Enzyme and Microbial Technology 51 (2012) 366–372

Contents lists available at SciVerse ScienceDirect

Enzyme and Microbial Technology

journal homepage: www.elsevier.com/locate/emt

Cellulosic ethanol production by combination of cellulase-displaying yeast cells

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ARTICLE INFO

Article history:
Received 29 May 2012
Received in revised form 6 August 2012
Accepted 10 August 2012

Keywords:
Cellulase
Cellulosic ethanol
Combination system
Metabolic burden
Yeast surface display

ABSTRACT

As an effort to find suitable endoglucanases to generate cellulolytic yeast strains, two fungal endoglucanases, *Thermoascus aurantiacus* EGI and *Trichoderma reesei* EGII, and two bacterial endoglucanases, *Clostridium thermocellum* CelA and CelD, were expressed on the yeast surface, and their surface expression levels, pH- and temperature-dependent enzyme activities, and substrate specificities were analyzed. *T. aurantiacus* EGI showed similar patterns of pH- and temperature-dependent activities to those of *T. reesei* EGII which has been widely used due to its high enzyme activity. Although EGII showed higher carboxymethyl cellulose (CMC) degradation activity than EGI, EGI showed better activity toward phosphoric acid swollen cellulose (PASC). For ethanol production from PASC, we combined three types of yeast cells, each displaying *T. aurantiacus* EGI, *T. reesei* CBHII (exoglucanase) and *Aspergillus aculeatus* BGLI (β-glucosidase), instead of co-expressing these enzymes in a single cell. In this system, ethanol production can be easily optimized by adjusting the combination ratio of each cell type. A mixture of cells with the optimized EGI:CBHII:BGLI ratio of 6:2:1 produced 1.3 fold more ethanol (2.1 g/l) than cells composed of an equal amount of each cell type, suggesting the usefulness of this system for cellulosic ethanol production.

1. Introduction

Cellulosic biomass is considered as one of the most promising alternatives to fossil fuels for the production of biofuels and other useful chemicals [1]. Hydrolysis of crystalline cellulose to glucose requires the sequential reactions of three groups of cellulases, endoglucanase (EG), exoglucanase (mostly cellobiohydrolase, CBH), and β-glucosidase (BGL), which are produced in cellulolytic fungi and bacteria [2,3]. EG randomly cleaves internal β-1,4-glycosidic bonds in the amorphous regions of crystalline cellulose. From the reaction products of EG, CBH produces cellodextrins such as cellobiose and cellotriose, which are finally hydrolyzed to glucose by BGL [4].

The high cost of cellulase is the main obstacle for the utilization of cellulosic biomass. Therefore, a great deal of effort has been made to screen and develop cellulases with high enzymatic activity and stability [5,6]. On the other hand, consolidated bioprocessing (CBP), combining cellulase production, cellulose degradation, and fermentation in a single step, is considered as the most cost-effective way to utilize cellulosic biomass [7,8]. One of the key requirements for cellulosic ethanol production by CBP is to develop microbial strains equipped with the properties of efficient cellulose degradation as well as ethanol production. *Saccharomyces cerevisiae*, having a high capacity of ethanol production, is one of the strong candidates for CBP. So far, to develop cellulolytic yeast strains, various fungal or bacterial cellulases have been expressed in yeast as secreted forms [9,10] or displayed on the yeast surface [11–15]. Especially, the surface display of cellulases enables to mimic cellulosome structure found on the surface of anaerobic cellulolytic bacteria such as *Clostridium thermocellum*, *Clostridium cellulovorans* and *Ruminococcus flavefaciens* [16,17]. Cellulosome is generated by assembly of multiple dockerin-containing cellulases and xylanases into a scaffoldin, an integrating protein containing cohesion domains, through interaction between dockerin and cohesion domains [18]. Such a multifunctional cellulosome structure allows highly efficient cellulose degradation through synergistic effects of various enzymes. To date, several fungal cellulases such as *Trichoderma reesei* EGII and CBHII, and *Aspergillus aculeatus* EGI and BGLI were expressed on the yeast surface [11,12,19]. In addition, some bacterial cellulases such as *C. thermocellum* CelA and BglA, and *Clostridium cellulolyticum* CelE and CelG were purified from *Escherichia coli* or secreted from yeast cells, and then attached to a chimeric scaffoldin expressed on the yeast surface [17,20].

To generate efficient cellulolytic yeast strains, it is important to select cellulases based on their enzymatic properties when displayed on the yeast surface. However, comparative analysis of various cellulases expressed on the yeast surface has not yet been conducted. Among the three types of cellulases, EG shows the

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http://dx.doi.org/10.1016/j.enzmictec.2012.08.005

Table 1
Primer sequences of cellulases used in this study (F, forward primer; R, reverse primer). Restriction enzyme sites are shown in bold and the sequences homologous to pCTCON vector are shown in italic.

