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Bioethanol production from alkaline-pretreated sugarcane bagasse by consolidated bioprocessing using *Phlebia* sp. MG-60

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ABSTRACT

Optimization of alkaline pretreatment of sugarcane bagasse for consolidated bioprocessing fermentation by the cellulose-fermenting fungus *Phlebia* sp. MG-60 was studied. The lignin and xylan contents of bagasse were decreased and ethanol production from each pretreated sugarcane bagasse by MG-60 was increased in an alkaline concentration-dependent manner. The fungus produced cellulase and xylanase rapidly over 120 h. When this fungus was cultured with 20 g L^{-1} of sugarcane bagasse pretreated with NaOH (0.8 wt%, 121 °C, 60 min), 4.5 g L^{-1} ethanol was produced, equivalent to 210 mg ethanol per gram of the original untreated bagasse after 240 h fermentation, giving ethanol yields of 65.7% of the theoretical maximum. These data suggest that *Phlebia* sp. MG-60 is a potential candidate for ethanol production from alkali-pretreated bagasse in a single bioreactor, without enzymatic or chemical hydrolysis.

1. Introduction

Sugarcane bagasse (SCB) is one of the most abundant agricultural residues in the world, creating 540 million tons of biomass per year (Satyanarayana et al., 2008). For the large-scale biological production of bioethanol, SCB is considered an attractive feedstock because of the concentration and abundance of low-cost raw materials, contributing to the reduction of greenhouse gas emissions and the improvement of food security. SCB typically contains (on a washed and dried basis) approximately 40% cellulose, 24% hemicelluloses, and 25% lignin (Saha, 2003). However, the primary limitation in SCB use is its high degree of complexity because of its mixed composition of cellulose, hemicelluloses, and lignin in extremely inhomogeneous fibers. Therefore, ethanol production from SCB is often complex, with three main steps, including pretreatment, saccharification, and fermentation. Pretreatment can loosen the crystalline structure and lignin of SCB, making them more accessible to the saccharification enzymes. It is necessary to reduce the lignin content to facilitate carbohydrate hydrolysis by enzyme systems. Several pretreatment methods have been studied for various biomass sources (Sun and Cheng, 2002; Conde-Mejía et al., 2012; Suhardi et al., 2013), including steam explosion, solvent extraction, and thermal pretreatment using acids or bases (Mosier et al., 2005). Pretreatment methods investigated for bagasse include acid pretreatment with various acids (Rodriguez-Chong et al., 2004; Gámez et al., 2006; Cheng et al., 2008; Hernández-Salas et al., 2009) steam explosion (Hernández-Salas et al., 2009), alkali treatment (Hernández-Salas et al., 2009), alkali dewaxing (Peng et al., 2009), biological treatment (Li et al., 2002), wet oxidation (Martín et al., 2007), organic solvent pretreatment (Pasquini et al., 2005; Pereira et al., 2007), and liquid hot water pretreatment (Laser et al., 2002). Although various physical (comminution, hydrothermolysis), chemical (acid, alkali, solvents, ozone), and biological pretreatment methods have been investigated over the years (Kumar et al., 2009), thermo-chemical pretreatment of biomass has been the pretreatment of choice to enhance substrate accessibility for efficient enzymatic hydrolysis (Himmel et al., 2007). Alkaline pretreatment dissolves most of lignin and various uronic acid substitutions responsible for

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inhibiting accessibility of the cellulose to enzymatic saccharification (Chang and Holtzapple, 2000) and removes part of hemicellulose, swelling cellulose micro fibrils (Pandey et al., 2000). Moreover, alkaline pretreatment has been reported to increase the biodegradability of plant cell walls through cleavage of the lignin bonds with hemicellulose and cellulose (Spencer and Akin, 1980).

After pretreatment, various ways have been described for saccharification and fermentation, including separate enzymatic hydrolysis and fermentation (SHF), simultaneous saccharification and fermentation (SSF), non-isothermal saccharification and fermentation (NSSF), simultaneous saccharification and co-fermentation (SSCF), and consolidated bio-processing (CBP) (Taherzaderh and Karimi, 2007). Emzymatic hydrolysis of cellulose consists of the absorption of cellulase onto the surface of cellulose, the biodegadation of cellulose to fermentable sugars and desorption of the cellulose. Three classes of hydrolytic cellulases are recognized on the basis of specific substrate: Endo-glucanases, exo-glucanases and β-glucosidases. Endo-1, 4-β-glucanases (EG) cleave randomly at 1, 4-/3-linkages within the cellulose chain. The endo-glucanses attack the low crystallinity regions of cellulose and create free chain-ends. Exo-1,4-β-glucanases (cellobiohydrolases, CBH) of *Phanerochaete chrysosporium*, releases both glucose and cellobiose from the non-reducing ends of cellulose chains (Taherzaderh and Karimi, 2007). Both endoglucanases and cellobiohydrolases are active on amorphous part of cellulose. Fungal cellulases have frequently been reported to act synergistically in the degradation of crystalline cellulose.

