

Article

Efficient Production of Enantiopure D-Lysine from L-Lysine by a Two-Enzyme Cascade System

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Abstract: The microbial production of D-lysine has been of great interest as a medicinal raw material. Here, a two-step process for D-lysine production from L-lysine by the successive microbial racemization and asymmetric degradation with lysine racemase and decarboxylase was developed. The whole-cell activities of engineered *Escherichia coli* expressing racemases from the strains *Proteus mirabilis* (LYR) and *Lactobacillus paracasei* (AAR) were first investigated comparatively. When the strain BL21-LYR with higher racemization activity was employed, L-lysine was rapidly racemized to give DL-lysine, and the D-lysine yield was approximately 48% after 0.5 h. Next, L-lysine was selectively catabolized to generate cadaverine by lysine decarboxylase. The comparative analysis of the decarboxylation activities of resting whole cells, permeabilized cells, and crude enzyme revealed that the crude enzyme was the best biocatalyst for enantiopure D-lysine production. The reaction temperature, pH, metal ion additive, and pyridoxal 5'-phosphate content of this two-step production process were subsequently optimized. Under optimal conditions, 750.7 mmol/L D-lysine was finally obtained from 1710 mmol/L L-lysine after 1 h of racemization reaction and 0.5 h of decarboxylation reaction. D-lysine yield could reach 48.8% with enantiomeric excess (ee) \geq 99%.

Keywords: D-lysine; racemase; decarboxylase; two-enzyme cascade system

1. Introduction

D-Amino acids are being increasingly used as a starting raw material in the production of valuable pharmaceuticals [1]. Among them, D-lysine has been employed for the synthesis of luteinizing-hormone-releasing hormone analog, or as a drug carrier in the form of polylysine [2,3]. Nowadays, several chemical or biochemical synthesis methods for D-lysine synthesis have been described [3]. In a previous study, D-lysine was successfully prepared from L-lysine by chemical racemization and microbial asymmetric degradation [4]. However, the chemical racemization of amino acids to prepare DL-amino acids is a complex process and requires severe reaction conditions, such as high temperature, strong acid, or alkali [3,4]. Meanwhile, the chemical resolution of DL-amino acids is inefficient with low optical purity of products, and requires expensive chiral resolving agent. Therefore, an efficient bio-based process for the commercial production of D-lysine is highly desirable.

The enzymatic synthesis of D-amino acids can be performed by hydrolases, or D-amino acid aminotransferases with synthetic intermediates and prochiral substrates as starting materials [5]. Currently, industry has an overcapacity for L-lysine with an annual production of more than 2 million tons [6]. We therefore focused on the use of L-lysine as a raw material for D-lysine production. Considering the current preparation process, a two-step reaction containing racemization and asymmetric degradation of L-lysine was regarded as a simple and economical process to

synthesize D-lysine effectively (Figure 1). In the first step, amino acid racemase was employed to catalyze racemization of L-lysine to DL-lysine. Amino acid racemases, which are widely prevalent in many living organisms, are divided into two groups: pyridoxal 5'-phosphate (PLP)-dependent and PLP-independent enzymes [7]. However, lysine racemase, one of the PLP-dependent enzymes, has only been described in a small number of bacteria, such as *Proteus mirabilis* [8] and *Oenococcus oeni* [9]. The heterologous expression of lysine racemase for large-scale production of DL-lysine has rarely been reported.

