Biological pretreatment of corn stover with white-rot fungus for improved enzymatic hydrolysis

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A B S T R A C T

Biological pretreatment of lignocellulosic biomass by white-rot fungus can represent a low-cost and eco-friendly alternative to harsh physical, chemical or physico-chemical pretreatment methods to facilitate enzymatic hydrolysis. However, fungal pretreatment can cause carbohydrate loss and it is, therefore, necessary to use the appropriate fungal strain-biomass type combination. In this work, 26 white-rot fungal strains were evaluated under solid state cultivation at 74% moisture level at 28 °C for 30 days for fermentable sugar production from corn stover after enzymatic hydrolysis of each fungal pretreated corn stover using a cocktail of 3 commercial enzyme (cellulase, β -glucosidase, hemicellulase) preparations. The best result was obtained with *Cyathus stercoreus* NRRL-6573 which gave a sugar yield of 394 ± 13 mg/g of pretreated stover followed by *Pycnoporus sanguineus* FP-10356-Sp (393 ± 17 mg/g) and *Phlebia brevispora* NRRL-13108 (383 ± 13 mg/g).

Keywords: White-rot fungus, Biological pretreatment, Enzymatic hydrolysis, Solid state cultivation, Corn stover

1. Introduction

Lignocellulosic biomass has great potential for generating useful bio-products such as fuels and chemicals. However, due to the recalcitrant nature of lignocellulosic materials relating to the structural integrity of lignin, hemicellulose and cellulose, it is essential to apply a pretreatment to facilitate conversion of lignocellulose to sugars or other value-added products. Pretreatment, as the first step towards conversion of lignocellulosic feedstocks to fuels and chemicals, makes up to one-third of the total production costs and remains one of the main barriers to commercial success (Saha, 2004). Typically, harsh methods using steam, acid or alkali and high temperature

are used to pretreat lignocellulosic biomass prior to its breakdown to sugars by enzymes, which also result in the formation of fermentation inhibitors (Saha, 2003). An alternative to harsh chemicals and high temperature is microbial pretreatment employing fungi and their enzymes to break down or remove lignin from the holocellulose (cellulose and hemicellulose) surface. Lignin is a polyaromatic polymer that provides rigidity to lignocellulose. The principal microorganisms responsible for lignin degradation in nature are white-rot fungi which belong to the basidiomycete and have complex ligninolytic systems (Leonowicz et al., 1999; Martinez et al., 2011). The excreted ligninolytic enzymes, mainly lignin peroxidase (EC 1.11.1.14), manganese peroxidase (EC 1.11.1.13) and laccase (EC 1.10.3.1) are responsible for delignification by white-rot fungi (Wan and Li, 2012).

Since fungal pretreatment is an environmentally friendly and low cost pretreatment approach for enhancing enzymatic saccharification and fermentation of lignocellulosic biomass, it is attracting increased attention in recent years (Martinez et al., 2009; Tian et al., 2012). However, the fungal pretreatment has two inherent disadvantages – low efficiency and long residence periods. Moreover, a majority of white-rot fungi degrade lignin and polysaccharide simultaneously, while some degrade lignin selectively (Kuhad et al., 1997; Guerra et al., 2003). The most investigated white-rot fungus for lignin degradation is *Phanerochaete chrysosporium* (Bak et al., 2009; Zeng et al., 2014). The structure and chemical composition are different between woody materials and agricultural residues, so the ligninolytic systems and the suitable strains implicated in the lignocellulose degradation process may be different between these two substrates (Li et al., 2008). It is, therefore, necessary to evaluate white-rot fungal strains in order to identify the most potent strain for very powerful delignification ability while possessing little or no metabolic activity for utilization of cellulose and hemicellulose components from corn

stover, a low cost abundant agricultural residue in the USA especially in the Mid-West region. The goal of this research is to study the feasibility of developing a small scale on-farm biological pretreatment of corn stover using a highly effective lignin degrading white rot fungal strain which leaves most of the carbohydrates intact during pretreatment for conversion to fermentable sugars by enzymatic hydrolysis.

