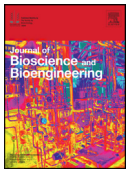

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NOTE

Expression of neutral β-glucosidase from *Scytalidium thermophilum* in *Candida glabrata* for ethanol production from alkaline-pretreated rice straw

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We successfully expressed the neutral β-glucosidase (BGL4) from *Scytalidium thermophilum* in the thermotolerant yeast *Candida glabrata*. Compared to the strain expressing *Aspergillus* acidic β-glucosidase (BGL1), the BGL4-expressing strain showed a higher cellobiose fermentation ability at pH 6.0 and 40°C, leading to a higher ethanol production from alkaline-pretreated rice straw.

[**Key words:** β-Glucosidase; Bioethanol; *Candida glabrata*; Cellobiose; *Scytalidium thermophilum*]

Internationally, bioethanol production has been mainly produced from sugar or corn starch. The efficient conversion of lignocellulosic biomass into fuel ethanol has become a world priority for producing an environmentally friendly renewable energy. It is essential for the bioethanol production to move away from food and grain crops and into alternative renewable feedstocks such as non-food lignocellulosic biomass. We have developed an alkaline pretreatment process, called the calcium capturing by carbonation (CaCCO) process as a pretreatment method for the efficient bioethanol production from rice straw (1). In this process, rice straw was pretreated with lime ($Ca(OH)_2$) and neutralized by carbonation. Unlike other alkaline pretreatment methods that use acid as a neutralizer, the CaCCO process did not require a solid–liquid-separation step or washing step, which was necessary for removing the soluble lignin and yeast growth inhibitors from the pretreated materials. Thus, this process was able to prevent the loss of solubilized carbohydrates, such as xylan, starch, and sucrose, resulting in a high recovery yield of fermentable monosaccharides (1).

In the CaCCO process, the pH value of the lime-treated rice straw samples was reduced from 10.8 to 6.8 by the neutralization with CO_2 bubbling and then maintained around 6.0–6.3 in a closed jar filled with CO_2 (1). Therefore, the saccharification enzymes used in the CaCCO process are required to function at neutral pH, whereas available commercial cellulases, most of which are derived from fungi, show the optimal activity at an acidic pH. Since cellobiose is a strong inhibitor of cellobiohydrolase, a sufficiently high level of β-glucosidase is crucial for both the overall saccharification rate and for the high glucose yield. A β-glucosidase from *Aspergillus niger* (BGL1) is often added to the *Trichoderma* cellulases, and its optimum pH for the conversion of cellobiose into glucose is reported to be 4.3 (2). On the other hand, *Scytalidium thermophilum* (a synonym of *Humicola grisea* var. *thermoidea*) is known to produce six different characterized β-glucosidases (3). One of them, BGL4, exhibited the optimum pH of 6.0 (4), which is considered to meet the requirement of the CaCCO process. In this study, we transformed *Candida glabrata*, which is a thermotolerant yeast, to express the *BGL4* gene. In order to examine the applicability of the neutral β-glucosidase BGL4 in the CaCCO process, the ethanol productions from cellobiose and rice straw using the transformant were compared to *C. glabrata* that expresses the acidic β-glucosidase BGL1, which is one of the most common β-glucosidases used for saccharification of lignocellulosic biomass.

S. thermophilum ATCC 26908, which was obtained from the American Type Culture Collection (ATCC; Manassas, VA, USA) was cultured at 25°C for 3 days on a mixed cellulose ester membrane filter (pore size, 0.20 μm) placed on surface of an agar medium containing 10 g/L yeast extract (Difco, Franklin Lakes, NJ, USA), 20 g/L Bacto peptone (Difco), 20 g/L glucose, 20 g/L cellobiose, and 20 g/L Bacto agar (Difco). The cells were next disrupted in liquid nitrogen to isolate the total RNA with an RNeasy Mini kit (Qiagen, Hilden, Germany). The *BGL4* gene (1431 bp; GenBank accession no. AB003109) was amplified by reverse transcription (RT)-PCR using primers of 5bglu-EcoRI (5′-CCGGAATTCATGTCTCTTCCTCCGG-3′) and 3bglu-HindIII (5′-CCCAAGCTTGGATCCTTACTCCTTGC-3′). *A. niger* NBRC 31125, which was obtained from the Biological Resource Center, NITE (Kisarazu, Japan) was cultured and used for the total RNA preparation as already described. The *BGL1* gene (2583 bp; GenBank accession no. AJ132386) was amplified by RT-PCR using primers of 5Aspbgl-EcoRI (5′-CGGAATTCATGAGGTTCACTTTGATCGAGG-3′) and 3Aspbgl-HindIII (5′-CCCAAGCTTTTAGTGAACAGTAGGCAGAG-3′). Each PCR product was digested with *Eco*RI and *Hin*dIII and cloned into pTEF-MF (Dualsystems Biotech, Zurich, Switzerland) to express as a fusion protein with the *Saccharomyces cerevisiae* α mating factor secretion signal under

