

Research Article

Ethanol production from cellulosic materials using cellulase-expressing yeast

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We demonstrate direct ethanol fermentation from amorphous cellulose using cellulase-co-expressing yeast. Endoglucanases (EG) and cellobiohydrolases (CBH) from *Trichoderma reesei*, and β -glucosidases (BGL) from *Aspergillus aculeatus* were integrated into genomes of the yeast strain *Saccharomyces cerevisiae* MT8-1. BGL was displayed on the yeast cell surface and both EG and CBH were secreted or displayed on the cell surface. All enzymes were successfully expressed on the cell surface or in culture supernatants in their active forms, and cellulose degradation was increased 3- to 5-fold by co-expressing EG and CBH. Direct ethanol fermentation from 10 g/L phosphoric acid swollen cellulose (PASC) was also carried out using EG-, CBH-, and BGL-co-expressing yeast. The ethanol yield was 2.1 g/L for EG-, CBH-, and BGL-displaying yeast, which was higher than that of EG- and CBH-secreting yeast (1.6 g/L ethanol). Our results show that cell surface display is more suitable for direct ethanol fermentation from cellulose.

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1 Introduction

Bioenergy represents the utilization of biomass as starting material for the production of sustainable fuels and chemicals [1]. Environmental concerns and the depletion of oil reserves have resulted in governmental actions and incentives to establish greater energy independence by promoting research on environmentally friendly and sustainable biofuels. Ethanol is the most widely used biofuel, either as a direct fuel or in gasoline blends.

World ethanol production has reached about 51 000 million liters, with the USA and Brazil being the largest producers (Renewable Fuels Association, 2007, Industry statistics. Available online: <http://www.ethanolrfa.org/industry/statistics>).

Cellulose is a major component of the cell wall of plants and the most abundant and renewable carbohydrate; therefore, practical and cost-effective processes for bioethanol production from cellulose are highly desired.

The yeast *S. cerevisiae* is a highly suitable microorganism for bioethanol production because of its high ethanol productivity, high ethanol tolerance, high endurance in industrial processes, and the simplicity of genetic engineering [2–4]. However, *S. cerevisiae* cannot utilize cellulosic materials, and a cellulose saccharification process to produce glucose is necessary before fermentation [5].

For cellulose degradation to glucose, cellulolytic enzymes have been widely used. The filamentous fungus *Trichoderma reesei* is known as a strongly

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Abbreviations: BGL, β -glucosidase; CBH, cellobiohydrolase; CMC, carboxymethyl-cellulose; EG, Endoglucanase; PASC, phosphoric acid swollen cellulose

cellulolytic microorganism. *T. reesei* extracellularly secretes three types of cellulolytic enzymes: endoglucanases (EG), cellobiohydrolases (CBH), and β -glucosidases (BGL). EG acts randomly against the amorphous region of the cellulose chain to produce reducing and nonreducing ends containing sugars. CBH acts against crystalline cellulose and produces reducing or nonreducing ends containing sugars [6–9]. Cellulose chains are thus efficiently degraded to soluble cellobiose and cellooligosaccharides by the endo-exo synergy of EG and CBH. In the last step of enzymatic cellulose degradation, cellooligosaccharides are hydrolyzed to glucose by BGL. Although enzymatic degradation is low-energy and environmentally friendly, the preparative costs of enzymes prevents their direct use for the production of bioethanol.

To reduce the ethanol production cost and simplify the production process, the construction of a cellulase expression yeast is an attractive approach. To facilitate direct ethanol fermentation from cellulose, we previously developed yeast strains displaying cellulolytic enzymes on the cell surface [5, 10]. In this study, to increase ethanol productivity, we optimized the cellulase expression system. We utilized a genome integrated expression system for stable expression of cellulases [11] and the PGK promoter for overexpression [12, 13]. Using EG-, CBH-, and BGL-displaying yeast, direct ethanol fermentation from amorphous cellulose was demonstrated. We constructed cellulase-secreting expression yeast or cellulase-displaying expression yeast and investigated their enzymatic

and ethanol fermentation abilities. Finally, direct and efficient ethanol fermentation from amorphous cellulose was demonstrated.

