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Significant enhancement of methionol production by co-expression of the aminotransferase gene *ARO8* and the decarboxylase gene *ARO10* in *Saccharomyces cerevisiae*

Methionol production by co-expression of *ARO8* and *ARO10*

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Abstract

Methionol is an important volatile sulphur flavour compound, which can be produced via the Ehrlich pathway in *Saccharomyces cerevisiae*. Aminotransferase and decarboxylase are essential enzymes catalyzing methionol biosynthesis. In In this work, two aminotransferase genes *ARO8* and *ARO9* and one decarboxylase gene *ARO10* were introduced into in *S. cerevisiae* S288c, respectively, via an expression vector. Over-expression of *ARO8* resulted in higher aminotransferase activity than that of *ARO9*. And the cellular decarboxylase activity was remarkably increased by over-expression of *ARO10*. A co-expression vector carrying both *ARO8* and *ARO10* was further constructed, to generate the recombinant strain S810. Shaking flask experiments showed that the methionol yield from S810 reached 1.27 g L^{-1}, which was increased by 51.8% and 68.8% compared to that from the wild type strain and the control strain harboring the empty vector. The fed-batch fermentation by strain S810 produced 3.24 g L^{-1} of methionol after 72 h of cultivation in a bioreactor. These results demonstrated that co-expression of *ARO8* and *ARO10* significantly boosted the methionol production. It is the first time that more than 3.0 g L^{-1} of methionol produced by genetically engineered yeast strain was reported by co-expression of the aminotransferase and decarboxylase via the Ehrlich pathway.

Keywords: methionol, aminotransferase, decarboxylase, co-expression, *Saccharomyces cerevisiae*

Introduction

Methionol (3-methylthio-1-propanol) is an important volatile sulphur flavour compound found in a wide array of food and beverages, which provides the distinct taste of meat, boiled potato, onion, cauliflower, cabbage, and cheese (Mestres *et al*., 2000; Burdock, 2010; Seow *et al*., 2010). As it contributes to characteristic flavors, sustained attention has been paid on the synthesis of this potent aroma compound. Currently, traditional organic synthesis remains the main method to generate methionol. Recently, growing interests have been shown in producing "natural" methionol through biosynthesis technology, which is recognized as safe, environment-friendly, and easily accepted by customers (Berger, 2009).

The biosynthesis of methionol has been reported in various microorganisms including *Brevibacterium*, *Arthrobacter* spp., lactic acid bacteria and yeasts (López del Castillo-Lozano *et al*., 2007). Previous studies indicated that methionol production from methionine is a common feature of *Saccharomyces* species (Moreira *et al*., 2005; Perpète *et al*., 2006; López del Castillo-Lozano *et al*., 2007; Liu & Crow, 2010). Moreover, as the microorganisms widely applied for industrial fermentation, the budding yeast *S. cerevisiae* possesses considerable potential due to its high capacity, robustness, and tolerance to pH fluctuation (Kondo *et al*., 2012). Hence, continuous efforts have been made to improve the methionol production in *S. cerevisiae* (López del Castillo-Lozano *et al*., 2007; Etschmann *et al*., 2008; Liu & Crow, 2010; Seow *et al*., 2010).

The conversion of methionine to methionol via the Ehrlich pathway in *S. cerevisiae* has been extensively studied for decades (Fig. 1). L-methionine is first converted to

methional (3-methylthio-propionaldehyde) by transamination and decarboxylation reactions, and the resulting derivative aldehyde is then reduced to methionol by dehydrogenase (Perpète *et al*., 2006; Etschmann *et al*., 2008). Thus, transaminase and decarboxylase should play essential roles in the biosynthesis pathway (Ghaemmaghami *et al*., 2003; Hazelwood *et al*., 2008). Although Aro8p and Aro9p have been characterized as aminotransferases with broad-substrate specificity in the Ehrlich pathway, their catalytic efficiency is still not clear (Iraqui *et al*., 1998; Urrestarazu *et al*., 1998; Iraqui *et al*., 1999; Hazelwood *et al*., 2008). On the other hand, five genes including *ARO10*, *THI3*, *PDC1*, *PDC5*, and *PDC6* encoding thiamine diphosphate-dependent decarboxylase activity have been found in *S. cerevisiae*. Vuralhan *et al*. (2003) reported that *ARO10* was sufficient to encode phenylpyruvate decarboxylase activity when the four other genes were inactivated. In addition, Aro10p has been reported to function specifically in methionine catabolism (Perpète *et al*., 2005; Vuralhan *et al*., 2005; Perpète *et al*., 2006).