Primer	Sequence
CelA: F	*GTGGTGGTGGTTCTGGTGGTGGTGGTTCT***GCTAGC**GCAGGTGTGCCTTTTAACACA
CelA: R	*AAGTCCTCTTCAGAAATAAGCTTTTGTTC***GGATCC**ATAAGGTAGGTGGGGTATGCT
CelD: F	*GTGGTGGTGGTTCTGGTGGTGGTGGTTCT***GCTAGC**GCAAAAATAACGGAGAATTAT
CelD: R	*AAGTCCTCTTCAGAAATAAGCTTTTGTTC***GGATCC**TATTGGTAATTTCTCGATTAC
EGI: F	*TGGTGGTGGTGGTTCTGGTGGTGGTGGTTCT***GCTAGC**ATGAAGCTCGGCTCTCTCGT
EGI: R	*ACAAGTCCTCTTCAGAAATAAGCTTTTGTTC***GGATCC**AAGATACGGAGTCAAAATAG
EGII: F	CCG**GCTAGC**CAGCAGACTGTCTGGGGC
EGII: R	AAT**GGATCC**CTTTCTTGCGAGACACGA
CBHII: F	CCG**GCTAGC**CAAGCTTGCTCAAGCGTC
CBHII: R	AAT**GGATCC**CAGGAACGATGGGTTTGC
BGLI: F	*GTGGTGGTGGTTCTGGTGGTGGTGGTTCT***GCTAGC**GATGAACTGGCGTTCTCTCCT
BGLI: R	*AAGTCCTCTTCAGAAATAAGCTTTTGTTC***GGATCC**TTGCACCTTCGGGAGCGCCGC

highest diversity, which might be necessary to degrade various types of cellulose in nature. Therefore, as an effort to search for enzymes suitable to develop cellulolytic yeast strains, we compared the activities of fungal and bacterial EGs expressed on the yeast surface. For fungal EGs, we selected *T. reesei* EGII, which has been widely studied on account of its high enzymatic activity, and EGI from a thermophilic fungus, *Thermoascus aurantiacus*. For bacterial EGs, we selected CelA and CelD from *C. thermocellum*, anaerobic and thermophilic bacteria. Except for EGII, other EGs have not yet been directly expressed on the yeast surface. Based on the characterized enzymatic properties, we chose *T. aurantiacus* EGI for ethanol production from PASC in combination with *T. reesei* CBHII and *A. aculeatus* BGLI. Previously, multiple cellulases were co-displayed in a single cell for ethanol production [11,14]. However, in this study, we adapted a new strategy of combining three types of yeast cells each displaying different cellulase, which allows convenient optimization of ethanol production by adjusting the combination ratio of each cell type.

2. Materials and methods

2.1. Strains and culture conditions

S. cerevisiae EBY100 strain (*MATa GAL1-AGA1::URA3 ura3-52 trp1 leu2Δ1 his3Δ200 pep4::HIS2 prb1Δ1.6R can1* GAL) containing pCTCON plasmid were grown in a selective SD-CAA medium (20 g/l dextrose, 6.7 g/l yeast nitrogen base, 5 g/l casamino acids, 5.4 g/l Na_2HPO_4 and 8.56 g/l $NaH_2PO_4{\cdot}H_2O$). For the induction of cellulase gene cloned in pCTCON vector, OD_{600} of 1 cells grown in SD-CAA medium were harvested and resuspended in SG-CAA medium containing galactose instead of glucose, and further incubated for 18 h at 30 °C.

2.2. Plasmids

The yeast surface display vector pCTCON was kindly provided by Dr. Y.-S. Kim (Ajou University, Korea) and *T. reesei* EGII, CBHII and *A. aculeatus* BGLI cDNAs were provided by Dr. H. Zhao (University of Illinois at Urbana-Champaign, USA). For the surface display, *T. reesei* EGII and CBHII genes lacking a signal sequence were amplified by PCR, and then cloned into the Nhe1 and BamH1 sites of pCTCON vector. The surface display vectors for CelA, CelD, EGI, and BGLI were generated by homologous recombination in yeast cells. *C. thermocellum* CelA and CelD genes lacking signal sequences were amplified from genomic DNA (ATCC 27405) and EGI and BGLI genes were amplified from each cDNA by using PCR primers, each containing 35 nt or 37 nt region homologous to the pCTCON vector. Each PCR product was transformed into *S. cerevisiae* EBY100 together with the pCTCON vector digested with Nhe1 and BamH1, leading to an insertion of the PCR product into a pCTCON vector by homologous recombination. The plasmid was isolated from the transformant and confirmed by DNA sequencing. The primer sequences used for each EG are shown in Table 1.

