Biotechnology and Biological Transformations

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J. Agric. Food Chem., **Just Accepted Manuscript** • DOI: 10.1021/acs.jafc.8b00627 • Publication Date (Web): 21 Mar 2018

Mimicking a new 2-phenylethanol production pathway from *Proteus mirabilis* JN458 in *Escherichia coli*

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Abstract

Bacteria rarely produce natural 2-phenylethanol. We verified a new pathway from *Proteus mirabilis* JN458 to produce 2-phenylethanol using *Escherichia coli* to co-express L-amino acid deaminase, α-keto acid decarboxylase, and alcohol dehydrogenase from *P. mirabilis*. Based on this pathway, a glucose dehydrogenase coenzyme regeneration system was constructed. The optimal conditions of biotransformation by the recombinant strain E-pAEAKaG were at 40°C and pH 7.0. Finally, the recombinant strain E-pAEAKaG produced 3.21±0.10 g/L 2-phenylethanol in M9 medium containing 10 g/L L-phenylalanine after a 16 h transformation. Furthermore, when the concentration of L-phenylalanine was 4 g/L (24 mM), the production of 2-phenylethanol reached 2.88±0.18 g/L and displayed a higher conversion rate of 97.38mol%.

Key words:

2-phenylethanol, L-phenylalanine, whole cells, *Proteus mirabilis,* L-amino acid deaminase

Introduction

Many flavors from fruits and flowers are derived from aromatic compounds. 2-Phenylethanol (2-PE, β-Phenylethanol) has a delicate rose fragrance and is an important aromatic compound that is used in the processing of foods, pharmaceuticals, cosmetics, cigarettes, and daily chemical products.[1] 2-PE is primarily used to enhance other flavors and is a primary ingredient in scent of foods and flowers.[2, 3] It is used as an additive in cigarettes and a preservative in soaps, and also acts as an inhibitor against tyrosinase.[4] 2-PE is an aroma that is widely used in cosmetic products and diverse foods. Therefore, sources of 2-PE that can be considered "natural" have been the focus of widespread attention by the flavor and fragrance industry.[1]

Currently, there are several biosynthetic pathways to obtain "natural" 2-PE: one derived from yeast and others from plants.[3, 5-7] Several strategies for the biotechnological production of 2-PE have been developed, and pathways from *S. cerevisiae* and plants are increasingly becoming economic alternatives to the traditional process. In *S. cerevisiae* and other yeasts, L-phenylalanine (L-Phe) participates in synthesizing 2-PE via the Ehrlich pathway,[5, 6] which produces phenylpyruvate (PPA) by transaminating the α-amino of L-Phe to α-ketoglutarate. Then, phenylacetaldehyde (PAAL) is produced by phenylpyruvate decarboxylase. Finally, alcohol dehydrogenase (ADH) transforms PAAL to produce 2-PE.[8] In plants, there are two different pathways that produce 2-PE. In one pathway, PAAL is directly produced by phenylacetaldehyde synthase using the substrate L-Phe, such as in

petunias;[9] the other pathway is via decarboxylation and deamination to PPA by amino acid decarboxylases and amine oxidation, respectively, such as in tomatoes.[3] In addition, a recent trend is to modify known metabolic pathways using biotechnology. Glucose has been used as a substrate to produce 2-PE via an expanded shikimate pathway, which converts the combined phosphoenolpyruvate (PEP) and erythrose-4-phosphate (E4P) from glucose to 3-deoxy-D-arabino-heptulosonate-7-phosphate (DAHP). Then, DAHP is converted to chorismate, which takes part in forming various amino acids. Subsequently, L-Phe, the product of the shikimate pathway, is involved in the Ehrlich pathway to produce 2-PE.[8, 10] Although there are many pathways to obtain natural 2-PE by microorganisms, the yield of 2-PE is low. Moreover, there are few wild microorganisms that can produce 2-PE, especially bacteria.

P. mirabilis and *P. vulgaris* are well known to be human opportunistic pathogens,[11] and studies have shown that 2-PE produced by the *P. vulgaris* in cheeses can increase their aromatic odor.[12] Recently, a novel α-keto acid decarboxylase from *P. mirabilis* JN458 using PPA as the substrate was identified.[13] The enzyme showed broad substrate specificity. Additionally, an earlier study found that L-Phe could undergo oxidative deamination into PPA.[14] That study indicated that *P. mirabilis* contained a novel pathway to produce 2-PE, which was different from those of yeasts and plants. In this study, we used *E. coli* to mimic this pathway from *P. mirabilis* JN458 to produce 2-PE (Fig. 1) and construct a cofactor regeneration system to obtain

a high production level.

