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FULL PAPER

Efficient Synthesis of Phenylacetate and 2-Phenylethanol via Modular Cascade Biocatalysis

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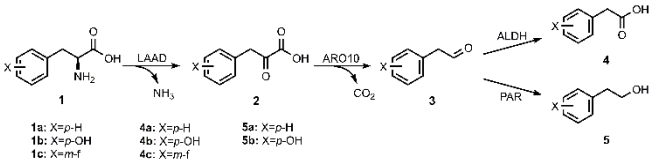
Abstract: Green and sustainable synthesis of chemicals from renewable feedstocks via biotransformation approach has gained an increasing attention in the recent years. In this report, we aimed to develop enzymatic cascades to efficiently convert L-phenylalanine (L-Phe) into 2-phenylethanol (2-PE) and phenylacetic acid (PAA), L-tyrosine (L-Tyr) into tyrosol (*p*-hydroxyphenylethanol, *p*-HPE) and *p*-hydroxyphenylacetic acid (*p*-HPAA). The enzymatic cascade was casted into aromatic aldehyde formation module, followed by aldehyde reduction module, or aldehyde oxidation module, to achieve one-pot biotransformation using the recombinant *Escherichia coli*. Biotransformation of 50 mM L-Phe produced PAA of 6.76 g/L with more than 99% conversion and 2-PE of 5.95 g/L with 97% conversion. And the bioconversion efficiency of *p*-HPAA and *p*-HPE from L-Tyr reached up to 88% and 94%, respectively. In addition, *m*-fluoro-phenylalanine was further employed as a non-natural aromatic amino acid substrate to obtain *m*-fluoro-phenylacetic acid, and above 96% conversion was achieved. In conclusion, our results demonstrated high-yielding and potential industrial synthesis of above aromatic compounds by one-pot cascade biocatalysis.

Introduction

Due to the oil consumption and consequent environmental pollution, green and sustainable manufacturing of fine chemicals from renewable feedstocks is drawing an increasing attention[1]. There are numerous excellent works on chemical or enzymatic conversion of biomass into bulk chemicals[2]. Due to mild reaction conditions and its capacity for performing cascade reactions, enzymatic conversion is considered as a promising alternative to chemical synthesis[3]. The development of synthetic biology and metabolic engineering has promoted the fermentative production of biobased bulk chemicals from (hemi)cellulose-derived sugars[4]. However, its ability to achieve high titer is limited by the toxicity of chemicals to the engineered microbial host cells. In comparison, biotransformation using the whole-cell biocatalyst is an efficient method to produce chemicals without the need to consider product toxicity issues[5]. Recently, a number of studies about generating natural[6] or non-natural products[7] from biobased substrates via cascade biotransformation have been reported. Therefore, there is a tremendous effort in developing efficient enzyme cascades to convert biobased substrates into target products.

Aromatic amino acids (AAAs) such as L-phenylalanine (L-Phe) and L-tyrosine (L-Tyr) are attractive biobased substrates since they can be manufactured from inexpensive and renewable biomass in a large quantity[8]. AAAs can be converted into a variety of value-added aromatic compounds, which are important in chemical, food, polymer and pharmaceutical industries[9]. Herein we report the development of efficient one-pot cascade biotransformation to convert AAAs into phenylacetic acid (PAA) **4a,** 2-phenylethanol (2-PE) **5a**, *p*-hydroxyphenylacetic acid (*p*-HPAA) **4b** and *p*-hydroxyphenylethanol (*p*-HPE) **5b** using recombinant *E. coli* (Scheme 1). In particular, the biocatalytic cascades for synthesizing the above aromatic compounds were designed as following. Module 1: AAAs undergoes the deamination and decarboxylation by employing L-amino acid deaminase (LAAD) from *Proteus mirabilis*[10] and α-keto acid decarboxylase (ARO10) from *Saccharomyces cerevisiae*[11]. Module 2: aldehyde oxidation using aldehyde dehydrogenase (ALDH) from *E. coli*[12]. And Module 3: aldehyde reduction using phenylacetaldehyde reductase (PAR) from *Solanum lycopersicum*[13].



