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RESEARCH ARTICLE

Construction of a recombinant yeast strain converting xylose and glucose to ethanol

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Abstract *Candida shehatae* gene *xyl1* and *Pichia stipitis* gene *xyl2*, encoding xylose reductase (XR) and xylitol dehydrogenase (XD) respectively, were amplified by PCR. The genes *xyl1* and *xyl2* were placed under the control of promoter *GAL* in vector pYES2 to construct the recombinant expression vector pYES2-P12. Subsequently the vector pYES2-P12 was transformed into *S. cerevisiae* YS58 by LiAc to produce the recombinant yeast YS58-12. The alcoholic ferment indicated that the recombinant yeast YS58-12 could convert xylose to ethanol with the xylose consumption rate of 81.3%.

Keywords recombinant *Saccaromyces cerevisiae*, *xyl1*, *xyl2*, xylose, glucose, ethanol

1 Introduction

With the rapid development of the global economy, the magnitude of fuel consumption has been increasing very fast, resulting in environmental pollution and scarcity of natural resources. Therefore, the exploitation and utilization of clean and renewable energy have become very necessary. At present, the most abundant renewable energy around the world is stored in plants and most of which is lignocellulose (Bruce, 2000). Lignocellulose is a complex material. About 30% of its weight is accounted for by cellulose, lignose and hemicellulose. It can be hydrolyzed by acids or enzymes and converted into pentoses (xylose and arabinose) and hexoses (glucose, galactose and mannose). Hexoses and pentoses account for 2/3 and 1/3 of the total sugars, respectively. Xylose, however, accounts for about 90% of hemicellulose hydrolysates (Gong et al., 1999). *Saccharomyces cerevisiae*, an excellent strain for industrial ethanol production, has a greater resistance to ethanol and lignocellulose hydrolysates than bacteria (Xie et al., 2001). *Saccharomyces cerevisiae* can easily ferment hexoses, but hardly xylose in lignocellulose hydrolysates, because *S. cerevisiae* lacks enzymes that convert xylose to xylulose. However, *S. cerevisiae* can ferment xylulose. Therefore, if genetic engineering strains and mix sugars were used as substrates, the total ethanol output will increase by 25% theoreticall,y and the cost of production will be reduced (Aristos and Merja, 2000). To ferment xylose easily, a metabolic pathway (converting xylose into xylulose) was introduced to *S. cerevisiae*. Through the metabolic system including pyruvic acid decarboxylase (PDC) and alcohol dehydrogenase (ADH), yeast and filamentary fungi can ferment xylose. First, xylose is reduced to xylitol by xylose reductase (XR) that can accept either NADH or NADPH as a cofactor. Xylitol can be oxidized to xylulose by NAD^+-dependent xylitol dehydrogenase (XDH). Some bacteria can use xylose isomerase (XI) to convert xylose into xylulose directly. Xylulose kinase (XKS) can phosphorylate xylulose to xylulose-5-phosphate, which enters the pentose phosphate pathway (PPP). Glyceraldehyde-3-P, an intermediate of PPP, enters the EMP (Embden-Meyerhof-Parnas pathway) and is converted to pyruvic acid through glycolytic steps. Pyruvic acid can finally be converted to ethanol by PDC and ADH (Zaldivar et al., 2001).

Here, the gene *xyl1* deriving from xylose-fermenting yeast *Candida shehatae*, was cloned for the first time, which coexpressed in *S. cerevisiae* with the gene *xyl2* from *Pichia stipitis*. This recombinant *S. cerevisiae* can ferment both xylose and glucose and produce ethanol, which is useful to further improve recombinant *S. cerevisiae*.

2 Materials and methods

2.1 Materials

2.1.1 Strains and vectors

Escherichia coli Top10, *S. cerevisiae* YS58, *Candida shehatae*, *Pichia stipitis* and yeast expression vector

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pYES2 (containing a GAL promoter) were stored in our lab. The clone vector pMD18-T was purchased from TaKaRa Biotech Co., Ltd.

2.1.2 Media and cultivation

E. coli Top10 and *E. coli* transformants were cultivated in LB medium at 37°C. *S. cerevisiae* and its transformants were cultivated in YPD or YNB medium at 30°C. Sugars in fermentation media for recombinant *S. cerevisiae* consisted of 5% xylose, 5% glucose, 3% glucose and 2% xylose.