2 Materials and Methods

2.1 Strains and media

The yeast strains used are summarized in Table 1. *S. cerevisiae* MT8-1 transformants were selected and maintained on SD medium plates (6.7 g/L yeast nitrogen base w/o amino acids, 20 g/L glucose) with amino acids as required.

2.2 Plasmid construction

The plasmid for cell surface display of the *A. aculeatus* BGL1 gene was constructed as follows. The DNA fragment encoding the secretion signal sequence derived from *Rhizopus oryzae* glucoamylase, BGL1, and α -agglutinin was amplified by PCR using pIBG13 [14] as the template with the following primers: 5'-GCTCTAGAATGCAACTGTTCAA TTTGCCATTGAAAG-3' and 5'-GCTCATGATTTG ATTATGTTCTTTCTATTTGAATGAGATATG-3'. The amplified fragment was digested with *Xho*I and ligated into pGK403 [15]. The resultant plasmid was named pIHAGBGL. Then the promoter region was amplified by PCR using pIHAGBGL as the template with the following primers: 5'-ATGCGCT AGCGCGGCCGCCGATTTGGGCGCGAATCC TTTA-3' and 5'-GCATGCTAGCTGTTTATATTT

Table 1. Strains and plasmids used in this study

Strains or plasmids	Relevant features
<i>S. cerevisiae</i> yeast strains	
MT8-1	<i>MATa ade his3 leu2 trp1 ura3</i>
MT8-1/AGEGII	EGII display
MT8-1/EGII	EGII secretion
MT8-1/AGCBHII	CBHII display
MT8-1/CBHII	CBHII secretion
MT8-1/AGBGL	BGL display
MT8-1/AGEGII-AGCBHII	EGII and CBHII display
MT8-1/EGII-CBHII	EGII and CBHII secretion
MT8-1/AGEGII-AGCBHII-AGBGL	EGII, CBHII and BGL display
MT8-1/EGII-CBHII-AGBGL	EGII and CBHII secretion and BGL display
Plasmids	
pIHAGBGL- <i>Not</i> I	BGL1 display with <i>HIS3</i> marker gene
pIWAGEGII	EGII display with <i>TRP1</i> marker gene
pIWEGII	EGII secretion with <i>TRP1</i> marker gene
pIHAGCBHII	CBHII display with <i>HIS3</i> marker gene
pIHCBHII	CBHII secretion with <i>HIS3</i> marker gene
pIWAGEGIIAGBGL	EGII and BGL display with <i>TRP1</i> marker gene
pIWEGIIAGBGL	EGII secretion and BGL display with <i>TRP1</i> marker gene

GTTGTAAA-3'. The amplified DNA fragment was digested by *NheI* and introduced into the *NheI* site of the plasmid pIHAGBGL. The resulting plasmid was named pIHAGBGL-*NotI*.

The plasmid for cell surface display of the *T. reesei* EGII gene was constructed as follows. The DNA fragment encoding the secretion signal sequence from *R. oryzae*, the EGII gene, and the 3' half of α -agglutinin was prepared by PCR using pEG23u31H6 [10] with the following primers: 5'-CATGCTAGCATGCAACTGTTCAATTTGCCATTGAAAG-3' and 5'-TCCCCCGGGTTTGATTATGTTCTTTCTATTTGAATGAGATATG-3'. The amplified fragment was digested with *NheI* and *XmaI* and ligated into pGK404 [15]. This plasmid was named pIWAGEGII. The plasmid for EGII secretion was constructed as follows. The DNA fragment encoding the secretion signal sequence from the *R. oryzae* and the EGII gene was amplified by PCR using pEGII3u31H6 as the template with the following primers: 5'-GCTCTAGAATGCAACTGTTCAATTTGCC and 5'-GCTCTAGACTACTTTCTTGCAGACACG. The amplified fragment was digested with *XbaI* and ligated into the *SpeI* site of pGK404. The resultant plasmid was named pIWEGII.

