ISSN print: 1224-5984
ISSN online: 2248-3942

Rom Biotechnol Lett. 2022; 27(5): 3669-3681
doi: 10.25083/rbl/27.5/3669.3681

Received for publication, January, 12, 2023
Accepted, January, 21, 2023

Original article

Synthesis of l-DOPA catalyzed by recombinant *Escherichia coli* expressing tyrosine phenol lyase gene from *Desulfitobacterium hafniense*

YI FENG[1], LIJUAN YU[1], DAYONG JIN[1], SUXIANG ZHENG[1], JIE LI[1], YONGHUI JIANG[1]

[1]School of Life Sciences, Nantong University, Nantong 226019, China

Abstract

L-DOPA (L-dihydroxyphenylalanine) is a potential drug for the treatment of Parkinson's disease, and the demand is increasing every year. Tyrosine phenol-lyase (TPL) is a valuable biocatalyst for the biosynthesis of L-tyrosine and its derivatives, which are valuable intermediates in the pharmaceutical industry. In this study, a new TPL gene (*Dh-TPL*) was cloned from *Desulfitobacterium hafniense*, ligated into pJJDuet30 vector, and successfully expressed in *Escherichia coli*. To increase the yield and stability of L-DOPA, as well as decrease the by-product formation, the enzyme production conditions and the catalytic reaction conditions were studied. The optimal TPL production conditions were as follows: the concentration of IPTG was 0.2 mmol/L and the induction temperature was controlled at 18°C. The optimum medium composition for recombinant bacteria includes yeast extract 64 g/L, tryptone 36 g/L, glycerol 4 g/L, KH_2PO_4 2.31 g/L and K_2HPO_4 9.4 g/L. The best biosynthesis of L-DOPA was performed in a reaction mixture containing 10.0 g/L catechol, 20 g/L sodium pyruvate and 20 g/L recombinant *E. coli* resting cells. The optimal reaction temperature and pH were determined to be 18°C and pH 8.5, respectively. Under these conditions, the yield of L-DOPA was 0.21 mol/L (41.7 g/L) after 3 hours reaction.

Keywords Tyrosine phenol lyase, l-DOPA, Biotransformation, *Desulfitobacterium hafniense*, catechol

To cite this article: YI FENG. Synthesis of l-DOPA catalyzed by recombinant *Escherichia coli* expressing tyrosine phenol lyase gene from *Desulfitobacterium hafniense*. *Rom Biotechnol Lett.* 2022; 27(5): 3669-3681 DOI: 10.25083/rbl/27.5/3669.3681

*Corresponding author: Lijuan Yu; email: ylj200908@ntu.edu.cn

Introduction

L-DOPA (3,4-dihydroxyphenyl-L-alanine), an amino acid produced by the oxidation of L-tyrosine, is a precursor of the neurotransmitter dopamine. L-DOPA has been an essential commodity for the pharmaceutical companies since the 1960s as it is used as a therapeutic agent for dopamine-responsive dystonia and Parkinson's Disease.

The traditional method to obtain L-DOPA is direct extraction from plants, which is usually restricted by the limitation of raw material sources and the complexity of extraction steps. Monsanto [1] developed a method for the production of L-DOPA by asymmetric hydrogenation. However, the chemical synthesis of L-DOPA has many problems, such as poor conversion, low optical selectivity, high catalysts cost (e.g., Rb-complex), and harsh operational conditions [2, 3]. The main enzymes used for L-DOPA synthesis include tyrosinase, p-hydroxyphenylacetate 3-hydroxylase (PHAH), aminoacylases, and tyrosine phenol lyase (TPL).

L-DOPA can be synthesized from L-tyrosine by tyrosinase, and at the same time, a large number of by-products (mainly dopaquinone) are produced. To reduce L-DOPA to dopaquinone, chemical reducing agents are usually employed [3]. Lee and Xun [4] reported another process for L-DOPA production from L-tyrosine based on PHAH. However, the reaction requires NADH as the cofactor. The high cost of NADH limits the industrial applications of this method. Pragati Agarwal *et al*. [5] used aminoacylases to produce L-DOPA. Aminoacylases are a class of enzymes that specifically hydrolyze the amide bonds in N-acetyl-L-amino acids, and have high enantioselectivity. However, the reaction conversion is low, and the final replacement step of hydrogen bromide will generate a large amount of methyl bromide, which can destroy ozone and causes serious environmental pollution.

TPL can degrade L-tyrosine to pyruvate, ammonia, and phenol. This reaction is reversible. Therefore, L-DOPA can be produced if phenol is substituted by catechol as shown in Scheme 1. Hitoshi Enei *et al*. [6] used *Erwinia herbicola* ATCC 21434 whole cells with high TPL activity to synthesize L-DOPA. However, the yield of L-DOPA was limited (only 11.5 g/L after 24 hours of reaction).

The microbial transformation based on TPL activity usually shows higher productivity than that based on tyrosinase activity [7-10]. Consequently, we use TPL to produce L-DOPA from catechol, sodium pyruvate, and ammonia. In this study, we cloned and expressed TPL gene from *Desulfitobacterium hafniense* DP7 (DSM 13498) in *E. coli*, and investigated the enzyme production conditions and the catalytic reaction conditions in order to synthesize L-DOPA more efficiently.

Materials and methods

Strains, plasmids, and reagents

E. coli DH5α, pJJDuet30, and *E. coli* BL21 (DE3) were purchased from Addgene. Luria-Bertani (LB) medium (10 g/L tryptone, 5 g/L yeast extract, 10 g/L NaCl) was used for the cultivation of *E. coli* strains. L-DOPA, sodium pyruvate, catechol, ammonium acetate, sodium sulfite, EDTA were purchased from Sigma-Aldrich. Plasmid Mini Preparation Kit, DNA Gel Extraction Kit, SDS-PAGE Gel Quick Preparation Kit, and restriction endonucleases were purchased from Beyotime Biotechnology.

Analytical methods

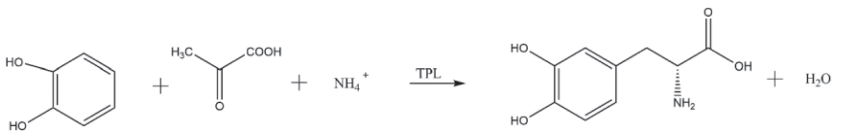
The conversion reaction solutions were filtered with 0.22 μm polytetrafluoroethylene membrane and analyzed by Agilent 1200 HPLC. The samples were separated on an Agilent Eclipse XDB-C18 column. The mobile phase consisted of 0.1% formic acid and methanol (6:4, v/v). The column temperature was maintained at 35°C and the detection wavelength was 280 nm. The injection volume of the samples was set at 20 μL and the flow rate was 0.8 mL/min.

Construction of plasmid pJJ-dhtpl

Standard operating measures were adopted for PCR, DNA purifications, enzyme digestions, ligations, and plasmid extractions. The *Dh*-TPL (GenBank: EHL08374.1) was chemically synthesized and inserted into a pJJDuet30 plasmid between *Sac* II site and BamH I site. The ligation product was transformed into *E. coli* DH5α and verified by colony PCR and DNA sequencing. The plasmid with the correct sequence was named pJJ-dhtpl.

