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Construction of a Starch-Utilizing Yeast by Cell Surface Engineering

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We have engineered the cell surface of the yeast *Saccharomyces cerevisiae* by anchoring active glucoamylase protein on the cell wall, and we have endowed the yeast cells with the ability to utilize starch directly as the sole carbon source. The gene encoding *Rhizopus oryzae* glucoamylase with its secretion signal peptide was fused with the gene encoding the C-terminal half (320 amino acid residues from the C terminus) of yeast α-agglutinin, a protein involved in mating and covalently anchored to the cell wall. The constructed plasmid containing this fusion gene was introduced into *S. cerevisiae* and expressed under the control of the glyceraldehyde-3-phosphate dehydrogenase promoter from *S. cerevisiae*. The glucoamylase activity was not detected in the culture medium, but it was detected in the cell pellet fraction. The glucoamylase protein transferred to the soluble fraction from the cell wall fraction after glucanase treatment but not after sodium dodecyl sulfate treatment, indicating the covalent binding of the fusion protein to the cell wall. Display of the fused protein was further confirmed by immunofluorescence microscopy and immunoelectron microscopy. The transformant cells could surely grow on starch as the sole carbon source. These results showed that the glucoamylase was anchored on the cell wall and displayed as its active form. This is the first example of an application of cell surface engineering to utilize and improve the metabolic ability of cells.

Display of heterologous proteins on the cell surface of microorganisms is an important objective for many applications in microbiology and molecular biology. For the past several years, the expression of proteins on the surfaces of bacteriophage and bacteria has been actively studied (5, 9). These systems are expected to be useful for the segregation of produced polypeptides and for construction of microbial biocatalysts, whole-cell adsorbents, and live vaccines. Expression of proteins on the cell surface of *Saccharomyces cerevisiae* offers several advantages: as *S. cerevisiae* is widely used in industrial production of proteins and chemicals, enzyme-coated yeast cells could be used as novel whole-cell biocatalysts, because surface-expressed proteins are covalently linked to glucan in the cell wall, rendering them resistant to extraction; as *S. cerevisiae* is safe for oral use, it can be used in food and pharmaceutical products and for oral vaccines.

Although starchy materials are available in abundance as carbon sources for cultivation, wild-type *S. cerevisiae* itself is unable to utilize starch. A number of strategies have been adopted for the construction of starch-utilizing systems, which include the addition of amylolytic enzymes in culture broth and the introduction of heterologous genes encoding amylases into yeast cells for secretive production of the enzymes (20). The present research has aimed to construct a novel starch-utilizable yeast by targeting glucoamylase (EC 3.2.1.3) to the cell wall of *S. cerevisiae*. The protein displayed on the cell wall was glucoamylase from *Rhizopus oryzae* (1), an exo-type amylolytic enzyme, cleaving α-1,4-linked and α-1,6-linked glucose effectively from starch. This cell surface-engineered yeast strain should be able to saccharify starch by glucoamylase on its cell wall and assimilate the released glucose to proliferate and ferment. To fix glucoamylase to the cell wall of *S. cerevisiae*, it was genetically fused to a part of the native cell wall-anchored protein α-agglutinin (17). α-Agglutinin is a mannoprotein involved in the sexual adhesion of mating type α *S. cerevisiae* cells with mating type **a** *S. cerevisiae* cells (13). α-Agglutinin has a glycosylphosphatidylinositol (GPI) anchor attachment signal, which is involved in anchoring cell wall proteins (12, 22). We employed the secretion signal of the glucoamylase precursor protein (1), as the glucoamylase must be transported across the cytoplasmic membrane to localize on the cell wall.

Here, we report on the construction of a novel starch-utilizing yeast, "cell surface-engineered" yeast, by display of the amylolytic enzyme on the cell wall of *S. cerevisiae*.

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MATERIALS AND METHODS

Strains and media. *Escherichia coli* DH5α [F$^-$ *endA1 hsdR17* (r_K^-/m_K^-) *supE44 thi-1* λ$^-$ *recA1 gyrA96* Δ*lacU169*(φ80*lacZ*ΔM15)] was used as a host for recombinant DNA manipulation. *S. cerevisiae* MT8-1 (*MAT***a** *ade his3 leu2 trp1 ura3*) (18) was used for cultivation. *E. coli* was grown in LB medium (1% tryptone, 0.5% yeast extract, 0.5% sodium chloride) containing 0.1% glucose. Yeast was precultivated in YPD medium (1% yeast extract, 2% peptone, 2% glucose) and cultivated aerobically at 30°C in modified Burkholder's medium (19) (containing 0.002% adenine sulfate, 0.002% L-histidine-HCl, 0.003% L-leucine, 0.002% uracil, and 1% Casamino Acids) to which 2% glucose or 1% soluble starch was added as a carbon source. Cell growth in the culture broth was measured by absorbance at 600 nm.