2. Materials and methods

2.1. Materials

Corn stover (~ 20%, w/w moisture) was collected from a corn field in the greater Peoria, Illinois area. It was air-dried to ~ 8% (w/w) moisture, chopped and milled in a Hammer mill so as to pass through a 4 mm screen. The milled corn stover was stored at ambient temperature in a tightly closed plastic bag. Celluclast 1.5 L (cellulase) and Novozyme 188 (β -glucosidase) were purchased from Brenntag Great Lakes, Milwaukee, WI, USA. Fiberzyme (hemicellulase) was supplied by Dyadic corp., Jupiter, FL, USA. Membrane Filter Unit (0.2 μ m) was purchased from Nalge Company, Rochester, NY, USA. Aminex HPX 87P column (300 x 7.8 mm), Aminex HPX 87H column (300 x 7.8 mm), De-ashing cartridge (30 x 4.6 mm), Carbo-P micro-guard cartridge (30 x 4.6 mm), and Cation H micro-guard cartridge (30 x 4.6 mm) were purchased from Bio-Rad Laboratories, Inc., Hercules, CA, USA. All other chemicals used were of standard analytical grades.

2.2. White-rot fungal strains

A total of 14 basidiomycete strains were obtained from ARS Culture Collection, Peoria, IL, USA. These strains were: *Agaricus bisporus* NRRL-20762, *Coprinus* sp. NRRL-6463, *Cop.*

cinereus NRRL-20638, *Cop. stercoreus* NRRL-20953, *Cyathus berkeleyanus* NRRL-6520, *Cya. pallidus* NRRL-6529, *Cya. stercoreus* NRRL-6573, *Flammulina velutipes* NRRL-2367, *Marasmius oreades* NRRL-2602, *Phanerochaete chrysosporium* NRRL-6370, *Phlebia brevispora* NRRL-13108, *Polyporus compactus* NRRL-A-2351, *Pol. versicolor* NRRL-A-2352 and *Sporotrichum pulverulentum* NRRL-6361. In addition, the following 12 basidiomycete strains were obtained from USDA Forest Service, Northern Research Station, Madison, WI, USA: *Bjerkandera adusta* FP-101809-Sp, *B. fumosa* FP-135285-T, *Ceriporiopsis pannocincta* FT-100624-Sp, *Cer. subvermispora* FP-105752-Sp, *Ganoderma lobatum* ERT-276-R, *Irpex lacteus* FP-101234-Sp, *Panaeolus* sp. FP-102035-Sp, *Phellinus badius* AZ-8-T, *Pycnoporus cinnabarinus* FP-104138-Sp, *Pyc. sanguineus* FP-103506-Sp, *Rigidoporus crocatus* MJL-1465-Sp and *R. lineatus* ES-500-Sp. All these basidiomycete strains were maintained in the laboratory on Potato Dextrose Agar (PDA) plates at 4 °C.

2.3. Preparation of inoculum

Each fungus was cultured on tomato juice agar (200 mL V-8 juice, 3 g CaCO₃ and 20 g agar per L) plates at 28 °C for 3-10 days. The seed was grown in 200 mL liquid medium containing 10 g glucose, 1 g NH₄NO₃, 0.8 g KH₂PO₄, 2 g NaHPO₄, 5 g MgSO₄·7 H₂O and 2 g yeast extract per L in a 1-L baffled flask for 7-10 days at 225 rpm at 28 °C (Elisashvili et al., 2008). The medium was adjusted to pH 6.0 prior to sterilization by adding 2 M NaOH. The grown liquid culture was aseptically homogenized in a closed tissue grinder tube for three 15-s cycles and then used for inoculation. The seed culture was normalized to provide 3 mg cells per mL for all strains.

2.4. Biological pretreatment of corn stover

The biological pretreatment with a white-rot fungal strain was carried out in a 500-mL Erlenmeyer flask with foam plug. The flask containing 10 g corn stover and 23 mL tap water was autoclaved at 121 °C for 15 min and aseptically inoculated with 5 mL seed culture. All flasks were incubated statically at 28 °C for 30 days. The moisture content was kept at ~ 74% during the experimental period. Non-inoculated samples were treated under the same conditions and used as controls. All experiments were performed in triplicate.

2.5. Enzyme assays

The cellulase activity in terms of filter paper activity was assayed and expressed as filter paper unit (FPU) by the procedure described by Ghose (1987). Carboxymethyl cellulase (CMCase), β -glucosidase and xylanase activities were assayed by the procedures described previously (Saha et al., 2005). All enzyme assays were performed at pH 5.0 and 45°C and the activities were expressed in terms of international units (IU, μ mol product formed per min).

