



Fungal pretreatment of cornstalk with *Phanerochaete chrysosporium* for enhancing enzymatic saccharification and hydrogen production

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ABSTRACT

The feasibility of fungal pretreatment of cornstalk with *Phanerochaete chrysosporium* for enzymatic saccharification and H₂ production was investigated in this study. Firstly, cornstalk was pretreated with *P. chrysosporium* at 29 °C under static condition for 15 d, lignin reduction was up to 34.3% with holocellulose loss less than 10%. Microscopic structure observation combined FTIR analysis further demonstrated that the lignin and crystallinity were decreased. Subsequently, the fungal-pretreated cornstalk was subjected to enzymatic hydrolysis by the crude cellulase from *Trichoderma viride* to produce fermentable sugars which were then fermented to bio-H₂ using *Thermoanaerobacterium thermosaccharolyticum* W16. The maximum enzymatic saccharification was found to be 47.3% which was 20.3% higher than the control without pretreatment. Upon fermentation of enzymatic hydrolysate, the yield of H₂ was calculated to be 80.3 ml/g-pretreated cornstalk. The present results suggested the potential of using hydrogen-producing bacteria for high-yield conversion of cornstalk into bio-H₂ integrate with biological pretreatment and enzymatic saccharification.

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1. Introduction

Increased concerns over the excessive utilization of limited energy resources and environmental impacts of fossil fuel use have inspired worldwide research and development of hydrogen gas (H₂) as an alternative. Anaerobic hydrogen fermentation seems to be favorable among various hydrogen production methods, since hydrogen can be yielded at a high rate in an environmental benign and energy-saving process (Kumar and Das, 2000; Ren et al., 2009; Zhao et al., 2012). Currently, fermentative hydrogen production is primarily from simple carbohydrates, such as glucose, sucrose, starch and waste water contain these compounds (Akutsu et al., 2009; Davila-Vazquez et al., 2009; Yuan et al., 2009), which greatly increase the hydrogen production cost. Lignocellulosic biomass, mainly from agricultural residues, municipal wastes and forestry sources (Wan and Li, 2010), is particularly well-suited for hydrogen application because of its large-scale availability, low cost, and environmentally friendly production. Cornstalk, as the most abundant agricultural residue in China, has the greatest potential to be used for hydrogen production.

However, lignocellulose has a relatively complex structure, resulting in highly resistant to enzymatic hydrolysis and low cellulose conversion. Many researchers have reported that without appropriate pretreatment, only 20% theoretical maximum sugar yield of cornstalk can be obtained from enzymatic hydrolysis (Kim et al., 2006; Mosier et al., 2005). Therefore, the development of a pretreatment process to separate lignin and hemicellulose from cellulose, deconstruct the cellulosic polymers and disrupt the crystalline structure of lignocellulose for improving enzymatic hydrolysis efficiency is urgent. The current leading pretreatment processes (e.g. diluted acid, steam explosion, alkali extraction, hydrothermolysis, etc.) constantly require high energy (steam and electricity), corrosion resistant, and high-pressure reactors. During the pretreatment procedures, great amount of inhibitors are produced (Bak et al., 2009), as well as loss in holocellulose (cellulose and hemicelluloses), which would hinder the enzymatic saccharification and hydrogen fermentation. Biological pretreatment using white rot fungus is increasingly being advocated as a process which not only does not have the disadvantages mentioned above, but also requires less energy contribution for lignin removal from lignocellulosic biomass. This process has been receiving extensive attention for biodelignification of lignocellulosic biomass. Dias et al. (2010) found that the initial ratio of cellulose/lignin in the wheat straw of 2.7 was increased to 5.9 and 4.6 after fungal pretreatment by *Basidiomycetes* Euc-1 and *Irpex lacteus*, respectively.

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29% of lignin in bamboo was reduced after a 30 d pretreatment with *Echinodontium taxodii* 2538 (Zeng et al., 2011). *Trametes versicolor* pretreatment of *Acacia mangium* resulted in a maximum lignin loss of 26.9% after 60 d (Liew et al., 2011).

Therefore, this study was undertaken to determine if fungal pretreatment could improve enzymatic saccharification and H₂ production from cornstalk. The biodelignification characteristics of fungal-pretreated cornstalk were described first. After that the enzymatic saccharification efficiency of fungal-pretreated cornstalk by crude enzyme from *T. viride* was evaluated to produce higher amount of reducing sugars. At last, the fermentable sugars were fermented to H₂ by *T. thermosaccharolyticum* W16. This study is the first to combine the white rot fungus pretreatment with enzymatic digestibility to produce reducing sugars for H₂ production.

2. Methods

2.1. Raw material

Air-dried cornstalk was obtained from Northeast Agricultural University, Harbin, Heilongjiang province, China. The raw material was milled to pass through a 40-mesh screen, and then dried at 105 °C until constant weight was obtained.

