

Combination of phenylpyruvic acid (PPA) pathway engineering and molecular engineering of L-amino acid deaminase improves PPA production with an *Escherichia coli* whole-cell biocatalyst

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Abstract In our previous study, we produced phenylpyruvic acid (PPA) in one step from L-phenylalanine by using an *Escherichia coli* whole-cell biocatalyst expressing an L-amino acid deaminase (L-AAD) from *Proteus mirabilis* KCTC2566. However, the PPA titer was low due to the degradation of PPA and low substrate specificity of L-AAD. In this study, metabolic engineering of the L-phenylalanine degradation pathway in *E. coli* and protein engineering of L-AAD from *P. mirabilis* were performed to improve the PPA titer. First, three aminotransferase genes were knocked out to block PPA degradation, which increased the PPA titer from 3.3 ± 0.2 to 3.9 ± 0.1 g/L and the substrate conversion ratio to 97.5 %. Next, L-AAD was engineered via error-prone polymerase chain reaction, followed by site-saturation mutation to improve its catalytic performance. The triple mutant D165K/F263M/L336M produced the highest PPA titer of 10.0 ± 0.4 g/L, with a substrate conversion ratio of 100 %, which was 3.0 times that of wild-type L-AAD. Comparative kinetics analysis showed that compared with wild-type L-AAD, the triple mutant had higher substrate-binding affinity and catalytic efficiency. Finally, an optimal fed-batch biotransformation process was developed to achieve a maximal PPA titer of 21 ± 1.8 g/L within 8 h. This

study developed a robust whole-cell *E. coli* biocatalyst for PPA production by integrating metabolic and protein engineering, strategies that may be useful for the construction of other biotransformation biocatalysts.

Keywords L-Amino acid deaminase · Phenylpyruvic acid · Whole-cell biocatalyst · Error-prone PCR · Site-saturation mutagenesis · Fed-batch biotransformation

Introduction

α -Keto acids are vital intermediate compounds with a wide range of applications in the pharmaceutical, food, and chemical industries (Fuchs et al. 2009; Pailla et al. 2000). Among these compounds, phenylpyruvic acid (PPA) is particularly useful. In the pharmaceutical industry, PPA is used to produce D-phenylalanine, the raw material of antidiabetic, antineoplastic, anti-AIDS compounds, and peptide antibiotics (Pantaleone et al. 2001; Zhu et al. 2014). In addition, PPA is used to synthesize phenyllactic acid, an antimicrobial agent, food preservative, and flavor compound (Li et al. 2008; Zheng et al. 2011).

Currently, PPA is chemically synthesized with glycine, benzaldehyde, benzyl chloride, and carbonic oxide (des Abbayes and Salaun 2003; Villablanca and Cilento 1987). This chemical strategy is not only environmentally unfriendly but also produces various toxic by-products. PPA can also be produced via microbial fermentation with microorganisms such as *Zygosaccharomyces rouxii*, *Proteus vulgaris*, *Morganella morganii*, and *Corynebacterium glutamicum* (Coban et al. 2014). Although microbial fermentation is environmentally friendly, it produces relatively small amounts of PPA. Biotransformation is an alternative for PPA production.

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The production of PPA via the oxidation of D-phenylalanine by D-amino acid oxidase (EC 1.4.3.3) has been reported (Buto et al. 1994; Fernandez-Lafuente et al. 1998; Yoshimoto et al. 2014). This reaction produces toxic hydrogen peroxide; however, an additional catalase enzyme is necessary to minimize detrimental effects of that compound. On the contrary, L-amino acid deaminases (L-AADS) catalyze the stereospecific oxidative deamination of L-amino acids to generate their respective keto acids and ammonia without the formation of hydrogen peroxide (Geueke and Hummel 2002). Moreover, L-phenylalanine is cheaper than D-phenylalanine, which is an important consideration for industrial PPA production. Therefore, L-AADS are an ideal enzyme choice for PPA biosynthesis.

Proteus mirabilis contains two types of membrane-bound L-AADS. One type has broad substrate specificity and catalyzes the oxidation of aliphatic and aromatic L-amino acids to α -keto acids, typically L-phenylalanine. The other acts mainly on basic L-amino acids (Duerre and Chakrabarty 1975). In a previous study, we constructed an *E. coli* whole-cell catalyst expressing the first type of L-AAD from *P. mirabilis* KCTC2566 and produced PPA from L-phenylalanine with a maximal production and substrate conversion ratio of 3.3 ± 0.2 g/L and 82.5 ± 4 %, respectively (Hou et al. 2015). The relatively low PPA production of this system was attributed to low substrate specificity, significant substrate or product inhibition, and side reactions. In the present study, the metabolic pathway of PPA in *E. coli* was engineered to block PPA degradation and the L-AAD from *P. mirabilis* KCTC2566 was engineered for improved catalytic performance. Three aminotransferase genes, *tyrB*, *aspC*, and *ilvE*, were deleted to reduce PPA degradation, and L-AAD was then engineered through a combination of directed evolution and site-saturation mutagenesis to increase its catalytic performance. Finally, an optimal fed-batch biotransformation was developed to improve PPA production further.