Materials and methods

Chemicals

2-PE, L-Phe, PPA, PAAL and thiamine pyrophosphate (TPP) were purchased from Aladdin (Shanghai, China). Molecular reagents including Prime STAR Max DNA Polymerase, In-Fusion HD Cloning Kit, LA Taq polymerase, and restriction enzymes, ampicillin, chloramphenicol, and isopropyl β-D-1-thiogalactopyranoside (IPTG) were purchased from TaKaRa (Dalian, China). The plasmid miniprep kit, DNA gel extraction kit, and genomic DNA Purification Kit were purchased from Axygen (Shanghai, China). All other chemical reagents were of the highest purity available. The water used was distilled and deionized.

Strains, plasmids, and media

A stock culture of *P. mirabilis* JN458 screened from soil was stored in our laboratory. Its genome was used as the template in Polymerase Chain Reaction (PCR). *E. coli* BL21 (DE3) and *E. coli* DH5α purchased from TaKaRa (Dalian, China) were used for protein expression and gene cloning, respectively. The co-expression plasmids pACYCDuet-1 and pETDuet-1 were purchased from Novagen (Madison, USA). The pACYCDuet-1 plasmid was used for the protein expression of L-amino acid deaminase (PmLAAD) and α-keto acid decarboxylase (PmKDC) derived from *P. mirabilis* JN458. The pETDuet-1 plasmid was used for the protein expression of alcohol dehydrogenase (PmADH) derived from *P. mirabilis* JN458 and glucose

dehydrogenase (BsGDH) derived from *Bacillus subtilis* ATCC 13952 (ATCC, Manassas, USA). *E. coli* with pACYCDuet-1 or pETDuet-1 was grown in Luria-Bertani (LB) medium with 30 μg/mL chloramphenicol or 100 μg/mL ampicillin, respectively. The LB medium contained 10 g/L sodium chloride, 5 g/L yeast extract, and 10 g/L tryptone. *P. mirabilis* was inoculated into 50 mL of liquid LB medium containing 10% glucose and cultured at 37°C and 200 rpm for 24h. The biotransformation took place in M9 medium (glucose, 20 g/L; NH_4Cl, 0.1 g/L; NaCl, 0.5 g/L; $Na_2HPO_4 \cdot 7H_2O$, 11 g/L; $CaCl_2$, 10 mg/L; KH_2PO_4, 3 g/L; $MgSO_4$, 120 mg /L; pH 7.0~7.4).[15]

Plasmid construction for recombinant strains

PmaLaad (MG746627), *Pmkdc* (KY441412.1), and *Pmadh* (MG736298) from *P. mirabilis* JN458 were amplified by PCR with the restriction sites *Sac*I/*Pst*I, *Kpn*I/*Xho*I, and *Sac*I/*Pst*I, respectively. The *Bsgdh* (AIW32463.1) gene from *B. subtilis* ATCC 13952 was amplified by PCR with the restriction sites *Nde*I/*Kpn*I. We constructed three recombinant vectors, pACYCDuet-*PmLaad-Pmkdc* (pAAK), pETDuet-*Pmadh-Bsgdh* (pEaG), and pETDuet-*Pmadh* (pEa). The recombinant strains were constructed as shown in Table 1. The primes are listed in Table 2.

Analysis methods

Cell density was monitored by measuring optical density at 600 nm (OD_{600}, 1 OD/L = 2.56 g (wet cell weight)/L). The production of 2-PE, PPA, and PAAL were detected by reverse-phase HPLC, which was operated by a PerkinElmer Series 200

instrument using C18 (5 μm, 4.6×250 mm, Waters Technologies, Sunfire, Shanghai, China) with a UV array detector at 210 nm. The mobile phase was made up of water:methanol:formic acid = 60:40:0.1 at a flow rate of 1 mL/min. The analysis of 2-PE and PAAL was performed using a Trace1310-ISQ LT GC-MS (Thermo Fisher Technologies, Shanghai, China), equipped with a DB-WAX capillary column (60 m×0.25 mm i.d., 0.25 μm particle size; Agilent Technologies, Shanghai, China). Helium with high purity was used as a carrier gas at a constant flow of 1.0 mL/min. The oven temperature was initially 60°C for 2 min, and then ramped from 60°C to 250°C at a rate of 5 °C/min. The mass data acquired in the electron impact mode with ionization energy of 70 eV were applied to the full scan at m/z 33-350. The samples were diluted 1:30 (v/v) with octanol, and 1μL of the diluted samples was injected automatically in splitless mode. The ion source and interface temperatures were set at 250°C and 280 °C, respectively.

Over-expression of recombinant proteins

The recombinant strains were cultured in LB medium (50 mL) at 37°C with shaking at 200 rpm. After the optical density OD_{600} reached 0.4~0.6, the culture was cooled at 15°C for 30 min, then was induced by adding IPTG to a final concentration of 0.2 mM. Finally, the culture was continued at 15°C with shaking at 200 rpm for 24 h. Whole cells were harvested by centrifugation at 4°C and 10000×g for 10 min and then suspended with 20 mM pH 7.0 sodium phosphate buffers.

