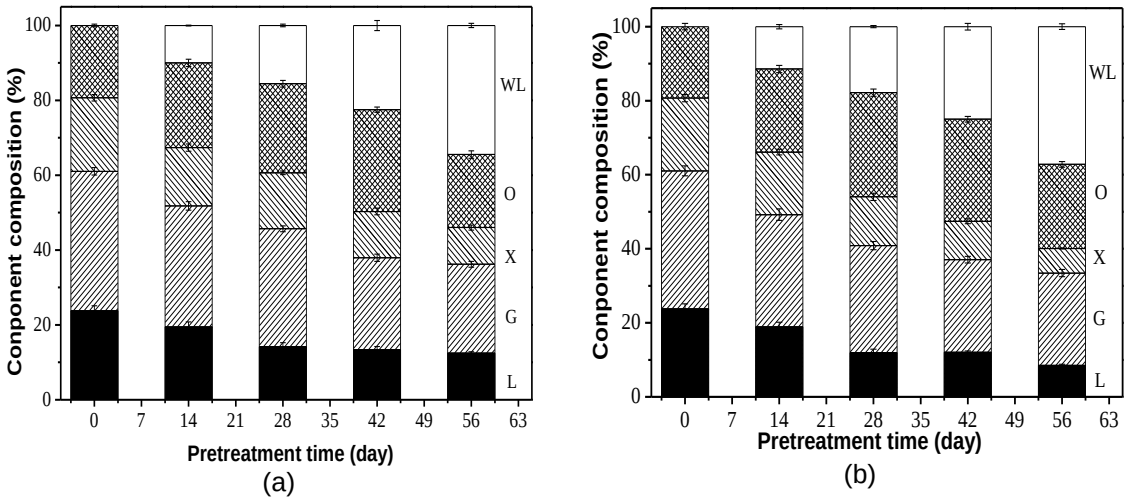

Fig. 1. The component composition of biologically pretreated corn stover under the conditions of (a) non-sterilization and (b) sterilization at different pretreatment periods (0, 14, 28, 42, 56 days); WL = weight loss; O = other; X = xylan; G = glucan; L = lignin

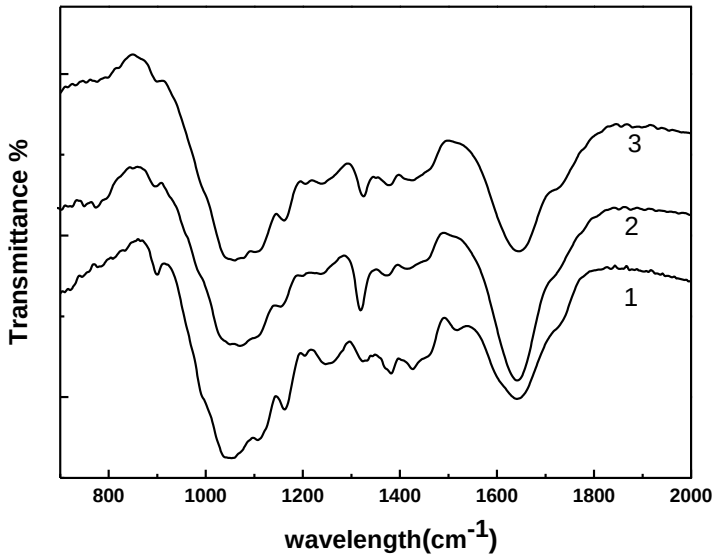

Fig. 2. FTIR spectra of corn stover: (1) raw corn stover, (2) bio-pretreated under sterile conditions, and (3) bio-pretreated under non-sterile conditions

