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Effect of microbial pretreatment on enzymatic hydrolysis and fermentation of cotton stalks for ethanol production

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ARTICLE INFO

Article history:
Received 30 May 2007
Received in revised form
22 April 2008
Accepted 24 April 2008
Available online 13 June 2008

Keywords:
Gossypium hirsutum L
Pretreatment
Phanerochaete chrysosporium
Lignin
Fuel ethanol
Cellobiose
Submerged cultivation
Solid state cultivation

ABSTRACT

The potential of microbial pretreatment of cotton stalks by *Phanerochaete chrysosporium* to degrade lignin and facilitate fuel ethanol production was investigated under two culture conditions: submerged cultivation (SmC) and solid state (SSC) cultivation. Although microbial pretreatments showed significant lignin degradation (LD) (19.38% and 35.53% for SmC and SSC, respectively), a study on hydrolysis and fermentation of the microbial-pretreated cotton stalks showed no increase in cellulose conversion (10.98% and 3.04% for SmC and SSC pretreated samples, respectively) compared to untreated cotton stalks (17.93%). Solid state cultivation demonstrated better selectivity of 0.82 than 0.70 with submerged pretreatment. Washing of pretreated cotton stalks did not significantly increase cellulose conversion. However, heating and washing remarkably improved ($P<0.05$) cellulose conversion to 14.94% and 17.81% for SmC and SSC 14 day pretreatment, respectively. Ethanol yields, up to 0.027 g ethanol g^{-1} initial cotton stalks, were low for all untreated and pretreated samples mainly due to the low cellulose conversion. Although potential and some critical aspects of fungal pretreatment using *P. chrysosporium* have been explored in this study, additional investigation is still required especially to improve the selectivity for preferential LD and to optimize hydrolysis efficiency. The mechanism of catalytic binding of cellulolytic enzymes to cotton stalks as affected by the presence of fungal mycelia also warrants further study.

1. Introduction

Increasing gas prices and environmental concerns, in recent years, have become the driving force for developing alternative energy sources, especially fuel ethanol for automobiles [1]. Currently, corn is the primary raw material for ethanol production in the United States, and fuels cars at the rate of 12.87 hm^3 per year [2]. However, sustainable production of ethanol from corn requires further investigation into environmental metrics [3]. Additionally, issues such as limited feedstock supply and use of corn as a livestock feed source need to be addressed. Lignocellulosic biomass has the potential to provide a more economical feedstock as a result of its widespread availability, sustainable production, and low starting value [4]. Pretreatment, as the first step towards conversion of lignocellulose to ethanol, makes up one-third of the total production costs and remains one of the main barriers preventing commercial success [5]. Existing pretreatment methods have largely been developed on the basis of physiochemical technologies such as steam explosion, dilute acid, alkali, and oxidant or their combinations [1]. However, typical physical and chemical pretreatments require high-energy (steam or electricity) and corrosion-resistant, high-pressure reactors, which increase the cost of pretreatment

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doi:10.1016/j.biombioe.2008.04.016

operations and need for equipment. Furthermore, chemical pretreatments can be detrimental to subsequent enzymatic hydrolysis and fermentation apart from generating acidic or alkaline waste water, which needs pre-disposal treatment to ensure environmental safety [6].

A benign alternative to harsh chemicals is microbial pretreatment, which employs microorganisms especially fungi and their enzyme systems to breakdown lignin present in lignocellulosic biomass. Fungal pretreatment has been previously explored to upgrade lignocellulosic materials for feed and paper applications [7–9], and recently this environment friendly approach has received renewed attention as a pretreatment technique for enhancing enzymatic saccharification and fermentation of lignocellulosic biomass to ethanol. Several basidiomycetes such as *Phanerochaete chrysosporium*, *Ceriporiopsis subvermispora*, *Phlebia subserialis*, and *Pleurotus ostreatus* have been examined on different lignocellulosic biomass to evaluate their delignification efficiencies [6,10–12]. It has been highlighted that microbial pretreatment has potential advantages over the prevailing physiochemical pretreatment technologies due to reduced energy and material costs, relatively simple equipment, and use of biological catalysts [6]. However, the feasibility of microbial pretreatment is still questioned, mainly due to the extremely long treatment time as well as the difficulty in selectively degrading lignin [10].