Construction of the plasmid and transformation of yeast. The plasmid pGA11 was constructed as follows. An *Xho*I site was generated at the end of the glucoamylase coding region on plasmid pYGA2269 (2) by site-directed mutagenesis (U.S.E. Mutagenesis Kit, Pharmacia Biotech. Co., Uppsala, Sweden) with the primers 5′-GCACCTGCCGCTGGCTCGAGAAATTTAAATGC-3′ and 5′-CTGTGACTGGTGACGCGTCAACCAAGTC-3′ as mutation and selection primer, respectively. A DNA fragment containing the glucoamylase coding region was isolated from the mutated plasmid by *Eco*RI-*Xho*I digestion. A DNA fragment of the α-agglutinin gene (*AG*α*1*) (12), containing the 3′ half of the coding region encoding 320 amino acids of the α-agglutinin and 446 bp of the 3′-flanking region, was prepared by PCR (primers, 5′-GTACCTCGAGCGCCA

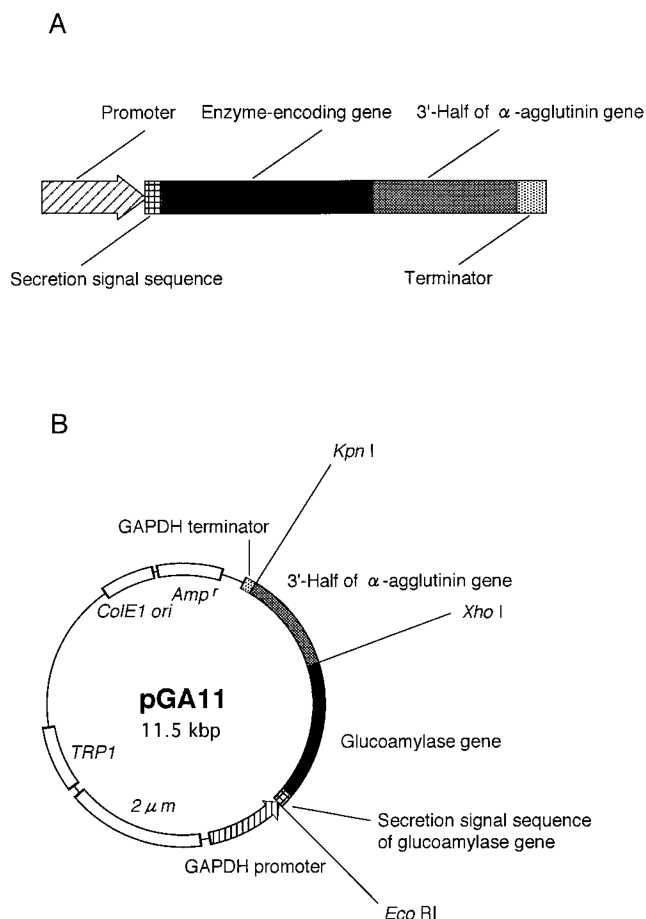

FIG. 1. General structure of the gene for cell surface display of an enzyme (A) and the constructed plasmid pGA11 for expression of the glucoamylase/α-agglutinin fusion gene (B).

AAAGCTCTTTTATC-3′ and 5′-GCGGTACCTTTGATTATGTTCTTTCTAT-3′) with genomic DNA from *S. cerevisiae* MT8-1 as a template, followed by digestion by *Xho*I and *Kpn*I. These two fragments were substituted for the *Eco*RI-*Kpn*I section between the glyceraldehyde-3-phosphate dehydrogenase (GAPDH) promoter and the GAPDH terminator of the yeast expression cassette vector pYE22m (16). The resulting plasmid was named pGA11.