2.3. Immunofluorescence microscopy

Yeast cells expressing cellulases were washed with wash buffer (PBS and 0.1% BSA) and incubated with anti-HA mouse monoclonal antibody (Roche) in PBSF buffer at room temperature. The cells were washed twice, and then incubated with goat anti-mouse IgG conjugated with fluorescein isothiocyanate (FITC, Sigma–Aldrich) for 10 min at 4 °C. After washing the cells, cellular localizations of the cellulases were observed by fluorescent microscope (Eclipse 80i, Nikon Instruments). The whole cell fluorescence intensity was measured by fluorescence microplate reader (AT/Genios Pro, TEKAN) at an excitation wavelength of 485 nm and an emission wavelength of 535 nm.

2.4. Endoglucanase activity assay

OD_{600} of 10 cells harboring the surface-display plasmid for EG were grown in SG-CAA medium, harvested, and washed with distilled water and then with a reaction buffer with various pH values. 50 mM citrate/sodium citrate buffer was used for the pH range of 3–6, and 50 mM Tris–HCl buffer was used for pH 7–9. Cells were resuspended in a reaction buffer containing 10 g/l carboxymethyl cellulose (CMC, Sigma–Aldrich), or 5 g/l phosphoric acid swollen cellulose (PASC) generated from Avicel PH-101 (Fluka), and then incubated for 6 h at various temperatures, from 30 to 80 °C. The amount of reducing sugar in the reaction supernatant was determined by dinitrosalicylic acid (DNS) method as described elsewhere [21]. One unit of enzyme activity was defined as the amount of enzyme releasing 1 μmol of reducing sugar from substrate per min.

2.5. Ethanol fermentation from PASC

Yeast cells, each harboring the surface-display plasmid for EG1, CBHII, or BGLI, were grown in SD-CAA medium and then transferred to SG-CAA medium for 48 h to express cellulase. OD_{600} of 50 of cells were washed with distilled water and cells expressing different cellulases were mixed in various ratios. A mixture of cells was incubated in 10 ml of YP medium (20 g/l peptone, 10 g/l yeast extract) for 1 h to remove residual carbon source, and then resuspended in YP-PASC medium (YP medium containing 10 g/l PASC as the sole carbon source). Ethanol fermentation was carried out at 30 °C in anaerobic condition. Cell mixture was incubated in a 30 ml serum bottle with 10 ml reaction volume. Ethanol concentration was measured by high performance liquid chromatography (HPLC) (Finnigan Surveyor Plus, thermo scientific, USA). HPLC was operated using a BioRad Aminex HPX-87H column (300 mm × 7.8 mm, 5 μm) at 60 °C with 5 mM H_2SO_4 as eluant at a flow rate of 0.8 ml/min. Refractive index (RI) detector (Finnigan Surveyor RI Plus detector, thermo scientific, USA) was used to determine the ethanol quantification.

3. Results

3.1. Expression of cellulases on the yeast surface

Two bacterial endoglucanases (EGs), *C. thermocellum* CelA and CelD, and two fungal EGs, *T. aurantiacus* EGI and *T. reesei* EGII, were expressed under the control of Gal1 promoter as Aga2-fusion proteins (Fig. 1). Aga2-fused EGs were displayed on the surface of *S. cerevisiae* EBY100 through a pair of disulfide bond between Aga2 and Aga1 [22], a yeast cell wall protein. CelA and CelD contain dockerin domains involved in the generation of cellulosome complex. The catalytic domains of CelA and CelD are classified as GH8 and GH9 glycoside hydrolase (GH) family, respectively. Both EGI and EGII have GH5 modules, and EGII has an additional carbohydrate binding module (CBM), promoting substrate binding. We also expressed *T. reesei* CBHII (exoglucanase) and *A. aculeatus* BGLI (β-glucosidase) on the yeast surface to be used for ethanol production.

After induction in a galactose medium for 18 h, the expression of cellulases was evaluated by western blotting (data not shown). Because of the fusion partners, each cellulase exhibited a molecular weight of about 30 kDa higher than the predicted size. We also confirmed the expression of cellulases on the yeast surface by

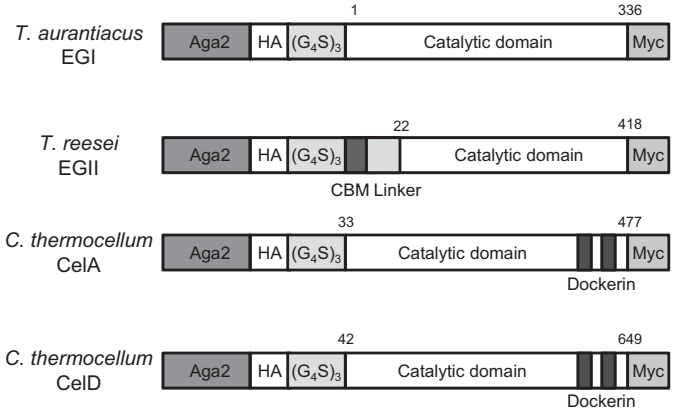

Fig. 1. Schematic diagram of endoglucanase structures for the yeast surface display. Each gene was cloned into pCTCON vector after deletion of a signal sequence except for *T. aurantiacus* EGI. The numbers indicate amino acid residues of each protein. Each EG is fused with Aga2 for linking to the yeast surface by a pair of disulfide bond, and contains an HA (N-terminal) tag, a Myc (C-terminal) tag, and a $(G_4S)_3$ linker domain. *C. thermocellum* CelA and CelD have a pair of dockerin (CelA, 417–440, 449–472; CelD, 585–608, 621–644), and *T. reesei* EGII contains a carbohydrate binding module (CBM) and a linker (CBM, 22–57; linker, 58–91).

immunofluorescent microscopy. As shown in Fig. 2, all cellulases were successfully displayed on the yeast surface.