2.6. Saccharification of pretreated corn stover

The enzymatic saccharification of the biologically pretreated corn stover was performed by shaking slowly (100 rpm) at 45°C for 72 h after diluting the pretreated material to 2% (w/v) solid level with 50 mM citrate-phosphate buffer, pH 5.0 and adding a filter sterilized enzyme cocktail of 3 commercial enzyme preparations. The enzyme cocktail contained 50 μ L Celluclast, 5 μ L Novozyme 188 and 50 μ L Fiberzyme per g of corn stover. After enzymatic hydrolysis, the residual solids were separated from the liquid by centrifugation ($12,000 \times g$; 10 min) before using the liquid portion as enzymatically saccharified corn stover hydrolyzate. The cocktail of commercial enzyme preparations used for enzymatic saccharification contained small quantities

of glucose. For simplification purpose, the quantity of glucose present in the enzyme cocktail was subtracted from the measured glucose in each case.

2.7. Ethanol production from glucose and xylose

The seed cultures of all 26 white-rot fungal strains were prepared as described above and the cell concentration was adjusted to 1 mg/mL for each case. For semi-aerobic fermentation, 40 mL of the liquid medium (1 g NH_4NO_3 , 0.8 g KH_2PO_4 , 0.2 g Na_2HPO_4 , 0.5 g MgSO_4 , 7 H_2O and 2 g yeast extract per L, pH 6.0) was used in 50 mL Erlenmeyer flask. The medium containing either 1% (w/v) glucose or 1% (w/v) xylose was inoculated with 4 ml of the seed culture and incubated at 130 rpm and 28 °C for 3 days. For aerobic fermentation, 10 mL of the same liquid medium was used in 50 mL Erlenmeyer flask. The medium containing either 1% (w/v) glucose or 1% (w/v) xylose was inoculated with 1 ml of the seed culture and incubated at 250 rpm and 28 °C for 3 days. Samples were taken at periodic intervals for analysis of substrate utilization and ethanol production by HPLC.

2.8. Analytical procedures

The composition of corn stover with respect to cellulose, hemicellulose, lignin and ash contents was determined using the standard laboratory analytical procedures for biomass analysis provided by National Renewable Energy Laboratory (NREL), Golden, CO, USA (Sluiter et al., 2008a, b). Each analysis was performed in duplicate. Moisture content was determined using a moisture analyzer (Mark 2, Sartorius Mechatronics Corp., Bohemia, NY, USA). Sugars, ethanol, furfural, and hydroxymethyl furfural (HMF) were analyzed by HPLC (Saha and Bothast, 1999). The separation system consisted of a fully integrated solvent delivery system (LC-20AD

Prominence, Shimadzu America, Inc., Columbia, MD, USA) equipped with an SIL-20AC autosampler, RID-10A refractive index detector, a SDP-20A UV detector, a CTO-10AS VP column heater and a computer software based integration system (LC solutions 1.23 SP1, Shimadzu). Two ion moderated partition chromatography columns (Aminex HPX-87P with De-ashing and Carbo-P micro-guard cartridges, Aminex HPX 87H with Cation H micro-guard cartridge) were used. The Aminex HPX-87P column was maintained at 85 °C, and the sugars, furfural and HMF were eluted with Milli-Q filtered water at a flow rate of 0.6 mL/min. The Aminex HPX-87H column was maintained at 65 °C, and the sugars and ethanol were eluted with 10 mM HNO₃ prepared using Milli-Q filtered water at a flow rate of 0.6 mL/min. Peaks were detected by refractive index or UV absorption (277 nm) and were identified and quantified by comparison to retention times of authentic standards (glucose, xylose, arabinose, galactose, ethanol, furfural and HMF). The yield of total sugars was calculated by the procedure provided by NREL (Sluiter et al., 2008a).

2.9. Statistical analysis

All experiments were performed in triplicate. Data were statistically analyzed by using SigmaPlot® 12 (Systat Software, Inc., San Jose, CA, USA). Mean values were presented with standard deviations. A single factor ANOVA was conducted on each measured variable to test for significant differences between species ($p < 0.05$). Following this, a Tukey's HSD (honest significant difference) means comparison was run to determine which species were significantly different from each other as well as which were significantly better or worse than control experiments. Reported values are the means and standard deviations from ANOVA. Letters next to each mean value indicate differences.

