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GENETICS AND MOLECULAR BIOLOGY OF INDUSTRIAL ORGANISMS - ORIGINAL PAPER

Regulation of crucial enzymes and transcription factors on 2-phenylethanol biosynthesis via Ehrlich pathway in *Saccharomyces cerevisiae*

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Abstract 2-Phenylethanol (2-PE) is widely used in food, perfume and pharmaceutical industry, but lower production in microbes and less known regulatory mechanisms of 2-PE make further study necessary. In this study, crucial genes like *ARO8* and *ARO10* of Ehrlich pathway for 2-PE synthesis and key transcription factor *ARO80* in *Saccharomyces cerevisiae* were re-regulated using constitutive promoter; in the meantime, the effect of nitrogen source in synthetic complete (SC) medium with L-phenylalanine (L-Phe) on Aro8/Aro9 and Aro10 was investigated. The results showed that aromatic aminotransferase activities of *ARO8* over-expressing strains were seriously inhibited by ammonia sulfate in SC + Phe medium. Flask fermentation test demonstrated that over-expressing *ARO8* or *ARO10* led to about 42 % increase in 2-PE production when compared with the control strain. Furthermore, influence of transcription factors Cat8 and Mig1 on 2-PE biosynthesis was explored. *CAT8* over-expression or *MIG1* deletion increased in the transcription of *ARO9* and *ARO10*. 2-PE production of *CAT8* over-expressing strain was 62 % higher than that of control strain. Deletion of *MIG1* also led to 2-PE biosynthesis enhancement. The strain of *CAT8* over-expression and *MIG1* deletion was most effective in regulating expression of *ARO9* and *ARO10*. Analysis of mRNA levels and enzyme activities indicates that transaminase in Ehrlich pathway is the crucial target of Nitrogen Catabolize Repression (NCR). Among the engineering strains, the higher 3.73 g/L 2-PE production in *CAT8* over-expressing strain without in situ product recovery suggests that the robust strain has potentiality for commercial exploitation.

Keywords 2-Phenylethanol · Regulation · *ARO8* · *ARO10* · *ARO80* · *CAT8* · *MIG1*

Introduction

2-Phenylethanol (2-PE) is a higher aromatic alcohol with a rose-like fragrance. It is naturally produced in many fermented foods and beverage [9, 15]. 2-PE is widely used in perfume and cosmetic industry [2, 7], and it can also be used as a substrate for the synthesis of other flavors or pharmaceutical compounds such as phenylethyl acetate ester, which is a valuable agent for calming the nerve [5]. Currently, most of 2-PE in the market is produced by chemical synthesis. However, as carcinogenic substances, the synthetic precursor benzene and styrene are harmful to health, and the by-products are difficult to be removed, which seriously affect the quality of 2-PE. Natural 2-PE extracted from plant essential oils is safe, but the production is hard to satisfy the market need. With high efficiency and low cost, bio-catalytic production of 2-PE using microbes is becoming an alternative to the traditional preparation [45]. Fungal species are mainly 2-PE producing microorganisms [13, 29]. As a significant eukaryotic model organism, *Saccharomyces cerevisiae* is universally recognized as a safe microbe, and it can also produce 2-PE in natural way. In addition, high tolerance towards many stress factors makes *S. cerevisiae* an ideal strain for 2-PE biosynthesis.

There are two main 2-PE synthesis pathways in 2-PE producing yeasts. One is Shikimate pathway and another is

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Ehrlich pathway. 2-PE can be produced by de novo synthesis via the Shikimate pathway. As metabolic substrates, phosphoenolpyruvate (PEP) and erythrose-4-phosphate (E4P), respectively, from the central metabolic pathways of the glycolysis and pentose-phosphate pathways, are firstly catalyzed to synthesize 3-deoxyl-D-arabino-heptulasonate-7-phosphate (DHAP), then via a series of intermediates such as shikimate, chorismate, prephenate, etc., phenylpyruvate is synthesized [5]. Shikimate pathway is a long biosynthetic pathway with multiple branches and a variety of feedback inhibitions; therefore, 2-PE production in this way is very low [8, 18]. Yeast can use aromatic amino acids as sole nitrogen source to produce the corresponding alcohols including 2-PE through Ehrlich pathway, which is a predominant pathway for 2-PE synthesis in *S. cerevisiae*. Ehrlich pathway consists of three steps. Two amino acid transaminase isoenzymes (Aro8 and Aro9) have been shown to catalyze the first step of Ehrlich pathway in transamination from L-phenylalanine (L-Phe) to phenylpyruvate [20, 39]. All three pyruvate decarboxylase isozymes (Pdc1, Pdc5, and Pdc6) and phenylpyruvate decarboxylase (Aro10) which is a thiamine pyrophosphate (TPP)-dependent decarboxylases can catalyze the second step of Ehrlich pathway to decarboxylate phenylpyruvate to form phenylacetaldehyde. The third step is reduction, and each of the five alcohol dehydrogenases Adh1, Adh2, Adh3, Adh4, Adh5 and formaldehyde dehydrogenase Sfa1 can catalyze the reduction of phenylacetaldehyde to form 2-PE [5, 13].

As a crucial pathway to synthesize 2-PE, regulation of Ehrlich pathway is very important. However, the regulation mechanism is not clear. Up to now, only a few regulatory factors have been reported. For example, Aro80, a zinc finger transcriptional activator in the Zn2Cys6 family, can activate transcription of aromatic amino acid catabolic genes *ARO9* and *ARO10* in the presence of aromatic amino acids in yeasts [19, 33]; it can also positively regulate 2-PE synthesis in other fungal species such as *Ashbya gossypii* [29]. *ARO9* and *ARO10* are also under the control of nitrogen catabolite repression, and their expression can be activated by such GATA factors as Gat1 and Gln3 [3]. Lack of the knowledge of the metabolic regulation of Ehrlich pathway for the aromatic products in *S. cerevisiae* makes the majority of the study on 2-PE biosynthesis with higher productivity focus on selection of species, traditional breeding of yeast strains, medium optimization, or in situ 2-PE removal [4, 6, 17, 25, 26, 36, 43].