Although the biosynthesis of methionol in *S. cerevisiae* has been extensively studied, research about genetic engineering of the strain to improve the methionol production has rarely been reported. In this work, we constructed a genetically engineered *S. cerevisiae* strain that exhibits enhanced expression level of both the aminotransferase gene and the decarboxylase gene, and the effect of co-expression of these two key enzyme on the methionol production was evaluated. This work provided a new strategy for the methionol production enhancement in *S. cerevisiae*.

Materials and methods

Strains, plasmids and culture conditions

Strains and plasmids used in this work are listed in Table 1. *Escherichia coli* TOP10 used as a cloning host was cultured at 37°C in Luria-Bertani (LB) medium with vigorous shaking. *S. cerevisiae* was grown in YPD medium at 30°C with vigorous shaking. When needed, antibiotics were added at the following concentration: 50 μg mL^{-1} kanamycin or 100 μg mL^{-1} ampicillin for *E. coli*; 200 μg mL^{-1} G418 for *S. cerevisiae*.

DNA manipulation techniques

Standard DNA manipulation techniques were performed as described by Sambrook & Russell (2001). Genomic and plasmid DNA from yeast was prepared using the TIANamp Yeast DNA Kit and the TIANprep Yeast Plasmid DNA Kit following the manufacturer's instructions (TIANGEN Biotech, CHN). Plasmid DNA from *E. coli* was isolated using the E.Z.N.A Plasmid Miniprep Kit according to the manufacturer's instructions (Omega, USA). Restriction endonuclease digestions and DNA ligation were conducted according to the supplier's instructions (Thermo Fisher Scientific, New England Biolabs, and Takara, CHN). Standard heat shock transformation method was used to introduce plasmids DNA to *E. coli* TOP10 (Sambrook & Russell, 2001) and the transformation of yeast was performed using the LiAc/PEG method as described by Amberg *et al*. (2005).

Vector construction

The vector construction strategy was shown in Fig. 2. Based on the *ARO8* gene (GenBank accession no. NM_001181067.1), the *ARO9* gene (GenBank accession no.

NM_001179267.1), and the *ARO10* gene (GenBank accession no. NM_001180688.3), specific primers P1/P2, P3/P4, and P5/P6 containing proper restriction enzyme sites were designed for PCR (Table 2). The three genes were amplified by PCR from the genomic DNA of *S. cerevisiae* S288c. Amplification reactions were performed using TakaRa Ex Taq DNA polymerase following the manufacturer's recommendations (Takara, CHN). The amplicons were then inserted into the MCS of an *E. coli* - *S. cerevisiae* shuttle vector pYES-pgk, respectively, to generate recombinant vectors pYES-pgk-ARO8, pYES-pgk-ARO9, and pYES-pgk-ARO10 (Fig. 2). The DNA ligation mixtures were transformed into *E. coli* TOP10 and transformants were screened on LB agar plates containing ampicillin. The recombinant plasmids were then sequenced and further analyzed with DNAMAN software package and BLAST Program at NCBI against the GenBank database. In order to construct a co-expression vector, the expression cassettes of *ARO8* and *ARO9* were amplified from plasmid pYES-pgk-ARO8 and pYES-pgk-ARO9 using primers P7/P8 (Table 2) and then inserted into plasmid pYES-pgk-ARO10, resulting in a new recombinant plasmid, designated as pYES-pgk-ARO810 and pYES-pgk-ARO910.

Protein expression and enzymatic activity assay

Plasmids pYES-pgk, pYES-pgk-ARO8, pYES-pgk-ARO9, pYES-pgk-ARO10, pYES-pgk-ARO810, and pYES-pgk-ARO910 were introduced into *S. cerevisiae* S288c by the LiAc/PEG method. Transformants were screened on YPD agar plates supplemented with G418. The corresponding recombinant strains *S. cerevisiae* SCK, *S.*

cerevisiae S8, *S. cerevisiae* S9, *S. cerevisiae* S10, *S. cerevisiae* S810, and *S. cerevisiae* S910 were further tested by plasmid profile and PCR.

For enzymatic activity assay, yeast cultures were grown at 30°C with vigorous shaking at 220 rpm in fermentation media containing 20.0 g L^{-1} of glucose, 1.24 g L^{-1} of KH_2PO_4, 0.16 g L^{-1} of K_2HPO_4, 1.7 g L^{-1} of yeast nitrogen base without amino acids and 10.0 g L^{-1} of methionine as the sole nitrogen source. Cells in the stationary growth phase after 36 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. 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, CHN) and cell debris was removed by centrifugation at 4°C for 20 min at 13000 *g*. The clear supernatant was used immediately for enzyme assays (Perpète *et al*., 2006).