In all of the processes mentioned above, an enzyme production is operated separately or the enzymes must be added externally, leading to increased production costs. In CBP, the ethanol and all the required enzymes are produced by a single microorganism strain in a single bioreactor. CBP reduces the ethanol production cost by eliminating the operating costs and capital investment associated with purchasing or producing the enzymes (Lynd et al., 2005). It is based on using mono- or co-cultures of microorganisms that directly ferment cellulose to ethanol. However, there are, as yet, no organisms or combinations of compatible microorganisms available that produce cellulase and other enzymes at the required high levels while also producing ethanol at the required high yields and concentrations. Recently, it was reported that when white rot fungus *Phlebia* sp. MG-60 was cultured with 20 g L^{-1} of unbleached hardwood kraft pulp (UHKP), 8.4 g L^{-1} ethanol was produced after 168 h incubation, giving ethanol yields of 0.42 g g^{-1} UHKP, equivalent to 71.8% of the theoretical maximum. When this fungus was cultured with waste newspaper, 4.2 g L^{-1} of ethanol was produced after 216 h incubation, giving ethanol yields of 0.20 g g^{-1} newspaper; 51.5% of the theoretical maximum (Kamei et al., 2012a). Subsequently, the delignification and simultaneous saccharification and fermentation of oak wood using only *Phlebia* sp. MG-60 was proposed (Kamei et al., 2012b). However, the process as presented requires extremely long times for delignification (Kamei et al., 2012b). Therefore, white-rot fungi *Phlebia* sp. MG-60, which has the ability to ferment cellulose directly, may be potentially applicable to ethanol production from thermo-chemical pretreated biomass by CBP fermentation. However, information regarding the influence of chemical pretreatment to improve fermentability by MG-60 is lacking.

In the present study, the combination between dilute alkaline-pretreatment and CBP fermentation using *Phlebia* sp. MG-60 was studied. Various concentrations of NaOH solutions were used to optimize the pretreatment of SCB, and the conversion rates into ethanol from the initial and pretreated SCB by *Phlebia* sp. MG-60 compared. The aim of this study was to evaluate the fermentability by *Phlebia* sp. MG-60 in CBP fermentation of alkaline-pretreated bagasse for ethanol production.

2. Materials and methods

2.1. Fungal strain and culture condition

Phlebia sp. MG-60 (MKFC40001: National Institute of Technology and Evaluation (NITE), Chiba, Japan) was used for the experiment. Stock cultures of *Phlebia* sp. MG-60 were maintained on a potato dextrose agar (PDA) slant at 4 °C in the dark. The mycelium from the slant was transferred to PDA plates and incubated at 28 °C for 5 days. Mycelial discs at the edge of the actively growing culture were used as inoculums.

2.2. Substrate

Sugarcane bagasse was obtained from Lam Son Company, Thanh Hoa City, Vietnam. The collected bagasse was crushed into pieces, sun and oven dried (at 60 °C), ground to fine particle size (42–100 mesh size bagasse). The bagasse was then extracted by an ethanol:benzene solution at a 2:1 ratio for 16 h in a Soxhlet apparatus (Sluiter et al., 2008) and stored in airtight plastic jars to avoid free moisture effect.

2.3. Alkali pretreatment

Five grams of extractive-free bagasse powder (42–100 mesh size, moisture content 10.3%) and 50 mL of 0.2, 0.4, 0.6, 0.8, 1, and 5% (w/v) alkali solutions were added to 300-mL Erlenmeyer flasks and autoclaved at 121 °C for 60 min. After cooling to room temperature, solid and liquid fractions were separated by filtration and the solid fraction (alkaline-pretreated bagasse) washed with water to neutral pH (approximately 6.5) and air-dried. Before use, the alkali-pretreated bagasse was weighed to measure the weight loss and the dried bagasse then defibrated using a mixer with mixing blades rotating at 20,000 rpm for 20 s.

2.4. Fermentation condition

After pretreatment, 0.4 g of alkali-pretreated bagasse and 20 mL of a basal liquid medium (10 g L^{-1} yeast extract, 10 g L^{-1} KH_2PO_4, 2 g L^{-1} $(NH_4)_2SO_4$, 0.5 g L^{-1} $MgSO_4.7H_2O$) (pH 6.0) were added to a 100-mL Erlenmeyer flask and autoclaved. Six-millimeter-diameter discs were punched from the peripheral region of the mycelium incubated on potato dextrose agar plates. A disc was placed into each of the 100-mL Erlenmeyer flasks mentioned above and sealed with a silicone plug stopper (semi-aerobic conditions). The sealed culture was then incubated at 28 °C in the dark for several days. When the basal liquid medium without bagasse was used for above-mentioned incubation, no production of ethanol was observed.

2.5. Analytical methods

After the direct fermentation step, the remaining bagasse powder and mycelium were removed by centrifugation at 13,000 × g for 10 min. The resulting supernatant was used to measure ethanol concentration by high-performance liquid chromatography (HPLC), after filtration through a membrane filter (0.45 μm). The HPLC system was a Shimadzu LC-10A instrument with a Shimadzu RID-10A differential refractive index detector, fitted with either a Shodex SUGAR KS-802 or a Shodex SUGAR SP0810 column, both with dimensions 8.0 × 300 mm (Showa Denko Inc., Tokyo, Japan). The supernatant was eluted with water at a flow rate of 1 mL min^{-1}. All cultures were conducted in triplicate (Kamei et al., 2012a).