Noda Institute for Scientific Research GRANT
2013 Young Investigator Research Grant
Outline of Research Result

Genetic engineering of *Kluyveromyces marxianus* for effective production of the rose-like odor 2-phenylethanol

Takashi KOYANAGI

Department of Food Science, Ishikawa Prefectural University

Research aims

2-Phenylethanol (2-PE) is found in various natural essential oils, particularly in that of rose and other related plants. Because the compound elicits a pleasant, rose-like odor, it is commonly used as an ingredient in artificial flavoring and in perfumery. Annually, more than several thousand tons of this compound is industrially produced by chemical synthesis, and consumed all over the world at inexpensive prices. However, there is currently a strong demand for natural aroma compounds that are not produced through chemical methods. Likewise, there is an increasing interest in white biotechnology and green chemistry. One valid approach for natural 2-PE production is direct extraction from plants; however, this process requires vast farmlands and long cultivation times. Another possibility is yeast fermentation, which is widely used for production of alcoholic compounds.

Kluyveromyces marxianus is a species of budding yeast that, owing to its vast metabolic capabilities and capacity for assimilation, has been used in a variety of bioindustrial processes, such as ethanol fermentation and whey bioconversion. *Kluyveromyces marxianus* is also known as a potent producer of 2-PE.[1, 2] In this species, 2-PE is produced via the same biosynthesis pathways that generate the aromatic amino acids: The pentose-phosphate pathway and the shikimic acid pathway. In this cascade, an intermediate product, phenylpyruvate, is converted into phenylacetaldehyde by the enzyme phenylpyruvate decarboxylase (Ppd, EC 4.1.1.43). Phenylacetaldehyde is then converted into 2-PE by alcohol dehydrogenase (Adh, EC 1.1.1.1) (Fig. 1),[3] and 2-PE is partially converted into phenylethylacetate by alcohol acetyltransferase (Atf, EC 2.3.1.84). It has also been shown that 2-PE is alternatively produced through the Ehrlich pathway, in which the a-amino group of phenylalanine is removed to produce phenylpyruvate through a bidirectionally regulated reaction, catalyzed by aromatic amino acid transaminase (EC 2.6.1.5 or 2.6.1.57).

In this study, we attempted to increase the levels of 2-PE production in *K. marxianus* NBRC1777 by genetic modification. In addition, we carried out metabolomic analyses to gain detailed insights into 2-PE production in relation to cellular metabolic flow.

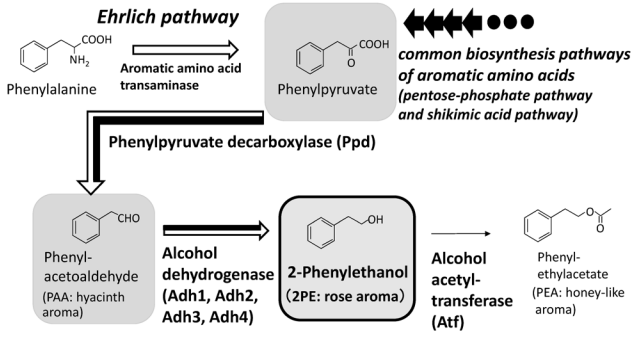

Fig. 1. 2PE biosynthesis pathway

Methods

Construction of a recombinant K. marxianus strain that overexpresses Ppd

The *E. coli*-yeast shuttle vector pDblet[4] was used to clone the structural gene of Ppd (*KmPPD*). The gene was amplified by polymerase chain reaction from genomic DNA of *K. marxianus* NBRC1777, and was placed under the control of the strong constitutive promoter of the *Saccharomyces cerevisiae* glyceraldehyde 3-phosphate dehydrogenase (GAPDH) gene. The Ppd-expression plasmid was then introduced into the auxotrophic strain, *K. marxianus* Δ*ura3* Δ*trp1* Δ*leu2* (NBRC1777 derivative), by electroporation, and cells were spread on plates of synthetic complete medium (SC) lacking uracil, to select for strains containing the plasmid. The production of 2-PE was measured photometrically using high-performance liquid chromatography (HPLC), equipped with Inertsil ODS3 (GL Science).

Metabolomic analysis

For metabolomic analysis, CE-TOFMS (cation and anion modes) was employed to evaluate the metabolic balance in *K. marxianus* cells overproducing 2-PE. The analysis was performed by the Human Metabolome Technologies Co. (Yamagata, Japan).

