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Molecules and Cells

Production of Cellulosic Ethanol in *Saccharomyces cerevisiae* Heterologous Expressing *Clostridium thermocellum* Endoglucanase and *Saccharomycopsis fibuligera* β-glucosidase Genes

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Heterologous secretory expression of endoglucanase E (*Clostridium thermocellum*) and β-glucosidase 1 (*Saccharomycopsis fibuligera*) was achieved in *Saccharomyces cerevisiae* fermentation cultures as an α-mating factor signal peptide fusion, based on the native enzyme coding sequence. Ethanol production depends on simultaneous saccharification of cellulose to glucose and fermentation of glucose to ethanol by a recombinant yeast strain as a microbial biocatalyst. Recombinant yeast strain expressing endoglucanase and β-glucosidase was able to produce ethanol from β-glucan, CMC and acid swollen cellulose. This indicates that the resultant yeast strain of this study acts efficiently as a whole cell biocatalyst.

INTRODUCTION

Cellulose is the most abundant biological polymer on the earth. Although the chemical composition of cellulose is very simple, consisting of only glucose residues connected by β-1,4-glucosidic bonds. (Schwarz, 2001; Teeri, 1997) The cellulose degraded by endoglucanase and exoglucanase results in cellobiose and some cello-oligosaccharides, which can be converted to glucose by β-glucosidase. The reaction catalyzed by β-glucosidase is also important step in the degradation of cellulose because it limits the efficiency of hydrolyzation and could relieve the cellobiose mediated inhibition of exoglucanase and endoglucanase. Since the yeast *Saccharomyces cerevisiae* cannot utilize cellulosic materials, these materials must undergo saccharification to glucose before ethanol production can take place. Various cellulose and β-glucosidase genes have been expressed in *S. cerevisiae* with the aim of direct ethanol production from cellulose (Okada et al., 1998).

Clostridium thermocellum is a gram-positive, acidogenic, thermophilic and anaerobic bacterium that produces a highly active extracellular cellulolytic complex called a cellulosome. The cellulosome is capable of a multi-step conversion of insoluble cellulosic substrate into fermentable end-products (Islam et al., 2008; Patni and Alexander, 1971). *C. thermocellum* possesses the highest rate of cellulose degradation among all known cellulolytic microorganisms (Lynd and Grethlein, 1987).

S. cerevisiae is the preferred organism for industrial fermentative ethanol production. It can ferment glucose, mannose, fructose and galactose at anaerobic and low pH conditions (Katahira et al., 2006; Van Maris et al., 2006). *S. cerevisiae* is more resistant to the inhibitors produced during cellulose hydrolysis than some other microorganisms that could potentially be used in ethanol production (Hahn-Hagerdal et al., 2001). Usually, new pathways can be easily constructed in *S. cerevisiae* with well-developed genetic technology. Therefore, the substrate range can be expanded as required (Van Maris et al., 2006). Previous report show that, *C. thermocellum celA* gene encoding endoglucanase A is expressed in *Saccharomyces cerevisiae* (Benitez et al., 1989).

In this study, we attempted to convert CMC and barley β-glucan into ethanol by constructing cellulose-degrading yeast cells which coexpress *C. thermocellum* cellulosomal Endoglucanase (EgE) and *Saccharomycopsis fibuligera* β-glucosidase (Bgl1).

MATERIALS AND METHODS

Strains and media

Escherichia coli DH5α was used for sub-cloning. The yeast strain YPH499 (Clontech Laboratories, Inc.) was used for producing EgE and Bgl1. Luria-Bertani medium (Sambrook, 1989) was used to culture *E. coli*, and 50 μg/ml of ampicillin was added for selecting transformants. Yeast was aerobically cultivated at 30°C in synthetic medium 0.17% of YNB (yeast nitrogen base without amino acid) per liter with 0.13% trp dropout

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amino acid to which 2% glucose per liter was added as the sole carbon source (SD medium) for selecting transformants. YPD medium (1% yeast extract, 2% peptone, 2% glucose) was used for culture (Lee et al., 2007). SG medium (2% galactose, 0.17% yeast YNB and 0.13% trp drop out amino acid) and YPG (1% yeast extract, 2% peptone, 2% galactose) were used for inducing gene expression. SG-CMC (0.02% galactose, 0.17% yeast YNB and 0.13% trp drop out amino acid, 10% CMC) and SG-Cellobiose (0.02% galactose, 0.17% YNB and 0.13% trp drop out amino acid, 5% cellobiose) (Stagoj et al., 2006) were used for fermentation.

