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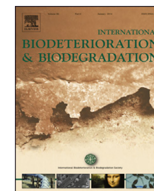

Biological pretreatment of corn stover with white-rot fungus for improved enzymatic hydrolysis

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ABSTRACT

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).

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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

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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 μm) was purchased from Nalge Company, Rochester, NY, USA. Aminex HPX 87P column (300 × 7.8 mm), Aminex HPX 87H column (300 × 7.8 mm), De-ashing cartridge (30 × 4.6 mm), Carbo-P micro-guard cartridge (30 × 4.6 mm), and Cation H micro-guard cartridge (30 × 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, *Bjerkandera fumosa* FP-135285-T, *Ceriporiopsis pannocincta*FP-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 *Rigidoporus 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 $CaCO_3$ 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 NH_4NO_3, 0.8 g KH_2PO_4, 2 g $NaHPO_4$, 5 g $MgSO_4 \cdot 7H_20$ 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, μmol 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 μL Celluclast, 5 μL Novozyme 188 and 50 μL Fiberzyme per g of corn stover. After enzymatic hydrolysis, the residual solids were separated from the liquid by centrifugation (12,000 × 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 $MgSO_4$, $7H_2O$ 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

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