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Synergistic Saccharification, and Direct Fermentation to Ethanol, of Amorphous Cellulose by Use of an Engineered Yeast Strain Codisplaying Three Types of Cellulolytic Enzyme

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A whole-cell biocatalyst with the ability to induce synergistic and sequential cellulose-degradation reaction was constructed through codisplay of three types of cellulolytic enzyme on the cell surface of the yeast *Saccharomyces cerevisiae*. When a cell surface display system based on α -agglutinin was used, *Trichoderma reesei* endoglucanase II and cellobiohydrolase II and *Aspergillus aculeatus* β -glucosidase 1 were simultaneously codisplayed as individual fusion proteins with the C-terminal-half region of α -agglutinin. Codisplay of the three enzymes on the cell surface was confirmed by observation of immunofluorescence-labeled cells with a fluorescence microscope. A yeast strain codisplaying endoglucanase II and cellobiohydrolase II showed significantly higher hydrolytic activity with amorphous cellulose (phosphoric acid-swollen cellulose) than one displaying only endoglucanase II, and its main product was cellobiose; codisplay of β -glucosidase 1, endoglucanase II, and cellobiohydrolase II enabled the yeast strain to directly produce ethanol from the amorphous cellulose (which a yeast strain codisplaying β -glucosidase 1 and endoglucanase II could not), with a yield of approximately 3 g per liter from 10 g per liter within 40 h. The yield (in grams of ethanol produced per gram of carbohydrate consumed) was 0.45 g/g, which corresponds to 88.5% of the theoretical yield. This indicates that simultaneous and synergistic saccharification and fermentation of amorphous cellulose to ethanol can be efficiently accomplished using a yeast strain codisplaying the three cellulolytic enzymes.

Biomass is the earth's most attractive alternative among fuel sources and most sustainable energy resource and is reproduced by the bioconversion of carbon dioxide. Ethanol produced from biomass is today the most widely used biofuel when blended with gasoline (e.g., E10 [gasoline containing 10% ethanol]). As the carbon dioxide released by combustion is recycled into biomass, the use of biofuels can significantly reduce the accumulation of greenhouse gas. Of the biomass materials, cellulose, a major component of the cell wall of plants, is the most abundant and renewable carbohydrate. In recent years, it has been proposed that waste cellulosic biomass could be used as a cheap and readily available sugar to replace starchy materials in fermentation. Many researchers have previously tried to develop an efficient and inexpensive process for ethanol production from such waste by using recombinant bacteria and yeast (e.g., *Saccharomyces cerevisiae*) (1, 2, 10, 12, 37), but so far without success. A process of this kind is needed to solve environmental problems such as global warming and to construct a society independent of fossil fuels.

The anaerobic bacteria *Clostridium thermocellum* and *Clostridium cellulovorans* and the filamentous fungus *Trichoderma reesei* are well known as strongly cellulolytic and xylanolytic microorganisms. *C. thermocellum* and *C. cellulovorans* produce a cellulosome complex consisting of cellulase and hemicellulase organized on the cell surface (5, 25); *T. reesei*, meanwhile,

extracellularly secretes three types of cellulolytic enzyme, including five endoglucanases (EG [EC 3.2.1.4]) (17, 19–22), two cellobiohydrolases (CBH [EC 3.2.1.91]) (9, 30), and two β -glucosidases (BGL [EC 3.2.1.21]) (3). Endoglucanases act randomly against the amorphous region of the cellulose chain to produce reducing and nonreducing ends for cellobiohydrolases, which produce cellobiose from reducing or nonreducing ends of crystalline cellulose. Cellulose chains are thus efficiently degraded to soluble cellobiose and celooligosaccharides by the endo-exo synergism of EG and CBH (9, 13, 29, 36). In the last step of enzymatic cellulose degradation, celooligosaccharides are hydrolyzed to glucose by β -glucosidase. In addition to endo-exo synergism, exo-exo synergism between two cellobiohydrolases has also been reported (9, 14, 28, 29).