Isolation of cell wall and protein extraction. Cells were harvested by centrifugation at 3,000 × *g* and washed in cooled buffer (10 mM Tris-HCl [pH 7.8], 1 mM phenylmethylsulfonyl fluoride). The cells, buffer, and glass beads (diameter, 0.45 to 0.50 mm) were mixed in a ratio of 1:2:1 (wet wt/vol/wt) in a glass tube and agitated vigorously, with a bench top vortex mixer, for 5 min at 0°C. The cell wall fraction was recovered by centrifugation of the homogenate at 1,000 × *g* for 5 min and was washed with the same buffer. Sodium dodecyl sulfate (SDS) extraction and subsequent glucanase extraction were carried out according to the previously reported method (17).

Glucoamylase assay. The substrate for glucoamylase reaction was prepared by adding soluble starch to boiling 20 mM sodium acetate buffer (pH 4.6) to give a concentration of 0.5%. After keeping 0.9 ml of the solution at 30°C for 5 min, 0.1 ml of enzyme solution was added and the mixture was incubated at the same temperature for 15 min. The reaction was stopped by boiling the mixture for 10 min and the concentration of glucose was determined by using the F-kit for glucose (Boehringer Mannheim, Mannheim, Germany). One unit of glucoamylase was defined as the amount of enzyme required to release 1 μmol of glucose/min from starch. To evaluate the amount of the glucoamylase protein expressed, the culture medium, the SDS extract, and the glucanase extract were also applied on a dot blotter (DP-96, Advantec Co., Tokyo), and proteins contained in each fraction were adsorbed onto a nitrocellulose filter. The glucoamylase protein on the filter was detected with anti-glucoamylase immunoglobulin G (IgG) and horseradish peroxidase-protein A (21).

Immunofluorescence microscopy. Immunofluorescence microscopy was performed as reported previously (11). Immunostaining was performed as follows. As the primary antibody, we used the antibody against *R. oryzae* glucoamylase at a dilution rate of 1:1,000. Cells and the antibody were incubated at room temperature for 1.5 h. After the cells were washed, the second antibody, fluorescein isothiocyanate-conjugated goat anti-rabbit IgG, diluted 1:300, was reacted with the cells at room temperature for 1 h (11). After washing, the images of cells were observed.

Immunoelectron microscopy. After yeast cells were fixed with 4% paraformaldehyde, immunostaining was done as follows. The antibody against glucoamylase diluted 1:100, as the primary antibody, was incubated with cells at 4°C for 2 h. The second antibody, goat anti-rabbit IgG conjugating 5-nm-diameter particles of gold diluted 1:40, was reacted with the cells at 4°C overnight, followed by fixation with 1% glutaraldehyde. Thereafter, embedding and microscopic observation were performed according to the method described previously (10).

Determination of starch and ethanol concentrations. Concentration of starch was determined according to the previously reported method (6). Concentration of ethanol was measured by using the F-kit for ethanol (Boehringer Mannheim).

RESULTS

Construction of the cell surface expression plasmid. The plasmid pGA11 was constructed as described in Materials and Methods (Fig. 1). The plasmid pGA11 was a multicopy plasmid for expression of the glucoamylase/α-agglutinin fusion gene containing the secretion signal sequence of the glucoamylase under the control of the GAPDH promoter. The plasmid pGA11 and the plasmid pYE22m, a control plasmid, were introduced into *S. cerevisiae* MT8-1.

Detection of amylolytic activity on plate. First we performed a plate assay to determine whether the transformants gained amylolytic activity. The amylolytic activity was detected by halo formation on an agar plate. Cells harboring the plasmid pGA11 or pYE22m, as a control, were inoculated on a plate of modified Burkholder's medium containing 2% glucose and 1% soluble starch. After incubation for 3 days at 30°C, the plate was stained with iodine vapor (3). The result shown in Fig. 2 demonstrated that the cells harboring the plasmid pGA11 hydrolyzed starch and produced a halo strictly around the colony, while no halo formation was observed around the cells harboring the plasmid pYE22m. This indicated that the former cells obtained amylolytic activity due to the expression of the glucoamylase/α-agglutinin fusion gene.

Localization of glucoamylase. We next determined whether glucoamylase was secreted in the culture medium or retained by the cells. Cells were cultivated in modified Burkholder's medium containing 2% glucose as a carbon source at 30°C for 24 h. Culture medium and cell pellets were isolated by centrifugation to measure the glucoamylase activities in both fractions. The result, shown in Table 1, demonstrated that the cells harboring the plasmid pGA11 had the cell-associated glucoamylase activity without secretion of the active enzyme.

