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Characterization of Cyclofructans from Inulin by *Saccharomyces cerevisiae* Strain Displaying Cell-Surface Cycloinulooligosaccharide Fructanotransferase

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Abstract The cycloinulooligosaccharide fructanotransferase (CFTase) gene (*cft*) from *Paenibacillus macerans* (GenBank access code AF222787) was expressed on the cell surface of *Saccharomyces cerevisiae* by fusing with Aga2p linked to the membrane-anchored protein Aga1p. The surface display of CFTase was confirmed by immunofluorescence microscopy and enzymatic assay. The optimized reaction conditions of surface-displayed CFTase were as follows; pH, 8.0; temperature, 50°C; enzyme amount, 30 milliunit; substrate concentration, 5%; inulin source, Jerusalem artichoke. As a result of the reaction with inulin, cycloinulohexaose was produced as a major product along with cycloinuloheptaose and cycloinulooctaose as minor products.

Keywords: Cycloinulooligosaccharide fructanotransferase, *Saccharomyces cerevisiae*, cell surface display, cyclofructan, inulin

Cycloinulooligosaccharides (cyclofructans, CFs) are the cyclic oligosaccharides that consist of six to eight molecules of β-(2→1)-linked D-fructofuranoses [cycloinulohexaose (CF6), cycloinuloheptaose (CF7), and cycloinulooctaose (CF8)] [11]. Cyclofructans have a characteristic crown ether in the central part of the molecule that can bind cationic molecules *via* charge-dipole electrostatic interactions [25, 27]. Cyclofructans also have stabilizing effects on various materials during the freezing and thawing process. Accordingly, cyclofructans are expected to be utilized extensively in the medical, food, and chemical fields [10]. Cyclofructans are synthesized from inulin by cycloinulooligosaccharide fructanotransferase (CFTase). Inulin is a polyfructan consisting of a linear β-(2→1) linked polyfructose chain with a terminal glucose residue. CFTase catalyzes the degradation of inulin into cycloinulooligosaccharides by intramolecular transfructosylation between β-(2→1)-fructo oligosaccharides by disproportionation and coupling reactions.

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 production of live vaccines, antibody libraries, and whole-cell biocatalysts and absorbents [1, 5, 7, 22, 23]. For the past several years, the expression of proteins on the surfaces of bacteriophage and bacteria has been actively studied [3]. These systems are expected to be useful for the segregation of polypeptides produced and for the construction of microbial biocatalysts. Expression of proteins on the cell surface of *Saccharomyces cerevisiae* offers more advantages than other microbial systems. First, since *S. cerevisiae* is widely used in the industrial production of proteins and chemicals, enzyme-coated yeast cells could be used as novel whole-cell biocatalysts, because surface immobilized proteins are covalently linked to glucan in the cell wall, rendering them resistant to extraction. Second, *S. cerevisiae* is safe for oral use in food and pharmaceutical products [17]. Third, it can be cultivated to high cell density using relatively inexpensive media. To immobilize protein on the cell surface of *S. cerevisiae*, α-agglutinin, a yeast cell wall-anchored protein, has been widely utilized [26]. α-Agglutinin is a mannoprotein involved in the sexual adhesion of mating-type a *S. cerevisiae* cells [21]. It has a glycosyl-phosphatidylinositol (GPI) anchor attachment signal, which is involved in anchoring cell wall proteins [18]. The 74-kDa Aga1p subunit of α-agglutinin is anchored to the cell wall via β-glucan linkage and the 69 amino acid

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binding subunit Aga2p is linked to Aga1p by two disulfide linkages [2].

Many studies have focused on the expression of various enzymes on the cell surface of *S. cerevisiae* using α-agglutinin [23, 29]. Alternatively, properties of the yeast cell surface, such as hydropathy and charges, might support the accessibility and affinity between enzymes and substrates in organic solvent. One of the advantages of enzymes displaying whole-cell biocatalysts is that enzymes are immobilized on the yeast cell surface by genetically modified anchor systems, which not only minimizes the risk of inactivation and inhibition, but also improves the performance of displayed enzyme. In our previous study, we characterized *P. macerans* CFTase in yeast episomal expression [14]. In this paper, a recombinant *S. cerevisiae* strain anchoring *P. macerans* CFTase on the cell surface was developed using Aga2p as a fusion partner, and its application as a novel whole-cell biocatalyst for the production of CFs was examined.

