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Overexpressing enzymes of the Ehrlich pathway and deleting genes of the competing pathway in *Saccharomyces cerevisiae* for increasing 2-phenylethanol production from glucose

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2-Phenylethanol (2-PE) is a higher aromatic alcohol that is used in the cosmetics and food industries. The budding yeast *Saccharomyces cerevisiae* is considered to be a suitable host for the industrial production of higher alcohols, including 2-PE. To produce 2-PE from glucose in *S. cerevisiae*, we searched for suitable 2-keto acid decarboxylase (KDC) and alcohol dehydrogenase (ADH) enzymes of the Ehrlich pathway for overexpression in strain YPH499, and found that overexpression of the *ARO10* and/or *ADH1* genes increased 2-PE production from glucose. Further, we screened ten BY4741 single-deletion mutants of genes involved in the competing pathways for 2-PE production, and found that strains *aro8*Δ and *aat2*Δ displayed increased 2-PE production. Based on these results, we engineered a BY4741 strain that overexpressed *ARO10* and contained an *aro8*Δ deletion, and demonstrated that the strain produced 96 mg/L 2-PE from glucose as the sole carbon source. As this engineered *S. cerevisiae* strain showed a significant increase in 2-PE production from glucose without the addition of an intermediate carbon substrate, it is a promising candidate for the large-scale production of 2-PE.

[**Key words:** 2-Phenylethanol; Ehrlich pathway; *ARO10*; *ARO8*; Glucose fermentation; *Saccharomyces cerevisiae*]

2-Phenylethanol (2-PE) is an aromatic alcohol with a rose-like fragrance and is commonly used as a flavor component in the perfumery, cosmetics, and food industries (1,2). In addition, due to its high-energy potential, 2-PE is a candidate molecule for biofuel production. Natural 2-PE is obtained by extraction from the essential oils of plants and flowers; however, this process is associated with low recovery rates and high production costs (3,4). Although 2-PE can be chemically synthesized, US and European legislation restrict the use of chemically synthesized 2-PE in food and cosmetic products (5). Thus, the bioproduction of 2-PE by microorganisms, such as the budding yeast *Saccharomyces cerevisiae*, which have the potential to produce 'natural' 2-PE in high yields, is a promising alternative to low-yielding and costly extraction and purification processes.

S. cerevisiae has suitable characteristics for industrial applications, such as bioethanol production, and has Generally Recognized as Safe (GRAS) status (6). Because *S. cerevisiae* naturally produces small amounts of 2-PE (1,7), several 2-PE-producing yeast strains have been obtained through the screening of randomly mutagenized strains. However, despite optimization of the medium and culture conditions (1,3,7), only slight increases in 2-PE production were achieved using this approach. Recent study reported that the metabolically engineered *Kluyveromyces marxianus* produced 1.3 g/L of 2-PE from 20 g/L glucose, and therefore it may be a suitable host strain for 2-PE production (8). However, *K. marxianus* might be still restricted for further engineering for 2-PE production, because there are few tools for gene expression and efficient gene deletion that can be easily accessible in the common resources (9,10). In other study, an engineered *S. cerevisiae* produced 4.8 g/L of 2-PE from the medium containing 10 g/L phenylalanine (not glucose) by triply overexpressing *ARO9* (amino acid transaminase), *ARO10* (2-keto acid decarboxylase) and *ARO80* (transcription factor for *ARO9* and *ARO10*) genes along with the deletion of *ALD3* (aldehyde dehydrogenase) (11). In this case, phenylalanine was used as a substrate in the optimized medium; however, it is not an ideal carbon substrate for cost-effective fermentation. Therefore, here, we focused on the production of 2-PE in *S. cerevisiae* from glucose, a major fermentable sugar that can be obtained from the hydrolysis of cellulosic biomass.

