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Improving 2-Phenylethanol Production via Ehrlich Pathway Using Genetic Engineered *Saccharomyces cerevisiae* Strains

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Abstract 2-phenylethanol (2-PE) is an important aromatic compound with a rose-like fragrance widely used in food industry and cosmetic manufacture. In order to obtain "natural" 2-PE, the genetically modified budding yeasts were developed and applied for the 2-PE production. The gene *ARO8* encoding transaminase and the gene *ARO10* encoding decarboxylase in the Ehrlich pathway were expressed in *Saccharomyces cerevisiae* S288c. The activities of transaminase and decarboxylase were both enhanced in the corresponding recombinant strains. Consequently, the 2-PE yield in the recombinant strains with *ARO8* and *ARO10* were increased by 9.3 and 16.3 %, respectively, than that in the wild strain. A co-expression vector harboring *ARO8* and *ARO10* was then introduced into *S. cerevisiae* S288c, generating the recombinant strain SPO810. The fed-batch fermentation results indicated that the 2-PE yield in SPO810 reached 2.61 g L^{-1} after 60 h of cultivation, which was 36.8 % higher than that in the wild strain. These results demonstrated that the 2-PE production was significantly improved by enhanced expression of the two key enzymes encoded by *ARO8* and *ARO10* in the Ehrlich pathway, providing new perspectives for enhancing "natural" 2-PE production in *S. cerevisiae*.

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Introduction

2-phenylethanol (2-PE) is an important volatile aromatic alcohol compound with enduring and elegant rose fragrance. It has been widely used in various essences modulation for application in food and cosmetics manufactures [5]. To meet the rapidly rising market demand, 2-PE is currently produced mainly by chemical synthesis. Although it has strong advantages in cost and yield, chemical synthesis would cause environmental pollution and toxic by-products. Hence, great interest has been attracted in biosynthesis of 2-PE [20]. Traditionally, natural 2-PE is extracted and refined from plant flowers and fruits, which is subjected to various limitations including season, region, cost, and efficiency of production [14]. By contrast, microbial synthesis method is a competitive alternative with obvious advantages and great potential in natural 2-PE manufacture.

Various microorganisms such as yeasts, *Aspergillus niger*, and *Hericium erinaceus* have capacity of de novo synthesis of 2-PE [4, 6, 9]. Yeast is commonly used for fusel alcohols including 2-PE production due to its capacity, robustness, and tolerance to low pH [12]. In *Saccharomyces cererisiae*, 2-PE biosynthesis is connected with TCA cycle, the Shikimate pathway, and the Ehrlich

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pathway [3, 7]. Although 2-PE can be de novo synthesized from glucose, the efficiency is quite low because of feedback regulations in many branched metabolic pathways [3]. The Ehrlich pathway is a relatively convenient and fast pathway to synthesize 2-PE. The conversion mainly includes three reaction steps using L-phenylalanine as the substrate [7]. L-phenylalanine is firstly converted to phenylpyruvate by transamination, which is then transformed to phenylacetaldehyde by decarboxylation; finally, the derivative aldehyde is finally reduced to 2-PE by dehydrogenation [7]. Various genes were involved in the catabolism of L-phenylalanine to 2-PE by the Ehrlich pathway in *S. cerevisiae*. Aro8p and Aro9p encoded by *ARO8* and *ARO9* were both characterized as aminotransferases with broad-substrate specificity [7, 10], but there is no experimental evidence on which one is more specific and efficient in the transamination reaction. For the decarboxylation reaction, five likely genes (*ARO10*, *THI3*, *PDC1*, *PDC5*, and *PDC6*) encoding thiamine diphosphate-dependent decarboxylases were found in *S. cerevisiae* [13, 18, 21], but Vuralhan et al. [19] proved that *ARO10* was sufficient to encode phenylpyruvate decarboxylase activity even when the other four genes were inactivated. These previous studies would help develop new methods to improve 2-PE production in *S. cerevisiae* by genetic engineering. Therefore, we constructed genetically engineered strains by expression of the aminotransferase gene *ARO8* and the decarboxylase gene *ARO10*. The results showed that co-expression of these two key enzymes in the Ehrlich pathway significantly increased 2-PE biosynthesis.

