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Direct fermentation of amorphous cellulose to ethanol by engineered *Saccharomyces cerevisiae* coexpressing *Trichoderma viride* EG3 and BGL1

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Direct ethanol fermentation from amorphous cellulose was achieved using an engineered industrial *Saccharomyces cerevisiae* strain. Two cellulase genes endoglucanase (*eg3*) and β -glucosidase (*bgl1*) were obtained from *Trichoderma viride* and integrated into the genome of *S. cerevisiae*. These two cellulases could be constitutively coexpressed and secreted by the recombinant strain *S. cerevisiae*-eb. The enzyme activities were analyzed in the culture supernatants, with the highest endoglucanase activity of 2.34 units/ml and β -glucosidase activity of 0.95 units/ml. The effects of pH, temperature and metal ions on enzyme activities were analyzed. The coexpression strain *S. cerevisiae*-eb could grow in carboxymethyl cellulose (CMC) and utilize it as the single carbon source. The 20 g/L CMC as a model substrate of amorphous cellulose was used in fermentation. The ethanol production reached 4.63 g/L in 24 h, with the conversion ratio of 64.2% compared with the theoretical concentration. This study demonstrated that the engineered industrial strain *S. cerevisiae*-eb could convert amorphous cellulose to ethanol simultaneously and achieve consolidated bioprocessing (CBP) directly.

Key words: bioethanol; cellulose; coexpression; consolidated bioprocessing; endoglucanase; β -glucosidase

Introduction

The rise of crude oil prices and the high emissions of greenhouse gasses have resulted in growing interest in renewable energy. Ethanol is an ideal renewable fuel substi-

tute which has been widely used in vehicles either as a neat fuel or in different combinations with gasoline (Fujita et al., 2004). Most of the fuel ethanol production is currently made from sugarcane and corn, which do not fit the bill as suitable alternative energy sources because of the global food shortage (Agbogbo and Coward-Kelly, 2008; Krugman, 2008; Kotaka et al., 2008). As a result, cellulosic substrates, such as agricultural and forestry residues, have become attractive raw materials for ethanol production because they are nonfood, renewable feedstock and exist in huge quantities (Demain et al., 2005; Lynd et al., 2005; Ragauskas et al., 2006; Den Haan et al., 2007).

The ethanol production from cellulose is performed in two steps: degradation of cellulose to celooligosaccharides and glucose, and conversion of glucose to ethanol by microorganisms. At the first step, acid treatment and enzyme hydrolysis are two major approaches used recently. The enzyme degradation of cellulose to sugar has received high attention because it will not generate an environmental load (Kotaka et al., 2008). Full enzymatic hydrolysis of crystalline cellulose requires at least three major cellulose components including β -1,4-endoglucanases (EG, EC3.2.1.4), β -1,4-cellobiohydrolases (CBH, EC3.2.1.91) and β -glucosidase (BGL, EC3.2.1.21) working together in a synergistic style (Terri, 1997). The expression ratios and synergistic effects of these enzymes both significantly influence the extent and specific rate of cellulose degradation (Sun et al., 2008; Yamada et al., 2011).

Some cellulases have been produced commercially for enzymatic degradation of cellulose, but they are expensive and much in demand. Nowadays, researchers focus on one-step conversion of cellulose to ethanol with just one organism. This method, defined as consolidated bioprocessing (CBP), combines cellulase production, cellulose hydrolysis and fermentation of sugars in one reactor (Lynd et al., 2005; van Zyl et al., 2007).

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There are two strategies for CBP: One is to engineer ethanol-producing organisms to express cellulases in order to obtain the ability to utilize cellulose, while the other is to engineer naturally cellulolytic microorganisms to improve the ethanol product-related property (la Grange et al., 2010). In the past decade, researchers used the first strategy to develop genetically engineered bacteria (Guedon et al., 2002; Zhou and Ingram, 2001) and fungi (Kotaka et al., 2008; Den Haan et al., 2007; Fujita et al., 2004; Fujita et al., 2002; Ribeiro et al., 2010; Yanase et al., 2010). Among them, *Saccharomyces cerevisiae* was a very attractive host because of its high ethanol productivity and tolerance (Den Haan et al., 2007). However, no ideal microorganisms have been identified so far (la Grange et al., 2010).

In this study, we attempted to coexpress two cellulases in an industrial strain, *S. cerevisiae* AS2.489, with high ethanol production ability and tolerance. *Trichoderma viride* *eg3* and *bgl1* were integrated into the genome and constitutively expressed and secreted. The transformants could directly produce ethanol from carboxymethyl cellulose (CMC).

