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Surface carbohydrate analysis and bioethanol production of sugarcane bagasse pretreated with the white rot fungus, *Ceriporiopsis subvermispora* and microwave hydrothermolysis

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ABSTRACT

Effects of pretreatments with a white rot fungus, *Ceriporiopsis subvermispora*, and microwave hydrothermolysis of bagasse on enzymatic saccharification and fermentation were evaluated. The best sugar yield, 44.9 g per 100 g of bagasse was obtained by fungal treatments followed by microwave hydrothermolysis at 180 °C for 20 min. Fluorescent-labeled carbohydrate-binding modules which recognize crystalline cellulose (*Cj*CBM3-GFP), non-crystalline cellulose (*Cj*CBM28-GFP) and xylan (*Ct*CBM22-GFP) were applied to characterize the exposed polysaccharides. The microwave pretreatments with and without the fungal cultivation resulted in similar levels of cellulose exposure, but the combined treatment caused more defibration and thinning of the plant tissues. Simultaneous saccharification and fermentation of the pulp fractions obtained by microwave hydrothermolysis with and without fungal treatment, gave ethanol yields of 35.8% and 27.0%, respectively, based on the holocellulose content in the pulp. These results suggest that *C. subvermispora* pretreatment could be beneficial part of the process to produce ethanol from bagasse.

1. Introduction

Bagasse is a by-product of the production of sugar from sugarcane and could be a potential raw material for the production of ethanol. Like other lignocellulosic materials, bagasse has to undergo pretreatment to facilitate the hydrolysis of cellulose and other polymers to provide sugars for ethanol-producing organisms.

Microwave irradiation is one of the methods that has been explored for pretreatment of lignocellulosics (Zhenhu and Zhiyou, 2008; Huan et al., 2009; Gong et al., 2010; Liu et al., 2010; Verma et al., 2011; Baba et al., 2011) because of its high and selective energy transfer efficiency.

Biological treatments with selective white rot fungi such as *Ceriporiopsis subvermispora* that preferentially degrade lignin but largely leave cellulose intact have also been investigated. For example, Zhang et al. (2007) treated bamboo culms with the white rot fungus *Echinodontium taxodii* 2538 for 4 weeks, and reported that this process decreased the lignin content by 29%, and increased the yield of fermentable sugars 8.8-fold. Treatment with the white rot fungus *C. subvermispora* prior to ethanolysis increased the ethanol yield from simultaneous saccharification and fermentation (SSF) of beech wood and Japanese cedar wood, the ethanol yields were 1.6 times and 23.4 times higher than that obtained without the fungal treatments, respectively (Ito et al., 2003; Baba et al., 2011).

The present study was undertaken to determine if treatment with *C. subvermispora* and microwave hydrothermolysis could be combined to improve enzymatic saccharification of sugarcane bagasse and ethanol production by *Saccharomyces cerevisiae* NBRC 0233. The effect of the treatments on exposure of cell wall polysaccharides was visualized with fluorescent-labeled recombinant carbohydrate-binding modules (CBMs) tagged with green fluorescent protein (GFP).

2. Methods

2.1. Raw bagasse

The bagasse was obtained from Kyuyo Sugar Co. Ltd., (Okinawa, Japan). The raw feedstock was cut into small fragments (1–3 cm in

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length and 0.1–0.3 cm in width), and was then air-dried. The chemical composition (based on dry material) of the bagasse was 70.4% holocellulose, 42.2% α-cellulose and 27.9% Klason lignin.

2.2. Fungal pretreatment

C. subvermispora ATCC 90467 was used for the fungal pretreatment. The strain was precultured on 2.4% potato-dextrose agar (PDA) plates at 28 °C for 5–6 days. Distilled water (130 ml) and 5.0 g of wheat bran (Nissin Flour Milling Inc., Tokyo, Japan) were added to the bagasse (50 g) in cultivation bags equipped with an air filter (20 × 45 cm). The solid medium was sterilized at 121 °C for 20 min. Ten pellets were taken from the preculture using a cork borer, and inoculated. The cultivation was performed stationarily at 28 °C at 70% relative humidity for 2–10 weeks.

2.3. Determination of bagasse components

The amount of moisture, holocellulose, α-cellulose, and Klason lignin present in the bagasse and pulp were determined according to the Technical Association of the Pulp and Paper Industry Standard. Holocellulose content was determined based on the weight of the residue obtained after repeated (3 times) delignification with $NaClO_2$ at 80 °C for 1 h. α-Cellulose content was determined as the amount of residue insoluble in a 17.5% NaOH aqueous solution at 20 °C. Klason lignin content was determined as the amount of residue obtained after treatment with 72% sulfuric acid at 20 °C for 4 h followed by with 3% sulfuric acid at 120 °C for 1 h. Neutral sugar composition of the bagasse and pulp was analyzed by gas chromatography after hydrolysis with H_2SO_4 and derivatization to alditol acetates (Sawardeker et al., 1965).

