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Short Communication

Candida utilis assimilates oligomeric sugars in rice straw hydrolysate *via* the Calcium-Capturing-by-Carbonation (CaCCO) process for glutathione- and cell-biomass production

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HIGHLIGHTS

- *Candida utilis* produced glutathione and yeast biomass from rice straw hydrolysate.
- The yeast degraded oligosaccharides in the hydrolysate as well as monosaccharides.
- Glucose- and xylose depletion by the yeast reached 99.1% and 84.2%, respectively.

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ABSTRACT

Rice-straw hydrolysate (RSH) prepared *via* the CaCCO (Calcium Capturing by Carbonation) process contains not only monosaccharides but also significant amounts of oligosaccharides. In this study, a glutathione-producing yeast, *Candida utilis* NBRC 0626, was found to assimilate those oligosaccharides. The yields of reduced glutathione (GSH) and dry cell weight (DCW) per consumed sugars in a medium with RSH after 72 h incubation were 10.1 mg/g-sugars and 0.49 g/g-sugars, respectively. The yields were comparative to those in a medium containing a model monosaccharide mix, suggesting that the assimilated oligosaccharides contribute to additional GSH and DCW production. Glycosyl linkage analysis indicated that the yeast could cleave xylose-, galactose-, and arabinose residues as well as glucose residues at the non-reducing ends. After 72 h incubation, 99.1% of the total glucose residues and 84.2% of the total xylose residues in RSH were depleted. Thus the yeast could be applied for efficient utilization of lignocellulosic hydrolysates.

1. Introduction

Biomass-utilization technologies have been expected to provide breakthroughs to reduce the dependence on non-renewable resources such as fossil fuels and naphtha, as biomass is a renewable resource that can be converted to fuels and chemicals by chemical/biological means (Hahn-Hägerdal et al., 2006; Zhou et al., 2011). Due to the existence of well-organized packaging- and logistic systems and little competition with the food supply, agricultural-waste biomass such as corn stover, wheat straw, and rice straw has been proposed as a promising feedstock for these products (Mussatto et al., 2010). These agricultural wastes mainly consist of lignocellulose fibers in which two polysaccharides, cellulose and hemicellulose, are the main sources for enzymatic- or physicochemical conversion to the fermentable monosaccharides glucose and xylose, respectively (Saha, 2003). In order to break the recalcitrant structures of the fibers prior to enzymatic saccharification, a number of chemical, physical and/or biological pretreatment technologies have been developed (Mussatto et al., 2010).

Enzymatic saccharification, however, poses a problem because the heterogeneous structures of hemicellulose result in oligosaccharides as apparent dead-end products, due to limited hydrolysis of the pretreated fibers by added enzymes (Bowman et al., 2012). The structures of these oligosaccharides could be affected by the feedstock and the pretreatment method used, as well as the properties of the enzymes, which would eventually reduce the yield of product unless the microorganism chosen for fermentation has the ability to utilize them (Appeldoorn et al., 2010). Therefore, for optimization of an integrated bioconversion system from feedstock to valuable product, the compatibility of the biomass hydrolysate as

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the feedstock for fermentation with the assimilation potential of the selected microorganism should be carefully evaluated.

In this study, rice straw hydrolysate (RSH) *via* the Calcium-Capturing-by-Carbonation (CaCCO) process (Park et al., 2010) was applied for glutathione- and cell-biomass production by *Candida utilis* in order to determine the consumption patterns of both monosaccharides and oligosaccharides in the RSH during the fermentation. The CaCCO process is a lime-pretreatment/enzymatic-saccharification system for fermentable sugar production, in which neutralization of calcium hydroxide after the lime pretreatment is performed with CO_2 in order to eliminate the subsequent washing- and solid/liquid-separation steps for removal of the alkali. In a large-scale conversion test with 10 kg of dry rice straw, the CaCCO process solubilized sugars from rice straw to give hydrolysate containing glucose and xylose as the main components, whereas significant amounts of oligosaccharides were still present in the solution (Ike et al., 2013a, 2013b).

Glutathione in a reduced form (γ-L-glutamyl-L-cysteinylglycine, GSH) is known to exhibit anti-oxidation activity, and it has been widely used in the pharmaceutical, food, and feed industries (Sies, 1999). Recently, it was shown that application of glutathione in an oxidized form (GSSG) to crops improves the biomass yield (Ogawa and Henmi, 2007), suggesting that the production of agriculture-grade glutathione might attract more interest in the near future. *C. utilis* is among several candidate microorganisms proposed for the industrial production of glutathione (Li et al., 2004). *C. utilis* is also known to be a useful yeast for cell biomass (so-called "single cell protein") production from carbon sources in hydrolysates of rice straw and sugarcane bagasse (Araujo and D'Souza, 1986; Holder et al., 1989). The presence of lignocellulose degradation activity in *C. utilis* was suggested using antibiotic-sterilized apple pomace (Villas-Boãs et al., 2002), whereas its assimilation potential for oligosaccharides in hydrolysates of lignocellulosic biomass has not been evaluated.

2. Methods

2.1. Microorganisms

C. utilis NBRC 0626 (the Biological Resource Center, National Institute of Technology and Evaluation, Chiba, Japan) was maintained on plastic plates containing YPD (10 g/L yeast extract, 20 g/L peptone and 20 g/L glucose) with 20 g/L agar at 30 °C. For seed-cultures, yeast colonies from YPD plates were inoculated into 10 mL YPD medium in 50 mL plastic tubes and cultured at 30 °C and 250 rpm for 24 h.