The CFTase gene, *cft*, consisting of 2.8 kb from *P. macerans*, was amplified by PCR using pECFTN [15] as a template. The sequences of the two primers were (5'-ACGGCTAGCATGTTCCAAGCAAGTGATAGG-3' and 5'-GGGATCCGTTGTTAGTTTTCTTCTTTACCTGAACA GGTAC-3'). The underlined sequences denote the NheI and BamHI restriction enzyme sites, respectively. After PCR amplification, the *cft* gene was digested with NheI and BamHI, and then subcloned into the surface display vector pCTcon (*GAL1*p MCS MFαt) [6], resulting in pCTECFTN plasmid. This plasmid was transformed into the competent *Escherichia coli* strain DH5α (*recA1 endA1 gyrA96 thi1 hsdR17 supE44 relA1 lacZΔM15*). The *E. coli* was grown in LB medium (1% tryptone, 0.5% yeast extract, 0.5% NaCl) containing 50 μg/ml ampicillin. The Aga2p was employed as a fusion partner to secrete and localize CFTase on the cell surface of *S. cerevisiae* EBY100 (*trp1 leu2Δ1 his3Δ200 pep4::HIS2 prbΔ1.6R an1 GAL*) [4]. The expression of the Aga2p/CFTase fusion protein was under the control of the *GAL1* promoter. The transformation of the plasmid pCTECFTN into *S. cerevisiae* EBY100 was carried out using the lithium acetate method [8]. The resulting transformant was named EBY100/pCTECFTN. To induce the yeast cells displaying CFTase on their surface, the yeast transformants were grown in a fermentor (KoBiotech Co., Incheon, Korea) containing a YPDG medium (1% yeast extract, 2% peptone, 1% glucose, and 1% galactose) at 20°C and pH 6.0.

The immunofluorescence microscopy and immunostaining method were performed to verify the surface-displayed CFTase as described previously [13, 19]. The immunostaining was conducted as follows: the cells were cultivated in the YPDG medium at 20°C for 48 h, collected by centrifugation at 8,000 rpm for 10 min, and washed with phosphate-buffered saline (pH 7.4, PBS). The cell suspension was then adjusted to OD_{600}=20 with PBS, and 100 μl of the cell suspension collected in a microtube. The cells and 100 μl of the primary antibody were further incubated in PBS containing 1% BSA for 1.5 h on ice. The primary antibody used was c-*myc* monoclonal antibody (mAb) 9E10 (Covance Inc., Berkeley, CA, U.S.A.) at a dilution rate of 1:500. The cells were then washed with PBS and incubated for 1 h on ice at a dilution rate of 1:100 with 100 μl of the secondary antibody, fluorecein-isothiocyanate (FITC)-labeled anti-mouse IgG (H+L) (KPL Co., Gaithersburg, MD, U.S.A.). After washing the cells again with PBS, the fluorescence image was observed under a confocal laser scanning microscope (LSM 510 META, Carl Zeiss, Jena, Germany).

An enzymatic reaction was carried out in 50 mM phosphate buffer (pH 7.0). Two % inulins from chicory, dahlia, and Jerusalem artichoke (Sigma Co., St. Louis, MO, U.S.A.) were used as the substrate. *S. cerevisiae* EBY100/pCTENIU and the control strain *S. cerevisiae* EBY100 were incubated in the YPDG medium for 48 h at 20°C, harvested by centrifugation, and washed twice with PBS. The cells were resuspended in the reaction buffer and added at final concentration of OD_{600}=20 for the enzymatic reaction. The residual concentration of sugar was measured by the dinitrosalicylic acid method [20]. One unit of endoinulinase activity was defined as the amount of the enzyme liberating 1 μmol reducing sugar from dahlia inulin per min at 50°C.