The plasmid for cell surface display of the *T. reesei* CBHII gene was constructed as follows. The DNA fragment encoding the secretion signal sequence from *R. oryzae*, the CBHII gene, and the 3' half of α -agglutinin was prepared by PCR using pFCBH2w3 [5] with the following primers: 5'-GCTCTAGAATGCAACTGTTCAATTTGCCATTGAAAG-3' and 5'-GCTCTAGATTTGATTATGTTCTTTCTATTTGAATGAGATATG-3'. The amplified fragment was digested with *XbaI* and ligated into pGK406 [15]. This plasmid was named pGK406CBHII. The plasmid pGK406CBHII was digested with *ApaI* and *NotI*, and the digested fragment was ligated into pRS403 (Stratagene). The resultant plasmid was named pIHAGCBHII. The plasmid for CBHII secretion was constructed as follows. The DNA fragment encoding the secretion signal sequence from *R. oryzae* and the CBHII gene was amplified by PCR using pFCBH2w3 as a template with the following primers: 5'-GCTCTAGAAATGCAACTGTTCAATTTGCC-3' and 5'-GCTCTAGATTACAGGAACGATGGGTTTGCATT-3'. The amplified fragment was digested with *XbaI* and ligated into the *SpeI* site of pGK403. The resultant plasmid was named pIHCBHII.

The plasmids for both EG and BGL expression were constructed as follows. The plasmid pIHAGBGL-*NotI* was digested with *NotI*, and the fragment encoding the PGK promoter, signal sequence, BGL1, α -agglutinin, and PGK terminator was ligated

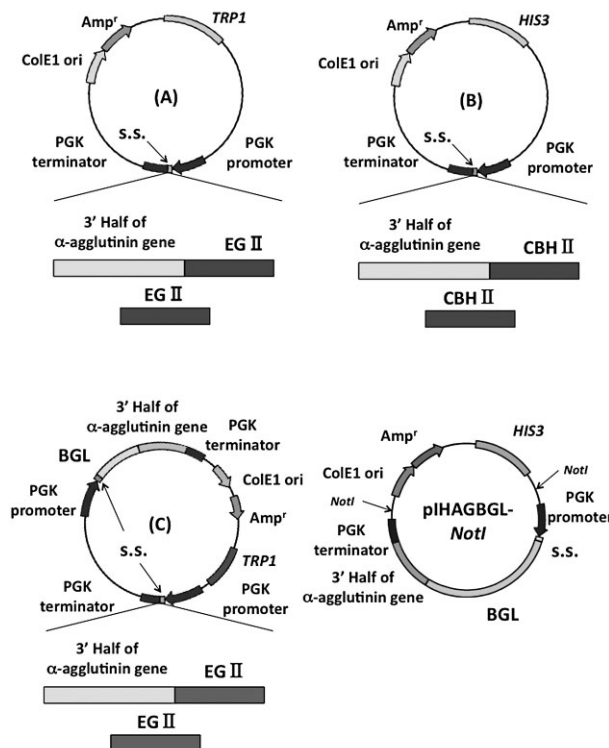


Figure 1. (A) Expression plasmids for cell surface display or secretion of *T. reesei* EGII (pIHAGEGII and pIWAGEGII). (B) Expression plasmids for cell surface display or secretion of *T. reesei* CBHII (pIHAGCBHII and pIWAGCBHII). (C) Expression plasmids for cell surface display or secretion of *T. reesei* EGII and cell surface display of *A. aculeatus* BGL1 (pIWAGEGIIAGBGL and pIWEGIIAGBGL).

ed into pIWAGEGII or pIWEGII. The resultant plasmids were named pIWAGEGIIAGBGL and pIWEGIIAGBGL, respectively. The plasmids are summarized in Table 1 and illustrated in Figure 1.

2.3 Yeast transformation

Plasmids pIHAGCBHII and pIHCBHII were linearized by *NdeI* digestion. Plasmids pIWAGEGII, pIWEGII, pIWAGEGIIAGBGL, and pIWEGIIAGBGL were linearized by *Bst1107I* digestion. Linearized fragments were transformed into *S. cerevisiae* MT8-1 by the lithium acetate method. The resultant transformants are summarized in Table 1. EGII-expressing yeasts (MT8-1/AGEGII, MT8-1/EGII, MT8-1/AGEGII-AGBGL, MT8-1/EGII-AGBGL, MT8-1/EGII-CBHII-EGBGL, and MT8-1/EGII-CBHII-EGBGL) were screened for their cellulose-degrading ability by incubating them on SDC medium plates containing 1 g/L AZCL-HE-CELLULOSE (Megazyme) with the required amino acids at 30°C for 1–2 days. CBHII-expressing yeasts (MT8-1/AGCBHII, MT8-1/CBHII, MT8-1/AGCBHII-AGBGL, MT8-1/CBHII-AGBGL, MT8-1/EGII-

