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MFα Signal Peptide Enhances the Expression of Cellulase *eg1* Gene in Yeast

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Abstract Ethanol production from lignocellulose by recombinant yeast with high level expression of heterologous cellulase genes has been a major anticipation. The native secretion signal sequence of the cellulase endoglucanase I (*eg1*) gene was replaced by *Saccharomyces cerevisiae* mating factor α prepro-leader sequence (MFα). The transformants containing native secretion signal (Y_1) and MFα secretion signal (Y_2) were characterized with respect to gene expression and growth on cellulose substrate. Increased enzyme activity and cellulose utilization were observed. The enzyme activity of Y_2 was 0.084 U/ml, 61.5% higher than Y_1 (0.052 U/ml). The sufficiency parameter (S value) was raised from 0.6 to 0.84. MFα signal peptide was more efficient than the native signal peptide of *eg1* gene, suggesting that signal peptide replacement is an efficient way to enhance the cellulase expression level in yeast, for cellulose-derived ethanol production.

Keywords Gene expression · Signal peptide · Endoglucanase I · Yeast

Introduction

Lignocellulose is an important source of biomass. Lignocellulose-derived ethanol was widely recognized as an attractive and promising substitute for gasoline [1, 2]. The main production processes involve the conversion of lignocellulose to glucose, in which cellulases are employed to convert the cellulosic biomass to glucose [3, 4]. However, the cellulases are currently too expensive to be widely used for lignocellulosic biomass production [5]. A promising strategy to meet this challenge involves expressing cellulase

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genes in yeast to endow it with a combination properties of cellulose hydrolysis and ethanol production without addition of cellulase [6, 7].

Cellulase complex consists of three types of enzymes: endoglucanase (EG) which cleaves the cellulose chain and produces nicks inside, cellobiohydrolase (CBH) which acts on the exposed ends and produces cellobiose units, and beta-glucosidase (BGL) which hydrolyzes the cellobiose units to glucose. To meet the requirement of cellulose-derived ethanol production, heterologous expression of cellulase genes in *Saccharomyces cerevisiae* has been an object of investigation and considerable success has been achieved enabling expression of cellulase genes in *S. cerevisiae*, with examples including EG, CBH, and BGL [8]. Fujita et al. [9] constructed a yeast strain codisplaying three types of cellulolytic enzyme including glucosidase I, endoglucanase II, and cellobiohydrolase II, with ethanol yield up to 0.45 g/g (in grams of ethanol produced per gram of carbohydrate consumed), which corresponds to 88.5% of the theoretical yield, indicating that fermentation of amorphous cellulose to ethanol can be efficiently accomplished using a yeast strain codisplaying the three cellulolytic enzymes. Den et al. [10] investigated the expression of four fungal cellulase-encoding genes in yeast. All genes were successfully expressed and similar extracellular activity was demonstrated. Their results suggested that although heterologous cellulase genes could be produced in *S. cerevisiae*, the titers of functionally secreted enzymes were relatively low. Future studies should aim to increase the expression level. Improvement of the expression levels of cellulase genes in yeast has been a major anticipation in the research field of cellulase-derived ethanol production.

As we know, many factors may affect protein expression. There are many alternatives to enhance the expression level of recombinant proteins, including codon bias selection, promoter selection, copies enhancement, and signal peptides designing [11]. Secretory proteins, which have been synthesized in cell, are needed to transverse the cell membrane and secret to the outerspace of the cell. Signal peptide plays an important role in the process of protein transfer and secretion. Three strategies are mainly used in the design of signal peptide. The first strategy is using the included signal peptide in the gene expressed. The second is using the hetero-signal peptide from other genes, and the third is using the artificially designed signal peptide. In the present work, the signal peptide of *eg1* gene with mating factor α prepro-leader sequence (MFα) signal peptide were replaced, and the effect of the replacement on gene expression, utilization of non-native substrate and ethanol production were investigated.