2.4. Microwave irradiation pretreatment

Microwave irradiation was performed at 2.45 GHz using an Initiator 60 instrument (Biotage Co. Ltd.,) equipped with a 30 ml reaction tube. The bagasse sample (0.6 g) was suspended in 20 ml of distilled water, heated at 180 or 200 °C for 10 or 20 min, respectively, cooled to room temperature and filtered. The pulp was washed with acetone followed by distilled water. The water-insoluble fraction was subjected to enzymatic hydrolysis. The water- and acetone-soluble fractions were combined and analyzed for total sugar content using the phenol–sulfuric acid method (Dubois et al., 1956).

Sugar recovery in the insoluble fraction was expressed as: Sugar recovery in the insoluble fraction = Recovery of insoluble fraction (%) × Enzymatic saccharification (%) of insoluble fraction.

2.5. Enzymatic hydrolysis

The pretreated pulp fraction was hydrolyzed using a commercial cellulase preparation, Meisellase, derived from *Trichoderma viride* (Meiji Seika Co. Ltd., 224 filter paper units (FPU)/g, β-glucosidase activity 264 IU/g). The cellulase load was 40 FPU/g substrate. Enzymatic hydrolysis was performed for 48 h in 0.05 M sodium acetate buffer (pH 4.5) at 45 °C, on a rotary shaker (NTS-4000C, Rikakikai, Japan) at 140 rpm. The saccharification ratio per gram of pulp was calculated according to the NREL LAP-009 procedure (Brown and Torget, 1996). The sugar yield was based on the weight percentage of reducing sugars in the original bagasse. All enzymatic hydrolysis experiments were performed in duplicate. Reducing sugar content was determined using the Somogyi–Nelson method (Somogyi, 1952).

2.6. Conditions for SSF

SSF was carried out in 10 ml of medium composed of 0.5 g of water-insoluble fraction of the pretreated bagasse, 9 ml of nutrient medium containing 10 g/l bacto-yeast extract and 20 g/l polypeptone, and 40 FPU of Meicellase. The water-insoluble fraction of the pretreated bagasse and the nutrient medium were autoclaved (121 °C, 20 min), while the enzyme solution was filter-sterilized. The medium was inoculated with 10% (v/v) inoculum of *S. cerevisiae* NBRC 0233. Cultivation was carried out for 48 h at 37 °C with shaking at 100 rpm. Aliquots of the samples (0.5 ml) were taken and assayed for ethanol concentration.

2.7. Determination of ethanol and residual glucose

Ethanol was determined using high-performance liquid chromatography with a Bio-Rad HPX-87H column at 65 °C. The mobile phase was 5 mM H_2SO_4 at a flow rate of 0.6 ml/min. The residual glucose concentration was measured by the mutarotase glucose oxidase method (Miwa et al., 1972).

2.8. Adsorption of CBM-GFPs

To construct plasmids of the GFP-tagged recombinant CBMs p*Cj*CBM3-GFP, p*Cj*CBM28-GFP, and p*Ct*CBM22-GFP, pRSET/EmGFP (Invitrogen) was used as a vector. The coding regions were amplified using polymerase chain reaction (PCR) with two pairs of synthetic oligonucleotide primers; the primers for p*Cj*CBM3-GFP and p*Cj*CBM28-GFP were previously described for the construction of p*Cj*CBM3-CFP and p*Cj*CBM28-CFP, respectively (Kawakubo et al., 2009). Primers for p*Ct*CBM22-GFP 5′-ggatccgcagctctgatttacgatg-3′ (*Bam*HI site underlined) and 5′-gaattcctcaatttcaggcaaatttgc3′ (*Eco*RI site underlined) were amplified from genomic DNA purified from *C. thermocellum* cells. Recombinant protein expression in *Escherichia coli* BL21 (DE3) (Novagen), and the expression and purification of each protein were performed as previously described (Kawakubo et al., 2009).

These CBM-GFPs were used for the analysis of surface carbohydrates in raw and bagasse pretreated with *C. subvermispora* for 8 weeks and subsequent microwave hydrothermolysis (200 °C for 10 min), and microwave hydrothermolysis without fungal treatment.

Nonspecific binding of the CBM-GFPs was prevented by incubating the samples for 3 h with 1 ml of aq. 5% (w/v) ovalbumin (Wako Pure Chemical Industries, Osaka, Japan). After washing the pretreated sample thrice with distilled water, solutions containing the prewashed substrate (1 μg) were transferred to new Eppendorf tubes, and the weight of the substrate was measured. An aqueous solution (100 μl) of the fusion protein (10 μM) was added to the tubes, and incubated for 1 h on ice with gentle mixing. All experiments were replicated thrice at a final working volume of 200 μl. After incubation, the sample was centrifuged at 10,000*g* for 5 min, and fluorescence of the supernatant was determined using a Shimadzu Spectrofluorophotometer RF-1500 (excitation 487 nm, emission 519 nm). The adsorption of the fusion protein was calculated from the supernatant fluorescence before and after incubation with the biomass sample using the following equation:

$$\text{Adsorption } (\%) = \frac{FI_{control} - FI_{final}}{FI_{control}} \times 100 \quad (1)$$

Each precipitated pulp/CBM-GFP sample was analyzed using LSM5 Pascal-Ver 2.8 confocal laser scanning microscope (Carl Zeiss), equipped with an argon–krypton and helium–neon laser using a filter BP505-530/LP505 for the green channel. GFP was excited at a 488 nm. Scanned stacks were processed using the Zeiss LSM software package.