JOURNAL OF BIOSCIENCE AND BIOENGINEERING
Vol. 105, No. 6, 622–627. 2008
DOI: 10.1263/jbb.105.622

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

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Received 26 December 2007/Accepted 29 February 2008

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

TABLE 2. PCR primers used in this study

Protein	Primer	Sequence (5′-3′)
β-Glucosidase B3 (AO090003000497)	B3-1F	GAACGTCGACATGGGTTCCACTTCAACATC
	B3-1R	AATCTGGTAACTCGCTGTTGCGAATCCCCA
	B3-2F	CAACAGCGAGTTACCAGATTGAAGGAGCCG
	B3-2R	AATGCGGGACCAAGAGAGTGAGAAACGGTA
	B3-3F	CACTCTCTTGGTCCCGCATTATTCCACTCG
	B3-3R	AGGTCGTCCACGAACTTTATATAATATTGC
	B3-4F	ATAAAGTTCGTGGACGACCTGCATGCTGCT
	B3-4R	GCCCAATCACCATTAAGAGTGATGCCAATC
	B3-5F	ACTCTTAATGGTGATTGGGCCGAGCCTTGG
	B3-5R	TTCGAAGTTATCCATCAAACTCCAAGCCAT
	B3-6F	GTTTGATGGATAACTTCGAATGGGCCGAGG
	B3-6R	CGTAGTCCACGTAAGTCACTCCGAATCGTG
	B3-7F	AGTGACTTACGTGGACTACGAGAATAACCA
	B3-7R	CTTAGTTAACCAGCTTTCTCAATGTATTGG
β-Glucosidase B6 (AO090113000148)	B6-1F	AGCAGTCGACATGAACGTGAATATGTTCAAGGCCGGGGACGACATCCTCCAG GATGTGGACCAATCATGTAAAGACCG
	B6-1R	AAAGGGAGGATTCTGGCCCAGGCGATCGAG
	B6-2F	GGGCCAGAATCCTCCCTTTAGGCGGGCGTG
	B6-2R	CGTCGCGTACGCCTGCACAGCAGCTGTATG
	B6-3F	CTGTGCAGGCGTACGCGACGGACTTTCAAC
	B6-3R	GATTGGTCGCCGATAGCGATCCCAGACCCA
	B6-4F	TCGCTATCGGCGACCAATCGTAGTCACGGA
	B6-4R	GCAGACCATTCAAAATTATCCATCAATGAC
	B6-5F	GATAATTTTGAATGGTCTGCTGGATATGGT
	B6-5R	AACCTCTAAGGCTTGTTGCCCTCCGTTTAT
β-Glucosidase B7 (AO090009000356)	B7-1F	CCACGTCGACATGAAGCTTGGTTGGATCGAGG
	B7-1R	CGAGATCATCCTTGGCACTGACTACTGAGG
	B7-2F	CAGTGCCAAGGATGATCTCGCGTACTCCCC
	B7-2R	TAGTTGCCATCCTGTTCCAGTCGTTAAGTT
	B7-3F	CTGGAACAGGATGGCAACTAGAGAGGTGTG
	B7-3R	GATGTTGAGTCTGGGAACACTGCCAGTTTG
	B7-4F	GTGTTCCCAGACTCAACATCCCCAGCTTGT
	B7-4R	TTGTAGTCCGAGAAACGAATACCAAGAGGA
	B7-5F	ATTCGTTTCTCGGACTACAATTCAGCTTTC
	B7-5R	CACGAACTCGTTGACCCTCTCGTAAGCACC
	B7-6F	AGAGGGTCAACGAGTTCGTGGACGTGCAAC
	B7-6R	ATAAACTCATGGATTCTTTCCAGCCCCTCC
	B7-7F	CTGGAAAGAATCCATGAGTTTATCTATCCC
	B7-7R	GGGAAACGTACAGCTGAGGAACTTCATCAC
	B7-8F	TCCTCAGCTGTACGTTTCCCTAGGCGGCCCGAA
	B7-8R	AACCCTGGGCCTTAGGCAGCGACGCCTGGAGCGGCAGTTT
Endoglucanase CelA (AO090026000102)	CA-1F	CCGCGTCGACATGAAGCTCTCATTGGCACT
	CA-1R	GCGTGCGAGCCAAATCATCAGTTCATAATC
	CA-2F	TGATGATTTGGCTCGCACGCTACGGTACTA
	CA-2R	AACTGCATATTGATCAAGTACTGGGCAGAA
	CA-3F	TACTTGATCAATATGCAGTTCGGAACTGAG
	CA-3R	CCGCGTTAACCGTTGACACTGGCAGTCCAG
Endoglucanase CelB (AO090010000314)	CB-1F	CCGCGTCGACATGATCTGGACACTCGCTCC
	CB-1R	GGAGGCGTCAACATCGAAGGTGAATTCCTG
	CB-2F	CTTCGATGTTGACGCCTCCACCCTCGTCTG
	CB-2R	GATTGAGTCAACAGTTTTTCCTCCAGAGGT
	CB-3F	AAAAACTGTTGACTCAATCACAAAGGACTT
	CB-3R	CCGCGTTAACCATGCCTGTAGGTAGATCCA