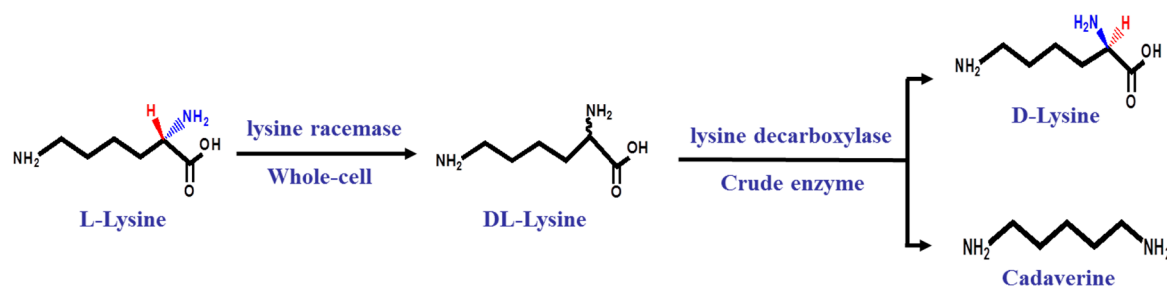


Figure 1. Scheme of enantiopure D-lysine production catalyzed by a two-enzyme cascade system of lysine racemase and lysine decarboxylase. The two enzymes were expressed in *Escherichia coli* (*E. coli*) respectively.

In order to obtain enantiopure D-lysine, chiral-selective degradation of L-lysine from the reaction mixture of DL-lysine is necessary. In previous reports, when DL-amino acids were employed as starting materials, D-amino acids, such as D-glutamate, D-arginine, or D-homoserine, have been synthesized by *N*-acyl-D-amino acid amidohydrolase, L-amino acid oxidase, or D-succinylase [10–12]. However, the related enzymes that are active with lysine were only found in few microorganisms, and their catalytic efficiency was too low to meet the demand of the high optical purity of D-lysine [13,14]. Therefore, it was vital to develop an efficient process that degraded L-lysine stereoselectively in the DL-lysine mixture to prepare the enantiopure D-lysine.

In our study, the two racemases from *Proteus mirabilis* (*P. mirabilis*) and *Lactobacillus paracasei* (*L. paracasei*), which have been reported to be active with L-lysine, were heterologously expressed in *Escherichia coli* (*E. coli*), and their activities to generate DL-lysine from L-lysine were compared. Subsequently, L-lysine was selectively catabolized to cadaverine by lysine decarboxylase, and the activities of resting whole cells, permeabilized cells, and crude enzyme were compared to identify the best biocatalyst for enantiopure D-lysine production. After defining the optimal conditions, an efficient bioconversion system for D-lysine production from L-lysine was described by racemization and asymmetric degradation with lysine racemase and decarboxylase, as shown in Figure 1.

2. Results

2.1. Construction of a Highly Efficient *E. coli* Whole-Cell Biocatalyst for DL-Lysine Production

In this study, amino racemases from *P. mirabilis* BCRC10725 (LYR) and *L. paracasei* (AAR), which have been reported capable of racemizing L-lysine to D-lysine, were selected [8,15]. The recombinant *E. coli* BL21 (DE3) harboring plasmid pET-28a-LYR or pET-28a-AAR was induced with IPTG (Figure 2a). The recombinant racemase expression was confirmed by SDS-PAGE analysis. It was found that protein LYR (45 kDa) and protein AAR (43 kDa) were all expressed in *E. coli* (Figure 2b), whose molecular weight was consistent with the prediction by gene sequencing. However, protein AAR was found to be highly insoluble in *E. coli*. Next, the specific activities of the two recombinant strains were compared. As shown in Figure 2c, the whole-cell BL21-LYR exhibited higher catalytic conversion activity of L-lysine to product D-lysine than BL21-AAR.