Construction of plasmids

The plasmids pαEgE, pαBG1 and pαEgEαBG1 for secretion of the *C. thermocellum* (ATCC 27405) EgE and *S. fibuligera* (ATCC 36309) Bgl1 enzymes were constructed as follows: The secretion signal sequence of the alpha mating factor gene from *S. cerevisiae* was prepared by PCR (primers 5′-aacggaattcatg-agatttccttcaatttttactgcagtt-3′ and 5′-gccagcggccgc gtcttttatccaa-agataccccttcttcttt ag-3′ with the genomic DNA of *S. cerevisiae*) and inserted into the *Eco*RI - *Not*I site of pESC-trp (Clontech Laboratories, Inc.). For construction of EgE and Bgl1 expressing vectors, the EgE gene was amplified by PCR with genomic DNA from *C. thermocellum* as a template with the primers 5′-gcgcggccgctcgggaacaaagcttttgga-3′ and 5′-gtgcggccgctattgctt-tttttaagaatgcaag-3′, and the Bgl1 gene was amplified by PCR with *S. fibuligera* genomic DNA with the primers 5′-ggcgcggcc-gcgtcccaattcaaaactatacc-3′ and 5′-ggcactagtcgaatagtaaacagg-acagatgtct-3′ (Van Rooyen et al., 2005). The fragments were inserted into the *Not*I-*Spe*I and *Bam*HI-*Xho*I sites, respectively. The resulting plasmids were named pαEgE and pαBG1, and the expression vector which contained both genes bidirectionally was named pαEgEαBG1.

Yeast transformation and protein expression

Transformation of the expression plasmid pαEgE, pαBG1 and pαEgEαBG1 into *S. cerevisiae* was carried out by the lithium acetate method by using a YEASTMAKER yeast transformation system (Clontech Laboratories, Inc.). Yeast transformants containing endoglucanase activity were selected on 0.3% CMC-YPG agar plate by the Congo-red halo test. For small-scale protein production, yeast cells were grown in 50 ml YPG medium at 30°C for 48 h. Growth medium supernatant was obtained by centrifugation at 700 × *g*, concentrated by an amicon ultra-15, 10 kDa cut off (Millipore, Co.). The concentrated enzyme was electrophoresed on 10% SDS-PAGE followed by Coomassie blue staining. Protein concentration was measured by using the method of Bradford (Bradford, 1976) with a Quick start protein assay kit (Bio-Rad Laboratories, Inc.), with bovine serum albumin as the standard.

Zymogram and Western blot analysis

After separation of the enzyme samples by means of SDS-PAGE, CMCase activity was detected in the separating gels (Schwarz et al., 1987). After SDS-PAGE, the gels were washed for 2 h at room temperature with two changes of 0.1 M succinate (pH 6.3) containing 1 mM dithiothreitol and 10 mM $CaCl_2$. The wash step allowed for renaturation of enzyme components. The gels were then incubated for 1 h at 60°C in 0.1 M succinate (pH 6.3) containing 1 mM dithiothreitol and 10 mM $CaCl_2$ and stained in a 0.1% Congo red solution for another 30 min. Yellow halos emerged against a red background after destaining with 1 M NaC1. For western blot, proteins were transferred onto PVDF membranes (Pall corporation, USA) and the membrane was blocked in 5% skim milk for 1 h at RT. ANTI-FLAG® antibody produced in rabbit (Sigma-Aldrich Co.,) in blocking buffer was used as the primary antibody. Goat Anti-rabbit IgG-HRP (Santacruz Biotechnology Inc.,) was used as secondary antibody and detection was performed with a western blotting luminal reagent (Santacruz Biotechnology Inc.).