Numerous metabolic pathways in living cells are harmonious based on the complicated regulatory net, because most of the metabolic pathways are generally regulated by a lot of factors. The same is true with Ehrlich pathway. With the development of bioinformatics and the study of yeast response to aromatic alcohol, a few regulators that might be related to Ehrlich pathway are predicted. By employing an integrated computational way of large-scale genomics datasets, Wuster and Babu [44] predicted that Cat8 and Mig1 might be key transcriptional regulators to control the differential expression of the genes affected by aromatic alcohol. In the previous reports, Cat8 and Mig1 were mainly considered to be related to carbon metabolism. Cat8 is a zinc cluster protein as well as a main effecter in glucose repression. As a transcription factor, Cat8 affected all key gluconeogenic enzymes whose genes were derepressed by binding of Cat8 upon carbon source-responsive element (CSRE) motifs [14, 27, 31]. Cat8 is a regulator for derepression of *ADH2*, glucose-repressible alcohol dehydrogenase II gene of the yeast *S. cerevisiae* [42]. It was shown that Cat8 was crucial for depression of a variety of genes under non-fermentative growth conditions [14, 27, 28, 31]. Mig1 is also a transcription factor with two Cys2His2 zinc finger motifs. Its major function is to repress the transcription of those genes encoding enzymes for utilization of the sucrose, galactose or maltose [1, 34]. It has been reported that Mig1 regulates *CAT8* and depends on the release of Mig1 from the promoter of *CAT8* [14, 34]. Cat8 could regulate at least 30 genes related to gluconeogenesis, ethanol utilization and the glyoxylate cycle shown from genomic studies [37], and Mig1 could regulate about 28 proteins shown in *Saccharomyces* Genome Database. Wuster and Babu [44] further proposed that Mig1 and Cat8 participated in quorum sensing, and they might be important for aromatic alcohol communication. However, the regulation of Mig1 and Cat8 to crucial enzymes in Ehrlich pathway and their influence on 2-PE synthesis have not been reported. Whether Mig1 and Cat8 can affect the performance of yeast, like utilization of non-preferred nitrogen sources, is still unknown.

Lower activity of transaminases and phenylpyruvate decarboxylase might be the reason for lowering 2-PE production in yeast; so in our study, we firstly modified crucial genes including *ARO8* and *ARO10*, and crucial activator *ARO80* from both laboratory haploid yeast strain YS58 and industrial diploid yeast strain MT2 by enhancing their expression and comparing their effect on 2-PE biosynthesis. In the meantime, we explored the regulation of different nitrogen sources on the above crucial genes. Then, we continued to study transcription factor Cat8 and its regulation on yeast growth and 2-PE metabolism. We also constructed Mig1 deletion strain and studied the performance of combined strain with both Mig1 deletion and Cat8 over-expression. The aim of this investigation is to explore some crucial regulatory elements of Ehrlich pathway in *S. cerevisiae* and further to elucidate regulatory mechanism for efficient 2-PE synthesis.

Materials and methods

Strains, culture media and cultivation conditions

Saccharomyces cerevisiae strains used in this study are shown in Table 1. *Escherichia coli* DH5α(*sup*E44Δ*lac*U1

Table 1 Strains and plasmids used in this study

Strains or plasmids	Relevant genotypes	References or sources
Strains		
E.coli. DH5α	*sup*E44 Δ*lac*U169(*φ*80*lac*ZΔM15) *hsd*R17 *rec*Al *end*Al *gyr*A96 *thi*-1,*rel*Al	Stratagene
S. cerevisiae YS58	*MATα flo1 ura3-52 leu2-3 112 his4-519 trp1-789*	[38]
MT2	Wild-type yeast strain	CGMCC
YS58-YEp	Recombined yeast strain	This work
YS58(ARO8-58)	Recombined yeast strain	This work
YS58(ARO8-2)	Recombined yeast strain	This work
YS58(ARO10-58)	Recombined yeast strain	This work
YS58(ARO10-2)	Recombined yeast strain	This work
YS58(ARO80-58)	Recombined yeast strain	This work
YS58(ARO80-2)	Recombined yeast strain	This work
YS58(Δmig1)	Recombined yeast strain	This work
YS58(CAT8)	Recombined yeast strain	This work
YS58(CAT8mig1)	Recombined yeast strain	This work
Plasmids		
YEp352	Cloning vector, *URA3 amp*	[16]
pUG6	Cloning vector, *kanMX amp*	[10]
YEpA-ARO8-58	Recombined plasmid, *URA3 amp*	This work
YEpA-ARO8-2	Recombined plasmid, *URA3 amp*	This work
YEpA-ARO10-58	Recombined plasmid, *URA3 amp*	This work
YEpA-ARO10-2	Recombined plasmid, *URA3 amp*	This work
YEpA-ARO80-58	Recombined plasmid, *URA3 amp*	This work
YEpA-ARO80-2	Recombined plasmid, *URA3 amp*	This work
YEpA-CAT8	Recombined plasmid, *URA3 amp*	This work

69(*φ*80*lac*ZΔM15)*hsd*R17*rec*Al*end*Al*gyr*A96*thi*-1*rel*Al) was used for harboring the recombined plasmids. *S. cerevisiae* YS58 (*MATαflo1ura3-52leu2-3, 112his4-519trp1-789*) was used as a parental strain, it was kindly given by Prof. Teunissen and was derived from diploid yeast YS60 [38]. MT2 is an industrial diploid yeast strain whose 2-PE production is greatly higher than that of YS58. Here, both YS58 and MT2 were, respectively, used as a donor for *ARO8*, *ARO10* and *ARO80* gene in order to be compared their contribution to 2-PE biosynthesis. YS58 was also used as a donor for *CAT8* gene.

Indicated quantities are per liter of media. In addition, solid medium contains 20 g of agar. Defined synthetic complete (SC) medium: 6.7 g of yeast nitrogen base (YNB), 20 g glucose, when needed, 20 mg each of uracil (Ura) and tryptophan (Trp), 100 mg of leucine (Leu) and histidine (His) are added. YPD: 10 g yeast extract, 20 g peptone and 20 g glucose. In SC + Phe and YPD + Phe, 5.5 g of L-phenylalanine is added into SC and YPD, respectively. Phe medium: 30 g glucose, 0.5 g magnesium sulfate, 0.5 g potassium dihydrogen phosphate, 5.5 g of L-phenylalanine (Phe).