The component modification and changes of functional groups were further evaluated by FTIR spectroscopy. As shown in Fig. 2, the aromatic ring stretch vibration of lignin at 1515 cm^{-1} decreased in intensity after biological pretreatment, indicating that *I. lacteus* effectively degraded lignin in both the sterile and non-sterile corn stover (Zhang *et al*. 2007). The band of 1226 cm^{-1}, the indicator of syringyl ring and C-O stretch in lignin and xylan (Mohebby 2008), decreased in intensity in the pretreated corn stover compared with raw samples. The intensity of 1226 cm^{-1} was weaker in sample 2 than sample 3, indicating that the indigenous microflora in raw corn stover inhibited the decomposition of syringyl lignin by *I. lacteus*, resulting in a smaller loss of lignin in non-sterile corn stover. The carbohydrate-related peaks at 1161 cm^{-1} and 898 cm^{-1} were assigned to the C-O-C vibration in cellulose and hemicellulose and C-H deformation in

cellulose, respectively (Liu *et al*. 2009). The intensities of carbohydrate bands also decreased after biological pretreatment (both in the sterile and non-sterile samples) compared to raw biomass. The results showed that during fungal pretreatment, some of the carbohydrates were also utilized as a source of nutrients and energy. The alteration of functional groups of lignocelluloses was confirmed by the results of composition analysis (Fig. 1). The changes of functional groups further proved that the decomposition of the chemical structures of corn stover by *I. lacteus* was extensive, although biological pretreatment was carried out under non-sterile conditions.

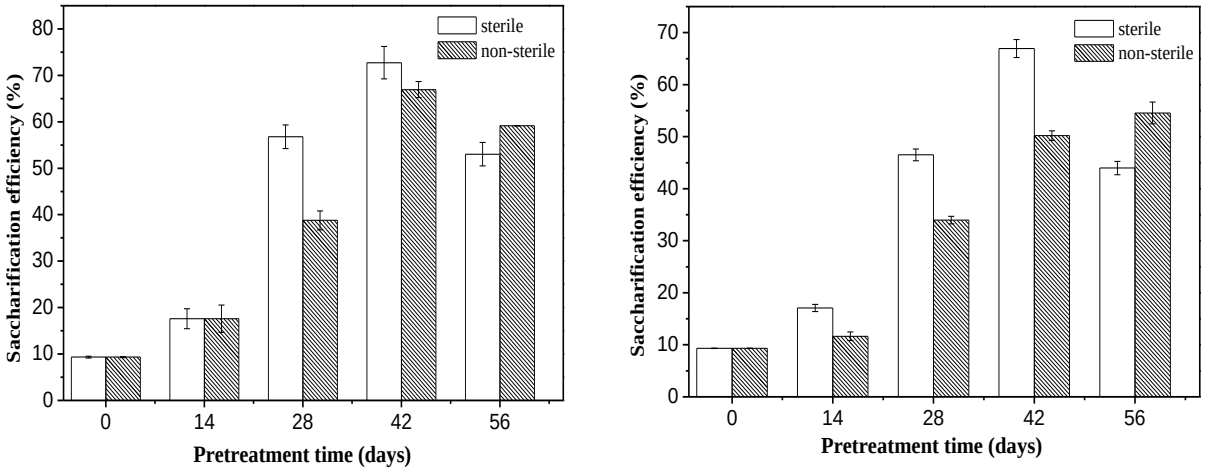

Fig. 3. The efficiency of enzymatic hydrolysis (a) when bio-pretreated corn stover was subjected to subsequent enzymatic hydrolysis and (b) when dried bio-pretreated corn stover was subjected to enzymatic hydrolysis

