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## Direct ethanol production from cellulosic materials using cellulase-displaying genes integrated with yeast

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1 Research Article ((3629 words))

2 **Direct ethanol production from cellulosic materials using**  
3 **cellulase-displaying genes integrated with yeast**

4  
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22  
23 List of abbreviations: EG; Endoglucanase, CBH; cellobiohydrolase,  
24 BGL;  $\beta$ -glucosidase, PASC; phosphoric acid swollen cellulose, CMC;

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For Peer Review

26 **Abstract**

27 We demonstrate direct ethanol fermentation from amorphous cellulose using  
28 cellulase-co-expressing yeast. Endoglucanases (EG), cellobiohydrolases (CBH) from  
29 *Trichoderma reesei*, and  $\beta$ -glucosidases (BGL) from *Aspergillus aculeatus* were  
30 integrated into genomes of the yeast strain *Saccharomyces cerevisiae* MT8-1. BGL was  
31 displayed on the yeast cell surface and both EG and CBH were secreted or displayed on  
32 the cell surface. All enzymes were successfully expressed on the cell surface or in  
33 culture supernatants in their active forms, and cellulose degradation was increased 3- to  
34 5-fold by co-expressing EG and CBH. Direct ethanol fermentation from 10 g/l  
35 phosphoric acid swollen cellulose (PASC) was also carried out using EG-, CBH-, and  
36 BGL-co-expressing yeast. The ethanol yield was 2.1 g/l for EG-, CBH-, and  
37 BGL-displaying yeast, which was higher than that of EG- and CBH-secreting yeast (1.6  
38 g/l ethanol). Our results show that cell surface display is more suitable for direct ethanol  
39 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 [2]. 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 *Saccharomyces 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 [3-5]. However, *S. cerevisiae* cannot utilize cellulosic materials, and a cellulose saccharification process to produce glucose is necessary before fermentation [6].

For cellulose degradation to glucose, cellulolytic enzymes have been widely used. The filamentous fungus *Trichoderma reesei* is known as a strongly cellulolytic microorganism. *T. reesei* extracellularly secretes three types of cellulolytic enzymes: endoglucanases (EG), cellobiohydrolases (CBH), and  $\beta$ -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 [7-10]. Cellulose chains are thus efficiently degraded to soluble cellobiose and cellooligosaccharides by the

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6 65 endo-exo synergy of EG and CBH. In the last step of enzymatic cellulose degradation,  
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8 66 celooligosaccharides are hydrolyzed to glucose by BGL. Although enzymatic  
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10 67 degradation is low-energy and environmentally friendly, the preparative costs of  
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12 68 enzymes prevents their direct use for the production of bioethanol.

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15 69 To reduce the ethanol production cost and simplify the production process, the  
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17 70 construction of a cellulase expression yeast is an attractive approach. To facilitate direct  
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19 71 ethanol fermentation from cellulose, we previously developed yeast strains displaying  
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21 72 cellulolytic enzymes on the cell surface [6, 11]. In this study, to increase ethanol  
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23 73 productivity, we optimized the cellulase expression system. We utilized a genome  
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25 74 integrated expression system for stable expression of cellulases [12] and the PGK  
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27 75 promoter for overexpression [13, 14]. Using EG-, CBH-, and BGL-displaying yeast,  
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29 76 direct ethanol fermentation from amorphous cellulose was demonstrated. We  
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31 77 constructed cellulase-secreting expression yeast or cellulase-displaying expression yeast  
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33 78 and investigated their enzymatic and ethanol fermentation abilities. Finally, direct and  
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35 79 efficient ethanol fermentation from amorphous cellulose was demonstrated.  
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## 45 82 **2. Materials and Methods**

### 46 83 47 48 49 50 84 **2.1 Strains and media**

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52 85 The yeast strains used are summarized in Table 1. *S. cerevisiae* MT8-1  
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54 86 transformants were selected and maintained on SD medium plates (6.7 g/l yeast  
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56 87 nitrogen base w/o amino acids, 20 g/l glucose) with amino acids as required.  
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## 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  $\alpha$ -agglutinin was amplified by PCR using pIBG13 [15] as the template with the following primers: 5'-GCTCTAGAATGCAACTGTTCAATTTGCCATTGAAAG-3' and 5'-GCTCATGATTTGATTATGTTCTTTCTATTTGAATGAGATATG-3'. The amplified fragment was digested with *Xho*I and ligated into pGK403 [16]. The resultant plasmid was named pIHAGBGL. Then the promoter region was amplified by PCR using pIHAGBGL as the template with the following primers: 5'-ATGCGCTAGCGCGGCCGCGATTGGGCGCGAATCCTTTA-3' and 5'-GCATGCTAGCTGTTTTATATTTGTTGTAAA-3'. The amplified DNA fragment was digested by *Nhe*I and introduced into the *Nhe*I site of the plasmid pIHAGBGL. The resulting plasmid was named pIHAGBGL-*Not*I.