Transformation and screening of transformants

The recombinant plasmid pJJ-dhtpl was transformed into *E. coli* BL21 (DE3) to obtain the genetically engineered strain BL21/pJJ-dhtpl. The heat shock method [11] was used to transfer the plasmid pJJ-dhtpl into *E. coli* competent cells. Single colonies on kanamycin-resistant plates were verified by colony PCR, restriction enzyme digestion (QuickCut *Sac* II/ QuickCut BamH I), and sequencing.



Scheme 1. Schematic diagram of l-DOPA synthesis catalyzed by TPL from pyruvate, ammonia and catechol.

Expression of Dh-TPL and SDS-PAGE analysis

The recombinant strains were routinely cultivated in LB medium and 50 μg/mL of antibiotics (kanamycin) were added. For *Dh-TPL* expression, a single colony was inoculated in 50 mL of LB medium and cultured at 37°C in a rotary shaker at 200 r/min. The overnight seed culture was inoculated in 2 L LB medium. When the optical density of the culture at 600 nm (OD_{600}) reached 0.6, IPTG was added to a final concentration of 0.1 mmol/L to induce the gene expression at 18°C for 12 h. The cells were then collected by centrifugation at 4,000 r/min for 10 min and washed twice with double-distilled water. The harvested cells were suspended in 5 mL double distilled water and disrupted by ultrasonication at 200 W for 10 min (ultrasonicated for 2 sec/gap 2 seconds). Supernatants and sediments were collected by centrifugation at 12,000 r/min for 5 minutes at 4°C and analyzed by 4-20% BeyoGel™ SDS-PAGE Precast Gel.

Determination of the *Dh*-TPL activity

One unit (U) of *Dh*-TPL activity was defined as the amount of enzyme that catalyzed the formation of 1 μmol L-DOPA per minute under the described assay conditions.

$$Dh\text{-TPL activity (U/g)} = \frac{\rho V \times 10^6}{mtM_r}$$

ρ, the mass concentration of L-DOPA, g/L. V, the reaction volume, L. M_r, the molecular weight of L-DOPA (197.19), g/mol. t, the reaction time, min. m, the wet weight of the injected bacteria, g.

Synthesis of L-DOPA catalyzed by recombinant *E. coli* cells

The recombinant *E. coli* cells expressing *Dh-TPL* were used for L-DOPA biosynthesis with catechol, sodium pyruvate, and ammonium acetate as substrates. The cells were collected by centrifugation at 4,000 r/min for 10 min and washed twice with double-distilled water. The harvested cells (20 g/L) were suspended in the L-DOPA transformation system. The L-DOPA transformation system (Ammonia adjustment pH 8) was composed of 5 g/L sodium pyruvate, 8 g/L catechol, 50 g/L ammonium acetate, 2 g/L sodium sulfite, and 1 g/L EDTA. The synthesis reaction were conducted at 16°C for 3 h at 150 r/min. After centrifugation (12000 r/min, 4°C, 2 min), the supernatant was analyzed using HPLC.

Optimization of *Dh*-TPL production conditions

Effects of medium type on Dh-TPL activity

Using LB medium as the initial fermentation medium, six nutritive sources were added at 2.5 g/L (glucose, lactose, sucrose, glycerol, sodium acetate and ammonium sulphate). Terrific Broth TB medium (24g/L yeast extract, 20g/L tryptone, 4ml/L glycerol, 0.017 M KH_2PO_4, 0.072 M K_2HPO_4)as well as LB medium were used as controls. After the induction, the *Dh*-TPL activity was determined. All experiments were carried out in triplicates and L-DOPA was detected and quantified by HPLC.

Effects of medium composition on Dh-TPL activity

By comparing these eight media, TB medium was determined to be the optimum medium. Further, the yeast extract concentrations were set to 4, 24, 44, 64 and 84 g/L. After the induction, the *Dh*-TPL activity was determined. All experiments were carried out in triplicates and L-DOPA was detected and quantified by HPLC.

At the optimum yeast extract concentration, the tryptone concentration was further set to 6, 12, 24, 36, 48 g/L. After the induction, the *Dh*-TPL activity was determined. All experiments were carried out in triplicates and L-DOPA was detected and quantified by HPLC.

At the optimum yeast extract and tryptone concentration, the glycerol addition was set to 2, 4, 8, 10, 12, 14, 16, 18 and 20 g/L. After the induction, the *Dh*-TPL activity was determined. All experiments were carried out in triplicates and L-DOPA was detected and quantified by HPLC.

Effects of IPTG concentration on Dh-TPL activity

IPTG as an inducer can influence the *Dh-TPL* expression. The cells were cultured at 37°C until the OD_{600} was 0.6, then IPTG was added until the final IPTG concentrations in the medium were 0.05, 0.1, 0.2, 0.4, and 0.6 mmol/L, respectively. The induction was carried out at 18°C for 12 h. After the induction, the *Dh*-TPL activity was determined. All experiments were carried out in triplicates and L-DOPA was detected and quantified by HPLC.

Effects of induction temperature on Dh-TPL activity

The cells were cultured at 37°C until the OD_{600} was 0.6, then IPTG (0.2 mmol/L) was added. The induction was carried out at different temperatures ranging from 12 to 36°C for 12 h. After the induction, the *Dh*-TPL activity was determined. All experiments were carried out in triplicates and L-DOPA was detected and quantified by HPLC.

Optimization of the synthesis conditions of L-DOPA

Effects of initial catechol concentration on l-DOPA synthesis

To optimize the initial catechol concentration, the reaction was performed in a shake flask (pH 8.0, 16°C, 150 r/min) based on the fixed sodium pyruvate concentration of 5 g/L, including different concentrations of catechol, 50 g/L ammonium acetate, 2 g/L sodium sulfite, 1 g/L EDTA, and 20 g/L recombinant *E. coli* resting cells. All experiments

were carried out in triplicates and the concentration of L-DOPA, residual catechol and the by-product were detected and quantified by HPLC.

Effects of initial main substrates ratio on l-DOPA synthesis

To optimize the initial main substrates ratio, the reaction was performed in a shake flask (pH 8.0, 16°C, 150 r/min) based on the fixed catechol concentration of 10 g/L, including different concentrations of sodium pyruvate, 50 g/L ammonium acetate, 2 g/L sodium sulfite, 1 g/L EDTA, and 20 g/L recombinant *E. coli* resting cells. All experiments were carried out in triplicates and the concentration of L-DOPA, residual catechol and the by-product were detected and quantified by HPLC.

Effects of reaction pH on L-DOPA synthesis

The optimal pH for L-DOPA biosynthesis was analyzed. The reaction system containing 20 g/L sodium pyruvate, 10 g/L catechol, 50 g/L ammonium acetate, 2 g/L sodium sulfite, 1 g/L EDTA sodium, and 20 g/L resting recombinant *E. coli* cells was mixed evenly. The synthesis was performed at pH ranging from 7.0 to 9.0 (adjusted by ammonia) at 16°C for 3 h. All experiments were carried out in triplicates and the concentration of L-DOPA, residual catechol and the by-product were detected and quantified by HPLC.