Materials and methods

Plasmids, bacterial strains, and chemicals

The strains and plasmids used in this study are listed in Table 1. Enzymes (PrimeSTAR HS DNA polymerase, 2 \times Taq polymerase chain reaction [PCR] mix) and kits (Genomic Extraction Kit, DNA Purification Kit, and Competent Cell Preparation Kit) were supplied by TaKaRa (Dalian, China). With the exception of phenylpyruvic acid (Sigma-Aldrich, Shanghai, China), all chemical reagents were purchased from Shanghai Sangon Biological Engineering Technology and Services Co. Ltd. (Shanghai, China).

Deletion of three aminotransferase genes *tyrB*, *aspC*, and *ilvE* in *E. coli*

The gene knockout strains were constructed with a one-step disruption protocol (Datsenko and Wanner 2000). The upstream and downstream regions (approximately 1000 bp) flanking the target gene were designed and amplified from *E. coli* BL21 (DE3) genome DNA. The disruption cassettes were amplified from vector pKD13 and fused to the flanking regions via fusion PCR. The primers are shown in Table 2. The purified PCR products were transformed via electroporation into *E. coli* BL21 (DE3). Kanamycin-resistant (Kan^r) transformants were selected in the presence of kanamycin (Shanghai Sangon Biological Engineering Technology and Services Co. Ltd). The resistance marker cassettes were removed from the chromosome with FLP recombinase by using the temperature-inducible plasmid pCP20. The single-deletion mutants were designated *E. coli* BL21 (DE3) (Δ *tyrB*), *E. coli* BL21 (DE3) (Δ *aspC*), and *E. coli* BL21 (DE3) (Δ *ilvE*). The double- and triple-deletion strains were constructed in the same way and designated *E. coli* BL21 (DE3) (Δ *tyrB Δ *aspC*), *E. coli* BL21 (DE3) (Δ *tyrB Δ *ilvE*), *E. coli* BL21 (DE3) (Δ *aspC Δ *ilvE*), and *E. coli* BL21 (DE3) (Δ *tyrB Δ *aspC Δ *ilvE*).*****

The amount of PPA degradation by the parental and engineered *E. coli* BL21 (DE3) mutants was determined under optimal biotransformation conditions (4 g/L PPA, 1.2 g/L cells (dry cell weight), pH 7.4, 40 °C, 6 h). The recombinant L-AAD was then transformed into the engineered strains, and whole-cell biotransformation was performed with various concentrations of L-phenylalanine (4, 12, and 20 g/L) to produce PPA.

Directed evolution of L-AAD via error-prone PCR

The L-AAD gene was amplified from the recombinant plasmid pET 20b (+)-AAD via error-prone PCR (ep-PCR) with a GeneMorph II Random Mutagenesis Kit (Agilent Technologies, Santa Clara, USA). The 50- μ L reaction mixture for ep-PCR contained 5 μ L 10 \times Mutazyme II reaction buffer, 1 μ L 40 mM deoxyribonucleotide triphosphate mix (200 μ M each, final), 0.5 μ L primer mix (250 ng/ μ L each primer), 1 μ L Mutazyme II DNA polymerase (2.5 U/ μ L), 1 μ L template, and 41.5 μ L water.

To produce high amplification yields after 30 cycles and a mutation frequency of 2–20 nucleotide changes per mutant, we used 0.1–100 ng target DNA as the template. The mutant library was transformed into *E. coli* BL21 (DE3) and cultured on Luria-Bertani (LB) solid medium containing 100 μ g/mL ampicillin at 37 °C for 8 h. Variants from the mutant library were selected and cultured in 0.5-mL LB broth containing 100 μ g/mL ampicillin and grown on 96-well plates at 37 °C overnight. Seed broth was inoculated into Terrific broth

Table 1 Strains and plasmids used in this study

Strains or plasmids	Characteristics	Source or reference
Wild type	BL21(DE3) harboring the recombinant L-AAD	Hou et al. (2015)
BL21(DE3) ($\Delta tyrB$)	$\Delta tyrB::FRT$	This study
BL21(DE3) ($\Delta aspC$)	$\Delta aspC::FRT$	This study
BL21(DE3) ($\Delta ilvE$)	$\Delta ilvE::FRT$	This study
BL21(DE3) ($\Delta tyrB\Delta aspC$)	$\Delta tyrB::FRT, \Delta aspC::FRT$	This study
BL21(DE3) ($\Delta tyrB\Delta ilvE$)	$\Delta tyrB::FRT, \Delta ilvE::FRT$	This study
BL21(DE3) ($\Delta aspC\Delta ilvE$)	$\Delta aspC::FRT, \Delta ilvE::FRT$	This study
BL21(DE3) ($\Delta tyrB\Delta aspC\Delta ilvE$)	$\Delta tyrB::FRT, \Delta aspC::FRT, \Delta ilvE::FRT$	This study
pET 20b(+)-AAD	The parent strain	Hou et al. (2015)
pKD13	<i>bla</i> FRT- <i>ahp</i> -FRT	Datsenko and Wanner (2000)
pKD46	<i>bla</i> $\alpha\beta$ exo (red recombinase), temperature conditional replicon	Datsenko and Wanner (2000)
pCP20	<i>bla cat</i> , temperature-sensitive replicon, temperature-inducible FLP recombinase	Datsenko and Wanner (2000)

medium containing 0.2 % L-phenylalanine and 100 $\mu\text{g}/\text{mL}$ ampicillin, and the cells were grown at 37 °C for 2 h, induced

by the addition of isopropyl- β -D-thiogalactopyranoside (IPTG; 0.04 mM), and grown for an additional 5 h at 30 °C.

