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Construction of a Recombinant *S. cerevisiae* Expressing a Fusion Protein and Study on the Effect of Converting Xylose and Glucose to Ethanol

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Abstract Gene *XYL1* from *Candida shehatae* and gene *XYL2* from *Pichia stipitis* were amplified by polymerase chain reaction (PCR), and the two genes were both placed under the strong promoter of alcohol dehydrogenase (ADH) of plasmid pAD2 to produce the recombinant expression vector pAD2-P12. Because the amplified *XYL1* fragment lacks the stop codon UAA, the polypeptide expressed in yeast cells should be a fusion protein, which is a fusion of xylose reductase and xylitol dehydrogenase. Subsequently, the pAD2-P12 vector was transformed into *Saccharomyces cerevisiae* YS58 to produce a recombinant *S. cerevisiae* YS58-12. It was indicated that *S. cerevisiae* YS58-12 has the ability of metabolizing xylose to produce ethanol by fermentation experiment. The result of cofermentation of glucose and xylose by using this recombinant *S. cerevisiae* YS58-12 showed a relatively satisfactory result. The highest percentage of xylose consumption rate reached 81.3% and the ethanol yield was equal to 67.14% of the ideal value.

Keywords Recombinant *Saccaromyces cerevisiae* · *XYL1* · *XYL2* · Fusion protein · Xylose · Ethanol

Introduction

With the booming economy of the world, the need of fuel is increasing very fast. The uncontrolled use of fossil fuels results in environmental pollution and the scarcity of natural resources. Therefore, the exploitation of clean and renewable energy is of great importance. Ethanol production from renewable lignocellulosic material represents an environmentally sustainable alternative to fossil-derived gasoline [1]. Lignocellulose is a complex material consisting of cellulose, lignose, and hemicellulose, and can be hydrolyzed into pentoses (xylose and arabinose) and hexoses (glucose, galactose and mannose) when it is hydrolyzed by acids or enzymes. Xylose is abundant in the hydrolysates of hemicellulose. It accounts

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for about 30% of the total carbohydrates and is only less than glucose in lignocellulosic hydrolysates of hardwoods and crop residues. Thus, it is very important to establish a yeast strain that could efficiently metabolize both glucose and xylose to ethanol, then the cost of bioethanol could be reduced significantly [2].

As a prominent microorganism, *Saccharomyces cerevisiae* is able to ferment glucose to ethanol efficiently, but it has the drawback of not being able to utilize xylose. The first successful approach to construct xylose-utilizing recombinant *S. cerevisiae* strain has been to express the xylose reductase (XR) and xylitol dehydrogenase (XDH) encoding genes *XYL1* and *XYL2*, respectively, from *Pichia stipitis* in *S. cerevisiae* [3]. The yield of ethanol from xylose was improved by overexpression of the endogenous gene *XKS1* encoding xylulokinase (XK) of *S. cerevisiae* [4]. In addition to overexpression of genes *XYL1* and *XYL2*, modifications of other relative genes of carbohydrate metabolism were performed. Some genes were deleted such as *ZWF1* (encoding glucose-6-phosphate) and *GND1* (encoding 6-phosphogluconate dehydrogenase) [5] and some were overexpressed such as *TAL1* (encoding transaldolase), *TKL1*(encoding transketolase) [6] and *GDP1* (encoding for a fungal $NADP^+$-dependent glyceraldehyde-3-phosphate) [7]. However, it should be noticed that the expression of *XYL1* and *XYL2* is the basis for the further modification and is crucial for the utilization of xylose. The two genes expressed in various recombinant *S. cerevisiae* strains were originally from *P. stipitis*, which is able to ferment xylose to ethanol under anaerobic conditions and whose ratio of the specific XR activity utilizing different co-factors has been determined to be NADH/NADPH approximately 0.65 [8]. *Candida shehatae* is also known as one of the best native ethanol producers together with *P. stipitis* from xylose. Bruinenberg et al. has proved that its xylose reductase (XR) activity ratio (NADH/NADPH) utilizing different co-factors is approximately 0.40 [9] and this indicated the gene *XYL1* encoding XR of *C. shehatae* could also be expressed in *S. cerevisiae* without too much redox imbalance.

