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ORIGINAL ARTICLE

Genetically modified *Saccharomyces cerevisiae* for one-step fermentation of bioalcohol using corncob as sole carbon source

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Abstract The corncob is an important biomass for bioalcohol production. However, there is a minor but complicated pretreatment process before it is used for bioalcohol fermentation. In this study, three genetically modified *Saccharomyces cerevisiae* Y33 strains containing endoglucanase (EG), cellobiohydrolase (CBH), and β-glucosidase (BG) genes were constructed. A one-step fermentation process was carried out with the recombinants using corncob as the sole carbon source. In a 3-L fermentation system, the concentration of alcohol reached 2.02 g/L and the concentration of glycerine reached 0.85 g/L after 96 h. The results prove that corncob powder can be utilized effectively by genetically modified *Saccharomyces cerevisiae* without any chemical pretreatment. The mixed recombinant *Saccharomyces cerevisiae* cells show effective synergism in the one-step fermentation system. It is feasible that corncob can be used as the sole carbon source in bioalcohol production with a one-step fermentation process.

Keywords Cellulase · Genetically modified yeast · Corncob cellulose · One-step fermentation

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Introduction

It has been estimated that 1.3 billion mega-tons of terrestrial plants are produced annually on a world-wide basis (Demain et al. 2005). Due to its renewability, abundance, and eco-friendliness, lignocellulosic biomass is the only feedstock that could potentially replace fossil fuels (Lynd et al. 2005). Nowadays, fermentation engineering with natural biomass has been applied in many fields, such as new energy (Liu et al. 2012), the food industry (Battcock and Azam-Ali 1998), chemical materials (Wang et al. 2012), and so on. Cellulose is an important natural biomass, but efficient utilization of this resource needs improvement.

Lignocellulosic biomass is efficiently degraded by the synergistic action of the cellulolytic enzymes endoglucanase (EG), cellobiohydrolase (CBH), and β-glucosidase (BG). Although there are numerous reports of lower-cost alcohol production from cellulosic material by consolidating hydrolyzing and fermentation steps using recombinant *Saccharomyces cerevisiae* strains expressing cellulolytic enzymes (Fujita et al. 2004; Tsai et al. 2009; Wen et al. 2010), the efficiency of cellulose degradation by this method has not been sufficiently improved, which may be due to the lack of synergy between different cellulolytic enzymes. Several filamentous fungi that express various cellulolytic enzymes and simultaneously control their expression in response to their environment are capable of effective cellulose degradation. The various cellulase proteins interact synergistically, so it is important that the ratios of the cellulases are appropriately balanced to achieve the maximum hydrolysis rate (Dashtban et al. 2009; Stricker et al. 2008).

Ethanol is one of the most important and prevailing products in fermentation engineering. Many researchers have tried to find a suitable method to improve ethanol productivity and productive technology. There are several fermentation pathways for producing ethanol, including direct fermentation, indirect fermentation, mixed bacteria fermentation (MBF),

separate hydrolysis and fermentation (SHF), simultaneous saccharification and fermentation (SSF) (Olofsson et al. 2008), etc.

SSF is a process option for the production of ethanol from lignocelluloses (Hari et al. 2001). In a previous study, the combination of the SSF and pentose fermentation (PF) processes resulted in the production of 144 mg/g of ethanol from non-pretreated Napier grass powder (Yasuda et al. 2012). SSF is currently the most efficient and chief process for producing bioalcohol from corn (Bothast and Schlicher 2005). Many studies have shown that most cellulosic biomass is not fermentable without appropriate pretreatment. Pretreatment with dilute sulfuric acid and steam explosion is widely used, but enzymatic pretreatment is a good alternative method (Gomathi et al. 2012). Mutreja et al. (2011) used recombinant cellulase to produce ethanol by SSF and Yu Shen et al. expressed β-glucosidase in *Saccharomyces cerevisiae* for ethanol fermentation by SSF (Yu et al. 2008). However, partial acid pretreatment was still performed in the most current studies.

Fermentation in combination with pretreatment by the fungal strains themselves in the same system, without any acid pretreatment, is an effective means of producing ethanol while conserving energy and protecting the environment. In this study, three genetically modified strains of cellulose secreting *Saccharomyces cerevisiae* were constructed. One-step fermentation of bioalcohol using corncob as the sole carbon source was subsequently carried out with these genetically modified *Saccharomyces cerevisiae*.

