RESEARCH LETTER

Cellulosic alcoholic fermentation using recombinant *Saccharomyces cerevisiae* engineered for the production of *Clostridium cellulovorans* endoglucanase and *Saccharomycopsis fibuligera* β-glucosidase

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Abstract

In this study, *Saccharomyces cerevisiae* was engineered for simultaneous saccharification and fermentation of cellulose by the overexpression of the endoglucanase D (EngD) from *Clostridium cellulovorans* and the β-glucosidase (Bgl1) from *Saccharomycopsis fibuligera*. To promote secretion of the two enzymes, the genes were fused to the secretion signal of the *S. cerevisiae* α mating factor gene. The recombinant developed yeast could produce ethanol through simultaneous production of sufficient extracellular endoglucanase and β-glucosidase. When direct ethanol fermentation from 20 g L^{-1} β-glucan as a substrate was performed with our recombinant strains, the ethanol concentration reached 9.15 g L^{-1} after 50 h of fermentation. The conversion ratio of ethanol from β-glucan was 80.3% of the theoretical ethanol concentration produced from 20 g L^{-1} β-glucan. In conclusion, we have demonstrated the construction of a yeast strain capable of conversion of a cellulosic substrate to ethanol, representing significant progress towards the realization of processing of cellulosic biomass in a consolidated bioprocessing configuration.

Introduction

Bioethanol is one of the most important renewable fuels on the market (Purwadi *et al.*, 2007). Ethanol is now primarily produced from sugar sources such as sugar cane juice and starch sources such as corn and wheat grains (Gil *et al.*, 1991). Because these materials are also foods for humans and animals, the limitations of stock and price may prevent these materials from being widely used in the future as feedstock for ethanol production. On the other hand, lignocellulosic materials, for example wastes from nonfood energy crops, for example switchgrass, are abundant and represent low-price feedstocks for bioethanol production (Ladisch & Tsao, 1986). Sugars for fermentation are obtained from lignocellulosic material through enzymatic or chemical hydrolysis processes (Galbe & Zacchi, 2002; Jorgensen *et al.*, 2003). There are great advantages in the application of enzymatic hydrolysis because of its distinguishing products and high yield (Percival Zhang *et al.*, 2006). The goal of cellulose-derived ethanol remains a highly attractive one in terms of environmental, economic and social sustainability (Greene *et al.*, 2004; Farrell *et al.*, 2006). Ethanol generated or converted from sugars in an efficient manner is the central technological challenge to develop its commercial possibilities (Lynd *et al.*, 2008).

Cellulose comprises a major part of biomass on the earth, and its synthesis and utilization contributes to the environmental cycle. Cellulases, which play a major role in the turnover of cellulosic materials, have been found either as free enzymes that work synergistically or as an enzyme complex known as the cellulosome (Doi & Tamaru, 2001). Cellulose is a fibrous, insoluble and crystalline polysaccharide made up of β-1-4-linked D-glucopyranosyl residues and constitutes the major structural component of plant cell walls. Cellulose can be degraded by the cooperative action of

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cellulolytic enzymes, such as endoglucanase, exoglucanase and β-glucosidase (Bhat, 2000).

Clostridium cellulovorans, an anaerobic, mesophilic, spore-forming bacterium (Sleat *et al.*, 1984), produces a cellulosome with a molecular weight of about 1 million and in which several cellulases interact tightly with the scaffolding protein, CbpA (Cho *et al.*, 2004). *Clostridium cellulovorans* produces not only cellulosomes but also noncellulosomal free cellulases, for example, endoglucanase D (EngD), that are not physically associated with the cellulosomes (Foong & Doi, 1992). One of the characteristics of these enzymes is the existence of a cellulose-binding domain (CBD) that enables them to bind quite strongly to amorphous cellulose (Gundllapalli *et al.*, 2007). EngD also has xylanase activity, and is thus able to hydrolyze cellulose and xylan. (Doi & Tamaru, 2001).

One of the most effective ethanol-producing yeasts, *Saccharomyces cerevisiae*, has several advantages owing to its high ethanol production from hexose and high tolerance to ethanol and other inhibitory compounds present in the acid hydrolysates of lignocellulosic biomass (Olsson & Hahn-Hägerdal, 1996). Because *S. cerevisiae* cannot make use of cellulosic materials, these materials must first undergo saccharification to glucose, followed by ethanol production (Fujita *et al.*, 2002). Generally, new pathways can be easily constructed in *S. cerevisiae* with well-defined genetic engineering. Consequently, the substrate assortment can be expanded as required (Van Maris *et al.*, 2006).