Table 2 Primers used in this study

Primer name	Nucleotide sequence (5'–3')
<i>tyrB</i> -Left-S	TAGCTGGACGAGCCGGTTAC
<i>tyrB</i> -Left-A	AGCTCCAGCCTACACGCGATGGTTCTCCAGGTTTACG
<i>tyrB</i> -Middle-S	TGGAGAACCATCGCGTGTAGGCTGGAGCTGCTTCG
<i>tyrB</i> -Middle-A	CAGCCTGCTTTCCTGCACTGTCAAACATGAGAATTAATCCGG
<i>tyrB</i> -Right-S	TTCTCATGTTTGACAGTGCAGGAAAGCAGGCTGGAGC
<i>tyrB</i> -Right-A	CTTCGCTTTCGCGGAGAAGGT
<i>aspC</i> -Left-S	CGCGGGTCTTAAATACGCTGACG
<i>aspC</i> -Left-A	AGCTCCAGCCTACACGACGAGGTTCCATTATGGTTACAGAAG
<i>aspC</i> -Middle-S	ATGGAACCTCGTCTGTAGGCTGGAGCTGCTTCG
<i>aspC</i> -Middle-A	CATTGTTTTTAATGCCTGTCAAACATGAGAATTAATCCGG
<i>aspC</i> -Right-S	TTCTCATGTTTGACAGGCATTAATAAACAATGAAGCCCGCT
<i>aspC</i> -Right-A	AGCAATATTCGCAAAGCCGCGTTAT
<i>ilvE</i> -Left-S	ATTACAAGGTGATTTAAATGCTCTG
<i>ilvE</i> -Left-A	AGCTCCAGCCTACACTTTTATATTCCTTTTGCCTCAGGCGCG
<i>ilvE</i> -Middle-S	CAAAAGGAATATAAAAGTGTAGGCTGGAGCTGCTTCG
<i>ilvE</i> -Middle-A	GTCCCATTTTTGTATCTGTCAAACATGAGAATTAATCCGG
<i>ilvE</i> -Right-S	TTCTCATGTTTGACAGATACAAAAATGGGACGGCACGC
<i>ilvE</i> -Right-A	ACACAGCTGCGGAACCTTGC GGGA
D165 forward	AGGTTTT GAT ACACCATTAATACTCGCATCAT
D165 reverse	GCCGCTTCTTTAGCTGTTTTGATCCACG
S179 forward	AGAGCTAT CAA ATCGCTTAGTCGGTGCTCAAAC
S179 reverse	TCACCTTTAATGATGCGAGTATTTAATGGTGTATC
F263 forward	GCGTTATTTTATGGGCAATATGGGTATTGATAT
F263 reverse	GACCAGATACCCCGAGCGAGTACAACCTG
L336 forward	TTATGCACTTAT TAG GTGGCGGAGAGTTAC
L336 reverse	ATTTAGGCCCTAGCAGGAAGCTATCTTTGACTAT

Underlined text represents the homologous sequences for fusion PCR. The bold codons (corresponding to D165, S179, F263, and L336) were replaced by codons of 19 other amino acids: A (GCT), R (CGT), N (AAC), D (GAC), C (TGT), E (GAA), Q (CAG), G (GGT), H (CAC), I (ATT), L (TTA), K (AAA), M (ATG), F (TTC), P (CCG), S (TCT), T (ACC), W (TGG), Y (TAC), and V (GTT)

Ferric chloride solution (1 %, w/v) was added to assess the presence of PPA (Massad et al. 1995). Positive mutants were selected and used for whole-cell transformation to check the PPA titer.

Site-directed mutagenesis of L-AAD

Site-directed mutagenesis was performed with a one-step PCR method by using a MutanBEST kit (TaKaRa). The reactions contained recombinant plasmid pET 20b (+)-AAD as the template and mutagenic oligonucleotides as primers (see Table 2). After purification, the PCR product was blunted with Blunting Kination Enzyme Mix and ligated with Ligation Solution I (TaKaRa). The mutant plasmids were transformed into *E. coli* BL21 (DE3) and confirmed through PCR and sequencing. The PPA titers of the successful transformants were measured under the optimal whole-cell biotransformation conditions (10 g/L L-phenylalanine, 4.2 g/L cells, pH 7.4, 40 °C, 6 h).

