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Display of *Bacillus macerans* Cyclodextrin Glucanotransferase on Cell Surface of *Saccharomyces cerevisiae*

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Abstract *Bacillus macerans* cyclodextrin glucanotransferase (CGTase) was expressed on the cell surface of *Saccharomyces cerevisiae* by fusing with Aga2p linked to the membrane-anchored protein, Aga1p. The surface display of CGTase was confirmed by immunofluorescence microscopy and its enzymatic ability to form α-cyclodextrin from starch. The maximum surface-display of CGTase was obtained by growing recombinant *S. cerevisiae* at 20°C and pH 6.0. *S. cerevisiae* cells displaying CGTase on their surface consumed glucose and maltose, inhibitory byproducts of the CGTase reaction, to enhance the purity of produced cyclodextrins. Accordingly, the experimental results described herein suggest a possibility of using the recombinant *S. cerevisiae* anchored with bacterial CGTase on the cell surface as a whole-cell biocatalyst for the production of cyclodextrin.

Key words: CGTase, surface display, *Saccharomyces cerevisiae*, whole-cell catalyst

Cyclodextrin glucanotransferases (EC 2.4.1.19) catalyze the formation of cyclodextrins (CDs) from starch by forming an α (1→4) linkage through a transglycosylation reaction [18]. Three different CDs have been so far identified, α-, β-, and γ-CDs, which consist of six, seven, and eight α (1→4) linked D-glucose units, respectively. All of these molecules have a doughnut-shaped structure, with a hydrophilic shell and hydrophobic central cavity, which enables them to form inclusion complexes with organic substances, such as fatty acids, vitamins, and flavor compounds.

Bacillus macerans CGTase mainly produces α-CD from starch [6, 9, 13]. α-CD is more soluble than β-CD and can be widely used for food and pharmaceutical purposes. The current authors previously constructed recombinant *E. coli* and *B. subtilis* systems for the production of *B. macerans* CGTase and investigated the influences of environmental factors on its expression in an effort to develop an optimized fermentation process [6, 8, 9, 13]. CDs are produced by cyclization reaction of CGTase which also transfers glycosyl residues to the acceptor molecules such as monosaccharide or oligosaccharide. Such a transglycosylation reaction involves coupling and disproportionation reactions. Therefore, the final products of CGTase reaction are generally mixture of CDs and maltooligosaccharides containing glucose, maltose, and other oligosaccharides [21]. Maltooligosaccharides not only participate in the intermolecular transglycosylation, but also function as strong inhibitors for cyclization reaction. Among them, glucose and maltose are the most remarkable inhibitors acting as both competitive inhibitors in the formation CD and acceptors in the coupling reaction, which eventually reduce the purity and productivity of CD.

A display of heterologous proteins on the cell surface of microorganisms such as yeast and bacterial cells has become one of the most interesting research areas with applications in live vaccines, antibody libraries, and whole-cell biocatalysts and adsorbents [1, 4, 5, 11, 12]. The expression of proteins on the cell surface of *Saccharomyces cerevisiae* may be more advantageous than bacterial display systems [1, 11, 12, 19]. Since this yeast benefits from a 'GRAS' (generally regarded as safe) status and can be cultivated to high cell density using relatively inexpensive media [14, 19], surface-displayed yeast cells could be developed as novel whole-cell biocatalysts or live-vaccines [19]. α-Agglutinin is linked covalently to the cell wall by the C-terminus [3]. Fusion to the C-terminus of α-agglutinin has been frequently used to anchor enzymes and antibody fragments on the yeast cell surface [11, 12, 17]. The 73-kDa Aga1p subunit of α-agglutinin is anchored to the cell wall via a β-glucan linkage and the 69-amino acid binding subunit Aga2p is linked to Aga1p by two

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disulfide linkages [3]. Recently, the fusion of a heterologous protein to the C-terminus of Aga2p has been tried for the directed evolution of target enzymes, selection of an antibody fragment showing high affinity, and to predict the secretion efficiency of an expressed protein [2, 7, 16, 17].

In the current study, a recombinant *S. cerevisiae* strain anchoring *B. macerans* CGTase on the cell surface was developed using Aga2p as a fusion partner and its application as a novel whole-cell catalyst for the production of CD was examined.