Effects of reaction temperature on L-DOPA synthesis

The product L-DOPA is easily oxidized and degraded at high temperature. Therefore, in this study, the synthesis of L-DOPA was conducted at lower temperatures (11°C-36°C), and the yields of L-DOPA at different temperatures were measured. The reaction system (pH 8.5) contained 20 g/L sodium pyruvate, 10 g/L catechol, 50 g/L ammonium acetate, 2 g/L sodium sulfite, 1 g/L EDTA sodium, and 20 g/L resting recombinant *E. coli* cells. All experiments were carried out in triplicates and the concentration of L-DOPA, residual catechol and the by-product were detected and quantified by HPLC.

Results and discussion

Construction of plasmid pJJ-dhtpl

To obtain active TPL, the target gene was cloned to the *Sac* II/BamH I site of pJJDuet30. The construction of the pJJ-dhtpl plasmid was shown in Fig. 1. The pJJ-dhtpl

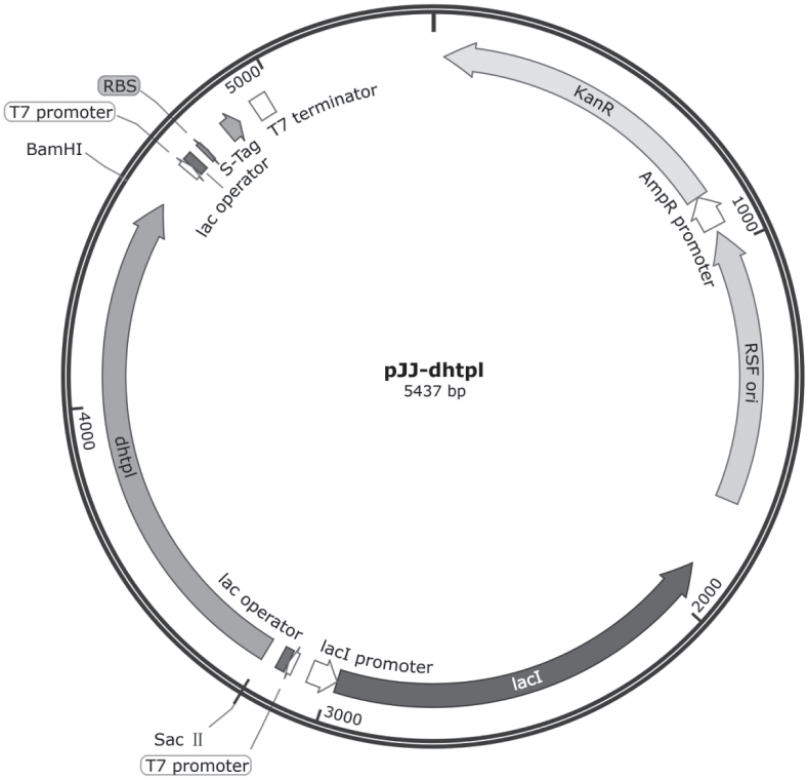


Figure 1. Construction of the pJJ-dhtpl plasmid.

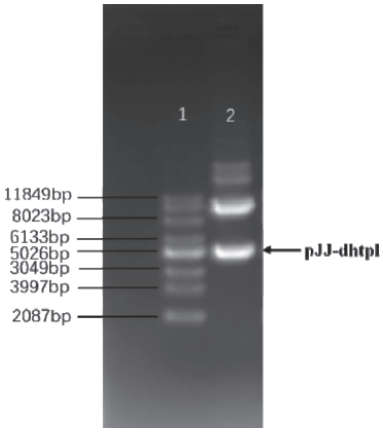


Figure 2. The electrophoresis of recombinant pJJ-dhtpl. Lane 1: the supercoiled DNA ladder; Lane 2: pJJ-dhtpl plasmid.

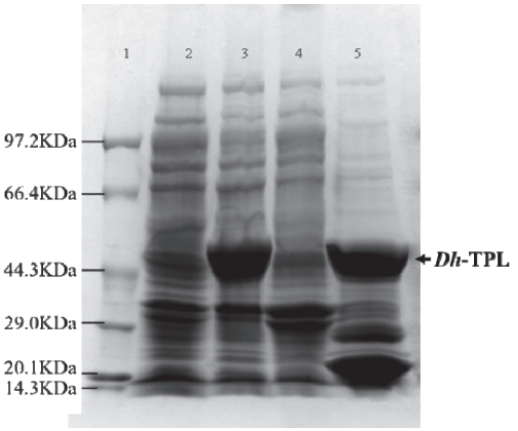


Figure 3. SDS-PAGE analysis of supernatants and sediments after ultrasonication and centrifugation. Lane 1: the Premixed Protein Marker (Low) standard; Lane 2 and Lane 4: the supernatant and sediment of BL21; Lane 3 and Lane 5: the supernatant and sediment of BL21/pJJ-dhtpl

plasmid was transformed into *E. coli* DH5α and the transformants selection was made on LB plates containing kanamycin. The plasmid size (Fig. 2) was consistent with the theoretical 5437 bp. The pJJ-dhtpl plasmid with correct sequence was used to transform *E. coli* BL21 (DE3) competent cells.

Expression of Dh-TPL and SDS-PAGE analysis

To achieve a large amount of TPL, the expression of TPL encoding gene was induced by 0.1 mmol/L IPTG for 12 h. The induction need to be carried out at 18°C to reduce the production of other proteins. The expression products were analyzed by SDS-PAGE. As shown in Fig. 3, *Dh-TPL* was expressed within the host cell with soluble and insoluble forms. Due to the large amount of insoluble TPL, the expression conditions need to further optimize to increase the TPL activity. The molecular weight of target band was about 50 KDa, which was in accordance with the molecular mass calculated by its primary amino acid sequence.

The rest cells expressing *TPL* were used in the synthesis of L-DOPA. The HPLC chromatogram of the conversion reaction solution was shown in Fig. 4. The retention time of the product L-DOPA was 2.900 min, the retention time of the by-product was 3.536 min and the retention time of the substrate catechol was 5.425 min.

Effects of medium type on Dh-TPL activity

The nutritive sources are the essential energy supply for the vital activity of bacteria and is the basis for bacterial cell formation and target protein synthesis. The initial medium

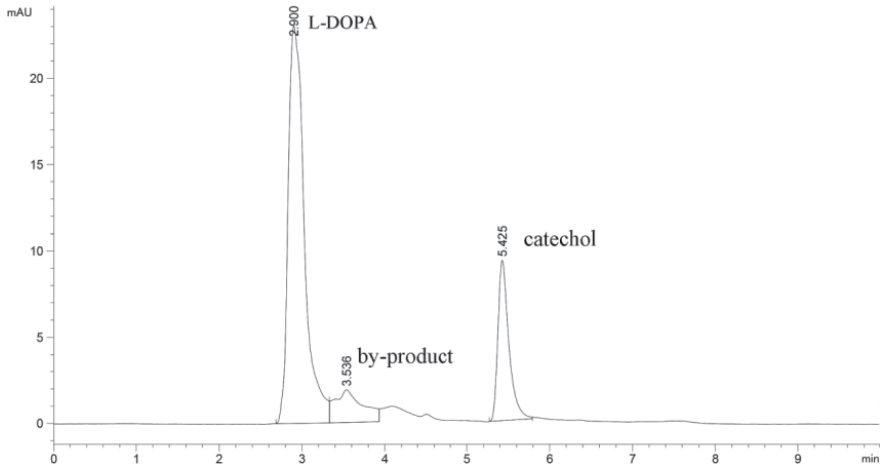


Figure 4. The HPLC chromatogram of the conversion reaction solution catalyzed by recombinant *E. coli* cells.