Enzyme assays

CMCase assay was investigated for CMC-saccharifying activity by incubating 0.5 ml of enzyme solution with 0.5 ml of CMC (1%) in sodium acetate buffer (50 mM, pH 5.0) for 30 min at 65°C (Arikan et al., 2002). Released sugar was measured as D-glucose equivalents, by the Somogyi-Nelson assay method (Wood and Bhat, 1988). One unit of enzyme activity was defined as the amount of enzyme releasing 1 μM of reducing sugar per min. β-glucosidase activity was measured as described previously (Fujita et al., 2002). The reaction mixture was composed of 2 mM ρ-nitrophenyl-β-D-glucopyranoside (pNPG) (Sigma-Aldrich Co.-) in 50 mM sodium acetate buffer (pH 5.0). The OD_{600} of the reaction mixture was adjusted to 1.0. After the reaction, supernatants were separated by centrifugation at 20,000 × *g* for 3 min at 4°C, and the released ρ-nitrophenol was measured spectrophotometrically as an increase in the absorbance at 400 nm after adding 1 ml of 1 M sodium carbonate to raise the pH and stop the reaction (Murai et al., 1998). One unit of enzyme activity was defined as the amount of enzyme required for producing 1 μM of ρ-nitrophenol from the substrate per 30 min.

Preparation of phosphoric acid swollen cellulose

PASC was prepared as described by Zhang et al. (2006) with slight modifications. Approximately 2 *g* of Avicel (FMC Co.) was soaked with 6 ml of distilled water. Subsequently, 50 ml of 86.2 % phosphoric acid was added slowly to the tube and mix well, followed by another 50 ml of phosphoric acid and mixing. The transparent solution was kept at 4°C overnight to allow complete solubilization of the cellulose, until no lumps remained in the reaction mixture. Next, 200 ml of ice-cold distilled water was added to the tube and mixed, followed by another 200 ml of water and mixing. The mixture was centrifuged at 3,500 rpm for 15 min and the supernatant removed. Addition of distilled water and subsequent centrifugation were repeated. Finally, 10 ml of 2 M sodium carbonate and 450 ml of water were added to the cellulose, followed by 2 or 3 washes with distilled water, until a final pH of 5-7 was obtained.

Fermentation

The transformants were aerobically precultivated for 24 h and then cultivated in SG medium for 72 h by adding 2% galactose every 24 h at 30°C and 200 rpm. The cells were harvested by centrifugation and resuspended with minimal medium (0.16% YNB, 0.13% trp drop out powder) and then cultivated 1 h at 30°C, at 200 rpm to remove residual galactose. The cells were harvested by centrifugation again and resuspended in 50 g/L cellobiose (pH 5.0), 20 g/L CMC (pH 5.0), 20 g/L β-glucan (pH 5.0) and 10 g/L PASC with optical density of 5.0, 20, 20 and 20 at 600 nm, respectively. Fermentation was carried out in a 50 ml closed bottle with a 20 ml reaction volume (Kotaka et al., 2008). Fermentations were performed at 30°C with mild agitation at 100 rpm. The ethanol concentration was measured by gas chromatography. The gas chromatograph (model GC7890; Agilent, USA) fitted with a flame ionization detector was operated under the following conditions: DB-WAXetr; temperatures of column and injector, 120 and 250°C, respectively; Helium gas flow rate, 40 ml/min.

RESULTS

Heterologous expression of endoglucanase and β-glucosidase

S. cerevisiae transformed with the pαEgE, pαBG1 and pαEgE-αBG1 (Fig. 1) secreted active endoglucanase into the medium. Halos were detected on CMC plates which absorbed 5 μl of culture supernatant (data not shown). SG^{-trp} plate containing 5 mM ρ-nitrophenyl-β-D-glucopyranoside was used to detect Bgl1 activity by adding 1 M sodium carbonate anhydrous (Machida et al., 1988); yellow circle zones appeared around positive colonies (data not shown). To confirm the extracellular expression of rEgE and rBgl1, we designed fusion proteins with a FLAG tag at its C-terminus and concentrated proteins from the culture supernatant of *S. cerevisiae* harboring each expression plasmid. As shown Fig. 2, about a 50 kDa and 96 kDa bands appeared on SDS-PAGE gels stained with Coomassie blue and endoglucanase activity was detected by a halo band on 0.1% CMC-SDS-PAGE gel stained with 0.25% Congo red followed by destaining with 1 M NaCl.