Materials and Methods

Strains, Plasmids, and Culture Conditions

Strains and plasmids used in this work are listed in Table 1. *S. cerevisiae* was grown in yeast extract peptone dextrose (YPD) medium at 30 °C with vigorous shaking. For enzymatic activity assay and shaking flask fermentation, yeast cultures were grown at 30 °C with vigorous shaking at 220 rpm in fermentation media I (40.0 g L^{-1} of sucrose, 0.5 g L^{-1} of Na_2HPO_4, 1.8 g L^{-1} of yeast nitrogen base without amino acids and 7.0 g L^{-1} of L-phenylalanine as the sole nitrogen source, pH 5.0). When needed, antibiotics were added at the following concentration: 50 μg mL^{-1} of kanamycin or 100 μg mL^{-1} of ampicillin for *E. coli*; 200 μg mL^{-1} of G418 for *S. cerevisiae*.

DNA Manipulation Techniques

Standard DNA manipulation techniques were performed as described by Sambrook and Russell [16]. The transformation of yeast was performed using the LiAc/PEG method as described by Amberg et al. [1]. The vector used in this work is shown in Fig. 1. The *ARO8* gene (GenBank accession no. NM_001181067.1) and the *ARO10* gene (GenBank accession no. NM_001180688.3) were amplified using primers P1/P2 (P1: CGGGATCCATGACTTTACC TGAATC; P2: GCTCTAGACTATTGGAAATACCAA AT; *Bam*HIand *Xba*Isites were underlined) and P3/P4 (P3: TTCGAGCTCATGGCACCTGTTACAATT; P4: GCTCT AGA CTATTTTTTATTTCTTTTAAG; *Sac*Iand *Xba*Isites were underlined) by PCR from *S. cerevisiae* S288c and inserted into the MCS of an *E. coli—S. cerevisiae* shuttle vector pYES-pgk, respectively, generating recombinant vectors pYES-pgk-ARO8 and pYES-pgk-ARO10. The expression cassettes of *ARO8* from plasmid pYES-pgk-ARO8 were inserted into pYES-pgk-ARO10, resulting in pYES-pgk-ARO810. Plasmids pYES-pgk, pYES-pgk-ARO8, pYES-pgk-ARO10, and pYES-pgk-ARO810 were introduced into *S. cerevisiae* S288c by the LiAc/PEG method, respectively. Transformants were screened on YPD agar plates supplemented with G418.

Enzymatic Activity Assay

Cells in the stationary growth phase after 24 h of cultivation were harvested by centrifugation at 4 °C for 10 min at 6000×*g*, and washed twice with ice-cold potassium phosphate buffer (50 mM, pH 7.5) containing 2 mM EDTA, 2 mM DTT, and 0.1 mM pyridoxal phosphate. After that, 0.2 g of cells (wet weight) were collected and resuspended in 5 mL of ice-cold potassium phosphate buffer. Cell extracts were prepared by sonication at 0 °C for 20 min at 2 s intervals with a sonicator (225 W output, SCIENTZ JY88-IIN, China) and cell debris was removed by centrifugation at 4 °C for 20 min at 13,000×*g*. The clear supernatant was used immediately for enzyme assays [15].

Assay of aminotransferase activity was conducted as previously described with some modifications [2, 8]. Briefly, 0.5 mL of mixture containing 50 mM potassium phosphate buffer, 0.2 mM α-ketoglutarate, and 0.1 mM L-phenylalanine was mixed with 0.1 mL of cell extract supernatant; the reaction was performed at 30 °C for 1 h; 0.6 mL of 1 mM 2, 4-dinitrophenylhydrazine (DNPH) was then added into the mixture and the reaction was performed at 30 °C for 10 min. The reaction was terminated by adding 5 mL of 0.4 M NaOH. The formation of 2, 4-dinitrophenylhydrazone of phenylpyruvic acid was measured using a spectrophotometer (Shimadzu UV-3600, Japan) at 460 nm. The amount of phenylpyruvic acid was calculated and 1 unit of aminotransferase activity was defined as formation of 0.1 μM phenylpyruvic acid in 1 h.