Materials and Methods

Strains, Growth Media, and Cultivation

Escherichia coli strain DH5α was used for plasmid transformation and propagation. It was grown in LB medium (5 g/L yeast extract, 10 g/L NaCl, 10 g/L tryptone) supplemented with ampicillin (100 mg/L). *S. cerevisiae* strain M15 (MATα, leu2-3/112, ura3-52, trp1-9) was used for *eg1* gene expression. *S. cerevisiae* M15 transformants were selected and maintained on YSD medium plates (6.7 g/L yeast nitrogen base, 20 g/L glucose, 0.3 g/L L Leucine, 0.05 g/L adenine, 0.2 g/L inositol, 20 g/L glucose, 0.3 g/L histone, 0.3 g/L Tryptone). For cellulose fermentation experiments, 10 g/L sodium carboxymethyl cellulose (CMC; DS=0.7) was used as carbon source in YSD medium without glucose. Plasmid pPIC9K was purchased from Invitrogen corporation, and Plasmid pRUL129 was kindly

provided by Prof. Steensma, Delft University of Technology [12]. *Trichoderma viride* CICC13038 was used for *eg1* gene cloning, and was cultivated at 30 °C on PDA medium. For cellulase expression and RNA isolation, it was grown on cellulase-inducing medium, containing 8 g/L microcrystalline cellulose, 1.4 g/L $(NH_4)_2SO_4$, 0.3 g/L urea, 2.0 g/L KH_2PO_4, 0.3 g/L $CaCl_2$, 0.3 g/L $MgSO_4{\cdot}7H_2O$, 1.0 g/L tryptone, 2.0 g/L Tween 80; 5.0 mg/L $FeSO_4{\cdot}7H_2O$, 1.4 mg/L $ZnSO_4{\cdot}7H_2O$, 1.6 mg/L $MnSO_4{\cdot}H_2O$, 2.0 mg/L $CoCl_2$, pH 5~6). Recombinant enzyme production was carried out in 150 mL shake flask cultures. Yeast strains were grown at 30 °C for 5 days on concentrated YPD media (10 g/L yeast extract, 20 g/L peptone, and 50 g/L glucose). The cultures were centrifuged at 3,000 rpm for 10 min to remove yeast cells, the supernatants were filtered through a glass fiber filter, and eg1 activity was tested.

Strain Construction

Sambrook and Russell's Standard protocols [13] were followed for DNA and RNA manipulations. Restriction endonucleases, T_4 DNA ligase and pfu DNA polymerase were purchased from Promega Corporation and used as recommended by the manufacturer. Details of the primers used in this study are given in Table 1. For expression of eg1 encoding genes in *S. cerevisiae*, the plasmids pRUL-eg1 and pRUL-MFα-eg1 were constructed. To construct pRUL-eg1, the *eg1* gene was amplified using PCR primers EGI-F and EGI-R. The resulted fragments were digested with *Xba*I and *Sal*I and then cloned into the corresponding restriction sites of pRUL129 by replacing the SFA1 cassette of pRUL129. For construction of pRUL-MFα-eg1, the 273-bp MFα secretion signal sequence was amplified from pPIC9k using PCR primers MF-F and MF-R. The *eg1* gene without secretion signal sequence was amplified from first strand cDNA prepared from *T. viride* with primers EGI-F and EGI-R. Overlap PCRs with primers MF-F, MF-R, EGI-F, and EGI-R were taken to replace the native secretion signal with MFα secretion signal sequence. The resulted fragments were digested with *Xba*I and *Sal*I and then cloned into the corresponding restriction sites of pRUL129.

Measurement of Growth, Substrates, and Products

For evaluation of yeast growth, the yeast cells were centrifuged and the dry weights were taken. Ethanol concentration was determined by gas chromatography (GC) with a micro-packed column and a flame ionization detector. The GC oven temperature was maintained at 150 °C. The injector and detector temperature was held at 240 °C. Glucose concentration was determined by 3,5-dinitrosalicylic acid (DNS) reagent. Add 3 mL of 1% DNS reagent

Table 1 The primers used in plasmid construction.

Primers	Sequence	
EGI-F	CCCTCTAGATCTTAGTCCTTCTTGT	*Xba*I
EGI-R	TTTGTCGACCATTTGGTAATGTAGA	*Sal*I
MF-F	TCCTCTAGATGAGATTTCCTTCAAT	*Xba*I
MF-R	CCGGTTGCTGAGCTTCAGCCTCT CTTTTCT	Overlap sequence
EGS-F	GGCTGAAGCTCAGCAACCGGGT ACCAGCAC	Overlap sequence