MATERIALS AND METHODS

Strains and Plasmids

E. coli DH5α was used for the propagation of the plasmid DNA and *S. cerevisiae* EBY100 (*trp1 leu2Δ1 his3Δ200 pep4::HIS2 prbΔ1.6R can1 GAL*) [2] was used for the expression and display of *B. macerans* CGTase. Plasmid pTCGT1 [13] was used as the template DNA for the isolation of the *B. macerans cgt* gene. Plasmid pYD1 (Invitrogen, Carlsbad, CA, U.S.A.) was employed for the expression of the *cgt* gene in *S. cerevisiae*. Recombinant *E. coli* BL21 (DE3) pLysE harboring the pTCGT1 plasmid was also used to produce CGTase.

Media and Cultivation

A Luria-Bertani (LB) medium [15] was used for the cultivation of recombinant *E. coli*. The yeast transformants were selected and maintained on a YNB plate (0.67% YNB, 20% glucose, and a 1.5% agar) that was supplemented with 30 mg of leucine per liter. A seed culture grown in the YNB-leucine medium was transferred to a shake flask containing a YPG medium (1% g yeast extract, 2% peptone and 2% g galactose) and further incubated at 20°C and pH 6.0 to obtain yeast cells displaying CGTase on their surfaces. The recombinant *E. coli* BL21 (DE3) strain growing exponentially in the LB medium at 37°C (pH 7.0) was induced with 1 mM IPTG (isopropyl-β-D-thiogalactopyranoside) to produce CGTase [13]. The CGTase was purified from the *E. coli* lyzate by affinity chromatography [8].

DNA Manipulation and Yeast Transformation

The *B. macerans cgt* gene truncated with its nascent bacterial signal sequence was obtained from the plasmid pTCGT1 by polymerase chain reaction (PCR) amplification. The sequences of the two PCR primers [(5'-3') AAA GGA CCT CAC CCG ATA CGA GCG TGG AC and (5'-3') AAA GAA TTC TTA ATT TTG CCA GTC CAC CGT CAC] (Bioneer, Cheongwon, Korea) were used to isolate the approximately 2.0-kb *cgt* gene. The PCR amplification of the template DNA was carried out in 100 μl of a reaction mixture containing a Pwo DNA polymerase buffer (Roche, Mannheim, Germany), 2 mM $MgSO_4$, 0.2 mM each dNTPs, 5 pmole of each primer, and Pwo DNA polymerase (5 units, Roche). Thirty cycles of PCR amplification were performed in a thermal cycler (Perkin-Elmer 9600, Boston, MA, U.S.A.) as follows: 2 min at 95°C for the denaturation step, 15 sec at 95°C, 30 sec at 55°C, and 2 min at 72°C for the amplification of the target fragment followed by elongation at 72°C for 7 min as the last cycle. The yeast transformation was carried out using an Alkali-Cation kit (BIO101, Palo-Alto, CA, U.S.A.) according to the manufacturer's instructions.

Immunofluorescence Microscopy

The recombinant *S. cerevisiae* grown in the YPG medium was harvested by centrifugation and washed twice with a PBS buffer (pH 7.4). The cell pellet was then incubated in a PBS buffer containing BSA (1 mg/ml) and an antibody (1 μg) for 1.5 h on ice. The antibody against *B. macerans* CGTase [6] was used as the primary antibody at a dilution ratio of 1:1000. After washing with PBS, the cells were incubated with a secondary antibody, fluorecein-isothiocyanate (FITC)-labeled goat anti-rabbit IgG, at a dilution ratio of 1:600 for 1 h at room temperature. After washing the cells again with PBS, the fluorescence image was observed under a laser confocal microscope (Bio-Rad MRC-1024, Hercules, CA, U.S.A.).

Enzymatic Reaction

An enzymatic reaction was carried out in an imidazole-HCl (0.1 M) buffer supplemented with 5mM $CaCl_2$ at 30°C and pH 6.0 [10]. Soluble starch (0.2%) was used as the substrate. *S. cerevisiae* EBY 100/pMDISCGT and the control strain harboring the pYD1 plasmid were incubated in the YPG medium for 48 h at 20°C, harvested by centrifugation, and washed twice with a PBS (pH 7.4) buffer. The cells were resuspended in the reaction buffer and added at a final concentration of 30 g/l for the enzymatic reaction. Finally, 1 unit of the CGTase purified from the *E. coli* cell lyzate was added. One unit of CGTase activity was defined as the amount of CGTase required for the formation of 1 mmol of α-CD per minute.

A mixture of glucose, maltose, and α-CD was used as a standard solution to identify the reaction products formed by the purified CGTase and CGTase immobilized on the surface of *S. cerevisiae*.

