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Ethanol production via simultaneous saccharification and fermentation of sodium hydroxide treated corn stover using *Phanerochaete chrysosporium* and *Gloeophyllum trabeum*

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HIGHLIGHTS

- Innovative coupling of mild alkaline pretreatment and fungal saccharification.
- Ethanol production from corn stover without the addition of costly enzymes.
- Improved ethanol production via cultivation of fungal and bacterial fermentation.
- Ethanol production more sustainable on fungal pretreated and cultivated corn stover.

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ABSTRACT

Ethanol was produced via the simultaneous saccharification and fermentation (SSF) of dilute sodium hydroxide treated corn stover. Saccharification was achieved by cultivating either *Phanerochaete chrysosporium* or *Gloeophyllum trabeum* on the treated stover, and fermentation was then performed by using either *Saccharomyces cerevisiae* or *Escherichia coli* K011. Ethanol production was highest on day 3 for the combination of *G. trabeum* and *E. coli* K011 at 6.68 g/100 g stover, followed by the combination of *P. chrysosporium* and *E. coli* K011 at 5.00 g/100 g stover. SSF with *S. cerevisiae* had lower ethanol yields, ranging between 2.88 g/100 g stover at day 3 (*P. chrysosporium* treated stover) and 3.09 g/100 g stover at day 4 (*G. trabeum* treated stover). The results indicated that mild alkaline pretreatment coupled with fungal saccharification offers a promising bioprocess for ethanol production from corn stover without the addition of commercial enzymes.

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1. Introduction

Ethanol is an important transportation fuel, either by itself, or as additive to gasoline (Rodríguez-Antón et al., 2013). According to a study by Fukuda et al. (2009), it is estimated that approximately 73% of global ethanol production is used in the transportation sectors. Usage increased from a little over 18.2 billion liters in 2000 to approximately 83 billion liters in 2012, with bioethanol contributing to about 85% of the total amount (Sainz, 2009; GRFA, 2013).

Biofuel research has been intensified on lignocellulosic materials such as cotton stalk, corn stover, baggase (sugar cane waste), rice straw, wood chips or other "energy crops" (fast-growing trees and grasses), as the primary sustainable bioethanol feedstock (Mosier et al., 2005; Murphy et al., 2007; Dwivedi et al., 2009; Binod et al., 2012). Meyer et al. (2013) expects approximately 78.75 billion liters of cellulosic ethanol to be produced by 2022. However, lignocellulosic ethanol production faces many obstacles as the individual feedstocks such as cellulose and hemicellulose have to be liberated from the encasing lignin barriers. These are usually achievable via pretreatment that include Ammonia Fiber Explosion/Expansion (AFEX), Ammonia Recycle Percolation (ARP), dilute acid and lime pretreatments (Aden and Foust, 2009; He et al., 2010; Kim et al., 2009). These pretreatments, although effective to some degree, are highly unfavorable in the mass production of

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fuel ethanol, especially the more severe ones that may introduce unwanted inhibitory compounds, and, the need for downstream post treatment, usually associated with disposal of leftovers (Talebnia et al., 2005; Binod et al., 2011). Furthermore, according to several other reports, severe pretreatments can be expensive with costs as high as 30 ¢/gallon of ethanol produced (Mosier et al., 2005; Wyman et al., 2005). Nonetheless, these pretreatments are crucial for effective hydrolysis of lignocellulosic materials (Binod et al., 2011). Thus, more studies are needed to optimize their use in sustainable and commercial production of lignocellulosic ethanol (Hendriks and Zeeman, 2009; Meyer et al., 2013).

One possible solution to reduce the severity of the pretreatment and cost of enzymes is to complement a mild pretreatment process with a fungal bioprocess, as suggested by Shrestha et al. (2009). The approach in this study was initial pretreatment of corn stover with mild sodium hydroxide (NaOH) solution, followed by a whole-cell fungal saccharification, employing the lignocellulolytic wood-rot fungi *Phanerochaete chrysosporium* and *Gloeophyllum trabeum*. Both these fungal species have been studied extensively for their abilities to degrade, depolymerize and modify all major components of plant cell walls including cellulose, hemicellulose, and the more recalcitrant lignin (Kerem et al., 1999; Cohen et al., 2005; Wymelenberg et al., 2005; Suzuki et al., 2008). Like most other wood-degrading fungi, *P. chrysosporium* and *G. trabeum* effectively perform these biomass degradations via the secretion of various cellulases and hemicellulases (Cohen et al., 2005; Wymelenberg et al., 2005; Vincent et al., 2011a,b). Finally, the saccharification products were fermented to ethanol using *Saccharomyces cerevisiae* or *Escherichia coli* K011.

2. Methods

2.1. Experimental setup

A flow-chart of the overall experimental setup is shown in Fig. 1. All experiments and analyses were done in triplicate ($n = 3$).