are three *A. oryzae* β-glucosidase genes in the glycoside hydrolases family 1 (GH1). For one of them, the initiation codon and intron could not be determined. We selected two β-glucosidases of GH1, β-glucosidases B3 and B6. β-Glucosidase B7 was selected as one of the GH3 enzymes that were homologous to the well-known β-glucosidase from *A. aculeatus* (15, 16). On the other hand, we cloned two endoglucanase genes, CelA and CelB, as previously reported for the enzymes that *A. oryzae* secretes. All of the cloned genes in this study except for *celB* were interrupted by introns. We cloned these genes by fusing each exon because the introns of *A. oryzae* cannot be removed in yeast cells. The lengths of the genes of the intronless β-glucosidases B3, B6 and B7, and endoglucanases CelA and CelB were 1317, 1521, 2586, 720 and 1251 bp, respectively. These gene fragments were inserted into the expression vector pK113 (Fig. 1), and then, to transform *S. cerevisiae* GRI-117-UK, the constructed plasmids were digested with *Stu*I. Each linear DNA fragment was introduced into the yeast chromosomal DNA by homologous recombination using the lithium acetate method.

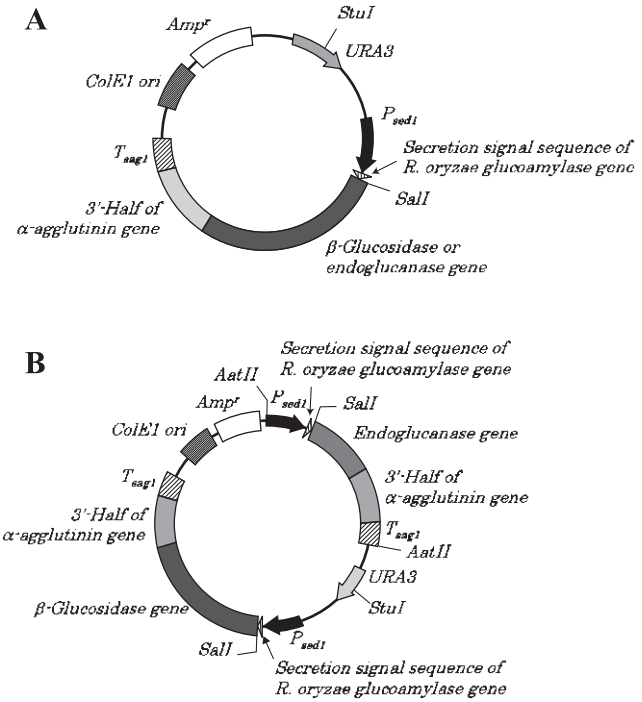

FIG. 1. Expression plasmids for cell surface display of β-glucosidase and/or endoglucanase. (A) Cell surface expression plasmid of β-glucosidase or endoglucanase; (B) pUDB7CB, cell surface expression plasmid of both β-glucosidase and endoglucanase.

Enzyme activities The β-glucosidase activities on the cell surface of the yeast transformants are listed in Table 3. The β-glucosidase activity was determined using synthetic substrates and glucobioses. After the yeast transformants were cultivated aerobically at 30°C for 48 h in YPD medium, the yeast strain displaying β-glucosidase B7 (GRI-117-UK/pUDB7) showed the highest activity among these transformants against various substrates, particularly *p*-nitrophenyl β-D-glucopyranoside, cellobiose and gentiobiose, while no activity was detected in strain GRI-117-UK/pK113 used as a control (Table 3). In the experiments on ethanol production, we decided to use strain GRI-117-UK/pUDB7 that displays β-glucosidase B7.