Such cellulolytic enzymes have been expressed in bacteria (34, 38) and yeast (4, 7, 15, 31, 32) as a way of reducing the cost of cellulase production and other pretreatments in the process of ethanol production from cellulosic materials (12, 26, 37). Recently some researchers have developed ethanologenic bacteria (8, 34, 38) and yeast (4, 7) that can produce ethanol from cellulosic materials. The recombinant *Klebsiella oxytoca* SZ21 developed by Zhou et al. was able to directly produce ethanol from amorphous cellulose, although with insufficient ethanol yield (38). When using other recombinant ethanologenic bacteria or yeast to ferment cellulose, addition of commercial cellulase is necessary for ethanol production.

Previously, we reported direct and efficient ethanol production from the soluble cellulosic polysaccharide barley β -glucan with a yeast strain codisplaying on the cell surface *T. reesei* EGII (glycosyl hydrolase family 5) and *Aspergillus aculeatus*

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TABLE 1. Characteristics of strains and plasmids used in this study

Strain or plasmid	Relevant feature(s) ^a	Reference or source
Strains		
<i>S. cerevisiae</i> yeast strains		
MT8-1	<i>MATa ade his3 leu2 trp1 ura3</i>	27
MT8-1/pCAS1	No display (control strain)	24
MT8-1/pFCBH2w3	Display of CBHII	Present study
MT8-1/pEG23u31H6	Display of EGII	7
MT8-1/pBG211	Display of BGL1	15
MT8-1/pEG23u31H6/pFCBH2w3	Display of EGII and CBHII	Present study
MT8-1/pBG211/pEG23u31H6	Display of BGL1 and EGII	7
MT8-1/pBG211/pEG23u31H6/pFCBH2w3	Display of BGL1, EGII, and CBHII	Present study
Bacterial strain		
<i>E. coli</i> NovaBlue	<i>endA1 hsdR17 (r_{K12}⁻ m_{K12}⁺) supE44 thi-1 gyrA96 relA1 lac recA1/ F' {proAB⁺ lacI^qZΔM15::Tn10 (Tet^r)}</i>	Novagen
Plasmids		
pCAS1	<i>TRP1</i> No expression (control plasmid)	24
pFCBH2w3	<i>TRP1</i> Surface expression of <i>T. reesei</i> cellobiohydrolase gene (CBHII)	Present study
pEG23u31H6	<i>URA3</i> Surface expression of <i>T. reesei</i> endoglucanase gene (EGII)	7
pBG211	<i>HIS3</i> Surface expression of <i>A. aculeatus</i> β-glucosidase gene (BGL1)	15

^a Each gene was expressed as a fusion protein with the secretion signal sequence of *R. oryzae* glucoamylase gene and the C-terminal half region of the α-agglutinin gene under the control of the glyceraldehyde 3-phosphate dehydrogenase (GAPDH) promoter.

BGL1 (family 3) (7). In the present study, we attempted simultaneous and synergistic saccharification and fermentation of amorphous cellulose to ethanol with the use of only a recombinant yeast strain codisplaying three types of cellulolytic enzyme, namely, *T. reesei* EGII and CBHII (family 6) and *A. aculeatus* BGL1.

MATERIALS AND METHODS

Strains and media. The bacterial and yeast strains used are summarized in Table 1. *Escherichia coli* was grown in Luria-Bertani medium (10 g of tryptone per liter, 5 g of yeast extract per liter, 5 g of sodium chloride per liter) containing 100 μg of ampicillin per ml. Following precultivation in synthetic medium (SD medium; 6.7 g of yeast nitrogen base without amino acid [Difco Laboratories, Detroit, Mich.] per liter with appropriate supplements containing 20 g of glucose per liter), yeast cells were aerobically cultivated at 30°C in SD medium containing 20 g of Casamino Acids (Difco) per liter (SDC medium).