P. chrysosporium is one of the most investigated white rot fungus for pretreatment because of its high growth rate compared to many other basidiomycetes, exceptional oxidation potential, and efficiency for lignin biodegradation [13,14]. Research has shown that delignification abilities of *P. chrysosporium* could be improved by optimizing nutrient supplements and cultivation methods to enhance ligninolytic enzyme production [15]. Efforts have been made to stimulate ligninase production and delignification by *P. chrysosporium* in chemically defined medium [16,17]. However, very limited resources report its application for pretreatment of lignocellulosic materials and its effects on hydrolysis and fermentation, especially to meet the needs for generation of a sugar platform.

The objective of this study was to evaluate the effects of fungal pretreatment of cotton stalks by *P. chrysosporium*, using two cultivation strategies (submerged (SmC) and solid state (SSC) cultivation) under oxygen-enriched conditions on saccharification and ethanol production. The composition profile changes in lignin, cellulose, and hemicellulose after pretreatment, hydrolysis conversion efficiency, and ethanol yield from fermentation were measured to evaluate pretreatment effectiveness. The effects of washing and combination of heating and washing prior to hydrolysis on removal of potential inhibitory factors were also investigated.

2. Materials and methods

2.1. Strain and inoculation

The fungal strain, *P. chrysosporium* (ATCC 24725), was obtained from the Forest Products Laboratory of USDA Forest Service (Madison, WI) and maintained as a frozen culture (−80 °C) in 30% glycerol. The organism was propagated on potato dextrose agar (PDA) plates for 2 days at 39 °C. Spore suspensions were prepared by washing the agar surface with 10 ml of sodium acetate buffer (50 mM, pH 4.5). Spore counts were determined with a hemacytometer (Hausser Scientific, Horsham, PA), and an inoculum with a final spore concentration of 5×10^6 spores ml^{-1} was prepared.

2.2. Biomass preparation

Cotton stalks (variety Deltapine DP5415RR), shredded and baled in the field, were harvested in early October 2003 from Cunningham Research Station, Kinston, NC (N 35°15.861′, E −77°29.940′). The stalk material with an initial M.C. of 7% was ground through a 1 mm screen for composition analysis. The feedstock for pretreatment was ground through a 3 mm screen by a Thomas Wiley Laboratory Mill (Model No. 4, Thomas Scientific, Philadelphia, PA) and stored in air tight containers at room temperature until use for pretreatment.

2.3. Pretreatment

Two strategies, SmC and SSC, previously identified as optimum [18], were applied to pretreat cotton stalks. For SmC pretreatments, 1 g of cotton stalk (air dry) was supplemented with 18 ml acetate buffer (20 mM, pH 4.5) plus 1 ml spore inoculum to obtain a 5% solid loading. For SSC pretreatment, 3 g of the cotton stalks were mixed with 4.8 ml acetate buffer (20 mM, pH 4.5) plus 3 ml spore inoculum to obtain 75% substrate moisture content (wet basis). Control flasks, without fungal inoculation, were prepared along with the pretreatment flasks and destructively sampled on days 0 and 14 to quantify the effects of substrate preparation and soaking. Since no significant difference was observed in composition analysis data between day 0 and day 14 samples, only day 14 data are reported (SmC control and SSC control). Fungal pretreatments were carried out in 250 ml Erlenmeyer flasks capped by a silicon stopper with inlet and exit lines connected to 0.2 μm filters (Acro® 37 TF, Pall Co., NY). Flasks with cotton stalks were autoclaved for 20 min (121 °C, 15 psi), cooled, mixed with buffer, and inoculated with spore suspension (5×10^6 spores g^{-1} cotton stalk). Pretreatments were performed in an air convection incubator at 39 °C and flasks were flushed with oxygen (125 ml min^{-1}) for 10 min every 3 days starting from day 0. In between flushing events, the culture flasks were closed by clamping off inlet and exit tubing lines. Triplicate flasks were destructively sampled after 14 days (SmC 14d and SSC 14d) and stored at 4 °C before composition analysis and hydrolysis.

2.4. Wash and heat wash pre-hydrolysis treatments

Prior to hydrolysis, flasks from the 14-day pretreated samples (for both SmC and SSC) were washed three times with 100 ml sterile distilled water (SmC 14d+W, SSC 14d+W) or autoclaved (121 °C, 15 psi) for 10 min, and then washed (SmC 14d+HW, SSC 14d+HW) as 'wash' and 'heat-wash' treatments, respectively. Pretreated samples (SmC 14d and SSC 14d) that were not washed and/or heated were also used in hydrolysis studies.