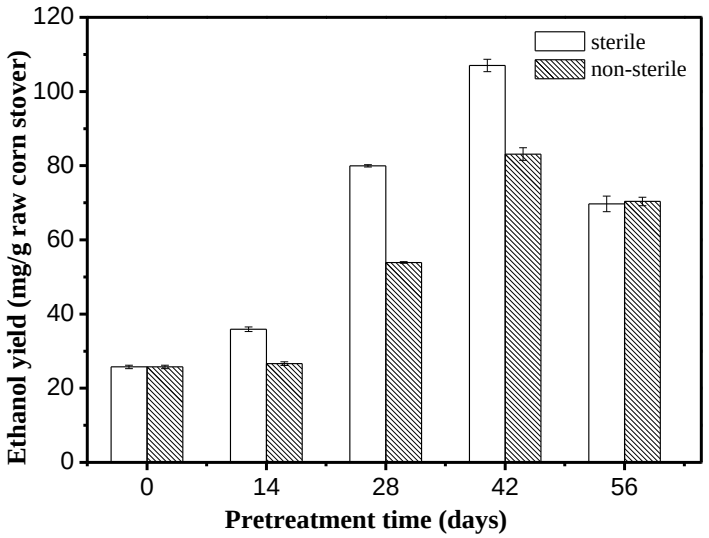

Fig. 4. Ethanol yield of pretreated corn stover under sterile and non-sterile conditions

Enzymatic Hydrolysis of Corn Stover and the Production of Ethanol

Pretreatment with white-rot fungi modifies the structure of corn stover and enhances the efficiency of enzymatic hydrolysis (Lee 1997). As shown in Fig. 3, without pretreatment, the saccharification efficiency of raw corn stover was only 9.3%. The recalcitrance of untreated corn stover to enzymatic hydrolysis was highly significant.

Even after 14-day pretreatment, the production of glucose was still very low. However, the efficiency of enzymatic hydrolysis was greatly improved after biological pretreatment for 28 days, due to an increase of lignin degradation. The maximum yield of glucose was obtained after 42-day biological pretreatment. *I. lacteus* selectively degraded lignin and xylan, contributing positively to enzymatic hydrolysis. However, without sterilization, pretreated corn stover still yielded a high content of sugars.

According to the results of subsequent enzymatic hydrolysis, the maximum saccharification efficiency was 66.9% under non-sterile conditions, which was 7-fold higher than that of raw biomass. In comparison to the results of sterilized pretreated corn stover (72.7%), the slight decrease of saccharification efficiency was mainly caused by the interference of indigenous microflora in the delignification and modification of corn stover. Moreover, the saccharification efficiency of non-sterile bio-pretreatment was even higher than bio-pretreatment under sterile conditions. The non-sterile biological pretreatment with *I. lacteus* in this study obtained high hydrolysis efficiency, which showed great potential for large-scale biological pretreatment. However, there was a decrease in the sugar yield after corn stover was dried, and greater losses of sugars occurred, in the non-sterile, dried, pretreated corn stover (Fig. 3b). Drying caused the deactivation of by-products (mainly enzymes) in corn stover, which reduced the yield of fermentable sugars after enzymatic hydrolysis. Additionally, it has been proven that there was a reduction in available pore volume in the fiber after drying, resulting in the decrease of sugar yield (Grous *et al*. 1986). Non-sterile bio-pretreated corn stover adapted to the subsequent enzymatic hydrolysis without drying, which could reduce the consumption of energy. Additionally, the relationship between lignin content and saccharification efficiency was obtained to evaluated the non-sterile fungal pretreatment. A greater correlation was found between both variables in non-sterile pretreatment ($r = -0.9282$) than that of bio-pretreatment in sterilized substrates ($r = -0.8620$). It can be concluded that in the non-sterile pretreatment, lignin was the main barrier to enzymatic hydrolysis, although more glucan was conserved.

Ethanol production is necessary to quantify the process' final performance. Ethanol yield, based on the amount of ethanol (mg) from the initial corn stover (g), was evaluated in the non-sterile bio-pretreatment. As shown in Fig. 4, the maximum yields of ethanol were 83.1 and 107.1 mg/g corn stover after bio-pretreatment for 42 days under non-sterile and sterile conditions, respectively, which, based on the initial glucose in the corn stover, were 275.3 and 270.6 mg/g corn stover, respectively. Results showed that the production of ethanol had a tight correlation with the efficiency of enzymatic hydrolysis. The decrease of ethanol yield from non-sterile corn stover was mainly due to the resistance of lignin to the production of glucose. Although there was a decrease in ethanol yield because of non-sterile biopretreatment, an ethanol conversion of 60.1%.was achieved. According to Ballesteros and his colleagues (2004), ethanol conversion from various lignocellulosic woody and herbaceous biomasses can be achieved in the range of 50-72%, during which biomass materials were treated using steam explosion. The use of fungal pretreatment obtained a high efficiency of ethanol conversion that was comparable with the results of steam explosion. Additionally, the process of pretreatment occurred under non-sterile conditions. After non-sterile biological pretreatment, the maximum yield of ethanol was 3.2-fold higher than that of raw corn stover. Compared to fungal pretreatment under strict pure culture conditions (Shi *et al*. 2009), the efficiency of ethanol production after non-sterile pretreatment using *I. lacteus* was much higher.