3.2. Characterization of the enzyme activities of EGs displayed on the yeast surface

3.2.1. Effect of pH on the activities of the surface-displayed EGs

In order to measure the EG activity displayed on the yeast surface, the same number of cells (OD_{600} = 10) were harvested after galactose induction, and then enzyme assay was carried out by using carboxymethyl cellulose (CMC) as a substrate. To evaluate the effect of pH on each EG, activity assay was performed at 37 °C and over a broad range of pH (Fig. 3A). Among the four EGs, *T. reesei* EGII showed the highest activity in overall pH range except for pH 9. *T. aurantiacus* EGI showed the second highest activity up to pH 6. In general, the two fungal EGs showed highest activities at pH 3 and 4, but their activities decreased rapidly at higher pH.

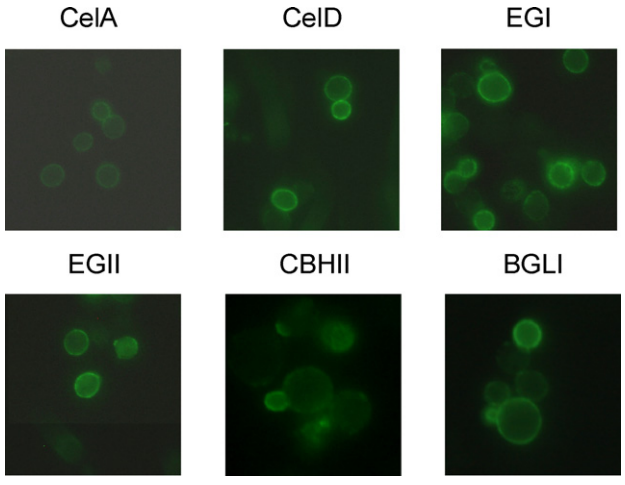

Fig. 2. Immunofluorescence microscopy of cellulases displayed on the yeast surface. Expression of cellulases in yeast EBY100 strain was induced in galactose media for 18 h. Cellulases were labeled with mouse anti-HA antibody and goat anti-mouse IgG conjugated with FITC, and their cellular localizations were detected by fluorescence microscope. Since BGL1 showed the highest fluorescence intensity, the brightness of other images was increased by 60% compared with that of BGL1.

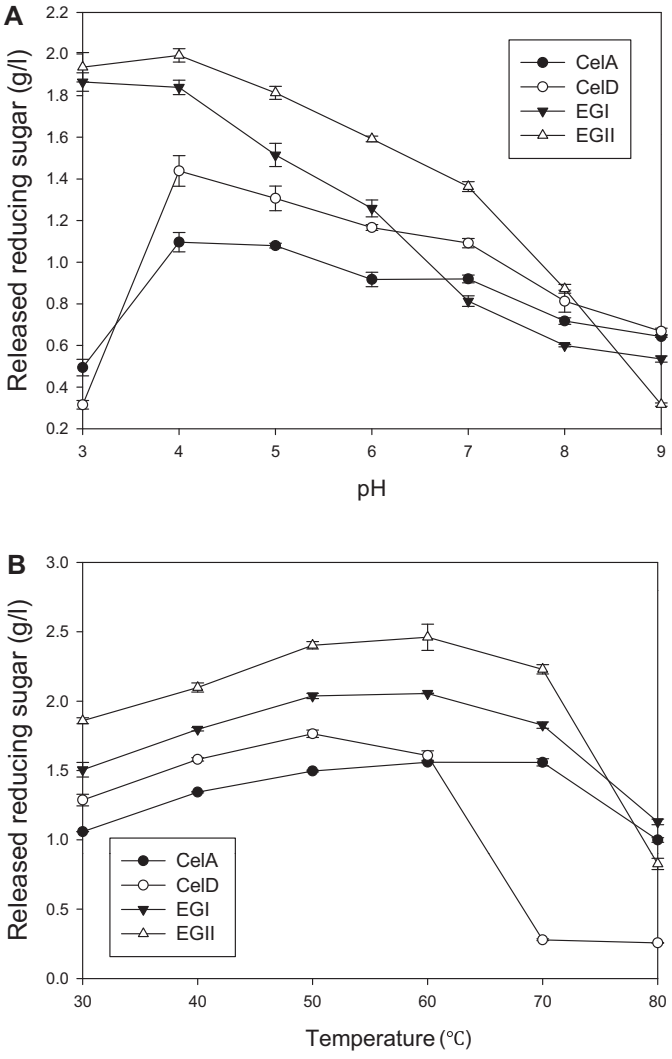

Fig. 3. Effects of pH and temperature on the activities of the surface-displayed EGs. (A) Activity of the surface-displayed EG was determined by using CMC as a substrate. After 6 h incubation of the EG-displaying yeast cells at 37 °C in a buffer with the indicated pH, the released reducing sugars were measured by DNS method. (B) Activity of the surface-displayed EG was determined at pH 5.2 and the indicated temperatures using CMC as a substrate. The experiments were performed independently at three times.