Thin Layer Chromatography (TLC)

A TLC analysis was performed to identify the cyclodextrins and sugars produced by the recombinant *S. cerevisiae* and purified CGTase. After activating the TLC plate (K5F, Whatman, Clifton, U.S.A.) for 1 h at 110°C, an aliquot of a sample was spotted and then developed in a solvent [water: nitromethane:n-propanol=3:4:10 (v/v)]. After drying, the plates were dipped in a methanol/sulfuric acid [5:95] solution

supplemented with 0.3% α-naphtol. Color images were obtained by incubating at 110°C for 10 min and analyzed using a densitiometer (GS700, Bio-Rad, Richmond, CA, U.S.A.).

RESULTS AND DISCUSSION

A PCR amplification was carried out to obtain the 2.0-kb DNA fragment truncated with its nascent bacterial signal sequences. The amplified DNA fragment was digested with *Bam*HI and *Eco*RI restriction endonucleases, and placed in a frame with the Aga2p gene in the pYD1 vector to construct the pMDISCGT (7.1 kb) plasmid. Aga2p was employed as a fusion partner to secret and localize CGTase on the cell surface of *S. cerevisiae*. The expression of the Aga2p/CGTase fusion protein was found to be under the control of the *GAL1* promoter (Fig. 1).

Immunofluorescence microscopy was used to confirm the expression of *B. macerans* CGTase on the cell surface of *S. cerevisiae*. Thus, the immunofluorescence labeling of the recombinant yeast cells was performed using anti-CGTase IgG as the primary antibody and FITC-IgG (goat) as the secondary antibody, and the *S. cerevisiae* cells harboring the pYD1 and pMDISCGT plasmids were observed under a laser confocal microscope. As expected, the cells were uniformly labeled with FITC, while the control strain harboring the pYD1 vector was not (Fig. 2). This observation, therefore, strongly indicated the successful expression and localization of CGTase on the cell surface of *S. cerevisiae*.

The patterns of the cell growth and expression of the surface-displayed CGTase in the recombinant *S. cerevisiae* were examined at various incubation temperatures and initial medium pHs to optimize the culture conditions. While the specific growth rate did not vary much from 25

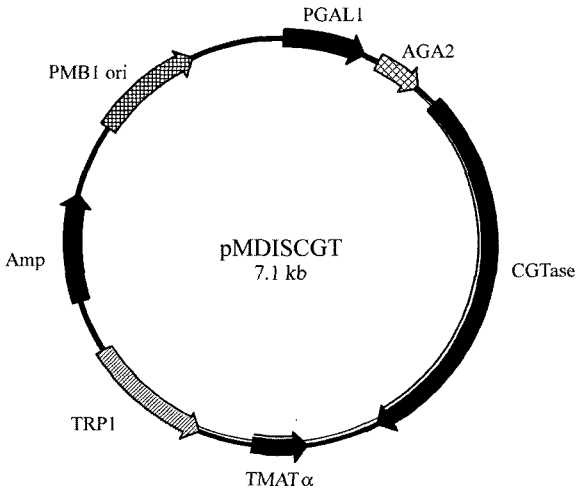

Fig. 1. Schematic illustration of pMDISCGT plasmid.

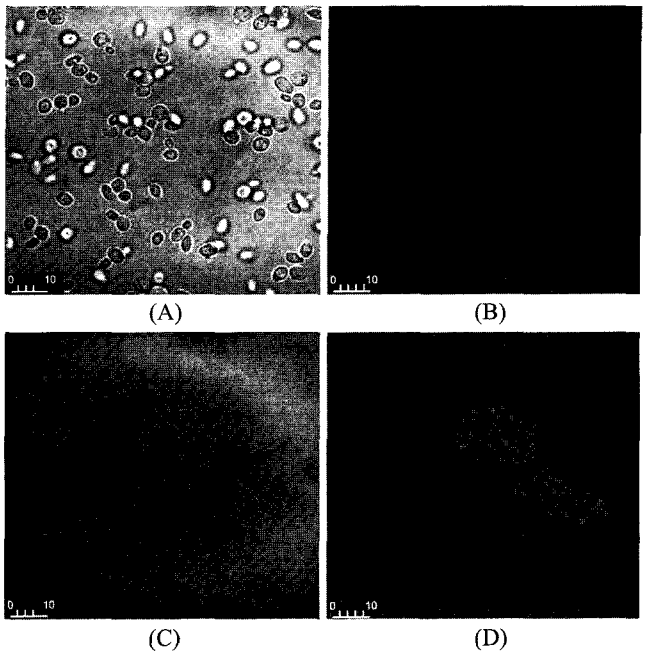

Fig. 2. Confocal microscopic images of yeast *Saccharomyces cerevisiae* harboring pYD1 (A, B) and pMDISCGT plasmids (C, D).
Cells were labeled with FITC-conjugated anti-goat IgG.