Preparation of the biocatalyst

The wild-type and mutants of L-AAD were transformed into the metabolically engineered *E. coli* BL21 (DE3). The recombinant *E. coli* BL21 (DE3) was inoculated into 20-mL LB medium containing ampicillin (100 µg/mL) and grown in a rotary shaker (200 rpm) at 37 °C overnight. A seed culture (1 %, v/v) was inoculated into 500-mL Terrific broth medium containing ampicillin (100 µg/mL) for expression. The overall optimal induction conditions were as follows: pH 8, 0.04 mM IPTG, optical density at 600 nm of 0.6, and induction at 20 °C for 12 h. The cells were obtained via centrifugation (Beckman Instruments, San Jose, CA) at 8000×g and 4 °C for 20 min, and washed twice with 20 mM phosphate buffer (pH 7.0). The cells were then resuspended in 20 mM phosphate buffer (pH 7.0). Biomass concentration was checked spectrophotometrically (UV-2450 PC; Shimadzu Co., Kyoto, Japan) at an optical density of 600.

Purification of L-AAD and its mutants

The solubilization and purification of the wild-type L-AAD and its mutants were performed according to a previously published method (Hou et al. 2015). Briefly, the induced cell pellets were suspended in purification buffers containing *n*-dodecyl-β-D-maltoside (0.01 %). After disruption with ultrasonication at 4 °C for 20 min, the detergent-solubilization solution was filtered through a 0.45-µm pore size membrane, and the filtrate was purified on a HisTrap™ FF 5 mL column with AKTA Explorer (GE Healthcare, Piscataway, NJ). Then, desalting was performed with an Ultra-4 Centrifugal Filter Device (Amicon, Shanghai, China). The concentration of protein was determined with a BCA protein assay kit (TianGen, Beijing, China).

Determination of kinetic parameters

The kinetic analysis of L-phenylalanine transformation to PPA with wild-type L-AAD and its mutants was performed according to a previously published method (Hou et al. 2015). The parameters were calculated with the Lineweaver-Burk plotting method shown in Eq. (1):

$$1/V = (K_m/V_{max} \times 1/[S]) + 1/V_{max} \quad (1)$$

where V is the reaction rate (the amount of PPA produced by 1 mg recombinant L-AAD per min; µmol PPA min⁻¹ mg⁻¹ protein), V_{max} is the maximum reaction rate (µmol PPA min⁻¹ mg⁻¹ protein), K_m is the Michaelis constant (mM), and $[S]$ is the L-phenylalanine concentration (mM).

Protein analysis

Protein was analyzed with sodium dodecyl sulfate-polyacrylamide gel electrophoresis. The cells were prepared in denaturing buffer at 95 °C for 10 min, and 15 µL of the samples was loaded onto a gel composed of a 5 % (m/v) polyacrylamide stacking gel and an 8 % (m/v) polyacrylamide separating gel (Bio-Rad Laboratories, Hercules, CA).