Previously, *Aspergillus oryzae*, which coexpresses both endoglucanase and β-glucosidase, was able to produce ethanol from 20 g L^{-1} β-glucan as a substrate by direct ethanol fermentation (Kotaka *et al.*, 2008). The ethanol concentration reached 7.94 g L^{-1} after 24 h of fermentation. In this research, we attempted to ferment barley β-glucan and carboxymethyl-cellulose (CMC) into ethanol by constructing cellulose-degrading yeast strains that coexpress *C. cellulovorans* noncellulosomal endoglucanase (EngD) and *Saccharomycopsis fibuligera* β-glucosidase (Bgl1).

Materials and methods

Strains and media

Escherichia coli DH5α was used for subcloning. The yeast strain YPH499 (Clontech Laboratories Inc., Palo Alto, CA) was used for expressing endoglucanase and the β-glucosidase gene. Luria–Bertani medium (Sambrook *et al.*, 1989) was used to culture *E. coli*, and 50 μg mL^{-1} of ampicillin was added for selecting transformants. *Saccharomyces cerevisiae* was aerobically cultivated at 30 °C in a synthetic SD medium [0.17% of yeast nitrogen base without amino acid (MP Biomedical, Solon, OH) per liter with a 0.13% trp dropout amino acid (Clontech, Mountain View, CA), to which 2% glucose L^{-1} was added as the sole carbon source for selecting transformants]. YPD medium (1% yeast extract, 2% peptone and 2% glucose) was used for culture of *S. cerevisiae*. SG medium (2% galactose, 0.17% yeast nitrogen base without amino acids and 0.13% trp dropout amino acid) and YPG medium (1% yeast extract, 2% peptone and 2% galactose) were used for inducing gene expression in transformants. SG-CMC medium (0.02% galactose, 0.17% yeast nitrogen base without amino acids and 0.13% trp dropout amino acid, 20% CMC), SG-β-glucan medium (0.02% galactose, 0.17% yeast nitrogen base without amino acids and 0.13% trp dropout amino acid, 20% β-glucan) and SG-cellobiose medium (0.02% galactose, 0.17% yeast nitrogen base without amino acids and 0.13% trp dropout amino acid, 5% cellobiose) (Stagoj *et al.*, 2006) were used for fermentation.

Construction of plasmids

The plasmids pαEngD, pαBG1 and pαEngDαBG1 for secretion of the *C. cellulovorans* (ATCC 35296) EngD and the *S. fibuligera* (ATCC 36309) Bgl1 gene were constructed as follows: the secretion signal sequence of the α mating factor gene from *S. cerevisiae* was prepared by PCR (primers 5′-aacggaattcatgagatttccttcaatttttactgcagtt-3′ and 5′-gccagcggcc gcgtcttttatccaaagataccccttcttctttag-3′ with the genomic DNA of *S. cerevisiae*) and inserted into the EcoRI–NotI site of pESC-trp (Clontech Laboratories Inc.). For construction of cellulase production vectors, the EngD gene was amplified by PCR with genomic DNA from *C. cellulovorans* as a template with the primers 5′-ggcggccgctctactgcttttacaggtgtacg-3′ and 5′-gactagtgcttttactgtgcattcagtaccat-3′, and the Bgl1 gene was amplified by PCR with *S. fibuligera* genomic DNA with the primers 5′-ggcgcggccgcgtcccaattcaaaactatacc-3′ and 5′-ggcactagtcgaatagtaaacaggacagatgtct-3′ (Van Rooyen *et al.*, 2005). The fragments were inserted into the NotI–SpeI and BamHI–XhoI sites. The resulting plasmids were named pαEngD and pαBG1, and the expression vector that contained both genes bidirectionally was named pαEngDαBG1.

Yeast transformation and protein expression

Transformation of the expression plasmids pαEngD, pαBG1 and pαEngDαBG1 into *S. cerevisiae* was carried out using the lithium acetate method using a YEASTMAKER yeast transformation system (Clontech Laboratories Inc.). Yeast transformants containing endoglucanase activity were selected on a 0.3% CMC-YPG agar plate using 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. The growth medium supernatant was obtained by centrifugation at 700 ***g***, concentrated by an Amicon ultra-15, 10-kDa cut-off filter (Millipore Co., Cork, Ireland). The concentrated enzyme was electrophoresed on 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE),

followed by Coomassie blue staining. For purification of proteins, the supernatant was loaded onto an anti-FLAG M2 affinity gel. The protein concentration was measured using the Bradford method (Bradford, 1976) using a Quick start protein assay kit (Bio-Rad Laboratories Inc., Hercules, CA), with bovine serum albumin as the standard.