To study the change of product composition during the inulin hydrolysis, enzyme reactions were carried out under the optimal conditions. During the course of dahlia inulin hydrolysis by CFTase, aliquots of the reaction mixture were analyzed for the hydrolysis products by TLC (60 F_{254}, Merck Co., Berlin, Germany). Aliquots (5 μl) of the reaction mixtures were spotted and then developed in a solvent [*n*-butanol:isopropanol:water=3:12:4 (v/v)]. The products were detected by spraying the plate with a urea reagent (93.22 ml of *n*-butanol, 6.78 ml of phosphoric acid, 5 ml of ethanol, and 3 g urea) and heating at 110°C for 10 min [9]. A standard set of fructooligosaccharides, including 1-kestose, nystose, and 1^F-fructosylnystose (Wako, Wako, Japan), and reagent-grade sugars, such as glucose, fructose, and sucrose (Junsei, Tokyo, Japan), were used to identify the reaction products.

As shown in Fig. 1, *S. cerevisiae* EBY100/pCTECFTN was labeled by FITC and its fluorescence was observed on the cells, whereas no fluorescence was shown on the cells of the control strain, *S. cerevisiae* EBY100. This observation, therefore, strongly indicated the successful expression and localization of CFTase on the cell surface of *S. cerevisiae*. When EBY100/pCTECFTN was cultured on a fermentor containing YPDG at 20°C, pH 6.0, for 48 h, the total CFTase activity in the cell pellet was about 0.4 unit/g-dry cell weight, whereas no CFTase activity was detected in the culture supernatant. The result revealed that the

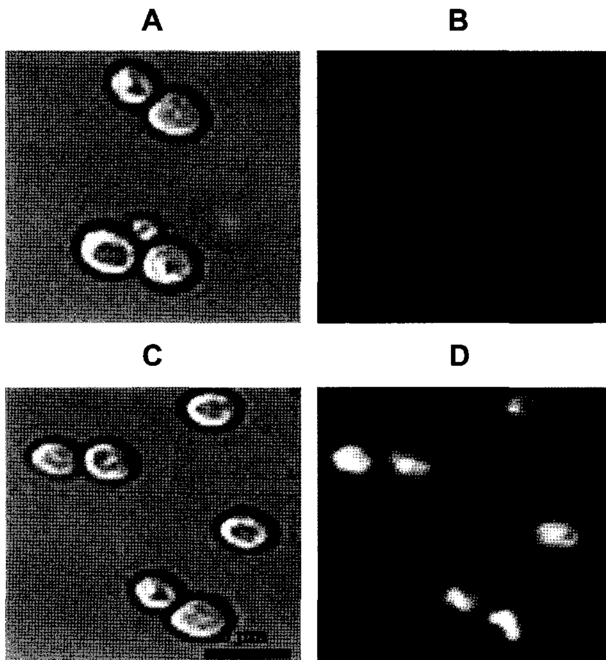

Fig. 1. Confocal microscopic images of yeast *Saccharomyces cerevisiae* EBY100 (**A**, **B**) and EBY100/pCTECFTN (**C**, **D**).
Optical micrographs (**A**, **C**) and confocal laser scanning micrographs (**B**, **D**).

CFTase is displayed on the cell surface in an active form. Furthermore, the CFTase activity of *P. macerans* in yeast episomal expression was detected at about 1.1 unit/g-dry cell weight. It seems probable that the restricted expression of α-agglutinin on the cell surface leads to the lower activity of the surface-displayed enzyme than episomal enzyme. In addition to this, as the enzyme of surface-displayed linked a various mannoprotein decreased the chance of binding of the substrate.

To characterize the CFTase displayed on the cell surface, the effects of pH (4.0–10.0) and temperature (30–70) were examined on CFTase activity of EBY100/pCTECFTN. The optimal pH and temperature for surface-displayed CFTase activity were found to be pH 8.0 and 50°C (data not shown). As previously reported, the optimal condition for wild-type *B. macerans* CFTase activity was observed at pH 7.5 and 45°C [16]. The enzyme was stable in the pH range of 6.0 to 9.5, and at temperatures up to 45°C for 1 h. Furthermore, the optimal pH and temperature of *P. macerans* CFTase in yeast episomal expression were pH 8.0 and 45°C [14]. We assume that the difference of optimal temperature and pH between the wild-type *B. macerans* and surface-displayed CFTase results from the addition of oligosaccharide by glycosylation in yeast. The effect increases the optimal pH and resistance of temperature of the enzyme.