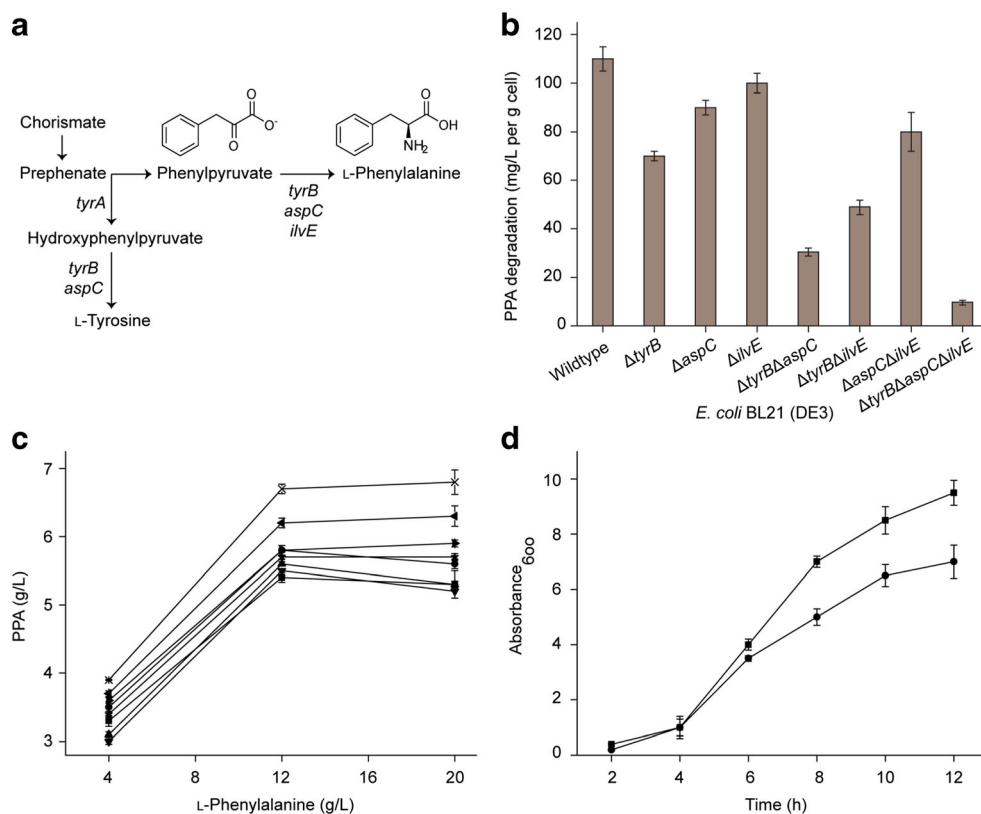
Results

Metabolic engineering of the PPA degradation pathway in *E. coli*

Product degradation via side reactions often occurs in whole-cell biocatalyst systems (Fig. 1a), and substrates are inefficiently utilized to produce the target products (de Carvalho 2011). Measurements show that the rate of PPA degradation by *E. coli* BL21 (DE3) is approximately 110±4 mg/L per gram cell (dry cell weight) (Fig. 1b). In *E. coli*, three aminotransferases—an aromatic amino acid aminotransferase encoded by *tyrB*, an aspartate aminotransferase encoded by *aspC*, and an isoleucine aminotransferase encoded by *ilvE*—participate in the degradation of PPA (see Fig. 1a) (Müller et al. 2006; Sun et al. 2011). The aromatic amino acid aminotransferase and aspartate aminotransferase are also involved in the synthesis of L-tyrosine (see Fig. 1a). Single-, double-, and triple-deletion mutants were constructed in *E. coli* BL21 (DE3) to determine the amount of PPA degradation that occurs in these strains (see Fig. 1b) and evaluate the effect of gene deletion on the growth of recombinant *E. coli* BL21 (DE3) (Fig. 1d).

The final cell concentration of the parental strain was 1.4 times than that of the recombinant *E. coli* BL21 (DE3) ($\Delta tyrB \Delta aspC \Delta ilvE$)—perhaps because the gene deletion mutants became auxotrophic for both L-phenylalanine and L-

Fig. 1 Construction of deletion mutants of *E. coli* BL21 (DE3). **a** L-Phenylalanine pathway in *E. coli* BL21 (DE3). **b** The amount of PPA degradation by the wild type and its knockout mutants. **c** PPA production of whole-cell biotransformation by the wild-type and engineered *E. coli* BL21 (DE3) with different concentrations of L-phenylalanine. *Square*, wild type; *circle*, BL21 (DE3) ($\Delta tyrB$); *up triangle*, BL21 (DE3) ($\Delta aspC$); *down triangle*, BL21 (DE3) ($\Delta ilvE$); *left triangle*, BL21 (DE3) ($\Delta tyrB\Delta aspC$); *right triangle*, BL21 (DE3) ($\Delta tyrB\Delta ilvE$); *star*, BL21 (DE3) ($\Delta aspC\Delta ilvE$); *cross*, BL21 (DE3) ($\Delta tyrB\Delta aspC\Delta ilvE$). **d** Cell growth profile of the recombinant *E. coli* BL21 (DE3). *Square*, wild type; *circle*, BL21 (DE3) ($\Delta tyrB\Delta aspC\Delta ilvE$)



tyrosine. For the single-deletion mutants, the PPA degradation rate of *E. coli* BL21 (DE3) ($\Delta tyrB$) was 64.5 % that of the parental strain, whereas deletion of *aspC* or *ilvE* had little effect on PPA degradation. As for the double-deletion mutants, the PPA degradation rate of *E. coli* BL21 (DE3) ($\Delta tyrB\Delta aspC$), *E. coli* BL21 (DE3) ($\Delta tyrB\Delta ilvE$), and *E. coli* BL21 (DE3) ($\Delta aspC\Delta ilvE$) was 29.1, 43.6, and 73.6 % that of the parental strain. For the triple-deletion mutant *E. coli* BL21 (DE3) ($\Delta tyrB\Delta aspC\Delta ilvE$), PPA degradation rate was only 10 ± 0.3 mg/L per gram cell, which indicated that compared with other mutants, the triple-deletion mutant had less PPA degradation. Then, whole-cell biotransformation of L-phenylalanine with these mutants was performed (Fig. 1c). Higher PPA production in the mutant strains was consistent with the lower PPA degradation rate. Deletion of *tyrB* increased PPA production in the single-deletion mutant compared with the parental strain, whereas deletion of *aspC* and *ilvE* had little effect on PPA production. Among all the mutant strains, recombinant *E. coli* BL21 (DE3) ($\Delta tyrB\Delta aspC\Delta ilvE$) had the highest PPA production of 3.9 ± 0.1 , 6.7 ± 0.7 , and 6.8 ± 0.5 g/L with 4, 12, and 20 g/L of L-phenylalanine, which was 1.2, 1.2, and 1.3 times than that of the wild type, respectively. We also found that at the lowest tested concentration of L-phenylalanine (4 g/L), the substrate conversion ratio was 97.5 %. This ratio decreased to 55.8 and 34.0 % at higher concentration of L-phenylalanine (12 and 20 g/L) due to low substrate specificity, substrate inhibition, and product inhibition.

Directed evolution and site-directed mutagenesis of L-AAD

Due to the low similarity (19 %) of L-AAD to other L-amino oxidases, it is difficult to develop a homology model to improve the catalytic performance. Therefore, to improve the catalytic performance of L-AAD and the biotransformation efficiency of L-phenylalanine, we performed directed evolution of L-AAD with ep-PCR and constructed a mutant library. First, the ep-PCR conditions were optimized by varying the initial amount of template. Low, medium, or high mutation rates can be produced by varying the initial amount of target DNA or the number of amplification cycles with minimal mutational bias (Neylon 2004). Compared with low mutation rates, high mutation rates produce a greater number of unique sequences (Drummond et al. 2005).