On the other hand, *C. thermocellum* CelA and CelD were almost inactive at pH3, but showed the highest activities at pH 4. In addition, although CelA and CelD also showed pH-dependent inactivation over a pH range from 4 to 9, their inactivation was less sensitive to the increase in pH compared with EGI and EGII.

3.2.2. Effect of temperature on the activities of the surface-displayed EGs

We also examined the optimal temperature of each EG displayed on the yeast surface. All the EGs showed increase in activity upon increase in temperature from 30 to 50 °C (Fig. 3B). CelA showed the highest temperature optimum of 70 °C, while CelD activity was maximized at 50 °C and dropped rapidly after reaching 60 °C. The temperature optimums for EGI and EGII were about 60 °C, similar to the previously reported temperature optimums measured using purified enzymes [23,24], and their activities were still maintained up until 70 °C.

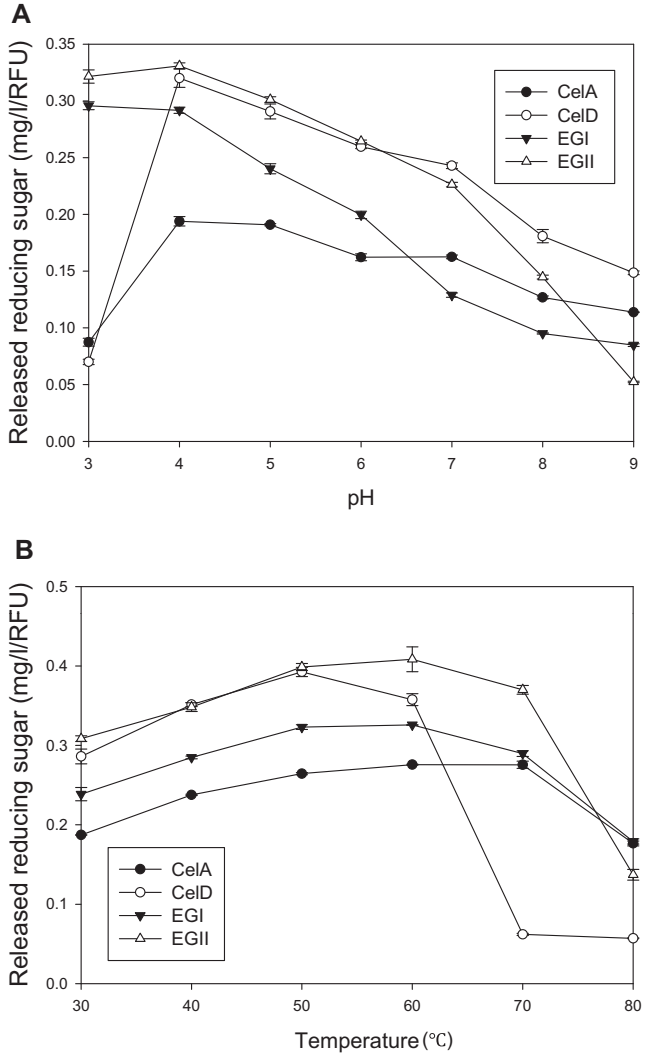

Fig. 4. Specific activities of EGs normalized to the protein expression levels on the yeast surface. (A) The enzyme activities shown in Fig. 3A were normalized to the whole cell fluorescent intensities of the corresponding EGs, which were measured by fluorescence microplate reader after labeling the cells with mouse anti-HA antibody and FITC-conjugated anti-mouse IgG antibody. (B) The enzyme activities shown in Fig. 3B were normalized to the whole cell fluorescent intensities of the corresponding EGs.