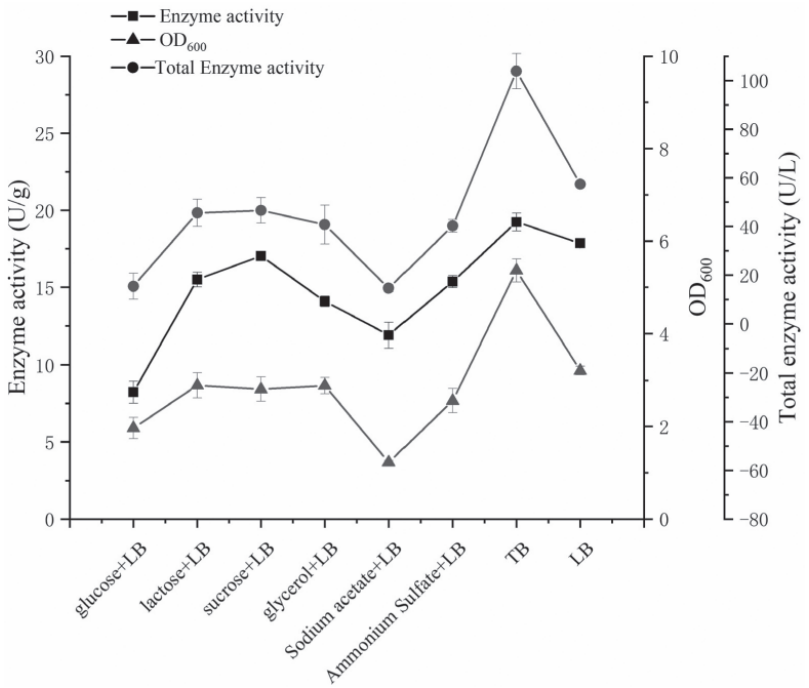


Figure 5. The effects of medium type on *Dh*-TPL activity.

LB was enriched with peptone and yeast extract. Based on the initial medium LB, six different types of nutritive sources were added and the results are shown in Fig. 5. It can be seen that the type of nutritive source has a great influence on the growth of recombinant bacteria and the enzyme activity, and the amount of bacteria in the medium with various nutritive sources added on top of LB medium was less than that of the original LB medium. The enzyme activity of TB medium was much higher than that of the other mediums, which indicated that TB medium was not only beneficial to

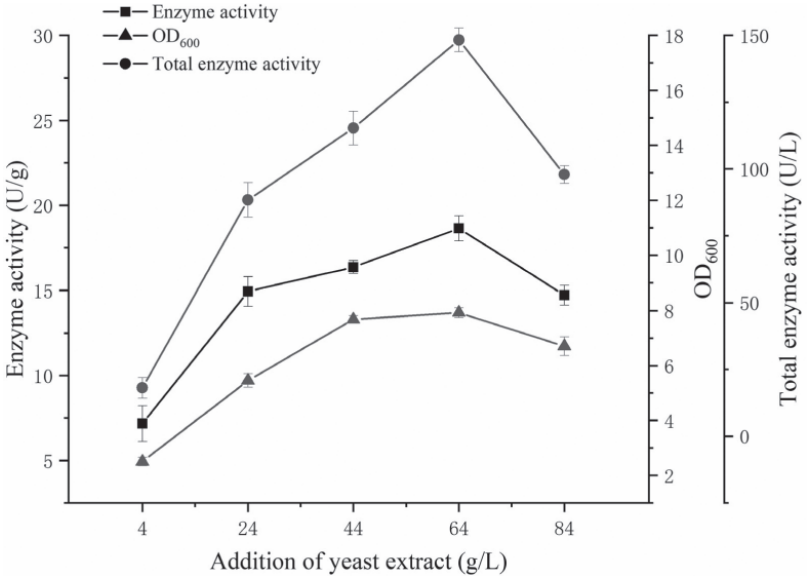


Figure 6. The effects of yeast extract addition on *Dh*-TPL activity.

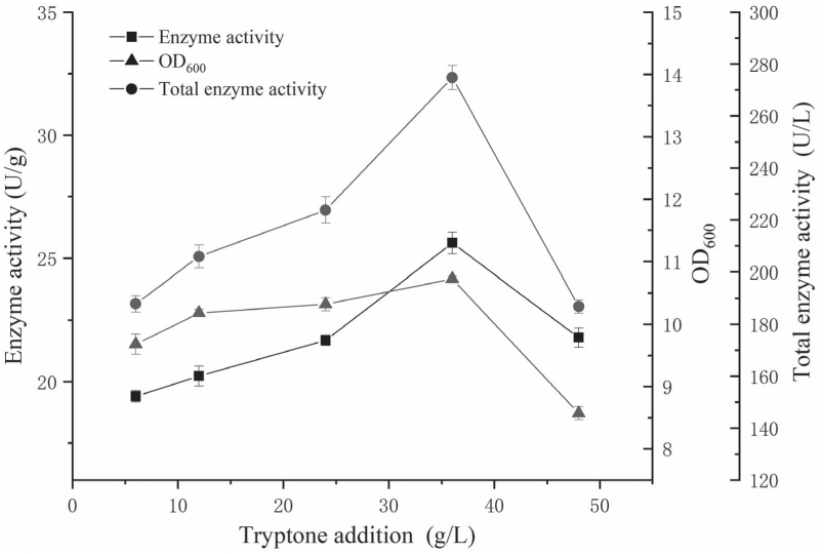


Figure 7. The effects of tryptone addition on *Dh*-TPL activity.

the growth of the bacteria but also to the enzyme production of the bacteria, which led to a significant increase in L-DOPA production.

Effects of medium composition on Dh-TPL activity

On the basis of TB medium, the addition of yeast extract was further optimized, and it can be seen from Fig. 6 that when the concentration of yeast extract was only 4 g/L, the enzyme activity per unit TPL was much lower than other concentrations, and with the increase of yeast extract, the enzyme activity per unit TPL gradually increased, but the bacterial volume showed a rapid increase, and when the addition of yeast extract reached 64 g/L, at which time the amount of bacteria, unit enzyme activity and total enzyme activity all reached the peak. When the addition of yeast extract increased to 84 g/L, the amount of bacteria, unit enzyme activity and total enzyme activity all decreased, indicating that excessive yeast extract inhibited the growth and metabolism of bacteria.