The results showed that the PCR conditions resulted in an average of 1–6 amino acid substitutions (2–20 nucleotide changes) per mutant. The resulting library of approximately 10,000 colonies was screened to identify the mutants with higher biotransformation efficiencies. One mutant had PPA production (8.1 ± 0.25 g/L) that was 2.1 times that of the wild-type (3.9 ± 0.1 g/L; see Fig. 2a). The catalytic properties of wild-type L-AAD and this mutant were analyzed (Table 3) and compared with the wild type, the mutant exhibited a 30.0 % increase in K_{cat} and an 8.0 % decrease in K_m . The overall catalytic efficiency (as indicated by K_{cat}/K_m) of the

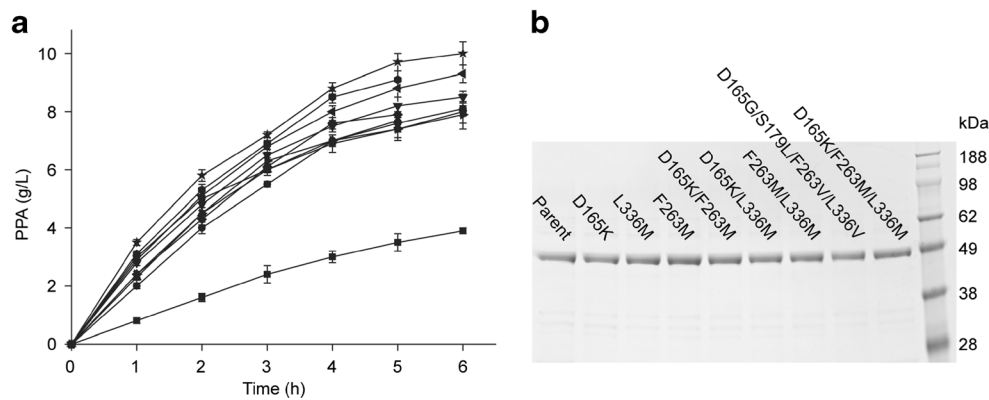


Fig. 2 PPA production of whole-cell biotransformation by different mutants and SDS-PAGE analysis of the purification results. **a** PPA production of whole-cell biotransformation by different mutants under 10 g/L of L-phenylalanine. Results were obtained with a whole-cell *E. coli* biocatalyst containing different mutants of L-AAD. *Square*,

mutant was $76 \text{ s}^{-1} \text{ M}^{-1}$, a 43.4 % improvement over the catalytic efficiency of the wild type. Sequence analysis showed that the mutant had four amino acid substitutions (D165G, S179L, F263V, and L336V). Two hydrophobic amino acids (phenylalanine and leucine) had changed to a different hydrophobic amino acid (valine), and one hydrophilic amino acid (aspartic acid) had changed to a different hydrophilic amino acid (glycine). However, one hydrophilic amino acid (serine) had changed to a hydrophobic amino acid (leucine). These results indicated that the amino acids D165, S179, F263, and L336 are critical to the catalytic efficiency of L-AAD. Hence, they were selected as targets for further evolution via site-saturation mutagenesis for the fine-tuning of the PPA production capacity of the mutants.

All L-AAD mutants were expressed in *E. coli* BL21 (DE3) ($\Delta\text{tyrB}\Delta\text{aspC}\Delta\text{ilvE}$), and similar expression levels were observed in sodium dodecyl sulfate gel analysis (data not shown).

Time courses for PPA production with the wild-type and mutants were compared (Fig. 2a). The D165K, F263M, and L336M mutants produced PPA titers of 7.7 ± 0.3 , 8.5 ± 0.2 , and

wild-type; *circle*, D165G/S179L/F263V/L336V; *up triangle*, D165K; *down triangle*, F263M; *diamond*, L336M; *left triangle*, D165K/F263M; *right triangle*, D165K/L336M; *hexagon*, F263M/L336M; *star*, D165K/F263M/L336M; *pentagon*, wild type *E. coli* (D165K/F263M/L336M). **b** SDS-PAGE analysis of the purification results of different mutants

$7.9 \pm 0.3 \text{ g/L}$ —2.0, 2.2, and 2.0 times that of the wild type, respectively. By contrast, any change to S179 resulted in PPA titers lower than that of the wild type, so S179 was not employed for further study. Based on these results, double and triple mutants were constructed, and their biotransformation capabilities were measured. The PPA titers of the double mutants D165K/F263M, D165K/L336M, and F263M/L336M were 2.4, 2.0, and 2.3 times that of the wild type, respectively. The triple mutant D165K/F263M/L336M produced the highest PPA titer of $10.0 \pm 0.4 \text{ g/L}$, with a substrate conversion ratio of 100 %, which was 3.0 times that of the wild-type. Compared with the wild type, the triple mutant exhibited a 60.7 % increase in K_{cat} and a 16.0 % decrease in K_m . Furthermore, it displayed the highest catalytic efficiency of $102 \text{ s}^{-1} \text{ M}^{-1}$, which was 1.9-fold that of wild type. Furthermore, the best L-AAD mutant D165K/F263M/L336M was expressed in *E. coli* BL21 (DE3) and *E. coli* BL21 (DE3) ($\Delta\text{tyrB}\Delta\text{aspC}\Delta\text{ilvE}$), and similar expression levels were observed in sodium dodecyl sulfate gel analysis (data not shown). The engineered *E. coli* produced PPA titers of 1.25 times that of the wild type.