The whole cells were lysed using a sonicator (Sonics, Xiamen, China; with

pulses of 3 s on and 2 s off for 15 min), and then were centrifuged at 4°C and 10000×g for 10 min. Subsequently, the supernatant and precipitation of broken cells were confirmed by 12% sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE).[16]

Bioconversion conditions

Whole cells were collected by centrifugation at 4°C and 10000×g for 10 min, washed twice with 20 mM pH 7.0 sodium phosphate buffers, and then suspended in 3 mL (OD_{600}=6.5) M9 medium (pH 7.0) containing 10 g/L L-Phe. The bioconversion reactions were initiated at 40 °C with shaking at 200 rpm for 24 h. The samples were collected at regular intervals (8h) and analyzed by HPLC. Experiments were performed in triplicates.

Optimizing the whole cell transformation conditions of 2-PE production

The optimal temperature was determined from 20°C to 50°C. The effect of pH was analyzed by modifying the pH from 5-9 with 1N NaOH or HCl, under the optimal temperature. The optimal biomass was measured by adding different biomass (OD_{600}=6.5 to 32.5) in the mixture, under the optimal conditions of pH and temperature. Time course of whole cell transformation was determined, ranging from 0 h to 28 h at regular intervals, under the optimized conditions and L-Phe concentration.

Results

Construction of recombinant strains

The recombinant strain E-pAAK and E-pAEAKa were constructed by corresponding recombinant plasmids (Table 2). After the recombinant proteins over-expressed, the whole cells collected were used for further study.

The whole cells of E-pAAK and E-pAEAKa were broken up for SDS-PAGE analysis. As Fig. 2 shows, the expression quantity of soluble protein (lane 2) from PmLAAD in E-pAAK was approximately the same as that of the insoluble protein. Additionally, the expression quantity of soluble protein (lane 5) of PmLAAD in E-pAEAKa was also nearly the same as that of the insoluble protein. On the other hand, the expression quantity of insoluble protein of PmKDC (lane 3) in E-pAAK was less than that of the soluble protein. However, the expression quantity of soluble protein of PmKDC (lane 5) in E-pAEAKa was much greater than that of the insoluble protein. In addition, the expression level of PmADH (lane 5) was less.

Detection of the production of 2-PE by recombinant strains and *P. mirabilis*

When the transformation was finished, we detected that the recombinant strain E-pAAK produced 0.03±0.02 g/L 2-PE and 0.41±0.05 g/L PPAL (Table 3). However, the recombinant strain E-pAEAKa produced 0.39±0.03 g/L 2-PE and had hardly any production of PAAL. These results revealed that the catalytic capacity of over-expressed alcohol dehydrogenase from *P. mirabilis* JN458 was better than that of *E. coli* to transform PAAL into 2-PE.[10] The wild type *P. mirabilis* JN458 produced 0.08±0.01 g/L 2-PE. However, the negative control (*E. coli* harboring pACYCDuet-1 without a gene inserted) and *E. coli* BL21 could not produce any 2-PE. The deficiency

of aromatic amino acid decarboxylase led to a block of the pathway in *E. coli*.[10] To the best of our knowledge, this is the first report on *P. mirabilis* producing 2-PE. In order to verify the accuracy of our results, a sample transformed by recombinant strain E-pAEAKa should be further detected by GC-MS.

Validation of 2-PE synthesis in the recombinant strain E-pAEAKa by GC-MS

As shown in Fig. 3, 2-PE transformed by the E-pAEAKa whole cells was detected by GC-MS. In addition, we also detected a little PAAL by GC-MS. The results revealed that the recombinant strain E-pAEAKa was able to synthesize 2-PE. In order to avoid the accumulation of product intermediates, it is necessary to ensure that each of the subsequent enzymes has a higher activity than the first one.[17] The residual quantity of PAAL revealed that the activity of ADH was suitable to transform PAAL.

Construction of recombinant strain E-pAEAKaG with a cofactor regeneration system

In order to reduce the cost of cofactors, the BsGDH was introduced to construct a cofactor regeneration system. Finally, the recombinant strain E-pAEAKaG harboring pAAK and pEaG was constructed. As shown in Fig. 4, the recombinant strain E-pAEAKaG produced 1.16±0.09 g/L 2-PE after 16 h. Meanwhile, there was hardly any PAAL remaining after the biotransformation. The recombinant strains E-pAEAKaG and E-pAEAKa produced the same production of 2-PE before 4 h, subsequently, the recombinant strain E-pAEAKa was influenced by the amount of

NADH. On the contrary, the recombinant strain E-pAEAKaG used glucose and NAD^+ to produce NADH; therefore, the production of 2-PE kept increasing. Consequently, a cofactor regeneration system applied to the recombinant strain was crucial to increase the production of 2-PE.[18] Subsequently, the recombinant strain E-pAEAKaG was chosen for further research.