Assay of aminotransferase activity was conducted as previously described with some modifications (Lee *et al*., 1985; Perpète *et al*., 2005; Brammer & Meyers, 2009; Hu *et al*., 2012). Briefly, a 0.9 mL mixture containing 50 mM potassium phosphate buffer, 2 mM α-ketoglutarate, 0.1 mM pyridoxal phosphate, and 0.2 mM methionine was mixed with 0.1 mL of cell extract supernatant and 0.2 mL of potassium phosphate buffer; the reaction was performed at 30°C for 10 min; 1.5mL of 1mM 2, 4-dinitrophenylhydrazine (DNPH) was then added into the mixture and the reaction was performed at 30°C for 20 min. The reaction was terminated by adding 10 mL of

0.4 M NaOH. The formation of 2, 4-dinitrophenylhydrazone of 2-keto-4-thiomethylbutyric acid (KMBA) was measured using a spectrophotometer (Shimadzu UV-3600, Japan) at 460 nm. The amount of KMBA was calculated and 1 unit of aminotransferase activity was defined as formation of 0.1 μM KMBA in 10 min.

Pyruvate decarboxylase activity assay was performed as described by Flikweert *et al*. (1996) and Vuralhan *et al*. (2003) with some modifications. Briefly, a 0.9 mL mixture containing 0.2 M citrate buffer (pH 6.0), 0.2 mM thiamine pyrophosphate (TPP), 5 mM $MgC1_2$, 2 mM NADH, 2 mM KMBA, and 0.35 U of yeast aldehyde dehydrogenase (Sigma-Aldrich, USA) dissolved in 1 mM dithiothreitol was mixed with 0.3 mL of cell extract supernatant and 3 mL of citrate buffer; 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

Strains S288c, SCK, S8, S10, and S810 were grown in YPD media with agitation at 150 rpm at 30°C for overnight. Then the fresh cells were harvested, washed with distilled water and inoculated into 200 mL of fermentation media (20.0 g L^{-1} of glucose, 1.24 g L^{-1} of KH_2PO_4, 0.16 g L^{-1} of K_2HPO_4, 1.7 g L^{-1} of yeast nitrogen base without amino acids and 10.0 g L^{-1} of methionine; pH 5.0) in a 500 mL shaking flask

(Adjusted initial OD600 was 0.25), followed by cultivation with vigorous agitation at 150 rpm at 30°C for 6 days. Samples were collected at 6, 12, 24, 48, 72, 96, 108, 120, and 132 h for measurement of methionol concentration.

Bioreactor fermentation

Fresh cultures of the strains S288c and S810 were inoculated into 1 L of fermentation media (60.0 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 10.0 g L^{-1} of methionine; pH 5.0) for fermentation. The cultivations were carried out at 30°C for 72 h in a 2.5 L bioreactor Minifors (INFORS, Switzerland) which was aerated at 0.5 vvm (air volume/culture volume/min) and maintained a dissolved oxygen concentration of 100% saturation with air. At 24, 36 and 48 h of cultivation, additional 20 g of glucose was fed into the media each time. At the end of fermentation, samples were collected for measurement of methionol concentration.

Measurement of methionol concentration

Methionol was quantified by a high-performance liquid chromatograph system (Shimadzu LC-20A, Japan) equipped with a Wondasil C18 column (Diameter, 4.6 mm; length, 250 mm; particle size, 5 μm; Shimadzu, Japan) as described by Bonnarme *et al*. (2001a). The mobile phase was methanol-water (30:70, v/v) and the operating conditions were as follows: flow rate of 0.1 mL/min at 30°C and detection at 215 nm. All analyses were performed in triplicate. Data were expressed as means ± standard

deviations.

Results and discussion

Cloning of the *ARO8, ARO9,* and *ARO10* genes and construction of recombinant expression vectors

The *ARO8*, *ARO9*, and *ARO10* genes were amplified from the genomic DNA of *S. cerevisiae* S288c using specific primers. Sequence analysis showed that the 1503-bp *ARO8* and 1542-bp *ARO9* shared a 100% identity with the 2-aminoadipate transaminase gene (GenBank accession no. NM_001181067.1) and the 2-oxoglutarate transaminase gene (GenBank accession no. NM_001179267.1), respectively. The *ARO10* gene with size of 1908-bp shared a 100% identity with the phenylpyruvate decarboxylase gene (GenBank accession no. NM_001180688.3). The amplicons were digested by corresponding restriction enzymes and inserted into pYES-pgk, generating recombinant expression vectors: 7.8-kb pYES-pgk-ARO8, 7.9-kb pYES-pgk-ARO9, and 8.2-kb pYES-pgk-ARO10. For co-expression of aminotransferase and decarboxylase, the expression cassettes of *ARO8* and *ARO9* were amplified and inserted into pYES-pgk-ARO10, respectively, resulting in 10.5-kb pYES-pgk-ARO810 and 10.7-kb pYES-pgk-ARO910. This strategy allowed each gene on the same expression vector pYES-pgk was controlled by an independent set of the 3-phosphoglycerate kinase gene promoter Ppgk1 and the CYC1 transcriptional terminator, which would prevent the potential interference from the over-expression

of each gene on the same vector.