Scheme 1. Cascade biotransformation of aromatic amino acids into their corresponding acids and alcohols. LAAD, L-amino acid deaminase from *P. mirabilis*; ARO10, α-keto acid decarboxylase from *S. cerevisiae*; ALDH, aldehyde dehydrogenase from *E. coli*; PAR, phenylacetaldehyde reductase from *S. lycopersicum*.

Results and Discussion

Synthesis of PAA via cascade biotransformation

PAA **4a** is an important aromatic compound due to its widely application in medicine, spices, pesticides, and dyestuff[14]; and it is also a precursor for the industrial production of penicillin G[15]. In the early research, aminotransferase such as TyrB has been used to produce phenylpyruvate **2** from L-Phe **1a**[16]. However, the requirement of additional co-substrate and low efficiency limit its application. In addition, it's also reported that L-amino acid oxidase (LAAO) can convert L-amino acids to α-keto acids by

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catalyzing the stereospecific oxidative deamination. However, hydrogen peroxide produced during the process of deamination is toxic to the cells[17]. As LAAD does not generate hydrogen peroxide, it is an ideal candidate to catalyze the deamination of L-amino acids into the corresponding *α*-keto acids. As shown in Figure 1A, combination of LAAD and ARO10 (module 1: pET-LAAD-ARO10) was used to convert L-Phe **1a** into phenylacetaldehyde **3**, then it was further oxidized by the endogenous ALDH (module 2: pRSF-ALDH) to form PAA **4a**. In order to minimize alcohol byproduct formation, *E. coli* MG1655 RARE with deletion of alcohol dehydrogenases (ADHs) and aldo-keto reductases (AKRs) was used to conduct PAA **4a** synthesis from L-Phe **1a**. As can be seen from Figure 1B, PAA **4a** production rapidly reached 49 mM after 8 h and bioconversion of 50 mM L-Phe **1a** eventually resulted in 49.7 mM PAA **4a** (6.8 g/L) after 21 h with more than 99% conversion. Furthermore, there was nearly no detectable amount of intermediates or byproducts in the biotransformation system (Figure S1).

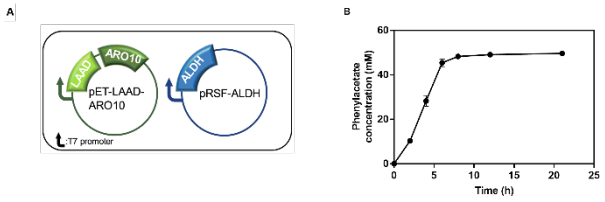

Figure 1. Biotransformation of L-Phe into PAA. (**A**) *E. coli* PAA01 containing module 1 (pET-LAAD-ARO10) and module 2 (pRSF-ALDH) for PAA **4a** synthesis. (**B**) Time course of biotransformation of L-Phe **1a** (50 mM) to PAA **4a** with resting cells of *E. coli* PAA01 (10 g-cdw/L). All experiments were performed in 2 mL KP buffer (200 mM, pH 8.0, 1% glucose) at 30°C. Experiments were performed in triplicate, and the data represent the mean value with standard deviation.

Synthesis of PAA analogs via cascade biotransformation

To explore the substrate scope of PAA biocatalytic pathway, L-Tyr **1b** was employed as the substrate for synthesizing *p*-HPAA **4b** with the recombinant *E. coli* (LAAD-ARO10-ALDH). *p*-HPAA **4b** is considered to be an important pharmaceutical owing to its anti-inflammatory and antioxidative action[18]. Due to poor solubility of L-Tyr **1b** in KP buffer, 5 mM of L-Tyr **1b** was used during the biotransformation. A maximum *p*-HPAA **4b** titer of 4.4 mM (0.67 g/L) with 88% conversion was obtained after reacting for 24 h (Figure 2A). We found that L-Tyr **1b** was efficiently converted to *p*-HPAA **4b** using the same enzymatic cascade without noticeable amounts of byproducts (Figure S2). These results indicated this biocatalytic pathway shows the good activity towards L-Tyr **1b** as the substrate.