Construction of plasmids. The plasmid pFCBH2w3 for cell-surface display of the *T. reesei* CBHII gene was constructed as follows: with first-strand cDNA prepared from *T. reesei* QM9414 as the template, a 1.39-kbp *SacII*-*BglII* DNA fragment of the mature region of the *T. reesei* CBHII gene fused with the gene encoding the FLAG peptide tag at the N terminus was prepared by PCR as described previously (18) with the two primers 5'-GAGCCGCGGGAGACTA CAAGGATGACGATGACAAGCAAGCTTGCTCAAGCGTCTGGGGCC-3' and 5'-CGAAGAGATCTAGGAACGATGGGTTTGCCTTTGTGAGAAG C-3'. The DNA fragment was digested by *SacII* and *BglII* and introduced into the *SacII*-*BglII* site of the cell-surface expression plasmid pCAS1 (24) containing the genes encoding the secretion-signal sequence of the glucoamylase gene from *Rhizopus oryzae* and the 3'-half region of the α-agglutinin gene (the gene encoding the C-terminal 320 amino acid residues and 446 bp of the 3'-flanking region) (11). The resulting plasmid was named pFCBH2w3 (Fig. 1). The plasmids used are summarized in Table 1.

Yeast transformation. Transformation of the expression plasmids into *S. cerevisiae* MT8-1 was carried out by a lithium acetate method using a YEASTMAKER yeast-transformation system (Clontech Laboratories, Inc., Palo Alto, Calif.). The transformants constructed and used in the study are summarized in Table 1.

Immunofluorescence labeling of cells. Immunofluorescence labeling of cells was carried out according to a method described previously (7). As the primary antibody, mouse anti-RGS(His)₄ immunoglobulin G (IgG) (Qiagen, Valencia, Calif.), rabbit anti-FLAG IgG (Sigma Chemical Co., St. Louis, Mo.) and rabbit anti-*A. aculeatus* BGL1 IgG were used at dilution rates of 1:100, 1:100, and 1:500 with the RGS(His)₆-EGII-α-agglutinin, FLAG-CBHII-α-agglutinin, and BGL1-α-agglutinin fusion proteins, respectively. As secondary antibody, goat anti-mouse IgG conjugated with Alexa Fluor 488 and goat anti-rabbit IgG conjugated with Alexa Fluor 546 (Molecular Probes, Inc., Eugene, Oreg.) were used at a dilution rate of 1:250.

Enzyme assay. Endoglucanase and cellobiohydrolase activities were determined by hydrolysis of 1 g of amorphous cellulose per liter in 50 mM sodium acetate buffer (pH 5.0) at 30°C. Phosphoric acid-swollen cellulose was prepared from Avicel PH-101 (Fluka Chemie GmbH, Buchs, Switzerland) as amorphous cellulose (33). After precultivation in SD medium for 24 h and aerobic cultivation in SDC medium for 72 h at 30°C, cells were collected by centrifugation at 6,000 × g for 10 min at 4°C, washed with distilled water twice, and resuspended in a reaction mixture with the optical density at 600 nm (OD₆₀₀) adjusted to 10. After a hydrolysis reaction, the supernatant was separated by centrifugation for 3 min at 20,000 × g and 4°C and used for measurement of the amount of reducing sugar, total soluble sugar, and insoluble sugar and for high-performance liquid chromatography (HPLC) analysis of hydrolysis products as described below. The amount of reducing sugar and total soluble sugar released from insoluble substrate and insoluble sugar was measured using a Somogyi-Nelson method (35) and a phenol-sulfuric acid method (6) to determine the number of glucose equivalents.

β-Glucosidase activity was measured using *p*-nitrophenyl-β-D-glucopyranoside (Nacalai Tesque, Inc., Kyoto, Japan) as the substrate according to a previously described method (7) but with an OD₆₀₀ of 0.1 in the reaction mixture.

Determination of hydrolysis products. The end products released from the phosphoric-acid swollen cellulose were analyzed according to a previously described method (7).

Fermentation. After precultivation in SD medium for 24 h, yeast cells were aerobically cultivated for 72 h at 30°C in SDC medium, collected by centrifugation for 10 min at 6,000 × g and 4°C, and washed with distilled water twice. The cell pellets were then inoculated into fermentation medium (6.7 g of yeast nitrogen base without amino acid [Difco] per liter with appropriate supplements, 20 g of Casamino Acids per liter, 10 g of nonsterilized phosphoric acid-swollen cellulose per liter as the sole carbon source), and ethanol fermentation was anaerobically performed at 30°C with the OD₆₀₀ of the fermentation medium

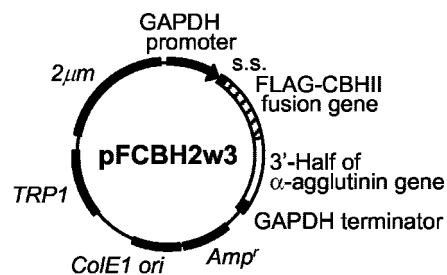


FIG. 1. Expression plasmid for display of *T. reesei* CBHII on the yeast cell surface (pFCBH2w3). s.s., secretion signal sequence of *R. oryzae* glucoamylase gene.