Materials and Methods

Microbial strains and medium. *Escherichia coli* JM109 was used as the host strain for recombinant DNA manipulations (New England Biolabs Beijing, Ltd., Beijing, China). *S. cerevisiae* AS2.489 was obtained from China General Microbiological Culture Collection Center. *T. viride* AS3.3711 was purchased from Microbial Culture Collection Center of Guangdong Province, China.

E. coli was grown in Luria-Bertani medium containing 10 g/L tryptone, 5 g/L yeast extract, 10 g/L sodium chloride (supplemented with 100 mg/L ampicillin). *S. cerevisiae* was cultivated aerobically at 30°C in YPD medium containing 20 g/L tryptone, 10 g/L yeast extract and 20 g/L glucose. *T. viride* was cultivated and conserved on potato dextrose agar (PDA) (Chand et al., 2005), followed by Medels medium to

induce cellulase expression (van Wyk and Mohulatsi, 2003).

Plasmid construction. Standard protocols were followed for DNA manipulations (Sambrook and Russell, 2001). Total RNA of *T. viride* was isolated using TRIzol Reagent (Invitrogen) and cDNA synthesis was performed using ReverTra Ace- α -[®] kit (Toyobo, Ltd., Osaka, Japan) according to the manufacturer's instructions. Then the cDNA-mixture was used as a template to amplify *T. viride* *eg3* and *bgl1* genes containing their own signal peptides by the gene-specific primers P_{eg3U}, P_{eg3D}, P_{bgl1U} and P_{bgl1D}, respectively (Table 1). The primers were designed on the basis of *Trichoderma reesei* *eg3* (GenBank accession No. M19373) (Saloheimo et al., 1988) and *bgl1* (GenBank accession No. TRU09580) (Mach, 1993) gene sequences submitted to Genbank, respectively.

The two amplified genes were inserted into a shuttle plasmid, pScIKP, constructed by our research group in a previous study (Gong et al., 2010) to generate two single-gene expression plasmids, pScIKP-*eg3* and pScIKP-*bgl1*. These plasmids contained an entire expression cassette under the control of a yeast constitutive phosphoglycerate kinase (*PGK1*) promoter and terminator (McNabb et al., 2005; Ribeiro et al., 2010). Then the pScIKP-*eg3* was digested with *Nhe* I and *Xba* I, which were isocaudomers, and released a 2.6 kb fragment. This *eg3* fragment was inserted into *Xba* I-digested pScIKP-*bgl1* and generated pScIKP-eb containing both *eg3* and *bgl1* genes (Fig. 1). This coexpression plasmid contained these two genes in the same direction and both had an independent expression cassette. The identity of the plasmid construction was confirmed by restriction enzyme analysis and DNA sequencing.

Yeast transformation. The pScIKP vector contains the *S. cerevisiae* rDNA sequence and can integrate into the yeast chromosomal DNA with multiple copies by homologous recombination (Gong et al., 2010). The single gene expression plasmids pScIKP-*eg3* and pScIKP-*bgl1* and the co-expression plasmid pScIKP-eb were digested with *Apa*I.

Table 1. Microbial strains, plasmids and primers used in this study.

Strains	Features	Source
<i>E. coli</i> JM109	<i>F'</i> traD36 <i>proA</i> + <i>B</i> + <i>lacIq</i> Δ (<i>lacZ</i>)M15/ Δ (<i>lac-proAB</i>) <i>glnV44 e14</i> – <i>gyrA96 recA1</i>	NEB
<i>S. cerevisiae</i> AS2.489	<i>relA1 endA1 thi hsdR17</i>	China General Microbiological Culture Collection Center
<i>T. viride</i> AS3.3711		Microbial Culture Collection Center of Guangdong, China
<i>S. cerevisiae</i> - <i>eg3</i>	<i>S. cerevisiae</i> expressing EG3	This work
<i>S. cerevisiae</i> - <i>bgl1</i>	<i>S. cerevisiae</i> expressing BGL1	This work
<i>S. cerevisiae</i> -eb	<i>S. cerevisiae</i> coexpressing EG3 and BGL1	This work
Plasmids	Features	Reference
pScIKP	G418, expression cassette under the <i>PGK1</i> promoter	(Gong et al., 2010)
pScIKP- <i>eg3</i>	Expression of EG3	This work
pScIKP- <i>bgl1</i>	Expression of BGL1	This work
pScIKP-eb	Expression of both EG3 and BGL1	This work
Primers	Sequence (5'-3') with restriction sites underlined	Source accession number and reference
<i>eg3</i>		AY343987 (Li et al., 2011)
P _{eg3U}	CGGGATCCATGAACAAGTCCGTGGCTCCAT	
P _{eg3D}	GGACTAGTCTACTTTCTTGCGAGACACGAG	
<i>bgl1</i>		FJ882071 (This work)
P _{bgl1U}	ATGCGTTACCGAACAGCAGCTGCGCTGGCACTTGCCACTGGTCCCTTTG	
P _{bgl1D}	GACTAGTCTACGCTACCGACAGAGTGCTCGTCAGCC

Fig. 1. The coexpression plasmid pScIKP-eb.