Considering LAAD from *P. mirabilis* has broad substrate specificity towards other L-amino acids, we further investigated a non-natural amino acid of *m*-fluoro-phenylalanine (*m*-f-Phe) as the substrate for synthesizing *m*-fluoro-phenylacetic acid **4c**, which can be used as a medical intermediate[19]. As LAAD only had little activity on the D-form substrate[10], 10 mM racemic mixture of D- and L-form of *m*-f-Phe used in the present study is expected to produce a maximum of 5 mM *m*-fluoro-phenylacetic acid **4c**. As shown in Figure 2B, although the conversion rate was slower than that of PAA synthesis, 10 mM *m*-f-D/L-Phe eventually gave 4.8 mM *m*-fluoro-phenylacetic acid **4c** after 24 h, which corresponds to 48% conversion. Therefore, the result demonstrated the cascade biotransformation could obtain near 50% conversion of racemic *m*-f-Phe into its corresponding product (Figure S3), suggesting the potential for synthesizing other functionalized PAA analogs by our biocatalytic system.

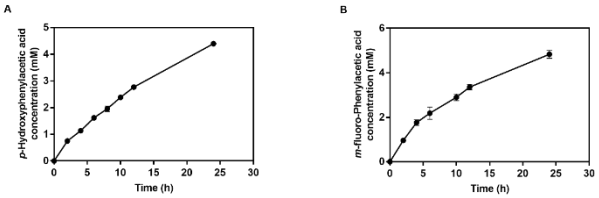

Figure 2. Biotransformation of L-Tyr and *m*-f-Phe into PAA analogs. (**A**) Time course of biotransformation of L-Tyr **1b** (5 mM) to *p*-hydroxyphenylacetic acid 4b (*p*-HPAA) with resting cells of *E. coli* PAA01 cells (10g-cdw/L). (**B**) Time course of biotransformation of *m*-fluorine-L-phenylalanine **1c** to *m*-fluoro-phenylacetic acid **4c** with resting cells of *E. coli* PAA01 cells (10 g-cdw/L). All experiments were performed in 2 mL KP buffer (200 mM, pH 8.0, 1% glucose) at 30°C for 24 h. Experiments were performed in triplicate, and the data represent the mean value with standard deviation.

Synthesis of 2-PE and *p*-HPE via cascade biotransformation

2-PE **5a** is widely applied in perfume, cosmetics, and food additives because of its rose-like fragrance. For 2-PE **5a** production, module 3 (pRSF-PAR) was designed to reduce phenylacetaldehyde **3** into 2-PE **5a**. Hence, combination of module 1 (pET-LAAD-ARO10) and module 3 (pRSF-PAR) was conducted in *E. coli* MG1655 RARE for 2-PE **5a** synthesis from L-Phe **1a** (Figure 3A). We found that 2-PE **5a** production at 37°C was 26% higher over that of the reaction carried out at 30°C (Figure 3B). Therefore, all the subsequent experiments were performed at 37°C. According to the time course during biotransformation of L-Phe **1a** into 2-PE **5a** as shown in Figure 3C, L-Phe **1a** was rapidly converted to 2-PE **5a** in the early 6 h. 50 mM L-Phe **1a** produced 48.7 mM (5.9 g/L) 2-PE **5a** with 99% conversion after 16 h reaction.