Results

The *KmPPD* gene was introduced into the *K. marxianus* Δ*ura3* Δ*trp1* Δ*leu2* strain, and the accumulation of 2-PE in the culture supernatants was monitored (Fig. 2). The results

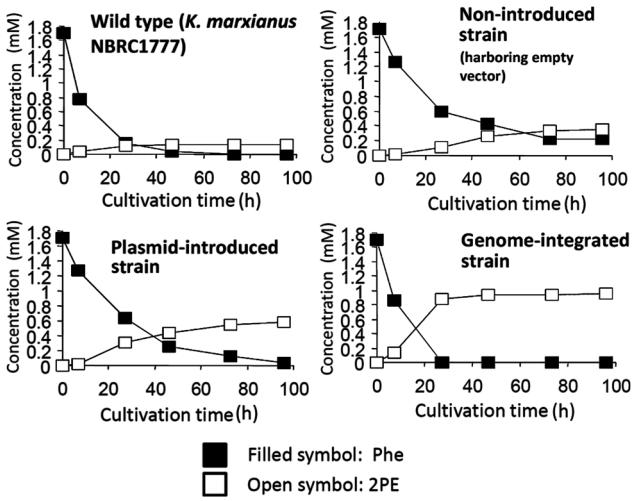

Fig. 2. Accumulation of 2-PE in culture supernatants.
Upper left: Wild type (NBRC1777), upper right: Strain containing the empty vector, lower left: Strain containing plasmid-encoded *KmPPD* lower right: Strain containing genome-integrated *KmPPD*

revealed that an increase in the copy number of *KmPPD* greatly affected the production of 2-PE. This was especially true when the genome- integrated *KmPPD* was present (8-fold increase, compared to the wild-type strain NBRC1777).

We then carried out the metabolomic analysis to elucidate the flow of cellular metabolism in the 2-PE-overproducing cells. For these analyses, the auxotrophic strain (*K. marxianus* Δ*ura3* Δ*trp1* Δ*leu2*), the strain containing a genome-integrated copy of *KmPPD* (Δ*ura3* Δ*trp1* Δ*leu2* $URA4^{+}_{S.\ pombe}$-$KmPPD^{+}$), and the control strain, harboring only the auxotrophic marker gene (Δ*ura3* Δ*trp1* Δ*leu2* $URA4^{+}_{S.\ pombe}$), were cultivated in SC medium at 37°C with shaking (140 rpm), and cell extracts were harvested. The strain carrying an integrated copy of *KmPPD* exhibited a marked increase in the accumulation of the glycolysis pathway intermediates, glucose-6-phosphate, fructose-6-phosphate, fructose-1,6-diphosphate, and dihydroxyacetone phosphate, in comparison with the control strain. A significant increase in sedoheptulose 7-phosphate accumulation, an important intermediate of pentose-phosphate pathway, was also observed. These results indicate that overexpression of Ppd may result in an increase in the metabolic flow of aromatic amino acid biosynthesis, including the shikimic acid pathway, which in turn also promotes glycolysis and the pentose phosphate pathway.

Conclusion

The introduction of an additional copy of the *KmPPD* gene into *K. marxianus* resulted in an increase in the level of intracellular 2PE accumulation. Furthermore, this increase in 2-PE was found to be attributable to a change in metabolic flow, including glycolysis and the pentose-phosphate pathway, as revealed by a metabolomic analyses. These results indicate that the level of Ppd expression is a critical component in the regulation of pathway flux and can tip the balance towards the 2-PE specific production pathway.

References

1) Wittmann, C., Hans, M., and Bluemke, W. (2002) Metabolic physiology of aroma-producing *Kluyveromyces marxianus*. *Yeast* **19**: 1351–1363.
2) Etschmann, M.M.W., Bluemke, W., Sell, D., and Schrader, J. (2002) Biotechnological production of 2-phenylethanol. *Appl. Microbiol. Biotechnol.* **59**: 1–8.
3) Lertwattanasakul, N., Sootsuwan, K., Limtong, S., Thanonkeo, P., and Yamada, M. (2007) Comparison of the gene expression patterns of alcohol dehydrogenase isozymes in the thermotolerant yeast *Kluyveromyces marxianus* and their physiological functions. *Biosci. Biotechnol. Biochem.* **71**: 1170–1182.
4) de Souza Junior, C.G. and de Morais Junior, M.A. (2000) The use of the replicating pDblet plasmid as a cloning vector with enhanced stability in *Kluyveromyces marxianus*. *Biotechnol. Lett.* **22**: 43–45.