Table 3 Comparison of kinetic parameters of the wild-type L-AAD and its mutants

Enzymes	K_m (mM)	V_{max} ($\mu\text{mol min}^{-1} \text{ mg}^{-1}$)	K_{cat}^a (s^{-1})	K_{cat}/K_m ($\text{s}^{-1} \text{ M}^{-1}$)
Wild type	26.2 ± 0.1	1.64 ± 0.1	1.40	53
D165G/S179L/F263V/L336V	24.1 ± 0.4	2.13 ± 0.2	1.82	76
D165K	24.8 ± 0.8	1.95 ± 0.1	1.67	67
L336M	24.5 ± 0.9	1.98 ± 0.1	1.69	69
F263M	23.8 ± 0.7	2.19 ± 0.4	1.87	79
D165K/F263M	22.6 ± 0.4	2.53 ± 0.6	2.16	96
D165K/L336M	24.3 ± 0.9	2.01 ± 0.4	1.72	71
F263M/L336M	22.4 ± 0.4	2.59 ± 0.5	2.21	99
D165K/F263M/L336M	22.0 ± 0.9	2.63 ± 0.8	2.25	102

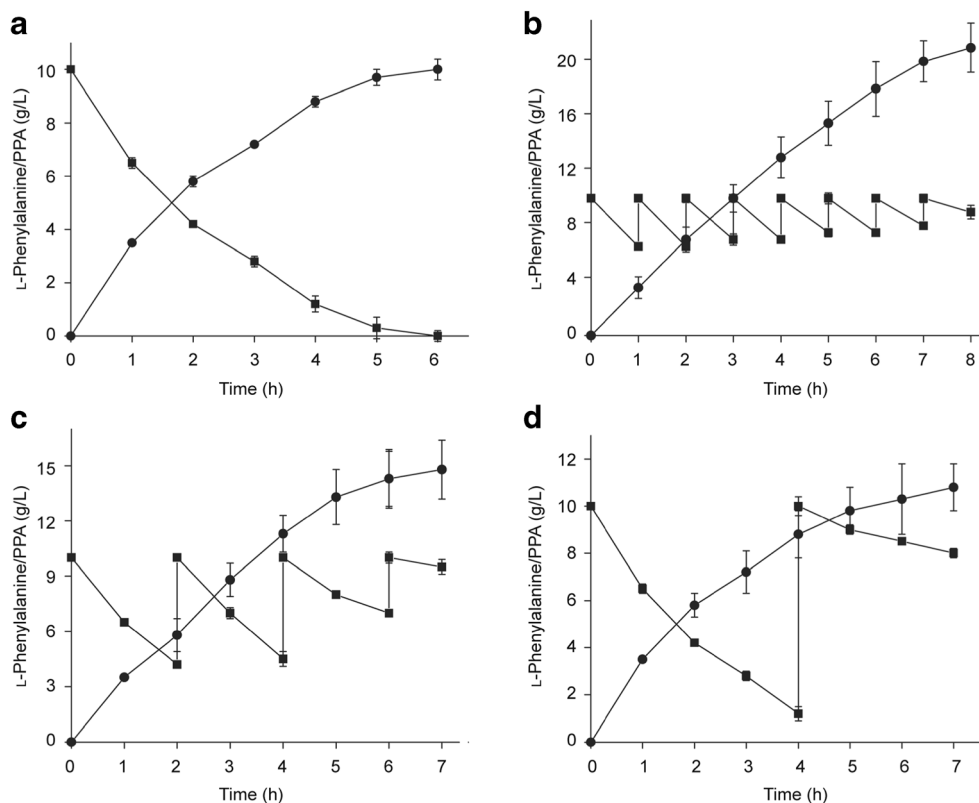
Each value was calculated from three independent experiments

^aThe average molecular weight of L-AAD was 52,030 Da

Fed-batch biotransformation process for PPA production

Although we deleted the PPA degradation pathway and improved the catalytic performance of L-AAD via molecular engineering, the PPA titer cannot increase further with more than 10 g/L L-phenylalanine due to substrate inhibition. To overcome this inhibition, we developed a fed-batch biotransformation strategy to increase the PPA titer by maintaining the L-phenylalanine concentration below 10 g/L. A specific amount of L-phenylalanine was added to the reaction mixture at various times. According to the time profiles of PPA synthesis and L-phenylalanine consumption in the batch biotransformation (Fig. 3a), almost all of the L-phenylalanine was used to produce PPA, and thus, substrate feeding can be performed by measuring PPA concentration. Beginning with 10 g/L of L-phenylalanine, the feeding interval was optimized. When feeding occurred every hour, the total L-phenylalanine added to the system was 31 ± 2.5 g/L and the maximal PPA production was 21 ± 1.8 g/L within 8 h. When feeding occurred every 2 h, the total L-phenylalanine added to the system was 24.3 ± 1.2 g/L and the maximal PPA production was 14.8 ± 1.6 g/L within 7 h. When feeding occurred every 4 h, the total L-phenylalanine added to the system was 18.8 ± 1.1 g/L and the maximal PPA production was 10.8 ± 1.0 g/L within 7 h. Therefore, feeding every hour was the optimal fed-batch method for PPA production (Fig. 3).