The linear DNA fragments were introduced into *S. cerevisiae* AS2.489 by the lithium acetate (LiAc) and dithiothreitol (DTT) method as described previously (Thompson et al., 1998).

The two genes inserted into the yeast genome of *S. cerevisiae*-eb were detected using PCR. In addition, transformants *S. cerevisiae*-eg3, *S. cerevisiae*-bgl1 and *S. cerevisiae*-eb were subcultured in G418-containing YPD plates for several cultural rounds. Wild-type strain *S. cerevisiae* AS2.489 could grow in 200 µg/ml G418, so the transformants were selected in a starting concentration of G418 from 300 µg/ml, and then screened in increasingly higher concentrations. The transformants with the highest activity were selected using CMC or *p*-nitrophenyl β-D-glucopyranoside (*p*NPG) as the substrate (Kotaka et al., 2008).

The recombinant and wild-type yeast cell growth were monitored by measuring cell counts during the culture in YPD medium.

The strains, transformants, plasmids and primers constructed or used in this study are summarized in Table 1.

Enzyme activity assays. The recombinant yeast colonies *S. cerevisiae*-eg3 and *S. cerevisiae*-eb were detected on YPD plates containing 0.5% (w/v) CMC followed by Congo-red (0.1%) staining for endoglucanase activity screening (Lee et al., 2007). The recombinant *S. cerevisiae*-bgl1 and *S. cerevisiae*-eb colonies were detected on YPD plates containing 0.1% (v/v) esculin hydrate (6,7-dihydroxycoumarin 6-β-glucoside hydrate) and 0.25% (v/v) ferric ammonium citrate for β-glucosidase activity screening (Liu et al., 2011).

The recombinant yeasts were aerobically cultivated in YPD medium at 30°C for 48 h, and the supernatants were collected by centrifugation and used for the measurement of extracellular endoglucanase and β-glucosidase activities. The measurements were repeated three times. Assays for the activity of individual enzymes were conducted as follows:

Endoglucanase assay. The CMC solution (2%) was prepared in 50 mmol/L sodium citrate buffer, pH 4.8. Two milliliters of CMC solution was incubated with 0.5 ml of the test enzyme solution at 50°C for 30 min. Three milliliters of 1% DNS reagent was added and the solution was incubated in a 100°C water bath for 10 min to terminate the reaction. The reducing sugar concentration produced from the enzymatic reaction was then measured at 540 nm and the absorbance of the samples was translated into glucose

production using a glucose standard curve (Ghose, 1987). The endoglucanase activity (unit/ml) was defined as the amount of enzyme required to liberate 1 mg glucose per hour.

β-Glucosidase assay. β-Glucosidase activity was estimated using *p*NPG as a substrate. Then 0.1 ml of enzyme solution was added to 0.9 ml phosphate/citrate buffer (0.2 mol/L, pH 4.5) and the mixture was prewarmed at 50°C for 10 min. Then 1 ml of *p*NPG (5 mmol/L) was added to the assay mixture and incubated at 50°C for 10 min. The *p*-nitrophenol liberated was measured at 410 nm after adding 1 ml of sodium carbonate (1 mol/L) to stop the reaction (Den Haan et al., 2007). One unit of enzyme activity was defined as the amount of enzyme required to liberate 1 µmol of *p*-nitrophenol per min.

Zymogram. The wild-type *S. cerevisiae* and recombinant yeast colony *S. cerevisiae*-eb were cultured in YPD medium for 60 h, and the culture samples were centrifuged at 12,000 × *g* for 5 min. The 25 µl supernatant was loaded on 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) followed by Coomassie Brilliant Blue R250 staining. The protein molecular weight standard was PageRuler Prestained Protein Ladder purchased from Thermo Scientific (Waltham, MA).

The zymogram of secreted proteins of recombinant *S. cerevisiae*-eb was generated as described by Romano et al. with some modifications (Romano et al., 2013). The 10% SDS-PAGE gel containing 0.5% (w/v) CMC was used for endoglucanase activity detection. After electrophoresis, renaturation was carried out in the gel by washing with solution I (10 mM Tris-HCl, 5 mM β-mercaptoethanol, 20% (v/v) isopropanol, pH 7.5) for 30 min. Then, the gel was incubated in solution II (50 mM Tris-HCl, 5 mM β-mercaptoethanol, 1 mM EDTA, pH 7.5) at 4°C for 18 h. After that, the gel was incubated at 50°C for 1 h, and stained with Congo red as described above. For β-glucosidase activity detection, 10% SDS-PAGE gel was renaturated as described above, and stained with 0.1% (v/v) esculin hydrate and 0.25% (v/v) ferric ammonium citrate.