2.1.3 Enzymes, antibiotics and reagents

Restriction enzymes were purchased from TaKaRa Biotech Co., Ltd. and Promega Co., Ltd., antibiotics from Beijing JingKeHongDa Biotech Co., Ltd., DNA extraction kit from Shanghai Huashun Bioengineering Co., Ltd, Ex Taq polymerase and T4 DNA ligase from TaKaRa Biotech Co., Ltd., DNA agarose gel electrophoresis purification kit from Beijing Dingguo Biotech Co., Ltd. The fragment was sequenced by TaKaRa Biotech Co., Ltd.

2.2 Methods

2.2.1 Genome extraction from *C. shehatae* and *P. stipitis*

Extraction was carried out by using yeast DNA extraction kit.

2.2.2 Amplification of genes *xyl1* and *xyl2*

The primers used for PCR of *xyl1* and *xyl2* were designed based on the report of Kötter et al. (1990) and Hallborn et al. (1991). Two recognition sites for *Bam*HI and *Hind*III (*xyl1*), *Bam*HI and *Sph*I (*xyl2*), respectively, were incorporated into primes. The primes are as follows:

(1) gene *xyl1*: upper: 5′-ATCCCGGGTTCTTTC-TGTAATCTACTAACT-3′;

lower: 5′-TAGCTAGCTCCTCCACCTCCAACGAA-CAT-3′.

(2) gene *xyl2*: upper: 5′-CGGGATCCCTATGTCGT-ATCTTTGCCTC-3′;

lower: 5′-CGGCATGCATTATGGTGCTCTTTTGTTA-3′.

A 50-μL PCR mixture contained 34.75 μL ddH_2O, 5 μL 10 × Ex Taq Buffer, 4 μL dNTP mixture, 4 μL templates DNA, 1 μL upstream primer, 1 μL downstream primer and 0.25 μL Ex Taq DNA polymerase (Sambrook et al., 2001). The reaction mixture was denatured for 5 min at 94°C, followed by 35 cycles under the conditions of 94°C, 1 min; 55°C, 1 min; 72°C , 2 min and completed at 72°C for 10 min.

2.2.3 Subcloning of *xyl1* and *xyl2*, transformation of *E. coli* and screening of positive transformants

The PCR products were characterized by agarose gel electrophoresis (0.8% w/v) and purified with DNA purification kit. The purified products were ligated into the vector pMD18-T and transformed into *E. coli* Top 10. The *E. coli* Top 10 transformants were selected on LB plates through the blue-white screening system (containing 100 μg/μL ampicillin, 400 μL X-gal and 6.7 μL IPTG). The positive transformants were inoculated into LB liquid medium (containing 100 μg/μL ampicillin). The recombinant colony was analyzed by PCR, and plasmids were extracted and digested with the restriction enzymes *Bam*HI and *Hind*III (*xyl1*) or *Bam*HI and *Sph*I (*xyl2*) respectively. PCR and the digestion products were verified by agarose gel electrophoresis (0.8% w/v). The subclone products were sequenced by TaKaRa Biotech Co., Ltd.

2.2.4 DNA recombination, *E. coli* transformation and screening of positive transformants

The subclone was digested by *Bam*HI and *Hind*III (*xyl1*) or *Bam*HI and *Sph*I (*xyl2*) respectively. After agarose gel electrophoresis of the digestion products, 1.1 kb and 1.7 kb fragments were obtained. The two fragments were ligated into the vector pYES2. The ligation products were transformed into *E. coli* Top 10. The *E. coli* Top 10 transformants were selected on LB plates (containing 100 μg/μL ampicillin). The positive transformants were inoculated into LB liquid medium (containing 100 μg/μL ampicillin), then cultivated in shaking flasks. The positive recombinants were analyzed by PCR, and the plasmids were digested simultaneously by two enzymes (*Bam*HI and *Hind*III (*xyl1*) or *Bam*HI and *Sph*I (*xyl2*). PCR and the digestion were verified by agarose gel electrophoresis (0.8% w/v). The verification of inserting target fragment into pYES2 was confirmed by DNA sequencing (finished by TaKaRa Biotech Co., Ltd.)

2.2.5 Transformation of *S. cerevisiae* and screening of positive transformants

S. cerevisiae was transformed through the lithium acetate method (Gietz et al., 1995). The transformation products were selected on YNB glucose plates without uracil and cultivated for fermentation.

2.2.6 Fermentation of recombinant *S. cerevisiae* YS58-12

The positive transformants were cultivated (with the flasks shaking at 80 rpm to activate the strains) in 20 mL YNB (without uracil) glucose medium for 24 hours at 30°C. This operation was repeated once. The cell contents of culture (OD value) were measured. Log-phase cells were chosen and centrifuged for 5 min at 4°C,

and washed twice with 0.9% NaCl solution. The obtained *S. cerevisiae* cells were divided into equal parts and inoculated into three kinds of fermentation medium.

