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An efficient strategy for enhancing enzymatic saccharification with delignified fungus *Myrothecium verrucaria* and solid acid

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ABSTRACT

In this work, corn stover was studied as a lignocellulosic material with the potential of serving as a feedstock in the field of bioenergetics. To improve the enzymatic digestibility of carbohydrates in the lignocellulosic biomass, corn stover was pretreated using *Myrothecium verrucaria* to deplete the lignin, combined with a solid acid pretreatment method to deplete the hemicellulose. The effects of lignin degradation conditions and solid acid concentration on cellulose conversion were investigated. The two-stage pretreatment with *M. verrucaria* and solid acid under optimal conditions gave 81.54% cellulose conversion yield, 214.22% higher than that of untreated corn stover. Significant lignin and hemicellulose removal rates were observed with the two-stage pretreatment. Scanning electron microscope (SEM), X-ray diffractometer (XRD) and Fourier transform infrared spectroscopy (FTIR) analyses showed major structural changes in the pretreated corn stover. This study provided an efficient strategy to enhance the enzymatic saccharification of cellulose in corn stover.

1. Introduction

Lignocellulosic material is an abundant and carbon-neutral energy resource (Tang et al., 2017). Sugars derived from lignocellulose are key platform chemicals that can be further converted into biofuels and other high-value chemicals via biological and/or chemical routes (Wettstein et al., 2012). High-efficient utilization of lignocellulose has great potential to solve the problems of the growing risk of fossil fuel shortages and the high degree of global pollution (Saha et al., 2016; Silva et al., 2018). Among the available reusable lignocellulose materials, corn stover is an abundant resource that has not been well-utilized, leading to both resource waste and environmental pollution (Perlack et al., 2005). Corn stover is composed of about 60 wt% of carbohydrates, which can be converted into sugars (Lee, 1997). Cellulose, hemicellulose and lignin are the main components of corn stover. Cellulose and hemicellulose bound together by an impermeable lignin layer (Liu et al., 2015).

Enzymatic hydrolysis is considered as a promising method to obtain fermentable sugars from cellulose due to its mild processing conditions and high selectivity (Chaturvedi and Verma, 2013). However, cellulose is hard to digest due to the covalent interactions and linkages between lignin and hemicellulose. Lignin provides a physical barrier that limits the accessibility of cellulase to cellulose and thus reduces the hydrolysis efficiency (Kumar et al., 2015a,b; Várnai et al., 2010). It is essential to apply an efficient pretreatment method to facilitate conversion of cellulose to sugars or other high-value products (Saha et al., 2016).

At present, corn stover pretreatments for cellulose biotransformation can be classified into physical, chemical, biological and combined methods. It was reported that sulfuric acid, hydrochloric acid, solid acid and inorganic salts pretreatments can effectively reduce the content of hemicellulose in biomass (Lloyd and Wyman, 2005; Wang et al., 2017a,b). The conversion yield of cellulose significantly increased after pretreatment with these kinds of acid. Lignin depletion of lignocellulose is also a key factor affecting the cellulose saccharification rate. Most alkali pretreatments, such as NaOH solution and ammonia pretreatments, can effectively remove lignin from biomass (Zheng et al., 2009). Another efficient method of depleting lignin is biological pretreatment, which is widely applied due to the advantages of being environmentally friendly and low energy requirement (Hakala et al., 2004). The most investigated type of fungus for lignin degradation is white-rot fungus, such as *Phanerochaete chrysosporium, Pleyrotus ostreatus* and *Coridus versicolor* (Bak et al., 2009; Taniguchi et al., 2005; Wang et al., 2013a,b). While some white-rot fungi can simultaneously degrade lignin and polysaccharides, resulting in the loss of carbohydrates (Guerra et al., 2003). *Myrothecium verrucaria* has been reported as a novel lignin depletion fungus that leads to lignin degradation reaching 45.50 ± 2.12% in birch sawdust, and it was effective in removing lignin selectively (Wang et al., 2017a,b). Combined pretreatment methods have also been adopted to enhance pretreatment effects. Ramadoss and Muthukumar (2015) proved that the performance of

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dilute acid pretreatment could be enhanced when combined with salts pretreatment. Xu et al. (2017) employed dilute aqueous ammonia combined with ultrasonic pretreatment and reported that the largest enzymatic hydrolysis sugar yield was 80.6%, 66.2% and 56.9% for corn cob, corn stover and sorghum stalk, respectively. However, major drawbacks to most pretreatment technologies still exist, such as toxicity and energy demand for physical and chemical methods and time consumption for biological methods (Keller et al., 2003; Wang et al., 2017a,b). Furthermore, a washing step is needed to separate biomass and mycelium for most of the biological methods (Li et al., 2014; Zhou et al., 2015). Therefore, new strategies which significantly increase cellulose conversion yield, avoid toxicity and energy demand requires further study.

In this study, a reported fungus, *M. verrucaria*, with high lignin depletion capacity was adopted to pretreat corn stover via forms of solid-state fermentation (SSF), and the lignin degradation conditions were optimized. Then, a hemicellulose depletion pretreatment process was carried out with several acids. The effect of enzymatic hydrolysis was evaluated after each step of pretreatment. An efficient strategy for enhancing the enzymatic saccharification of corn stover was developed.