In the present study, we innovatively used *C. shehatae* as one of the gene sources and attempted at establishing a vector that could express the two genes in the form of a fusion protein. Gene *XYL1* from *C. shehatae* and gene *XYL2* from *P. stipitis* were amplified by PCR. However, the amplified fragment of *XYL1* lacks stop codon UAA, and then the incomplete fragment was linked with the entire fragment of gene *XYL2*. Therefore, the combined fragment was under the control of strong constitutive promoter of alcohol dehydrogenase (ADH), which suggested a fusion protein, a fusion of xylose reductase and xylitol dehydrogenase, could be synthesized in host cell.

Materials and Methods

Material

Strains and Plasmids *Escherichia coli* Top10, *S. cerevisiae* YS58, *C. shehatae*, *P. stipitis*. All strains were stored in our lab. Yeast expression vector pAD2, which has an ADH promoter, was also stored in our lab. Clone vector pMD18-T was purchased from TaKaRa Biotech Co. (Dalian, China).

Media and culture conditions *Escherichia coli* Top10 and *E. coli t*ransformants were cultured in shaking or still Luria-Bertani medium at 37°C. *S. cerevisiae* YS58 and its transformants were cultured in shaking or still yeast extract–peptone-–dextrose or synthetic medium(20 g of glucose/liter and 6.7 g of yeast nitrogen base [YNB] without amino acids supplemented with amino and nucleic acids [omitting uracil used as selection marker]) medium at 30°C.

Concentration of sugars in fermentation media for recombinant *S. cerevisiae* contained 50 g of xylose/liter, 50 g glucose/liter, 30 g glucose and 20 g xylose per liter, respectively.

Enzymes, Antibiotics, and Reagents Restriction enzymes were purchased from TaKaRa Biotech Co., Ltd. and Promega Co., Ltd., Beijing, China. Antibiotics were purchased from Beijing JingKeHongDa Biotech Co., Ltd., Beijing, China. DNA extraction kit was purchased from Shanghai Huashun Engineering Co., Ltd., Shanghai, China. Ex Taq polymerase and T4 DNA Ligase were purchased from TaKaRa Biotech. DNA agarose gel electrophoresis purification kit was purchased from Beijing Dingguo Biotech Co., Ltd., Beijing, China. The fragment was sequenced by TaKaRa Biotech Co.

Methods

Genome Extraction from C. shehatae and P. stipitis The extraction was performed by using Yeast DNA extraction kit.

Amplification of the genes XYL1 and XYL2 Primers used for amplifying *XYL1* and *XYL2* were designed based on the information of a publication [10, 11]. The primers used for amplifying *XYL1* (Fig. 1) contained the recognition sites of *Bam*HI and *Hin*dIII, the primers used for amplifying *XYL2* (Fig. 2) contained the recognition sites of *Bam*HI and *Sph*I. All reactions were performed as below [12]: 50 μl reaction volume containing 34.75 μl ddH_2O, 5 μl 10× Ex Taq Buffer, 4 μl dNTPs Mixture, 4 μl yeast genome DNA, 1 μl upstream primer, 1 μl downstream primer and 0.25 μl Ex Taq DNA polymerase. A predenaturation step was at 94°C for 5 min and then followed by 35 PCR cycles of 94°C for 1 min, 55°C for 1 min, 72°C for 2 min, and a final elongation step was at 72°C for 10 min.

Subcloning of XYL1 and XYL2, Transformation of E. coli and Screening Out of Positive Transformants Amplicons were separated and visualized by electrophoresis on agarose gel (0.8% *w*/*v*) and purified with DNA purification kit. pMD18-T vector was mixed with the purified fragments. Afterward, the ligation product was used to transform the competent cells *E. coli* Top 10. Positive transformants were identified through the blue-white screening system on LB plates containing 100 μg/μl Ampicillin, 40 μl X-gal, and 6.7 μl IPTG and then inoculated into LB liquid medium. The positive recombinants were confirmed by the methods of colony PCR and restriction digestion of the plasmids using