Aminotransferase and decarboxylase activity assay

The wild type strain S288c and the recombinant strains SCK, S8, S9, S10, S810, and S910 were grown to the stationary phase in fermentation media for aminotransferase and decarboxylase activity detection. As shown in Fig. 3a, strains S8 (4680 ± 77 U) and S810 (4062 ± 40 U) exhibited the strongest aminotransferase activity, which were more than 4-fold higher compared to the control strain SCK (1007 ± 158 U). The result suggested that the significant increase in enzyme activity was a consequence of the introduction of extra copy of the *ARO8* gene. Besides, the aminotransferase activities in strains S9 (1962 ± 112 U) and S910 (1645 ± 45 U) were less than 2-fold higher than that in SCK and at the same level compared to the wild type strain S288c (1780 ± 31 U), indicating that over-expression of the *ARO9* gene only caused a slight enhancement in aminotransferase activity. Although Aro8 and Aro9 have been identified as isoenzymes that catalyze the first transamination step of amino acids catabolism in the Ehrlich pathway (Iraqui *et al*., 1998), the difference in aminotransferase activity between strain S9/S910 and strain S8/S810 probably resulted from the substrate specificity, as *ARO9* encoded an aromatic aminotransferase Ⅱ that prefer to catalyze the first step of tryptophan, phenylalanine, and tyrosine catabolism (Johnston *et al*., 1994). Though Karsten *et al*. (2011) categorized Aro8 the α-aminoadipate aminotransferase rather than the aromatic aminotransferase, the importance of Aro8 for aromatic amino acids catabolism via the Ehrlich pathway

should be highlighted. On the other hand, the decarboxylase activity in strains S10 (3538 ± 73 U), S810 (3448 ± 19 U) and S910 (3286 ± 27 U) were almost 6-fold higher, respectively, than that of the control strain SCK (Fig. 3b), suggesting that over-expression of the *ARO10* gene significantly enhanced decarboxylase activity.

Moreover, the control strain SCK showed lower enzymatic activities than the wild type strain S288c, indicating the negative influence caused by introduction of the empty vector. But this fact also reflected that enzymatic activities enhancement were fulfilled in recombinant strains by gene over-expression. Besides, co-expression of two genes did not affect the expression of each enzyme, as no obvious difference in enzyme activity between strains with single gene over-expression and two genes co-expression.

Methionol production in recombinant strains

In order to investigate the effect of co-expression of the aminotransferase and decarboxylase genes on methionol production, strains S288c, SCK, S8, S10 and S810 were cultivated continuously in fermentation media containing 10.0 g L^{-1} of methionine in 500 mL flasks. As shown in Fig. 4, the dynamics of methionol production over time in each strain followed a similar pattern. During the first 24 h, the methionol production sharply increased in the exponential growth phase until it reached the plateau. After 132 h of fermentation, strain S810 produced the most methionol with a yield of 1.27 g L^{-1}, followed by strain S10, in which the methionol yield reached 1.12 g L^{-1}. In other strains the methionol yield hardly exceeded 1.0 g L^{-1}.

These fermentation data was consistent with the aforementioned aminotransferase and decarboxylase activity results. The strain S810 that exhibited the strongest aminotransferase and decarboxylase activity produced 68.8% and 51.8% more methionol than the control strain SCK and the wild type strain S288c. And the strains S10 and S8 with either higher decarboxylase or aminotransferase activity also produced more methionol compared to the strains SCK and S288c. Besides, it's noteworthy that the strain S10 with over-expression of *ARO10* produced much more methionol than the strain S8, suggesting that the decarboxylation reaction catalyzed by Aro10 probably outweighed the transamination step catalyzed by Aro8 in promoting the conversion of L-methionine into methionol. The strain S9 was not included for flask fermentation since it did not show higher aminotransferase activity and in preliminary experiment the methionol production in the strain S9 was at the same level as that in the strain SCK, which was much lower than that in the strain S8 (data not shown).