The prediction of protein molecular weight and potential *N*-glycosylation sites was carried out by Compute pI/Mw and NetNGlyc online (<http://www.expasy.org/tools>), respectively.

Characterization of enzyme activities. The influence of the pH value on endoglucanase activity was determined at 60°C in citrate buffer with a pH range from 3.0 to 7.0. The influence of the temperature on endoglucanase activity was measured at pH 6.0 at different temperatures ranging from 30 to 70°C. The influence of the pH value on β-glucosidase activity was determined at 60°C in phosphate/citrate buffer with a pH range from 3.5 to 6.0. The influence of the temperature on β-glucosidase activity was measured at pH 4.5 with incubation temperatures ranging from 30 to 70°C.

To evaluate the effect of metal cations such as Fe³⁺, Mn²⁺, Ba²⁺, Mg²⁺, Ca²⁺, Zn²⁺, Cu²⁺, Co²⁺ and EDTA, these elements were added separately to the enzyme activity assay to a final concentration of 1 and 10 mM, respectively. Relative enzyme activities were calculated by taking control enzyme activity without adding metal cations as 100%. All the enzyme activity tests were performed using the standard assay as described above.

The significance of difference between controls and treatments was tested using ANOVA, SPSS 13.0 software. The level of statistical significance was accepted as $p < 0.05$.

Fermentation. The direct fermentation of cellulose was carried out using CMC as the single carbon source and performed as described previously with modifications (Karimi et al., 2006). The fermentation medium Y-CMC contained CMC as the single carbon source with a concentration of 20 g/L CMC, 5 g/L yeast extract, 7.5 g/L $(\text{NH}_4)_2\text{SO}_4$, 3.5 g/L K_2HPO_4 , 1 g/L $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$, 0.75 g/L $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ and 0.05 mol/L buffer citrate at pH 5.5. Volumes of 50 ml Y-CMC medium were autoclaved at 121°C and pH was adjusted to 5.0 by 2 mol/L NaOH.

The transformant *S. cerevisiae*-eb was aerobically pre-cultivated for 24 h and then cultivated in YPD medium at 30°C for another 12 h. The cells were harvested by centrifugation and transferred to 50 ml Y-CMC medium. The cell growth was monitored by measuring cell counts. The small-scale fermentation was carried out in a 250 ml flask and shaken at 150 rpm. The flasks were plugged with rubber stoppers which could discharge carbon dioxide to maintain the oxygen-limited conditions. The fermentation supernatant samples were collected every 12 h and the reducing sugar concentration was measured as described previously (Ghose, 1987).

Gas chromatography analysis of ethanol production. During the 60 h of fermentation, samples of fermentation supernatant were collected at 12 h intervals and the ethanol concentration was examined by gas chromatography (model GC7890; Agilent, Santa Clara, CA). The GC fitted using a flame ionization detector was operated with an HP-INNOWax GC column (30 m $\times$ 0.25 mm $\times$ 0.25 μm). The temperatures of the column and the injector were 200 and 250°C respectively, and hydrogen gas flow rate was 30 ml/min. The ethanol standards were prepared using commercial grade ethanol.

Nucleotide sequence accession numbers. The nucleotide sequence for *eg3* is in the GenBank database under the accession number AY343987 and *bgl1* under the accession number FJ882071.

Results

Cloning and coexpression of two types of cellulases in yeast

The *eg3* and *bgl1* genes were obtained from *T. viride* using RT-PCR. DNA sequencing and BLAST revealed that *T. viride eg3* was 1,257 nucleotides (nt) long and had the same nucleotide sequence as the *eg3* gene from *T. viride* previously reported (GenBank accession number AY343987). The *bgl1* was 2,235 nt long, and had the same nucleotide sequence as *T. reesei bgl1* (GenBank accession number U09580) but had not been reported in *T. viride* before. As a result, the *T. viride bgl1* gene sequence was submitted to GenBank with a new accession number of FJ882071.

The signal peptide analysis using Signal P identified that *T. viride eg3* and *bgl1* contained their native signal peptide cleavage sites (data not shown), which suggested that the expressed products could be secreted into the cell culture suspension.