Zymogram and SDS-PAGE analysis

SDS-PAGE was conducted using the method of Laemmli, (1970). The gel was stained for protein with Coomassie brilliant blue R-250 for concentrated protein from the culture supernatant of pαEngDαBG1 and silver staining for purified protein. 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 50 °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 l M NaC1.

Enzyme assays

The 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, using the Somogyi–Nelson assay (Wood & Bhat, 1988). One unit of enzyme activity was defined as the amount of enzyme releasing 1 μM reducing sugar min^{-1}. 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, St. Louis, MO) in 50 mM sodium acetate buffer (pH 5.0). The $OD_{600\,nm}$ 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 $A_{400\,nm}$ after adding 1 mL of 1 M sodium carbonate to increase 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.

Fermentation

The transformants were aerobically precultivated for 24 h and then cultivated in an SG medium for 72 h by adding 2% galactose every 24 h at 30 °C and 200 r.p.m. The cells were harvested by centrifugation and resuspended with minimal medium (0.16% yeast nitrogen base, 0.13% trp dropout powder) and then cultivated for 1 h at 30 °C and 200 r.p.m., to remove residual galactose. The cells were harvested by centrifugation again and resuspended in 50 g L^{-1} cellobiose (pH 5.0), 20 g L^{-1} CMC (pH 5.0) and 20 g L^{-1} β-glucan (pH 5.0) with ODs of 5.0, 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). The ethanol concentration was measured by GC. The gas chromatograph (model GC7890; Agilent, Santa Clara, CA) fitted using a flame ionization detector was operated under the following conditions: DB-WAXetr; temperatures of the column and the injector, 120 and 250 °C, respectively; and helium gas flow rate, 40 mL min^{-1}.

Results

Heterologous expression of endoglucanase and β-glucosidase in *S. cerevisiae*

To ferment cellulose to ethanol, we constructed a yeast strain coexpressing EngD and Bgl1 (Fig. 1). pαEngD, pαBG1 and pαEngDαBG1 were transformed into *S. cerevisiae* and the resultant transformants released active enzymes into the medium. Halos were detected on CMC plates, which absorbed 5 μL of the culture supernatant (data not shown). The SG^{-trp} plate containing 5 mM pNPG 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). As shown in Fig. 2, concentrated rEngD and rBgl1 from the culture supernatant of *S. cerevisiae* harboring each expression plasmid were detected on SDS-PAGE gels stained with Coomassie blue at about 50 and 90 kDa. To confirm the endoglucanase activity, we used a direct activity staining technique that allowed

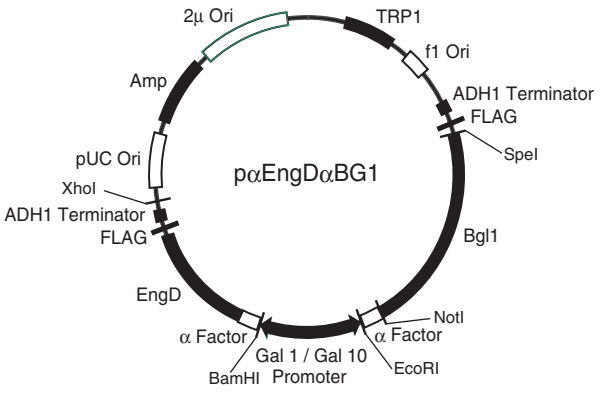

Fig. 1. Construction of the vector to express the *Clostridium cellulovorans* EngD gene and the *Saccharomycopsis fibuligera* Bgl1 gene bidirectionally.

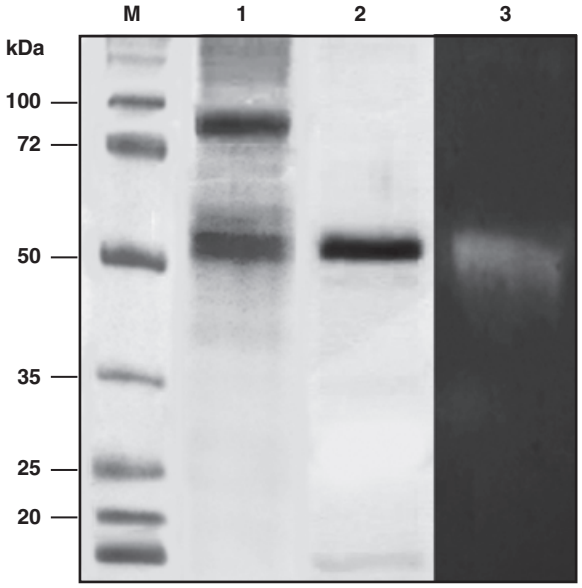

Fig. 2. SDS-PAGE and zymogram. Lane M, the molecular weight standards; lane 1, concentrated protein from the culture supernatant of pαEngDαBG1 on 10% SDS-PAGE visualized by Coomassie blue staining; lane 2, EngD protein purified by Flag affinity agarose on 10% SDS-PAGE visualized by silver staining; lane 3, zymogram analysis with 0.1% CMC incorporated into the polyacrylamide.

rapid, specific detection of endoglucanase on polyacrylamide gels. A halo band appeared on a 0.1% CMC-SDS-PAGE gel stained with 0.25% Congo red, followed by destaining with 1 M NaCl.