The cell wall of *S. cerevisiae* mainly consists of glucan and mannoproteins (8). Glucan is composed of β-1,3- and β-1,6-linked glucose (14, 15). Some mannoproteins seemed to be covalently linked with glucan, because they can be extracted by β-1,3- or β-1,6-glucanase (7). Therefore, we further deter-

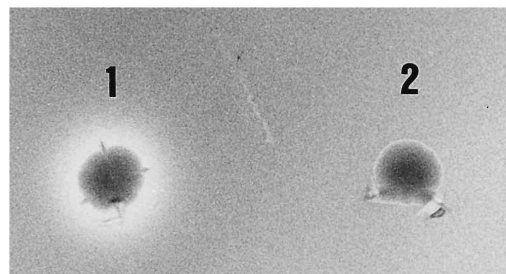

FIG. 2. Plate test for detection of amylolytic activity. Panels: 1, *S. cerevisiae* MT8-1/pGA11; 2, *S. cerevisiae* MT8-1/pYE22m (control).

TABLE 1. Distribution of glucoamylase

Strain	Glucoamylase activity (U/liter of culture broth)		Glucoamylase protein in cell wall[a]	
	Culture medium	Cell pellet	SDS extract	Glucanase extract
MT8-1/pGA11	ND[b]	30.0	6.8	93.2
MT8-1/pYE22m	ND	ND	ND	ND

[a] Percentage of the total extractable glucoamylase protein.
[b] ND, not detected.

mined the localization of the glucoamylase protein and its association with cell wall. Cells were disrupted to obtain cell wall, and glucoamylase was extracted from this fraction in a two-step procedure. First, noncovalently bound proteins or proteins bound through disulfide bridges could be extracted from cell wall with hot SDS. Following this, the SDS-treated cell wall was further digested with laminarinase (β-1,3-glucanase). To quantify the amount of the SDS-extracted and glucanase-extracted glucoamylase protein, intensities of signals from each fraction on the filter described in Materials and Methods were compared (Table 1). The sum of the amount of glucoamylase extracted through the two-step procedure was defined as 100%. The result revealed that even after treatment with SDS, 93.2% of the total extractable glucoamylase could still be extracted by glucanase from cell walls of the cells harboring the plasmid pGA11. This indicated that the glucoamylase was covalently attached to the cell wall.

Immunofluorescence microscopy. We conducted immunofluorescent labeling of cells with anti-glucoamylase IgG and found that cells expressing the glucoamylase/α-agglutinin fusion gene were uniformly labeled, although not all the cells were equally intensively labeled (Fig. 3a and 3b). This is probably due to differences in expression levels among the cells. The cells harboring the control plasmid were hardly labeled (Fig. 3c and 3d). Thus, it was confirmed that the expressed glucoamylase/α-agglutinin fusion protein was anchored on the cell wall of the cells harboring the plasmid pGA11.

Immunoelectron microscopy. We further investigated the localization of glucoamylase/α-agglutinin fusion protein by immunoelectron microscopy. Gold particles were detected on the

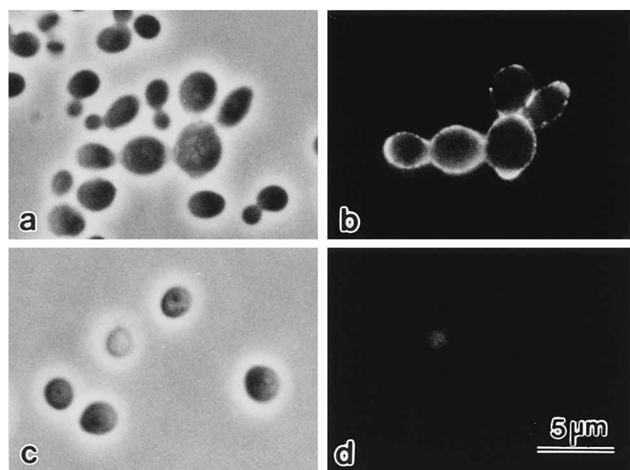

FIG. 3. Immunofluorescent labeling of transformed cells. Phase micrographs (a and c) and immunofluorescence micrographs (b and d). (a and b) *S. cerevisiae* MT8-1/pGA11; (c and d) *S. cerevisiae* MT8-1/pYE22m (control).