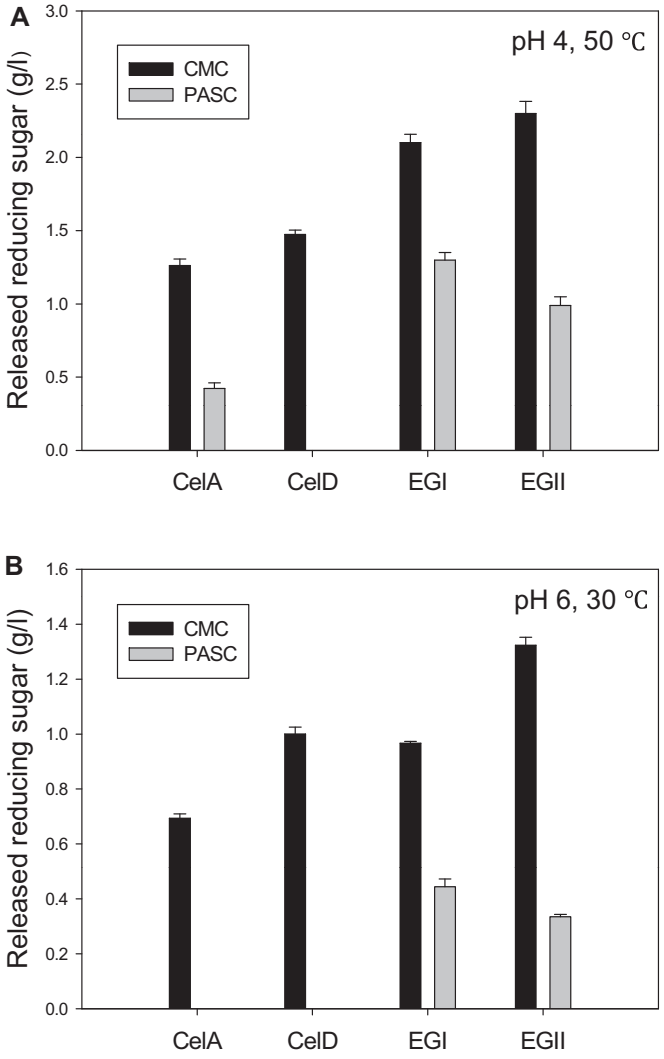

Fig. 5. Substrate specificities of the surface-displayed EGs. Enzyme activity was determined by using 10 g/l CMC and 5 g/l PASC at pH 4 and 50 °C (A), and at pH 6 and 30 °C (B).

3.2.3. *Specific activities of EGs displayed on the yeast surface*

Since the surface expression level of each enzyme can be different, the activity we measured by using the same amount of cells does not necessarily reflect the specific activity of each enzyme. Therefore, to normalize the enzyme activity to its protein level, protein expression levels on the surface were measured by detecting whole cell fluorescent intensities after treating the cells with mouse anti-HA antibody and FITC-conjugated anti-mouse IgG antibody. The fluorescence intensity was highest in cells expressing EGI (100%), followed by the cells expressing EGII (96%), CelA (90%), and CelD (71%). When the EG activities were normalized to their expression levels, CelD exhibited a comparable activity to EGII in overall pH range (Fig. 4A), and in the temperature range from 30 to 50 °C (Fig. 4B). Therefore, CelD could be as active as EGII if expressed at equal levels.

3.3. *Substrate specificities of the surface-displayed EGs*

Next, we examined the substrate specificities of the four EGs displayed on the yeast surface by using two types of cellulosic materials, CMC and PASC. Activity assays were performed under two different conditions, the optimal CMC hydrolysis condition (pH 4, 50 °C) (Fig. 5A) as determined above and general yeast culture condition (pH 6, 30 °C) (Fig. 5B). EGI and EGII could degrade both CMC and PASC under both conditions. Although EGII showed higher CMC hydrolysis activity than EGI, EGI showed better PASC hydrolysis activity than EGII.

On the other hand, PASC served as a poor substrate for bacterial EGs. Although CelA and CelD showed efficient CMC hydrolysis activity, CelD did not show any PASC hydrolysis activity, and CelA hydrolyzed PASC only under the condition of pH 4 and 50 °C (Fig. 5A and B). These results indicate that each EG has a unique substrate specificity, which might be affected by reaction conditions.

3.4. *Cellulosic ethanol production by combination of cells displaying different types of cellulases*

Since *T. aurantiacus* EGI seems to have enzyme activity comparable to the widely used *T. reesei* EGII, and showed better activity toward PASC, we explored the possibility of ethanol production from PASC by using EGI. For this purpose, we also expressed *T. reesei* CBHII (exoglucanase) and *A. aculeatus* BGLI (β-glucosidase) on

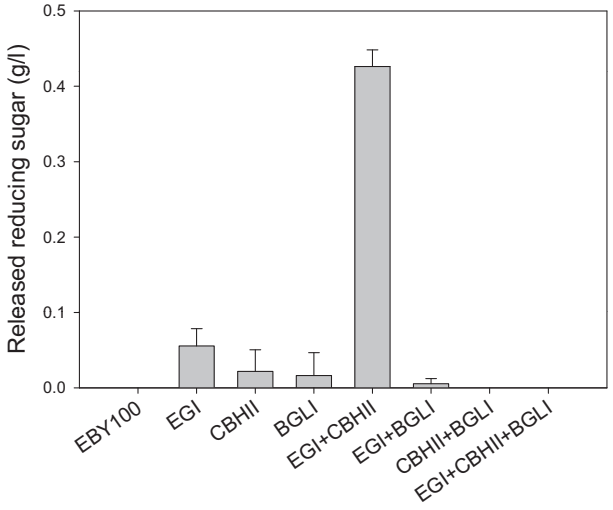

Fig. 6. Synergistic hydrolysis of PASC by combination of the surface-displayed cellulases. Equal number of cells, each displaying EG1, CBHII, and BGL1 were mixed to OD_{600} of 5 as the indicated combinations, and EBY100 host cells were added to adjust the cell density. Reducing sugars generated from the hydrolysis of 5 g/l PASC was measured at pH 4.7 and 30 °C.

the yeast surface (Fig. 2B), and investigated the interplay of EGI with CBHII and BGL in the hydrolysis of PASC.