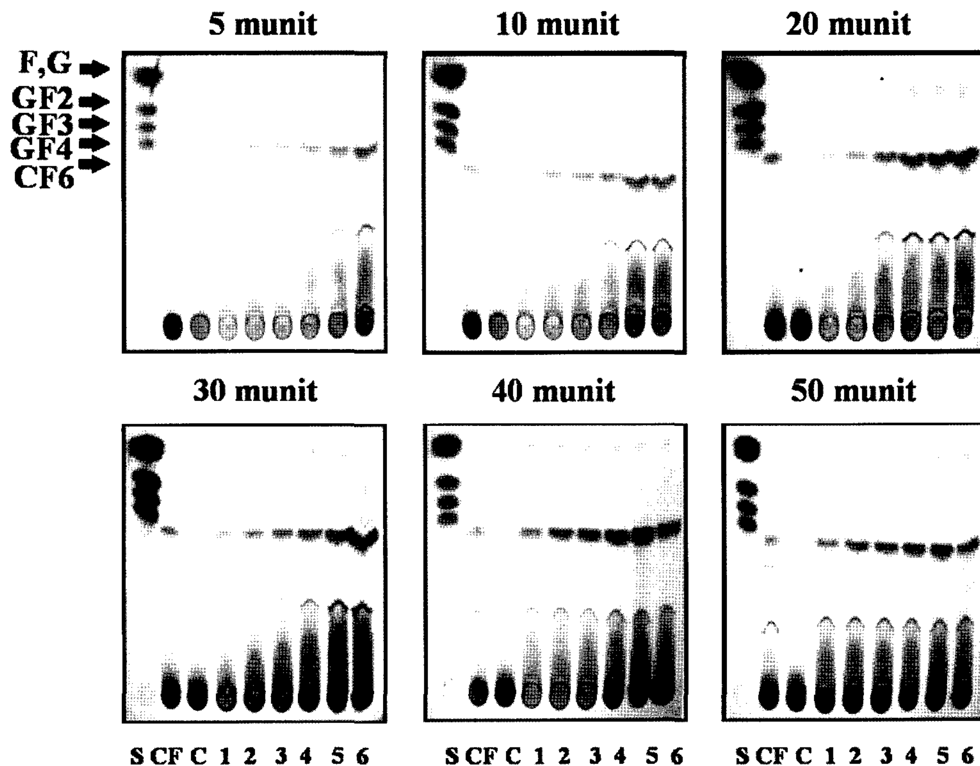

Fig. 2. Effects of enzyme amount on the production of cyclofructans.
Dahlia inulin (5%) was used as the substrate. Lanes 1, 5 min; 2, 10 min; 3, 30 min; 4, 1 h; 5, 3 h; 6, 6 h. F, fructose; G, glucose; GF2, 1-kestose; GF3, nystose; GF4, 1^F-Fructosyl Nystose; C, CF6.

To investigate the effect of amount of enzyme on the hydrolysis of inulin, 5% dahlia inulin was hydrolyzed at 50°C for 30 min with different amounts of surface-displayed enzymes (5–50 milliunits of CFTase). When more than 5 milliunit of enzyme was added, the formation of CFs was observed after 5 min, and thereafter at 1 h the hydrolysis of inulin was almost finished (Fig. 2). As shown in Fig. 2, 30 milliunit of enzymes represented the highest CFs production. In order to determine the optimal substrate concentration, dahlia inulin at different concentrations (1–7%) was hydrolyzed at 50°C by the surface-displayed CFTase. The higher the substrate concentration, the slower was the rate of hydrolysis obtained. When dahlia inulin of 5% was used, the highest CFs production was observed (data not shown).