Fig. 3 Time profile of fed-batch biotransformation for the production of PPA by the knockout strain with the engineered L-AAD. **a** No feeding. **b** Feeding every hour. **c** Feeding every 2 h. **d** Feeding every 4 h. *Square*, L-phenylalanine concentration; *circle*, PPA concentration



Discussion

Previously, we used both a purified enzyme and a whole-cell biocatalyst to produce PPA via one-step biotransformation from L-phenylalanine. Although the whole-cell biocatalyst provided substantial benefits such as simplified cell preparation, higher thermal stability, and the capacity to recycle, PPA production was not remarkably high owing to the degradation of PPA by side reactions. In this study, metabolic engineering of the PPA degradation pathway and protein engineering of L-AAD were performed to improve PPA production with an *E. coli* whole-cell biocatalyst.

Resting *E. coli* under nongrowing conditions suspended in buffer solution without carbon, nitrogen, or mineral sources is used as a biocatalyst to produce many compounds (Hara and Kino 2010; Narancic et al. 2013), but the enzymes in the cells still function under certain conditions, resulting in side reactions as well as degradation (Hossain et al. 2014b). In *E. coli* BL21 (DE3), three aminotransferases are involved in the degradation of PPA. To minimize PPA degradation by the whole-cell biocatalyst, we constructed single-, double-, and triple-gene deletion strains. Among all the strains, the triple-deletion mutant *E. coli* BL21 (DE3) ($\Delta tyrB \Delta aspC \Delta ilvE$) was the most effective in reducing PPA degradation, and therefore, PPA production improved. In general, the performance of the double-deletion strains was better than that of the single-deletion strains, which demonstrated the synergistic effect of these three genes on the degradation of PPA. We

concluded that the aromatic amino acid aminotransferase is the primary enzyme for transforming PPA to L-phenylalanine.

The biotransformation efficiency of *E. coli* BL21 (DE3) ($\Delta aspC$) was better than that of *E. coli* BL21 (DE3) ($\Delta ilvE$), which indicated that the aspartate aminotransferase was the second most significant enzyme in this reaction, whereas the isoleucine aminotransferase was the least vital. Although the deletion of *ilvE* had no obvious effect on PPA degradation, PPA degradation dramatically decreased when *ilvE* was deleted after the deletion of *tyrB* and *aspC*. The results also showed that the isoleucine aminotransferase was more effective without the aromatic amino acid and aspartate aminotransferases. A small amount of PPA degraded in the triple-deletion mutant, which may be attributed to unknown reactions in *E. coli*.

The results of directed evolution and site-directed mutagenesis of L-AAD showed that compared with the single mutants, the double mutants had higher substrate binding affinity and catalytic efficiency, and the triple mutant exhibited the highest catalytic efficiency overall. These outcomes indicated that the three mutations had a synergistic effect on enzyme activity toward L-phenylalanine. After fine-tuning the mutation sites, which were identified through random mutagenesis, we observed that the new mutants generated by site-saturation mutagenesis were more effective for biotransformation. Thus, a combination of directed evolution and site-directed mutation is an effective strategy for the quick and effective evolution of enzymes (Cherry et al. 1999; Hossain et al. 2014a).

For enzymatic transformation, the synthesis of PPA via the oxidation of D-phenylalanine (Brodellius et al. 1981) or D,L-phenylalanine (Szwajcer et al. 1982) by D-amino acid oxidases has been reported, but only 10–50 mM substrate could be catalyzed and the PPA production was low (Brodellius et al. 1981; Buto et al. 1994; Fernandez-Lafuente et al. 1998). Besides, these reactions produce hydrogen peroxide, which is toxic to D-amino acid oxidases. Many methods were used to eliminate hydrogen peroxide, like the addition of catalase (Fernandez-Lafuente et al. 1998), manganese oxide (Brodellius et al. 1981), and activated charcoal (Szwajcer et al. 1982), resulting in increased costs and complicated process. As for microbial fermentation, *P. vulgaris* was used to produce PPA and the titer reached 1.1 g L^{-1} under the optimal conditions (Coban et al. 2014). Compared with the PPA titers in the above studies, the production of PPA here was higher. Moreover, the reaction catalyzed by L-AAD from *P. mirabilis* does not produce hydrogen peroxide and L-phenylalanine is cheaper than D-phenylalanine.

In conclusion, we constructed a metabolically engineered *E. coli* whole-cell biocatalyst via the deletion of three aminotransferase genes to eliminate side reactions and increase PPA production. L-AAD was also engineered via ep-PCR and site-directed mutagenesis, and the final triple mutant showed increased substrate binding affinity and catalytic efficiency. Finally, fed-batch biotransformation was optimized to increase

PPA production. Substrate and product inhibition are expected to be decreased further through structural engineering of L-AAD and kinetic modeling of the biotransformation system in future studies.

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Compliance with ethical approval This article does not contain any studies with human participants or animals performed by any of the authors.

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