Optimal conditions for E-pAEAKaG whole cell transformation

The E-pAEAKaG whole cells showed an optimal temperature of 40°C. As Fig. 5 shows, the production of 2-PE clearly increased, ranging from 20°C to 40°C, and reached the highest production at 40°C, then showed a declining trend. As time went on, the production of 2-PE gradually increased steadily. The optimal pH for the E-pAEAKaG whole cells transformation was detected to be pH 7. As shown in Fig. 6, E-pAEAKaG whole cells have a preference for neutral pH. When the biomass of the E-pAEAKaG whole cells was beyond an OD_{600} of 13.0, the production of 2-PE showed a decreasing trend after 16 h (Fig. 6). The high concentration of cells easily formed an aggregate, which resulted in declining uptake of L-Phe and a deficiency in cell respiration.[19, 20] Finally, the production of 2-PE reached 3.21±0.10 g/L in the mixture under the optimum conditions.

The concentration of L-Phe was also an important factor for the production of 2-PE. With increasing concentration of L-Phe, the production of 2-PE also tended to increase. When the concentration of L-Phe was 4 g/L (24mM), the conversion rate of 2-PE reached 97.38mol%, which was the highest conversion rate observed.

Subsequently, the conversion rate started to decrease. The excess of phenylalanine might cause the binding of substrate prior to re-oxidation of the cofactor.[21] Previous study also suggested that the inhibition of LAAO at high substrate concentration could be due to binding of the substrate, resulting in the reduced flavin.[22] As shown in Fig. 7, the activity of the E-pAEAKaG whole cells showed an increasing trend and almost stabilized ranging from 4 g/L (24mM) to 10 g/L (60mM). Considering the conversion rate of L-Phe and the production of 2-PE, the concentration of L-Phe at 4 g/L (24mM) was chosen for biotransformation. Under the optimal L-Phe concentration, 2-PE production reached maximum (2.88±0.18 g/L) in 16 h (Fig. 8).

Discussion

In this study, we established a synthesis pathway for 2-PE in *E. coli* to mimic that of *P. mirabilis* JN458 from the substrate L-Phe. The results demonstrated that *P. mirabilis* JN458 contained a novel pathway to synthesize 2-PE. This pathway started with the deamination of L-Phe by PmLAAD to produce PPA. Then, PPA was decarboxylated by PmKDC to produce PAAL. Finally, in the pathway, PAAL was catalyzed by PmADH to produce 2-PE. With respect to cofactor-dependent enzymes, cofactors often account for a considerable proportion of the production cost.[23] Therefore, a cofactor regeneration system was constructed in this pathway to produce a large amount of 2-PE. As a consequence, *Proteus mirabilis* is a new class of bacteria that can synthesize 2-PE, except for *Microbacterium* sp. and the *Brevibacterium linens*.[24, 25]

In this study, L-amino acid deaminases were used to catalyze the first step in 2-PE synthesis. L-amino acid deaminase resembles the functionally known L-amino acid oxidase, but it uses a cytochrome b-like protein as an electron acceptor without producing hydrogen peroxide.[26-29] Compared to the first step in the Ehrlich pathway,[6, 30] whole cells need to supply aromatic amino acid transaminase to produce enough pyridoxal 5'-phosphate (PLP) and α-ketoglutarate, but a great deal of glutamic acid is produced after the transformation. In fact, intracellular α-ketoglutarate concentration was far below the demand for the continuous transamination.[31] In plants, one route for the transformation of L-Phe into PAAL is via phenylacetaldehyde synthase transformation, which shows a bifunctional character of both decarboxylation and oxidation, leading to a simpler process.[7] However, the phenylacetaldehyde synthase derived from plants has a problem of protein insolubility in *E. coli*. Additionally, it produces hydrogen peroxide coupled with the transformation.[7, 9] The other first step in plants is the decarboxylation of L-Phe by aromatic amino acid decarboxylases to produce phenylethylamine.[3] Except for the problem of protein insolubility in *E. coli*, the aromatic amino acid decarboxylase requires whole cells to produce pyridoxal 5'-phosphate to participate in the transformation.[3] As for the first step in other pathways, when glucose was used for a substrate alternative to L-Phe, some byproducts were produced along with the transformation.[10] Moreover, the efficiency of 2-PE production was quite low due to feedback regulation in other branched metabolic pathways.[32] Therefore, L-amino acid deaminase from *Proteus* sp. can be

used for biotechnological applications with broad prospects.[26]

Up to now, the co-expression of multiple enzymes has been used to investigate 2-PE biosynthesis in yeast or *E. coli*.[7, 30] Although yeast is the most common organism used to produce 2-PE, the fermentation process usually takes many days.[33] In addition, yeast, via the Ehrlich pathway, requires 2-oxoglutarate as an amino group acceptor and PLP as cofactor to produce 2-PE, which largely influences the production rate. As for *E. coli*, it is fast-growing and easy to manipulate.[31] *E. coli* can maintain several heterologous plasmids stably, which have compatible origins of replication and independent antibiotic selection.[34] Additionally, it has a high tolerance to alcohols and aldehydes.[35] Thus, a co-expression system was constructed in *E. coli* to mimic the 2-PE synthesis pathway in *P. mirabilis*. Finally, a cofactor regeneration system was constructed to reduce the cost and increase the production of 2-PE.