Pyruvate decarboxylase activity assay was performed as previously described with some modifications [17, 19].

Table 1 Strains and plasmids used in this work

Strains or plasmids	Relevant features	Reference or source
Plasmids		
pYES-pgk	pYES2-derived expression vector controlled by a 3-phosphoglycerate kinase promoter from *S. cerevisiae*, Amp^R, $G418^R$	Laboratory collection
pYES-pgk-ARO8	*Bam*HI-*Xba*Idigested P1/P2 PCR product of *ARO8* cloned in pYES-pgk, Amp^R, $G418^R$	Laboratory collection
pYES-pgk-ARO10	*Sac*I- *Xba*Idigested P3/P4 PCR product of *ARO10* cloned in pYES-pgk, Amp^R, $G418^R$	Laboratory collection
pYES-pgk-ARO810	*Ngo*MIV- *Age*Idigested P5/P6 PCR product of *ARO8* expression cassette cloned in pYES-pgk-ARO10, Amp^R, $G418^R$	Laboratory collection
Strains		
S. cerevisiae S288c (ATCC 204508)	*MATα SUC2 gal2 mal mel flo1 flo8-1 hap1 ho bio1 bio6*; donor of *ARO8*, *ARO9*, and *ARO10*; host for expression	Laboratory collection
S. cerevisiae SP	*S. cerevisiae* S288c harboring pYES-pgk, $G418^R$	This work
S. cerevisiae SPO8	*S. cerevisiae* S288c harboring pYES-pgk-ARO8, $G418^R$	This work
S. cerevisiae SPO10	*S. cerevisiae* S288c harboring pYES-pgk-ARO10, $G418^R$	This work
S. cerevisiae SPO810	*S. cerevisiae* S288c harboring pYES-pgk-ARO810, $G418^R$	This work

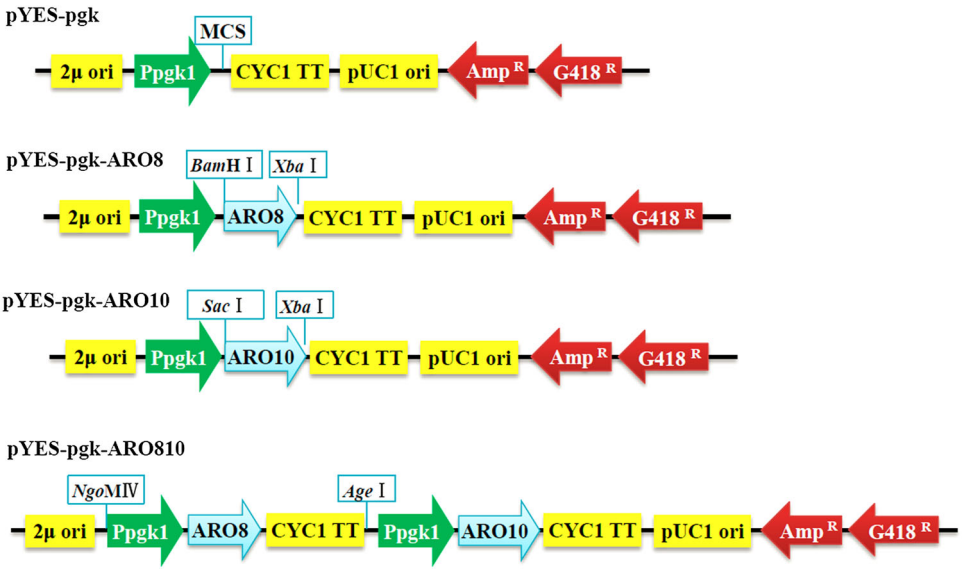

Fig. 1 Expression vectors used in this study. ORFs and promoters are indicated by arrows, replication origins and terminators are indicated by boxes, restriction enzyme sites are shown. MCS, multiple cloning site; pUC1 ori, replication origin of pUC1 from *E. coli*; 2μ ori, replication origin for high-copy plasmid maintenance in *S. cerevisiae*; CYC1 TT, CYC1 transcriptional terminator; Ppgk1, promoter of the 3-phosphoglycerate kinase gene from *S. cerevisiae*; Amp^R, ampicillin resistance gene; $G418^R$, G418, and kanamycin resistance gene; ARO8, the *ARO8* gene; ARO10, the *ARO10* gene