3. Results and discussion

3.1. Composition of corn stover and commercial enzyme preparations

Corn stover was used in this study without washing. It contained $36.3 \pm 0.2\%$ cellulose, $31.4 \pm 0.5\%$ hemicellulose ($24.5 \pm 0.1\%$ xylan, $4.9 \pm 0.4\%$ arabinan and $2.0 \pm 0.1\%$ galactan) (total carbohydrate content, $67.6 \pm 0.7\%$), $17.2 \pm 0.2\%$ lignin and $9.1 \pm 0.1\%$ ash on dry basis. Three commercial enzyme preparations were used for saccharification of pretreated corn stover. Celluclast contained 41 ± 1 FPU of cellulose (filter paper), 751 ± 35 U CMCase, 16 ± 2 U β -glucosidase and 781 ± 21 U of xylanase per mL. Novozyme 188 contained 1 ± 0 FPU of cellulose (filter paper), 41 ± 2 U CMCase, 380 ± 19 U β -glucosidase and 61 ± 1 U of xylanase per mL. The xylanase preparation (Fiberzyme) contained 7 ± 1 FPU of cellulose, 685 ± 23 U CMCase, 52 ± 3 U β -glucosidase and $9,837 \pm 190$ U of xylanase per mL.

3.2. Weight loss of corn stover after fungal pretreatment

Corn stover was pretreated by all 26 basidiomycete strains separately under solid state cultivation at $\sim 74\%$ moisture level and 28°C for 30 days. The final weight loss values of corn stover decayed by the white-rot fungal strains are given in Fig. 1. It ranged from $9.8 \pm 0.1\%$ to $52.8 \pm 1.3\%$. The maximum weight loss was $52.8 \pm 1.3\%$ for *Pha. chrysosporium* NRRL-6370 followed by *S. pulverulentum* NRRL-6361 ($50.6 \pm 0.8\%$), *B. adusta* FP-10809-Sp ($41.1 \pm 1.0\%$), *Pol. compactus* NRRL-A-2351 ($40.9 \pm 0.1\%$), *Cya. stercoreus* NRRL-6573 ($40.4 \pm 2.0\%$), *I. lacteus* FP-101234-Sp ($40.3 \pm 1.6\%$), *Cya. pallidus* NRRL-6529 ($39.2 \pm 0.9\%$) and *Pyc. sanguineus* FP-103506-Sp ($38.6 \pm 1.0\%$). The lowest weight loss was $9.8 \pm 0.1\%$ for *Coprinus* sp. NRRL-6463 followed by *M. oreades* NRRL-2602 ($10.0 \pm 0.6\%$), *Cop. stercoreus* NRRL-20953

(10.1±0.6%) and *Cer. subvermispora* FP-10572-Sp (11.8±0.1%). These data indicate that the corn stover degradation ability varied widely among these 26 white-rot fungal strains under the conditions used.

3.3. Component (lignin, hemicellulose and cellulose) loss of corn stover after fungal pretreatment

The lignin losses from corn stover by the 26 white-rot fungal strains are shown in Fig. 2A. The highest lignin loss (51.4±2.9%) occurred for *Pha. chrysosporium* NRRL-6370, followed by *Pyc. sanguineus* NRRL-FP-103506-Sp (51.0±1.2%), *I. lacteus* FP-101234-Sp (46.7±1.8%), *Cya. stercoreus* NRRL-6573 (46.2±0.8%), *Pol. compactus* NRRL-A-2351 (45.4±1.5%) and *Phl. brevispora* NRRL-13108 (39.6±1.6%). The lignin loss was below 10% for *A. bisporus* NRRL-20762 (7.1±0.6%), *Cop. cinereus* NRRL-20638 (6.6±0.8%), *B. fumosa* FP-135285-T (4.4±0.0%), *F. velutipes* NRRL-2367 (3.6±0.2%), *R. crocatus* MJL-1465-Sp (2.4±0.1%), *Panaeolus* sp. FP-102035-Sp (2.2±0.0%) and *Cer. pannocincta* FT-100624-Sp (1.5±0.0%). In addition, there was no loss of lignin from corn stover for 4 strains (*Coprinus* sp. NRRL-6463, *Cop. stercoreus* NRRL-20953, *G. lobatum* ERT-276-R and *M. oreades* NRRL-2602).