2.2. Fungal strain and bio-pretreatment of cornstalk

The white rot fungus *Phanerochaete chrysosporium* (CGMCC5.776) was obtained from China General Microbiological Culture Collection Center and maintained on Potato Dextrose Agar (PDA) plates at 4 °C. The seed culture for pretreatment was prepared as the method described by Zeng et al. (2011) at 29 °C, a rotation speed of 150 rpm for 3 d. Fungal pretreatment was performed in 250 ml flasks containing 10 g of cornstalk at a moisture content of 70%. Erlenmeyer flasks containing wet cornstalk were autoclaved at 121 °C for 20 min prior to inoculation with 10 ml seed culture. Pretreatment was carried out in a constant temperature incubator at 29 °C under static condition for 21 d. The samples were taken every third day for further analysis. All samples were subjected to enzymatic hydrolysis to evaluate saccharification rate. Samples without fungal inoculation were used as controls. All tests were performed in triplicate.

2.3. Enzymatic hydrolysis of fungal-pretreated cornstalk

Clarified supernatant of *Trichoderma viride* (CGMCC 3.2876), which was obtained from China General Microbiological Culture Collection Center and cultivated as described by Mandels and Reese (1957) for 4 d, was used as the source of cellulase for hydrolysis. The activity of cellulases and xylanase were analyzed according to the method reported by Ghose (1987). The international units (IU) of cellulases and xylanase were defined as the amount of enzyme required to liberate 1 μmol glucose or xylose equivalent per minute. Batch enzymatic hydrolysis of fungal-pretreated cornstalk was performed in 250 ml flasks at a solid concentration of 3.5% (w/v) in 100 ml mixture of 50 mM citrate buffer (pH 5.0) with cellulase loading of 30 FPA/g substrate. The hydrolysis was carried out at 50 °C and 130 rpm on a rotary shaker for 96 h. Simultaneously, untreated cornstalk was taken as a control, which was hydrolyzed at the same conditions as mentioned above. The hydrolyzed slurry was boiled to inactive cellulase. After cooling to room temperature, the slurry was centrifuged and the supernatant was kept at 4 °C for further use and analyze. The enzyme saccharification rate was calculated as (Li et al., 2009): enzymatic saccharification (%) = reducing sugars released (g) × 0.9 × 100/polysaccharides in substrate (g) where 0.9 is the correction coefficient for hydrolysis.

2.4. Biohydrogen production in batch culture

The cornstalk hydrolysate was used as carbon source to produce H₂ by *T. thermosaccharolyticum* W16 isolated by Ren et al. (2008). Hydrogen production was performed in 100 ml serum vials with working volume of 50 ml. The tested hydrolysate was supplemented with the following nutrients (L⁻¹): 1.0 g NH₄Cl, 3.0 g K₂HPO₄, 1.5 g KH₂PO₄, 0.5 g MgCl₂·6H₂O, 1.0 g NaCl, 0.2 g KCl, 0.5 g cysteine-HCl, 2.0 g yeast extract, and 2.0 g tryptone. The culture temperature and pH were 60 °C and 7.0, respectively. During the course of fermentation, cell density, pH, residual carbon substrate concentration, quantity and compositions of produced biogas, and metabolic products were monitored with respect to culture time. All tests mentioned above were performed in triplicate to determine the reproducibility of the experiments.

2.5. Analytical methods

The structural carbohydrates, lignin, and moisture contents of pretreated and unpretreated cornstalk were determined followed standard analytical methods established by the National Renewable Energy Laboratory (NREL) (Hames, 2005; Sluiter, 2006). Microstructural changes in the cornstalk following fungal pretreatment was observed by scanning electron microscopic (SEM). Briefly, cornstalk samples were rinsed with water and then dried in an air oven at 60 °C until constant weight, after which they were mounted on stubs and sputter-coated with gold prior to imaging with a JEOL JSM-840 SEM using 5 kV accelerating voltage and 10 mm distance. FT-IR spectroscopy analysis for detecting the functional groups change in fungal-pretreated cornstalk was carried out using a Magna-IR 750 (Nicolet Instrument Co., USA). Discs were prepared by first mixing 1.5 mg of powered sample with 200 mg of KBr in an agate mortar (Monrroy et al., 2011). The resulting mixture was successively pressed at 20 MPa. Spectra were recorded between 4000 and 400 cm⁻¹. The background spectrum of pure potassium bromide was subtracted from the sample spectrum.

The gas products (H₂ and CO₂) were analyzed by gas chromatography (4890D, Agilent Cooperation, USA) using a thermal conductivity detector. High performance liquid chromatography (HPLC) system (LC-10A, Shimadzu Corporation, Kyoto, Japan) was used to detect the initial sugar compositions of the hydrolysate, and the sugar consumption during fermentation (Ren et al., 2010). The soluble metabolites were detected by gas chromatography (4890D, Agilent Cooperation, USA) equipped with a hydrogen flame-ionization detector (FID).

3. Results and discussion

3.1. Physical and chemical characteristics of cornstalk after fungal pretreatment

To investigate the biodegradation characteristics of *P. chrysosporium* on cornstalk, the changes of chemical components were analyzed and the results are shown in Fig. 1, lignin content in cornstalk decreased with cultivation time, and a maximum loss of 35.3% was reached after 21 d fermentation, accompanying with slightly holocellulose reduction of 9.5%. The residue solid yield was high, almost above 72% of the initial weight. Nevertheless, after 15 d pretreatment, the decrease of lignin is not significant, considering both energy consumption and lignin removal efficiency, in the rest of this study, the cornstalk was pretreated by *P. chrysosporium* for 15 d.

The morphological changes that were induced by *P. chrysosporium* with varied pretreatment time were examined by SEM

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