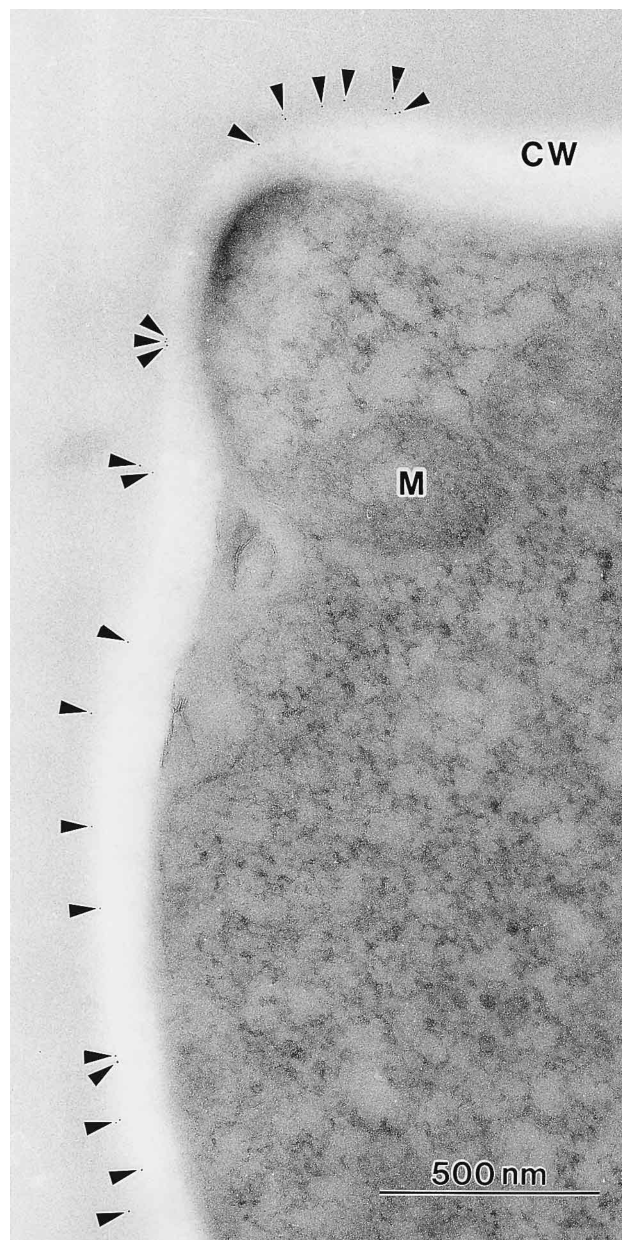

FIG. 4. Immunoelectron micrograph of *S. cerevisiae* MT8-1/pGA11. Cells were immunogold labeled with the antibody against glucoamylase. M, mitochondrion; CW, cell wall. Bar = 500 nm. Arrowheads indicate gold particles (diameter, 5 nm).

surface of the cell wall of the cells harboring the plasmid pGA11 (Fig. 4). Few gold particles were detected in the case of the control cells (data not shown). These results were good confirmation of the localization of the expressed glucoamylase/α-agglutinin fusion protein on the cell wall.

Growth in starch-containing medium. Cells were cultivated aerobically with 1% soluble starch as the sole carbon source, and cell growth, consumption of starch, and ethanol formation were measured (Fig. 5). The cells harboring the plasmid pGA11 utilized starch and proliferated to reach an absorbance of about 10, which was the same level as for the culture on 1% glucose (data not shown). These data show that the cell surface-anchored glucoamylase reacted sufficiently for the utiliza-