to 30°C, the maximum expression of CGTase was observed at 20°C. Optimum pH of the medium ranged from 5 to 6 (data not shown). No CGTase activity was detected in the supernatant, thereby suggesting the stable immobilization of CGTase over a prolonged period. Accordingly, the recombinant *S. cerevisiae* was cultivated at 20°C and pH 6.0 in all subsequent experiments.

A TLC analysis was undertaken to verify whether the surface-displayed CGTase was able to produce α-CD from soluble starch. Lane M in Fig. 3 shows that the α-CD was well separated from glucose, maltose oligosaccharides (G2-G7), and other CDs. As illustrated in Lane E, the free CGTase purified from the recombinant *E. coli* cell lyzate instantaneously reacted with soluble starch to produce an α-CD, however, with a slightly altered product distribution after 36 h of incubation time. The CGTase displayed on the cell surface of *S. cerevisiae* certainly synthesized an α-CD (Lane R). In contrast, the control strain harboring the pYD1 vector (Lane C) did not produce anything from the soluble starch, thus clearly demonstrating that the surface-displayed CGTase maintained its enzymatic ability.

Enzymatic properties of free and displayed CGTase were compared using a mixture of glucose, maltose, and α-CD as the substrate. In the case of free CGTase, maltoheptaose (G7) and maltooctaose (G8) were formed from α-CD by CGTase with glucose and maltose as acceptor molecules. Free CGTase continuously catalyzed

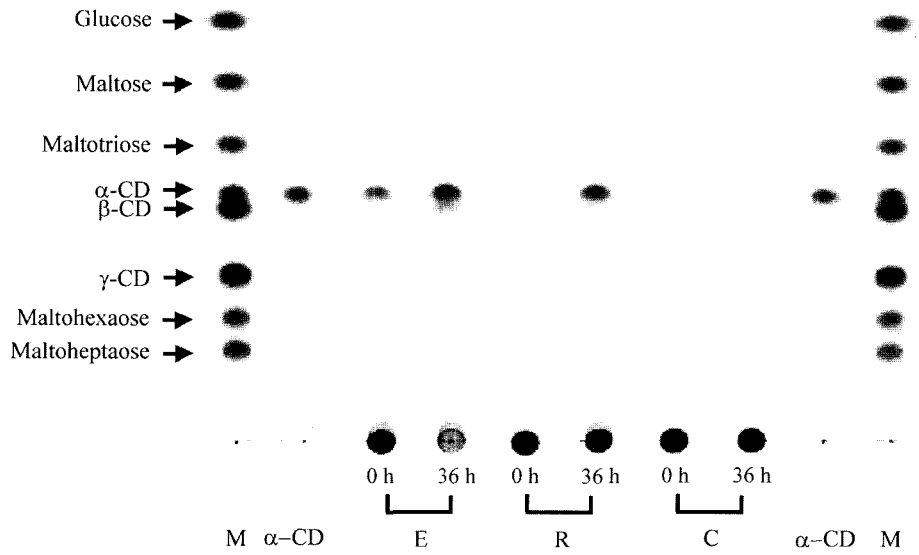

Fig. 3. Thin layer chromatograms of enzymatic reactions of CGTase purified from recombinant *E. coli* (free CGTase) and CGTase displayed on the surface of *S. cerevisiae*.
The reactions were conducted in 0.1 M immidazole buffer containing 2 g/l starch with 5 mM $CaCl_2$ at 30°C and pH 6.0. Lane E, free CGTase expressed in recombinant *E. coli*; lane R, *S. cerevisiae* EBY100/pMDISCGT; lane C, *S. cerevisiae* EBY100/pYD1.

intermolecular transglycosylation reaction to produce glucose and linear oligosaccharides from α-CD. This reaction increased the concentrations of maltooligosaccharides ranging from maltotriose (G3) to maltohexaose (G6), and concomitantly reduced the concentration of α-CD (Fig. 4A). For the displayed CGTase on the cell surface of *S.*