To confirm the endoglucanase activity, TLC analysis was performed. We measured the endoglucanase activity after the disruption of the harvested cells because glucose released from barley β-glucan was taken up by yeast cells as soon as degradation occurred. After the yeast transformants were cultivated aerobically in YPD medium at 30°C for 48 h, the disrupted cells (OD_{600}=5.0) were used in the reaction at 16 h, and the degradation of barley β-glucan to the smaller oligosaccharides and glucose was detected (Fig. 2). The yeast strain displaying CelB (GRI-117-UK/pUDCB) produced more cellobiose and glucose than the yeast strain displaying CelA (GRI-117-UK/pUDCA). No activity was detected in the yeast strain harboring the control plasmid (GRI-117-UK/pK113). To produce ethanol from cellulose, we decided to use strain GRI-117-UK/pUDCB that displays endoglucanase CelB.

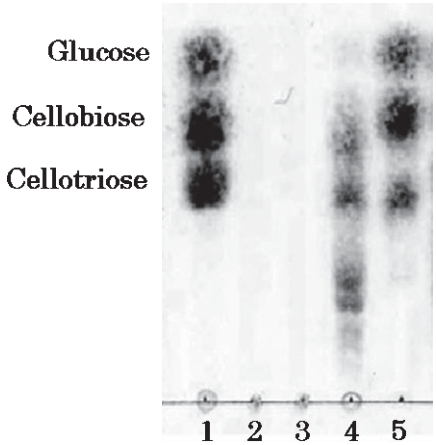

FIG. 2. TLC analysis of hydrolysis products released from barley β-glucan. Lane 1, Standards of glucose, cellobiose and cellotriose; lane 2, blank sample; lane 3, products after reaction with GRI-117-UK/pK113; lane 4, GRI-117-UK/pUDCA; lane 5, GRI-117-UK/pUDCB.

Ethanol production from cellobiose Direct fermentation from cellobiose to ethanol was performed using the yeast strain displaying β-glucosidase B7 (GRI-117-UK/pUDB7). The highest ethanol concentration was approximately 21.6 g/*l* after 48 h of fermentation (Fig. 3). The ethanol yield was 0.432 g/g of cellobiose added. This value corresponded to 80.3% of the theoretical ethanol concentration produced from 50 g/*l* cellobiose. No ethanol was produced by GRI-117-UK/pK113 used as the control.

Ethanol production from barley β-glucan Barley β-glucan was used as the model biomass of cellulosic material. For the direct fermentation of barley β-glucan to ethanol, a cellulose-utilizing yeast strain was constructed by integrating plasmid pUDB7CB, which made the yeast codis-

TABLE 3. Substrate specificity of each yeast strain displaying β-glucosidase

Substrate (1 mM)	Specific activity of enzyme (mU/g dry cell weight)			
	GRI-117-UK /pK113	GRI-117-UK /pUDB3	GRI-117-UK /pUDB6	GRI-117-UK /pUDB7
p-Nitrophenyl β-D-glucopyranoside	n.d.	1.92	1.91	20.9
p-Nitrophenyl β-D-cellobioside	n.d.	0.147	0.0421	7.65
p-Nitrophenyl β-D-cellotrioside	n.d.	0.0496	0.0230	4.91
o-Nitrophenyl β-D-xylopyranoside	n.d.	n.d.	n.d.	4.08
Cellobiose (β-1,4)	n.d.	6.24	5.30	52.0
Laminaribiose (β-1,3)	n.d.	2.78	1.64	11.7
Gentiobiose (β-1,6)	n.d.	6.65	6.96	73.7

Values represent the averages of three independent experiments. n.d., Not detected.