In previous studies, multiple cellulases were co-displayed in a single cell for ethanol production [11,14,17,19]. In that case, protein expression levels can be limited because of the metabolic burden. Furthermore, it is difficult to control the expression levels of each cellulase for optimal ethanol production. Therefore, instead of co-displaying the cellulases, we displayed only one type of cellulase in one cell, and then mixed the cells displaying different cellulases to induce a synergy in cellulose degradation. To confirm the cooperative actions of the three types of cellulases, we mixed an equal amount of cells, each displaying a different cellulase, and measured the released reducing sugars from PASC. Although CBHII itself showed little PASC hydrolysis activity, CBHII greatly enhanced reducing sugar production in combination with EGI (EGI + CBHII), indicating that CBHII can hydrolyze the degradation products generated by EGI (Fig. 6). However, the reducing sugars generated by EGI and CBHII were almost completely abolished by the addition of cells expressing BGLI (EGI + CBHII + BGLI) (Fig. 6). This result suggests that BGL1 converts the reducing sugar to glucose which can be immediately consumed by yeast cells. Taken together, PASC can be successfully degraded into glucose by combining cells, each displaying EGI, CBHII, and BGLI.

Next, we produced ethanol from PASC by using this cell combination system. First, we optimized the EGI:CBHII ratio for efficient generation of reducing sugars. Cells displaying EGI and CBHII were mixed in various ratios and the released reducing sugars from PASC were measured. As shown in Fig. 7A, EGI:CBHII in a ratio of 3:1 or 2:1 produced the highest amounts of reducing sugars compared with other mixing ratios. Therefore, we fixed the EGI:CBHII ratio to 3:1, and then added cells displaying BGLI with various ratios to find an optimal condition for ethanol production. A mixture of cells with EGI:CBHII:BGLI ratio of 6:2:1 produced the highest amount of ethanol (2.12 g/l) after 85 h incubation with 10 g/l PASC (Fig. 7B and C). A mixture of cells composed of an equal amount of each cell type produced 1.65 g/l ethanol after 85 h, indicating about 1.3-fold improvement of ethanol production by optimizing the cell ratio (Fig. 7B and C).