The hemicellulose losses from corn stover by the 26 fungal strains are shown in Fig. 2B. Seven strains lost more than 50% hemicellulose. The highest loss occurred for *Pha. chrysosporium* NRRL-6370 (56.5±2.9%) followed by *S. pulverulentum* NRRL-6361 (55.5±0.4%), *B. adusta* FP-101809-Sp (54.6±0.7%), *I. lactus* FP-101234-Sp (52.5±2.0%), *Pol. compactus* NRRL-A-2351 (52.1±1.3%), *Cya. stercoreus* NRRL-6573 (51.8±1.8%) and *Pyc. sanguineus* FP-103506-Sp (50.7±2.1%). In addition, 5 strains (*A. bispora* NRRL-20762, *Cya. pallidus* NRRL-6529, *Phl. brevispora* NRRL-13108, *Pyc. cinnabarinus* FP-104138-Sp and *R.*

lineatus ES-500-Sp) lost between 40-50% hemicellulose and 4 strains (*Cer. pannocincta* FT-100624-Sp, *Cop. cinereus* NRRL-20638, *F. velutipes* NRRL-2367 and *Phe. badius* AZ-8-T) lost between 30-40% of hemicellulose. Comparing the lignin loss, hemicellulose loss was more prevalent among the strains investigated.

The cellulose (glucan) losses from corn stover by the 26 white-rot fungal strains are shown in Fig. 2C. The highest glucan loss occurred for *Pha. chrysosporium* NRRL-6370 ($50.1 \pm 1.1\%$) followed by *S. pulverulentum* NRRL-6361 ($49.2 \pm 0.9\%$), *I. lacteus* FP-101234-Sp ($38.0 \pm 1.4\%$), *B. adusta* FP-101809-Sp ($37.0 \pm 1.5\%$), *A. bisporus* NRRL-20762 ($34.0 \pm 0.7\%$), *Pol. compactus* NRRL-A-2351 ($33.1 \pm 1.4\%$) and *Pyc. cinnabarinus* FP-104138-Sp ($32.8 \pm 1.8\%$). The lowest glucan loss occurred for *Cop. stercoreus* NRRL-20953 ($7.8 \pm 0.4\%$) followed by *R. crocatus* MJL-1465-Sp ($9.9 \pm 0.4\%$), *Coprinus* sp. NRRL-6463 ($9.9 \pm 0.9\%$), *Cer. pannocincta* FT-100624-Sp ($11.6 \pm 0.8\%$) and *Panaeolus* sp. FP-102035-Sp ($12.8 \pm 0.6\%$).

In this study, overall the hemicellulose component was more intensively degraded than lignin and cellulose components. This may indicate that hemicellulose was more easily attacked than other components in corn stover. This may be due to a lower degree of polymerization than cellulose (Isroi et al., 2011). White-rot fungus uses its enzymatic machinery to break down lignin and alter lignocellulose structure. Selectivity (ratio of lignin loss and cellulose loss) values for all 26 strains varied from 0 to 2.46 (Table 1). The low selectivity value indicates the relatively high cellulose loss during the biological pretreatment. The selectivity value of 1.0 means that both lignin and cellulose were equally lost during pretreatment. Only 8 strains showed higher lignin loss compared to cellulose loss. The pretreated corn stover for all 26 fungal strains did not contain typical sugar degradation products such as furfural and HMF at all as were evident from HPLC analysis (data not shown).