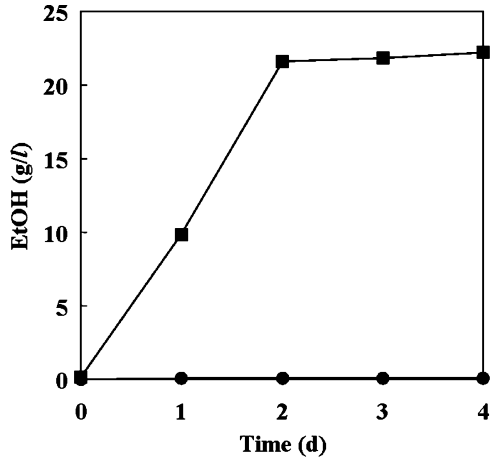

FIG. 3. Production of ethanol from 50 g/*l* cellobiose. Symbols: circles, GRI-117-UK; squares, GRI-117-UK/pUDB7.

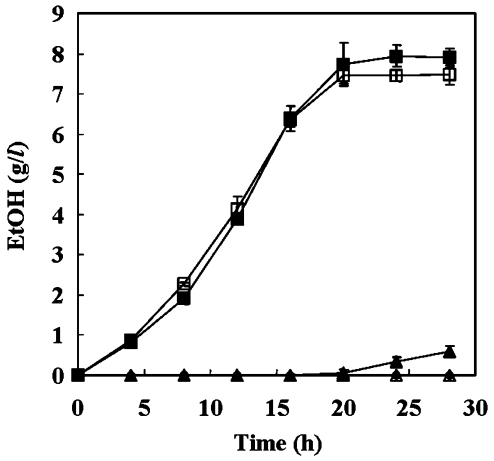

FIG. 4. Production of ethanol from 20 g/*l* β-glucan. Symbols: closed circles, GRI-117-UK; open triangles, GRI-117-UK/pUDB7; closed triangles, GRI-117-UK/pUDCB; open squares, mixture of GRI-117-UK/pUDB7 and GRI-117-UK/pUDCB; closed squares, GRI-117-UK/pUDB7CB.

play β-glucosidase B7 and endoglucanase CelB on the cell surface. With the resulting cellulose-utilizing yeast strain, GRI-117-UK/pUDB7CB, direct ethanol fermentation from cellulose was performed. The highest ethanol concentration was 7.94 g/*l* after 24 h of fermentation (Fig. 4). This corresponded to 69.6% of the theoretical ethanol concentration produced from 20 g/*l* barley β-glucan. A similar result was obtained when the yeast strains, GRI-117-UK/pUDB7 and GRI-117-UK/pUDCB were simultaneously used. GRI-117-UK/pUDCB produced 0.59 g/*l* ethanol, while GRI-117-UK/pK113 and GRI-117-UK/pUDB7 produced no ethanol.

DISCUSSION

Three genes encoding β-glucosidase were cloned from *A. oryzae* and the gene products were displayed on the yeast cell surface. We could obtain the useful genes efficiently by exploiting the *A. oryzae* genomic information. According to the genomic information, there are 26 β-glucosidase-encoding genes. However, only a few β-glucosidases were reported before (5, 6), and others are not considered to be produced by *A. oryzae* cells. β-Glucosidases displayed on the yeast cell surface showed various substrate specificities. The activity of GRI-117-UK/pUDB7 was higher than those of GRI-117-UK/pUDB3 and GRI-117-UK/pUDB6. β-Glucosidases B3 and B6 belong to GH1, and β-glucosidase B7 belongs to GH3. The structural difference among these enzymes would affect the activity. β-Glucosidase B7 shows high homology with *A. aculeatus* β-glucosidase 1 (79%) and *A. niger* β-glucosidase (77%), which have very high β-glucosidase activities (15–17). β-Glucosidase B7 showed high activities for all the substrates used in this study. Because β-glucosidase B7 showed high activity for cellobiose, it is considered suitable for degrading cellooligosaccharides. In contrast, there are some endoglucanase-encoding genes in the *A. oryzae* genomic DNA. The endoglucanases CelA and CelB could degrade barley β-glucan to cellooligosaccharides and glucose. CelB can degrade more efficiently than CelA (Fig. 2). GH7 enzymes, to which CelB belongs, exist only in eukaryotes, but GH12 enzymes, to which CelA belongs, are widely expressed in various species. It was reported that *Trichoderma viride* endoglucanase I, a GH7 enzyme, (18) and *Thermotoga neapolitana* endo-1,4-glucanase B, a GH12 enzyme, (19) can degrade cellooligosaccharides to glucose. In this study, *A. oryzae* CelB also produced smaller cellooligosaccharides and glucose, and it was considered that *A. oryzae* CelB is comparable to other enzymes such as the *T. viride* endoglucanase I. The sake yeasts displaying CelB showed high activity for barley β-glucan. This strain is expected to degrade cellulose.