As can be seen from Fig. 7, the amount of bacteria increased slowly with the addition of tryptone, but decreased rapidly when the addition amount exceeded 36 g/L. This indicates that the excessive amount of tryptone was not conducive to the massive expansion of the recombinant bacteria, which in turn led to a decrease in the total enzyme activity. At the stage of addition of 24-36 g/L, the unit enzyme activity increased rapidly and the total enzyme activity reached the peak at this time, indicating that the right amount of tryptone was conducive to the massive expression of the target protein by the recombinant bacteria.

As shown in Fig. 8, when glycerol was added at 4-20 g/L, there was no significant increasing trend in unit enzyme activity, OD_{600} and total enzyme activity, which remained in a relatively stable range, but the total enzyme activity reached the highest when glycerol was added at 4 g/L. The recombinant *E. coli* in this study showed the highest enzyme activity when only a small amount of glycerol was added, which greatly reduced the production costs for the subsequent industrial production of L-DOPA.

Effects of IPTG concentration on Dh-TPL activity

IPTG as an important and toxic inducer, regulates the expression of cloned *Dh-TPL*. The influences of IPTG concentration on TPL activity were shown in Fig. 9. The values of OD_{600} decreased with the increase of IPTG concentration. The enzyme activity of unit cells and the total enzyme activity reached the maximums when the IPTG concentration was 0.2 mmol/L. However, excessive addition of IPTG did not increase enzyme activity and total enzyme activity, a significant decline was observed when IPTG concentration exceeded 0.4 mmol/L. This due to the fact that high concentration of IPTG delayed the growth of cells and caused the metabolic burden to the host cells [12].

Effects of IPTG induction temperature on Dh-TPL activity

As shown in Fig. 10, when the temperature was 12°C, the OD_{600} value was only 10.3808. At 18°C, the OD_{600} value raised to 11.44703. Then, with the increase of temperature, OD_{600} value raised very slowly. The values of OD_{600} improved with the increase of temperature, which indicated

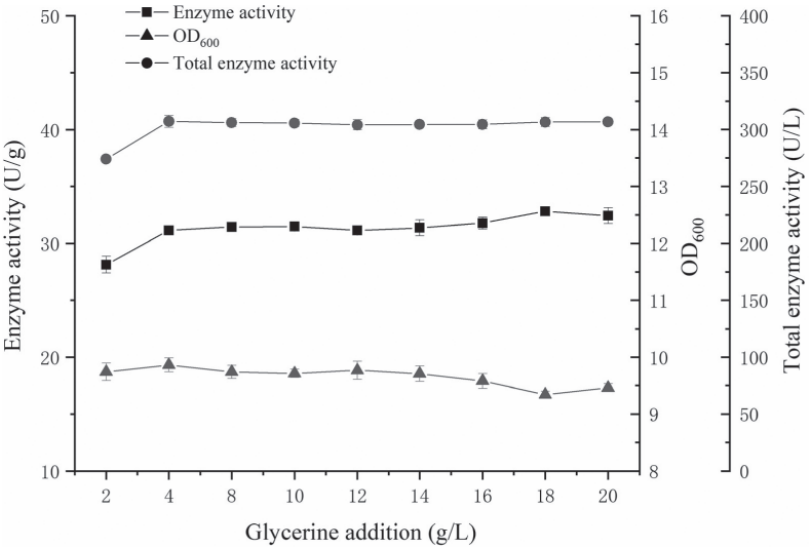


Figure 8. The effects of glycerol addition on *Dh*-TPL activity.

that high temperature was beneficial to the growth of bacteria to a certain degree. The enzyme activity of unit cells and the total enzyme activity increased significantly from 12°C to 18°C, then decreased above 18°C. Too low induction temperature (12°C) slowed down bacterial growth and affected the expression of foreign gene, while higher induction temperature would cause a large number of insoluble inclusion body to form in the cell, resulting in excessive accumulation of insoluble TPL and reducing enzyme activity. Therefore, the optimal induction temperature was fixed at 18°C.

Effects of initial catechol concentration on* l*-DOPA synthesis

Proper initial substrate concentration is not only conducive to the synthesis of L-DOPA, but also can reduce the formation of by-products. Under the condition of sufficient enzymes (cells), increasing the concentration of catechol can accelerate the initial reaction rate. However, the high concentration of catechol in the system has toxic and inhibitory effects on cells and enzyme activity [13, 14]. It has been reported that high concentration of catechol can destroy cell

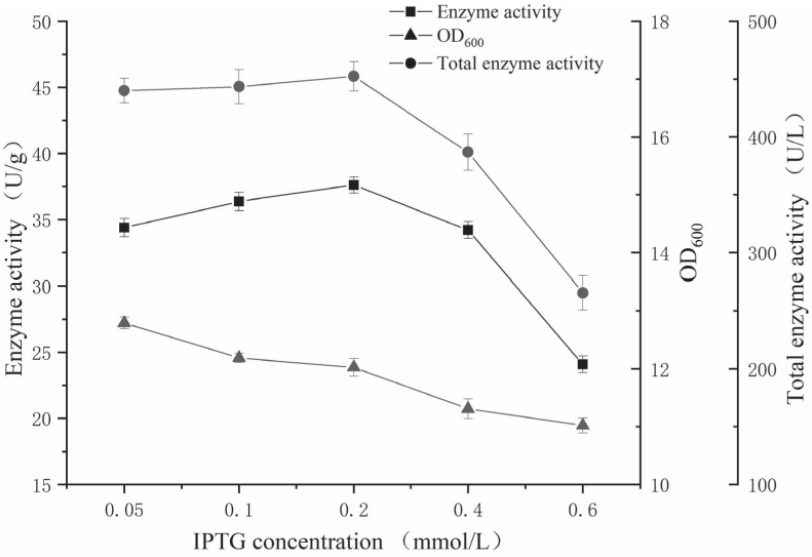


Figure 9. The effects of IPTG concentration on *Dh*-TPL activity.

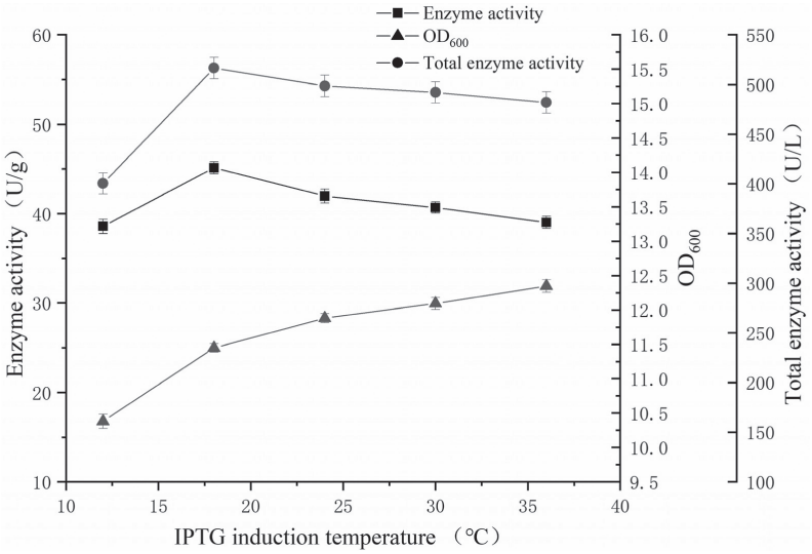


Figure 10. The effects of induction temperature on *Dh*-TPL activity and cell growth.