Moreover, after 56-day pretreatment, ethanol yields from sterile and non-sterile corn stover were similar.

Fungal pretreatment achieves substantial lignin degradation. However, the long pretreatment time imposes a barrier to biological pretreatment (Wan and Li 2011). On-farm wet storage associated with non-sterile fungal pretreatment will address the issue of long pretreatment time and provide feasible technology for improving the production of ethanol from lignocelluloses. Wet storage also presents an opportunity to add value to feedstock and reduce harvesting costs (Digman *et al*. 2010). In the long-term storage of feedstocks, white-rot fungus *I. lacteus* is a potential candidate for pretreating corn stover because it significantly decreased the recalcitrance of biomass and improved feedstock susceptibility to enzymatic hydrolysis. Non-sterile biological pretreatment appears to be novel, considering its low-cost bioconversion, and ensures a stable, secure, and environmentally friendly energy supply.

Table 1. Enzyme Activities from Water Extract of Pretreated Corn Stover*

Corn stover	0 day	14 days	28 days	42 days	56 days
FPA (IU/g corn stover)					
Non-sterile	0 (0.00)	0.63 (0.03)	0.39 (0.02)	0.64 (0.02)	1.23 (0.07)
Sterile	0 (0.00)	0.56 (0.01)	0.44 (0.03)	0.52 (0.04)	1.26 (0.06)
XYL (IU/g corn stover)					
Non-sterile	3.74 (0.14)	6.27 (0.24)	6.07 (0.10)	19.60 (0.75)	23.00 (0.90)
Sterile	3.38 (0.15)	5.53 (0.33)	6.24 (0.15)	13.64 (0.43)	24.68 (0.96)
EG (IU/g corn stover)					
Non-sterile	0.51 (0.01)	0.51 (0.02)	0.84 (0.03)	2.28 (0.11)	1.85 (0.14)
Sterile	0.74 (0.02)	0.35 (0.01)	0.89 (0.03)	1.84 (0.10)	3.10 (0.12)
CBH (IU/g corn stover)					
Non-sterile	1.79 (0.12)	1.61 (0.11)	1.54 (0.10)	1.21 (0.05)	0.90 (0.08)
Sterile	1.80 (0.17)	1.47 (0.06)	1.56 (0.12)	0.88 (0.08)	1.30 (0.09)
BG (IU/g corn stover)					
Non-sterile	0 (0.00)	0.23 (0.01)	0.49 (0.01)	1.42 (0.07)	2.50 (0.13)
Sterile	0 (0.00)	0.42 (0.01)	0.50 (0.01)	1.48 (0.08)	3.91 (0.15)
FeRA (A/min)					
Non-sterile	2.57 (0.09)	0.29 (0.02)	0.12 (0.01)	2.28 (0.14)	2.44 (0.09)
Sterile	1.22 (0.07)	0.18 (0.01)	0.08 (0.00)	0.76 (0.05)	2.76 (0.06)

* FPA = filter paper activity; XYL = xylanase; EG = endoglucanase; CBH = cellobiohydrolase; BG =β-glucosidase activity; FeRA = Fe^{3+}-reducing activity
Numbers in parentheses represent standard deviations