Enzyme activities

The EgE and Bgl1 activities of the yeast strains expressing EgE and Bgl1 are summarized in Table 1. EgE activity was determined by using CMC as the substrate after aerobic cultivation of yeast cells in SG medium for 48 h at 30°C. The yeast strain expressing EgE and the yeast strain coexpressing EgE and Bgl1 showed high levels of endoglucanase activity, while no activity was detected either in the yeast strain harboring control plasmid pESC-trp or in the strain expressing only Bgl1. The strain harboring pαEgE showed higher endoglucanase activity than the strain harboring pαEgEαBG1 but ethanol production is occurred only by the strain expressing both endoglucanase and β-glucosidase probably because PASC, CMC and β-glucan are hydrolyzed to glucose, a more powerful reducing agent than cellooligosaccharides, by the sequential reactions of EgE and Bgl1. Bgl1 activity was determined by using ρ-nitrophenyl-β-D-glucopyranoside as substrate. The yeast strain expressing Bgl1 and the strain coexpressing Bgl1 and EgE showed Bgl1 activity, but no activity was detected in strains harboring pESC-trp (empty vector).

Ethanol production from PASC, CMC, β-glucan and cellobiose

Direct fermentation of cellobiose to ethanol was performed using three strains transformed with expression plasmids constructed in this study. Fermentation was anaerobically performed at 30°C in fermentation medium. Higher production of ethanol occurred by the strain harboring pαBG1 or pαEgEBG1 than pESC-trp (control plasmid) (Fig. 3). For the direct fermentation of barley β-glucan to ethanol, a cellulose-utilizing yeast strain was constructed with expression plasmid pESC-trp, which made the yeast coexpress β-glucosidase Bgl1 and endoglucanase EgE. With the resulting cellulose-utilizing yeast strain, direct ethanol fermentation from cellulose was performed. The highest ethanol concentration by strain harboring pαEgEα BG1 reached approximately 9.67 g/L and 8.56 g/L from β-glucan and CMC, respectively. And 7.16 g/L ethanol was obtained from PASC by strain harboring pαEgEαBG1 (Figs. 4 and 5). The yield (in grams of ethanol produced per each substrate) was 0.48 g/g, 0.43 g/g and 0.72 g/g from β-glucan, CMC and PASC, respectively. This result suggests that CMC, β-glucan and PASC are hydrolyzed to glucose by sequential reactions of EgE and Bgl1 and that the resulting glucose, final product from recombinant yeast, is immediately utilized by the yeast.

Table 1. Enzyme activities of recombinant EgE and Bgl1

Strain (gene introduced)	Activities (U g^{-1}[dry wt] of cells)	
	BGase[a]	CMCase[b]
YPH499/pESC-trp (empty vector)	ND	ND[c]
YPH499/pαBG1 (β-glucosidase gene)	6.53	ND
YPH499/pαEgE (endoglucanase gene)	ND	1.54
YPH499/pαBG1αEgE (β-glucosidase and endoglucanase genes)	4.97	1.03

[a]β-Glucosidase activity
[b]CMCase activity
[c]ND, Not detected

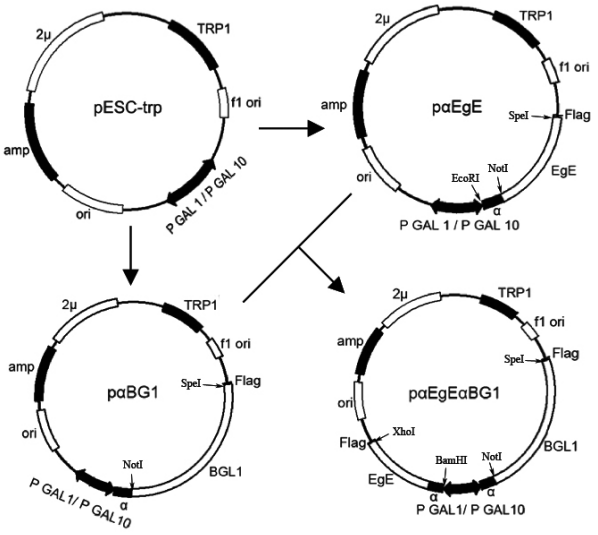

Fig. 1. Construction of the vector to express the *Clostridium thermocellum* EgE gene and *Saccharomycopsis fibuligera* Bgl1 gene bidirectionally.