These two genes were cloned into the shuttle plasmid

pScIKP, resulting in a coexpression plasmid, pScIKP-eb. The restriction enzyme analysis and DNA sequencing (data not shown) revealed that they were inserted in the same direction and each had an independent expression cassette under the control of *PGK1* promoter (Fig. 1). Then pScIKP-eb was integrated into the *S. cerevisiae* genome by rDNA homologous recombination. The positive transformants *S. cerevisiae*-eb could grow on the YPD plate with a G418 concentration of 3,000 $\mu\text{g/ml}$, while the wild type could grow in 200 $\mu\text{g/ml}$. The two genes' integration was further identified by PCR using *S. cerevisiae*-eb chromosomal DNA as templates (data not shown).

Expression and enzymes activity of cellulases produced in *S. cerevisiae*

The growth of recombinant yeast cells was monitored during 120 h cultivation in YPD medium. *S. cerevisiae*-eb and *S. cerevisiae*-eg3 both reached the highest cell density at 60 h, while *S. cerevisiae*-bgl1 and the wild type reached the highest cell density at a relatively later time of 98 h (data not shown). Thus, *S. cerevisiae*-eb and *S. cerevisiae*-eg3 grew faster than *S. cerevisiae*-bgl1 and the wild type, but in terms of the highest cell densities, all the recombinant yeasts were lower than the wild type.

The secretion of active endoglucanase was demonstrated by Congo-red staining after incubation of wild-type and recombinant strains in the CMC plate. The clearing zone was presented by *S. cerevisiae*-eb with an average diameter of 20.3 mm, while the average diameter of the clearing zone presented by *S. cerevisiae*-eg3 was 21.2 mm. The wild type and *S. cerevisiae*-bgl1 did not present a clearing zone, indicating the endoglucanase activity of recombinant strains with the ability to degrade CMC substrate.

The expression and secretion of endoglucanase were further measured according to the hydrolysis of CMC. The culture supernatants of the wild type and all the recombinant strains were collected every 12 h and assayed for endoglucanase activity (Fig. 2). The *S. cerevisiae*-bgl1 and wild type strains did not demonstrate any endoglucanase activity (data not shown). During the 120 h of measurement, *S. cerevisiae*-

Fig. 2. Endoglucanase activity from the supernatants of recombinant *S. cerevisiae* strains growing in YPD medium.

Symbols: $\square$, *S. cerevisiae*-eg3; $\circ$, *S. cerevisiae*-eb. Endoglucanase activity was measured with CMC assay. The data represent the averages of three independent determinations. Standard deviations ($\pm$ SD) are represented by bars when larger than the symbols.

eb reached the highest endoglucanase activity at 96 h while *S. cerevisiae*-eg3 did so at 84 h, with the highest activity values of 2.34 unit/ml and 4.31 unit/ml respectively. Therefore, *S. cerevisiae*-eb showed lower endoglucanase activity than *S. cerevisiae*-eg3.

The secretion of active β -glucosidase was demonstrated by esculin as a substrate after incubation of wild-type and recombinant strains on plates. The black zone was presented by *S. cerevisiae*-eb with an average diameter of 23.5 mm, while the average diameter of the black zone presented by *S. cerevisiae*-bgl1 was 20.2 mm. The wild type and *S. cerevisiae*-eg3 did not present a black zone. This result indicated the β -glucosidase activity of recombinant strains with the ability to degrade esculin substrate.

The expression and secretion of β -glucosidase were determined by the ability of hydrolysis of *p*NPG. The culture supernatants of all strains were also collected and tested at 12-h intervals (Fig. 3). The *S. cerevisiae*-eg3 and wild-type strains did not demonstrate β -glucosidase activity (data not shown). *S. cerevisiae*-eb reached the highest β -glucosidase activity at 108 h while *S. cerevisiae*-bgl1 did so at 84 h, with the highest activity values of 0.95 unit/ml and 0.81 unit/ml respectively. *S. cerevisiae*-eb showed higher β -glucosidase activity than *S. cerevisiae*-bgl1 did.

Thus, both genes were successfully coexpressed in yeast transformants *S. cerevisiae*-eb, and their native signal peptide sequences were able to direct the secretion of both proteins into the culture supernatants of *S. cerevisiae*-eb.

Optimal conditions for enzyme activities

The optimal enzyme conditions for these two expressed enzymes were tested and are shown in Fig. 4. EG3 demonstrated the maximum activity at pH 6.0 and 60°C. The relative enzyme activity remained above 60% at the pH range of 5.0–6.0 (Fig. 4a) and the temperature range of 50–70°C (Fig. 4b). BGL1 showed the maximum activity at pH 4.5 and 60°C. The relative enzyme activity remained

Fig. 3. β -Glucosidase activity from the supernatants of recombinant *S. cerevisiae* strains growing in YPD medium.