2. Materials and methods

2.1. Lignin-degrading pretreatment procedure

Corn stover was cleaned, milled, screened with 60–80 mesh and then dried to constant weight in an oven at 80 °C for 24 h. The process of fungal pretreatment was performed in a 250-mL Erlenmeyer flask under SSF. For this process, 10 g of corn stover and 30 mL of liquid fermentation medium (2.0 g/L $(NH_4)_2SO_4$, 2.0 g/L KH_2PO_4, 0.3 g/L $MgSO_4$, 0.3 g/L $CaCl_2$, 0.5 g/L NaCl, 0.005 g/L $FeSO_4$, 0.016 g/L $MnSO_4$, and 0.017 g/L $ZnCl_2$) were mixed and then sterilized at 121 °C for 20 min and cooled down to room temperature. 2% (v/w) conidia spore suspension of *M. verrucaria* was inoculated into the corn stover culture, and the spore concentration was 2×10^7 spores/g solid medium. The Erlenmeyer flask was sealed and cultivated at 29 °C for 14 d.

The solid residue was collected and washed with 0.1% NaOH solution (w/v) at 180 rpm for 2 h after fungal pretreatment. The solution was centrifuged, and the precipitate was washed with deionized water until a neutral pH was reached and then dried to constant weight in an oven at 80 °C for 24 h. Each fungus was inoculated in triplicate.

2.2. Optimization of lignin degradation by M. verrucaria pretreatment

The inoculation amount of *M. verrucaria* conidia spore suspension ranged from 1% to 5% (the spore concentration was 1×10^7–5×10^7 spores/g solid medium, accordingly), the fermentation time ranged from 7 to 35 d, the solid-to-liquid ratio ranged from 1:2.5 to 1:4.5, and the pH of the liquid fermentation medium ranged from 4.5 to 6.5, which prepared with 10 mM phosphate buffer solution. The optimization experiments were conducted in 250-mL Erlenmeyer flasks with 10 g of corn stover.

2.3. Comparison among different hemicellulose-depletion pretreatment methods

After fermentation at the optimum conditions with *M. verrucaria*, 1% sulfuric acid (CAS: 7664-93-6; H_2SO_4) solution (v/v) (Rocha et al., 2015), 2% hydrochloric acid (v/v) (CAS: 7647-01-0; HCl) solution (Zu et al., 2014) and 1.5% solid acid solution (w/v) (Wang et al., 2017a,b) were added to the blends of mycelium and corn stover according to a solid-to-liquid ratio of 1:20 (w/v) and then pretreated at the conditions of reported protocols for each kind of acid. After the second-step of pretreatment, corn stover was washed to neutral pH with deionized water and dried to constant weight for subsequent enzymatic hydrolysis in an oven at 80 °C for 24 h.

Solid acid solutions of 1.5%, 2.0%, 2.5% and 3.0% (v/v) were prepared and added to the corn stover at a solid-to-liquid ratio of 1:20 (w/v) and mixed well. The prepared solutions were treated at 120 °C for 80 min (Wang et al., 2017a,b). Then, the pretreated corn stover was washed to neutral pH with deionized water and dried to constant weight for subsequent enzymatic hydrolysis. Three parallel tests were performed for each sample.

2.4. Enzymatic hydrolysis assay

The conversion of cellulose to glucose was conducted to estimate the efficiency of the pretreatments. All experiments were performed in 40 mL of 50 mM sodium citrate buffer (pH 4.8) at solid consistency of 3% (w/w) cellulose in corn stover. The mixture was sterilized at 121 °C for 20 min. The suspension was further supplemented with 30 fpu/g cellulose from commercial cellulases (Novozymes, Cellic Ctec2) and 60 U/g β-glucosidase (Novozymes SP188). The flasks were incubated at 50 °C and 140 rpm in a water bath shaker for 72 h. The released glucose in the supernatants was determined by high-performance liquid chromatography (HPLC) to calculate the conversion yield of cellulose to glucose. The supernatant was filtered into HPLC vials using a polyethersulfone syringe filter (25 mm, 0.2 μm), then frozen at −20 °C. Three parallel tests were run for each sample.

2.5. Composition analysis of corn stover

The content of cellulose, hemicellulose and lignin was analysed according to the method of the National Renewable Energy Laboratory, Golden, CO, USA (Sluiter et al., 2008a,b) to evaluate the effect of pretreatment for corn stover. Untreated corn stover was selected as the control. Sugars were quantified by HPLC. Each analysis was performed in triplicate.

2.6. HPLC analysis

Sugars from enzymatic hydrolysis and compositions analysis samples were determined using a chromatographic column (Bio-Rad Aminex, HPX-87H column) equipped with a refractive index detector (Agilent 1200 Series). Filtered (0.2 μm) and degassed 0.005 M sulfuric acid solution was used as the mobile phase at a flow rate of 0.5 mL/min. The column temperature was 50 °C. Each analysis was performed in triplicate. The percent of glucan/xylan conversion was calculated as follows:

$$E_c(\%) = (m_s \times a)/P_s \times 100\% \quad (1)$$

where E_c is the polysaccharide enzymatic conversion yield (%), m_s is the monosaccharide released after enzymatic hydrolysis (g), P_s is the potential polysaccharide in corn stover (g), and a is the conversion factor in which the glucose to equivalent glucan is 0.9 and the xylose to equivalent xylan is 0.88 (Zhao et al., 2016).

2.7. Effects of pretreatment on the structure of corn stover

2.7.1. SEM analysis

The untreated and pretreated corn stover was dehydrated using a freeze dryer. The different surface morphologies of untreated and biopretreated corn stover were characterized by scanning electron microscopy (S–3400 N, Hitachi, Japan). Samples were gold sputter coated prior to analysis, and images were taken at a magnification of 1000×.

2.7.2. XRD analysis

The crystallinity of untreated and pretreated corn stover was obtained using an X-ray diffractometer (XRD-6000, Shimadzu, Japan) in conjunction with a radiation source (0.154 nm) operated at 40 kV and