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Direct Ethanol Production from Barley β-Glucan by Sake Yeast Displaying *Aspergillus oryzae* β-Glucosidase and Endoglucanase

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Three β-glucosidase- and two endoglucanase-encoding genes were cloned from *Aspergillus oryzae*, and their gene products were displayed on the cell surface of the sake yeast, *Saccharomyces cerevisiae* GRI-117-UK. GRI-117-UK/pUDB7 displaying β-glucosidase AO090009000356 showed the highest activity against various substrates and efficiently produced ethanol from cellobiose. On the other hand, GRI-117-UK/pUDCB displaying endoglucanase AO090010000314 efficiently degraded barley β-glucan to glucose and smaller cellooligosaccharides. GRI-117-UK/pUDB7CB codisplaying both β-glucosidase AO090009000356 and endoglucanase AO090010000314 was constructed. When direct ethanol fermentation from 20 g/*l* barley β-glucan as a model substrate was performed with the codisplaying strain, the ethanol concentration reached 7.94 g/*l* after 24 h of fermentation. The conversion ratio of ethanol from β-glucan was 69.6% of the theoretical ethanol concentration produced from 20 g/*l* barley β-glucan. These results showed that sake yeast displaying *A. oryzae* cellulolytic enzymes can be used to produce ethanol from cellulosic materials. Our constructs have higher ethanol production potential than the laboratory constructs previously reported.

[**Key words:** sake yeast, *Aspergillus oryzae*, β-glucosidase, endoglucanase, cell surface engineering, cellulosic materials]

Ethanol from biomass is called bioethanol, a substitute for fossil fuels (1). Particularly, cellulosic biomass, such as agricultural and forestry residues, exists in huge quantities and is remarkable because it is not a food material. Ethanol production from cellulose is performed via the degradation of cellulose to cellooligosaccharides and glucose, followed by the conversion of glucose to ethanol by microorganisms such as yeast.

Generally, to degrade cellulose, acid treatment with sulfate is carried out, and then the solution is neutralized and used for ethanol fermentation. However, the residues by neutralization generate environmental load. On the other hand, degradation by enzymes, such as endoglucanase (EC. 3.2.1.4), β-glucosidase (EC. 3.2.1.21) and others, proceeds under mild conditions and emits less residues. The fermentation of glucose to ethanol is performed by various microorganisms including yeast. To date, the direct production of ethanol from cellulosic materials has been under development; the laboratory strain of *Saccharomyces cerevisiae* displays cellulolytic enzymes on its cell surface and can simultaneously saccharify and ferment (2). However, the growth and ethanol productivity of laboratory yeasts are inferior to those of practical strains, including sake yeasts.

In Japanese sake brewing, *koji* mold (*Aspergillus oryzae*) and sake yeast (*S. cerevisiae*) are used. *Koji* mold saccharifies rice starch and sake yeast ferments glucose to ethanol. This method is called simultaneous saccharification and fermentation. The enzymes that the *koji* mold secretes are used in making Japanese sake, soy sauce, *miso*, and so on. *Koji* mold produces a large amount and many types of enzyme under various conditions. On the other hand, sake yeast rapidly proliferates, vigorously ferments, has high ethanol resistance, and is stable in terms of quality. Particularly, sake yeast can produce ethanol until the ethanol concentration reaches approximately 20% (v/v). However, *S. cerevisiae* cannot utilize cellulosic materials.

Previously, two endoglucanases were cloned from *A. oryzae*, and their gene products were overproduced in *A. oryzae* and characterized (3, 4), whereas two β-glucosidases produced by *A. oryzae* were also purified and characterized (5, 6). Recently, it has been revealed that *A. oryzae* has

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TABLE 1. Characteristics of microbial strains and plasmids

Strain or plasmid	Features
Strains	
E. coli DH5α	F⁻ *endA1 hsdR17* (r_K^-/m_K^-) *supE44 thi-1* λ⁻ *recA1 gyrA96* Δ*lacU169* (ϕ80*lacZ* ΔM15)
GRI-117-UK	Mutant of sake yeast Kyokai No. 9, *MAT***a**/α *ura3*/*ura3 lys2*/*lys2*
GRI-117-UK/pUDB3	GRI-117-UK displaying β-glucosidase B3
GRI-117-UK/pUDB6	GRI-117-UK displaying β-glucosidase B6
GRI-117-UK/pUDB7	GRI-117-UK displaying β-glucosidase B7
GRI-117-UK/pUDCA	GRI-117-UK displaying endo-1,4-glucanase CelA
GRI-117-UK/pUDCB	GRI-117-UK displaying endo-1,4-glucanase CelB
GRI-117-UK/pUDB7CB	GRI-117-UK displaying both β-glucosidase B7 and endo-1,4-glucanase CelB
Plasmids	
pUDB3	*URA3*, cell surface expression of *A. oryzae* β-glucosidase B3 gene
pUDB6	*URA3*, cell surface expression of *A. oryzae* β-glucosidase B6 gene
pUDB7	*URA3*, cell surface expression of *A. oryzae* β-glucosidase B7 gene
pUDCA	*URA3*, cell surface expression of *A. oryzae celA*
pUDCB	*URA3*, cell surface expression of *A. oryzae celB*
pUDB7CB	*URA3*, cell surface expression of both *A. oryzae* β-glucosidase B7 gene and *celB*

many cellulose degradation enzymes, because genomic information on *A. oryzae* had been disclosed (7).