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  $\alpha$ -agglutinin was prepared by PCR using pEG23u31H6 [11] with the following primers: 5'-CATGCTAGCATGCAACTGTTCAATTTGCCATTGAAAG-3' and 5'-TCCCCCGGGTTTGATTATGTTCTTTCTATTTGAATGAGATATG-3'. The amplified fragment was digested with *Nhe*I and *Xma*I and ligated into pGK404 [16]. 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

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6 113 with the following primers: 5'-GCTCTAGAATGCAACTGTTCAATTTGCC and  
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8 114 5'-GCTCTAGACTACTTTCTTGCAGACACG. The amplified fragment was digested  
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10 115 with *Xba*I and ligated into the *Spe*I site of pGK404. The resultant plasmid was named  
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12 116 pIWEGII.

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15 117 The plasmid for cell surface display of the *T. reesei* CBHII gene was  
16  
17 118 constructed as follows. The DNA fragment encoding the secretion signal sequence from  
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19 119 *R. oryzae*, the CBHII gene, and the 3' half of  $\alpha$ -agglutinin was prepared by PCR using  
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21 120 pFCBH2w3 [6] with the following primers:  
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23 121 5'-GCTCTAGAATGCAACTGTTCAATTTGCCATTGAAAG-3' and  
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25 122 5'-GCTCTAGATTTGATTATGTTCTTTCTATTTGAATGAGATATG-3'. The amplified  
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27 123 fragment was digested with *Xba*I and ligated into pGK406 [16]. This plasmid was  
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29 124 named pGK406CBHII. The plasmid pGK406CBHII was digested with *Apa*I and *Not*I,  
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31 125 and the digested fragment was ligated into pRS403 (Stratagene). The resultant plasmid  
32  
33 126 was named pIHAGCBHII. The plasmid for CBHII secretion was constructed as follows.  
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35 127 The DNA fragment encoding the secretion signal sequence from *R. oryzae* and the  
36  
37 128 CBHII gene was amplified by PCR using pFCBH2w3 as a template with the following  
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39 129 primers: 5'-GCTCTAGAATGCAACTGTTCAATTTGCC-3' and  
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41 130 5'-GCTCTAGATTACAGGAACGATGGGTTTGCATT-3'. The amplified fragment was  
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43 131 digested with *Xba*I and ligated into the *Spe*I site of pGK403. The resultant plasmid was  
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45 132 named pIHCBHII.

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48 133 The plasmids for both EG and BGL expression were constructed as follows.  
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50 134 The plasmid pIHAGBGL-*Not*I was digested with *Not*I, and the fragment encoding the  
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52 135 PGK promoter, signal sequence, BGL1,  $\alpha$ -agglutinin, and PGK terminator was ligated  
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54 136 into pIWAGEGII or pIWEGII. The resultant plasmids were named  
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6 137 pIWAGEGIIAGBGL and pIWEGIIAGBGL, respectively. The plasmids are summarized  
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8 138 in Table 1 and illustrated in Figure 1.  
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### 11 140 **2.3 Yeast transformation**