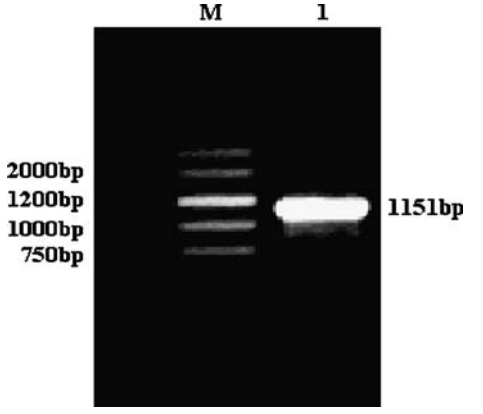

Fig. 1 PCR amplification of *XYL1* (M-marker; 1-amplification of *XYL1*)

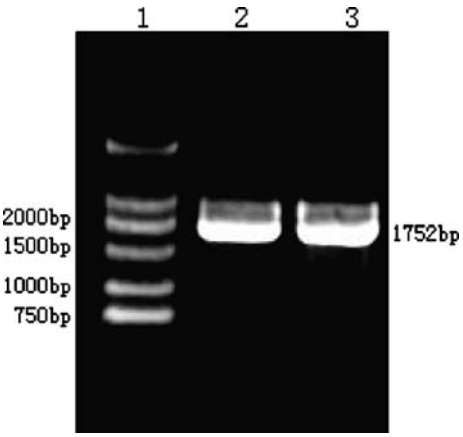

Fig. 2 PCR amplification of *XYL2* (1-marker; 2, 3-amplification of *XYL2*)

*Bam*HI, *Hin*dIII or *Bam*HI, *Sph*I, respectively. The results of colony PCR and double digestion were verified by agarose electrophoresis .The product of subcloning was sequenced by TaKaRa Biotech Co, Ltd.

DNA Recombination, E. coli Transformation, and Screening of Positive Transformants The subcloning product was digested by using *Bam*HI and *Hin*dIII or *Bam*HI and *Sph*I. The 1.1-kb and 1.7-kb fragments were separated and purified from the digestion product by gel-purification. Yeast expression vector pAD2 was cut with the same restriction enzymes and then ligated with the two fragments. This ligation product was then used to transform *E. coli* Top 10 cells. Screened out positive transformants on LB plates containing 100 μg/μl ampicillin and afterward the positive transformants were inoculated into LB liquid medium also containing Ampicillin and cultivated in shaking flasks. The positive recombinants were confirmed by double digestion of the plasmids by the two corresponding restriction endonuclease. The results of colony PCR and double digestion were also verified by agarose electrophoresis. The plasmids were extracted and the inserted fragment was sequenced again to assure the accuracy of the fragments

Transformation of *S. cerevisiae* was performed by lithium acetate method [13]. After transformation the positive transformants were screened out on YNB glucose plates without uracil. Positive transformants were picked out and kept for the fermentation experiment in next step.

Fermentation Experiment of Recombinant S. cerevisiae YS58-12 The positive transformants were inoculated into 20-ml YNB (without uracil) glucose medium and incubated for 24 h at 30°C with the flasks shaking at 80 rpm to activate the strains. The strains were inoculated into fresh medium and incubated again for 24 h under the same condition. The cell concentration of culture was measured by spectrophotometer and those cultures that were in their log phase, were elected out. The cells were collected by centrifugation for 5 min at 4°C and washed twice with 0.9 % NaCl solution. Then the cells were divided to equal quantities and inoculated into three different kinds of fermentation medium as the initial seeds.

Seeds were inoculated into 100 ml 5% xylose, 5% glucose, and 3%glucose + 2%xylose fermentation medium and incubated at 30°C, 80 rpm. Samples were taken out every 12 h. This was done six times. The concentrations of ethanol and sugar substrates were determined by using high-performance liquid chromatography.

Results and Discussion

Cloning of gene *XYL1*, *XYL2* from *C. shehatae* and *P. stipitis,* respectively

The genome DNA was extracted and used as a template for PCR. Primers were designed and the target genes were amplified. The amplicons were visualized and there were clear bands with the length of 1.1 kb and 1.7 kb, and no other specific bands could be seen.