CBHII-AGBGL, and MT8-1/AGEGII-AGCBHII-AGBGL) were also screened for their cellulose-degrading ability by incubating them on SDC medium (6.7 g/L yeast nitrogen base w/o amino acids, 20 g/L glucose, and 20 g/L casamino acids) plates containing 1 g/L β -glucan and supplemented with amino acids as required at 30°C for 2 days. After incubation, colonies were washed off the plate and the remaining β -glucan was stained with 0.1% Congo red and de-stained with 1 M NaCl.

2.4 Enzymatic assays

The EG activity of MT8-1/AGEGII or MT8-1/EGII was determined by hydrolysis of carboxymethyl-cellulose (CMC). Ten grams per liter of CMC in 100 mM citric acid buffer at pH 5.0 was mixed with EGII-displaying yeast whose OD_{600} was adjusted to 10 by distilled water. In the case of MT8-1/EGII, the OD_{600} was adjusted to 10 by culture supernatant after 72 h cultivation and mixed with 10 g/L CMC. The mixture was incubated for 6 h at 50°C. After the hydrolysis reaction, the supernatant was separated by centrifugation at $14\,000 \times g$ for 10 min at 4°C and the amount of reducing sugar was measured using the Somogyi–Nelson method [16] to determine the number of glucose equivalents. One unit was defined as the amount of enzyme required to produce 1 μ mol *p*-nitrophenol or reducing sugar per minute under the assay conditions.

The CBHII activity of MT8-1/AGCBHII or MT8-1/CBHII was determined by hydrolysis of phosphoric acid swollen cellulose (PASC). PASC was prepared according to a previous report [17]. One gram per liter of PASC in 50 mM sodium acetate buffer at pH 5.0 was mixed with CBHII-displaying yeast whose OD_{600} was adjusted to 10 by distilled water. In the case of MT8-1/CBHII, OD_{600} was adjusted to 10 by culture supernatant after 72 h cultivation and mixed with 1 g/L PASC. The mixture was incubated at 50°C for 6 h. After incubation, the activity was estimated by the Somogyi–Nelson method as described above.

BGL activity was determined using *p*-nitrophenyl- β -D-glucopyranoside as the substrate according to a previously described method [10].

In the case of several cellulase-expressing yeasts, the PASC degradation activity was evaluated similarly. Ten grams per liter of PASC in 50 mM sodium acetate buffer at pH 5.0 were mixed with EGII- and/or CBHII- and BGL-displaying yeast (MT8-1/AGEGII, MT8-1/AGCBHII, MT8-1/AGEGII-AGCBHII, and MT8-1/AGEGII-AGCBHII-AGBGL) whose OD_{600} was adjusted to 10 by distilled water and incubated at 30°C. In the case of MT8-1/EGII, MT8-1/CBHII, MT8-1/EGII-CBHII,

and MT8-1/EGII-CBHII-AGBGL, the OD_{600} was adjusted to 10 by the culture supernatant after 72 h and mixed with PASC and incubated at 30°C.

2.5 Fermentation

After precultivation in SD medium for 24 h, yeast cells were aerobically cultivated for 72 h at 30°C in YPD medium. The cells were collected by centrifugation for 5 min at $1000 \times g$ and 15°C, and washed with distilled water twice. The cell pellets were inoculated into fermentation medium (10 g/L yeast extract, 20 g/L polypeptone, 10 g/L PASC as the sole carbon source, and 0.5 g/L potassium disulfide). Ethanol fermentation was anaerobically performed at 30°C with the OD_{600} of the fermentation medium adjusted to 50 [5]. Ethanol and glucose concentrations were measured by HPLC and total sugar concentrations were measured by a phenol-sulfuric acid method as a glucose equivalent [18]. The sugar concentration including both PASC and yeast cells were measured and also the concentration of the sugar derived from yeast were determined. Total sugar concentrations of PASC were determined by subtracting the yeast cell-derived sugar from the culture medium containing the yeast cells and cellulose.