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 (Cho and Yoo, 1999; Guedon et al., 2002; Wood and Ingram, 1992; Zhou and Ingram, 2001). These whole-cell biocatalysts with the ability to degrade cellulose have several advantages. They produce ethanol by using glucose and cellobiose which inhibit endoglucanase and β-glucosidase activities. They also require lower sterilization and use a single reactor, because glucose is immediately taken up by cells and ethanol is produced. Yeast cells are not able to utilize cellulose or cellooligosaccharides, thus cellulose must first be degraded to glucose in the fermentation of cellulose with yeast cells.

The EgE is an endoglucanase from the thermophilic bacterium, *C. thermocellum.* EgE consists of a signal peptide, a catalytic domain, a flexible linker (Pro-Thr-box), and a C-end cellulosome-binding domain (CBD) (Abdeev et al., 2001; Hall et al., 1988).

The cellobiose-assimilating yeast *S. fibuligera* was found to produce a BglIand BglII which are extracellular β-glucosidases of different substraste specificities. BglI hydrolyzed cellobiose and cellooligosaccharides efficiently. Thus *S. cerevisiae* trans-

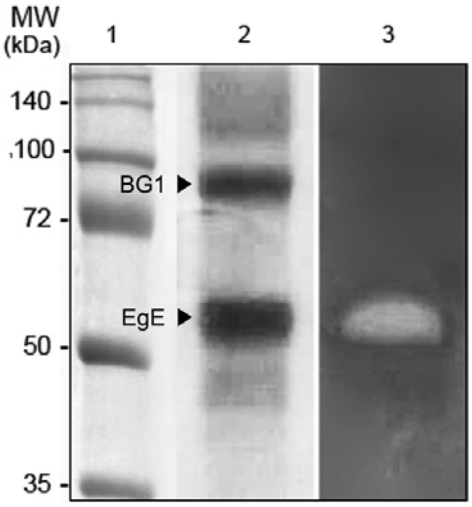

Fig. 2. SDS-PAGE and zymogram. Lane 1, The molecular weight standards; lane 2, concentrated protein from culture supernatant of pαEgEBG1 on 10% SDS-PAGE visualized by Coomassie blue staining; lane 3, Zymogram analysis with 0.1% carboxymethylcellulose (CMC) incorporated into the polyacrylamide.

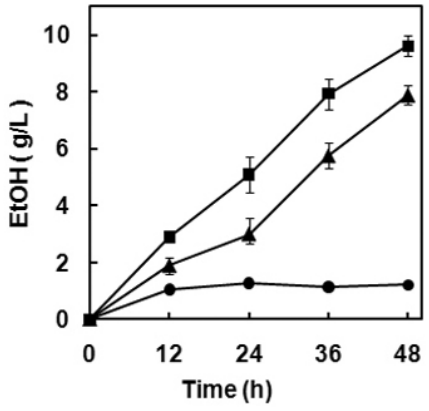

Fig. 3. Production of ethanol from 50 g/L cellobiose. The preculture of strains was inoculated into SG-cellobiose medium to give OD600 = 5.0, and further cultured at 30°C anaerobically. Symbols: closed circles, pESC-trp; closed triangles, pαEgEαBG1; closed square, pαBG1.

formant carrying BglI fermented cellobiose to ethanol. BglI encode proteins of 876 amino acid residues with molecular weights of 96 kDa (Machida et al., 1988).

A viable and cost-effective strategy for the production of bioethanol is dependent on the production of cellulolytic enzymes, hydrolysis of biomass, and conversion of resulting sugars to desired products via a cellulolytic microorganism or a consortium. Therefore we genetically engineered *S. cerevisiae* with bacterial cellulosomal endoglucanase and fungal β-glucosidase for anaerobic ethanol production from cellobiose, CMC, β-glucan or PASC as the sole carbon source (Figs. 3 and 4).