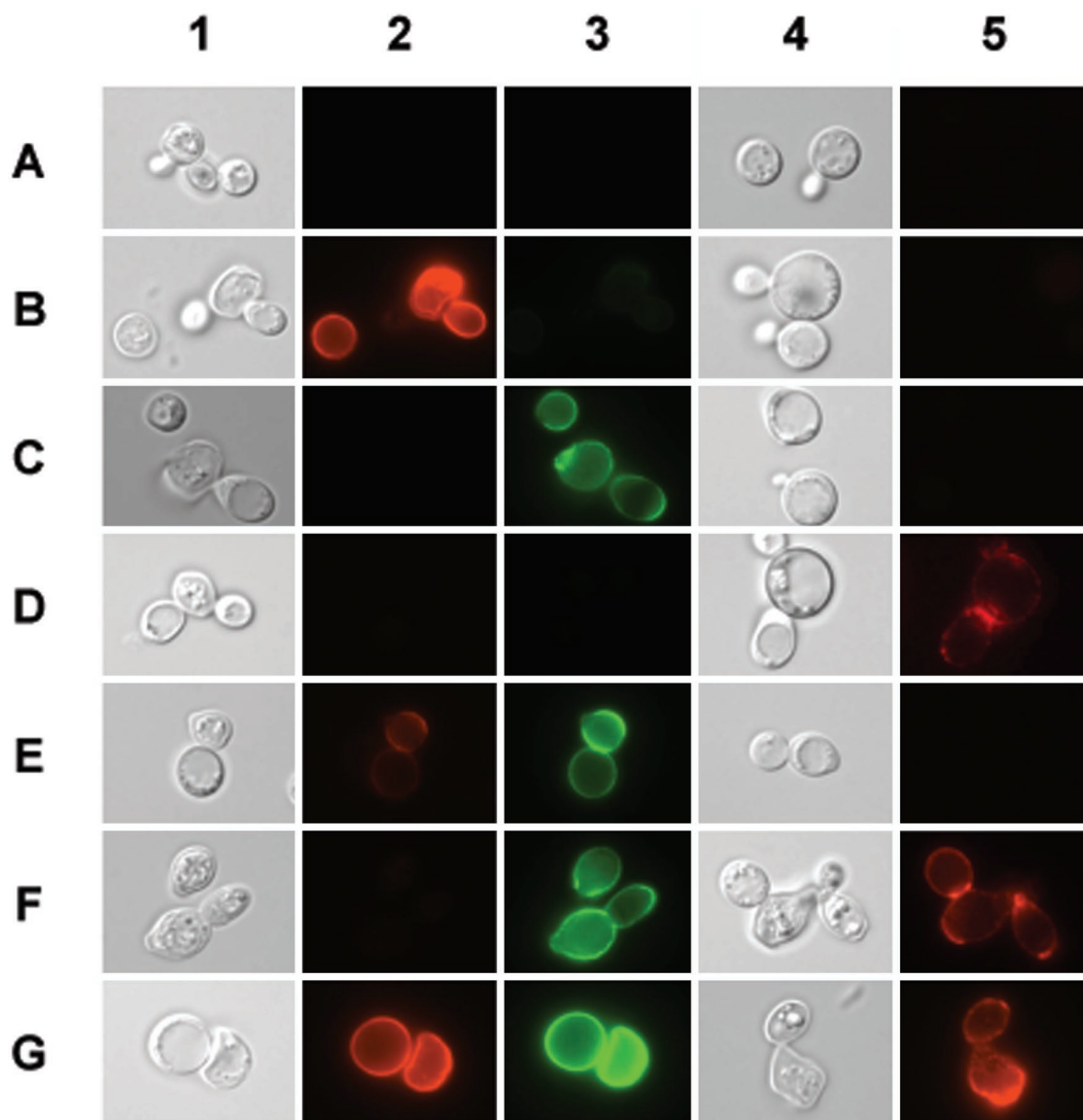


FIG. 2. Immunofluorescence labeling of transformants: Nomarski differential interference micrographs (columns 1 and 4) and immunofluorescence micrographs (columns 2, 3, and 5) of *S. cerevisiae* MT8-1/pCAS1 (control) (A), MT8-1/pFCBH2w3 (B), MT8-1/pEG23u31H6 (C), MT8-1/pBG211 (D), MT8-1/pEG23u31H6/pFCBH2w3 (E), MT8-1/pBG211/pEG23u31H6 (F), and MT8-1/pBG211/pEG23u31H6/pFCBH2w3 (G). Cells were labeled with rabbit anti-FLAG IgG antibody and goat anti-rabbit IgG conjugated with Alexa Fluor 546 (column 2), with mouse anti-RGS(His)₄ antibody and goat anti-mouse IgG conjugated with Alexa Fluor 488 (column 3), and with rabbit anti-*A. aculeatus* BGL1 antibody and goat anti-rabbit IgG conjugated with Alexa Fluor 546 (column 5).

adjusted to 50. Ethanol, total sugar, and glucose concentrations were measured using gas chromatography, a phenol-sulfuric acid method (6) as a glucose equivalent, and a Glucose CII test (Wako Pure Chemical Industries, Ltd., Osaka, Japan), respectively. The gas chromatograph (model GC-8A, Shimadzu, Kyoto, Japan) (fitted with a flame ionization detector) was operated under the following conditions: glass column (2.0 m by 3.2 mm) packed with Thermon-3000 (Shimadzu); temperature of column, injector, and detector, 180°C; nitrogen carrier gas flow rate, 25 ml/min. Total sugar concentrations were determined by subtracting the yeast cell-derived sugar from the culture medium containing the yeast cells and cellulose.