Symbols: $\square$, *S. cerevisiae*-bgl1; $\circ$, *S. cerevisiae*-eb. β -Glucosidase activity was measured with *p*NPG assay. The data represent the averages of three independent determinations. Standard deviations ($\pm$ SD) are represented by bars when larger than the symbols.

Fig. 4. Enzyme relative activities in culture supernatants of recombinant *S. cerevisiae*-eb at different pHs and temperatures.

Fig. 4a, EG3 relative activities at different pHs; b, EG3 relative activities at different temperatures; c, BGL1 relative activities at different pHs; d, BGL1 relative activities at different temperatures. Standard deviations ($\pm$ SD) are represented by bars when larger than the symbols.

above 60% at the pH range of 3.5–6.0 (Fig. 4c) and the temperature range of 50–60°C (Fig. 4d). Thus both of them worked well under faintly acidic conditions and demonstrated the maximum activity at 60°C.

The effects of metal ions on both enzyme activities were tested. At the concentration of 1 mM, only Mn^{2+} showed extreme significant enhancement of endoglucanase activity compared to the control reaction containing no metal ions ($p < 0.01$), while Fe^{3+} significantly inhibited it ($p < 0.05$). When the ion concentration was increased to 10 mM, Mn^{2+} showed extreme significant enhancement ($p < 0.01$) and Ca^{2+} showed significant enhancement of it ($p < 0.05$), while Fe^{3+} extremely significantly inhibited the activity ($p < 0.01$) and Cu^{2+} , Co^{2+} and the chelator EDTA significantly inhibited it ($p < 0.05$) (Table 2).

The metal ions showed relatively greater effects on β -glucosidase activity. At the concentration of 1 mM, only Ca^{2+} showed significant enhancement of β -glucosidase activity, while Fe^{3+} and Mn^{2+} showed significant inhibition of it ($p < 0.05$). When the ion concentration was increased to 10 mM, Ba^{2+} , Mg^{2+} , Ca^{2+} , Zn^{2+} and Cu^{2+} showed extreme significant enhancement, while Fe^{3+} and Co^{2+} showed extreme significant inhibition of it ($p < 0.01$), and Mn^{2+} and EDTA significantly inhibited it ($p < 0.05$) (Table 3).

Table 2. Effect of metal ions on the endoglucanase activity.

Metal	1 mM	10 mM
$\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$	$81 \pm 0.8^*$	$61 \pm 0.3^{**}$
$\text{MnSO}_4 \cdot \text{H}_2\text{O}$	$108 \pm 0.4^{**}$	$113 \pm 0.5^{**}$
$\text{BaCl}_2 \cdot 2\text{H}_2\text{O}$	86 ± 0.8	64 ± 0.8
MgCl_2	106 ± 0.3	119 ± 0.3
$\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$	109 ± 0.5	122 ± 0.4
CaCl_2	111 ± 0.5	$191 \pm 0.4^*$
ZnCl_2	102 ± 0.3	113 ± 0.3
$\text{CuCl}_2 \cdot 2\text{H}_2\text{O}$	85 ± 1.0	$56 \pm 0.2^*$
$\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$	92 ± 0.4	$60 \pm 0.9^*$
EDTA	98 ± 0.2	$89 \pm 0.2^*$
Control	100	100

The relative activities are expressed as percentage $\pm$ SD by normalization to the control activity.

*: significant difference compared to the control reaction containing no metal ions ($p < 0.05$). **: extreme significant difference compared to the control reaction ($p < 0.01$).

Table 3. Effect of metal ions on the β -glucosidase activity.

Metal	1 mM	10 mM
$\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$	$79 \pm 0.3^*$	$53 \pm 0.7^{**}$
$\text{MnSO}_4 \cdot \text{H}_2\text{O}$	$70 \pm 0.3^*$	$37 \pm 0.7^*$
$\text{BaCl}_2 \cdot 2\text{H}_2\text{O}$	105 ± 0.2	$113 \pm 0.4^{**}$
MgCl_2	106 ± 0.2	$110 \pm 0.3^{**}$
$\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$	104 ± 0.3	$113 \pm 0.3^{**}$
CaCl_2	$107 \pm 0.3^*$	$118 \pm 0.1^{**}$
ZnCl_2	103 ± 0.2	$107 \pm 0.1^{**}$
$\text{CuCl}_2 \cdot 2\text{H}_2\text{O}$	111 ± 0.2	$124 \pm 0.4^{**}$
$\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$	94 ± 0.2	$85 \pm 0.3^{**}$
EDTA	93 ± 0.7	$84 \pm 0.4^*$
Control	100	100

The relative activities are expressed as percentage $\pm$ SD by normalization to the control activity.