As it is economical to use natural substrates as fermentation media for industrial process, the methionol production in recombinant strains cultivated in YPD media was also investigated in this work. However, using YPD media did not result in a higher methionol yield. After 144 h of fermentation in YPD media supplemented with 10.0 g L^{-1} of methionine, the strains S810 and S10 produced about 1.4 g L^{-1} of methionol (Fig. S1). Considering the initial 0.4 g L^{-1} of methionol from the media, the net amount of methionol produced in S810 and S10 was just around 1.0 g L^{-1} (Fig. S1), which was lower than that obtained by cultivation in the fermentation media

containing methionine as the sole nitrogen source. When the additional 10.0 g L^{-1} of methionol was absent from YPD media, all the five strains could hardly produce more methionol during the whole fermentation process (Fig. S2). The possible reason for that lied in that the presence of other amino acids from YPD media interfered the methionol biosynthesis from methionine catabolism. Various amino acids including valine, leucine, isoleucine, methionine, and phenylalanine can be converted into fusel alcohols or acids via the Ehrlich pathway in *S. cerevisiae*, during which the aminotransferase Aro8 and the decarboxylase Aro10 get involved in catalysis reactions (Hazelwood *et al*., 2008). When methionine was not the sole nitrogen source, the catalytic efficiency of Aro8 and Aro10 would be diffused by other amino acids' catabolism, leading to lower methionol production.

According to the flask experiment results, a fed-batch fermentation was carried out in the strains S810 and S288c in bioreactors. A final methionol concentration of 3.24 g L^{-1} was obtained in the strain S810 after 72 h of cultivation, which was 1.3-fold higher than that in the wild strain S288c. The results demonstrated that increase of decarboxylase and aminotransferase activity significantly enhanced the methionol production in *S. cerevisiae*. To the authors' knowledge, it is the first time that more than 3.0 g L^{-1} of methionol produced by genetically engineered yeast strain is reported by co-expression of the aminotransferase and decarboxylase via the Ehrlich pathway. This work provided a new strategy for methionol production enhancement in *S. cerevisiae*.