(dinitrosalicylic acid 10 g, phenol 2 g, sodium sulfite 0.5 g, sodium hydroxide 10 g, add water to 1 L) to 3 mL of glucose sample in a lightly capped test tube, and then heat the mixture at 90 °C for 5–15 min to develop the red-brown color. Add 1 mL of a 40% potassium sodium tartrate solution to stabilize the color. After cooling to room temperature in a cold water bath, record the absorbance with a spectrophotometer (Molecular Devices Corporation) at 575 nm. CMC concentration was determined by acid precipitation test. Five milliliters CMC solution was taken and 5 mL 10% HCl was added. Centrifuged at 12,000 rpm for 30 min, and then dried in a baking oven and measure the weight. For graphing the standard curve, 0%, 0.2%, 0.4%, 0.6%, 0.8%, and 1% CMC solutions were prepared, plotting the CMC concentration on the *x*-axis, and dried weight on the *y*-axis. The same assay was then performed with samples of unknown concentration.

Measurement of Enzyme Activity

Endoglucanase I activity in culture supernatants was assayed. Quantitation of endoglucanase I activity was done by determining the amount of sugar generated from carboxymethylcellulose (CMC). Briefly, 0.5 mL of culture supernatant was incubated with 0.5 mL of 0.05 M citrate buffer, pH 4.8, containing 1% CMC, 10 mM dithiothreitol, and 0.02% (*w*/*v*) NaN_3. The assay was performed aerobically at 37 °C for 30 min. Generated sugars were assayed colorimetrically using dinitrosalicylic acid reagent.

Evaluation of the Sufficiency of eg1 Expression

For evaluation of the growth ability of the recombinant yeast strains on CMC, Sufficiency parameter *S* was undertaken as depicted by McBride et al. [14], using the equation $S=a/q_{max}$, where parameter a was glucose produced (grams per cell per hour) by CMC enzymolysis, representing the average activity of heterologous enzyme. "q_{max}" means substrate consumption rate of glucose (grams per cell per hour). q_{max} was calculated based on the specific growth rate μ_{max} (grams per cell per hour), and the cell yield $Y_{x/s}$ (grams per cell per grams per glucose) using equation $q_{max}=\mu_{max}/Y_{x/s}$.

The sufficiency parameter *S* with a value equal to 1 means that the glucose produced by CMC degradation are just the weigh needed for growth. The *S* less than 1 means that the glucose produced by CMC degradation is not enough for growth, in other words, the heterologous cellulase expressed are not sufficient.

Results

Cloning and Expression of eg1 Gene in *S. cerevisiae*

RNA was extracted from CICC13038. RT-PCR was done and the *eg1* gene was cloned. Sequence analysis (Genbank accession number: Eu587012) revealed that this gene had a similarity of 99% to the *eg1* gene reported (Genbank accession number: AY343986). Then the *eg1* gene was cloned into the pRUL129 vector with native eg1 secretion signal sequence (pRUL-eg1) and MFα secretion signal sequence (pRUL-MFα-eg1), respectively, as shown in Fig. 1. These two expression vectors were subsequently transformed into *S. cerevisiae* M15, to create recombinant strains Y_1 (pRUL-eg1) and Y_2 (pRUL-MFα-eg1). Y_0 (without eg1) was used as a control. Transformants were confirmed by PCR analysis on total DNA isolates.

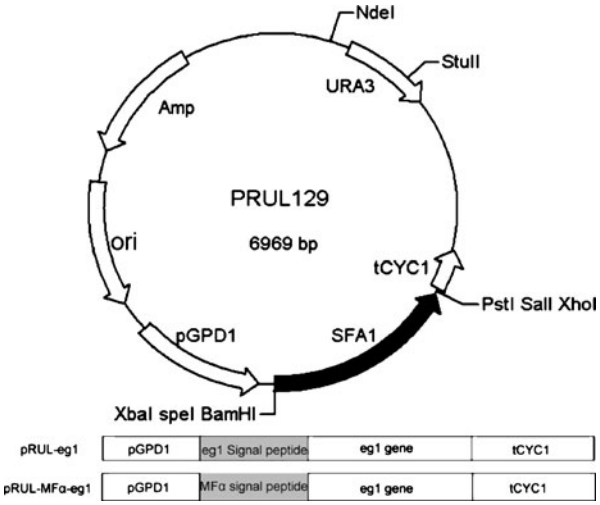

Fig. 1 Expression cassette for eg1 with native secretion signal and MFα secretion signal. The *eg1* gene and MFα-eg1 gene were cloned in the *Xba*I and *Sal*I sites, replacing the SFA1 gene of pRUL129

Effects of Signal Peptide on Enzyme Activity

Recombinant yeast clones were selected and cultured in YPD medium. The EGI activity was tested by method 2.4, as shown in Fig. 2. The two recombinants of Y_1 and Y_2 resulted in the highest enzyme activity after 72-h shake culture, with the activity of 0.052 U/ml and 0.084 U/ml, respectively. As compared with that of Y_1, the activity of Y_2 was improved by 61.5%.

