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Cofactor self-sufficient whole-cell biocatalysts for the production of 2-phenylethanol

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ABSTRACT

The efficiency of biocatalysis is often affected by an insufficient supply and regeneration of cofactors and redox equivalents. To alleviate this shortcoming, a cofactor self-sufficient system was developed for enhanced production of 2-phenylethanol (2-PE) in *E. coli*. A "bridge" between the amino acid and its corresponding alcohol was designed in the system using glutamate dehydrogenase. By coupling glutamate dehydrogenase with transaminase and alcohol dehydrogenase, the cosubstrate (2-oxoglutarate) and redox equivalents (NAD(P)H) were regenerated simultaneously, so that no external cofactor or redox source was required. Thus, a cofactor self-sufficient system was developed, which improved the biocatalyst efficiency 3.8-fold. The ammonium generated in this process was removed using zeolite, which further improved the biosynthetic efficiency and resulted in a cleaner system. To the best of our knowledge, this system yielded the highest titer of 2-PE ever obtained in *E. coli*. Additionally, the wider applicability of this self-sufficient strategy was demonstrated in the production of D-phenyllactic acid. This study thus offers a new method to resolve the cofactor/redox imbalance problem and demonstrates the feasibility of the cofactor self-sufficient strategy for enhanced production of diverse chemicals.

1. Introduction

2-Phenylethanol (2-PE), an aromatic alcohol with a pleasant rose-like fragrance, is widely used in the cosmetic and food industries. 2-PE is traditionally extracted from rose, the yield is typically low (Etschmann et al., 2002). Nowadays, 2-PE is mostly produced by chemical synthesis, which is environmentally unfriendly and produces unwanted by-products (Xu et al., 2007). On the other hand, biotechnologically produced flavors are currently considered as natural by European and U.S. food agencies (Xu et al., 2007). Therefore, biotechnological 2-PE production is highly desirable and holds promise to be the most commercially viable route to produce 2-PE (Hua and Xu, 2011).

2-PE can be produced by yeast *via* the Ehrlich pathway from L-phenylalanine (L-Phe) (Hazelwood et al., 2008). There are three reactions involved in this synthetic pathway (Fig. 1a). First, aromatic transaminase converts L-Phe to phenylpyruvate (PPA) using 2-oxoglutarate (2-OG) as an amine receptor. Second, PPA is converted to phenylacetaldehyde (PAAL) by phenylpyruvate decarboxylase (PDC). Finally, aldehyde reductase or alcohol dehydrogenase (ADH) converts PAAL to 2-PE, at the expense of the cell's reducing power. Great efforts have been made on yeast-based production of 2-PE, encompassing metabolic engineering, fermentation and process engineering, leading to the production of about 6 g/L 2-PE by yeast fermentation (Eshkol et al., 2009; Etschmann and Schrader, 2006; Kim et al., 2014; Morrissey et al., 2015; Stark et al., 2002). Furthermore, since cell growth is inhibited by the accumulated 2-PE (Etschmann and Schrader, 2006), *in situ* product removal (ISPR) has been used to extract 2-PE in the aqueous phase to circumvent the problem of product inhibition, which is proven to be a useful approach. Extractants such as polypropylene glycol 1200 and Hytrel® 8206 were used to remove 2-PE from the fermentation broth, and 10.2–20.4 g/L 2-PE was achieved in 30–36 h (Etschmann and Schrader, 2006; Gao and Daugulis, 2009). However the yeast fermentation process usually takes 30–50 h or even a few

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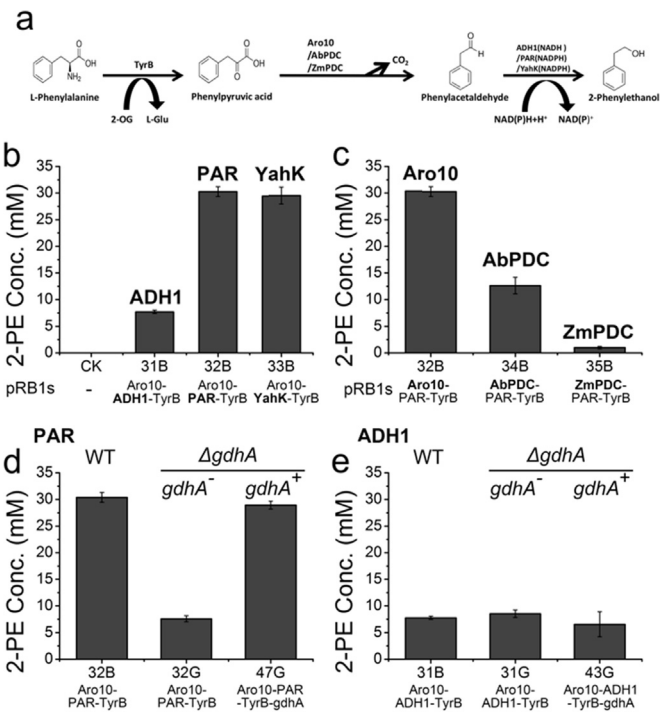

Fig. 1. 2-PE production from L-Phe by whole-cell bioconversion using different recombinant E. coli strains. (a) Biosynthesis of 2-PE from L-Phe *via* the Ehrlich pathway; (b) Production of 2-PE by strains with different reductases; (c) Production of 2-PE by strains with different phenylpyruvate decarboxylases; (d) NADPH-dependent reductase PAR with genomically or plasmid-expressed GDH; (e) NADH-dependent dehydrogenase ADH1 with genomically or plasmid-expressed GDH.

days, so 2-PE productivity is relatively low (Etschmann and Schrader, 2006; Mei et al., 2009).