3 Results and Discussion

3.1 Construction of yeast strains co-expressing three types of cellulolytic enzymes

We constructed yeast strains co-expressing three types of cellulolytic enzymes (EG, CBH, and BGL). Cellulosic materials were digested first by EG and CBH, and finally, the digested sugars were converted to glucose by BGL. Therefore, in all transformants, BGL was expressed on the cell surface in order for produced glucose to be easily taken up into the yeast. EG and CBH were introduced by secretion or displaying expression. The resultant transformants are summarized in Table 1.

3.2 Enzymatic activity of EGII, CBHII, and BGL

Transformants with high EG or CBH activity were screened by a halo assay. The enzymatic activities of screened transformants after 72 h cultivation are shown in Table 2. EGII activity was evaluated by CMC hydrolysis and CBHII activity was evaluated by PASC hydrolysis. The EGII activity on the cell surface of MT8-1/AGEGII (2.06 U/ OD_{600}) was higher than that of the supernatant (1.92 U/L); alternatively, the EGII activity of the supernatant of

Table 2. Enzyme activity of cell surface and supernatant

Cellulase	Enzyme activity Cells (U/OD ₆₀₀)	Supernatants (U/L)
EGII		
MT8-1/AGEGII	2.06 ± 0.045	1.92 ± 0.43
MT8-1/EGII	0.32 ± 0.21	3.56 ± 0.20
MT8-1	0.02 ± 0.0025	N.D.
CBHII		
MT8-1/AGCBHII	0.58 ± 0.19	N.D.
MT8-1/CBHII	0.28 ± 0.14	0.07 ± 0.087
MT8-1	0.11 ± 0.029	N.D.
BGLI		
MT8-1/AGBGLI	4.02 ± 0.042	1.98 ± 0.069
MT8-1	N.D.	0.73 ± 0.12

MT8-1/EGII (3.56 U/L) was higher than that of the cell surface (0.32 U/OD₆₀₀). These results show successful expression of active EGII on both the cell surface and in the culture supernatant. In the case of CBHII, active CBHII was also expressed both on the cell surface and culture supernatant; however, the activity of CBHII was significantly lower. Because CBHII acts mainly against crystalline cellulose, which is produced by EGII activity, CBHII activity without EGII was low. Active BGL was also expressed on the cell surface, corresponding to our previous report [19]. The BGL activities both MT8-1/AGEGII-AGCBHII-AGBGL and MT8-1/EGII-CBHII-AGBGL strains have almost the same (data not shown).

3.3 PASC degradation using EGII-, CBHII-, and BGL-co-expressing yeast

We then examined the amorphous cellulose degradation ability using two or three types of cellulase-co-expressing yeast. After cultivation in YPD medium for 72 h at 30°C, cells were mixed with PASC and reducing sugar was detected by the Somogyi–Nelson method. The time-course of reducing sugar concentration by PASC degradation is shown in Figure 2. The CBHII-secreting yeast MT8-1/CBHII cannot digest PASC as does MT8-1, because CBHII acts against crystalline cellulose produced by EGII digestion, and hence CBHII cannot digest PASC without EGII. The EGII-secreting yeast MT8-1/EGII partially digested PASC and produced reducing sugars. MT8-1/EGII-CBHII, both of which are EGII- and CBHII-secreting yeasts, can digest PASC efficiently and produce large amounts of reducing sugars, suggesting that synergetic effects of both EGII and CBHII increase cellulose degradation. As shown in Figure 2B, MT8-1/AGCBHII also cannot digest PASC, and MT8-1/AGEGII