3.4. Composition of biologically pretreated corn stover

The utilization of cellulose, hemicellulose and lignin by all 26 white-rot strains varied in different proportions during biological pretreatment. As shown in Fig. 3, the composition of the remaining corn stover after pretreatment varied with respect to cellulose, hemicellulose and lignin contents. It is evident that the highest total component loss (52.7%) occurred for *Pha. chrysosporium* NRRL-6370 followed by *S. pulverulentum* NRRL-6361 (49.1%), *I. lacteus* FP-101234-Sp (45.2%), *B. adusta* FP-101809-Sp (44.1%), *Pol. compactus* NRRL-2351 (42.6%), *Cya. stercoreus* NRRL-6573 (42.2%), *Pyc. Sanguineus* FP-103506-Sp (40.4%) and *Cya. pallidus* NRRL-6529 (35.5%). The lowest total component loss (6.2%) occurred for *Cer. subvermispora* FP-105752-Sp followed by *Coprinus* Sp. NRRL-20638 (7.4%), *Cop. stercoreus* NRRL-30953 (8.9%), *Panaeolus* Sp. FP-102035-Sp (13.2%), *M. oreades* NRRL-2602 (13.5%) and *R. crocatus* MJL-1465-Sp (14.7%). It was observed that fungal pretreatment can substantially densify the pretreated corn stover substrate (data not shown).

3.4. Saccharification of biologically pretreated corn stover

The results of enzymatic saccharification of fungal pretreated corn stover at pH 5.0 and 45 °C for 72 h using a cocktail of 3 commercial enzyme (cellulase, β -glucosidase and hemicellulase) preparations containing 2 FPU cellulase, 5 U β -glucosidase and 530 U xylanase per g of pretreated stover are presented in Fig. 4. The data clearly indicate that the efficiency of the enzyme cocktail for the saccharification of the pretreated corn stover differed greatly among these 26 pretreated corn stover materials. It can be inferred that enzymatic hydrolysis is closely correlated with the loss of lignin, the higher is the lignin loss, the greater is the sugar yield from the pretreated corn stover. The reduction of lignin after fungal pretreatment can increase pore

size in the substrate and therefore provide more accessible surface area to cellulase and hemicellulase (Taniguchi et al., 2005; Bak et al., 2009; Yu et al., 2009; Zhao et al., 2012). The maximum sugar yield (mg/g of pretreated corn stover) was 394 ± 13 for *Cya. stercoreus* NRRL-6573 followed by *Pyc. sanguineus* FP-103506-Sp (393 ± 17), *Phl. brevispora* NRRL-13108 (383 ± 13), *Pol. compactus* NRRL-A-2351 (333 ± 9) and *Cya. pallidus* NRRL-6529 (284 ± 13). The highest glucose yield (304 ± 10 mg/g pretreated stover) was obtained for *Cya. stercoreus* NRRL-6573 whereas the highest xylose yield was 113 ± 3 mg/g stover for *Phl. brevispora* NRRL-13108. The digestibility of the pretreated corn stover after enzymatic hydrolysis under the conditions tested was improved for only half of the strains investigated for pretreatment.

Summary of component losses after pretreatment and sugar yield after enzymatic hydrolysis of six superior white-rot fungal strains (based on total sugar yield) from the pretreated corn stover are presented in Table 1 and Table 2, respectively. These data are for a fixed (30 days) time period for all these strains. In addition, very low dose of cellulase (2 FPU/g pretreated stover) was used in this study for enzymatic hydrolysis with the assumption that fungal pretreated lignocellulose would be more easily degraded by enzymes. Moreover, the data indicate that the xylanase preparation used in this investigation was not completely effective in degrading corn stover hemicellulose.

It is expected that by optimizing the solid state cultivation conditions with respect to particle size of the feedstock, reactor design, inoculum size, temperature, moisture, nutrient supplements, aeration and duration, and enzymatic saccharification conditions with respect to the choice of commercial enzyme preparations and their dose levels, the sugar yield could be improved significantly (Tian et al., 2012; Wan and Li, 2012). Taniguchi et al. (2005) reported that the total sugar yields of rice straw following its pretreatment with *Pleurotus ostreatus* for 60 days were