E. coli is a widely used chassis for the production of fine chemicals because it is fast-growing and easy to manipulate. However, *E. coli* cannot intrinsically produce 2-PE because it lacks PDC, which converts PPA to PAAL (Koma et al., 2012). By heterogeneously overexpressing PDC from *Azospirillum brasilense*, the engineered *E. coli* was able to produce 6.9 mM 2-PE (Koma et al., 2012). To further improve the efficiency of the pathway, well-characterized genes encoding phenylacetaldehyde synthase and phenylacetaldehyde reductase from plants were introduced to optimize the 2-PE biosynthetic pathway in *E. coli* (Achmon et al., 2014; Sakai et al., 2007). The shikimic acid pathway was redesigned to increase the supply of the intermediate (PPA) and 2-PE production from glucose was improved to 2.33 mM (0.285 g/L) (Kang et al., 2014). Hwang et al. engineered *E. coli* for the whole-cell bioconversion of phenylalanine and 38 mM (4.7 g/L) 2-PE was produced from 100 mM L-Phe (Hwang et al., 2009). However, the yield of 2-PE produced by engineered *E. coli* remained low. For the biosynthesis of 2-PE, 2-OG and NAD(P)H are required for the transamination and dehydrogenation reactions, respectively, the limited cofactor supply and redox imbalance remain major bottlenecks.

Cofactor recycling is key not only to lower process costs, but also to drive the reaction of interest to completion. When reducing power is needed in a whole-cell biotransformation process, introducing an oxidoreductase is one solution to recycle NAD(P)H, and often leads to improved catalytic efficiency (Hummel and Groger, 2014). The enzymes most commonly exploited to recycle NAD(P)H are formate dehydrogenase and glucose dehydrogenase, which obtain reduction equivalents by oxidizing a sacrificial substrate (Kratzer et al., 2015). For example, by coupling the relevant reaction to the oxidation of formate to CO_2 for NADH regeneration, o-chloroacetophenone and xylose were reduced to (S)-1-(2-chlorophenyl)-ethanol and xylitol with high yield (Eixelsberger et al., 2013; Madje et al., 2012). For 2-PE biosynthesis, glucose dehydrogenase was overexpressed in engineered *E. coli* for NADPH regeneration, which resulted in a 3-fold increase of 2-PE yield (4.7 g/L), compared to the parent strain without glucose dehydrogenase (Hwang et al., 2009). This enzymatic approach thus offered a solution for NAD(P)H regeneration and resulted in higher yield. However, since enzyme-mediated cofactor recycling for NAD(P)H regeneration consumes a sacrificial substrate, and an additional coproduct is produced, this approach increases the complexity of product purification, and thus adds up the overall process costs. In addition, the high concentrations of sacrificial substrate or coproduct may inhibit enzyme activity. Recently, Mutti et al. developed an elegant redox self-sufficient system using alcohol and amine dehydrogenases, which operate in tandem to convert alcohols to amines with hydrogen shuttled by a nicotinamide coenzyme (Mutti et al., 2015). However, for a biosynthesis pathway consuming both cosubstrate and redox equivalents, such as 2-PE biosynthesis, the simultaneous regeneration of cosubstrate and redox equivalents remains a challenge. Metabolic engineering strategies have to be devised to generate a more efficient biocatalyst for enhanced production of 2-PE from the perspective of cofactor supply and regeneration.

In this study, a heterogeneous 2-PE biosynthetic pathway was constructed in *E. coli*, and the pathway flux was increased by substituting key enzymes. Glutamate dehydrogenase was found to play a crucial role in cofactor regeneration and in maintaining redox balance during 2-PE biosynthesis. Therefore, we designed a “bridge” between L-Phe and 2-PE using glutamate dehydrogenase, to develop a cofactor and redox self-sufficient system for efficient 2-PE production. The system was further improved by removing the by-product NH_3 to form a cleaner process. Finally, this cofactor self-sufficiency strategy was applied to the bioconversion of L-Phe into D-phenyllactic acid (D-PLA). The cofactor and redox self-sufficient strategy has the potential to enhance the production of a number of desirable chemicals with respect to cofactor regeneration and balance.

2. Materials and methods

2.1. Chemicals and reagents

L-Phe, 2-OG, L-Glu and zeolite were purchased from Shanghai ShengGong Bio-chemical Co. Ltd. (Shanghai, China). For high-performance liquid chromatography (HPLC) analysis, standards for L-Phe, PPA, PAAL, 2-PE and D-PLA were purchased from Sigma-Aldrich (Steinheim, Germany), Hytrel® 8206 was purchased from DuPont (Delaware, USA). Restriction endonucleases, DNA polymerases, T4 DNA ligase, and Gibson kits were purchased from New England Biolabs (USA).

2.2. Bacterial strains and culture conditions

All strains and plasmids used in this study are listed in Supplementary Table S1. P1 transduction based on Keio collection strains (Baba et al., 2006) enabled gene deletion. The mutant strains were verified by PCR and further confirmed by sequencing (Shenggong, China). *E. coli* strains were grown at 37 °C on a shaker at 220 rpm in LB (Luria–Bertani) medium with streptomycin (50 μg/mL) added as required. For protein expression, overnight cultures were inoculated into an auto-induction ZYM medium (Studier, 2005) with 1% inoculum and incubated under constant shaking at 20 °C (for 2-PE) or 37 °C (for D-PLA) for 16 h.

2.3. Cloning and expression

The genes encoding *Ab*PDC, *Zm*PDC, PAR, *Pa*GDH and hGDH were codon-optimized based on the *E. coli* codon preference; the other genes for 2-PE and D-PLA production were amplified from corresponding genomic DNA. The primers are listed in Supplementary Table S2. The