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15 141 Plasmids pIHAGCBHII and pIHCBHII were linearized by *NdeI* digestion.  
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18 142 Plasmids pIWAGEGII, pIWEGII, pIWAGEGIIAGBGL, and pIWEGIIAGBGL were  
19  
20 143 linearized by *BstI*107I digestion. Linearized fragments were transformed into *S.*  
21  
22 144 *cerevisiae* MT8-1 by the lithium acetate method. The resultant transformants are  
23  
24 145 summarized in Table 1. EGII-expressing yeasts (MT8-1/AGEGII, MT8-1/EGII,  
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26 146 MT8-1/AGEGII-AGBGL, MT8-1/EGII-AGBGL, MT8-1/EGII-CBHII-EGBGL, and  
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28 147 MT8-1/EGII-CBHII-EGBGL) were screened for their cellulose-degrading ability by  
29  
30 148 incubating them on SDC medium plates containing 1 g/l AZCL-HE-CELLULOSE  
31  
32 149 (Megazyme) with the required amino acids at 30°C for 1-2 days. CBHII-expressing  
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34  
35 150 yeasts (MT8-1/AGCBHII, MT8-1/CBHII, MT8-1/AGCBHII-AGBGL,  
36  
37 151 MT8-1/CBHII-AGBGL, MT8-1/EGII-CBHII-AGBGL, and  
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39 152 MT8-1/AGEGII-AGCBHII-AGBGL) were also screened for their cellulose-degrading  
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42 153 ability by incubating them on SDC medium (6.7 g/l yeast nitrogen base w/o amino acids,  
43  
44 154 20 g/l glucose, and 20 g/l casamino acids) plates containing 1 g/l  $\beta$ -glucan and  
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46 155 supplemented with amino acids as required at 30°C for 2 days. After incubation,  
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48 156 colonies were washed off the plate and the remaining  $\beta$ -glucan was stained with 0.1%  
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50 157 Congo red and de-stained with 1 M NaCl.  
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### 55 159 **2.4 Enzymatic assays**

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58 160 The EG activity of MT8-1/AGEGII or MT8-1/EGII was determined by  
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7 161 hydrolysis of carboxymethyl-cellulose (CMC). Ten grams per liter of CMC in 100 mM  
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9 162 citric acid buffer at pH 5.0 was mixed with EGII-displaying yeast whose OD<sub>600</sub> was  
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11 163 adjusted to 10 by distilled water. In the case of MT8-1/EGII, the OD<sub>600</sub> was adjusted to  
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13 164 10 by culture supernatant after 72 h cultivation and mixed with 10 g/l CMC. The  
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15 165 mixture was incubated for 6 h at 50°C. After the hydrolysis reaction, the supernatant  
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18 166 was separated by centrifugation at 14,000 × g for 10 min at 4°C and the amount of  
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20 167 reducing sugar was measured using the Somogyi-Nelson method [17] to determine the  
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22 168 number of glucose equivalents. One unit was defined as the amount of enzyme required  
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25 169 to produce 1 μmol *p*-nitrophenol or reducing sugar per minute under the assay  
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27 170 conditions.

28  
29 171 The CBHII activity of MT8-1/AGCBHII or MT8-1/CBHII was determined by  
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31 172 hydrolysis of phosphoric acid swollen cellulose (PASC). PASC was prepared according  
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34 173 to a previous report [18]. One gram per liter of PASC in 50 mM sodium acetate buffer at  
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36 174 pH 5.0 was mixed with CBHII-displaying yeast whose OD<sub>600</sub> was adjusted to 10 by  
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38 175 distilled water. In the case of MT8-1/CBHII, OD<sub>600</sub> was adjusted to 10 by culture  
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40  
41 176 supernatant after 72 h cultivation and mixed with 1 g/l PASC. The mixture was  
42  
43 177 incubated at 50°C for 6 h. After incubation, the activity was estimated by the  
44  
45 178 Somogyi-Nelson method as described above.

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48 179 BGL activity was determined using *p*-nitrophenyl-β-D-glucopyranoside as the  
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50 180 substrate according to a previously described method [11].

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53 181 In the case of several cellulase-expressing yeasts, the PASC degradation  
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55 182 activity was evaluated similarly. Ten grams per liter of PASC in 50 mM sodium acetate  
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57 183 buffer at pH 5.0 were mixed with EGII- and/or CBHII- and BGL-displaying yeast  
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59 184 (MT8-1/AGEGII, MT8-1/AGCBHII, MT8-1/AGEGII-AGCBHII, and  
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6 185 MT8-1/AGEGII-AGCBHII-AGBGL) whose  $OD_{600}$  was adjusted to 10 by distilled  
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8 186 water and incubated at 30°C. In the case of MT8-1/EGII, MT8-1/CBHII,  
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10 187 MT8-1/EGII-CBHII, and MT8-1/EGII-CBHII-AGBGL, the  $OD_{600}$  was adjusted to 10  
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12 188 by the culture supernatant after 72 h and mixed with PASC and incubated at 30°C.  
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## 18 190 **2.5 Fermentation**