Acknowledgments

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Figure legends

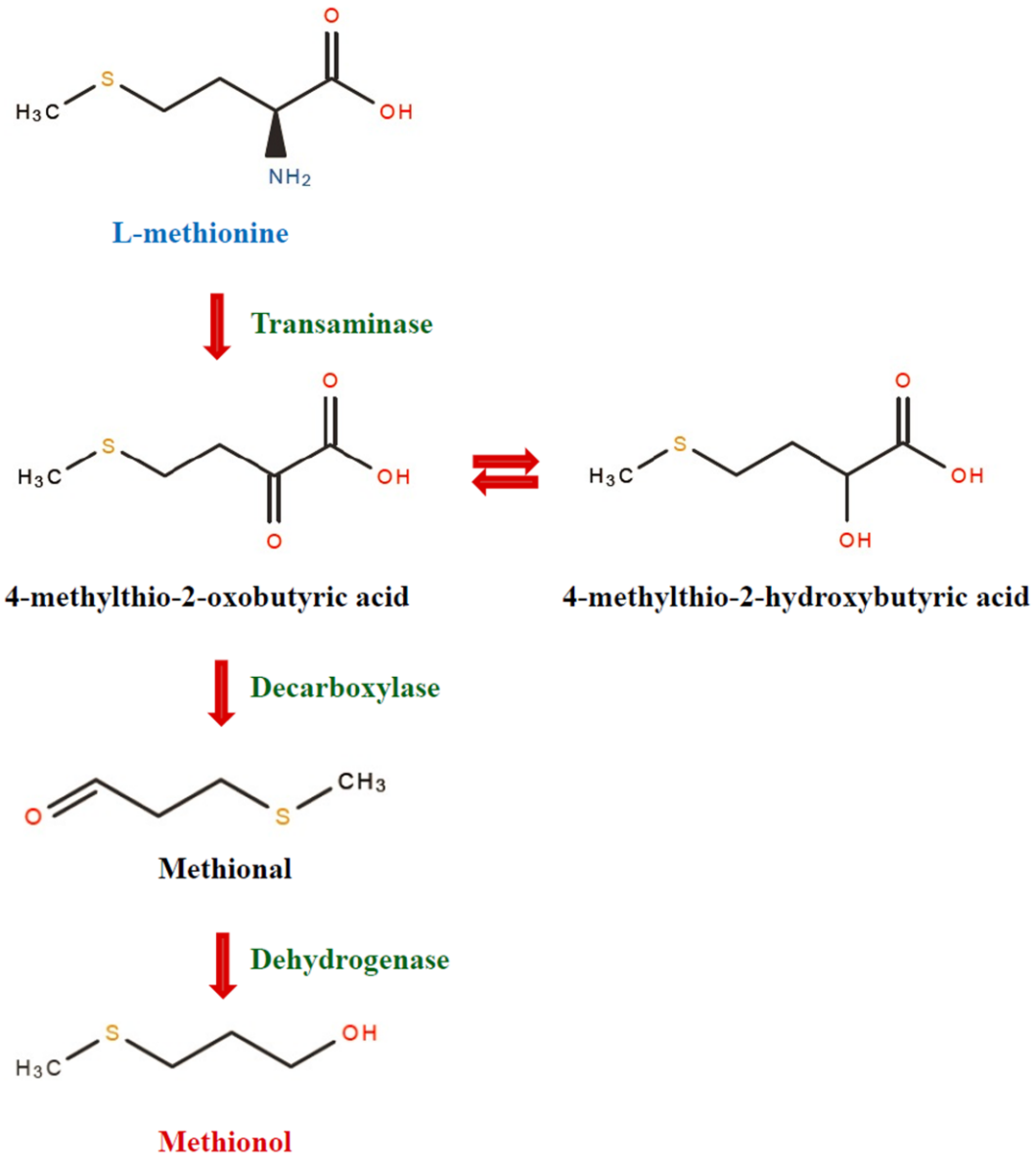


Fig. 1. The conversion of L-methionine into methionol via the Ehrlich pathway in *S. cerevisiae.*

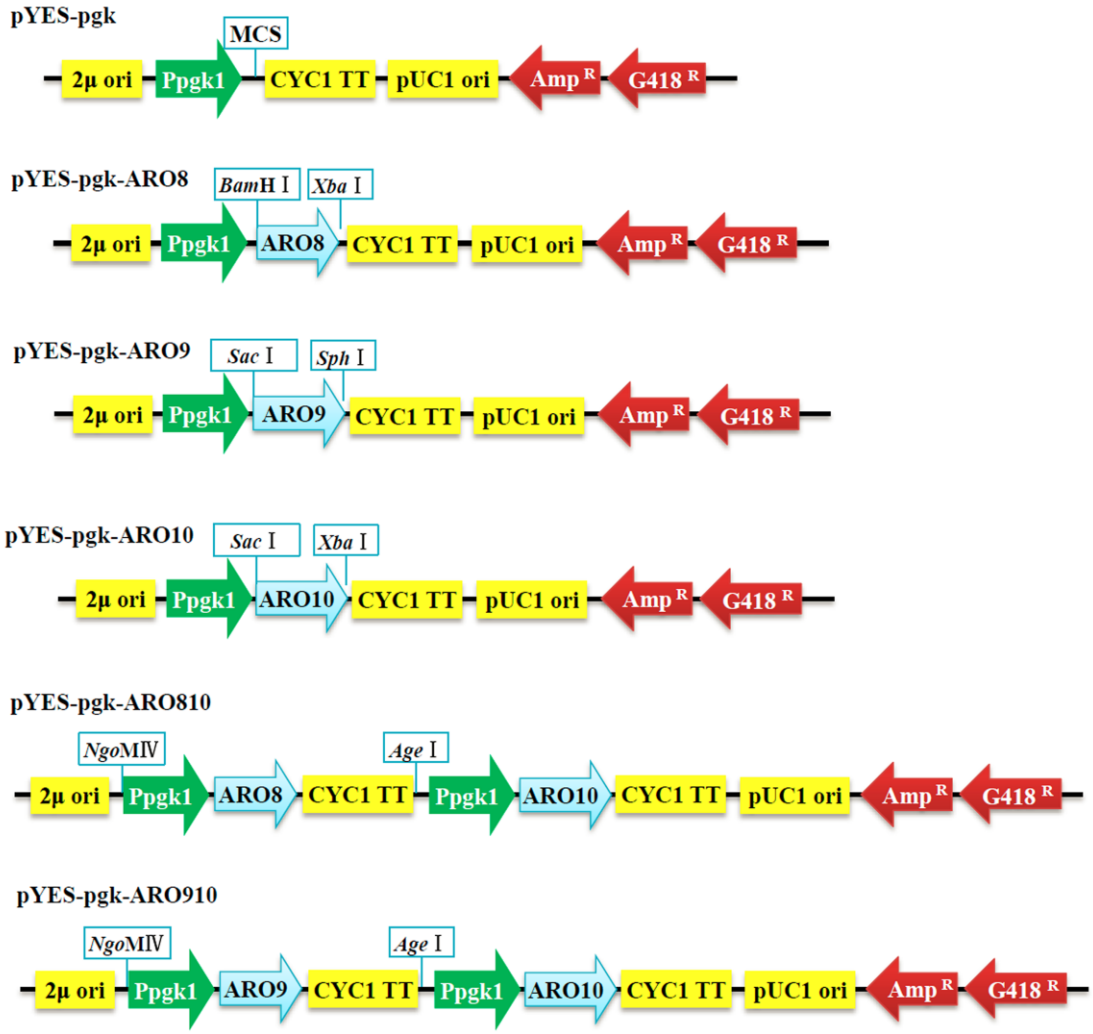
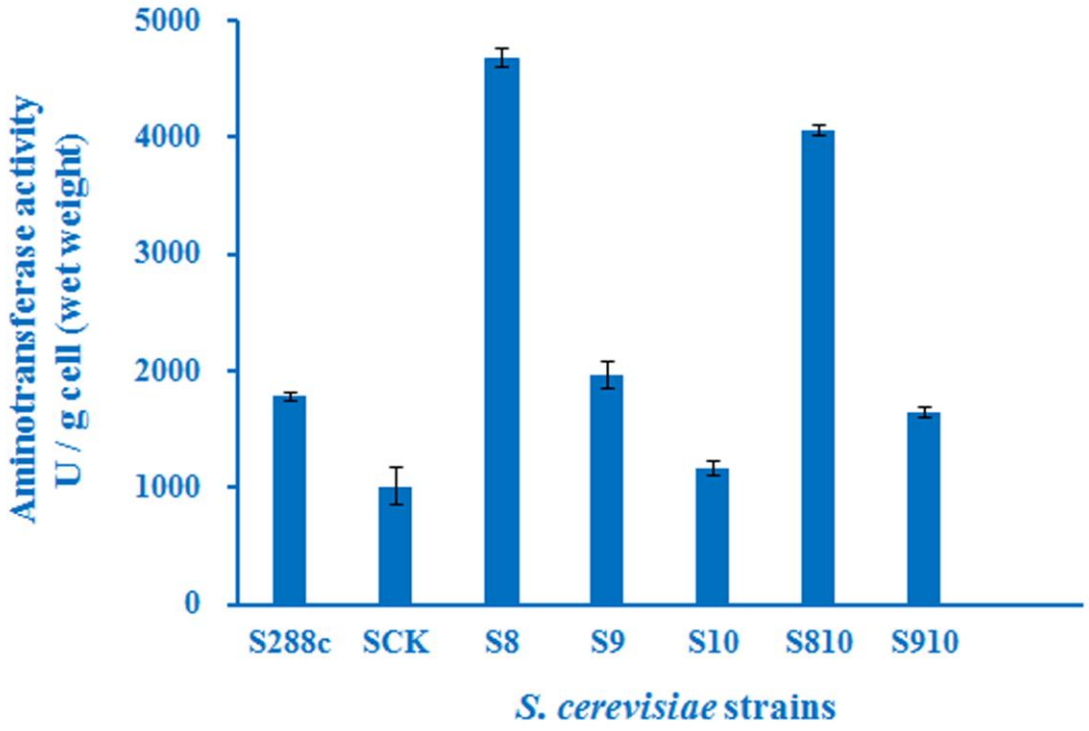