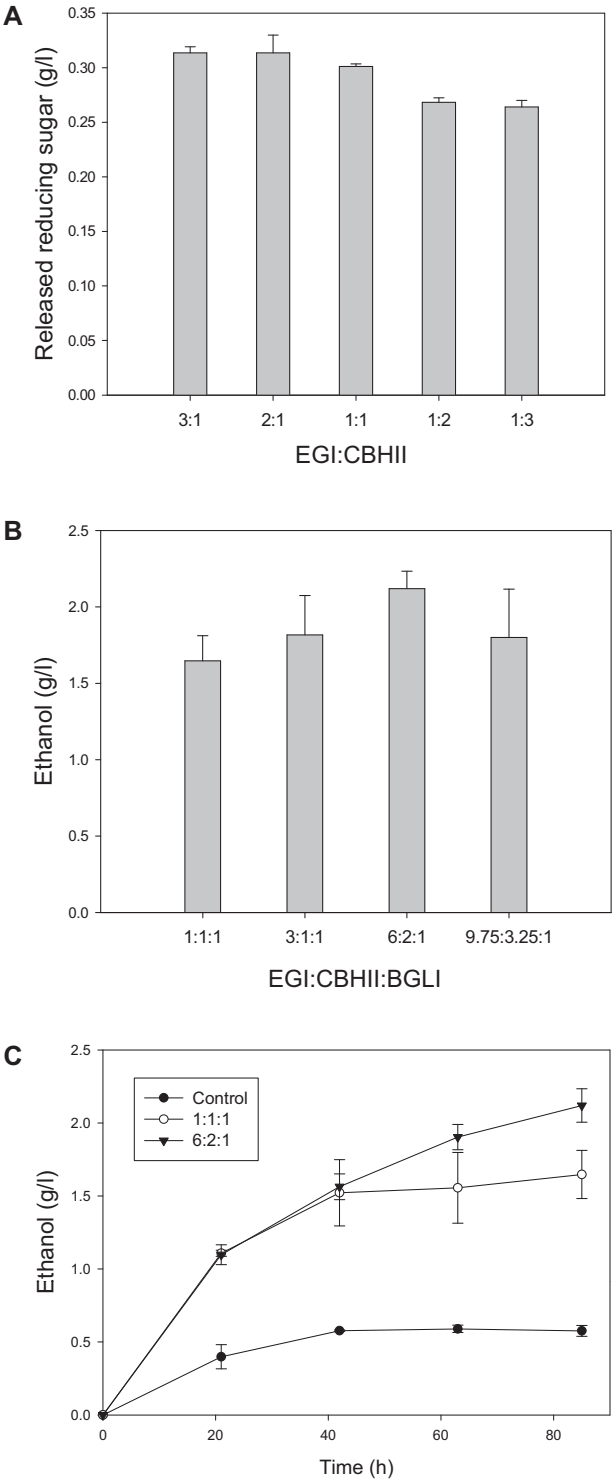

Fig. 7. Optimization of the cellulase combination ratio and ethanol production from PASC. (A) Optimization of EGI:CBHII ratio for PASC hydrolysis. (B) Optimization of EGI:CBHII:BGLI ratio for ethanol production from PASC. Cell mixtures composed of the indicated ratios of EGI, CBHII and BGLI were incubated with 10 g/l PASC and ethanol production was detected after 85 h. (C) Time course of ethanol production from negative control (closed circle), EGI:CBHII:BGLI ratio of 1:1:1 (open circle), and the optimized 6:2:1 ratio (closed triangle).

4. Discussion

In this study, we developed an ethanol production system in which yeast cells displaying different types of cellulases are combined in an optimized ratio. We first selected an EG suitable for the yeast surface display based on the characterization of the surface-immobilized enzyme activities, rather than those of free enzymes. We analyzed enzymatic properties of four fungal and bacterial EGs expressed on the yeast surface. The pH optimums of all the tested EGs attached on the yeast surface were around pH 4. To inhibit bacterial contamination, low initial pH was used for ethanol fermentation, and most yeast ethanol fermentation is operated in the pH range of 4.0–5.0 [29]. Therefore, the pH optimums of these EGs might be suitable for their future usage in CBP.

For the surface-displayed EGII, its pH-dependent changes in the enzyme activity are very similar to those reported for native EGII or recombinant EGII purified after secretion from *S. cerevisiae* [24]. Therefore, attachment of this enzyme on the yeast surface might not induce large changes in its overall pH-dependent activities. The previously reported pH optimums for CelA and CelD purified from *E. coli* are in the range of pH 5.5–6.5 [25,26], which are higher than the pH optimums observed for the surface-displayed enzymes in our study. In addition, although our surface-displayed CelA and CelD showed poor PASC degradation activity, especially at pH 6, CelA and CelD purified from *E. coli* could degrade both CMC and PASC at pH 4 [25,27]. However, in agreement with our results, both enzymes produced more reducing sugars from CMC than PASC [25,27]. Although there is no report about CelD activity expressed in yeast, CelA, secreted from yeast cells and attached to a chimeric scaffoldin expressed on the yeast surface, showed PASC degradation activity [17]. These differences in pH optimum and substrate specificity might be caused by various factors including enzyme immobilization and using a different host for expression. It has been shown recently that glycosylation is one of the factors influencing cellulase activity when expressed in yeast cells. CelA expressed in yeast showed lower activity than CelA purified from *E. coli* because of the glycosylation occurring in yeast [28].

All the EGs tested showed high temperature optimums ranging from 50 to 70 °C. Such high temperature optimums of these enzymes could be a disadvantage for the operation of CBP using yeast cells whose usual cultivation temperature is 30 °C. Therefore, to ensure higher enzyme activities of many cellulases with high temperature optimums, fermentation at higher temperature using heat-resistant yeast strains could be considered as one of the solutions.

Higher expression levels of fungal EGs than those of bacterial enzymes suggest that fungal EGs could have advantages in expression in yeast cells although the expression levels can also be affected by other gene-specific factors. Therefore, considering both expression level and activity, *T. aurantiacus* EGI might be as useful as *T. reesei* EGII which have been widely studied and used for ethanol production.

For cellulosic ethanol production, instead of expressing multiple cellulases in one cell, we adopted a new strategy of combining three types of cells, each displaying a different cellulase. Such a strategy allows optimization of the ethanol production by simply changing the ratio of cells expressing each type of cellulase. Furthermore, the number of different cellulases expressed in a single cell can be limited because of the difficulties in molecular cloning as well as poor expression levels due to the metabolic burden [30]. However, unlimited number of cellulases can be included for ethanol production by using this cell combination system. Further characterization of a wide range of cellulases on the yeast surface and improvement the surface expression efficiency would facilitate the development of CBP using cellulolytic yeast strains.

Acknowledgements

We are grateful to Drs. Y.-S. Kim and H. Zhao for providing plasmids. This work was supported by the National Research Foundation of Korea Grant funded by the Korean Government (MEST) (NRF-2010-C1AAA001-0028801).

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