Escherichia coli strain was grown at 37 °C in Luria-Bertani medium [32] supplemented with ampicillin (100 mg/L) when necessary. The yeast strains for transformation and for collection of yeast pellets for fermentation were grown at 28 °C in SC medium or YPD. For fermentation, the yeast strains were cultured in different media according to different experiment designs.

Gene cloning and construction of plasmids

Plasmids used in this study are shown in Table 1. The *ARO8* ORFs, amplified by PCR from haploid YS58 genomic DNA and diploid yeast strain MT2, respectively, were cloned into the *Sal*I/*Xba*I site of episomal vector YEpA (YEp352 with *ADH1* promoter and terminator sequence), resulting in expression vector named YEpA-ARO8-58 and YEpA-ARO8-2, respectively. The *ARO10* ORFs, respectively, amplified from YS58 and MT2 were inserted into the *Kpn*I/*Xba*I site of YEpA generating YEpA-ARO10-58 and YEpA-ARO10-2. The *ARO80* ORFs, respectively, amplified from YS58 and MT2 were inserted into the *Kpn*I/*Sac*I site of YEpA generating YEpA-ARO80-58 and YEpA-ARO80-2. The *CAT8* ORF from YS58 was cloned into the *Sma*I and *Xho*I sites of YEpA resulting in YEpA-CAT8. Primer sequences used for plasmid construction are shown in Table 2.

Table 2 Primers used in this study

Primers	Sequences (5′–3′)
For recombined plasmids	
P1-ARO8	ACGCGTCGACATGACTTTACCTGAATCAA (*Sal*I)
P2-ARO8	GCTCTAGACTATTTGGAAATACCAAATTC (*Xba*I)
P1-ARO10	GGGGTACCATGGCACCTGTTACAATTGAA (*Kpn*I)
P2-ARO10	GCTCTAGACTATTTTTTATTTCTTTTAAG (*Xba*I)
P1-ARO80	GGGGTACCATGTCTGCTAAGAAAAGGCCT (*Kpn*I)
P2-ARO80	CGAGCTCTTATTTACGCGTTATTGGCCTT (*Sac*I)
P1-CAT8	TCCCCCGGGATGGCAAATAATTCT (*Sma*I)
P2-CAT8	CCGCTCGAGTTATTTGGCGTTTTG (*Xho*I)
For gene knock-out	
mig1-kanMXF	AGCCCATATCCAATGACACAAGTGTCTAACGTTGATGATGGGTCACTATTGAAGGAGAGTCAGCTGAAGCTTCGTACGC
mig1-kanMXR	AGGGCAACGGTAAACTTCTTATGGGTGGTAATGTTTCCAGTTCTTGAACTTGTGAATCCGGCATAGGCCACTAGTGGATCTG
For qRT-PCR	
Q1-ARO8	TACCAAGATTATCCACGATT
Q2-ARO8	AATGTGCCTCAACTAAGAT
Q1-ARO9	GCCGACATTACTACAAGG
Q2-ARO9	TCATAGAAAGAGAGGACGAT
Q1-ARO10	AACGCTCACATCAATGGT
Q2-ARO10	GTTCCAAATGATAACTTC
Q1-ARO80	GAGATGGCACCACTCCTCTAGAG
Q2-ARO80	AAGCCCGATATCTTCACTAGC
Q1-CAT8	ATGGTATCCTCCGTAAGT
Q2-CAT8	CAGTGTAGAAGGTGTATCC

Construction and screening of transformants

Yeast strain YS58 was transformed with the expression plasmids carrying *ARO8*, *ARO10*, *ARO80* or *CAT8,* respectively, and the transformants were screened on SC medium supplemented with tryptophan, leucine and histidine. The resulting recombinant strains were named as YS58(ARO8-58), YS58(ARO8-2), YS58(ARO10-58), YS58(ARO10-2), YS58(ARO80-58), YS58(ARO80-2) and YS58(CAT8), respectively. The control strain YS58-YEp was obtained by transforming empty vector YEp352 into YS58. Deletion mutant YS58(Δmig1) was generated by recombination of the *mig1*::*kanMX* cassette, which was amplified by PCR with mig1-kanMXF and mig1-kanMXR primers using pUG6 as a template, and was screened on YPD medium with geneticin (G418) [10, 11]. Primer sequences used for gene knock-out are shown in Table 2 including homologous arm of *MIG1* and sequence or complementary sequence of pUG6. The combination strain YS58(CAT8mig1) was constructed by transforming plasmid YEpA-CAT8 into YS58(Δmig1).

Fermentation and analytical methods

The host was first grown in 2 mL of SC medium with His, Leu, Trp and Ura, and recombinants were grown in 2 mL of SC with His, Leu and Trp at 28 °C for 18 h. After enlargement culturing in the same condition in 5 mL of the same medium for 20 h, they were harvested by centrifugation, then the pellets were inoculated into 10 mL of Phe medium with initial OD_{600} at 1.0. Samples for qRT-PCR were assayed at 12 h, 24 h, 48 h and 60 h.

Total RNA was isolated from yeast cells and the amounts of mRNAs of *ARO8*, *ARO9*, *ARO10*, *ARO80* or *CAT8* were determined by qRT-PCR. 1 μg of total RNA was first incubated in a 10 μL DNA removing mixture containing DNase (TIANGRN Biotech Co., Ltd, Beijing, China) at 42 °C for 3 min. Then, RNA was subjected to reverse transcription in a 20 μL reaction mixture containing Quant Reverse Transcriptase (TIANGRN Biotech Co., Ltd) and 0.1 mg oligo-dT (M-biotech, Inc.) at 42 °C for 60 min. Reaction mixture containing 50 ng cDNA, 10 μL SuperReal PreMix Plus (with SYBR Green I), and gene-specific primers (Table 2) was subjected to qPCR. PCR was performed with 45 cycles of 95 °C for 10 s, 55 °C for 30 s, and 72 °C for 30 s using Roche Light Cylcer 96 real-time PCR system (Roche Diagnostics). Expression data were processed by the second-derivative maximum method of LightCycler96 software SW1.1. Delta cycle threshold (ΔC_T) values were calculated by the C_Ts of the target genes minus C_T of the *ACT1* housekeeping gene. $\Delta\Delta C_T$ values were calculated by ΔC_T values of the experimental samples $-\Delta C_T$ of the wild-type sample. Fold changes were calculated using the $2^{-\Delta\Delta CT}$ method [24].