Briefly, 3.2 mL of 0.2 M citrate buffer (pH 6.0), 0.3 mL of 2 mM phenylpyruvic acid, 0.1 mL of 100 mM β-NADH, and 0.1 mL of 200 U mL^{-1} yeast alcohol dehydrogenases (Sigma-Aldrich, USA) were mixed with 0.3 mL of cell extract supernatant; the reaction was performed at 30 °C for 60 min. The reduction of NAD was measured in a spectrophotometer (Shimadzu UV-3600, Japan) at 340 nm. The amount of NADH was calculated and 1 unit of decarboxylase activity was defined as consumption of 0.01 μM NADH in 30 min.

Shaking Flask Fermentation

The fresh cells of the strains S288c, SP, SPO8, SPO10, and SPO810 were inoculated into 100 mL of fermentation media I in a 500 mL shaking flask (Adjusted initial OD600 was 0.25), respectively, followed by cultivation with vigorous agitation at 150 rpm at 30 °C for 60 h. The fermentation samples of each strain were collected at 10, 24, 36, 48, and 60 h for measurement of 2-PE concentration.

Fed-Batch Fermentation

Fresh cultures of the strains S288c and SPO810 were inoculated into 750 mL of fermentation media I (80.0 g L^{-1} of glucose, 12.4 g L^{-1} of KH_2PO_4, 1.6 g L^{-1} of K_2HPO_4, 10 g L^{-1} of yeast nitrogen base without amino acids, and 7.0 g L^{-1} of L-phenylalanine; pH 5.0) in a 2.5 L bioreactor Minifors (INFORS, Switzerland). The bioreactor was aerated at 2.0 vvm (air volume/culture volume/min) and maintained a dissolved oxygen concentration of 100 % saturation with air. The cells were cultivated at 30 °C for 60 h. At 12, 24, 36, and 48 h of cultivation, additional 100 mL of fermentation media I and 8 g of glucose were fed into the media each time. At the end of fermentation, samples were collected for measurement of 2-PE concentration.

Measurement of 2-PE

2-PE was quantified by a high-performance liquid chromatograph system (Shimadzu LC-20A, Japan) equipped with a RP-C18 column (Diameter, 4.6 mm; length, 150 mm; particle size, 5 μm; Shimadzu, Japan). The mobile phase was methanol–water (50:50, v/v) and the operating conditions were as follows: flow rate of 1.0 mL min^{-1} at 30 °C and detection at 215 nm. All analyses were performed in triplicate. Data were expressed as means ± standard deviations.

Results and Discussion

Development of the Recombinant *S. cerevisiae* Strains and Enzymatic Activity Assay

The recombinant plasmids pYES-pgk, pYES-pgk-ARO8, pYES-pgk-ARO10, and pYES-pgk-ARO810 were introduced into *S. cerevisiae* S288c, respectively, generating recombinant strains *S. cerevisiae* SP, *S. cerevisiae* SPO8, *S. cerevisiae* SPO10, and *S. cerevisiae* SPO810. The wild type strain S288c and the recombinant strains were grown to the stationary phase in fermentation media I for