In order to improve the natural product yield, many strategies and methods aiming at improving green production of 2-PE have been investigated. As for wild strains, the production of 2-PE was relative low, including *Aspergillus oryzae*, yeast, *Enterobacter* sp., and so on.[15, 36, 37] Therefore, some genetic engineering techniques were applied to increase the 2-PE production. It is apparent that whole cell containing a co-expression system has been more deeply explored to produce a wide range of valuable chemical substances. An earlier study reached a maximum 2-PE concentration of 4.8 g/L with *Saccharomyces cerevisiae* via the Ehrlich pathway in a minimum medium containing 10 g/L L-Phe as a sole nitrogen source after 195 h.[6]

Considering the long culture period of that yeast host, the yield of 2-PE was relatively lower than that of *E. coli*. Moreover, some whole cells constructed by co-expression system involving different heterologous enzymes which derived from plants have a problem of protein insolubility.[7] On the contrary, the recombinant *E. coli* took about 2 h to produce 4.7 g/L 2-PE based on 40 mM L-Phe.[30] Although, the production of 2-PE was lower than that of several other recombinant strains, a high production yield could be obtained after the genes and vectors are further optimized.

To the best of our knowledge, a new pathway was verified using *E. coli* to mimic the 2-PE production in *P. mirabilis*. 2PE is widely used in the food, drink and cosmetic industry therefore making it a very valuable compound. Employing the recombinant strain E-pAEAKaG for 2-PE production would produce a higher titer of 2-PE for a broad market application. In addition, the recombinant strain E-pAEAKaG has the potential to produce higher aliphatic alcohols, like 3-methyl butanol, isobutanol, and n-propanol,[38] in addition to other aromatic higher alcohols, like tyrosol.[39]

Abbreviations

2-PE	2-Phenylethanol
L-Phe	L-phenylalanine
PPA	phenylpyruvate
PAA	phenylacetaldehyde
AAD	amino acid deaminase

317	KDC	α-keto acid decarboxylase
318	ADH	alcohol dehydrogenase
319	GDH	glucose dehydrogenase
320	IPTG	isopropyl β-D-1-thiogalactopyranoside
321	HPLC	High Performance Liquid Chromatography
322	GC-MS	Gas Chromatography-Mass Spectrometer
323	SDS-PAGE	sodium dodecyl sulphate-polyacrylamide gel electrophoresis

324 Acknowledgements

325 We thank the National Key Scientific Instrument and Equipment Development
326 Project of China (2013YQ17052504), the Program for Changjiang Scholars and
327 Innovative Research Team in the University of Ministry of Education of China
328 (IRT_15R55), the seventh group of Hundred-Talent Program of Shanxi Province
329 (2015), and The Key Project of Research and Development Plan of Shaanxi
330 (2017ZDCXL-SF-01-02-01) for financial support.

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449

450

Figure Captions

Fig. 1. The pathway for 2-Phenylethanol synthesis in *S. cerevisiae* (a), the proposed pathway in *P. mirabilis* (b), and the identified pathway in plants (c). L-AAD: L-amino acid deaminase, KDC: amino acid decarboxylase, ADH: alcohol dehydrogenase, AADC: aromatic amino acid decarboxylase.

Fig. 2. SDS-PAGE analysis of the co-expression proteins from the E-pAAK and E-pAEAKa. Lane M, the molecular-weight marker; lane 1, crude enzyme of *E. coli* BL21 (DE3) harboring the pACYCDuet-1 plasmid; lane 2, supernatant of the crude enzyme of E-pAAK; lane 3, precipitate of the crude enzyme of E-pAAK; lane 4, crude enzyme of *E. coli* BL21 (DE3) harboring the pACYCDuet-1 and pETDuet-1 plasmids; lane 5, supernatant of the crude enzyme of E-pAEAKa; lane 6, precipitate of the crude enzyme of E-pAEAKa.

Fig. 3. GC-MS profiles of 2-PE transformed by E-pAEAKa whole cells

Fig. 4. Effect of cofactor regeneration system on the production of 2-PE and PAAL.

Fig. 5. The optimum temperature and pH for the production of 2-PE transformed by the E-pAEAKaG whole cells. The reaction composed of 10 g/L L-Phe in 3 mL M9 mixture, was initiated by adding an OD_{600} of 6.5 wet cells for 24 h. (a) Effect of

different temperature on 2-PE production at a pH of 7, (b) Effect of different pH on 2-PE production at a temperature of 40°C. Data represent averages of three biological replicates. Standard deviations of average of the results are denoted by error bars.

