Note

Efficient and Direct Fermentation of Starch to Ethanol by Sake Yeast Strains Displaying Fungal Glucoamylases

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Aspergillus oryzae glucoamylases encoded by glaA and glaB, and Rhizopus oryzae glucoamylase, were displayed on the cell surface of sake yeast Saccharomyces cerevisiae GRI-117-UK and laboratory yeast S. cerevisiae MT8-1. Among constructed transformants, GRI-117-UK/pUDGAA, displaying glaA glucoamylase, produced the most ethanol from liquefied starch, although MT8-1/pUDGAR, displaying R. oryzae glucoamylase, had the highest glucoamylase activity on its cell surface.

Key words: sake yeast; Aspergillus oryzae; glucoamylase; cell-surface engineering; bioethanol

Ethanol production from starch is performed through saccharification of starch by amylolytic enzymes such as α-amylase (EC 3.2.1.1) and glucoamylase (EC 3.2.1.3), and the subsequent ethanol conversion of glucose by microorganisms such as yeast. High amylolytic activity of enzymes and high conversion rate of microorganisms make it possible to achieve high ethanol productivity. Various commercial enzymes are often used in the saccharification process in industrial ethanol production, because the yeast Saccharomyces cerevisiae cannot convert starch to ethanol directly. However, enzymes are expensive and difficult to recycle. To find a way to overcome this problem, cell-surface engineering was applied to a laboratory strain of S. cerevisiae.1) When amylolytic enzymes are displayed on the cell surface of the strain, starch can be directly converted to ethanol. While yeast cells displaying enzymes can be regarded as whole-cell biocatalysts, enzymes are simply collected by centrifugation and easily recycled, but investigation of more suitable host strain and enzyme has not previously been done.

In Japanese sake brewing, koji mold (Aspergillus oryzae) powerfully degrades rice starch, and sake yeast (S. cerevisiae) vigorously ferments glucose to ethanol. Previously, two glucoamylase-encoding genes, glaA and glaB, were cloned from A. oryzae; glaA is specifically expressed in submerged culture, and glaB in solid-state culture. It is thought that koji mold produces these glucoamylases, degrading starch in various states.2,3) On the other hand, sake yeasts show higher ethanol productivity and tolerance than laboratory strains of S. cerevisiae.4) We attempted a new combination of A. oryzae amylases as amylolytic enzymes with industrial sake yeast as the host strain. We found that ethanol was efficiently produced in large quantities directly from starch by the cell-surface engineered sake yeast strains.

Yeast strains S. cerevisiae GRI-117-UK (α/α ura3/ura3 lys2/lys2) and MT8-1 (α ade his3 leu2 trp1 ura3) were used as the hosts for cell-surface display. Sake yeast strain GRI-117-UK was obtained by ethyl methane sulfonate mutagenesis of the wild type of sake yeast Kyokai no. 9 (Brewing Society of Japan, Tokyo). Plasmid pUDGAA containing the glaA glucoamylase gene, pUDGAB containing the glaB glucoamylase gene, and pUDGAR containing the R. oryzae glucoamylase gene were constructed (Fig. 1). These constructed plasmids and pAKUD1 as a control plasmid were integrated into the chromosomal DNA of S. cerevisiae by the lithium acetate procedure.6) Glucoamylase-displaying laboratory yeast MT8-1/pGA11 and its control yeast MT8-1/pCAS1 were obtained previously.7) To ferment starch to ethanol directly, yeast episomal plasmids which made the yeast display glucoamylase were first introduced into the laboratory yeast. But this system requires selective pressure to retain yeast episomal plasmids. Furthermore, retaining the plasmids with a 2-μm origin of replication has been reported to be difficult in sake yeast,7) so yeast integration plasmids are usually used.

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A 1.5-kb fragment containing the signal sequence of *R. oryzae* glucoamylase gene, the 3'-half of α-agglutinin gene, and the terminator of α-agglutinin gene was obtained by digesting pCAS1 with EcoRI and KpnI. This fragment was ligated into plasmid pRS406 (Stratagene, La Jolla, CA) digested with EcoRI and KpnI. The resulting plasmid was designated pRS406/CAS1. The promoter region of SED1, whose expression increases in the stationary phase, was amplified from *S. cerevisiae* X2180-1A chromosomal DNA by PCR using primers 5'-CCG-CCCAGGGGAAAAACGACAACATTCCA-3' and 5'-CCGGAATTCCTTAATAGACGCAACGTATTTT-3', and then digested with SmaI and EcoRI. The resulting fragment was inserted into plasmid pRS406/CAS1 digested with SmaI and EcoRI, yielding pAKUD1. *A. oryzae* glaA and glaB were amplified by PCR with plasmids pYGA1 and pYGB1 as template using primers 5'-CCCAGATCTCGAAGCGACTGGATCTAGAT-3' and 5'-CCCAGATCTCCCCGCCAAACATCGCTCTGC-3', and 5'-CCCAGATCTCGAAGCGACTGGATCTAGAT-3' and 5'-CCCAGATCTCGAAGCGACTGGATCTAGAT-3', respectively. Subsequently, the fragments of glaA and glaB were digested with BglII, and then inserted into plasmid pAKUD1 digested with the same nuclease. The resulting plasmids were designated pUDGAA and pUDGAB. Plasmid pUDGAR was constructed as follows; a fragment of the *R. oryzae* glucoamylase gene was obtained from pGA11 digested with BglII, and then inserted into plasmid pAKUD1 digested with the same nuclease. The resulting plasmids contained glaA glucoamylase gene (1,761 bp), pUDGAA contained the *glaB* glucoamylase gene (1,401 bp), and pUDGAR contained the *R. oryzae* glucoamylase gene (1,737 bp).