Growth Data of the Recombinant Strain

The recombinant yeast strains Y_1 (pRUL-eg1) and Y_2 (pRUL-MFα-eg1) were cultured in the medium containing 1% CMC or glucose. Y_0 (without eg1) was used as a control. Concentrations of substrates, cells, and products throughout the experiment were

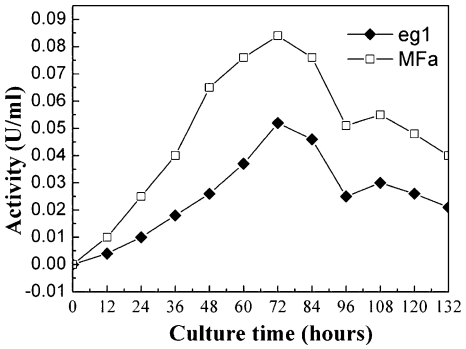

Fig. 2 Comparism of the enzyme activity for the recombinant yeast strains with the native signal peptide of endoglucanase I and MFα signal peptide. Endoglucanase I activity in culture supernatants was assayed by determining the amount of sugar generated from carboxymethylcellulose (*CMC*). The data shown were the mean value of three independent experiments ($p<0.05$)

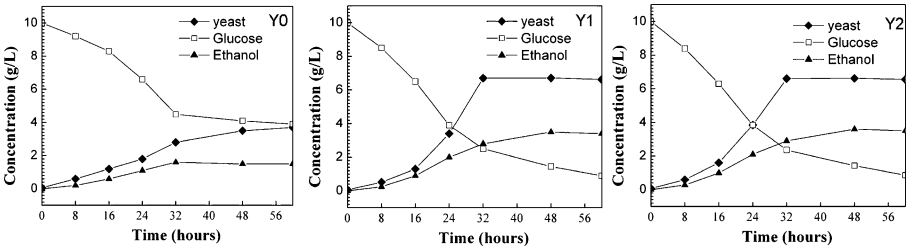

Fig. 3 Glucose consumption, cell mass, and ethanol yield for Y_0, Y_1, and Y_2 on glucose. The yeast strains Y_0 (without plasmid), Y_1 (with pRUL-eg1), and Y_2 (with pRUL-MFα-eg1)were cultured in the medium containing 10 g/L glucose. The data shown were the mean value of three independent experiments ($p<0.05$)

monitored. Very similar data sets were observed for Y_1 and Y_2 when they were cultured with glucose (Fig. 3). However, when grown on CMC, Y_2 exhibited faster substrate consumption and growth, and produced more ethanol than Y_1 (Fig. 4).

Specific Growth Rate and *S* Value

Based on the equation that the logarithmic value of cell concentration is directly proportional to growth time ($\ln X_t = \ln X_0 + \mu t$, X: cell concentration), the growth data of Y_0, Y_1, and Y_2 were fitted and the growth rates (μ values) of the three strains in glucose or CMC were obtained (Fig. 5).

Cell-specific activity of the heterologous enzyme, the parameter a, glucose produced by CMC, grams per hour, along with substrate conversion rate $Y_{x/s}$, grams cells per grams substrate, and the cell yield on glucose μ_{max}, grams per hour, were used to calculate the dimensionless sufficiency *S* (Table 2).

The sufficiency parameter *S*, which represents the expression level of eg1, was 0.12, 0.35, 0.82 for Y_0, Y_1, and Y_2, respectively. These data showed that the expression level for Y_2 were much higher than Y_1. In other words, the enzyme expressed by Y_2 was more sufficient for the yeast growth in the aspect of hydrolyzing CMC to glucose as growth carbon source.