membrane components and accelerate cell lysis [15]. In order to improve the yield of L-DOPA and decrease the toxicity of catechol to cells, the appropriate catechol concentration in the synthesis system was investigated. As shown in Fig. 11, when the catechol concentration increased, the yield of L-DOPA raised too, and when initial catechol concentration was 10 g/L, the greatest L-DOPA yield could be obtained. Subsequently, an excessive addition of catechol was obviously unfavorable to the production of L-DOPA, which indicated that high concentrations of catechol can irreversibly inhibit the activity of *Dh*-TPL. Meanwhile, when the concentrations of catechol were lower than 8 g/L, due to the relative shortage of catechol, the spontaneous reaction between newly formed L-DOPA and residual sodium pyruvate leads to a large amount of by-product. When the initial concentration of catechol was 10 g/L, the minimum amount of by-product was achieved. After 10 g/L, the activity of *Dh*-TPL was inhibited by catechol, the quantity of residual sodium pyruvate increased, and the amount of by-product also began to raise. Therefore, the initial con-

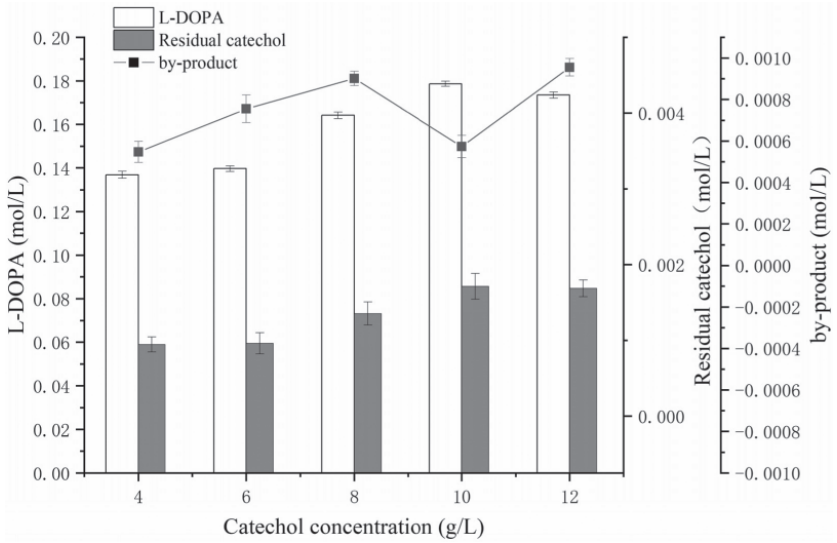


Figure 11. The effects of initial catechol concentration on L-DOPA synthesis.

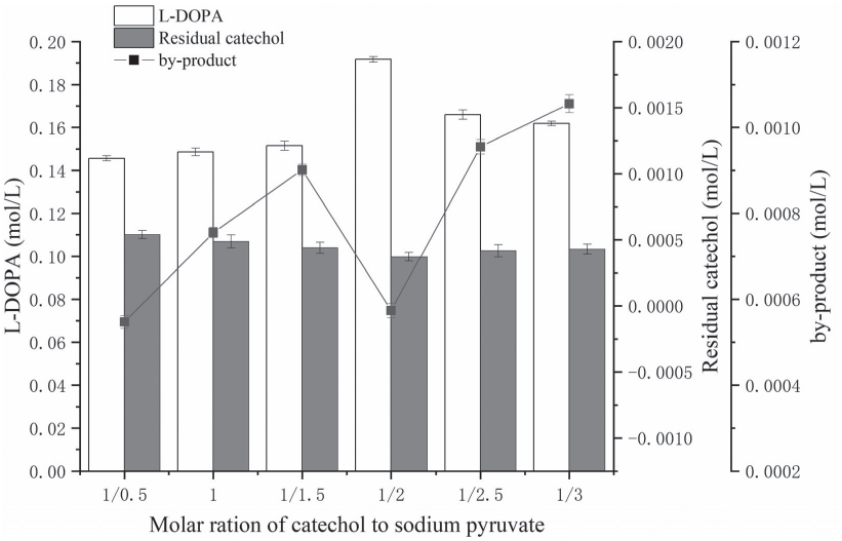


Figure 12. The effects of initial molar ratio of catechol to sodium pyruvate on L-DOPA synthesis.

centration of catechol was fixed at 10 g/L in the subsequent reaction, which is consistent with E313W and E313M TPL (from *Citrobacter freundii*) [16] and *Eh*-TPL (from *Erwinia herbicola*) [17]. This value is much higher than that of *Fn*-TPL (from *Fusobacterium nucleatum*, 5 g/L) [10]. Wei Yuan *et al.* [18] adopted 12.5 g/L of initial catechol concentration in 10 L fermenter, which was the highest level currently reported. Compared with them, the catechol tolerance of *Dh*-TPL is slightly lower, however, the synthesis reactions in this study were carried out in shake flasks. According to experience, there may be a large upside in the subsequent scale-up reactions.

Effects of initial main substrates ratio on* l*-DOPA synthesis

During the synthesis of L-DOPA catalyzed by *Dh*-TPL, the excessive sodium pyruvate can react with L-DOPA spontaneously without enzyme catalysis. Therefore, the proper initial ratio of catechol to sodium pyruvate is very important for the reaction. The effects of catechol/ sodium pyruvate ratios on L-DOPA synthesis were shown in Fig. 12. With the increase of sodium pyruvate, the conversion of catechol increased obviously. When the molar ratio of catechol to sodium pyruvate was set at 1:0.5, the yield of L-DOPA only reached 0.146 mol/L after 3 h. Further increas-

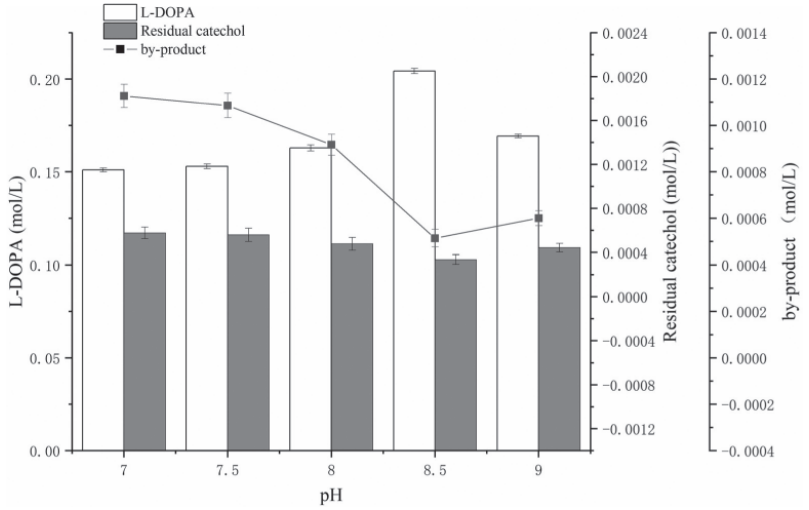


Figure 13. The effects of reaction pH on L-DOPA synthesis.