Previously, it was reported that the wild-type CFTase from *Bacillus macerans* produced CF6 and CF7 in an approximate ratio of 4:1 after 6 h incubation, and the yield of cyclofructans was estimated to be up to 80% of the amount of inulin [16]. In our previous study, *P. macerans* CFTase in yeast episomal expression produced CF6, CF7, and CF8 from inulin [14]. In the present work, after 18 h culture of EBY100/pCTECFTN cells, the reaction products started to be formed. The CFs consisting of CF6, CF7, and CF8 were produced, and among them, CF6 was the major product (Fig. 3A). As shown in the product profile with respect to the reaction time, CFs started to be produced after 5 min of enzymatic reaction (Fig. 3B). It is the same as shown in our previous study that the production of CFs was observed after 5 min [14].

In order to determine the optimal inulin source, chicory inulin, dahlia inulin, and Jerusalem artichoke inulin (5%) were hydrolyzed at 50°C and pH 8.0. The highest CFs production was observed with Jerusalem artichoke inulin (Fig. 4). The preferable substrate for the CFs production was the following order: Jerusalem artichoke>Dahlia> Chicory. This result demonstrates that Jerusalem artichoke inulin seems to have an industrial potential for CFs production. Considering the average degree of polymerization (DP) was 33.6 for chicory and 40.3 for dahlia, a DP value of much greater than 40 would to be estimated for Jerusalem artichoke [24]. The high DP of Jerusalem artichoke might result in increased cell growth, leading to an elevated production of inulooligosaccharide or ethanol [12, 28].

The current study developed a *S. cerevisiae* system anchored with *P. macerans* CFTase to produce CFs from inulin. Further research is in progress to improve the secretion efficiency of bacterial CFTase in *S. cerevisiae*, to select a clone harboring maximum CFTase activity via fluorescence-activated cell sorting (FACS), to compare product profiles for the whole-cell biocatalyst, and finally to optimize the reactor configurations of CFs production.

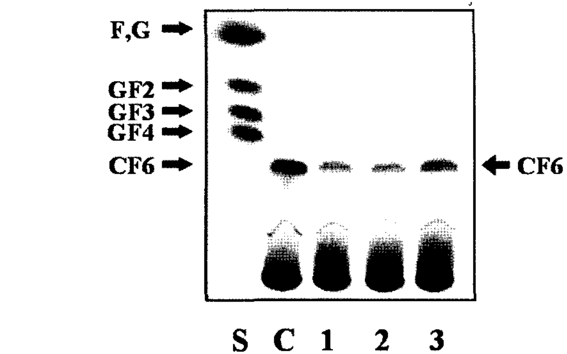

Fig. 4. Effect of inulin source on the production of cyclofructans.
Lane 1, dahlia; 2, chicory; 3, jerusalem artichoke. CFs production in 50 mM phosphate buffer (pH 8.0); 50°C was measured at the indicated inulin source. F, fructose; G, glucose; GF2, 1-kestose; GF3, nystose; GF4, 1F-Fructosyl Nystose; C, CF6.

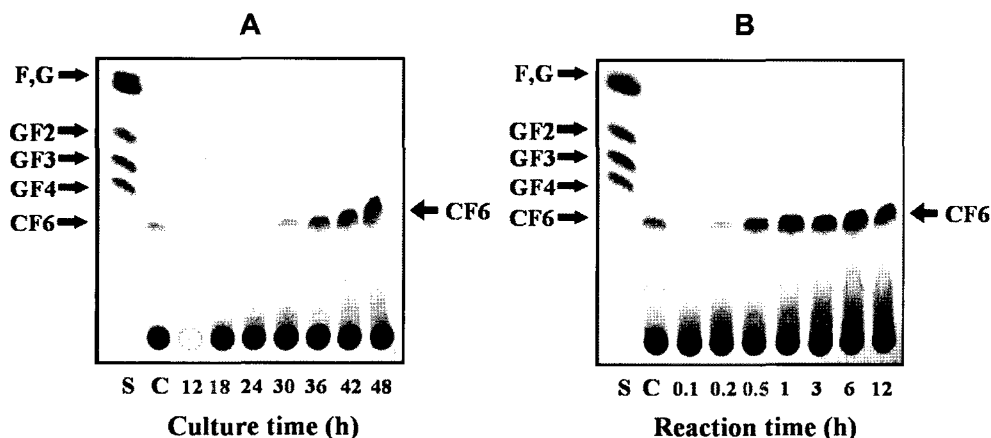

Fig. 3. Time profile of the reaction products from dahlia inulin by surface-displayed CFTase.
A. CF formation by EBY100/pCTECFTN cells at different culture times. **B.** CF formation at different reaction times. F, fructose; G, glucose; GF2, 1-kestose; GF3, nystose; GF4, 1^F-Fructosyl Nystose; C, CF6.