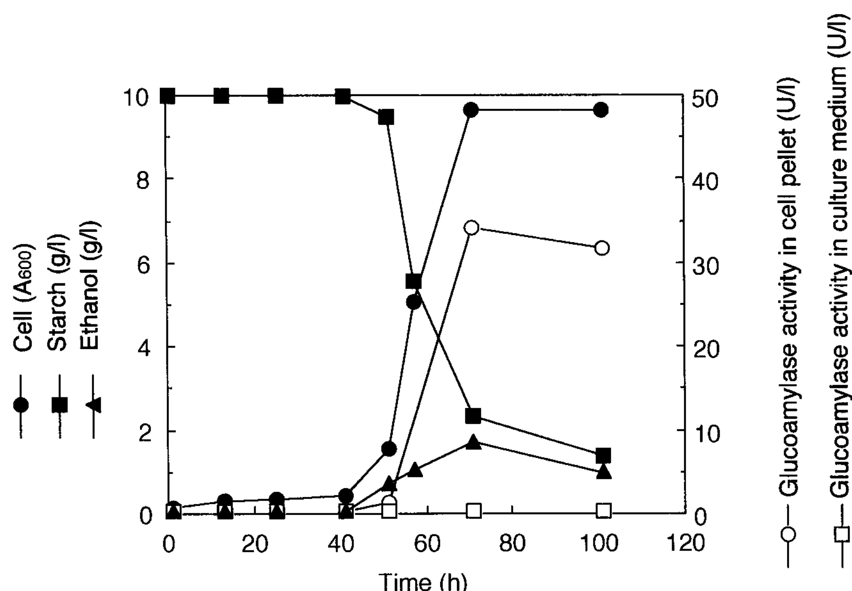

FIG. 5. Time courses of cell growth, consumption of starch, production of ethanol, and glucoamylase activity in cell pellet and in culture medium during aerobic cultivation of *S. cerevisiae* MT8-1/pGA11 in starch-containing medium. Symbols: ●, cell growth (absorbance at 600 nm); ■, starch concentration (in grams per liter); ▲, ethanol produced (in grams per liter); ○, glucoamylase activity in cell pellet (in units per liter); □, glucoamylase activity in culture medium (in units per liter).

tion of starch. No growth on starch was observed with the control cells.

Properties of the anchored enzyme. To examine the properties of the anchored enzyme, thermal stability, optimal temperature, and optimal pH of the glucoamylase anchored on the cell surface were compared with those of the secreted free glucoamylase. The cell wall fraction (see Materials and Methods) of the cells harboring the plasmid pGA11 was suspended in 20 mM sodium acetate buffer (pH 4.6). The cells harboring the plasmid for expression of the glucoamylase without being fused to α-agglutinin were cultivated in modified Burkholder's medium containing 2% glucose as a carbon source at 30°C for 24 h, and the supernatant of the culture broth was used as the source of free glucoamylase. Free glucoamylase solution was prepared by dialysis of the culture supernatant in 20 mM sodium acetate buffer (pH 4.6) at 4°C. The glucoamylase activities in the cell wall suspension and the dialyzed supernatant were measured. The properties of the anchored glucoamylase examined were similar to those of the free glucoamylase (data not shown), suggesting that the enzymatic function of the anchored glucoamylase was comparable to that of the free enzyme.

DISCUSSION

We have described the development of a novel yeast which can grow on starch, applying the technique of cell surface engineering. The data presented here have shown that by using yeast α-agglutinin as an anchor region and the secretion signal peptide from *R. oryzae* glucoamylase, we were able to transport and covalently bind glucoamylase protein to the cell surface. The results seen in Fig. 2, 3, and 4 indicated that the pGA11-transformed cells displayed glucoamylase on the outside of the cell wall and acquired the glucoamylase activity on their cell surface. As a result, they exhibited starch-utilizing ability (Fig. 5).

Schreuder et al. reported that they had succeeded in targeting α-galactosidase from *Cyamopsis tetragonoloba* seeds, used as a reporter enzyme, to the cell wall of *S. cerevisiae* (17). However, the engineered *S. cerevisiae* reported here is the first example of yeast in which protein was targeted to the cell surface and endowed the cells with a new beneficial property. The displayed enzyme is regarded as a kind of a self-immobilized enzyme on the cell surface, this phenomenon being passed on to daughter cells as long as the plasmid is retained by the cells. This display system could turn or remake *S. cerevisiae* into a novel and attractive microorganism as a whole-cell biocatalyst by surface expression of various enzymes, especially when target substrates are not able to be taken up by the cells, and will make it possible to produce renewable self-immobilizing catalysts.

In the studies of surface display of proteins, previously reported systems have been used mainly to obtain possible single-chain antibodies as in the phage display system or to explore the use of surface-displayed enzymes as catalysts (5, 9). On the other hand, in the studies of cellular engineering, studies have focused on changing or improving intracellular metabolic abilities by addition or deletion of certain enzymes (4). Here we have combined the cell surface display system and endowment of an additional metabolic reaction to the yeast cells for the first time: we have developed novel cell surface-engineered cells endowed with starch-utilizing ability by displaying glucoamylase on their cell surface. Thus, the cell surface can be regarded as a new target for giving additional characteristics of metabolic reactions. The research we have reported here will open a new frontier of cellular engineering.

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