2.2. Enzyme preparation

A mixture of culture filtrate from *Trichoderma reesei* M2-1 (Ike et al., 2010) and a commercial β-glucosidase preparation (Novozyme 188; Novozymes Japan, Chiba, Japan) was used as enzymes for saccharification of $Ca(OH)_2$-pretreated rice straw. *T. reesei* M2-1 was cultivated in a 5-L jar fermentor (BNR-5L, B. E. Marubishi Co. Ltd, Tokyo, Japan) with a continuous feeding strategy as described previously (Ike et al., 2013a), with some modifications in the procedure; (i) substrate sugar solution: a mixture of glucose (46 g/L), xylose (30 g/L), arabinose (4.0 g/L) and cellobiose (40 g/L), (ii) feeding rate: 2.15 g-sugar/hr and (iii) enzyme production period: 15-days. The resultant culture filtrate exhibited enzyme activities of 15.9 FPU (Filter Paper Units) for filter paper, 3.42 CbU (Cellobiase Units) for cellobiose, 560 U for birchwood xylan, 17.7 U for *p*-nitrophenyl (*p*NP) β-D-xylopyranoside and 38.5 U for *p*NP α-L-arabinofuranoside per mL at pH 5.0. Cellulolytic and hemicellulolytic activities were measured by the methods described in the previous report (Ike et al., 2013a).

2.3. Preparation of rice-straw hydrolysate

Sun-dried rice straw (*Cultivar Koshihikari*) containing cellulose (30.5% (w/w-of the dry matter of rice straw (DM)), xylan (17.3% (w/w-DM)), lignin (15.8% (w/w-DM)), ash (18.2% (w/w-DM)), β-1,3-1,4-glucan (0.32% (w/w-DM)) and starch (2.40% (w/w-DM)) as major components was used (Ike et al. (2013b)). Rice-straw hydrolysate (RSH) was prepared *via* the CaCCO (Calcium Capturing by Carbonation) process as described by Ike et al. (2013b) with some modifications. Briefly, chopped rice-straw (10 kg of DM) was mixed with 1.0 kg of $Ca(OH)_2$ and 9.1 L of distilled water. The mixture was put into a continuous wet-milling machine (Shokusenki, Shinko Engineering Co. Ltd., Hyogo, Japan), and the wet-milled sample was pretreated in an autoclave at 95 °C for 1 h. The $Ca(OH)_2$-pretreated sample was put in a 100-L, CO_2-pressurizing saccharification reactor (BMP-100K, coproduction by ABLE & Biott Co., Ltd., Tokyo, Japan and Tsukasa Industry Co., Ltd., Aichi, Japan) and neutralized by CO_2. A mixture of *T. reesei* M2-1 culture filtrate (4.94 mg-protein (equivalent to 5 FPU)/g-DM), Novozyme 188 (1.07 mg-protein (equivalent to 18 CbU)/g-DM) and 32.7 kg of water was added into a reactor. The reactor was pressurized with CO_2 up to 0.9 MPa, and enzymatic saccharification was performed at a slurry concentration of 20% (w/w) for 72 h at 40 °C with gently mixing (15–30 rpm). After the enzymatic saccharification, the produced slurry was subjected to centrifugation at 11,000×*g* for 10 min. The supernatant was recovered by decantation and heated at 70 °C for 2 h in order to inactivate enzymes. Then, the sample was cooled to 4 °C overnight, and the pH was adjusted to 4.0 with 14 N H_2SO_4 to precipitate calcium ions in the form of $CaSO_4$. The pH-adjusted suspension was subjected to centrifugation at 10,000×*g* for 10 min, and the supernatant was sterilized by passing through a 0.45 μm filter to obtain RSH.

2.4. Glutathione production by C. utilis

Two kinds of glutathione-production media were prepared according to Nie et al. (2005) with slight modifications. For the carbon source, either RSH or a stock solution of a model sugar mixture (42.0 g/L glucose, 25.0 g/L xylose and 2.5 g/L arabinose) was used at 16% by volume of the final volume of the medium. The other components were 0.8% (w/v) $(NH_4)_2SO_4$, 0.3% (w/v) KH_2PO_4 and 0.25% (w/v) $MgSO_4$. Seed cultures of *C. utilis* (10 mL) were inoculated into each glutathione-production medium (100 mL) in 500 mL Erlenmeyer-flasks. Glutathione production was carried out on a rotary-shaker at 250 rpm and 30 °C for 72 h. Aliquots (1 mL) were taken periodically for quantification of soluble sugars, glutathione, and dry cell weight (DCW).

2.5. Analytical methods

The amounts of monomeric sugars were quantified using a HPLC system (SPD-20A, Shimadzu Corporation, Kyoto, Japan) with a refractive index (RI) detector (RID-10A; Shimadzu Corporation). An Aminex HPX-87P column (300 × 7.8 mm, Bio-Rad Laboratories Japan, Tokyo, Japan) was used for separation of sugars, with a mobile phase of H_2O and a flow rate of 0.6 mL/min at 60 °C. Oligosaccharides present in RSH were hydrolyzed in 2 N H_2SO_4 at 100 °C for 2 h, and the hydrolysate was neutralized with an equal volume of 30% (w/w) $CaCO_3$. The neutralized hydrolysate was subjected to centrifugation at 10,000×*g* for 10 min, and the supernatant was filtered through a 0.45 μm filter for monosaccharide analysis. The amounts of sugars existing as oligosaccharides were indicated as the weights of monomeric-sugar equivalents.

The *C. utilis* cells were collected from 400 μL of culture by centrifugation at 10,000×*g* for 2 min, and the precipitate was washed twice with distilled water. The concentration of GSH in the cell