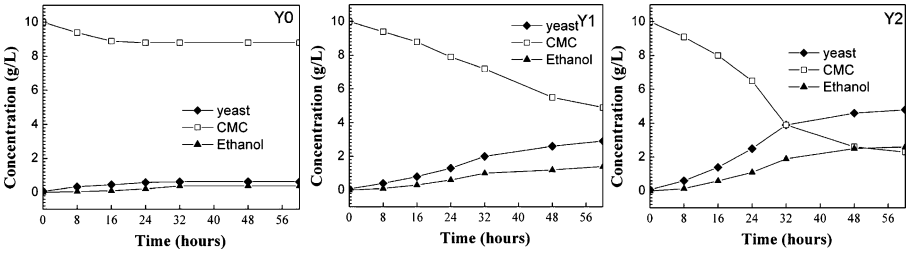

Fig. 4 Glucose consumption, cell mass, and ethanol yield for Y_0, Y_1, and Y_2 on carboxymethylcellulose (*CMC*). The yeast strains Y_0 (without plasmid), Y_1 (with pRUL-eg1), and Y_2 (with pRUL-MFα-eg1) were cultured in the medium containing 10 g/L CMC. The data shown were the mean value of three independent experiments ($p<0.05$)

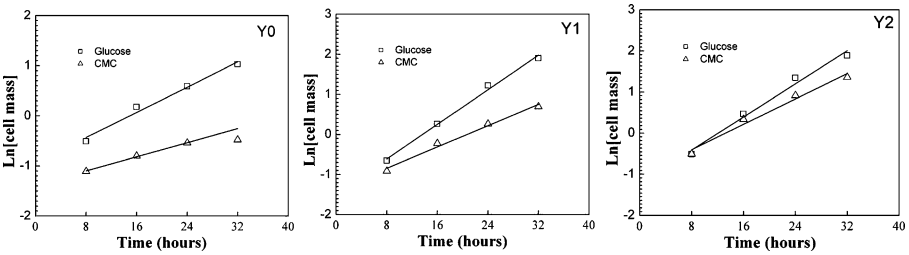

Fig. 5 Growth rate of Y_0, Y_1, and Y_2 on glucose and CMC. The logarithmic value of cell mass for Y_0, Y_1, and Y_2 in logarithmic phase from 8 to 32 h was taken. The data was fitted by the equation $\ln X_t = \ln X_0 + \mu t$ (X: cell concentration)

Discussion

The effects of the MFα secretion sequence on the expression level of the fungal EGI encoding genes in *S. cerevisiae* were showed in this research. The two yeast strains, which owned the plasmid of recombinant expression cassette of native secretion sequence and MFα secretion sequence were investigated in detail. Great enhancement of the enzyme activity was observed by the MFα signals. It was also demonstrated that the growth parameters of the recombinant yeast strains on the media of glucose and CMC were different. Finally, by employing sufficiency parameter, it was found that there was significant difference of the enzyme activity between the native and MFα signals.

In the authors' prior work, the cellulase genes of CBHI, EGI, and BGL had been cloned, and efforts had been made to express these genes in yeast for ethanol production, but the results of low expression level cellulase expression in yeast had been puzzling. The observation in this study that the activity of recombinant EGI could be enhanced by MFα signal peptide should be a fine progress for the problem. Consequently it is concluded that MFα signal peptide is more efficient than the native signal peptide sequence of *eg1* gene, and signal peptide replacement is an efficient way for the enhancement of the eg1 expression level in yeast. This result also shed lights on the expression of other cellulase genes such as CBH and BGL. The sufficient parameter S in this research reached 0.84, indicating that the glucose produced by cellulose hydrolysis is almost enough for yeast growth. However, there is still a long way towards the production of high concentration ethanol. More substantial effort is required for improvement of the expression level and realization of low-cost production of cellulose ethanol.

Table 2 The sufficiency parameter (S value) for Y_0, Y_1, and Y_2.

Strain	Substrate	Growth rate (g/h)	Glucose consumed (g/h)	Cell yield (g cells/g glucose)	Glucose produced by CMC (g/h)	Sufficiency parameter
Y_0	Glucose	0.0628	0.20	0.31	0.02	0.10
	CMC	0.0352				
Y_1	Glucose	0.1079	0.25	0.43	0.09	0.36
	CMC	0.0737				
Y_2	Glucose	0.1009	0.25	0.40	0.21	0.84
	CMC	0.0774				

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