Activity measurements from recombinant cellulases

The endoglucanase and β-glucosidase activities expressed in yeast cells were measured (Table 1). As expected, EngD activities were observed in the strains harboring pαEngD and pαEngDαBG1 while no activity was detected either in the yeast strain harboring an empty plasmid or in the strain expressing only Bgl1, and β-glucosidase activity was observed in the strains harboring pαBG1 and pαEngDαBG1 but no activity was detected in strains harboring an empty vector. The strain harboring the empty plasmid exhibited neither of these activities. These results suggest that endoglucanase and β-glucosidase proteins were efficiently synthesized in their active forms.

Fermentation of CMC, β-glucan and cellobiose with strains expressing the EngD and Bgl1 genes

We investigated whether recombinant strains can ferment CMC, β-glucan and cellobiose into ethanol. The time course of the fermentation experiments is shown in Figs 3 and 4. For the fermentation of barley β-glucan or CMC directly to ethanol, a yeast strain was constructed with the yeast expression plasmid pESC-trp, which made the yeast coexpress endoglucanase EngD and β-glucosidase Bgl1 and utilize cellulose. With the resultant cellulose-utilizing yeast strain, direct ethanol fermentation from 20 g L^{-1} CMC and β-glucan was observed. The recombinant yeast strain expressing endoglucanase and β-glucosidase simultaneously was only able to produce ethanol by fermentation of model cellulosic biomass, for example CMC, β-glucan (Fig. 3). Ethanol fermentation from cellobiose was also observed with 50 g L^{-1} cellobiose by yeast expressing the Bgl1 gene. As with the fermentation from CMC and β-glucan, yeast strains expressing β-glucosidase produced ethanol at higher levels than strains harboring pαEngD or pESC-trp (not containing cellulase genes) (Fig. 4). The highest ethanol concentration produced after 40 h of fermentation by the strain harboring pαEngDαBG1 reached approximately 11.03 g L^{-1} from CMC, 9.15 g L^{-1} from β-glucan, and after 50 h of fermentation by the strain harboring pαBG1 it reached 9.5 g L^{-1} and by the strain harboring pαEngDαBG1

Table 1. Enzyme activities of recombinant EngD and Bgl1

	Activities	
Strain (gene introduced)	BGase*	CMCase†
Y/pESC-trp (empty vector)	ND	ND
Y/pαBG1 (β-glucosidase gene)	6.531	ND
Y/pαEngD (endoglucanase gene)	ND	2.033
Y/pαEngDαBG1 (endoglucanase and β-glucosidase genes)	5.159	1.862

*β-Glucosidase activity (U g^{-1} dry cell weight).
†CMCase activity (U mL^{-1}).
ND, not detected.

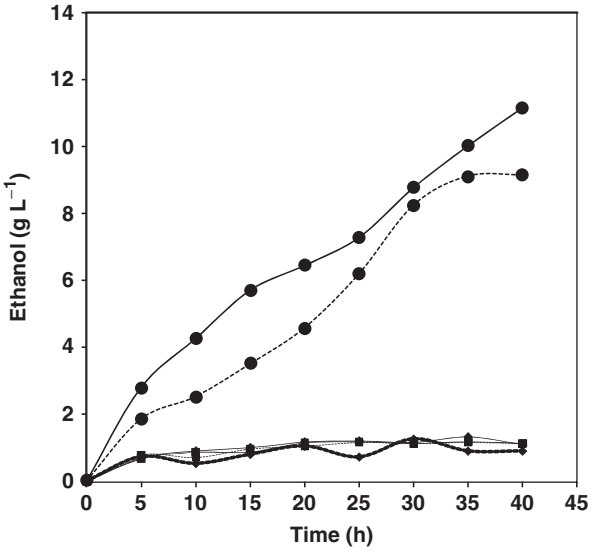

Fig. 3. Production of ethanol from 20 g L^{-1} CMC and β-glucan. ●, pαEngDαBG1; ■, pESC-trp; ♦, pαEngD. The line and the dotted line show the substrate CMC and β-glucan, respectively.