RESULTS

Codisplay of three types of cellulolytic enzyme on the yeast cell surface. To ferment amorphous cellulose to ethanol, we

constructed a yeast strain codisplaying three types of cellulolytic enzyme on the cell surface simultaneously. The expression plasmids pBG211, pEG23u31H6, and pFCBH2w3 (Fig. 1) (for display of the BGL1- α -agglutinin, RGS(His)₆-EGII- α -agglutinin, and FLAG-CBHII- α -agglutinin fusion genes) were transformed or cotransformed into the yeast *S. cerevisiae* MT8-1 strain simultaneously, and the resultant transformants were designated strains MT8-1/pFCBH2w3, MT8-1/pEG23u31H6, MT8-1/pBG211, MT8-1/pEG23u31H6/pFCBH2w3, MT8-1/pBG211/pEG23u31H6, and MT8-1/pBG211/pEG23u31H6/pFCBH2w3 (Table 1).

To confirm codisplay of BGL1, EGII, and CBHII on the yeast cell surface, immunofluorescence labeling of the cells

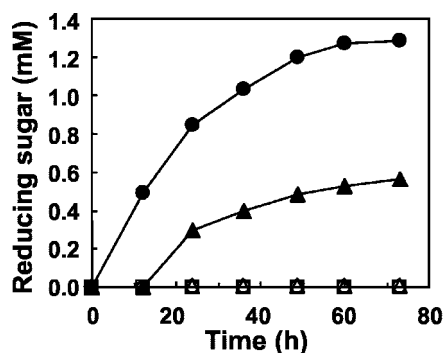


FIG. 3. Time course of synergistic hydrolysis of amorphous cellulose by *S. cerevisiae* MT8-1/pCAS1 (control) (open square), MT8-1/pFCBH2w3 (open triangle), MT8-1/pEG23u31H6 (closed triangle), MT8-1/pEG23u31H6/pFCBH2w3 (closed circle), and MT8-1/pBG211/pEG23u31H6/pFCBH2w3 (open circle). The data points represent the averages of five independent experiments.