Fig. 6. Effects of different E-pAEAKaG biomass on 2-PE production. The reaction composed of 10 g/L L-Phe in 3 mL M9 mixture, was initiated by adding different biomass (OD_{600} 6.5 to 32.5) at a temperature of 40°C and pH of 7 for 24 h. Data represent averages of three biological replicates. Standard deviations of average of the results are denoted by error bars.

Fig. 7. Effect of L-Phe concentration on the production of 2-PE. The reaction composed of different L-Phe concentration from 1 g/L (6 mM) to 10 g/L (60 mM)in 3 mL M9 mixture, was initiated by adding an OD_{600} of 13 wet cells at a temperature of 40°C and pH of 7 for 16 h. Data represent averages of three biological replicates. Standard deviations of average of the results are denoted by error bars.

Fig. 8. Time course of the transformation under the optimal conditions. The reaction composed of 4 g/L L-Phe in 3 mL M9 mixture, was initiated by adding an OD_{600} of 13 wet cells at a temperature of 40°C and pH of 7. Data represent averages of three biological replicates. Standard deviations of average of the results are denoted by error bars.

Tables

Table 1. Primers used in this study.

Primers	Nucleotide sequence (5'→3')	Restriction site
PmLaad-F	TCCGAATTCGAGCTCATGAACATTTCAAGGAGAAAGC	*Sac*I
PmLaad-R	GCTTGTCGACCTGCAGTTACTTCTTAAAACGATCCAAAC	*Pst*I
Pmkdc-F	CGCTGACGTCGGTACCGAGGTAATACACATGACAAACACAG	*Kpn*I
Pmkdc-R	CTTTACCAGACTCGAGTTAGAATGAATATAATGAACTTTTTG	*Xho*I
Pmadh-F	ATTCGAATTCGAGCTCATGGCCAGTTCAACATTTTATATC	*Sac*I
Pmadh-R	GCTTGTCGACCTGCAGTTACATTGCTTGGCGGAATAT	*Pst*I
Bsgdh-F	CATATGGCAGATCTCATGTATCCGGATTTAAAAGGAAAAGTC	*Nde*I
Bsgdh-R	AGACTCGAGGGTACCTTAACCGCGGCCTGCCTGGAAT	*Kpn*I

Table 2. Strains used in this study.

Strains	Descriptions	Source
P. mirabilis JN458	used for a PCR template	In this study
E. coli BL21 (DE3)	used for expressing	TaKaRa
E. coli DH5α	used for cloning	TaKaRa
B. subtilis ATCC 13952	used for a PCR template	ATCC
E-pAAK	*E. coli* BL21 (DE) carrying pACYCDuet-1 expressed PmLAAD and PmKDC	In this study
E-pAEAKaG	*E. coli* BL21 (DE) carrying pACYCDuet-1 and pETDuet-1 expressed PmLAAD and PmKDC, PmADH and BsGDH, respectively.	In this study
E-pAEAKa	*E. coli* BL21 (DE) carrying pACYCDuet-1 expressed PmLAAD and PmKDC, pETDuet-1 expressed PmADH	In this study

Table 3. The activity of different strains on the production of 2-PE and PAAL

Strains	PAAL (g/L)	2-PE (g/L)
E. coli BL21	ND	ND
E-pACYCDuet	ND	ND
P. mirabilis JN458	ND	0.08±0.01
E-pAEAKa	0.04±0.01	0.39±0.03
E-pAAK	0.41±0.05	0.03±0.02

Note: "ND" denoted the result was not detected. The reaction composed of 10 g/L L-Phe in 3 mL M9 mixture, was initiated by adding an OD_{600} of 6.5 wet cells at a temperature of 40°C and pH of 7 for 8 h. Data represent averages of three biological replicates. Standard deviations of average of the results are denoted by error bars.

Figure graphics

Fig. 1.