The recombinant *S. cerevisiae* was inoculated into 100 mL fermentation media 5% xylose, 5% glucose, 3% glucose + 2% xylose, respectively, and incubated at 30°C, 80 rpm. Samples were taken every 12 hours for a total of 72 hours. The concentrations of ethanol and residual sugars were determined.

3 Results and discussion

3.1 Cloning of genes *xyl1* and *xyl*2 from *C. shehatae* and *P. stipitis* respectively

The genome DNA was used as a template to amplify genes *xyl1* and *xyl2* by PCR from *C. shehatae* and *P. stipitis*. The results of elecotropheresis showed a clear band of 1.1 kb and 1.7 kb respectively and no further clear non-specific band (Figs. 1, 2).

The purified target fragments were linked to pMD18-T vector and this ligation product was used to transform the competent cells *E. coli* Top 10. Subsequently, the positive transformants were screened through the blue-white screening system. The extracted plasmids from the positive transformants were cut with the restriction enzymes *Bam*HI and *Hind*III or *Bam*HI and *Sph*I respectively to verify the results. The subclone of *xyl1, xyl2* showed that the restriction digestion products and PCR products were the same basically, which indicated that target genes cloning was achieved.

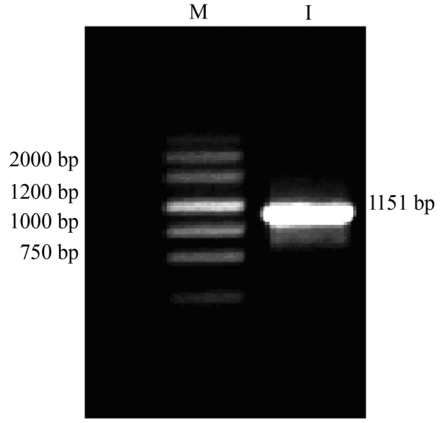

Fig. 1 PCR amplification of *xyl1*. Note: M: Marker; 1: amplification of *xyl1*

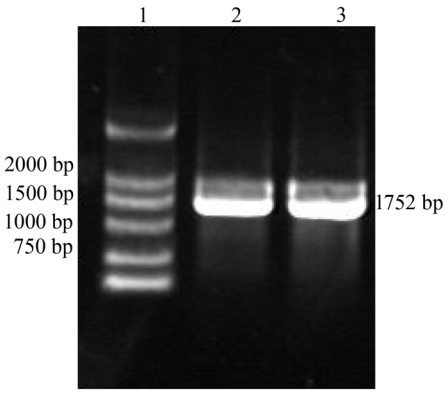

Fig. 2 PCR amplification of *xyl2*. Note: 1: Marker; 2, 3: amplification of *xyl2*

The verification of inserting fragment into the plasmid by DNA sequencing showed that the sequence of *xyl1* was correct. The cloned *xyl1* fragment had 99% and 100% homology with the sequence and protein from the GeneBank, respectively. For the cloned *xyl*2 fragment, the homology was 98% and 100% with the sequence and protein from the GenBank.

3.2 Construction of expression vector pYES2-P12 in recombinant *S. cerevisiae*

The plasmid pMD18-T1 was digested by restriction enzymes *Bam*HI and *Hind*III, and the gene *xyl1* fragment was obtained. At the same time, the yeast expression vector pYES2 was also digested by the same restriction enzymes. The recombinant vector pYES2-1 was constructed by the ligation of the gene *xyl1* and pYES2 large fragment using T4 DNA ligase. The vector pYES2-1 was transformed into *E. coli* Top10. The transformation products were subsequently inoculated on plates and incubated for one night. The positive clones were screened with ampicillin. The single colonies of positive transformants were selected and inoculated into LB liquid media in shaking flasks.

The recombinant plasmids pMD18-T2 and pYES2-1 were digested with *Bam*HI and *Sph*I, respectively. The digestion products were characterized and purified by agarose gel electrophoresis. The gene *xyl*2 and pYES2-1 large fragment were ligated using T4 DNA ligase. The ligation product was recombinant vector pYES2-12 that contained both *xyl1* and *xyl*2 (Fig. 3). The recombinant vector pYES2-12 was used for transforming *S. cerevisiae.*

3.3 Expression of *xyl*1 and *xyl*2 in recombinant *S. cerevisiae* YS58-12 and ethanol fermentation of recombinant *S. cerevisiae*

The vector pYES2-12 was transformed into uracil auxotroph *S. cerevisiae* YS58 using the lithium acetate method. The transformation products were selected by YNB glucose plates without uracil, and single colonies were selected again on the plates.