In recent years, *Escherichia coli* and *S. cerevisiae* have been metabolically engineered to produce a variety of fusel alcohols, including 2-PE, by overexpressing the Ehrlich pathway enzymes 2-keto acid decarboxylase (KDC) and alcohol dehydrogenase (ADH) (2,7,12). In *S. cerevisiae*, 2-PE biosynthesis from glucose is catalyzed by the shikimate and Ehrlich pathways (3,4). Phenylalanine is first synthesized via phosphoenolpyruvate (PEP) and erythrose 4-phosphate (E4P) through the shikimate pathway and is subsequently converted into the intermediate phenylpyruvate, which is then converted to 2-PE via phenylacetaldehyde through the Ehrlich pathway in reactions catalyzed by KDC and ADH (Fig. 1) (7,13). Phenylacetaldehyde is also converted to phenylacetate in a

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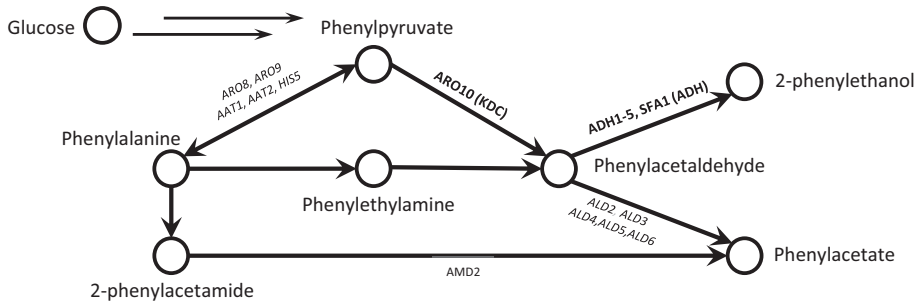

FIG. 1. Pathway for 2-phenylethanol (2-PE) production in *S. cerevisiae*. Bold letters indicate overexpressed genes and italicized letters indicate deleted genes.

competing reaction catalyzed by acetaldehyde dehydrogenase (ALD) (Fig. 1). In addition, phenylpyruvate is reversibly converted to phenylalanine by the catalysis of transaminase, leading to the generation of 2-PE via phenylethylamine through a different pathway (Fig. 1) (14).

In the present study, we first selected suitable KDC and ADH genes for overexpression in *S. cerevisiae* among several candidate enzymes for 2-PE production using glucose as the carbon source. Glucose was selected because it is readily fermented by *S. cerevisiae* and can be obtained from the hydrolysis of cellulosic biomass. We then screened for transaminase and acetaldehyde dehydrogenase gene-knockout strains with increased 2-PE production. Finally, we overexpressed the *ARO10* and/or *ADH1* genes in a *S. cerevisiae* strain with transaminase and acetaldehyde dehydrogenase gene deletions, and demonstrated a marked elevation of 2-PE production from glucose by the metabolically engineered strain with *ARO10* overexpression and *ARO8* transaminase deletion.

MATERIALS AND METHODS

Strains and plasmids *S. cerevisiae* YPH499 (*MAT***a** *ura3-52 lys2-801 ade2-101 trp1-Δ63 his3-Δ200 leu2-Δ1*; Stratagene, La Jolla, CA, USA), BY4741 (*MAT***a** *his3Δ1 leu2Δ0 met15Δ0 ura3Δ0*), and BY4741 single-gene deletion mutants (Yeast Deletion Clones Mat-A Complete Set; Invitrogen, Carlsbad, CA, USA) were used as host strains. All plasmids used in this study were derived from the pGK vector series, in which expression of the target gene is under control of the *PGK1* promoter (15). The expression plasmid pGK425-ARO10 was constructed by inserting the *BglII*/*NheI*–digested *ARO10* gene from pGK424-ARO10 (16) into the same restriction endonuclease sites of vector pGK425 (16). All plasmids were transformed into *S. cerevisiae* by the lithium acetate method (17). The yeast strains and plasmids generated in this study are listed in Tables 1 and 2, respectively.

2-PE fermentation The yeast strains were cultured for 72 h at 30°C in 5 ml SD (20 g/L glucose and 6.7 g/L yeast nitrogen base without amino acids) minimal medium (containing all required amino acids and nucleotides) or SD selective medium (containing the required amino acids and nucleotides for appropriate auxotrophy). The cells were centrifuged at 1000×*g* for 5 min, washed once with sterile water, and then further cultured in 5 ml fresh SD minimal medium or SD selective medium. After 48 h of fermentation, the concentrations of 2-PE in the medium were determined by GC–MS (GCMS-QP2010 Ultra; Shimazu, Kyoto, Japan) basically using a previously described procedure (16) (30–200 *m*/*z* scan mode was used in Fig. 2, and 91, 92 and 122 *m*/*z* SIM mode was used in Figs. 3–5).