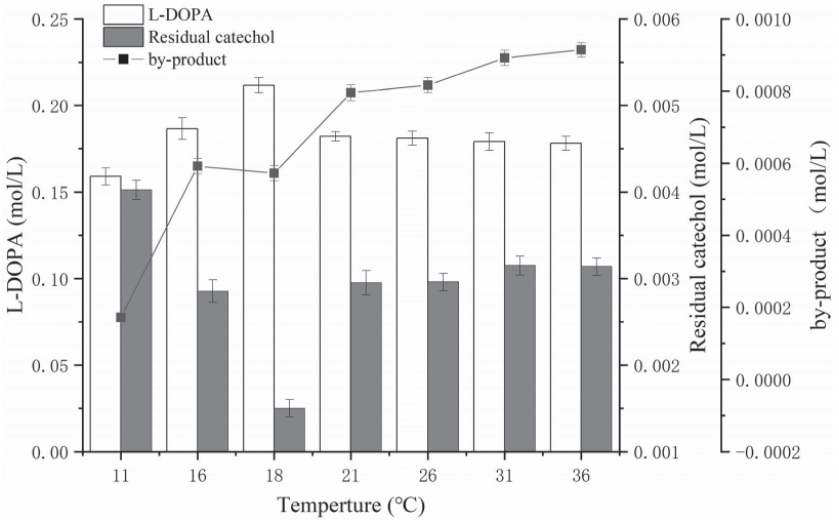


Figure 14. The effects of reaction temperature on L-DOPA synthesis.

ing the ratio of catechol to sodium pyruvate leads to an increase in sodium pyruvate consumption and an increase in L-DOPA production; when the ratio of catechol to sodium pyruvate reached 1:2, the yield of L-DOPA in the reaction solution was the largest, moreover the amount of by-products was the smallest. Further increasing the amount of sodium pyruvate, more by-products were produced by a spontaneous non-enzymatic reaction between excessive sodium pyruvate and L-DOPA, which resulted the rapid decrease of L-DOPA. So, the mole ratio of 1:2 between catechol and sodium pyruvate was determined as the best initial substrate proportion. Tang *et al.* [19] adopted 5 g/L catechol and 1:1.6 catechol to sodium pyruvate to produce L-DOPA, which was much lower than those in our studies. Thus the *Dh*-TPL constructed by us was more favorable for the synthesis of L-DOPA.

Effects of reaction pH on* l*-DOPA synthesis

The effects of pH on L-DOPA synthesis were investigated over a pH range from 7.0 to 9.0. As shown in Fig. 13, the maximum production of L-DOPA was observed at pH 8.5. When pH was lower than 8.5, the production of L-DOPA decreased significantly. It has been reported that the optimum pH of *Ki*-TPL (from *Kluyvera intermedia*), *Eh*-TPL (from *Erwinia herbicola*) and *Fn*-TPL (from *Fusobacterium nucleatum*) was 8.0 [6, 18, 19]. Meenakshi Chandel *et al*. [13] ever proposed that the optimal pH of *Cf*-TPL from *Citrobacter freundii* was 8.5. All the results indicated that weak alkaline environment was favorable for the synthesis of L-DOPA. However, too strong alkalinity can affect the activity of *Dh*-TPL, the production of L-DOPA was cut down. At the same time, the concentration of by-product was the lowest at pH 8.5. When the pH was increased from 8.5 to 9.0, or decreased from 8.5 to 7.0, the by-product concentration raised clearly. Hitoshi Enei *et al* [6] reported that the pH of the minimum by-products was 9, and Xiao-Ling Tang *et al*. [19] proposed that the pH of the minimum by-products was 8, respectively. Our results were essentially in agreement with the previous studies. From all above, it was recommended to perform the biosynthesis of L-DOPA at pH 8.5.

Effects of reaction temperature on* l*-DOPA synthesis

The substrate catechol and product L-DOPA were unstable at high temperatures. The influences of temperature during L-DOPA synthesis were investigated. The production of L-DOPA increased with the increase of temperature (Fig. 14). The maximum L-DOPA yield was obtained at 18°C and decreased at higher temperatures. In the process of L-DOPA biosynthesis catalyzed by *Dh*-TPL, it is proved that L-DOPA and pyruvate can form by-product through Pictet-Spengler reaction, which proved to be tetrahydroisoquinoline derivatives [6]. The formation of the by-product was affected by both temperature and pH [19]. As shown in Fig. 14, with the increase of temperature, the production of by-product was raised similarly. After 3 hours reaction, the amount of by-product at 36°C was the highest. In consideration of L-DOPA and by-product, the biosynthesis of L-DOPA should be carried out at 18°C. It has been recommended to perform the biosynthesis of L-DOPA at a temperature of 15 to 20°C [6], because high temperature promotes the formation of by-product. Wei Yuan *et al*. [18] employed 25°C to syn-

thetise L-DOPA with *Ki*-TPL (from *Kluyvera intermedia*), while Meenakshi Chandel *et al*. [13] prepare L-DOPA using *Cf*-TPL (from *Citrobacter freundii*) at 45°C. Compared with *Dh*-TPL, their reaction temperature was much higher and likely to produce more by-product. The synthesis of L-DOPA by *Dh*-TPL at the lower temperature (18°C) is very beneficial as low temperature greatly improves the stability of L-DOPA, reduces the generation of by-product and simplifies the subsequent purification process.

Under the optimal conditions (10.0 g/L catechol, 1:2 catechol to sodium pyruvate, pH 8.5 and 18°C), the yield of L-DOPA was 0.21 mol/L (41.7 g/L) after 3 hours reaction. Wei Yuan *et al.* [18] obtained 5.74 g/L/h L-DOPA by the wild-type YW000 (from *Kluyvera intermedia*, 25°C, fed batch process) and 7.52 g/L/h L-DOPA by mutant YW021 in 10 L fermenter (25°C, fed batch process), which was the highest level reported at present. In this study, the yield of L-DOPA obtained in the shake flask system was increased by 84.8% compared to the highest level reported so far, up to 13.9 g/L/h. In addition, the *TPL* gene is wild type. There may be a large space of improvement after amplifying the reaction scale and using the fed-batch mode or by genetic modification. These works are currently being studied in our lab. So, *Dh*-TPL may be a promising biocatalyst for the large-scale production of L-DOPA.