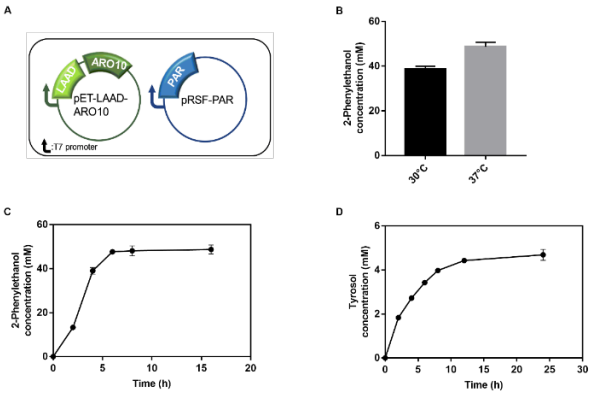

Figure 3. Biotransformation of L-Phe and L-Tyr into 2-PE and *p*-HPE. (**A**) *E. coli* 2-PE01 containing module 1 (pET-LAAD-ARO10) and module 3 (pRSF-PAR) for 2-PE **5a** synthesis. (**B**) 2-PE **5a** production from L-Phe **1a** at 30°C and 37°C for 24 h. (**C**) Time course of biotransformation of L-Phe **1a** (50 mM) to 2-PE **5a** with resting cells of *E. coli* 2-PE01 cells (10 g-cdw/L). (**D**) Time course of

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biotransformation of L-Tyr **1b** (5 mM) to *p*-HPE **5b** with resting cells of *E. coli* 2-PE01 cells (10 g-cdw/L). All experiments were performed in 2 mL KP buffer (200 mM, pH 8.0, 1% glucose) at 37°C. Experiments were performed in triplicate, and the data represent the mean value with standard deviation.

In the process of whole-cell biotransformation, no intermediates or accompanied byproducts were detected in the reaction mixture (Figure S4), indicating that L-Phe **1a** was completely converted into the corresponding product of 2-PE **5a**. Moreover, compared to the five catalytic steps of the previously reported cascade that comprises phenylalanine ammonia lyase (PAL), phenylacrylic acid decarboxylase (PAD), styrene monooxygenase (SMO), styrene oxide isomerase (SOI) and PAR[20], the three catalytic steps of LAAD-ARO10-PAR could also achieve similar catalytic efficiency and product concentration without using n-hexadecane overlayer. Therefore, our results suggested that the cascade biotransformation of LAAD-ARO10-PAR has great potential for the future industrial conversion of L-Phe **1a** into 2-PE **5a** product.

Tyrosol (*p*-Hydroxyphenylethanol, *p*-HPE, **5b**) is another important pharmaceutical intermediate that can be used to synthesize metoprolol, betaxolol, salidroside, etc.[21] According to previous studies that LAAD has very broad substrate scope[11], we further explored the possibility of biotransformation of L-Tyr **1b** into *p*-HPE **5b** by the enzymatic cascade LAAD-ARO10-PAR. As expected, *p*-HPE **5b** was successfully synthesized after reacting with recombinant *E. coli* (Figure S5). Bioconversion of L-Tyr **1b** (5 mM) produced 4.7 mM (0.65 g/L) *p*-HPE **5b** with 94% conversion (Figure 3D), which was much higher than the previously established enzymatic cascade of ARO8-ARO10-ADH (87.1%)[21b].

Conclusion

Artificial enzymatic cascades were designed and constructed for synthesis of high-value aromatic compounds from biobased L-Phe or L-Tyr. The cascade biotransformation with the whole-cell biocatalysts obtained the target aromatic compounds PAA, *p*-HPAA, 2-PE, and *p*-HPE in good productivity, suggesting our multienzyme cascades are highly versatile and efficient. Moreover, there were no noticeable amounts of intermediates or byproducts during the process of bioconversion, suggesting that it would remarkably reduce the cost for the downstream product separation and purification. Furthermore, our results also revealed the possibility of our enzymatic cascades for converting non-natural AAAs into their corresponding products, which would eventually result in the synthesis of numerous other functionalized aromatic compounds.

Experimental Section

Construction of plasmids and strains

Oligonucleotides applied for plasmids construction in this study are listed in Table S1. Target genes were PCR amplified using Phusion High-Fidelity DNA Polymerase. *LAAD* from *P. mirabilis* and *PAR* from *S. lycopersicum* were codon optimized and synthesized by GenScript. The fragment of *ARO10* was PCR amplified using *S. cerevisiae* genomic DNA and the fragment of *ALDH* was PCR amplified from *E. coli* genomic DNA. The fragments of *LAAD* and *ARO10* were cloned into pETDuet-1 to yield plasmid pET-LAAD-ARO10, and the fragments *ALDH* and *PAR* were cloned into pRSFDuet-1 to obtain plasmids pRSF-ALDH and pRSF-PAR, respectively. Plasmids were constructed by standard restriction enzyme and ligation approach, and confirmed by restriction enzyme digestion analysis and DNA sequencing. Plasmids were transformed into *E. coli* strain via standard heatshock approach or electroporation. All the plasmids and strains are listed in Table S2.