RESULTS AND DISCUSSION

Selection of KDC and ADH enzymes for increasing 2-PE production To increase 2-PE production by *S. cerevisiae*, we attempted to identify suitable KDC and ADH enzymes of the Ehrlich pathway for overexpression. Specifically, three types of KDC enzymes were examined: phenylpyruvate decarboxylase Aro10, a predicted alpha-ketoisocaproate decarboxylase (Thi3) derived from *S. cerevisiae* (18,19), and the decarboxylase Kivd derived from *Lactococcus lactis* (20). In addition, six types of ADH enzymes were evaluated: Adh1, 2, 5, 6, and 7, and Sfa1. Notably, mitochondrial enzyme Adh3 and zinc-dependent enzyme Adh4 derived from *S. cerevisiae* were excluded from the analysis (7,16,21,22). The expression plasmids for the selected ADH and KDC genes were introduced into *S. cerevisiae* strain YPH499 (Tables 1 and 2), and the obtained transformants were inoculated into SD selective medium containing 20 g/L glucose for 48-h fermentation (Fig. 2), because the yeast cells definitely consumed the glucose in the media and mostly showed maximum productivity for the 2-keto-acid-mediated fermentations until 48 h (8,11,12). The overexpression of *kivd* and *ARO10* led to an increase of 2-PE production by strain YPH499, whereas overexpression of *THI3* had no effect on 2-PE yields. This latter finding may have been due to the low enzymatic activity or low specificity of Thi3 for phenylpyruvate (23). Compared with *kivd* and *ARO10*, overexpression of the ADH-encoding genes appeared to have a less marked effect on 2-PE production. However, co-expression of the *ADH1*, *ADH2* or *ADH5* gene with the *ARO10* gene led to an increase of 2-PE production. In the strain overexpressing *ARO10* and *ADH1*, maximal 2-PE production in SD selective medium reached 48 mg/L after 48 h of fermentation.

In the recent report, the triple overexpression of *ARO9*, *ARO10* and *ARO80* in *ald3*Δ strain produced 4.8 g/L of 2-PE from the medium containing 10 g/L phenylalanine (11). In our case using 20 g/L glucose as a sole carbon source, *ARO10* also played important roles for 2-PE production. However, we never tested *ARO9* and *ARO80* overexpressions, because *ARO9* and *ARO80* had been predicted to enhance the conversion of phenylpyruvate to phenylalanine, which has high possibility to reduce the 2-PE production from glucose (Fig. 1).

Screening of gene-deletion strains of the competing pathway for 2-PE production The deletion of pathways that compete with the target metabolic process often improves production of the target chemical(s) (24). In *S. cerevisiae*, phenylpyruvate can be transaminated to phenylalanine, and phenylacetaldehyde can be oxidized to phenylacetate (14,25,26). As these reactions compete with the Ehrlich pathway for 2-PE production (Fig. 1), here, we performed 2-PE fermentation tests in SD minimal medium using ten BY4741-derived single-gene deletion strains of five aminotransferases (Aat1, mitochondrial aspartate aminotransferase; Aat2, cytosolic aspartate aminotransferase; His5, histidinol-phosphate aminotransferase; Aro8 and Aro9, aromatic aminotransferases) involved in the transamination of phenylpyruvate to phenylalanine (14,27–29), and five aldehyde dehydrogenases (Ald2 and Ald3, cytosolic aldehyde dehydrogenases; Ald4, Ald5 and Ald6, mitochondrial aldehyde dehydrogenases) involved in the oxidation of phenylacetaldehyde to phenylacetate (Fig. 3) (25,26). Most gene deletion strains showed similar productivities of 2-PE compared with the wild-type strain, with the exception of strains *aat2Δ* and *aro8Δ*. 2-PE production by the *aat2*Δ and *aro8*Δ single-deletion strains increased by 174% and 127%, respectively, compared to that of wild-type BY4741. As the single deletion of the *ALD* genes did not affect 2-PE production, the oxidation of