For comparison of cells growth and 2-PE biosynthesis of yeast strains in different culture media, the experiments were carried out in 30-mL test tubes, the enlargement culturing was similar to the above; after collecting the cell pellets, they were, respectively, inoculated into 10 mL of different media which were, respectively, SC, SC + Phe,

YPD, YPD + Phe and Phe medium. All initial OD_{600} was about 1. After 60 h fermentation, cell density and 2-PE concentration were tested. 2-PE was quantified by high performance liquid chromatography (Agilent 1260 HPLC, America) equipped with diode array detector (DAD) using C-18 column (guige; Agilent Technologies, America). The solvent composition changed from 99 % water and 1 % acetonitrile to 70 % water and 30 % acetonitrile within 10 min followed by a 2 min hold time and a successive gradual increase to the initial conditions within 10 min. 1 mL/min of constant flow rate was kept. Elution time of 2-PE was 14.6 min under these conditions.

Two enzyme activities were tested. Aromatic aminotransferases activity: The reaction mixtures (1 mL) contained potassium phosphate buffer (0.1 mM, pH 8.0), pyridoxalphosphate (0.1 mM), L-phenylalanine (1 mM), pyruvate (10 mM) as amino acceptor and cell free extract (0.2–0.6 mg of protein). The reactions were started by the addition of L-phenylalanine. Two parallel reactions of aminotransferase activities were stopped after 2 and 10 min at 30 °C by the addition of 1 mL 1 M NaOH on ice. Activity was measured by monitoring the increment of phenylpyruvate, the reaction product of the phenylalanine aminotransferase at 320 nm, $\varepsilon = 1.7500 \times 10^4$ cm^2 mol^{-1} [22]. Phenylpyruvate decarboxylase activity: The reaction mixtures (1 mL) contained potassium phosphate buffer (70 mM, pH 7.0), NAD^+ (2 mM), thiamine pyrophosphate (0.2 mM), yeast acetaldehyde dehydrogenase (0.35 U, dissolved in 1 mM dithiothreitol)), phenylpyruvic acid (2 mM) and cell free extract (about 0.5 mg of protein). The reactions were started by the addition of phenylpyruvic acid. Activity was measured by monitoring the reduction of NAD^+ at 340 nm, $\varepsilon = 6.2 \times 10^3$ cm^2 mol^{-1} [41]. International unit (IU) was adopted for the above two enzyme activities: the amount of enzyme required to convert 1 μmol of substrate to product per minute under a designated condition.

For flask fermentation, the host and recombinants were first grown in 5 mL of YPD at 28 °C for 18 h; after enlargement culturing in the same condition in 30 mL of the same medium for 20–24 h, they were harvested by centrifugation, then the pellets were inoculated into 50 mL Phe medium in 250-mL flask. The initial OD_{600} was about 5. Samples were assayed at 36, 60 and 84 h, respectively.

All data are presented as the averages of the results of three independent experiments. Error bars show standard deviations.

Accession numbers of nucleotide sequences

ARO8 of MT2: KU050080. *ARO10* of MT2: KU050081. The sequences of *ARO8*, *ARO10*, *ARO80* and *CAT8* of YS58 are the same as that of *Saccharomyces cerevisiae* S288C on the National Center for Biotechnology Information (NCBI) whose accession numbers are, respectively, NM_001181067.1 (*ARO8*), NM_001180688.3 (*ARO10*), NM_001180729.1 (*ARO80*) and NM_001182787.1 (*CAT8*).

Results

A series of study including sequence analysis, expression and regulation of *ARO8*, *ARO10* and *ARO80* on 2-PE biosynthesis of *S. cerevisiae* in different culture media

Sequences of *ARO8* in plasmids YEpA-ARO8-58 and YEpA-ARO8-2 were analyzed. The results indicated that the homology of *ARO8* originated from laboratory haploid yeast strain YS58 and industrial diploid yeast strain MT2 was 99.63 %. There was one amino acid of difference between Aro8 of YS58 and that of MT2: 381(Lys, K) in YS58 and 381(Gln, Q) in MT2. Similarly, sequences of *ARO10* in YEpA-ARO10-58 and YEpA-ARO10-2 were analyzed. There was also one amino acid of difference between Aro10 of YS58 and that of MT2: 377(Ala, A) in YS58 and 377(Val, V) in MT2. There was no difference between *ARO80* from YS58 and MT2.

After over-expression of *ARO8*, *ARO10* or *ARO80* from different yeast strains in *S. cerevisiae* YS58, mRNA levels of *ARO8*, *ARO10* and *ARO80* were tested by qRT-PCR. *ACT1*, one of the most commonly used housekeeping genes whose expression is assumed to remain unchanged over a wide range of conditions [35, 40], was used as the reference gene in this study. The results of qRT-PCR indicated that the expression reached the maximum at 24 h. *ARO8* expression levels in strains YS58(ARO8-58) and YS58(ARO8-2) were, respectively, up to 13.2- and 14.4-fold higher than that of the control strain. *ARO10* expression levels in YS58(ARO10-58) and YS58(ARO10-2) were, respectively, 10.3- and 11.1-fold higher than that of the control. Similarly, both mRNA levels of *ARO80* in YS58(ARO80-58) and YS58(ARO80-2) enhanced about 13.5-fold compared to control strain (Fig. 1).