Enzyme Assays

Enzyme activities were detected in water extracted from pretreated corn stover. The activities of extracellular enzymes from *I. lacteus* were all very low, with the exception of xylanase (Table 1). High activity of xylanase corresponded to the selective degradation of xylan by *I. lacteus* (Fig. 1). Additionally, no ligninolytic enzymes (Lac, Mnp, and Lip) were detected in the pretreated corn stover. The occurrence of maximum sugar yields coincided with an increase of enzyme activities in the non-sterile corn stover pretreated for 42 days. The activities of FPA, xylanase, EG, CBH, and FeRA increased by 23.1%, 43.7%, 23.9%, 37.5%, and 200%, respectively, when compared with those of pretreated corn stover under sterile conditions. The increase of enzyme activities in the non-sterile pretreated corn stover showed that microorganisms generated abundant by-products (enzymes and low molecular mass mediators) with high activities. The by-

products of microorganisms associated with cellulase had a synergistic effect on corn stover hydrolysis. Additionally, the activity of FeRA corresponded to the delignification of biomass by *I. lacteus*, since ligninolytic enzymes could not be detected. *I. lacteus* has been found to secrete a low molecular mass mediator that catalyzes a redox reaction to produce the hydroxyl radical (Tanaka *et al*. 1999). The Fe^{3+}-reducing activity in the extract represented the generation of hydroxyl radicals by *I. lacteus*, and it improved the process of delignification (Morgavi *et al*. 2000; Novotný *et al*. 2009; Xu *et al*. 2009). Abundant by-products were obtained from indigenous microflora in the non-sterile biomass, which were beneficial to the subsequent enzymatic hydrolysis and increased the yield of glucose. On the other hand, it has been reported that the degradation of glucan by white-rot fungus depended on the activity of β-glucosidase (Sternberg *et al.* 1977; Wen *et al.* 2005). The coexistence of indigenous microflora with *I. lacteus* in non-sterile corn stover generated lower activities of β-glucosidase (BG), which resulted in less extensive degradation of glucan and more carbohydrates being conserved during pretreatment. In general, non-sterile biological pretreatment removed lignin to result in high sugar recovery. More importantly, it greatly reduced the cost of special equipment and decreased the consumption of energy.

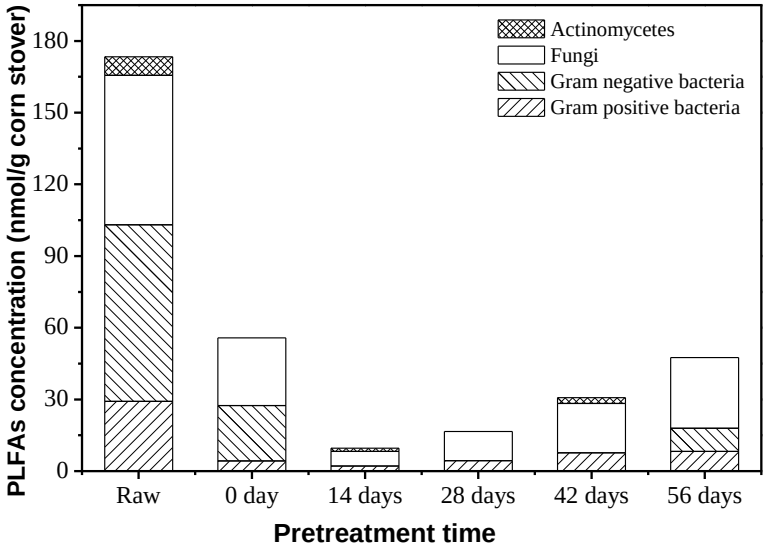

Fig. 5. Changes of the microbial community during non-sterile biological pretreatment (raw = raw corn stover, 0 day = lime-disinfected corn stover)