19 191 After precultivation in SD medium for 24 h, yeast cells were aerobically  
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21 192 cultivated for 72 h at 30°C in YPD medium. The cells were collected by centrifugation  
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23 193 for 5 min at 1,000 × *g* and 15°C, and washed with distilled water twice. The cell pellets  
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25 194 were inoculated into fermentation medium (10 g/l yeast extract, 20 g/l polypeptone, 10  
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27 195 g/l PASC as the sole carbon source, and 0.5 g/l potassium disulfide). Ethanol  
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29 196 fermentation was anaerobically performed at 30°C with the  $OD_{600}$  of the fermentation  
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31 197 medium adjusted to 50 [6]. Ethanol and glucose concentrations were measured by  
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33 198 high-performance liquid chromatography (HPLC) and total sugar concentrations were  
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35 199 measured by a phenol-sulfuric acid method as a glucose equivalent [19]. The sugar  
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37 200 concentration including both PASC and yeast cells were measured and also the  
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39 201 concentration of the sugar derived from yeast were determined. Total sugar  
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41 202 concentrations of PASC were determined by subtracting the yeast cell-derived sugar  
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43 203 from the culture medium containing the yeast cells and cellulose.  
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## 55 206 **3. Results and discussion**

### 56 207 57 208 **3.1 Construction of yeast strains co-expressing three types of cellulolytic enzymes**

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7 209 We constructed yeast strains co-expressing three types of cellulolytic enzymes  
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9 210 (EG, CBH, and BGL). Cellulosic materials were digested first by EG and CBH, and  
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11 211 finally, the digested sugars were converted to glucose by BGL. Therefore, in all  
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13 212 transformants, BGL was expressed on the cell surface in order for produced glucose to  
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15 213 be easily taken up into the yeast. EG and CBH were introduced by secretion or  
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18 214 displaying expression. The resultant transformants are summarized in Table 1.  
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### 22 216 **3.2 Enzymatic activity of EGII, CBHII, and BGL**

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25 217 Transformants with high EG or CBH activity were screened by a halo assay.  
26  
27 218 The enzymatic activities of screened transformants after 72 h cultivation are shown in  
28  
29 219 Table 2. EGII activity was evaluated by CMC hydrolysis and CBHII activity was  
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31 220 evaluated by PASC hydrolysis. The EGII activity on the cell surface of MT8-1/AGEGII  
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33 221 (2.06 U/OD<sub>600</sub>) was higher than that of the supernatant (1.92 U/l); alternatively, the  
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35 222 EGII activity of the supernatant of MT8-1/EGII (3.56 U/l) was higher than that of the  
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37 223 cell surface (0.32 U/OD<sub>600</sub>). These results show successful expression of active EGII on  
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39 224 both the cell surface and in the culture supernatant. In the case of CBHII, active CBHII  
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41 225 was also expressed both on the cell surface and culture supernatant; however, the  
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43 226 activity of CBHII was significantly lower. Because CBHII acts mainly against  
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45 227 crystalline cellulose, which is produced by EGII activity, CBHII activity without EGII  
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47 228 was low. Active BGL was also expressed on the cell surface, corresponding to our  
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49 229 previous report [20]. The BGL activities both MT8-1/AGEGII-AGCBHII-AGBGL and  
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51 230 MT8-1/EGII-CBHII-AGBGL strains have almost the same (data not shown).  
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### 233 **3.3 PASC degradation using EGII-, CBHII-, and BGL-co-expressing yeast**

234 We then examined the amorphous cellulose degradation ability using two or  
235 three types of cellulase-co-expressing yeast. After cultivation in YPD medium for 72 h  
236 at 30°C, cells were mixed with PASC and reducing sugar was detected by the  
237 Somogyi-Nelson method. The time-course of reducing sugar concentration by PASC  
238 degradation is shown in Figure 2. The CBHII-secreting yeast MT8-1/CBHII cannot  
239 digest PASC as does MT8-1, because CBHII acts against crystalline cellulose produced  
240 by EGII digestion, and hence CBHII cannot digest PASC without EGII. The  
241 EGII-secreting yeast MT8-1/EGII partially digested PASC and produced reducing  
242 sugars. MT8-1/EGII-CBHII, both of which are EGII- and CBHII-secreting yeasts, can  
243 digest PASC efficiently and produce large amounts of reducing sugars, suggesting that  
244 synergetic effects of both EGII and CBHII increase cellulose degradation. As shown in  
245 Figure 2B, MT8-1/AGCBHII also cannot digest PASC, and MT8-1/AGEGII can  
246 partially digest PASC in a similar manner as described above.  
247 MT8-1/AGEGII-AGCBHII can also digest cellulose efficiently due to synergetic effects  
248 of both CBHII and EGII. In the case of MT8-1/EGII-CBHII-AGBGL and  
249 MT8-1/AGEGII-AGCBHII-AGBGL, reducing sugars were not detected because  
250 cellulose was digested to glucose by BGL, and produced glucose was immediately  
251 consumed by the yeast. These results also show that the rate-limiting step is EGII and  
252 CBHII mediated cellulose degradation; hence, improvement of EGII and CBHII  
253 activities is important for efficient ethanol fermentation from cellulose.