Materials and methods

Strains, plasmids and media

Escherichia coli DH5α, used as the recipient strain for recombinant plasmids, was grown in LB medium (1 % tryptone, 0.5 % yeast extract, 1% NaCl, pH 7.0) at 37°C. *Saccharomyces cerevisiae* Y33 (*His*$^-$ *leu*$^-$ *ura*$^-$ *Ade*$^-$) was used as the host for expression of cellulase genes. The yeast cells were grown in YPD (1 % yeast extract, 2 % peptone, 2 % dextrose) medium at 28 °C. The recombinants were selected in MD (13.4 % YNB, 0.0004 % biotin, 20 % glucose) with 0.02 % His, Leu, and Ade. Galactose was used as the inducing substance for cellulase expression. The expression vector pHBM368 (P_{Gal1}, α-Factor Signal Sequence, *Kan*^{r}, *CYC1* TT, rDNA, *Amp*^{r}, *Col*E1 ori, *URA*3) was conserved in our lab.

Nucleic acid manipulation and yeast transformation

DNA was purified and manipulated essentially as described by Sambrook and Russell (2001). After analyzing the exoglucanase gene (CBH, GeneBank: AY861348) from *Chaetomium thermophilum* and the endoglucanase (EG, GeneBank: EU169241) and β-glucosidase genes (BG, GeneBank: AF163097) from *Thailand red sandalwood*, the three cellulase genes were synthesized, respectively (Generay Biotech Co., Ltd, Shanghai). The restriction enzyme sites *Hin*dIII and *Xba*I were included in the 5'- and 3'- terminals of CBH and BG, respectively, and the restriction enzyme site *Hin*dIII and *Spe*I were included in the 5'- and 3'- terminals of EG.

The *Saccharomyces cerevisiae* Y33 was transformed with linearized plasmid, based on the method described by Kawai et al. (2010). 10 mL of YPD medium was inoculated with a colony of Y33 and shaken overnight at 30 °C. The OD_{600} of the overnight culture was determined. The culture was diluted to an OD_{600} of 0.4 in 50 mL of YPD medium and grown an additional 2–4 h. The cells were pelleted at 1500×*g* and the pellet resuspended in 40 mL 1× TE; then the cells were pelleted at 1500×*g* and the pellet resuspended in 2 mL of 1× LiAc/0.5× TE. Before the cells were used for transformation, they were incubated at room temperature for 10 min. For each transformation, 1 μg plasmid DNA and 100 μg denatured sheared salmon sperm DNA with 100 μL of the yeast suspension were mixed together.

One-step fermentation

After being shattered into powder or tiny particles, the corncobs were used as the sole carbon source in the fermentation medium. The recombinants and the control were cultured in 50 mL YPD for 16 h at 28 °C, shaking at 200 rpm. After the OD_{600} reached 2.0, they were used as inoculation seed. The fermentation was performed at pH 7.6 and 28 °C, shaking at 250 rpm. The inoculation amount was 10 % (v/v).

Analytical methods

The reducing sugar concentration was assayed using 3, 5-dinitrosalicylic acid (DNS) method (Gusakov et al. 2011), and the cellulose activity was calculated according to reducing sugar concentration. Because glycerine and Cu^{2+} generated mazarine complex under alkaline conditions (Yan and Qiu 2004), the complex exhibited the maximum absorbance at wavelength 630 nm. The concentration of glycerine was determined as described by Pflügl et al. (2012). The concentration of ethanol was determined using the method described by Choi et al. (2012).

The standard deviation was calculated in data analysis, and could reflect dispersion degrees of a data set. In the figures, the error bars were added on trend lines where the standard deviation was less than 5 %.