Culturing *E. coli* cells

E. coli MG1655 RARE strains harbouring the plasmids were cultivated in LB medium supplemented with appropriate antibiotics (100 μg/mL ampicillin, 50 μg/mL kanamycin, 34 μg/mL chloramphenicol) at 37°C and 250 rpm. The overnight culture (1 mL) was transferred and grown into a 250 mL baffled culture flask with Terrific Broth medium (12 g/L tryptone, 24 g/L yeast extract, 0.4% glycerol, 12.54 g/L $K_2HPO_4 \cdot 3H_2O$, 2.31 g/L KH_2PO_4 and appropriate antibiotics). After the optical density at 600 nm (OD600) of the culture reached ~0.6, IPTG (Isopropyl *β*-D-1-thiogalactopyranoside) was added to a final concentration of 1 mM to induce the protein expression. The cells were harvested by centrifugation (4000 g, 10 min, and 4°C) after 16 h induction at 20°C.

Culture of *E. coli* strains for biotransformation

The harvested cell pellets were washed twice with ice-cold ddH_2O, and then resuspended with KP buffer (200 mM, pH 8.0) to a cell density of 20 g-cdw/L. L-Phe **1a** of 200 mM, L-Tyr **1b** of 12.5 mM, and *m*-f-Phe of 50 mM stock solutions were prepared in KP buffer, respectively. For biotransformation of PAA **4a** and 2-PE **5a**, 1 mL cell suspension, 500 μL L-Phe **1a** solution, 100 μL glucose (20%), 400 μL KP buffer were added together to give 2 mL mixture containing 10 g-cdw/L cells, 50 mM L-Phe **1a**. The addition of 1% glucose in final concentration was used for regenerating NAD(P)H. For biotransformation of *p*-HPAA **4b** and *p*-HPE **5b**, 1 mL cell suspension, 800 μL L-Tyr **1b** solution, 100 μL glucose (20%) and 100 μL KP buffer were added together to give 2 mL mixture containing 10 g-cdw/L cells and 5 mM L-Tyr **1b**. For *m*-f-PAA **4c** production, 1 mL cell suspension, 400 μL *m*-f-Phe solution, 100 μL glucose (20%), 500 μL KP buffer were added together to give 2 mL mixture containing 10 g-cdw/L cells and 10 mM *m*-f-Phe. The reactions were performed at 30°C or 37°C and 250 rpm for 24 h. All of the samples were collected at regular intervals and then analyzed by HPLC.

Analytical methods

The target products were analyzed by Shimadzu Prominence HPLC system with a photodiode array detector under reversed-phase condition at a temperature of 40°C. Column: Shimadzu C18 column (150 mm × 4.6 mm × 5 μm). Flow rate: 1 mL/min. Mobile phase for the quantitation of PAA **4a**, 2-PE **5a**, *p*-HPAA **4b** and *m*-f-PAA **4c**: 70% water with 0.1% TFA, 30% acetonitrile. Retention time: PAA **4a** 6.3 min, 2-PE **4a** 6.5 min, *p*-HPAA **4b** 2.7 min, *m*-f-PAA **4c** 7.6 min. Mobile phase for the quantitation of *p*-HPE **5b**: 90% water with 0.1% TFA, 10% acetonitrile. Retention time: *p*-HPE **5b** 8.3 min. The standard curves were plotted using the peak area or peak height. The concentration of products was calculated by HPLC based on the standard curves.

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Keywords: biotransformation • 2-phenylethanol • phenylacetic acid • aromatic amino acids • biocatalysis

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