Fig. 2. Schematic representation of construction of the recombinant expression vectors. ORFs and promoters are indicated by closed arrows, replication origins and terminators are indicated by closed boxes. Some restriction enzyme sites are shown. MCS, multiple cloning site; pUC1 ori, replication origin 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; ARO9, the *ARO9* gene; ARO10, the *ARO10* gene.

A



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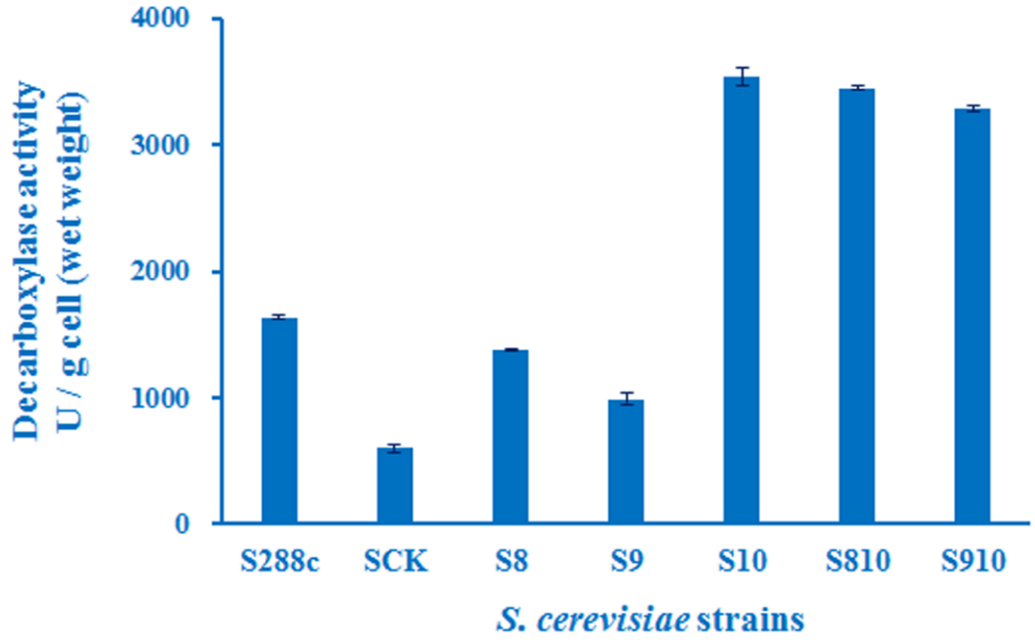