Conclusion

In summary, a new TPL from *D. hafniense* was constructed and its potential application for L-DOPA synthesis was explored. Types and composition of enzyme-producing media, the IPTG concentration and induction temperature both had an obviously influences on the enzyme activity, which provided important information for further study of *Dh*-TPL. The most suitable medium for the growth and expression of target proteins in recombinant *E. coli* was explored. The biosynthesis conditions of L-DOPA were discussed. The optimised enzyme-producing medium consists of yeast extract 64 g/L, tryptone 36 g/L, glycerol 4 g/L, KH_2PO_4 2.31 g/L and K_2HPO_4 9.4 g/L. The optimal reaction pH and temperature were 8.5 and 18°C, respectively. The best catechol concentration was 10.0 g/L and the mole ratio of catechol to sodium pyruvate was 1:2. Under these conditions, the yield of L-DOPA was 0.21 mol/L after 3 hours reaction, which was much higher than the highest level reported so far. However, the reactions were conducted in shaking flask reaction system, which may have a large space of improvement. Furthermore, the whole-cell catalysis exhibits many advantages including ease of operation, convenient downstream separation and low cost of industrialization. Consequently, the expression of *Dh-TPL* in *E. coli* provides another cheap and efficient biocatalyst for large-scale production of L-DOPA.

Conflict of Interest The authors declare that they have no conflict of interest.

References

1. Knowles William S. in Asymmetric Catalysis on Industrial Scale. (ed. W.S. Knowles) 21-38 (Wiley-VCH Verlag GmbH & Co. KGaA, Weinheim, FRG; 2003).
2. Sayyed Iliyas Ali,Sudalai Arumugam. Asymmetric synthesis of l-DOPA and (R)-selegiline via, OsO4-catalyzed asymmetric dihydroxylation. *Tetrahedron: Asymmetry*. 2004; 15 (19):3111-3116. doi: 10.1016/j.tetasy.2004.08.007
3. Valdés Ricardo Hernández, Puzer Luciano, Gomes Marlito, Marques Carlos E. S. J., Aranda Donato A. G., Bastos Marcelo L., Gemal André L.,Antunes O. A. C. Production of L-DOPA under heterogeneous asymmetric catalysis. *Catalysis Communications*. 2004; 5 (10):631-634. doi: 10.1016/j.catcom.2004.07.018
4. Lee Jang-Young,Xun Luying. Novel biological process for L-DOPA production from L-tyrosine by p-hydroxyphenylacetate 3-hydroxylase. *Biotechnology Letters*. 1998; 20 (5):479-482. doi: 10.1023/a:1005440229420
5. Agarwal Pragati, Pareek Nidhi, Dubey Swati, Singh Jyoti,Singh R. P. Aspergillus Niger PA2: A novel strain for extracellular biotransformation of l-tyrosine into l-DOPA. *Amino Acids*. 2016; 48 (5):1253-1262. doi: 10.1007/s00726-016-2174-7
6. Enei Hitoshi, Nakazawa Hidetsugu, Okumura Shinji,Yamada Hideaki. Synthesis of L-Tyrosine or 3, 4-Dihydroxyphenyl-L-alanine from pyruvic acid, ammonia and phenol or pyrocatechol. *Agricultural and Biological Chemistry*. 1973; 37 (4):725-735. doi: 10.1271/bbb1961.37.725
7. Li Tao,Li Xuan. Comprehensive mass analysis for chemical processes, a case study on L-Dopa manufacture. *Green Chemistry*. 2014; 16 (9):4241-4256. doi: 10.1039/c4gc00565a
8. Li Weiping, Wang Shuying, Yan Qun,Li Huazhong. Construction of tyrosine phenol lyase over-expression recombinant E. coli for the biosynthesis of levodopa. *Zhongguo Yiyao Gongye Zazhi*. 2005; 36 (12):743-746.
9. Min K., Park K., Park D. H.,Yoo Y. J. Overview on the biotechnological production of L-DOPA. *Appl Microbiol Biotechnol*. 2015; 99 (2):575-584. doi: 10.1007/s00253-014-6215-4
10. Zheng Ren Chao, Tang Xiao Ling, Suo Hui, Feng Li Lin, Liu Xiao, Yang Jian,Zheng Yu Guo. Biochemical char-

acterization of a novel tyrosine phenol-lyase from Fusobacterium nucleatum for highly efficient biosynthesis of L-DOPA. *Enzyme and Microbial Technology*. 2018; 112:88-93. doi: 10.1016/j.enzmictec.2017.11.004

11. Panja Subrata, Aich Pulakesh, Jana Bimal,Basu Tarakdas. How does plasmid DNA penetrate cell membranes in artificial transformation process of Escherichia coli? *Molecular Membrane Biology*. 2008; 25 (5):411-422. doi: 10.1080/09687680802187765
12. Malakar Pushkar,Venkatesh K. V. Effect of substrate and IPTG concentrations on the burden to growth of Escherichia coli on glycerol due to the expression of Lac proteins. *Applied Microbiology and Biotechnology*. 2012; 93 (6):2543-2549. doi: 10.1007/s00253-011-3642-3
13. Chandel M.,Azmi W. Purification and characterization of tyrosine phenol lyase from Citrobacter freundii. *Appl Biochem Biotechnol*. 2013; 171 (8):2040-2052. doi: 10.1007/s12010-013-0491-9
14. Kim D. Y., Rha E., Choi S. L., Song J. J., Hong S. P., Sung M. H.,Lee S. G. Development of bioreactor system for L-tyrosine synthesis using thermostable tyrosine phenol-lyase. *Journal of Microbiology and Biotechnology*. 2007; 17 (1):116-122. doi:
15. De Smet Marie Jose, Kingma Jaap,Witholt Bernard. The effect of toluene on the structure and permeability of the outer and cytoplasmic membranes of escherichia coli. *BBA - Biomembranes*. 1978; 506 (1):64-80. doi: 10.1016/0005-2736(78)90435-2
16. Han Hongmei, Zeng Weizhu, Du Guocheng, Chen Jian,Zhou Jingwen. Site-directed mutagenesis to improve the thermostability of tyrosine phenol-lyase. *Journal of Biotechnology*. 2020; 310:6-12. doi: 10.1016/j.jbiotec.2020.01.005
17. Zeng Weizhu, Xu Bingbing, Du Guocheng, Chen Jian,Zhou Jingwen. Integrating enzyme evolution and high-throughput screening for efficient biosynthesis of l-DOPA. *Journal of Industrial Microbiology and Biotechnology*. 2019; 46 (12):1631-1641. doi: 10.1007/s10295-019-02237-8
18. Yuan Wei, Zhong Shuang, Xiao Yanming, Wang Zhao,Sun Jie. Efficient biocatalyst of L-DOPA with Escherichia coli expressing a tyrosine phenol-lyase mutant from Kluyvera intermedia. *Applied Biochemistry and Biotechnology*. 2020; 190 (4):1187-1200. doi: 10.1007/s12010-019-03164-1
19. Tang Xiao Ling, Liu Xiao, Suo Hui, Wang Zhi Chao, Zheng Ren Chao,Zheng Yu Guo. Process development for efficient biosynthesis of l-DOPA with recombinant Escherichia coli harboring tyrosine phenol lyase from Fusobacterium nucleatum. *Bioprocess and Biosystems Engineering*. 2018; 41 (9):1347-1354. doi: 10.1007/s00449-018-1962-8