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REFERENCES

1. Boder, E. T. and K. D. Wittrup. 1997. Yeast surface display for screening combinatorial polypeptide libraries. *Nat. Biotechnol.* **15:** 553–557.
2. Cappellaro, C., R. R. Baldermann, and W. Tanner. 1994. Mating type-specific cell-cell recoginition of *Saccharomyces cerevisiae*: Cell wall attachment and active sites of a- and α-agglutinin. *EMBO J.* **13:** 4737–4744.
3. Chiswell, D. J. and J. McCafferty. 1992. Phage antibodies: Will new 'coliclonal' antibodies replace monoclonal antibodies? *Trends Biotechnol.* **10:** 80–84.
4. Cho, B. K., M. C. Kieke, E. T. Boder, K. D. Wittrup, and D. M. Kranz. 1998. A yeast surface display system for the discovery of ligands that trigger cell activation. *J. Immunol. Methods* **220:** 179–188.
5. Choi, J. H., J. I. Choi, and S. Y. Lee. 2005. Display of proteins on the surface of *Escherichia coli* by C-terminal deletion fusion to the *Salmonella typhimurium* OmpC. *J. Microbiol. Biotechnol.* **15:** 141–146.
6. Cochran, J. R., Y. S. Kim, M. J. Olsen, R. Bhandari, and K. D. Wittrup. 2004. Domain-antibody epitope mapping through yeast surface display of epidermal growth factor receptor fragments. *J. Immunol. Methods* **287:** 147–158.
7. Ezaki, S., M. Tsukio, M. Takagi, and T. Imanaka. 1998. Display of heterologous gene products on the *Escherichia coli* cell surface as fusion proteins with flagellin. *J. Ferment. Bioeng.* **86:** 500–503.
8. Ito, H. Y., Y. Fukuda, K. Murata, and A. Kimura. 1983. Transformation of intact yeast cells treated with alkali cations. *J. Bacteriol.* **153:** 163–168.
9. Jeon, S. J., D. J. You, H. J. Kwon, S. Kanaya, N. Kunihiro, K. H. Kim, Y. H. Kim, and B. W. Kim. 2002. Cloning and characterization of cycloinulooligosacchride fructano-transferase (CFTase) from *Bacillus polymyxa* MGL21. *J. Microbiol. Biotechnol.* **12:** 921–928.
10. Kanai, T., N. Ueki, T. Kawaguch, Y. Teranishi, H. Atomi, C. Tomorbaatar, M. Ueda, and A. Tanaka. 1997. Recombinant thermostable cycloinulooligosaccharide fructano-transferase produced by *Saccharomyces cerevisiae. Appl. Environ. Microbiol.* **63:** 4956–4960.
11. Kawamura, M., T. Uchiyama, T. Kuramoto, Y. Tamura, and K. Mizutani. 1989. Formation of a cycloinulooligosaccharide from inulin by an extracellular enzyme of *Bacillus circulans* OKUMZ31B. *Carbohydr. Res.* **192:** 83–90.
12. Kim, D. H., Y. J. Choi, S. K. Song, and J. W. Yun. 1997. Production of inulo-oligosaccharides using endo-inulinase from *Pseudomonas* sp. *Biotechnol. Lett.* **19:** 369–371.
13. Kim, H. C., H. J. Kim, W. B. Choi, and S. W. Nam. 2006. Inulooligosaccharide production from inulin by *Saccharomyces cerevisiae* strain displaying cell-surface endoinulinase. *J. Microbiol. Biotechnol.* **16:** 360–367.
14. Kim, H. C., J. H. Jeong, S. J. Jeon, W. B. Choi, and S. W. Nam. 2005. Expression of *Paenibacillus macerans* cycloinulooligosaccharide fructanotransferase in *Saccharomyces cerevisiae. J. Life Sci.* **15:** 317–322.