Results

Construction of genetically modified *Saccharomyces cerevisiae*

The cellulase gene *CBH* was amplified with the primers CBHf-*Hin*dIII and CBHr-*Xba*I (CBHf-*Hin*dIII 5′-tcaaagcttcttgcggccatttcgctg-3′; CBHr-*Xba*I 5′-tattctagatcagaacggagggttggca-3′), and the gene *BG* was amplified with the primers BGf-*Hin*dIII and BGr-*Xba*I (BGf-*Hin*dIII 5′-attaagcttctattgactttgcaaaaga-3′; BGr-*Xba*I 5′-atatctagactaaaagccttcaatgcccct-3′). Both products were digested with *Hin*dIII and *Xba*I. The gene *EG* was amplified with EGf-*Hin*dIII and EGr-*Spe*I (EGf-*Hin*dIII 5′-tcaaagcttttaccagaacggttccgaaatc-3′; EGr-*Spe*I 5′-tatactagtttaggagctagattgtggagca-3′), and the product digested with *Hin*dIII and *Spe*I. Then they were ligated with the expression vector pHBM368, which was digested with *Hin*dIII and *Xba*I for CBH and BG cloning, or *Hin*dIII and *Spe*I for EG cloning. The cellulase genes *CBH*, *BG*, and *EG* were cloned into the expression vector pHBM368 with the ORF of the mature gene cloned in-frame and downstream of the α-factor signal sequence, which had been identified by sequencing, for secreting recombinant protein into the medium. The recombinant plasmids with *CBH*, *BG*, and *EG* were named pHBM368-CBH, pHBM368-BG, and pHBM368-EG, respectively (Fig. 1).

The recombinant plasmids were linearized with *Hpa* I and transformed into *Saccharomyces cerevisiae* Y33 with chemical transformation, respectively, as described in the Methods section. The recombinants were screened by modified MD (13.4 % YNB, 0.0004 % biotin, 20 % galactose, 0.02 % His, 0.02 % Leu, 0.02 % Ade) plates supplemented with carboxymethyl cellulose (CMC) as the substrate and trypan blue as the indicator. The expression patterns were determined by halo around the colony (Fig. 2). The transformants with the maximum cellulase activity (bigger halo around the colony implied better cellulase activity) were inoculated and named

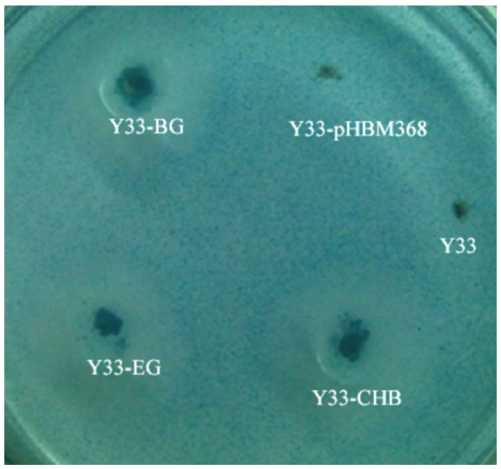

Fig. 2 Recombinants screened by modified MD plates, carboxymethyl cellulose, and trypan blue. Y33-CBH, recombinant *Saccharomyces cerevisiae* Y33-CBH; Y33-EG, recombinant *Saccharomyces cerevisiae* Y33-BG; Y33-EG, recombinant *Saccharomyces cerevisiae* Y33-EG; controls: Y33, original strain *Saccharomyces cerevisiae* Y33; Y33-pHBM368, *Saccharomyces cerevisiae* Y33 transformed with the control plasmid pHBM368. The recombinants were determined by halo around the colony

Saccharomyces cerevisiae Y33-CBH, *Saccharomyces cerevisiae* Y33-BG, and *Saccharomyces cerevisiae* Y33-EG, respectively. By using the total DNA of *Saccharomyces cerevisiae* Y33-CBH, *Saccharomyces cerevisiae* Y33-BG, and *Saccharomyces cerevisiae* Y33-EG as the template, DNA fragments with the same sizes as cellulase genes *CBH*, *BG*, and *EG* were obtained by PCR amplification, confirming that the cellulase genes were integrated into *Saccharomyces cerevisiae* Y33, respectively.

Expression and detection of cellulase

Expression of cellulase was performed in 500-mL conical flasks. Aliquots of culture supernatants were taken daily and analyzed by SDS-PAGE, and the cellulase activity was