2.2. Corn stover pretreatment and analysis

Corn stover used in this study was obtained from the Department of Agronomy, Iowa State University. The field dried corn stover was ground and then screened using a 20 mesh sieve. To perform the dilute NaOH pretreatment of the corn stover, 98 g of stover was mixed with 800 ml solution of 0.25% (w/v) NaOH in water, thereby using 2% (w/w) NaOH per g solids. The mixture was autoclaved at 121 °C for 1 h followed by rinsing with distilled water. The pH was then adjusted to a final pH of 4.8–5.0

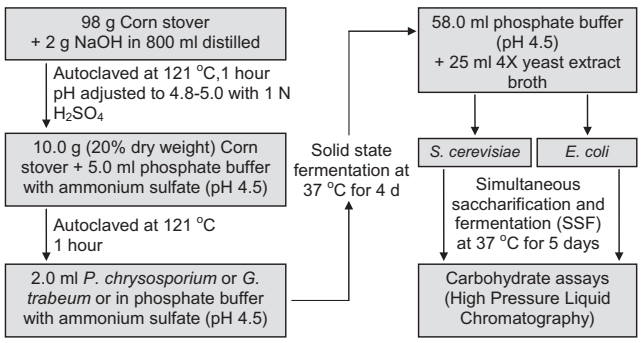

Fig. 1. Flow-chart of process outlining the steps for dilute NaOH treatment of corn stover, followed by solid state fermentation of *P. chrysosporium* and *G. trabeum* on corn stover, and simultaneous saccharification and fermentation (SSF) using *S. cerevisiae* and *E. coli* K011.

with 1 N (0.5 M) H_2SO_4 at room temperature. The acidified corn stover was then press dried in cheese cloth to a 20% (w/w) solid content.

For compositional determination, oven-dried corn stover and NaOH treated corn stover were subjected to cellulose and hemicellulose analysis using the ANKOM method as described previously (Vogel et al., 1999). Lignin content was determined by using a modified Klason lignin assay, where by glass fiber filters (1.6 μm) (Fisherbrand, Fisher Scientific, Pittsburgh, PA) were used instead of Whatman No. 1 filter papers for capturing the solid lignin residues (Crawford and Pometto, 1988).

2.3. Microorganism culture preparation

Stock cultures of *P. chrysosporium* (ATCC 24725), *G. trabeum* (ATCC 11539), *S. cerevisiae* (ATCC 24859) and *E. coli* K011 (ATCC 55124) used in this study were obtained from the American Type Culture Collection (Rockville, MD). *P. chrysosporium* and *G. trabeum* cultures were grown in 1 L of Yeast Mold (YM) Broth (Difco Laboratories, Detroit, MI) and incubated at 30 °C, agitated at 150 rpm. After 7 days of growth, the fungal mycelia were harvested via centrifugation at 7277*g* for 20 min using a Sorvall-RC3B Plus centrifuge (Thermo Fisher Scientific, Wilmington, DE) (Vincent et al., 2011b). The collected fungal mycelia were rinsed with a mineral salt solution (pH 4.5–4.8) containing 50 mm phosphate buffer, 0.5% $(NH_4)_2SO_4$ and basal medium (0.25 g of KH_2PO_4, 0.063 g of $MgSO_4{\cdot}7H_2O$, 0.013 g of $CaCl_2{\cdot}2H_2O$, and 1.25 ml of trace element solutions (Sigma Aldrich, St. Louis, MO) in 1 L of deionized water).

S. cerevisiae and E. coli K011 culture inocula were prepared in sterile 50 ml of YM Broth at 32 °C with constant agitation (120 rpm). The respective yeast and bacterial cells were harvested via centrifugation in a 50 ml conical centrifuge tubes (BD Falcon, BD, Franklin Lakes, NJ) at 2852*g* for 10 min using a Beckman J2-21 centrifuge (Beckman Coulter Inc., Brea, CA) and cell density was adjusted by using sterile YM Broth to 10^7–10^8 CFU/ml as determined at 600 nm via a standard curve (Vogel et al., 1999).

2.4. Solid state fermentation

Solid-state fermentation was performed on the sterilized corn stover (10 g, 20% dry solid weight) that was mixed with 5 ml mineral salt medium, mentioned as mentioned above, and three marble balls to assist in the even distribution of the fermentation mixtures (Shrestha et al., 2008; Vincent et al., 2011b). Next, rinsed *P. chrysosporium* or *G. trabeum* inocula were mixed with fungal mineral salt solution, and 2 ml of the mixture was added to the sterilized corn stover, followed by incubation at 37 °C in a humidity controlled chamber for four days to induce enzyme production (Shrestha et al., 2009; Vincent et al., 2011b).

2.5. Simultaneous saccharification and fermentation (SSF)

SSF experiments were carried out in batch cultures of 100 ml, consisting of 25 ml of 4× Yeast Extract Broth and buffered basal medium as mentioned previously (Shrestha et al., 2009; Vincent et al., 2011b). The flasks were then aseptically inoculated with *S. cerevisiae* or *E. coli* K011 cultures. These SSF culture bottles were incubated for 5 days at 37 °C under static conditions.

2.6. Protein and enzyme activities assays

Protein production by *P. chrysosporium* or *G. trabeum* cultured on corn stover was measured via the NanoDrop™ 1000 spectrophotometer (Thermo Fisher Scientific). This system measures a loading of 2 μl sample size and calculates the protein