*: significant difference compared to the control reaction containing no metal ions ($p < 0.05$). **: extreme significant difference compared to the control reaction ($p < 0.01$).

Fig. 5. SDS-PAGE and zymogram of the secreted proteins of recombinant *S. cerevisiae*-eb.

Fig. 5a, SDS-PAGE of the secreted proteins of recombinant *S. cerevisiae*-eb. Fig. 5b and 5c, Zymogram of the secreted proteins of recombinant *S. cerevisiae*-eb in SDS-PAGE with CMC substrate followed by renaturation and Congo red staining (b), or SDS-PAGE followed by renaturation and esculin hydrate and ferric ammonium citrate staining (c). Lane M, molecular weight standard; lane 1, 3 and 5, secreted proteins of wild-type *S. cerevisiae*; lane 2, 4 and 6, secreted proteins of recombinant *S. cerevisiae*-eb. The arrows show a protein of 100 kDa with endoglucanase activity (b) and a protein of 120 kDa with β -glucosidase activity (c), respectively.

Zymogram in recombinant *S. cerevisiae*-eb

The EG3 protein has 418 amino acid residues with a theoretical molecular weight of 44.15 kDa and isoelectric point of 4.96, while the BGL1 protein has 744 amino acid residues with a theoretical molecular weight of 78.43 kDa and isoelectric point of 6.38. Two potential *N*-glycosylation sites were found in each protein, at residues 2 and 124 in EG3 and residues 239 and 341 in BGL1, respectively.

The zymogram of the secreted proteins in SDS-PAGE followed by renaturation showed that the protein with endoglucanase activity had a molecular weight of 100 kDa (Fig. 5b), while that with β -glucosidase activity had a molecular weight of 120 kDa (Fig. 5c). These molecular weight differences might be caused by glycosylation at the potential *N*-glycosylation sites.

Fermentation and ethanol production

Direct fermentation from CMC to ethanol was performed using the yeast strain *S. cerevisiae*-eb coexpressing *eg3* and *bgl1*. The cells could grow in CMC as the single carbon source according to cell counts (Fig. 6). The highest ethanol concentration was 4.63 g/L at 24 h of fermentation, while the reducing sugar remained at a low level during the process. These indicated that the strain simultaneously carried on saccharification and fermentation. Considering the conversion of CMC to glucose and then ethanol, the ethanol yield achieved 64.2% of the theoretical value. In addition, the reducing sugar in the fermentation broth remained at a very low level, with the highest concentration of 0.389 g/L at 48 h. This indicated that the reducing sugar produced by EG3 and BGL1 was consumed immediately by the strain to produce ethanol directly. No ethanol was produced by wild-type *S. cerevisiae*.

Fig. 6. Fermentation and ethanol production from CMC as a carbon source by recombinant strain *S. cerevisiae*-eb.

Symbols: ■, ethanol concentration; ▲, reducing sugar concentration; ●, cell counts.

Discussion

The yeast *S. cerevisiae* is a very attractive host microorganism for the one-step strategy to convert cellulosic materials to ethanol. It has high ethanol productivity close to theoretical yields (0.51 g ethanol produced/g glucose used), high ethanol tolerance, natural robustness in industrial processes and ease of genetic manipulation. In addition, it's generally regarded as a safe resource due to its long association with the food and beverage industries (Den Haan et al., 2007; Byrne et al., 2005). However, *S. cerevisiae* cannot decompose or assimilate cellulose due to the lack of cellulases. In this study, metabolic engineering was applied to the industrial strain *S. cerevisiae* AS2.489, which showed a high tolerance to ethanol and also a strong ability to produce ethanol in previous studies of our group (Tai, 2007).

The endoglucanases are active on the amorphous regions of cellulose and yield cellobiose and cello-oligosaccharides, while β -glucosidases degrade cellobiose and some cello-oligosaccharides to glucose (Den Haan et al., 2007). So the integration of these two cellulase genes in the genome of *S. cerevisiae* AS2.489 enabled the strain to degrade amorphous cellulosic materials such as CMC. In a similar previous study, phosphoric acid swollen cellulose (PASC) was used as a model substrate of amorphous cellulosic materials (Den Haan et al., 2007). The *eg3* and *bgl1* genes from *T. viride* were selected because they showed relatively higher activity compared with homologs from other fungi resources using the same expression method in *S. cerevisiae* AS2.489 (Tang, 2010).