Fig. 3. Detection of aminotransferase (a) and decarboxylase (b) activity in recombinant strains. Enzymatic activity is expressed in U per g of cells (wet weight)

in the stationary growth phase after 36 h of cultivation in fermentation media. The results correspond to the mean value of three parallel assays. Error bars represent standard deviations. S288c, the wild strain *S. cerevisiae* S288c; SCK, *S. cerevisiae* SCK harboring the empty vector pYES-pgk ; S8, *S. cerevisiae* S8 with over-expression of *ARO8*; S9, *S. cerevisiae* S9 with over-expression of *ARO9*; S10, *S. cerevisiae* S10 with over-expression of *ARO10*; S810, *S. cerevisiae* S810 with co-expression of *ARO8* and *ARO10*; S910, *S. cerevisiae* S910 with co-expression of *ARO9* and *ARO10*.

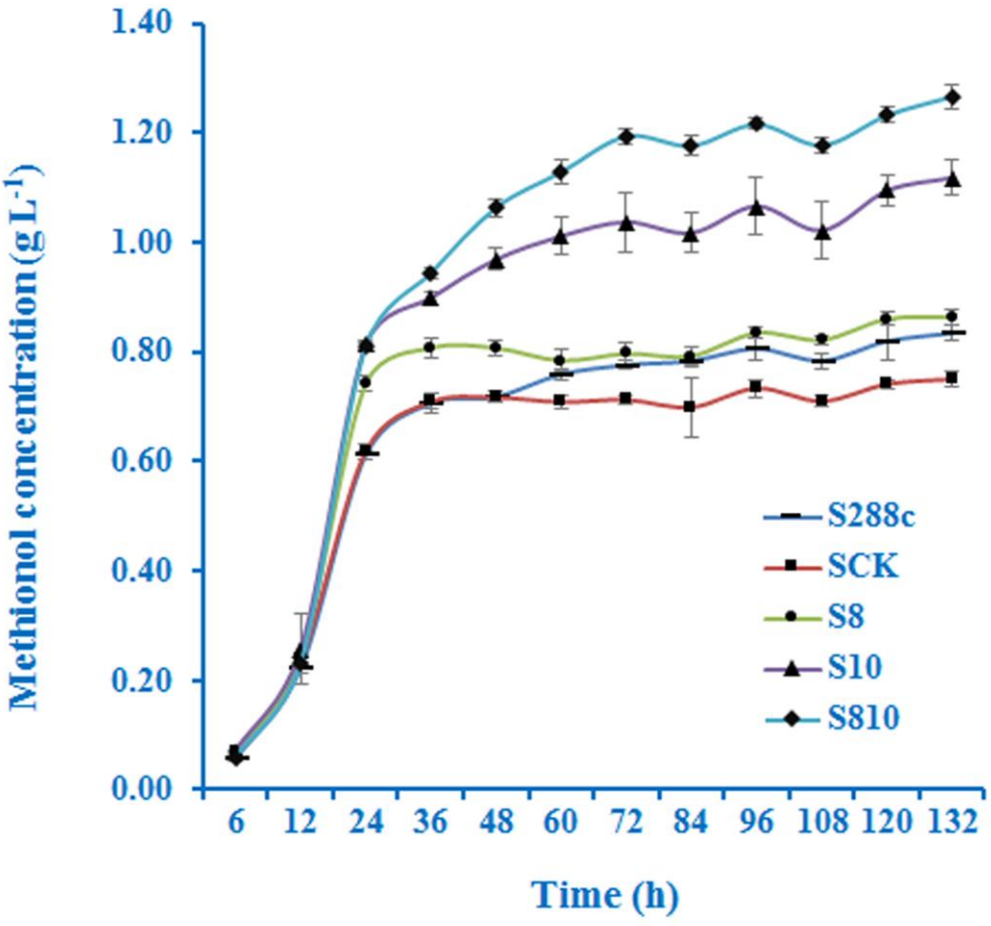

Fig. 4. Measurement of the methionol production in recombinant strains. The results correspond to the mean value of three parallel assays. Error bars represent standard

deviations. S288c, the wild strain *S. cerevisiae* S288c; SCK, *S. cerevisiae* SCK harboring the empty vector pYES-pgk ; S8, *S. cerevisiae* S8 with over-expression of *ARO8*; S10, *S. cerevisiae* S10 with over-expression of *ARO10*; S810, *S. cerevisiae* S810 with co-expression of *ARO8* and *ARO10*.

Graphical Abstract