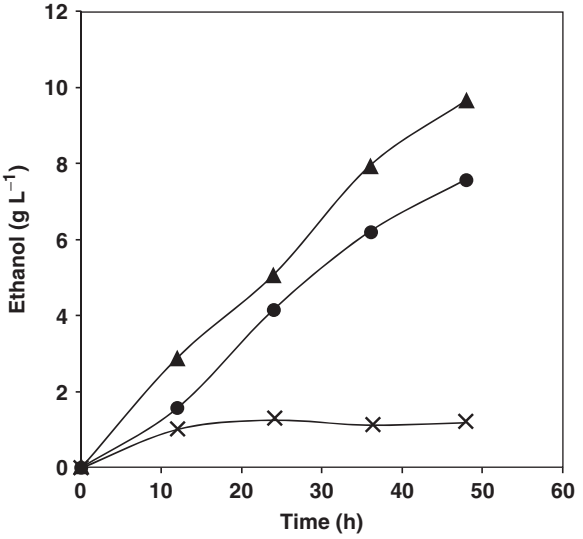

Fig. 4. Production of ethanol from 50 g L^{-1} cellobiose. ▲, pαBG1; ●, pαEngDαBG1; ✖, pESC-trp.

it reached 7.5 g L^{-1} from cellobiose. Fermentation from CMC or β-glucan is about three times more efficient than from cellobiose. β-Glucan fermentation showed a higher ethanol concentration than that of the previous work (Kotaka *et al.*, 2008). This corresponded to 80.3% of the theoretical ethanol concentration produced from 20 g L^{-1} β-glucan. These results suggest that CMC and β-glucan are hydrolyzed to glucose by sequential reactions of EngD and Bgl1 and that the resulting glucose is immediately utilized by the yeast.

Discussion

The utilization of low-cost and abundant cellulose feedstock is an attractive option for bioethanol production. A number of previous attempts have been made to enable the utilization of cellulosic substrate by expressing cellulolytic enzymes in yeasts. These whole-cell biocatalysts, with the ability to degrade cellulose, were able to convert cellobiose and glucose, which inhibit cellulase and β-glucosidase activities, in a single reactor (Cho *et al.*, 1999; Fujita *et al.*, 2002, 2004; Tokuhiro *et al.*, 2008).

Cellulose can be hydrolyzed by cellulolytic enzymes and the released fermentable sugars can be converted to ethanol by *S. cerevisiae*. Simultaneous saccharification and fermentation produces higher ethanol yields, while requiring lower amounts of enzyme (Hari Krishna *et al.*, 2001; Shen *et al.*, 2008). 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 engineered *S. cerevisiae* genetically with anaerobic bacterial noncellulosomal endoglucanase and fungal β-glucosidase for ethanol production from CMC, β-glucan and cellobiose.

EngD, a *C. cellulovorans* protein, possesses multiple enzymatic activities such as endoglucanase, xylanase and exoglucanase activities. This enzyme does not appear to interact with the scaffolding protein of CbpA and probably exists as an independent enzyme (Foong & Doi, 1992; Hamamoto *et al.*, 1992; Yeh *et al.*, 2005). Its proline–threonine linker or 'hinge' region is thought to be a flexible region for linking its catalytic domain to its C-terminal CBD that allows EngD to bind to cellulose. The substrate-binding domains increase the enzyme concentration at the substrate surface and help to target the enzyme. The binding efficiency of the cellulases is considerably enhanced by the presence of the CBD and the enhanced binding clearly seems to correlate with better activity towards insoluble cellulose (Limon *et al.*, 2001). This differs from the cellulosomal enzymes, which generally do not have a CBD (Doi *et al.*, 1998).

We successfully transformed this bacterial CBD-containing noncellulosomal endoglucanase, *C. cellulovorans* EngD, into *S. cerevisiae*, so that the resultant yeast strain secreted the EngD efficiently and fermented CMC and β-glucan into ethanol with the aid of β-glucosidase. As this enzyme was released by the recombinant yeast strain through the fusion of *S. cerevisiae* α mating factor secretion signal to this EngD gene in-frame, this construction yielded a resultant yeast strain that was capable of releasing EngD protein into the culture medium. It efficiently hydrolyzed cellulose to smaller fragments for efficient fermentation through its CBDs' affinity to the substrate. The production of extracellular cellulases by this yeast strain resulted in the effective utilization of CMC, β-glucan and cellobiose. In the present study, we have demonstrated the construction of a yeast strain capable of consolidated conversion of amorphous cellulose to ethanol.

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