The growth of yeast strains over-expressing *ARO8* or *ARO10* was much better than the control strain in different culture media including SC, SC + Phe, Phe medium, YPD + Phe and YPD. These results showed that highly efficient expression of *ARO8* or *ARO10* was helpful for yeast growth (Fig. 2a). As for 2-PE production, there was no significant difference between the control strain and the engineering strains in SC, YPD + Phe and YPD (Fig. 2b). In Phe medium, the 2-PE production of *ARO8* over-expressing strains YS58(ARO8-58) and YS58(ARO8-2) was, respectively, 4.42- and 4.55-fold higher than that of the control, and 2-PE production of

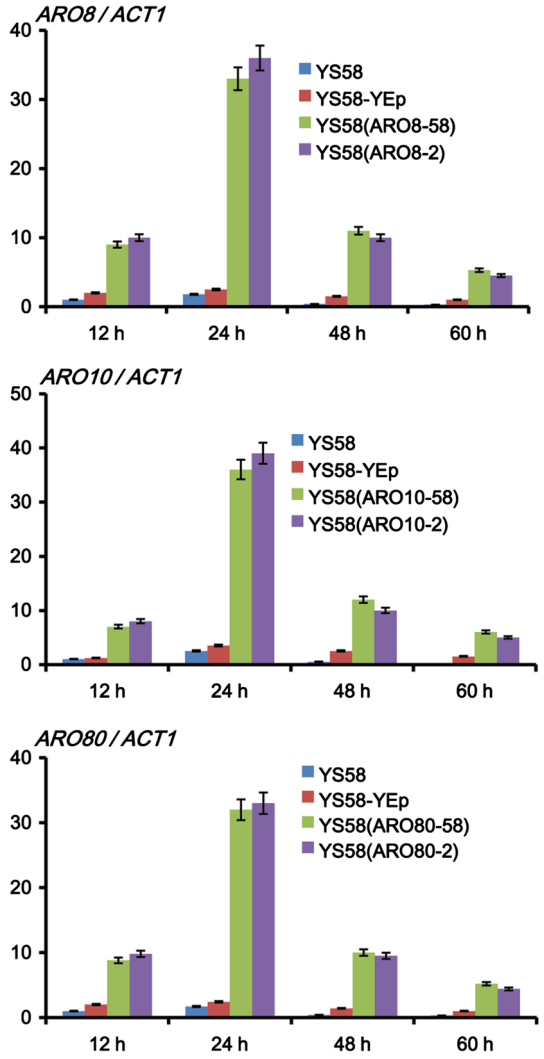

Fig. 1 Expression levels of *ARO8*, *ARO10* and *ARO80* detected by qRT-PCR. Data are presented as the averages of the results of three independent experiments. *Error bars* show standard deviations

ARO10 over-expressing strains YS58(ARO10-58) and YS58(ARO10-2) was, respectively, 4.47- and 4.57-fold higher than that of the control (Fig. 2b). But, in SC + Phe, the 2-PE production of *ARO8* over-expressing strains was, respectively, 2.13- and 2.19-fold higher than that of the control, and 2-PE titer of *ARO10* over-expressing strains was, respectively, 4.17- and 4.34-fold higher than that of the control (Fig. 2b). In our study, over-expression of *ARO80* from different yeast strains did not cause the significant change in yeast growth and 2-PE production when compared with control strain with empty vector YEp352 (Data not shown). Although the sequences of *ARO8* from YS58 and MT2 were different, there was no significant difference in cell growth and 2-PE concentration between YS58(ARO8-58) and YS58(ARO8-2); the same is true with YS58(ARO10-58) and YS58(ARO10-2) (Fig. 2b).

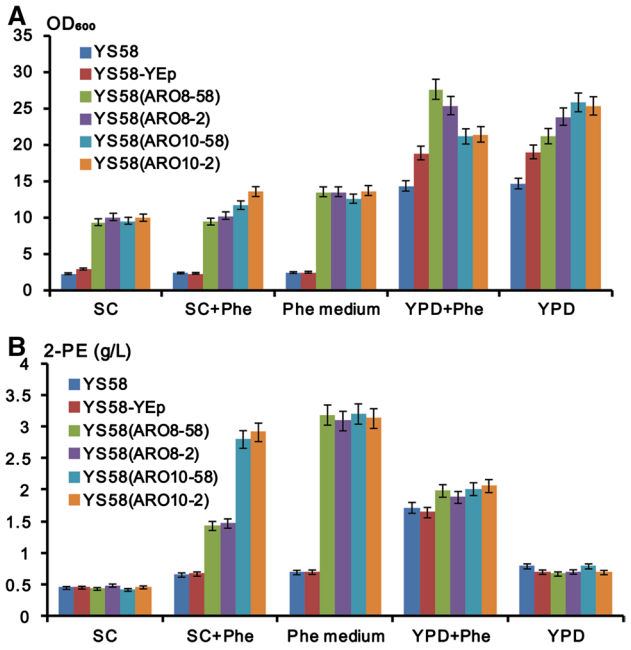

Fig. 2 Comparison of growth and 2-PE production of yeast strains in different culture media. Data are presented as the averages of the results of three independent experiments. *Error bars* show standard deviations

Effect of ammonia sulfate in SC + Phe medium on transaminase activity

To analyze the difference of 2-PE of *ARO8* over-expressing strains in SC + Phe and Phe medium shown in Fig. 2b, mRNA levels of *ARO8* in YS58(ARO8-58) and YS58(ARO8-2) were tested throughout the fermentation in SC + Phe. The expression results were close to that in Phe medium similarly as Fig. 1 shows, indicating that ammonia sulfate in SC medium did not affect the expression of *ARO8* when inducer L-Phe existed. However, transaminase activities of YS58(ARO8-58) and YS58(ARO8-2) in SC + Phe were less than half of that in Phe medium (Table 3). The lower enzyme activities might result in the lower 2-PE synthesis.