Integration results and enzyme activity analysis confirmed that both genes were successfully integrated into the genome of *S. cerevisiae* and could be stably expressed and secreted into culture supernatants without induction (Figs. 2, 3, 5). The zymogram of secreted proteins showed that the expressed EG3 and BGL1 demonstrated a higher molecular weight than the theoretical one (Fig. 5). That might be because *S. cerevisiae* has a relatively stronger glycosylation mechanism compared with other eukaryotic systems such as *Pichia pastoris*. Thus, the site-directed mutagenesis in two potential *N*-glycosylation sites may identify whether they can affect

the enzyme activities. Some researchers chose a cell surface displaying method to express cellulases in *S. cerevisiae* (Yanase et al., 2010; Kotaka et al., 2008; Fujita et al., 2002; Fujita et al., 2004). A comparison study assumed that the ethanol yield of cellulase-displaying yeast (2.1 g/L) was higher than that of secreting yeast (1.6 g/L) (Yanase et al., 2010). However, using secreting expression, enzymes could be released into culture supernatants, and it would bring an easy retrieval and purification of cellulases for further potential application.

The time course of cell growth showed that the integration of two cellulase genes or one single gene did not significantly change the characteristics of *S. cerevisiae* cell growth. However, when comparing two genes' coexpression with individual expression, the enzyme activity level changed. The coexpression strain *S. cerevisiae*-eb showed lower endoglucanase activity than an *eg3* individual expressing strain (2.34 unit/ml and 4.31 unit/ml respectively) (Fig. 2), but its β -glucosidase activity slightly increased from 0.81 unit/ml to 0.95 unit/ml compared with a *bgl1* individual expressing strain (Fig. 3). Similar results occurred in coexpressing multiple cellulase genes using the same methods in our group. The coexpressing protein level and enzyme activity were lower than that of single gene expressing strains (Tai, 2007). This is a common problem occurring in multiple gene coexpression when each gene contains its independent expression cassette. There are some possible explanations for this phenomenon. One is called "promoter suppression," which affects heterogenous proteins' expression level depending on the types of vectors and promoters (Emmerman and Temin, 1986). Other factors such as the transcription level of mRNA, the limit of protein-folding efficiency, and/or the proteins' own stability must have major effects on the final yields of different proteins (Thill et al., 1990; Romanos, 1995).

The effects of pH, temperature and metal ions on both enzyme activities were analyzed. The optimal conditions of EG3 at pH 6.0 and temperature 60°C were a bit different from those expressed in recombinant baculoviruses, in which the maximum activity was exhibited under the conditions of pH 8.0 and temperature 50°C (Li et al., 2011). Therefore, the optimal conditions might change if the recombinant enzyme is expressed in different expression systems. Both enzymes maintained high activities at pH 5.0–6.0 (Fig. 4a, c), a condition close to the optimal growth pH of *S. cerevisiae* (pH 4.5–5.0) (Arroyo-López et al., 2009). However, the optimal temperature for both enzymes was 60°C, much higher than the growth and fermentation temperatures for yeast. Thus, the enzymes will work more efficiently if they are expressed in *S. cerevisiae* strains with stress tolerance to high temperature (Benjaphokee et al., 2012). Given the effects of the metal ions tested, only Ca^{2+} could enhance both enzyme activities (Tables 2 and 3). Therefore, it will be feasible to improve the activities by adding 10 mM Ca^{2+} to the co-expression system.

The fermentation results showed that the coexpression strain *S. cerevisiae*-eb could successfully grow in Y-CMC medium which only contained CMC, yeast extract and inorganic salts (Fig. 6). It demonstrated the ability of both utilizing CMC as single carbon source and converting it to ethanol directly. This method exactly fitted the one-step

strategy of converting cellulose to ethanol with only one microorganism. The ethanol concentration reached 4.63 g/L at 24 h (Fig. 6). In a previous study, a strain coexpressing *T. reesei* *egl* and *Saccharomycopsis fibuligera* *bgl1* converted PASC to ethanol with the highest concentration of 1 g/L at the far later time of 192 h (Den Haan et al., 2007). Comparatively, the ethanol production in our study was higher and more efficient. Another study reported fermentation of pretreated corn stover by yeast expressing three cellulase genes (Khramtsov et al., 2011). One similar study engineered yeast expressing two cellulase genes and the strain converted β -glucan to ethanol with a concentration of 9.15 g/L at 50 h (Jeon et al., 2009). The ethanol yield was higher than that in this study. The difference might result from lower enzyme activity in our study, and at the fermentation temperature of 30°C, EG3 and BGL1 only demonstrated 40.33% and 34.20% of the highest activities (Fig. 4). In conclusion, the strain *S. cerevisiae*-eb constructed in the present study demonstrated simultaneous saccharification and fermentation, as well as ethanol production ability. Such a finding might indicate its potential wide applications in industry.

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