We successfully transformed this thermophilic bacterial endoglucanase into *S. cerevisiae,* so that the recombinant yeast strain could release the EgE efficiently, and ferment CMC or β-glucan into ethanol with the aid of β-glucosidase. It's the first try that produce ethanol by recombinant yeast transformed with *C.*

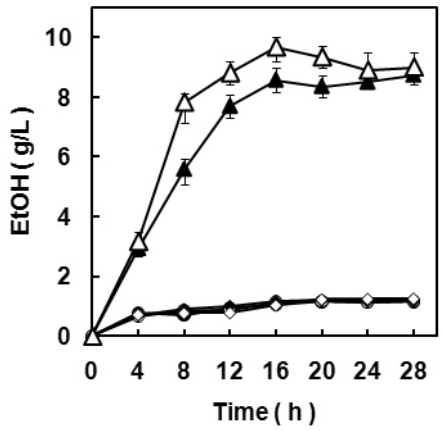

Fig. 4. Production of ethanol from 20 g/L CMC and β-glucan. The preculture of strains was inoculated into SG-CMC and SG-β-glucan medium to give OD600 = 20.0, and further cultured at 30°C anaerobically. Symbols: closed circles, pESC-trp (CMC); open circles, pESC-trp (β-glucan); closed diamonds, pαEgE (CMC); open diamonds, pαEgE (β-glucan); closed triangles, pαEgEαBG1 (CMC); open triangles, pαEgEαBG1 (β-glucan).

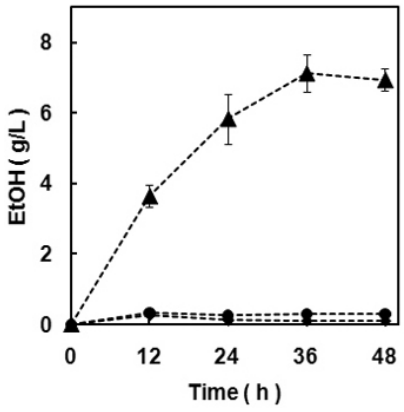

Fig. 5. Production of ethanol from 10 g/L PASC. The preculture of strains was inoculated into SG-PASC medium to give OD600 = 20.0, and further cultured at 30°C anaerobically. Symbols: circles, pESC-trp; diamonds, pαEgE, triangles, pαEgEαBG1.

thermocellum EgE. We have constructed an in-frame α factor-endoglucanase E gene fusion that directs secretion of endoglucanase E and expressed it from a multi copy yeast expression vector under transcriptional control of the GAL1, GAL10 promoter, C-terminal FLAG tag and ADH terminator. The recombinant *S. cerevisiae* strains were cultivated in selective medium.

In this report, we describe a stable and efficient system for cellulase production by the yeast. The strains in this study produce enzymes not on the cell surface but extracellularly by secreting the enzymes to the culture medium. This work describes the successful construction and characterization of a recombinant *S. cerevisiae* strain that is able to effectively utilize cellobiose or β-glucan.

As described above, efficient direct fermentation of cellulosic materials to ethanol was achieved by developing a yeast strain coexpressing two types of cellulolytic enzyme. These results indicate that the hydrolysis of cellulosic materials by cellulolytic enzyme is major factor for producing of ethanol by recombinant

yeast. It has previously been reported that endoglucanase of *Trichoderma reesei* and β-glucosidase of *S. fibuligera* coexpressing *S. cerevisiae* was able to grow and capable of one-step conversion of cellulosic material to ethanol (Den Haan et al., 2007). However, the result of our study showed significantly higher concentration of ethanol than that of previously work. The cellulase system of the anaerobic cellulolytic bacterium *C. thermocellum* was shown to consist of a discrete multi-enzyme complex (Tokatlidis et al., 1991). Construction of multi-enzyme complex affected cellulose hydrolysis, suggesting that combination of cellulases with scaffolding subunit is effective in producing efficient degradation. Further work is needed to analyze the synergic effect of the multi-enzyme complex, cellulosome and to construct recombinant yeast with an improved ability to degrade cellulosic materials and produce ethanol by direct fermentation.

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