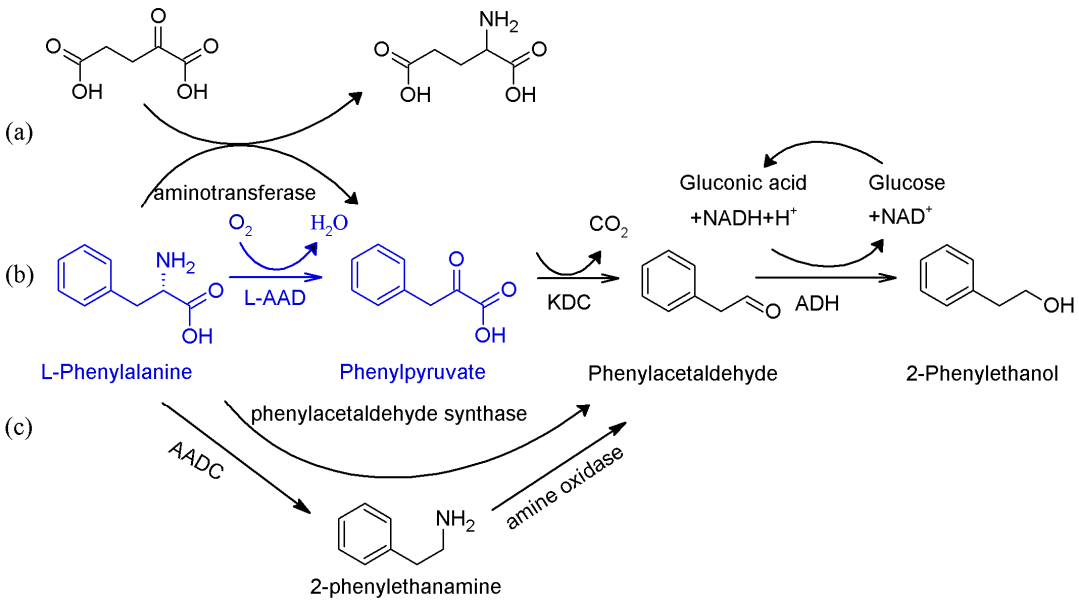


Fig. 2.

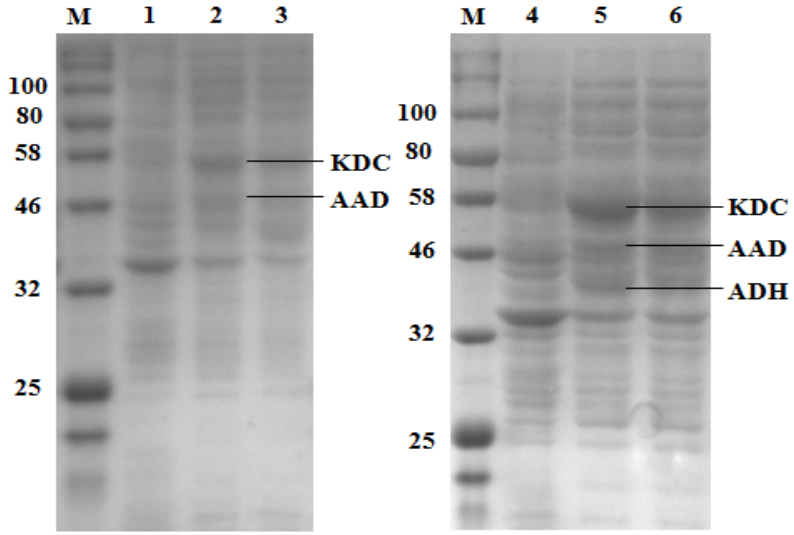


Fig. 3.

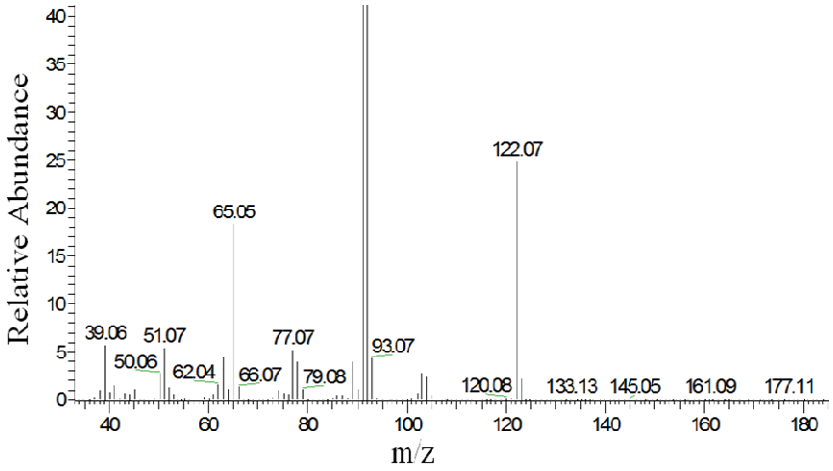

Relative Abundance
m/Z
122.07
65.05
39.06
51.07
77.07
93.07
50.06
62.04
66.07
79.08
120.08
133.13
145.05
161.09
177.11

Fig. 4.