The recombinant *S. cerevisiae* YS58-12 was inoculated into 5% xylose, 5% glucose and 3% glucose + 2% xylose fermentation medium respectively.

The fermentation results indicated in 5% xylose medium that xylose consumption rate was 33.8% after fermenting

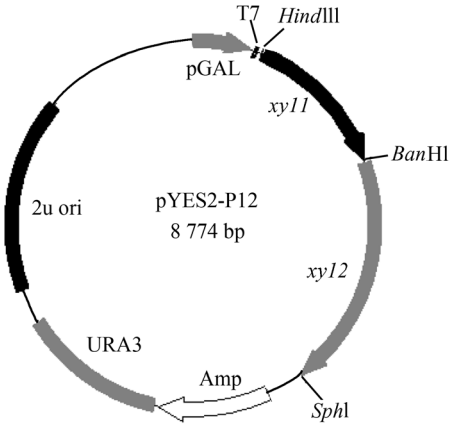

Fig. 3 Recombinant expression vector pYES2-P12

for 24 hours, 67.6% after 48 hours, and 72.4% after 72 hours. However, ethanol yield was relatively low. After fermenting for 72 hours, ethanol yield was 1.76 g/L, which was 9.6% of the ideal value. In 3% glucose+2% xylose medium, the xylose consumption rate of *S. cerevisiae* YS58-12 reached 33.5% after fermenting for 24 hours. During the first 24 hours, xylose was consumed relatively fast, and xylose consumption rate reached 81.3% after fermenting for 48 hours. This indicated that when glucose was added as co-substrate, the consumption of xylose would increase and ethanol yield would improve too. After fermenting for 12 hours, ethanol yield was 10.03 g/L, 14.48 g/L after 24 hours, and 17.12 g/L after 72 hours, which was equal to 67.14% of the ideal value (Figs. 4 and 5).

4 Discussion

Genes *xyl1* and *xyl*2 (derived from *C. shehatae* and from *P. stipitis*, respectively) could express in recombinant *S. cerevisiae* YS58-12. YS58-12 could grow using xylose as the exclusive carbon source, but could not ferment xylose

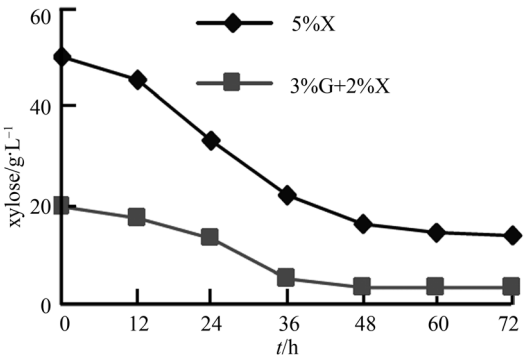

Fig. 4 The consumption of sugar with the time

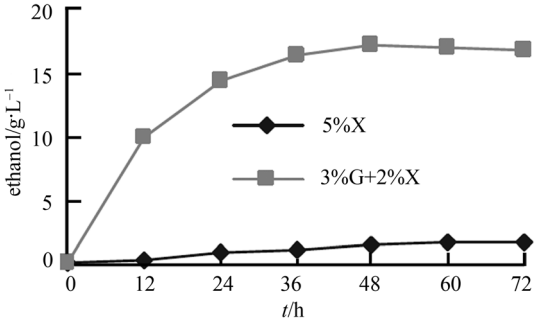

Fig. 5 The yield of ethanol with the time

to produce ethanol efficiently. The xylose was converted to ethanol, xylitol and other coproducts simultaneously. When *S. cerevisiae* YS58-12 grew in a medium containing xylose and glucose, all of the glucose was consumed during the first 24 hours and totally converted to ethanol. When all of the glucose was used up, the consumption rate of xylose would increase. Therefore, when glucose and xylose were supplied simultaneously, *S. cerevisiae* YS58-12 preferred glucose to xylose. During the consumption of xylose by *S. cerevisiae* YS58-12, ethanol yield was still relatively low, but the content of xylitol increased. This could be caused by some blocks in metabolic pathways of xylose or a redox imbalance in cells during fermentation.

Theoretically, to improve ethanol production by xylose, it is feasible to reconstruct *S. cerevisiae* by gene engineering technology. Moreover, recombinant *S. cerevisiae* YS58-12 is already constructed, and it can efficiently convert xylose and glucose to ethanol indeed. However, *S. cerevisiae* YS58-12 still cannot be used commercially. Therefore, it is very important to detect the mechanism of xylose metabolism in microbes and to reconstruct strains with better characteristics of fermentation. How to obtain an excellent industrial strain and reduce the cost of ethanol production is an urgent topic for ethanol production from lignocellulose.

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