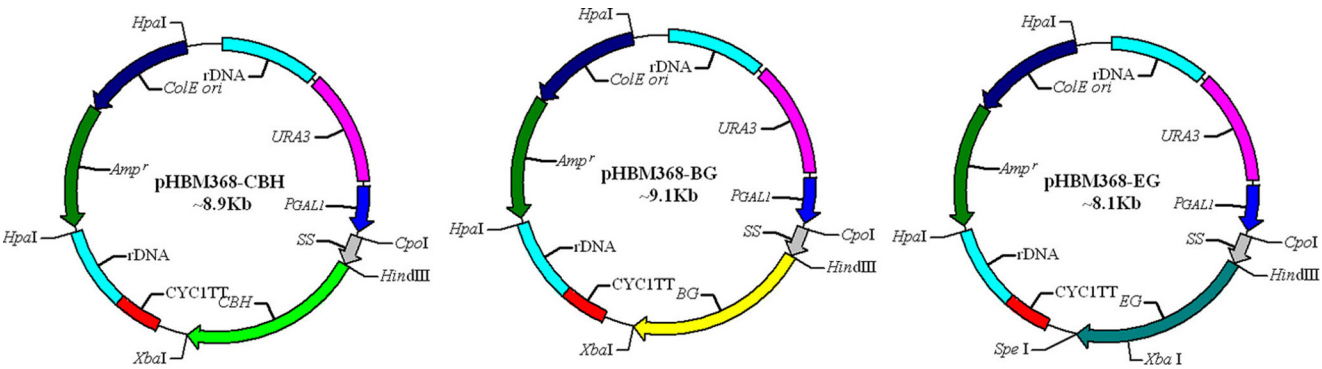

Fig. 1 The structure of the recombinant plasmids. P_{GAL1}, gal1 promoter; *SS*, α-factor signal sequence; *CBH*, *BG*, *and EG*, cellulase genes cloned in frame and downstream of the α-factor signal sequence; CYC1 TT, transcriptional terminator from *CYC1* gene; rDNA, rDNA fragment from *Saccharomyces cerevisiae* for heterogeneous gene integration; *Amp*^{r}, Ampicillin resistance gene; *CoE1*: *Escherichia coli* origin of replication; *URA3*, selection marker

assayed at the same time. The proteins expressed by recombinant strains were analyzed by SDS-PAGE, and the expected apparent molecular weight heterologous bands could be observed. The cellulase activity reached its maximum (3.96 U/mL, 3.01 U/mL, and 3.89 U/mL, respectively, assayed at 50 °C, pH 7.0) after 3 days of induction.

Simulation fermentation and scale-up fermentation

Three recombinant yeast strains (*Saccharomyces cerevisiae* Y33-CBH, *Saccharomyces cerevisiae* Y33-BG, and *Saccharomyces cerevisiae* Y33-EG), the original yeast strain (*Saccharomyces cerevisiae* Y33), and an empty vector control (*Saccharomyces cerevisiae* Y33 transformed with the control plasmid pHBM368) were activated as fermentation seed. When the OD_{600} reached 2.0, the seed was inoculated into 1-L conical flasks, which included 100 mL liquid medium with corncob powder as the sole carbon source. Meanwhile, a mixed inoculation of the three recombinant yeasts, with equal ratios of each, was carried out. The biomass, ethanol concentration, and glycerine concentration were determined every 12 h and reduplicated three times.

The biomass of recombinant yeasts, evaluated by OD_{600}, was substantially higher than the blank control and original yeast (Fig. 3). The OD_{600} of the original and the blank control groups could only maintain an OD_{600} of about 0.3, meaning no growth. The OD_{600} of the three recombinants reached above 1.1, meaning better growth, while the OD_{600} of the mixed inoculation group reached 2.1, indicating the best growth. The original and the blank control yeast could not use the corncob as a carbon source to grow, but the genetically modified yeasts could. The result of the mixed inoculation group also indicated that the synergistic action of the different cellulases was positive and effective.

The ethanol and glycerine concentrations of the various groups showed a pattern similar to the biomass. The ethanol and glycerine concentrations of the mixed inoculation group were higher than those of the other groups (Figs. 4 and 5). The concentration of alcohol reached 2.02 g/L, and the concentration of glycerine reached 0.85 g/L after 96 h. The results showed that the synergistic effect of different cellulases was necessary and sufficient for one-step bioalcohol fermentation using corncob as the sole carbon source.

Scale-up fermentation of the simulation fermentation process was performed in a 3-L triangular flask, and a similar result was achieved.

Discussion

In simulation fermentation, the biomass of the three recombinants increased substantially. The maximum biomass of the recombinants could reach three times that of the controls, and the growth curves of the controls decreased over time (Fig. 3), illustrating that the yeast of the controls could not grow and that some of them died out, perhaps because of the lack of a carbon source.