Yeast *S. cerevisiae* cannot decompose and assimilate cellulose. To address these problems, the cell surface engineering was applied to an industrial strain, sake yeast. In our study, sake yeast strains displaying β-glucosidase and endoglucanase from *A. oryzae* on the cell surface were constructed. Displaying multiple types of enzyme on the cell surface is useful. We could use only one strain to degrade barley β-glucan because a codisplaying strain was constructed. GRI-117-UK/pUDB7CB, displaying both β-glucosidase B7 and endoglucanase CelB, had as much β-glucosidase activity as GRI-117-UK/pUDB7, but the endoglucanase activity was not determined because the degradation products of barley β-glucan (cellooligosaccharides and glucose) were not found (data not shown). It is assumed that the cellooligosaccharides were degraded to glucose by the β-glucosidase on the cell surface and the yeast immediately consumed glucose. The endoglucanase of GRI-117-UK/pUDB7CB indirectly showed ethanol production from barley β-glucan (Fig. 4).

In the previous report (20), the laboratory yeast MT8-1 displaying *A. aculeatus* β-glucosidase (MT8-1/BGL) was constructed. The transformant (OD_{600} of 20) produced 19.3 g/*l* ethanol from 50 g/*l* cellobiose in 24 h. In contrast, in this study, the sake yeast GRI-117-UK/pUDB7 (OD_{600} of 5.0) produced 21.6 g/*l* ethanol from 50 g/*l* cellobiose in 48 h. The theoretical ethanol productions from 50 g/*l* cellobiose were 71.7% (MT8-1/BGL) and 80.3% (GRI-117-UK/pUDB7). The conversion rate of ethanol from cellobiose was higher using GRI-117-UK/pUDB7. In another report, MT8-1 displaying both *A. aculeatus* β-glucosidase and *Trichoderma*

reesei endoglucanase (MT8-1/pBG211/pEG23u31H6) was constructed (2). The transformant produced 16.5 g/*l* ethanol from 45 g/*l* barley β-glucan in 50 h, and this corresponded to 64.2% of the ethanol concentration theoretically produced from 45 g/*l* β-glucan. In contrast, in this study, the sake yeast we constructed (GRI-117-UK/pUDB7CB) produced 7.94 g/*l* ethanol from 20 g/*l* barley β-glucan in 24 h, and this corresponded to 69.6% of the ethanol concentration theoretically produced from 20 g/*l* β-glucan. The conversion rate of ethanol from barley β-glucan was higher using GRI-117-UK/pUDB7CB. These results raise the possibility that cellulose-utilizing sake yeasts are useful for ethanol production. GRI-117-UK/pUDCB displaying CelB produced only 0.59 g/*l* ethanol from 20 g/*l* barley β-glucan because it could not release glucose sufficiently. Therefore, the codisplaying strain GRI-117-UK/pUDB7CB was demonstrated to be useful for the direct ethanol production from cellulose.

Now, in the United States and Brazil, ethanol is produced from sugarcane and corn, and used as fuel on a large scale. However, these carbon sources could be eaten, so it is hoped that the biomass that could not be eaten, such as cellulose, is used. Actually, food prices are rising because bioethanol is produced from starch. In this study, our results revealed that the combination of sake yeast and *koji* mold enzymes is very attractive for biomass conversion to energy. In addition, the cellulose-utilizing sake yeast GRI-117-UK/pUDB7CB still has lysine auxotrophy that can be used for displaying more β-glucosidases and endoglucanases, or other glycoside hydrolases. The genes encoding other cellulose-degrading enzymes such as cellobiohydrolase and exoglucanase also exist in the *A. oryzae* genome. We now attempt to display cellobiohydrolase and/or exoglucanase on the cell surface of GRI-117-UK/pUDB7CB. The enhancement of cellulose-degrading enzyme activities will lead more efficient ethanol production. The cellulose-utilizing sake yeasts constructed in this study are expected to produce ethanol from cellulose efficiently.

ACKNOWLEDGMENTS

This work was financed by the Japan Science and Technology Agency (JST).

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