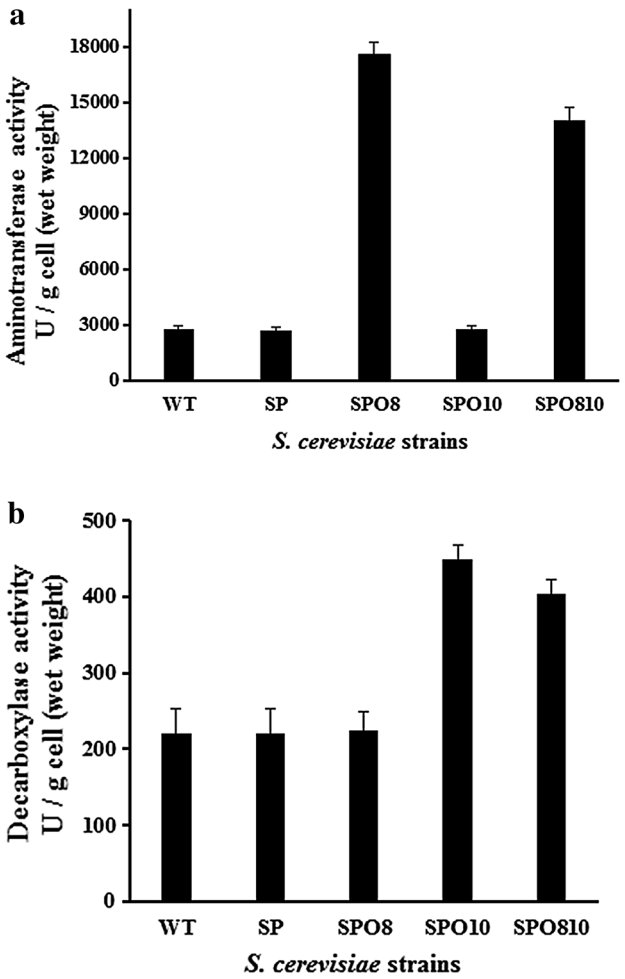

Fig. 2 Detection of aminotransferase (**a**) and decarboxylase (**b**) activity in recombinant strains. Enzymatic activity is expressed in U per g of cells (wet weight). WT, *S. cerevisiae* S288c; SP, *S. cerevisiae* SP; SPO8, *S. cerevisiae* SPO8; SPO10, *S. cerevisiae* SPO10; SPO810, *S. cerevisiae* SPO810

enzymatic activity detection. As shown in Fig. 2a, strains SPO8 and SPO810 exhibited the strongest aminotransferase activity, which were more than six-fold higher than the wild strain, the strains SP and SPO10. It is reasonable to conclude that the significant increase in enzyme activity resulted from expression of the *ARO8* gene. Similarly, with respect to decarboxylase activity, the strains SPO10 and SPO810 showed the highest catalyzing capacity, almost two-fold higher than that of other strains (Fig. 2b), resulting from expression of the *ARO10* gene. The enzymatic activity assay results strongly indicated that *ARO8* and *ARO10* were successfully expressed in the recombinant strains SPO8, SPO10, and SPO810. Moreover, compared with the strains SPO8 and SPO10, the strain SPO810 showed slightly lower aminotransferase and decarboxylase activities, which possibly because of the competition for the host's RNA polymerase between the transcription of

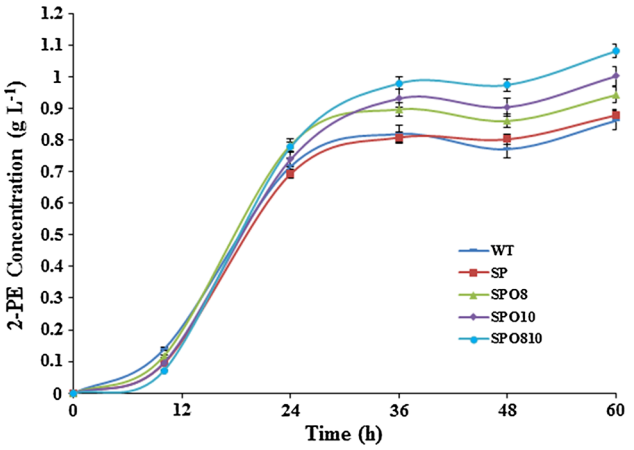

Fig. 3 The 2-PE production in recombinant strains in the flask fermentation. WT, *S. cerevisiae* S288c; SP, *S. cerevisiae* SP; SPO8, *S. cerevisiae* SPO8; SPO10, *S. cerevisiae* SPO10; SPO810, *S. cerevisiae* SPO810