Gene expression, yeast growth and 2-PE production of *CAT8* over-expression strain, *MIG1* deletion strain and combination strain

The qRT-PCR test showed that mRNA level of *CAT8* in strain YS58(CAT8) increased rapidly to 19-fold than that of the control strain YS58-YEp after 24 h fermentation. In strain YS58(CAT8), *ARO9* and *ARO10* mRNA levels rose, respectively, to 10- and eightfold higher than that of the control strain (Fig. 3). When comparing the growth and 2-PE concentration of the strain after fermentation

Table 3 Activity of aromatic aminotransferase

Strains[a]	Enzyme activities (IU mg^{-1} protein)[b]			
	In Phe medium		In SC + Phe	
	24 h	60 h	24 h	60 h
YS58	0.27 ± 0.01	0.19 ± 0.01	0.14 ± 0.01	0.1 ± 0.01
YS58-YEp	0.30 ± 0.01	0.21 ± 0.01	0.16 ± 0.01	0.1 ± 0.01
YS58 (ARO8-58)	0.78 ± 0.02	0.69 ± 0.01	0.38 ± 0.01	0.32 ± 0.01
YS58 (ARO8-2)	0.77 ± 0.01	0.70 ± 0.02	0.37 ± 0.02	0.31 ± 0.01

[a] YS58, host strain; YS58-YEp, strain with control vector; YS58(ARO8-58) and YS58(ARO8-2), strains with *ARO8* expression cassette whose gene was from YS58 and MT2, respectively

[b] Significance of difference $P < 0.01$

in different media, we found that although the strain YS58(CAT8) exhibited obvious faster growth in 5 kinds of medium (Fig. 4a), it produced higher 2-PE only in SC + Phe and Phe medium, which was, respectively, 2.8- and 4.6-fold than that of the control strain (Fig. 4b).

qRT-PCR analysis indicated that *MIG1* deletion not only enhanced *ARO9* and *ARO10* expression, but also boosted *CAT8* expression. In YS58(Δmig1), the mRNA level of *CAT8* was 10-fold higher than that of parental strain YS58, and the mRNA levels of *ARO9* and *ARO10* were up to, respectively, 10.5- and 8.3-fold than that of YS58 after 24 h fermentation (Fig. 3). The growth of YS58(Δmig1) in SC + Phe, YPD + Phe, Phe medium and YPD was a bit lower than that of YS58, and the growth of YS58(Δmig1) in SC medium was similar to that of YS58 (Fig. 4a). 2-PE biosynthesis of YS58(Δmig1) was higher than that of YS58 (Fig. 4b).

The combination strain YS58(CAT8mig1) with both *CAT8* over-expression and *MIG1* deletion showed dramatic increase in mRNA level of *CAT8*, which was as many as 22-fold compared with the control strain YS58-YEp. mRNA levels of *ARO9* and *ARO10* in YS58(CAT8mig1) were about, respectively, 12- and 10-fold higher than that of YS58-YEp (Fig. 3). The highest expression of *CAT8, ARO9* and *ARO10* in the combined strain might owe to overlapping role of *CAT8* over-expression and *MIG1* deletion. Growth rate of YS58(CAT8mig1) was close to that of YS58(Δmig1) (Fig. 4a). 2-PE titer of this strain in different culture media indicated that YS58(CAT8mig1) produced higher 2-PE in SC + Phe and Phe medium which was about twice that of the control (Fig. 4b).

Comparison of 2-PE synthesis ability of different engineering strains in flask fermentation and comprehensive analysis among different strains

Based on the above results, cell density and the performance of 2-PE synthetic ability of some of the recombined

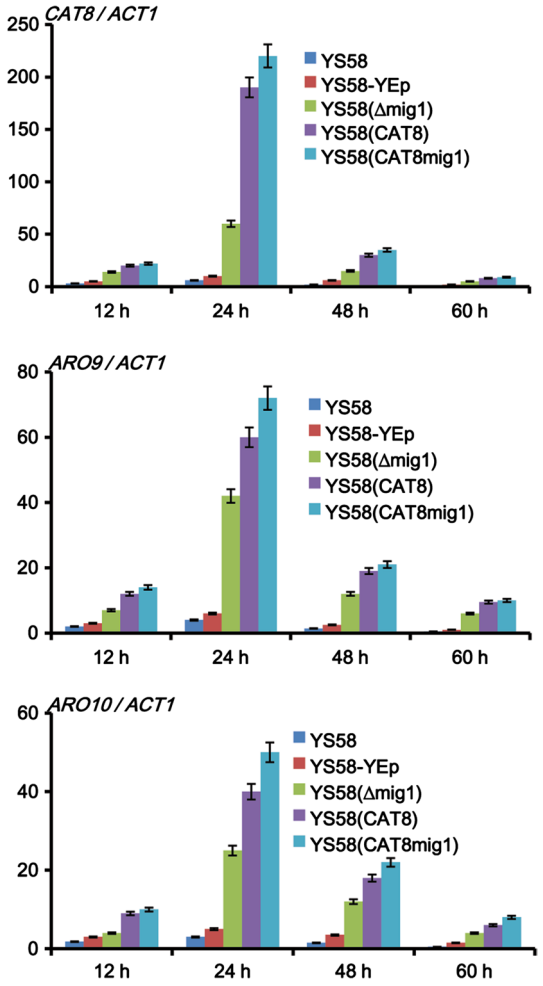

Fig. 3 Expression levels of *ARO9*, *ARO10* and *CAT8* detected by qRT-PCR. Data are presented as the averages of the results of three independent experiments. *Error bars* show standard deviations

strains were further tested on a larger scale during fermentation in flask. The results indicated that YS58(ARO8-58) and YS58(ARO10-58) grew faster than the parental strain YS58 and the control strain YS58-YEp with empty vector (Fig. 5a). 2-PE synthetic ability of these two recombinant strains was similar, and the 2-PE production after 60 h and 84 h fermentation was also similar, about 3.2 g/L, 42 % higher than that of the control strain (Fig. 5b).

MIG1 deletion strain YS58(Δmig1), *CAT8* over-expression strain YS58(CAT8) and the combination strain YS58(CAT8mig1) grew differently in flask fermentation. Among these three engineering strains, growth rate of YS58(CAT8) was the fastest, and YS58(Δmig1) was the slowest (Fig. 5c). After 84 h fermentation, YS58(CAT8) synthesized 3.73 g/L of 2-PE which was 62 % higher than the control strain YS58-YEp, and YS58(CAT8mig1) produced 3.58 g/L 2-PE which was 55 % higher than the control. The 2-PE concentration of YS58(Δmig1) was