15. Kim, H. Y. and Y. J. Choi. 2001. Molecular characterization of cyclo-inulooligosaccharide fructanotransferase from *Bacillus macerans. Appl. Environ. Microbiol.* **67:** 995–1000.
16. Kim, H. Y. and Y. J. Choi. 1998. Purification and characterization of cyclo-inulooligosaccharide fructanotransferase from *Bacillus macerans* CFC1. *J. Microbiol. Biotechnol.* **8:** 251–257.
17. Kim, J. J., S. W. Kim, C. O. Jeon, J. Y. Yun, H. S. Lee, and H. S. Ro. 2006. Screening of yeast diauxic promoters for production of foreign proteins. *J. Microbiol. Biotechnol.* **16:** 1459–1463
18. Kim, Y. H., S. W. Nam, and B. H. Chung. 1998. Simultaneous saccharification of inulin and ethanol fermentation by recombinant *Saccharomyces cerevisiae* secreting inulinase. *Biotechnol. Bioproc. Eng.* **3:** 55–60.
19. Kobori, H., M. Sato, and M. Osumi. 1992. Relationship of actin organization to growth in the two forms of the dimorphic yeast *Candida tropicalis*. *Protoplasma* **167:** 193–204.
20. Kongruang, S., M. J. Han, C. I. Breton, and M. H. Penner. 2004. Quantitative analysis of cellulose-reducing ends. *Appl. Biochem. Biotechnol.* **113:** 213–231.
21. Lipke, P. N. and J. Kurjan. 1992. Sexual agglutination in budding yeasts: Structure, function, and regulation of adhesion glycoproteins. *Microbiol. Rev.* **56:** 180–194.
22. Murai, T., M. Ueda, H. Atomi, Y. Shibasaki, N. Kamasawa, M. Osumi, T. Imanaka, and A. Tanaka. 1999. Development of an arming yeast strain for efficient utilization of starch by co-display of sequential amylolytic enzymes on the cell surface. *Appl. Microbiol. Biotechnol.* **51:** 65–70.
23. Murai, T., M. Ueda, H. Atomi, Y. Shibasaki, N. Kamasawa, M. Osumi, T. Kawaguichi, M. Arai, and A. Tanaka. 1997. Genetic immobilization of cellulase on the cell surface of *Saccharomyces cerevisiae. Appl. Microbiol. Biotechnol.* **48:** 499–503.
24. Ohta, K., S. Hamada, and T. Nakamura. 1993. Production of high concentrations of ethanol from inulin by simultaneous saccharification and fermentation using *Aspergillus niger* and *Saccharomyces cerevisiae. Appl. Environ. Microbiol.* **59:** 729–733.
25. Sawada, M., T. Tanaka, Y. Takai, T. Hanafrsa, T. Taniguchi, M. Kawamura, and T. Uchiyama. 1991. The crystal structure of cycloinulohexaose produced from inulin by cycloinulooligosaccharide fructanotransferase. *Carbohydr. Res.* **217:** 7–17.

26. Schreuder, M. P., S. Brekelmans, H. van den Ende, and F. M. Klis. 1993. Targeting of a heterologous protein to the cell wall of *Saccharomyces cerevisiae. Yeast* **9:** 399–409.
27. Uchiyama, T., M. Kawamura, T. Uragami, and H. Okuno. 1993. Complexing of cycloinulo-oligosaccharides with metal ions. *Carbohydr. Res.* **241:** 245–248.
28. Vandamme, E. J. and D. G. Derycke. 1983. Microbial inulinase: Fermentation process, properties, and applications. *Adv. Appl. Microbiol.* **29:** 139–176.
29. Yasuya, F., K. Satoshi, M. Ueda, A. Tanaka, J. Okada, Y. Morikawa, H. Fukuda, and A. Kondo. 2002. Construction of whole-cell biocatalyst for xylan degradation through cell-surface xylanase display in *Saccharomyces cerevisiae. J. Mol. Catal. B: Enzym.* **17:** 189–195.