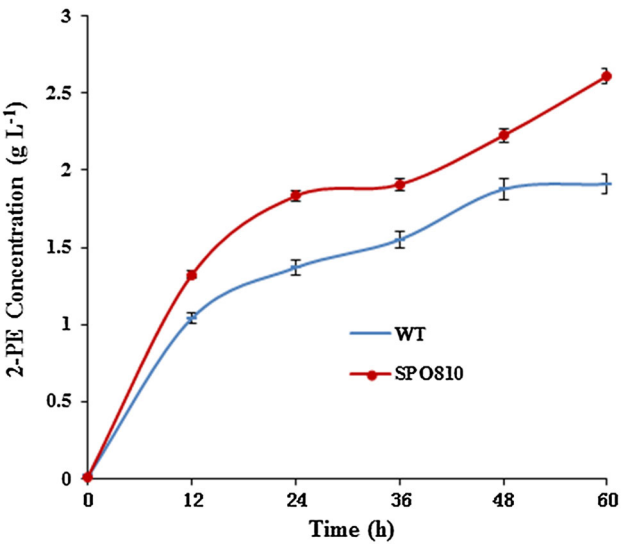

Fig. 4 The 2-PE production in strains S288c and SPO810 in the fed-batch fermentation. WT, the wild strain *S. cerevisiae* S288c; SPO810, *S. cerevisiae* SPO810

ARO8 and *ARO10* when the two genes were expressed simultaneously in the same vector in the strain SPO810.

2-PE Production in Recombinant Strains

As shown in Fig. 3, a similar pattern of 2-PE production by time was observed in each strain. During the first 24 h, the 2-PE sharply increased as cells propagated quickly in the exponential growth phase. Then the concentration of 2-PE kept increasing slowly. After 60 h of fermentation, strain SPO810 produced the highest 2-PE yield of 1.10 g L^{-1}, followed by strain SPO10 with a yield of 1.0 g L^{-1}. Although the yield in strain SPO8 did not exceed 1.0 g L^{-1}, it was still much higher than that in the wild strain WT and the control strain SP. In the strains WT and SP, the 2-PE content was almost in the same level, indicating that introduction of the empty expression vector did not affect 2-PE production.

The flask fermentation results were consistent with the aforementioned enzymatic activity assay results. The recombinant strain SPO810 exhibiting the strongest aminotransferase and decarboxylase activity produced the highest yield of 2-PE; the strains SPO10 and SPO8 with either enhanced decarboxylase or aminotransferase activity also produced more 2-PE than the control strain SP and the wild strain. This strongly indicated that expression of the decarboxylase and aminotransferase genes *ARO10* and *ARO8* could significantly enhanced biosynthesis of 2-PE. Moreover, the strain SPO10 produced more 2-PE than the strain SPO8, implying that improving the decarboxylation reaction might be more efficient than the transamination reaction in promoting 2-PE biosynthesis in *S. cerevisiae.*

According to these findings mentioned above, a fed-batch fermentation was carried out in the strains S288c and SPO810. After 60 h of cultivation in bioreactors, the 2-PE production in the strain S288c hardly reached 2.0 g L^{-1}; while a yield of 2.61 g L^{-1} was achieved in the strain SO810, which was 36.8 % higher than that in the strain S288c (Fig. 4). The results indicated that enhancing activities of the decarboxylase and aminotransferase by co-expression of the genes *ARO8* and *ARO10* remarkably increased the 2-PE production in *S. cerevisiae*.

Kim et al. [11] also reported enhanced 2-PE production in *S. cerevisiae* using metabolic engineering approach. The engineered strain with *ALD3* deletion and over-expressing *ARO9*, *ARO10*, and the transcription factor *Aro80* produced 4.8 g L^{-1} 2-PE in a batch culture system. However, we think it is debatable that the authors chose *ARO9* instead of *ARO8* as the transaminase gene for over-expression. Actually, in our preliminary experiments, we also constructed a recombinant strain expressing *ARO9* and found it hardly showed enhanced aminotransferase activity and improved 2-PE biosynthesis (data not shown). Therefore, though *ARO8* and *ARO9* encode isoenzymes that catalyze the first transamination step of amino acids catabolism in the Ehrlich pathway [10], *ARO8* acts more efficiently and specifically as aminotransferase in 2-PE biosynthesis from L-phenylalanine catabolism in *S. cerevisiae*. And it would be a better strategy to improve 2-PE production if *ALD3* deletion is combined with co-expression of *ARO8*, *ARO10*, and the transcription factor *Aro80* in *S. cerevisiae*.

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