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 out by the blue-white screening system. The extracted plasmids from the positive transformants were cut with the restriction endonucleases *Bam*HI and *Hin*dIII or *Bam*HI and *Sph*I to verify the results. The length of the fragments derived from restriction digestion and from PCR were almost the same. The positive transformants were incubated in liquid medium and then colony PCR was performed. The results of agarose electrophoresis showed that the results of colony PCR and original PCR were basically the same. The results above indicated that TA cloning was successful. The result of sequencing the inserted fragment in the plasmid showed that the sequence of *XYL1* was correct, the homology between the cloned *XYL1* fragment and sequence from GeneBank was 99%, and the homology of protein was 100%. The homology between the cloned *XYL2* fragment and sequence from GeneBank was 98%, the homology of protein was 100%.

Establishment of recombinant yeast expression vector pAD2-P12

The fragment of gene *XYL1* was obtained by cutting the recombinant plasmid pMD18-T1 with *Bam*HI and *Hin*dIII and purifying the target fragment. At the same time, the yeast expression vector pAD2 was cut with the same restriction endonuclease. Ligation of the fragment containing open reading frame (ORF) of gene *XYL1* but without stop codon UAA and pAD2 large fragment using T4 DNA ligase established the recombinant vector pAD2-P1. Competent cells *E. coli* Top10 were transformed with recombinant vector pAD2-P1.

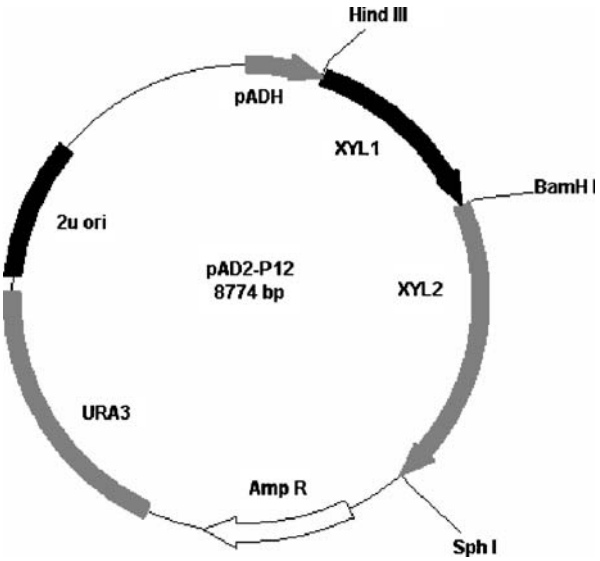

Fig. 3 Recombinant expression vector pAD2-P12

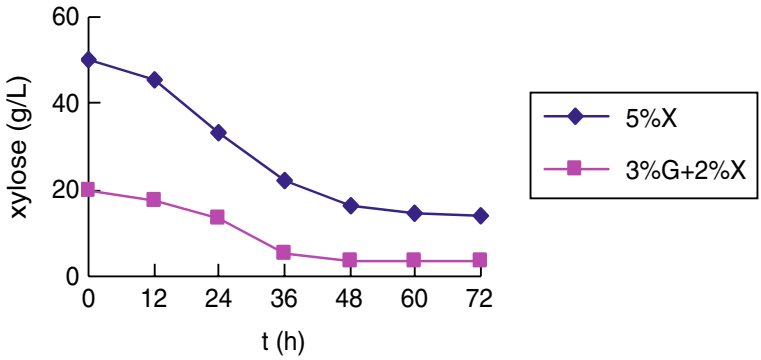

Fig. 4 The time course of xylose consumption

The positive clones were screened out by antibiotics ampicillin. The colony of positive transformants was picked out and then inoculated into LB liquid media in shaking flasks. The plasmids were extracted from *E. coli* cells for ligation of gene *XYL2* in the next step.

The recombinant plasmids pMD18-T2 and pAD2-P1 were both cut by *Bam*HI and *Sph*I. After electrophoresis, the corresponding bands were recycled and purified. The fragment containing entire ORF of gene *XYL2* and pAD2-1 large fragment were ligated again by T4 DNA ligase, which led to the establishment of recombinant vector pAD2–P12 (Fig. 3). The recombinant vector pAD2-P12 was used for transforming *S. cerevisiae* YS58.