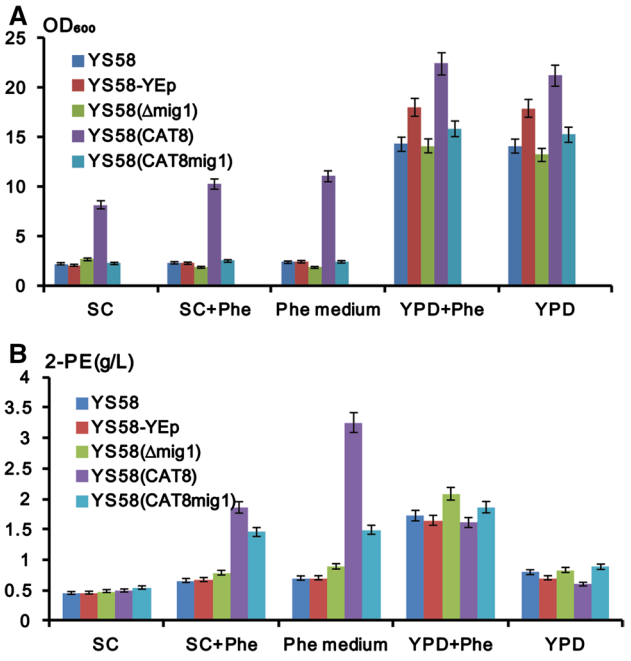

Fig. 4 Comparison of growth and 2-PE concentration of yeast strains in different culture media. Data are presented as the averages of the results of three independent experiments. *Error bars* show standard deviations

3.05 g/L (Fig. 5d). The combination of *CAT8* over-expression and *MIG1* deletion made the greatest contribution to Ehrlich pathway regulation in YS58(CAT8mig1) resulting in 1.241 g/g dried cells of 2-PE synthesis after the fermentation was finished, which was higher than that of both YS58(CAT8) and YS58(Δmig1) (Table 4). YS58(CAT8) was found to be the best one when compared with the total 2-PE production and transformation ratio at 60 and 84 h (Table 4).

Discussion

Aromatic aminotransferase and phenylpyruvate decarboxylase in Ehrlich pathway and transcription factor like Aro80 are important to 2-PE synthesis in some fungal microbes [13, 19, 29, 33]. In previous studies on *S. cerevisiae*, Kim et al. reported that over-expression of *ARO9*, *ARO10* and *ARO80* enhanced 2-PE production in synthetic complete (SC) medium [21]; Yin et al. reported that *ARO8* and *ARO10* over-expression in Phe medium promoted 2-PE synthesis [46]; Romagnoli et al. proved a positive role of *ARO10* over-expression, and also reported that deletion of *ARO8* resulted in 2-PE increase when using ammonium as

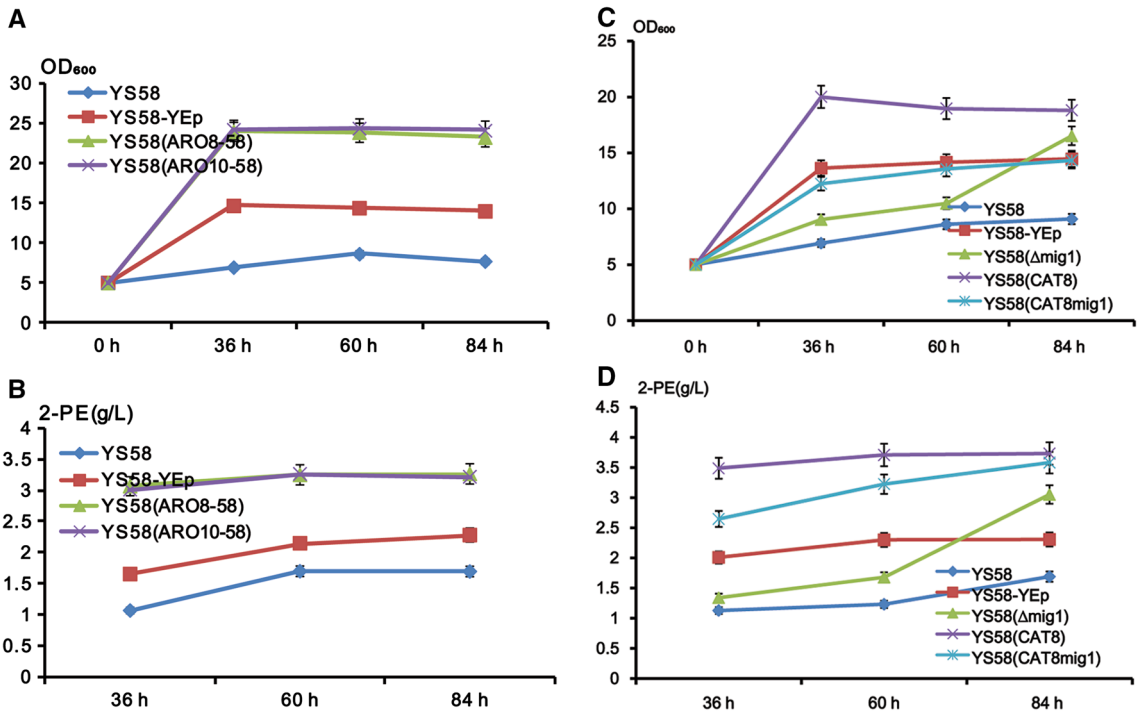

Fig. 5 Cell density and 2-PE concentration of different yeast strains during flask fermentation. Data are presented as the averages of the results of three independent experiments. *Error bars* show standard deviations

Table 4 2-PE synthesis ability and transformation ratio

Strains	2-PE synthesis ability (g/g dried cells)	Transformation ratio (%)	
	84 h	60 h	84 h
YS58	0.800 ± 0.001	30	42
YS58-YEp	0.796 ± 0.002	57	57
YS58 (Δmig1)	0.991 ± 0.002	41	75
YS58 (CAT8)	1.045 ± 0.003	91	91.7
YS58 (CAT8mig)	1.241 ± 0.004	79	88

Values are means of three replications. Transformation ratio is the percent conversion

sole nitrogen source [30]. Based on the above results and our previous investigation, we put forward our hypothesis that crucial factors of Ehrlich pathway might regulate 2-PE synthesis differently, especially in different media and using different strains. Aro8 and Aro9 are aromatic aminotransferase isoenzymes. Considering that aromatic aminotransferase II Aro9 is an inducible enzyme and mainly participates in tryptophan degradation, and aromatic aminotransferase I Aro8 is mainly responsible for phenylalanine [22, 39] but its expression level is low; we chose *ARO8* as our target gene. Since different yeast strains in our laboratory presented significant difference in 2-PE synthesis, we chose two strains including industrial diploid strain MT2 and laboratory haploid strain YS58 as gene donors. Furthermore, five different media were used for comparing their different effects on crucial factors in Ehrlich pathway.