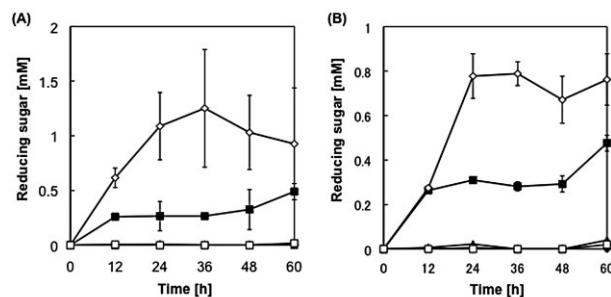


Figure 2. (A) Time-course of synergistic hydrolysis of amorphous cellulose by *S. cerevisiae* MT8-1 (closed diamond), MT8-1/EGII (closed square), MT8-1/CBHII (closed triangle), MT8-1/EGII-CBHII (open diamond), and MT8-1/EGII-CBHII-AGBGL (open square). (B) Time-course of synergistic hydrolysis of amorphous cellulose by *S. cerevisiae* MT8-1 (closed diamond), MT8-1/AGEGII (closed square), MT8-1/AGCBHII (closed triangle), MT8-1/AGEGII-AGCBHII (open diamond), and MT8-1/AGEGII-AGCBHII-AGBGL (open square). The data points represent the averages of three independent experiments.

can partially digest PASC in a similar manner as described above. MT8-1/AGEGII-AGCBHII can also digest cellulose efficiently due to synergetic effects of both CBHII and EGII. In the case of MT8-1/EGII-CBHII-AGBGL and MT8-1/AGEGII-AGCBHII-AGBGL, reducing sugars were not detected because cellulose was digested to glucose by BGL, and produced glucose was immediately consumed by the yeast. These results also show that the rate-limiting step is EGII and CBHII mediated cellulose degradation; hence, improvement of EGII and CBHII activities is important for efficient ethanol fermentation from cellulose.

3.4 Direct fermentation of amorphous cellulose to ethanol using cellulase-expressing yeast

Direct production of ethanol from amorphous cellulose was performed using the yeast strain co-displaying EGII, CBHII, and BGL (MT8-1/AGEGII-AGCBHII-AGBGL), or the yeast strain secreting EGII and CBHII and displaying BGL (MT8-1/EGII-CBHII-BGL). Fermentation was anaerobically performed at 30°C from 10 g/L PASC. Time-courses of ethanol and total sugar concentrations are shown in Figure 3. Both strains digested PASC and efficiently produced ethanol. The final ethanol yield was 2.1 g/L for MT8-1/AGEGII-AGCBHII-AGBGL and 1.6 g/L for MT8-1/EGII-CBHII-AGBGL. This result clearly shows that cellulase-co-displaying yeast is more suitable for direct ethanol fermentation from PASC compared to cellulase-secreting yeast. Cell surface display technology allows the accumulation of each cellulase on the cell surface, which accelerates the synergetic effects of CBHII, EGII, and BGL for PASC degradation.

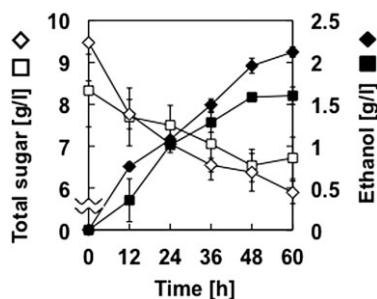


Figure 3. Time-course of ethanol fermentation from amorphous cellulose by MT8-1/AGEGII-AGCBHII-AGBGL (diamond) and MT8-1/EGII-CBHII-AGBGL (square). Closed symbols represents ethanol and open symbols represents total sugar. The data points represent the averages of three independent experiments.

In our previous report, 2.9 g/L ethanol was produced from PASC by cellulase-co-displaying yeast. Because a multi-copy vector system was utilized in the previous report, and the expression level of enzymes was higher than in this study, the present ethanol yield based on gene integration was a little lower (2.1 g/L). In general, gene integration expression systems reduce the amount of expressed proteins; however, from a practical point of view, a gene integration system is more suitable due to gene stability and prevention of the loss of plasmids. To improve the cellulase activity, one possible strategy is increasing the integrated gene copy number. For example, δ -integration method is available to introduce many numbers of genes into yeast genome [20], which may improve the cellulases activity by increasing the amount of expressed cellulases.

4 Concluding Remarks

We successfully demonstrated direct and efficient ethanol fermentation from amorphous cellulose using cellulase-co-expressing yeast. Cellulase-displaying yeast more efficiently produced ethanol from PASC compared to cellulase-secreting yeast. However, cellulase activities are not enough for complete cellulose digestion. Further improvements of cellulase activity and/or expression levels are ongoing studies in our laboratory.

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