The lignocellulose of corncob was utilized effectively by genetically modified yeast, and the concentration of ethanol and glycerine showed a positive correlation with the synergistic effect of the three cellulases. The ethanol concentration of the mixed inoculation group increased about 25 % more than the single recombinant yeast. The mixed fermentation of genetically modified yeast was a potential process for bioalcohol production from various lignocellulose sources.

In previous reports, pretreatment with dilute acid or steam explosion was widely applied to hydrolyze the corncob for bioalcohol fermentation. The corncob used in this study was only ground into powder by micromill, and there was no other pretreatment with acid or steam explosion. It was a green and low energy-consuming process for cellulose bioalcohol, and it has a potential industrial application with low cost and low environmental impact. Optimization of the fermentation process is necessary to improve the utilization ratio of corncob and the production coefficient of bioalcohol in future work.

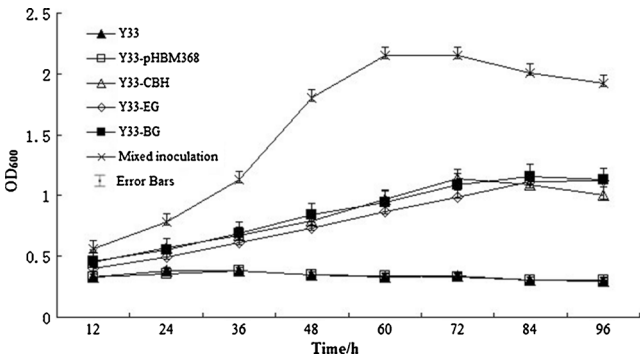

Fig. 3 Biomass curve of one-step fermentation. The biomass of each fermentation system was evaluated by OD_{600}, and was determined every 12 h. The OD_{600} of the mixed inoculation group reached 2.1, indicating the best growth

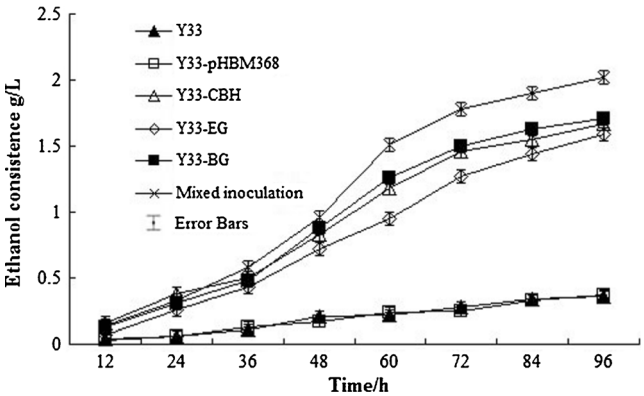

Fig. 4 Ethanol concentration curve of one-step fermentation . The ethanol concentration of each fermentation system was determined every 12 h. The concentration of alcohol reached 2.02 g/L after 96 h

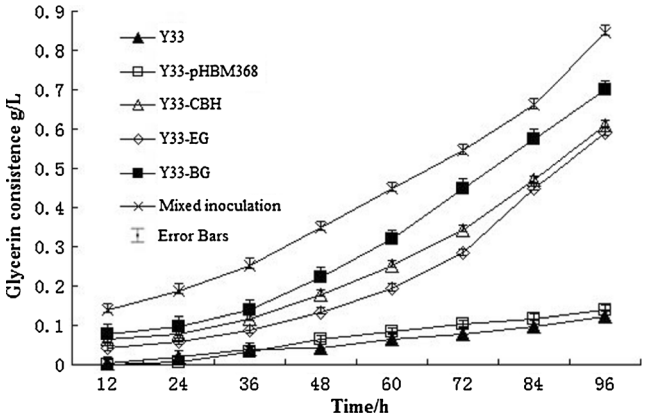

Fig. 5 Glycerine concentration curve of one-step fermentation. The glycerine concentration of each fermentation system was determined every 12 h. The concentration of glycerine reached 0.85 g/L after 96 h

In this study, three genetically modified *Saccharomyces cerevisiae* Y33 strains were constructed. The three recombinant strains were used in one-step fermentation of bioalcohol using corncob as the sole carbon source. The results proved that the corncob powder could be utilized effectively by genetically modified *Saccharomyces cerevisiae* without acid pretreatment. It is feasible that corncob could be used as the sole carbon source in bioalcohol production with a one-step fermentation process. This tactic could be extended to other cellulose biomass for bioalcohol.

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