33% based on mass of untreated straw after enzymatic hydrolysis. They showed that the increase in susceptibility of rice straw to enzymatic hydrolysis by pretreatment with *Ple. ostreatus* is caused by partial degradation of the lignin seal. The presence of the lignin seal prevents penetration by degrading enzymes. The pretreatment of rice straw by *Dichomitus squalens* for 15 days resulted in the enzymatic digestibility of 58.1% of the theoretical glucose yield for the remaining glucan (Bak et al., 2010). Hatakka (1983) reported that *Ple. ostreatus* converted 35% of wheat straw to reducing sugars after 35 days of cultivation. A saccharification yield of 36% was obtained from corn stover pretreated with *Cya. stercoreus* for 29 days when enzymatic hydrolysis was conducted at 60 FPU/g glucan for 136 h (Keller et al., 2003). The glucose yield was doubled in comparison with control. However, pretreatment of corn stover with *Pha. chrysosporium* for 29 days did not significantly increase the saccharification yield compared to the control, probably due to too long pretreatment time. Liu et al. (2013) reported that the enzymatic hydrolysis efficiency for switch grass with 36 days pretreatment using *Pycnoporus* sp. SYBC-L3 was about 50% greater than the untreated one. Zhang et al. (2007) obtained a maximum saccharification yield of 37% from *Coriolus versicolor* pretreated bamboo residues. Lee et al. (2007) reported a 21% sugar yield from Japanese red pine pretreated with *Stereun hirsutum* for 8 weeks, of which 14% was glucose. It was shown that combination of fungal pretreatment with a second pretreatment with mild acid or liquid hot water can enhance the enzymatic hydrolysis of lignocellulosic biomass further (Ma et al., 2010; Wang et al., 2012).

The use of ligninolytic enzymes, which can partially degrade the lignin and loosen its molecular structure, for pretreatment of lignocellulosic biomass, which only takes several hours to a couple of days has the potential to overcome the problems associated with carbohydrate loss and long pretreatment time (Ramos et al., 2004; Wang et al., 2013). Recently, Larran et al.

(2015) found that pretreatment of *Spartina argentinensis* (C4 grass) with *Pyc. sanguineus* supernatant improves fermentable carbohydrate availability, compared with a conventional chemical pretreatment. About 57% of cellulose can be hydrolyzed using this pretreatment. This area deserves further investigation.

3. 5. Ethanol production ability by white-rot fungal strains

Consolidated bioprocessing such as single stage bioconversion of lignocellulosic feedstock to ethanol is gaining importance because of process simplicity and reduction in cost (Saha, 2004). It is, therefore, of interest to evaluate the ability of the white-rot fungal strains for production of ethanol from glucose and xylose. The ethanol production from glucose (1%, w/v) and 1% (w/v) xylose by all 26 white rot fungal strains was investigated under semi-aerobic conditions at 28 °C for 3 days. Only three strains were able to produce ethanol from glucose. *A. bisporus* NRRL-20762, *B. adusta* FP-101809-Sp and *I. lacteus* FP-101234-Sp produced 0.31 ± 0.01 , 0.24 ± 0.10 and 0.13 ± 0.01 g ethanol per g glucose, respectively. None of the 26 strains produce ethanol or xylitol from xylose even though most of them used xylose. Under aerobic condition, 2 strains – *F. velutipes* NRRL-2363 and *Cya. stercoreus* NRRL-6573 produced 0.30 ± 0.00 and 0.21 ± 0.00 g ethanol per g glucose, respectively. None of the 26 strains produced ethanol from xylose and only *F. velutipes* strain produced xylitol (0.57 ± 0.00 g/g) from xylose.

4. Conclusions

This study demonstrates the feasibility of using white-rot fungal pretreatment of corn stover to improve the efficiency of enzymatic hydrolysis. Based on increased digestibility, a number of strains such as *Cya. stercoreus* NRRL- 6573, *Pyc. sanguineus* FP-103506-Sp, *Phl. brevispora*

NRRL- 13108, *Pol. compatus* NRRL- 2351 and *Cya. pallidus* NRRL- 6529 were found to be suitable for further optimization of biological pretreatment of corn stover for production of fermentable sugars. While an increase in digestibility was achieved, it was also at the expense of carbohydrate lost during pretreatment. Optimization of the pretreatment conditions, especially duration of pretreatment, may improve the sugar yield by a selected white-rot fungal strain.

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Figure Captions

Fig. 1. Weight loss (%) of corn stover after pretreatment with 26 white-rot fungal strains for 30 days at 28 °C and 74% moisture level. The data presented is average $\pm$ standard deviation for triplicate experiments for each fungal strain. Different letters above the bars are significantly ($p < 0.05$) different.

Fig. 2. Lignin (A), hemicellulose (B) and cellulose (B) loss (%) of corn stover after pretreatment with 26 white-rot fungal strains for 30 days at 28 °C and 74% moisture level. The data presented is average $\pm$ standard deviation for triplicate experiments for each fungal strain. Different letters above the bars are significantly ($p < 0.05$) different.