was carried out using rabbit anti-FLAG antibody, mouse anti-RGS(His)₄ antibody, and rabbit anti-*A. aculeatus* BGL1 antibody as the primary antibody. As shown in Fig. 2, the red fluorescence of Alexa Fluor 546-conjugated goat anti-rabbit IgG was observed for strains MT8-1/pFCBH2w3, MT8-1/pEG23u31H6/pFCBH2w3, and MT8-1/pBG211/pEG23u31H6/pFCBH2w3 (Fig. 2, column 2); that of Alexa Fluor 546-conjugated anti-rabbit IgG was observed for strains MT8-1/pBG211, MT8-1/pBG211/pEG23u31H6, and MT8-1/pBG211/pEG23u31H6/pFCBH2w3 (Fig. 2, column 5); and the green fluorescence of Alexa Fluor 488-conjugated goat anti-mouse IgG was observed for strains MT8-1/pEG23u31H6, MT8-1/pEG23u31H6/pFCBH2w3, MT8-1/pBG211/pEG23u31H6, and MT8-1/pBG211/pEG23u31H6/pFCBH2w3 (Fig. 2, column 3). These results confirm single display and codisplay of BGL1, EGII, and CBHII. Specifically, three types of cellulase (namely, BGL1, EGII, and CBHII) were successfully codisplayed on the cell surface of strain MT8-1/pBG211/pEG23u31H6/pFCBH2w3.

Degradation of amorphous cellulose by yeast strain codisplaying EGII and CBHII. To examine the effect of codisplay of EGII and CBHII on hydrolysis activity, a hydrolysis experiment was performed using strains MT8-1/pFCBH2w3, MT8-1/pEG23u31H6, and MT8-1/pEG23u31H6/pFCBH2w3 after

aerobic cultivation of cells in SDC medium for 72 h at 30°C. In the yeast strain displaying CBHII (MT8-1/pFCBH2w3), reducing sugar was not detected by a Somogyi-Nelson method (Fig. 3) and only a little sugar was detected by a phenol-sulfuric acid method (data not shown). However, the yeast strain codisplaying EGII and CBHII (MT8-1/pEG23u31H6/pFCBH2w3) showed much higher activity than the yeast strain displaying EGII alone (MT8-1/pEG23u31H6) (Fig. 3), and a significant reduction in the insoluble cellulose in the reaction mixture was observed (Fig. 4). This result suggests that CBHII is active on the yeast cell surface and plays a very important role in amorphous cellulose degradation. The activities of EGII and CBHII tagged with RGS(His)₆ and FLAG were nearly equal to those of nontagged EGII and CBHII (data not shown).

HPLC analysis was carried out to examine the hydrolysis products released from amorphous cellulose by strains MT8-1/pEG23u31H6 and MT8-1/pEG23u31H6/pFCBH2w3. Samples subjected to a hydrolysis reaction for 72 h were used for HPLC analysis. Cellobiose and cellotriose were detected as the main products of strain MT8-1/pEG23u31H6, while a large amount of cellobiose was detected as the main product of strain MT8-1/pEG23u31H6/pFCBH2w3 (data not shown). Only a small amount of cellobiose was detected as the main product of strain MT8-1/pFCBH2w3, and no hydrolysis product was observed in the reaction mixture of the control strain MT8-1/pCAS1 (data not shown).

Degradation of amorphous cellulose by a yeast strain codisplaying three types of cellulolytic enzyme. The ability of the yeast strain MT8-1/pBG211/pEG23u31H6/pFCBH2w3 to hydrolyze amorphous cellulose was examined using the same method as described above. In spite of codisplay of three types of enzyme on the cell surface, no reducing sugar was detected in the reaction mixture of the strain (Fig. 3). However, the residual amount of insoluble sugar in the reaction mixture of this strain was smaller than that seen with yeast strain MT8-1/pEG23u31H6/pFCBH2w3 (Fig. 4). The reducing sugar produced by the hydrolysis of amorphous cellulose was thus immediately taken up by the yeast cells.