Analysis of Microbial Community During Non-sterile Process

The composition of the microbial community was measured by an analysis of phospholipid fatty acids (PFLAs). There were a total of 10 different kinds of PLFAs extracted from the non-sterile bio-pretreated corn stover. The markers of PLFAs that were analyzed within the dataset included 10me17:0 (indicative of actinomycetes), 18:2ω (indicative of fungi), a summation of i15:0, a15:0, i16:0, i17:0, and a17:0 (indicative of gram positive bacteria, G^+ bacteria), and a summation of 16:1ω7, 18:1ω7c, and 16:1ω9 (indicative of gram negative bacteria, G^- bacteria) (Drenovsky *et al*. 2004; McKinley *et al*. 2005). Figure 5 shows that raw corn stover contained a large number of microbes, the majority of which were G^- bacteria and fungi. The total amount of phospholipid fatty acids rapidly decreased when corn stover was disinfected with hydrated lime (0 day). The

contents of G^- bacteria decreased from 29.2% to 4.3%, and actinomycetes were removed after lime disinfection. Disinfection with hydrated lime effectively removed most of the indigenous microflora in raw corn stover, while it kept *I. lacteus* in a dominant and stable state during non-sterile biological pretreatment. In the early stages of pretreatment (14 days), the amount of microbes was the lowest among all samples, mainly because of the competition among microbial species. After the inoculum was added to the non-sterile substrates, the indigenous microflora and *I. lacteus* competed for the readily available compounds for utilization and colonization. Additionally, it has been proven that *I. lacteus* stresses the bacterial community greatly in a natural environment (Byss *et al*. 2008). Because of the exhaustion of easily available carbohydrates, there was an increase of bacteria and fungi community coincided with an increase of lignin degradation (Marschner *et al*. 2011). During non-sterile biological pretreatment, the colonization of G^- bacteria was greatly inhibited. Therefore, it could be concluded that *I. lacteus* stressed the G^- bacterial community. During the process of non-sterile biological pretreatment, there were bacteria and fungi that coexisted with *I. lacteus* in the substrates. An analysis of this microbial community could be of use for further study on the function of the microbial community, as well as the relationship between microbes and the degradation of biomass in a natural environment.

It has been reported that some white-rot fungi can grow on non-sterile lignocelluloses. *Phanerochaete chrysosporium*, a typical white-rot fungus, efficiently degraded lignin on non-sterile lignocellulosic materials during biopulping (Chen *et al*. 1998). An efficient scale-up process of non-sterile biopulping using white-rot fungi is affected by factors such as the species of fungus, inocula, disinfection of biomass, sources of feedstocks, nutrients supplement, *etc*. (Hofrichter and Steinbüchel 2001; Lee 1997). In this study, non-sterile biological pretreatment was used to produce glucose and ethanol from corn stover, and the efficiency was comparable to some fungal pretreatments under strict sterile conditions. Non-sterile biological pretreatment using *I. lacteus* was successfully carried out and the results could be adequately replicated on a laboratory scale. Additionally, it was reported that *Irpex lacteus* colonized the non-sterile soil and grew competitively with indigenous soil microflora (Novotný *et al*. 2000). The abilities of robust growth, selective degradation of lignin, and inhibition of bacteria made *I. lacteus* suitable to be the functional fungus for non-sterile biological pretreatment. However, the degradation of lignocelluloses by *I. lacteus* is impacted by various environmental factors. It is necessary to evaluate the stability of non-sterile biopretreatment in a natural environment in future research.

CONCLUSIONS

1. During non-sterile biological pretreatment, *I. lacteus* selectively degraded lignin and xylan, leaving more glucan for saccharification.
2. After 42-day non-sterile biological pretreatment, the efficiency of enzymatic hydrolysis and ethanol yield were 7-fold and 3.2-fold higher than that of raw corn stover, respectively.
3. In the process of non-sterile biological pretreatment, more abundant and higher activities of by-products were obtained, which greatly improved the subsequent enzymatic hydrolysis.

4. During non-sterile biological pretreatment, microorganisms coexisted and the colonization of G^- bacteria was greatly suppressed by *I. lacteus*.
5. This original technology reveals great potential for the production of biofuels by integrating on-farm wet storage of feedstocks, because it is costless, environmentally friendly, and improves corn stover susceptibility to enzymatic hydrolysis.

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