Fig. 3. Composition (cellulose, hemicellulose and lignin) of the remaining corn stover after pretreatment with 26 white-rot fungal strains for 30 days at 28 °C and 74% moisture level. The data presented is average for triplicate experiments for each fungal strain. Different letters above the bars are significantly ($p < 0.05$) different with respect to total components (cellulose + hemicellulose + lignin).

Fig. 4. Sugar (glucose, xylose, arabinose and galactose) yield from 26 white rot fungi pretreated (30 days) corn stover after enzymatic hydrolysis at pH 5.0 and 45 °C for 72 h using a cocktail of 3 commercial enzyme (cellulase, β -glucosidase, hemicellulase) preparations. The data presented is average for triplicate experiments for each fungal strain. Different letters above the bars are significantly ($p < 0.05$) different with respect to yield of total sugars.

Fig. 1.

Fig. 2.

Fig. 3.

Fig. 4.

Table 1

Component loss of corn stover after pretreatment with white-rot fungal strains for 30 days at 28°C and 74% moisture level.

Strain	Weight loss (%)	Lignin loss (%)	Hemicellulose loss (%)	Cellulose loss (%)	Selectivity value ¹
<i>Coprinus stercoreus</i> NRRL-20953	10.1±0.6 ^a	0.0±0.0 ^a	14.9±1.2 ^a	7.8±0.4 ^a	0.00 ^a
<i>Cyathus pallidus</i> NRRL-6529	39.2±0.9 ^c	38.1±3.0 ^b	42.6±2.3 ^{bc}	28.3±0.6 ^{de}	1.35 ^b
<i>Cyathus stercoreus</i> NRRL-6573	40.4±2.0 ^c	46.2±0.7 ^{cd}	51.8±1.8 ^c	32.1±1.6 ^{ef}	1.44 ^{bc}
<i>Phlebia brevispora</i> NRRL-13108	32.2±2.7 ^b	39.6±1.6 ^b	42.1±1.0 ^b	16.1±1.8 ^b	2.46 ^e
<i>Polyporus compactus</i> NRRL-A-2351	40.9±0.1 ^c	45.4±1.5 ^{cd}	52.1±1.3 ^c	33.1±1.4 ^f	1.37 ^{bc}
<i>Pycnoporus sanguineus</i> FP-103506-Sp	38.6±1.0 ^c	51.0±1.2 ^d	50.7±2.1 ^c	25.4±0.3 ^{cd}	2.01 ^d

The data presented are mean ± standard deviation for triplicate experiments. Different letters in superscript following the means indicate significant difference. Statistical significance was determined using ANOVA and Tukey's HSD means comparison ($p < 0.05$).

¹Ratio of lignin loss and cellulose loss.

Table 2

Sugar yield after enzymatic hydrolysis (at pH 5.0, 45 °C for 72 h) of corn stover pretreated with white-rot fungal strains for 30 days at 28 °C and 74% moisture level.

Strain	Glucose yield (mg/g) ¹	Xylose yield (mg/g) ¹	Total sugar yield (mg/g) ¹
<i>Coprinus stercoreus</i> NRRL-20953	169±4 ^a (41%)	75±0 ^{ab} (29%)	265±4 ^a (36%)
<i>Cyathus pallidus</i> NRRL-6529	202±9 ^b (46%)	70±3 ^a (28%)	284±13 ^a (38%)
<i>Cyathus stercoreus</i> NRRL-6573	304±10 ^d (64%)	80±2 ^b (35%)	394±13 ^c (52%)
<i>Phlebia brevispora</i> NRRL-13108	252±8 ^c (51%)	111±3 ^d (48%)	383±13 ^c (48%)
<i>Polyporus compactus</i> NRRL-A-2351	224±7 ^b (50%)	91±1 ^c (40%)	333±9 ^b (46%)
<i>Pycnoporus sanguineus</i> FP-103506-Sp	272±14 ^c (57%)	106±0 ^d (45%)	393±17 ^c (52%)

The data presented are mean ± standard deviation for triplicate experiments. The number in parenthesis indicates the yield in percentage. Different letters in superscript following the means indicate significant difference. Statistical significance was determined using ANOVA and Tukey's HSD means comparison ($p < 0.05$).

¹Yield in mg/g of pretreated corn stover.