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255

### 256 **3.4 Direct fermentation of amorphous cellulose to ethanol using**

**257 cellulase-expressing yeast**

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

270 In our previous report, 2.9 g/l ethanol was produced from PASC by  
271 cellulase-co-displaying yeast. Because a multi-copy vector system was utilized in the  
272 previous report, and the expression level of enzymes was higher than in this study, the  
273 present ethanol yield based on gene integration was a little lower (2.1 g/l). In general,  
274 gene integration expression systems reduce the amount of expressed proteins; however,  
275 from a practical point of view, a gene integration system is more suitable due to gene  
276 stability and prevention of the loss of plasmids. To improve the cellulase activity, one  
277 possible strategy is increasing the integrated gene copy number. For example,  
278  $\delta$ -integration method is available to introduce many numbers of genes into yeast  
279 genome [21], which may improve the cellulases activity by increasing the amount of  
280 expressed cellulases.

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281**4. Concluding Remarks**

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

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297  
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358 **7. Figure legends**

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360 Figure 1. (A) Expression plasmids for cell surface display or secretion of *T. reesei* EGII  
361 (pIHAGEGII and pIHEGII). (B) Expression plasmids for cell surface display or  
362 secretion of *T. reesei* CBHII (pIHAGCBHII and pIHCBHII). (C) Expression plasmids  
363 for cell surface display or secretion of *T. reesei* EGII and cell surface display of *A.*  
364 *aculeatus* BGL1 (pIWAGEGIIAGBGL and pIWEGIIAGBGL).

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

374

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

379

380 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>NotI</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

381

382 Table 2 Enzyme activity of cell surface and supernatant

cellulase	Enzyme activity	
	Cells [U/OD600]	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.
BGL1		
MT8-1/AGBGL1	4.02 ± 0.042	1.98 ± 0.069
MT8-1	N.D.	0.73 ± 0.12

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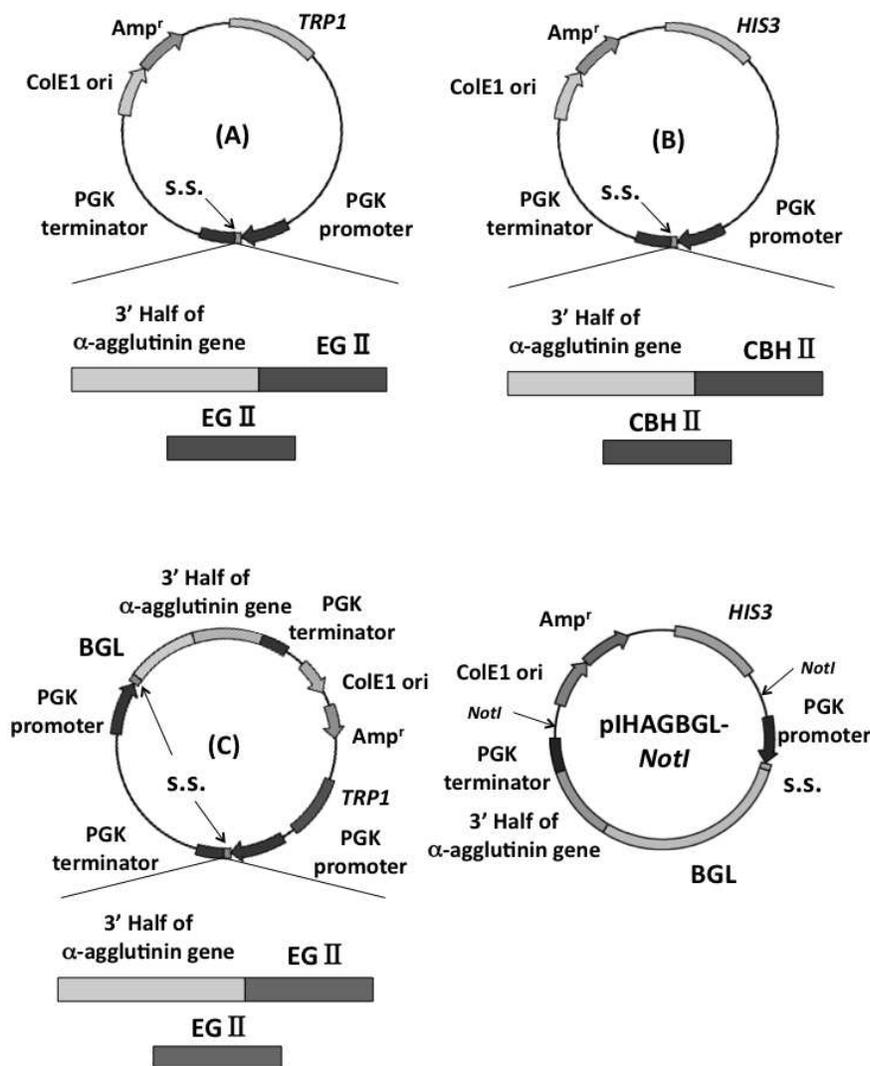