In this study, the genes of *A. oryzae* cellulolytic enzymes were cloned on the basis of genomic information. In addition, the cell surface display technique is applied to sake yeast, and then the ethanol production was achieved using constructed strains that display β-glucosidase and endoglucanase from *A. oryzae* on their cell surface.

MATERIALS AND METHODS

Strains and media The microbial strains and plasmids used in this study are listed in Table 1. The sake yeast strain GRI-117-UK was obtained by ethyl methane sulphonate mutagenesis of the wild type of sake yeast Kyokai no. 9 (Brewing Society of Japan), and then selected using the 5-fluoroorotic acid medium (8) and α-aminoadipate medium (9). *E. coli* was grown in Luria–Bertani medium containing 10 g/*l* Polypepton, 5 g/*l* yeast extract, 10 g/*l* sodium chloride and 100 mg/*l* ampicillin. Yeast was cultivated aerobically at 30°C in YPD medium (10 g/*l* yeast extract, 20 g/*l* Polypepton and 20 g/*l* glucose) or SD medium (6.7 g/*l* yeast nitrogen base without amino acids, 20 g/*l* glucose and appropriate supplements).

Construction of plasmids To acquire the genes whose introns were eliminated, junction PCR was performed using the primers listed in Table 2. At first, each set of primers and *A. oryzae* chromosomal DNA were used to amplify a part of the gene by PCR. Next, the first PCR products and the primers annealing the 5′-end and 3′-end of the gene were used to amplify the full length of the gene. The second PCR product was digested with *Sal*I and *Hpa*I and then inserted into plasmid pK113, containing the *SED1* promoter (10), the secretion signal sequence of *Rhizopus oryzae* glucoamylase gene, 3′-half of α-agglutinin gene as the anchor (11) and *URA3* selectable marker, which was digested with *Sal*I and *Sma*I. The plasmid for displaying both β-glucosidase B7 and endoglucanase CelB was constructed as follows. A DNA fragment containing the *SED1* promoter, CelB gene and 3′-half of α-agglutinin was amplified from pUDCB by PCR, using the primers, Psed800(AatII)F (5′-GGCGACGTCTTGGATATAGAAAATTAA-3′) and CAS1(AatII)R (5′-CGGCGACGTCTTTGATTATGTTCTTTCTA-3′), and then digested with *Aat*II. The resulting fragment was inserted into plasmid pUDB7 digested with *Aat*II, yielding pUDB7CB.

Yeast transformation The expression plasmids were introduced into *S. cerevisiae* GRI-117-UK by the lithium acetate method (12). We selected the transformants that showed the highest activity using *p*-nitrophenyl β-D-glucopyranoside or barley β-glucan as the substrate.

Enzyme assays β-Glucosidase activity was measured in 50 mM sodium acetate buffer (pH 5.0) at 37°C with 1 mM *p*-nitrophenyl β-D-glucopyranoside, *p*-nitrophenyl β-D-cellobioside, *p*-nitrophenyl β-D-cellotrioside, cellobiose, gentiobiose and laminaribiose as the substrates. The cells were aerobically cultivated in YPD medium at 30°C for 48 h, harvested by centrifugation, washed with distilled water, and resuspended in a reaction mixture with the optical density of 1.0 at 600 nm. After the reaction, the supernatants were separated by centrifugation, and the *p*-nitrophenol released or the reducing sugar remaining was determined by measuring the absorbance at 415 nm or by the Somogyi–Nelson method (13), respectively. One unit of enzyme activity was defined as the amount of enzyme that released 1 μmol of *p*-nitrophenol or glucose from the substrate per min.

Endoglucanase activity was confirmed by analyzing the products released from barley β-glucan by the sake yeast displaying endoglucanases. After cultivation in YPD medium for 48 h at 30°C, the cells were collected by centrifugation and washed with distilled water. Then the cells were disrupted using CelLytic-Y (Sigma-Aldrich, St. Louis, MO, USA) and resuspended in a reaction mixture containing 20 g/*l* barley β-glucan (Sigma-Aldrich) and 50 mM sodium acetate buffer (pH 5.0). After the hydrolysis reaction at 37°C, the supernatants were separated by centrifugation. The products were detected by thin-layer chromatography (TLC) (14).

Ethanol production The transformants were aerobically precultivated for 24 h and then cultivated in YPD medium at 30°C for 48 h. The cells were harvested by centrifugation and resuspended in 50 g/*l* cellobiose (pH 5.0) or 20 g/*l* barley β-glucan (pH 5.0) with the optical density of 5.0 or 20 at 600 nm, respectively. The small-scale fermentation (100 ml of cellobiose solution in a 250-ml closed bottle or 20 ml of barley β-glucan solution in a 50-ml closed bottle) was carried out anaerobically at 30°C. The ethanol concentration was measured by gas chromatography. The gas chromatograph (model GC-8A; Shimadzu, Kyoto) was operated under the following conditions: Gasukuropack54; temperatures of column and injector, 180 and 250°C, respectively; N_2 carrier gas flow rate, 60 ml/min.

RESULTS

Display of β-glucosidase and endoglucanase *A. oryzae* β-glucosidase (B3, B6 and B7) and endoglucanase (CelA and CelB) genes were selected on the basis of *A. oryzae* genomic information (http://www.bio.nite.go.jp/dogan/). There