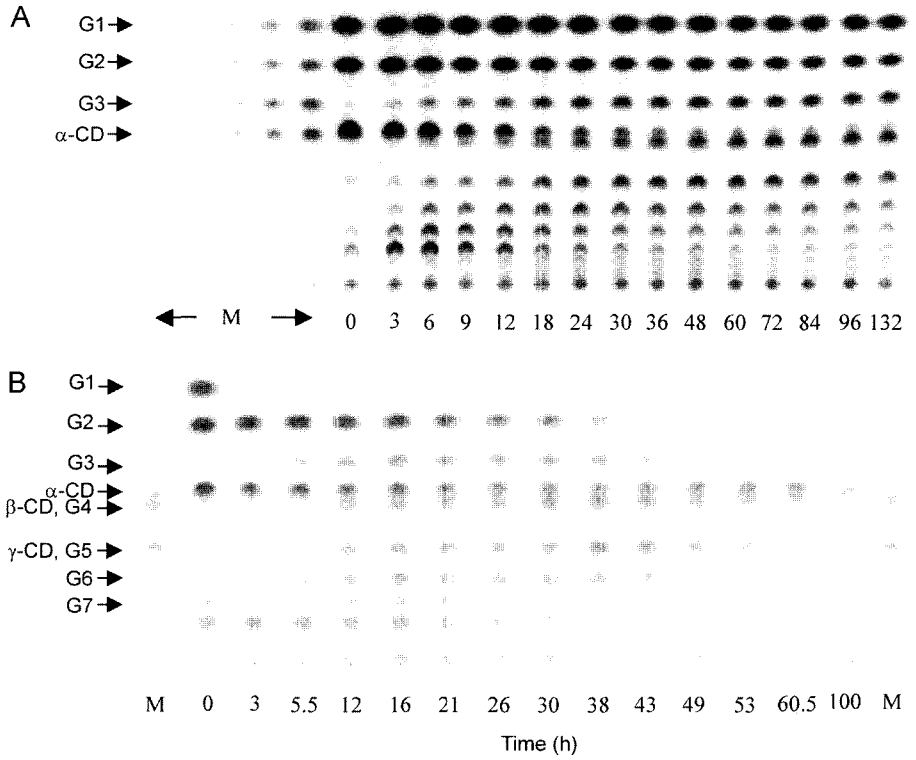

Fig. 4. Thin layer chromatogram of enzymatic reactions of free CGTase (A) and CGTase displayed on the surface of *S. cerevisiae* (B).
The reactions were conducted in 0.1 M immidazole buffer containing 2 g/l α-CD, 1 g/l glucose, and 1 g/l maltose with 5 mM $CaCl_2$ at 30°C and pH 6.0.

cerevisiae, glucose added initially as the substrate was completely consumed in 60 h of incubation. As observed in the free CGTase, glucose and maltose served as acceptors for the coupling reaction to produce G7 and G8 compounds in the early phase of enzymatic reaction (Fig. 4B). Maltose was present until 49 h of reaction, so that coupling and disproportionation reactions utilizing maltose as an acceptor occurred, and hence, the concentration of oligosaccharides increased. However, as maltose concentration became limited after 49 h of incubation, concentrations of oligosaccharides gradually decreased, enhancing the purity of finally produced CDs. Consequently, this study indicated that the consumption of glucose and maltose by *S. cerevisiae* cells had positive effects on enhancing the purity of CGTase reaction product.

Murai *et al.* [11, 12] studied the α-amylase of *B. stearothermophilus* on the cell surface of *S. cerevisiae* using α-agglutinin and observed that the surface-engineered yeast cells utilized soluble starch as a sole carbon source for growth. They further observed that *S. cerevisiae* cells harboring both glucoamylase and α-amylase grew faster than glucoamylase-immobilized cells [20].

The current study developed a *S. cerevisiae* system anchored with *B. macerans* CGTase to produce CD(s) from soluble starch. The results described above strongly suggest that the CGTase immobilized on the cell surface may have potential as a whole-cell biocatalyst for the production of CD from starch. Furthermore, it was revealed that instantaneous consumption of glucose and maltose, which are inhibitory byproducts of CD formation from starch [21], by *S. cerevisiae* was advantageous for the economical production of CD by alleviating the inhibitory effects and also simplifying the purification steps involved in removing contaminating products. Further research is in progress to improve the secretion efficiency of bacterial CGTase in *S. cerevisiae*, select a clone harboring maximum CGTase activity via fluorescence-activated cell sorting (FACS), compare product profiles for the whole-cell catalyst, and finally optimize the reactor configurations for CD production.

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