Expression of *XYL1* and *XYL2* in recombinant *S. cerevisiae* YS58-12 and ethanol fermentation of recombinant *S. cerevisiae*

The recombinant expression vector pAD2-12 was transformed into uracil auxotroph *S. cerevisiae* YS58 cells by using the lithium acetate method. Positive transformants were screened out on YNB glucose plates without uracil and single colony was separated again on YNB plates. After incubation of identified positive recombinant YS58-12, the strain was inoculated and the fermentation experiment was performed. Recombinant *S. cerevisiae* YS58-12 was inoculated into fermentation medium containing 5% xylose, 5% glucose, and 3% glucose plus 2% xylose. The result of fermentation is shown in Figs. 4 and 5.

The result indicated that the consumed xylose accounted for 33.8% of total amount after fermenting for 24 h, for 67.6% after 48 h, and 72.4% after 72 h in the medium containing

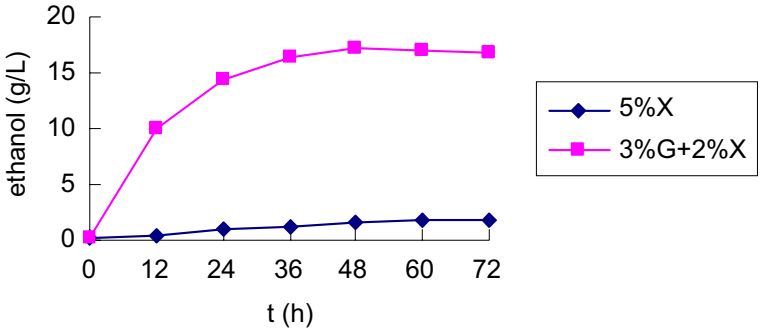

Fig. 5 The time course of ethanol yield

5% xylose. However, ethanol yield is relatively low. After fermenting for 72 h, ethanol yield was 1.76 g/l, which was 9.6% of the ideal value. In the medium containing 3% glucose and 2% xylose, the consumed xylose accounted for 33.5% after fermenting for 24 h. During these first 24 h, xylose was consumed relatively fast and 81.3% of the total xylose was consumed after fermenting for 48 h. It indicated that when glucose was added as co-substrate, the consumption of xylose would increase and ethanol yield could also be improved. When fermented in the medium containing 3% glucose and 2% xylose, ethanol yields were 10.03 g/l after 12 h, 14.48 g/l after 24 h, and 17.12 g/l after 72 h, which was equal to 67.14% of the ideal yield.

Recombinant *S. cerevisiae* YS58-12, which was considered to express a fusion protein, consists of XR from *C. shehatae* and XDH from *P. stipitis*, could grow on a medium with xylose as the exclusive carbon source, but could not ferment xylose as the only carbohydrate to produce ethanol efficiently. The possible reason could be that the consumed xylose was partly converted to xylitol as a main co-product as well as ethanol. When *S. cerevisiae* YS58-12 was fermented in the medium containing xylose and glucose as substrates, all of the glucose was consumed during the first 24 h and was totally converted to ethanol. When all of the glucose was used up, the consumption rate of xylose increased. Therefore, when both glucose and xylose were supplied as substrates, *S. cerevisiae* YS58-12 still preferred glucose to xylose. During the process of xylose consumption by *S. cerevisiae* YS58-12, ethanol yield was still relatively low. That phenomenon could be caused by some blocks in metabolic pathways of xylose or a redox imbalance inside the cells during the process of fermentation.

Conclusion

Theoretically, it is feasible to reconstruct *S. cerevisiae* by gene engineering technology to improve its capability of converting xylose to ethanol. In the present study, recombinant *S. cerevisiae* YS58-12 is already constructed, and it has a relatively satisfactory capability of converting xylose and glucose to ethanol and is able to grow on xylose. Moreover, a fusion protein functioned as XR and XDH is innovatively introduced into the host cell. Although there have not been some molecular experiments to prove the quantity and function of this fusion protein, it is obvious that the *S. cerevisiae* YS58 cannot grow and produce ethanol in the medium with xylose as the sole carbon source if the key xylose metabolic enzymes do not exist. Therefore, this method provided another possible way to establish the recombinant *S. cerevisiae* fermenting xylose to ethanol. In this way, the problems happening when two plasmids were transformed together, such as plasmid incompatibility, could be avoided and the possibility of success of establishing recombinant *S. cerevisiae* may be improved.

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