In our previous study, we found that 2-PE concentration in yeast strain MT2 was greatly higher than that of strain YS58; in this study, we also found that there exist differences in amino acids sequence between Aro8-58 and Aro8-2, or between Aro10-58 and Aro10-2, which are, respectively, from YS58 and MT2; however, their over-expression did not bring the difference in 2-PE production (Fig. 2b), suggesting that higher 2-PE production of MT2 might have other regulators and more research on the global regulation should be conducted.

Nitrogen catabolize repression (NCR) seriously limits 2-PE synthesis, so exploring the crucial target elements of NCR in Ehrlich pathway is essential, and investigating the effect of these crucial elements on yeast performance is also necessary. The results of Fig. 2a showed that the enhanced expression of *ARO8* or *ARO10* was helpful to yeast growing in 5 different culture media, which might result from the acceleration of utilization of nitrogen sources including both preferred and non-preferred nitrogen sources. However, Fig. 2b shows that fermentation with *ARO8* or *ARO10* over-expressing strains in YPD + Phe did not give rise to higher 2-PE production, indicating that activities of multi-copy of *ARO8* and *ARO10* might be inhibited completely in rich medium. In SC + Phe, mRNA levels of *ARO10* and phenylpyruvate decarboxylase activity in *ARO10* over-expressing strains were close to that in Phe medium, resulting in similar 2-PE production, which suggested that ammonia sulfate in SC + Phe medium almost did not affect the activity of Aro10. Differently, aromatic aminotransferase activities and 2-PE production of *ARO8* over-expressing strains in SC + Phe shown in Table 3 and Fig. 2b were significantly lower than that in Phe medium though their mRNA levels of *ARO8* were similar, meaning that Aro8 activity was dramatically inhibited by sulfate ammonia. The obvious different affection of nitrogen source in SC + Phe on Aro8 and Aro10 activities suggested that Aro8 was the crucial target of Nitrogen Catabolize Repression when inducer Phe existed, and regulation of Aro8 might mainly happen in a post-translational level.

In our study, over-expression of *ARO80* did not present positive role on 2-PE synthesis, which was different with that reported by Kim et al. [21]. In one respect, this may be due to different responses from different yeast strains to gene modification. On the other hand, since Aro80 is constitutively bound to its target promoters [23], over-expression of *ARO80* may be redundant in some strains and cannot promote 2-PE synthesis.

To understand the regulation of Ehrlich pathway in a comprehensive way, studying some crucial regulatory elements is necessary. Although Cat8 and Mig1 were reported to be closely related to carbon utilization [12], based on Wuster and Babu's [44] proposal about Mig1 and Cat8's role in aromatic alcohol sensing, we presented our hypothesis that Cat8 might regulate 2-PE synthesis dramatically through Ehrlich pathway. Considering the influence of Mig1 on Cat8 biosynthesis [14, 28], we further hypothesized that deletion of Mig1 might be helpful to efficient expression of Cat8 which will further affect 2-PE metabolism.

The results of this study proved our hypothesis. An efficient expression of *CAT8* up-regulated transcriptional levels of *ARO9* and *ARO10*, enhancing 2-PE production (Figs. 3, 4b, 5d). Over-expression of *CAT8* also promoted yeast growing (Figs. 4a, 5c). The significant difference between 2-PE titer of *CAT8* over-expression strain in SC + Phe and that in Phe medium (Fig. 4b) and other analysis also helped us further verify Nitrogen Catabolize Repression on transaminase. *MIG1* deletion promoted *ARO9* and *ARO10* to express as well as to *CAT8*, suggesting that Mig1 participated in the transcriptional regulation of *CAT8*. Increased transcription of *CAT8* might be the result of derepression caused by *MIG1* deletion and then Cat8 further activated the transcription of *ARO9* and *ARO10*. Deleting *MIG1* might prolong the lag phase of yeast; therefore, YS58(Δmig1) did not present its advantage in 2-PE

biosynthesis after 36 and 60 h fermentation because of lower cell density (Figs. 4a, 5c). But after 84 h of fermentation, cell density of YS58(Δmig1) increased, and the positive regulation of *MIG1* deletion on 2-PE metabolism was displayed (Fig. 5d).

Theoretically, combining strain YS58(CAT8mig1) with both *CAT8* over-expression and *MIG1* deletion could make more contribution to 2-PE biosynthesis. Expression levels of *ARO9* and *ARO10* in YS58(CAT8mig1) were definitely the highest compared with that in YS58(CAT8) (Fig. 3), but 2-PE in YS58(CAT8mig1) was not as high as that in YS58(CAT8), which might be due to less cell density caused by *MIG1* deletion (Figs. 4a, 5c). When we inspected the 2-PE concentration under the equal cell weight, YS58(CAT8mig1) produced the highest 2-PE compared with other strains, suggesting that the synergistic action of *CAT8* over-expression and *MIG1* deletion played a significant role in the regulation of 2-PE biosynthesis (Table 4).

In summary, efficient expression of aromatic aminotransferase and phenylpyruvate decarboxylase greatly enhanced 2-PE biosynthesis, but the effect of nitrogen source on these two enzymes was significantly different. When inducer-like L-Phe existed, transaminase in Ehrlich pathway was the crucial target of Nitrogen Catabolize Repression (NCR). Among transcription factors, Cat8 over-expression and Mig1 deletion could affect 2-PE biosynthesis greatly by up-regulating transcription levels of crucial enzyme genes in Ehrlich pathway. Combination of *CAT8* over-expression and *MIG1* deletion contributed a lot to 2-PE biosynthesis when compared the ratio of 2-PE product per cell mass. Considering the comprehensive performance of the engineering strains, we found that YS58(CAT8) was excellent because its growing speed, total 2-PE production (3.73 g/L) (Fig. 5d), and transformation ratio (91 %) (Table 4) were the highest. All these results are not only useful to help us to understand the regulatory mechanism of Ehrlich pathway for 2-PE biosynthesis, but also meaningful to guide us to construct engineering strains for industrial application.

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