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the control of the constitutive *S. cerevisiae TEF1* promoter, and the resultant plasmids were designated pTEF-MF-*BGL4* and pTEF-MF-*BGL1*. These plasmids were linearized by digesting with *Sse*8387I and then transformed into a *C. glabrata* uracil-auxotrophic mutant by the lithium acetate method of Gietz and Woods (5). The uracil-auxotrophic mutant was isolated from *C. glabrata* NFRI 3163 (National Food Research Institute, Tsukuba, Japan) by spontaneous mutation on a medium containing 5-fluoro-orotic acid (6). The strains transformed with pTEF-MF-*BGL4* and pTEF-MF-*BGL1* were isolated by restoration of the uracil prototrophy as a marker and designated 3163-BGL4 and 3163-BGL1, respectively.

To characterize the transformants, their β-glucosidase activities were determined (Table 1). The recombinant and parent strains were cultured in YPD medium (10 g/L yeast extract, 20 g/L Bacto peptone, and 20 g/L glucose) for 20 h at 30°C or 40°C with shaking at 200 rpm. The β-glucosidase activities in the cultures were evaluated based on the *p*-nitrophenyl β-D-glucopyranoside (pNPG)-hydrolyzing activity. The culture supernatants were separated from the cells by centrifugation at 5000 ×*g* for 5 min. These samples were incubated for 10 min at 50°C in 50 mM sodium acetate buffer (pH 4.0) or 50 mM sodium phosphate buffer (pH 6.0) with 1 mM pNPG. The reactions were then terminated by the addition of 1 M Na_2CO_3, and then the *p*-nitrophenol content was measured at 405 nm. When cultured at 30°C, the entire 3163-BGL4 culture exhibited pNPG-hydrolytic activity (49.0 U/L) at pH 6.0, but not at pH 4.0. In contrast, the entire 3163-BGL1 culture exhibited an activity not only at pH 4.0 (122.4 U/L), but also at pH 6.0 (32.3 U/L), suggesting that the recombinant BGL1 remains active over a wider pH range than BGL4. The parent strain NFRI 3163 did not show any β-glucosidase activity. The kinetic parameters of the purified BGL1 for pNPG hydrolysis at pH 4.8 have been determined to be K_m of 0.57 mM and k_{cat} of 26.4 s^{-1} (7). On the other hand, the purified BGL4 hydrolyzed pNPG at pH 6.0 with the K_m of 0.34 mM and k_{cat} of 7.84 s^{-1} (3). (The k_{cat} value was estimated based on the V_{max} value of 8.70 μmol min^{-1} mg $protein^{-1}$ (3) and the calculated molecular mass of 54.1 kDa) The rate constant for BGL4 is lower than that for BGL1, corresponding to a lower hydrolysis activity in 3163-BGL4.

The culture supernatant of 3163-BGL4 exhibited the β-glucosidase activity of 38.7 U/L at pH 6.0 and 30°C, indicating that most (79%) of the recombinant β-glucosidase protein was secreted into the medium. In contrast, only a low β-glucosidase activity (4.5 U/L) was detected in the culture supernatant of 3163-BGL1 at pH 4.0 and 30°C. Unlike BGL4, most of the recombinant BGL1 protein was associated with the cells. Increases in the cell densities of the transformants, which were determined by measuring the optical density at 600 nm (OD_{600}), were similar to that of the parent strain when they were cultured at 30°C (Table 1). In addition, NFRI 3163 and 3163-BGL4 exhibited OD_{600} values at 40°C similar to those at 30°C. However, 3163-BGL1 exhibited a 2-fold lower OD_{600} value at 40°C than at 30°C. These results indicated that the expression of both exogenous β-glucosidase genes did not adversely affect the cell growth of the transformants at 30°C, but the expression of *BGL1* inhibited the cell growth at 40°C. The 3163-BGL4 strain grown at 40°C exhibited a similar β-glucosidase activity to that at 30°C, whereas 3163-BGL1 grown at 40°C resulted in three-fold decreased activities compared with those at 30°C. Since the BGL1 protein (93 kDa) has a 1.7-fold higher molecular mass than the BGL4 protein (54 kDa), the BGL1 could tend to be aggregated in the cells, resulting in inhibition of the secretion and the cell growth at high temperature.