Figure 1. (A) Expression plasmids for cell surface display or secretion of *T. reesei* EGII (pIHAGEGII and pIHEGII). (B) Expression plasmids for cell surface display or secretion of *T. reesei* CBHII (pIHAGCBHII and pIHCBHII). (C) Expression plasmids for cell surface display or secretion of *T. reesei* EGII and cell surface display of *A. aculeatus* BGL1 (pIWAGEGIIAGBGL and pIWEGIIAGBGL). 266x355mm (72 x 72 DPI)

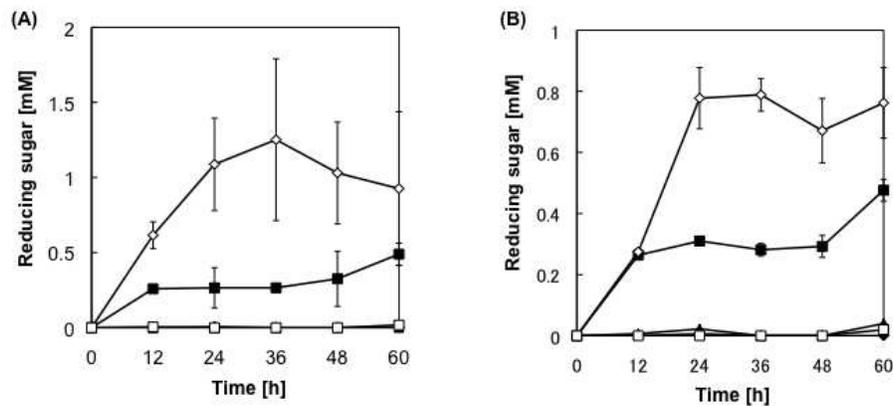


Figure 2. (A) Time-course of synergistic hydrolysis of amorphous cellulose by *S. cerevisiae* MT8-1 (closed diamond), MT8-1/EGII (closed square), MT8-1/CBHII (closed triangle), MT8-1/EGII-CBHII (open diamond), and MT8-1/EGII-CBHII-AGBGL (open square). (B) Time-course of synergistic hydrolysis of amorphous cellulose by *S. cerevisiae* MT8-1 (closed diamond), MT8-1/AEGEGII (closed square), MT8-1/AGCBHII (closed triangle), MT8-1/AEGEGII-AGCBHII (open diamond), and MT8-1/AEGEGII-AGCBHII-AGBGL (open square). The data points represent the averages of three independent experiments  
254x190mm (72 x 72 DPI)

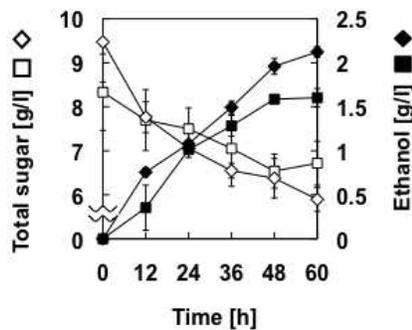


Figure 3. Time-course of ethanol fermentation from amorphous cellulose by MT8-1/AGEGII-AGCBHII-AGBGL (diamond) and MT8-1/EGII-CBHII-AGBGL (square). Closed symbols represents ethanol and open symbols represents total sugar. The data points represent the averages of three independent experiments.  
254x190mm (72 x 72 DPI)