Direct fermentation of amorphous cellulose to ethanol. Direct production of ethanol from amorphous cellulose was performed using the yeast strain codisplaying BGL1, EGII, and CBHII (MT8-1/pBG211/pEG23u31H6/pFCBH2w3). Fermentation was anaerobically performed at 30°C in fermentation

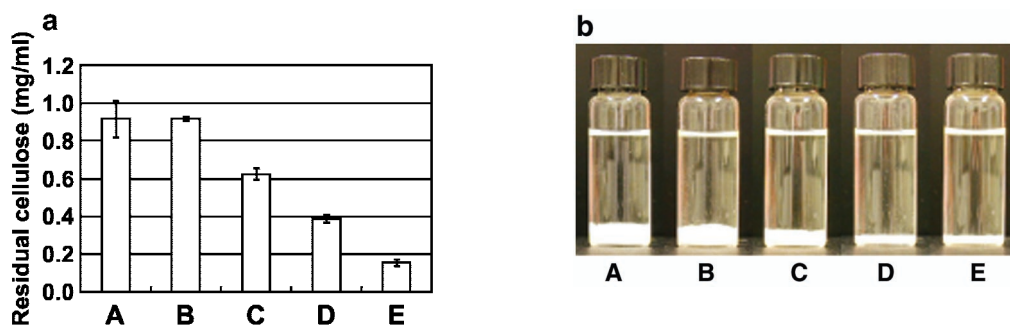


FIG. 4. Graph (a) and photograph (b) representing the residual amount of cellulose in hydrolysis reaction mixture after 72 h of reaction with *S. cerevisiae* MT8-1/pCAS1 (control) (A), MT8-1/pFCBH2w3 (B), MT8-1/pEG23u31H6 (C), MT8-1/pEG23u31H6/pFCBH2w3 (D), and MT8-1/pBG211/pEG23u31H6/pFCBH2w3 (E). The data represent the averages of three independent experiments.

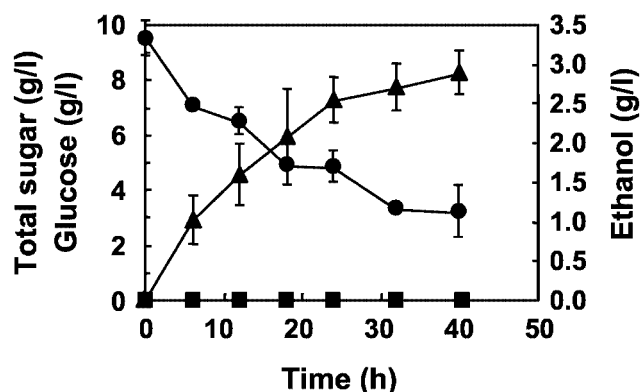


FIG. 5. Time course of production of ethanol from amorphous cellulose as the sole carbon source with strain MT8-1/pBG211/pEG23u31H6/pFCBH2w3. Symbols: triangle, ethanol; circle, total sugar; square, glucose in culture broth. The data points represent the averages of seven independent experiments.

medium containing 10 g of phosphoric acid-swollen cellulose per liter as the sole carbon source and using yeast cells ($OD_{600} = 50$) subjected to aerobic cultivation in SDC medium for 72 h at 30°C. Ethanol was not produced from amorphous cellulose when the yeast strain codisplaying EGII and BGL1 (MT8-1/pBG211/pEG23u31H6) was used (data not shown), but with strain MT8-1/pBG211/pEG23u31H6/pFCBH2w3, ethanol was efficiently produced and the maximum concentration of around 2.9 g/liter was reached within 40 h of commencing fermentation (Fig. 5). When fermentation was started, the ethanol concentration increased and the total sugar concentration decreased without a time lag. Glucose was not detected in the culture broth during fermentation. The yield (in grams of ethanol produced per gram of sugar consumed) was 0.45 g/g, which corresponds to 88.5% of the theoretical yield for 40 h of fermentation.