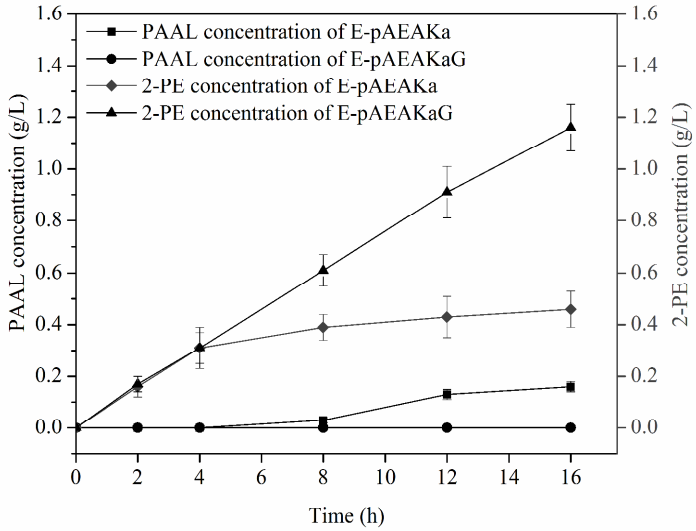

PAAL concentration of E-pAEAKa
PAAL concentration of E-pAEAKaG
2-PE concentration of E-pAEAKa
2-PE concentration of E-pAEAKaG
PAAL concentration (g/L)
2-PE concentration (g/L)
Time (h)

Fig. 5.

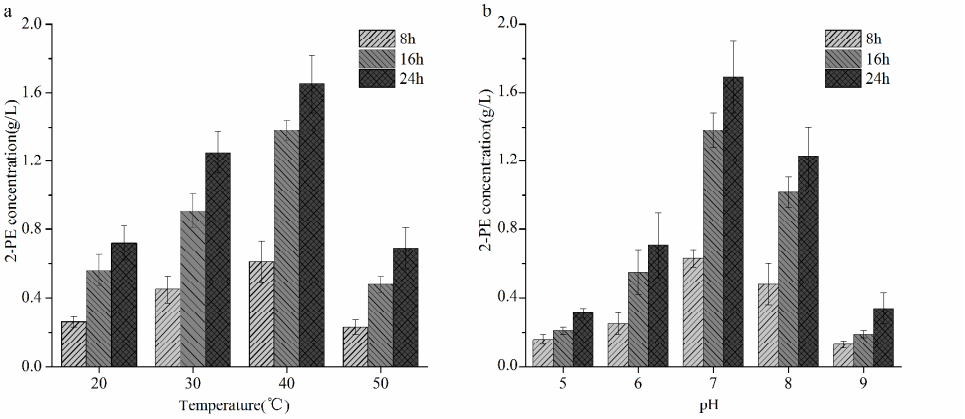

a
b
8h
16h
24h
2-PE concentration(g/L)
Temperature(℃)
pH

Fig. 6.

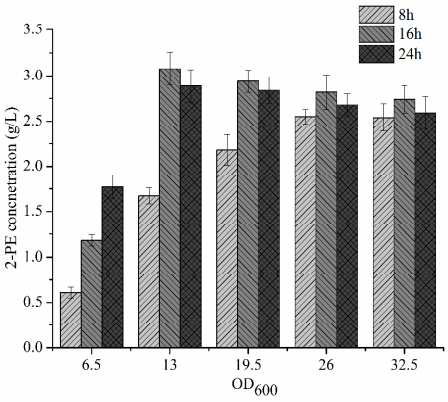

8h
16h
24h
2-PE concnetration (g/L)
OD600

Fig. 7.

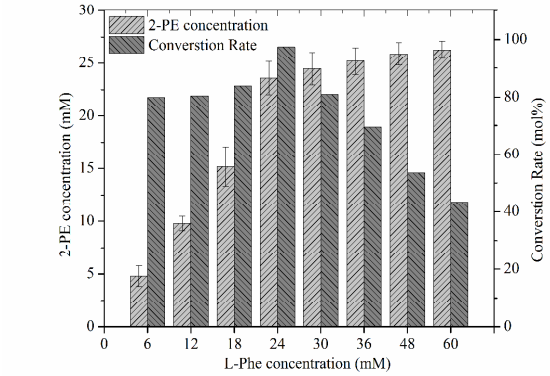
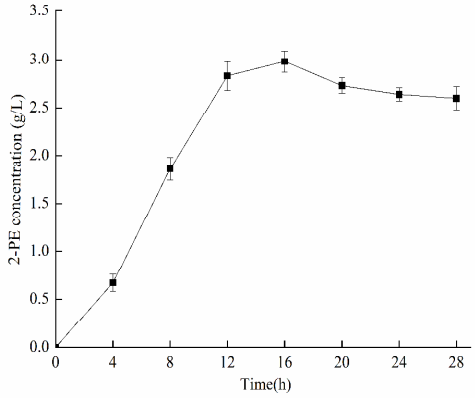

2-PE concentration
Converstion Rate
2-PE concentration (mM)
Conversion Rate (mol%)
L-Phe concentration (mM)
0
6
12
18
24
30
36
48
60
0
5
10
15
20
25
30
0
20
40
60
80
100

Fig. 8.


2-PE concentration (g/L)
Time(h)
0
4
8
12
16
20
24
28
0.0
0.5
1.0
1.5
2.0
2.5
3.0
3.5

Graphic for table of contents