TABLE 1. β-Glucosidase activities in the transformants.

Strain	Culture temperature (°C)	OD_{600}	Activity (U/L)			
			pH 6.0		pH 4.0	
			Entire culture	Supernatant	Entire culture	Supernatant
3163-BGL4	30	23	49.0	38.7	n.d.	n.d.
	40	23	58.6	33.0	n.d.	n.d.
3163-BGL1	30	22	32.3	n.d.	122.4	4.5
	40	11	11.0	n.d.	43.6	10.5
NFRI 3163	30	22	n.d.	n.d.	n.d.	n.d.
	40	22	n.d.	n.d.	n.d.	n.d.

One unit (U) of the activity was defined as the amount of enzyme that formed one μmol *p*-nitrophenol per minute. n.d., not detected.

We tested the cellobiose fermentation abilities of the transformants using the synthetic media. Cells of 3163-BGL4 and 3163-BGL1 were aerobically precultured in the YPD medium for 16 h at 30°C and washed with sterile water before seeding into the fermentation media. The initial cell density of the fermentation was adjusted to 0.6 g dry cell weight (DCW)/L. The DCW of *C. glabrata* was converted from the OD_{600} values by the equation, 0.3 g/L DCW = 1 OD_{600}. The fermentation media contained 100 mM citrate-potassium phosphate buffer (pH 4.0 or 6.0), 6.7 g/L yeast nitrogen base without amino acids (Difco), 0.79 g/L CSM (Sunrise Science Products), and 20 g/L cellobiose. The fermentation was anaerobically performed using 10-ml vials capped with rubber stoppers and aluminum crimp seals. The vials were incubated at 30°C or 40°C with shaking at 200 rpm without pH control during the fermentation. Samples were periodically withdrawn with needles to determine the concentration of ethanol and cellobiose by HPLC using a Shodex SP0810 column (8.0 mm I.D. x 300 mm, Showa Denko, Tokyo, Japan) and a refractive index detector (RID-10A, Shimadzu, Kyoto, Japan). As shown in Fig. 1a and c, 3163-BGL4 exhibited ethanol production from cellobiose at pH 6.0, but not at pH 4.0. On the other hand, 3163-BGL1 consumed cellobiose and produced ethanol at both pH values (Fig. 1b and d). These results are well consistent with the β-glucosidase activities of the strains (Table 1). The 3163-BGL4 strain produced 2.7 g/L and 8.3 g/L ethanol at 30°C and 40°C, respectively, when incubated in the medium at pH 6.0 for 72 h, showing a higher ethanol production at 40°C than at 30°C. On the other hand, 3163-BGL1 produced 8.5 g/L (30°C) and 1.2 g/L (40°C) ethanol in the medium at pH 6.0 and produced 8.6 g/L (30°C) and 5.3 g/L (40°C) ethanol at pH 4.0. The 3163-BGL1 strain showed a higher and faster ethanol production than 3163-BGL4 even at pH 6.0 when incubated at 30°C. The ethanol production by 3163-BGL1, however, decreased at 40°C, especially at pH 6.0. The reduction in the cellobiose fermentation ability of 3163-BGL1 at 40°C appears to be caused by the inhibited cell growth and decrease in the β-glucosidase activity (Table 1). Therefore, 3163-BGL4 is expected to produce more ethanol at 40°C under near neutral pH conditions such as the CaCCO process. The parent strain produced no ethanol from cellobiose under any of the tested conditions (data not shown).

The β-glucosidase-expressing strains were applied to the ethanol production from rice straw by the simultaneous saccharification and fermentation (SSF) process. Rice straw (cv. Koshihikari) was pretreated by the CaCCO method as described previously (1). Briefly, 500 mg rice straw powder (0.5-mm ground), 100 mg $Ca(OH)_2$ and 3.33 ml water were added to a 10-ml vial and autoclaved at 120°C for 1 h after capping with a rubber stopper and aluminum crimp seal. The lime-treated rice straw was then neutralized by blowing CO_2 gas into the vials through a needle at 1 L/min for 5 min and then incubated overnight at room temperature. Cells of NFRI 3163, 3163-BGL4 and 3163-BGL1 were aerobically precultured at 30°C in the YPD medium, of which initial pH value was 6.0. The initial cell density of the fermentation was adjusted to 1.2 g DCW/L. Cellulase was prepared from the *Trichoderma reesei* strain M-2 (8) and a three-filter-paper degrading unit was loaded into the vials. Since *C. glabrata* cannot utilize sucrose, invertase (Megazyme, Wicklow, Ireland) was also added. The SSF