DISCUSSION

To reduce the cost of ethanol production from cellulosic biomass, recombinant microorganisms with the ability to ferment cellulose have been developed by many researchers (4, 8, 34, 38). These whole-cell biocatalysts with the ability to degrade cellulose have several advantages: conversion of cellobiose and glucose, which inhibit cellulase and β -glucosidase activities; lower sterilization requirements, as glucose is immediately taken up by cells and ethanol is produced; and a single reactor. In the fermentation of cellulose with yeast cells, cellulose must first be degraded to glucose, as yeast cells are not able to utilize cellulose or cellooligosaccharides. Enzymatic degradation of cellulose requires three types of cellulolytic enzyme (endoglucanase, cellobiohydrolase, and β -glucosidase), and a synergistic effect between endoglucanase and cellobiohydrolase is essential for efficient hydrolysis of cellulose (9, 13, 29, 36). We therefore constructed a yeast strain in which the three types of cellulase necessary to efficiently degrade cellulose are codisplayed on the cell surface, with α -agglutinin as an anchor (Fig. 2). While codisplay of two proteins through the use of a cell surface display system based on α -agglutinin has been reported (7, 15, 16, 23), there is no report of codisplay of

three or more proteins. As demonstrated by the result shown in Fig. 5, we succeeded in directly producing ethanol from amorphous cellulose without the addition of cellulase enzymes by developing a yeast strain codisplaying *T. reesei* EGII and CBHII and *A. aculeatus* BGL1 on the cell surface. This is the first report of codisplay on the yeast cell surface of three functional proteins.

It has previously been reported that EG and CBH act synergistically on cellulose chains to produce soluble cellooligosaccharides and that CBH is the key cellulase in cellulose hydrolysis (9, 13, 29, 36). Although surface-displayed CBHII had only a little activity with respect to amorphous cellulose, the yeast strain codisplaying EGII and CBHII showed significantly higher activity than the yeast strain displaying only EGII and produced cellobiose as the main product (Fig. 3). This result indicates that CBHII plays a very important role in cellulose degradation and that synergism between EGII and CBHII is successfully induced on the yeast cell surface.

In addition to codisplay of EGII and CBHII, BGL1 was simultaneously codisplayed to produce ethanol from amorphous cellulose. The β -glucosidase activity of the yeast strain codisplaying EGII, CBHII, and BGL1 (164.5 U/g [dry weight] of cells) was approximately 2.0 and 1.6 times higher, respectively, than that of the strain displaying BGL1 and the strain codisplaying BGL1 and EGII (80.8 and 129.8 U/g [dry weight] of cells). Interestingly, flow cytometric analysis of fluorescence-labeled yeast cells confirmed that the total protein of yeast strains displaying two or three enzymes was greater than that of single-display strains, as measured by mean fluorescence intensity (data not shown). This result indicated that the sum of the number of BGL1, EGII, and CBHII molecules in a codisplay strain is larger than that seen in a single-display strain.

In the hydrolysis experiment, although the reducing sugar produced by the yeast strain codisplaying EGII, CBHII, and BGL1 was not detected in the supernatant of the reaction mixture (Fig. 3), a decrease in insoluble cellulose was observed (Fig. 4). This is because the cellobiose produced by the synergistic reaction of EGII and CBHII is further converted to glucose by BGL1 and the glucose is immediately taken up by cells. The yeast strain MT8-1/pBG211/pEG23u31H6/pFCBH2w3 was thus able to directly produce ethanol from amorphous cellulose (Fig. 5). On the other hand, the yeast strain codisplaying BGL1 and EGII could not ferment amorphous cellulose to ethanol. Through codisplay of the three types of cellulolytic enzyme, amorphous cellulose was synergistically and sequentially hydrolyzed to glucose on the yeast cell surface and immediately converted to ethanol by intracellular metabolic enzymes. These results indicate that codisplay of CBHII is very effective in inducing hydrolysis and direct fermentation of amorphous cellulose. The ethanol production was also efficient, as there was no time lag in the decrease in insoluble cellulose and the ethanol yield was high (88.5% of theoretical yield). Moreover, glucose was not detected in the fermentation medium, which is advantageous to prevent or minimize contamination.

As described above, efficient direct fermentation of amorphous cellulose to ethanol was achieved by developing a yeast strain codisplaying three types of cellulolytic enzyme. Codisplay of CBHII significantly affected cellulose hydrolysis, sug-

gesting that combination of cellulases with various functions is effective in producing efficient degradation. Further work is needed to analyze the synergistic reaction of the cellulases codisplayed on the cell surface and to construct a yeast whole-cell biocatalyst with an improved ability to catalyze cellulose degradation and fermentation.

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