The activity was measured using *p*-nitrophenyl β-maltoside as the substrate. One unit of glucoamylase activity was defined as the amount of enzyme that released 1 μmol of *p*-nitrophenol per min. After aerobic cultivation of yeast in YPD medium (10 g/l yeast extract, 20 g/l polypeptone, and 20 g/l glucose) at 30°C for 48 h, cells were harvested by centrifugation, washed with distilled water, and resuspended in a reaction mixture with the optical density 5.0 at 600 nm.
Fig. 3. Direct Production of Ethanol from Liquefied Corn Starch.
A, GRI-117-UK with the yeast integrating plasmid; B, MT8-1 with the yeast integrating plasmid; C, MT8-1 with the yeast episomal plasmid. After precultivation in SD medium (6.7 g/l of yeast nitrogen base without amino acids, 20 g/l of glucose and appropriate supplements) for 16 h, yeast cells were aerobically cultivated for 48 h at 30°C in YPD medium, harvested by centrifugation, and washed with distilled water. The cells were placed into liquefied corn starch containing 50 g/l corn starch and 50 mM acetate buffer (pH 5.0), and ethanol fermentation was anaerobically demonstrated at 30°C with the optical density 20 at 600 nm. The ethanol concentration was measured by gas chromatography. The gas chromatograph (model GC-8A, Shimadzu, Kyoto, Japan) was operated under the following conditions: Gasukuropack54; temperatures of column and injector, 180°C and 250°C, respectively; N₂ carrier gas flow rate, 60 ml/min. Open circle, strain harboring pUDGAA; closed circle, strain harboring pUDGAB; open triangle, strain harboring pUDGAR; closed triangle, strain harboring pAKUD1; open square, strain harboring pGA11; closed square, strain harboring pCAS1.
The glucoamylase activity of the yeast strains was determined using the glucose-forming activity assay kit (Kikkoman, Chiba, Japan). The yeast transformants were cultivated aerobically at 30°C for 48 h in YPD medium; their glucoamylase activities are shown in Fig. 2. MT8-1/pUDGAR, displaying R. oryzae glucoamylase, had the highest glucoamylase activity on its cell surface among the constructed transformants. The transformants of laboratory yeast displaying glaB glucoamylase or R. oryzae glucoamylase showed somewhat higher activity than those of sake yeast. The laboratory yeast displayed these enzymes on the cell surface more efficiently than the sake yeast because of the differences in the cell-surface structures. Sake yeasts have Awa1p, a glycosylphosphatidylinositol (GPI)-anchored protein, which gives the cell surface hydrophobicity, and does not appear in laboratory yeasts. The cell-surface structure of sake yeast might be more complicated than that of laboratory yeasts. Moreover, Awa1p might compete with the glucoamylase displayed on the cell surface.

Direct fermentation of corn starch liquefied by autoclaving to ethanol was performed using these glucoamylase-displaying yeasts. The highest ethanol concentration was around 18.5 g/l after 48 h of fermentation using strain GRI-117-UK/pUDGAA (Fig. 3), and the ethanol conversion efficiency was 64.9% of the theoretical ethanol concentration produced from 50 g/l of liquefied starch. The ethanol production of sake yeasts displaying glucoamylases was more efficient than those of laboratory yeasts displaying glucoamylases. It is suggested that glucose released from starch was almost completely converted to ethanol by the sake yeasts, whereas the glucose was partially used in other aspects of metabolism by laboratory yeasts.

A. oryzae glucoamylases encoded by glaA and glaB differ in protein structure. Glucoamylase encoded by glaA consists of two domains for enzyme catalysis and starch binding at the N and C termini, respectively, while glaB consists of a single domain only for enzyme catalysis. R. oryzae glucoamylase also has two domains for enzyme catalysis and starch binding at the C and N termini in reverse order of glaA glucoamylase. When three fungal glucoamylases were displayed by this method with the 3′-half of α-agglutinin, the catalytic domains of glaB and R. oryzae glucoamylases were adjacent on the cell-surface anchor region, α-agglutinin, but that of glaA glucoamylase was kept at a distance from the anchor region by the insertion of a starch-binding domain. In a previous report, the insertion of linker peptide as a spacer in the C-terminal portion of the enzyme on the cell surface changed its activity. If a suitable linker peptide is inserted into the glucoamylase displayed, activity might increase. On the other hand, glaA and glaB glucoamylases show different substrate specificities. These differences cause glaA glucoamylase displayed on the cell-surface to prefer starch degradation (Figs. 2 and 3). From this reason, GRI-117-UK/pUDGAA produced ethanol from 50 g/l of liquefied corn starch in high yield, although it had lower activity by the method of measurement using p-nitrophenyl β-maltoside.

The sake yeast strains constructed in this study are expected to produce bioethanol from starchy materials such as corn. Furthermore, to improve the efficiency of hydrolysis, a combination of sake yeast and various enzymes that cleave α-glucoside bonds should be used. This procedure based on our sake yeast might lead to more practical production of bioethanol from starchy materials and contribute to the development of the ethanol fermentation industry.

References