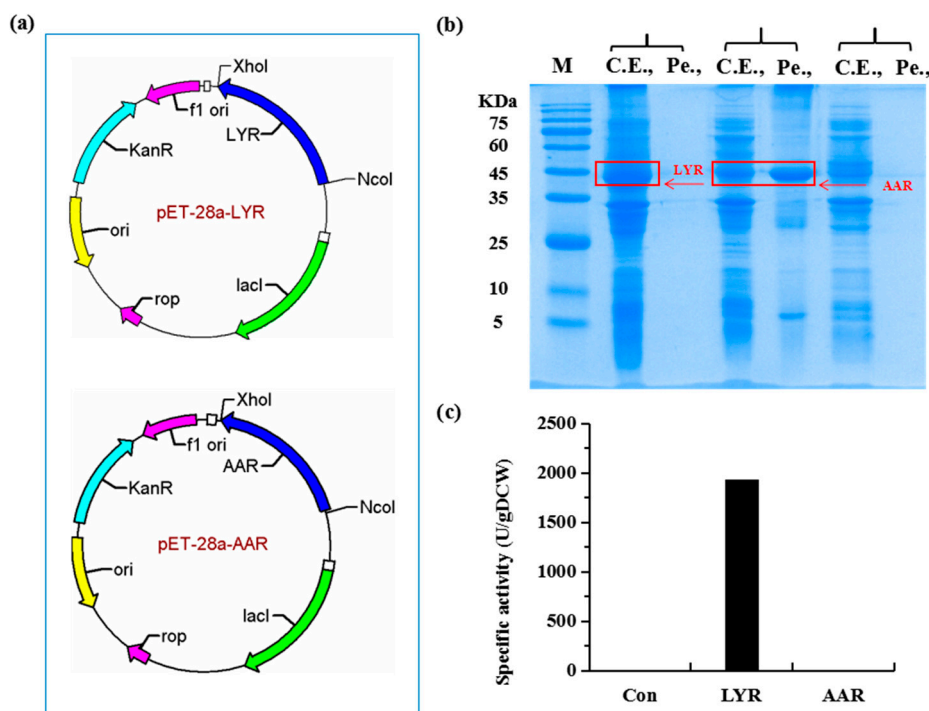


Figure 2. Comparison of specific activities of the whole cell BL21-LYR and BL21-AAR. (a) The racemases from *Proteus mirabilis* BCRC10725 (lysine racemase, LYR) and *Lactobacillus casei* (amino acid racemase, AAR) were cloned into plasmid pET-28a to generate plasmid pET-28a-LYR and pET-28a-AAR and transformed into *E. coli* BL21 (DE3) respectively; (b) Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) of cell extracts of *E. coli* BL21-LYR and BL21-AAR grown in Luria–Bertani (LB) medium at 30 °C. Equal amounts (20 µg) of protein were applied to each lane. Vertical lanes are labeled by M (protein size marker), C.E. (crude extract), or Pe. (pellet); (c) The specific activities of the whole cell BL21-LYR and BL21-AAR.

2.2. A Two-Step Process for Enantiopure D-Lysine Preparation

Using the whole-cell biocatalyst BL21-LYR, L-lysine was transformed to DL-lysine. Subsequently, to prepare the enantiopure D-lysine, it was necessary to selectively degrade L-lysine to a compound that could be easily separated from D-lysine. In our research, the recombinant *E. coli* ATS3, which has been engineered for the efficient bioconversion of lysine to cadaverine [16], was employed for the asymmetric degradation of L-lysine to cadaverine. As shown in Figure 3, L-lysine was rapidly racemized to generate DL-lysine with a D-lysine yield of 48% at 0.5 h. Next, whole cells of *E. coli* ATS3 (5.0 of OD₆₀₀) were added into the reaction mixture. Within 0.5 h, 85.3% of L-lysine (215 mmol/L) could be converted to cadaverine. However, 14.7% of L-lysine still remained in the reaction solution after 3 h. The enantiomeric excess of D-lysine was only 83%.

To improve the purity, we developed two other processes that permeabilized cells, and crude extracts of the strain ATS3 were used as the biocatalysts to compare their effects on D-lysine enantiomeric excess (Figure 3). Whole cells of AST3 (5.0 of OD₆₀₀) were treated with 0.5% Triton X-100 to obtain permeabilized cells, while the crude enzyme was prepared by sonic disruption. The permeabilized cells were found to exhibit higher activities than whole cells, and the enantiomeric excess of D-lysine reached 92.2% (Figure 3). Then, 0.2 g/L crude enzyme, which was equal to the protein amount in 5.0 of OD₆₀₀ of whole cells, was used to perform the asymmetric degradation experiment. As shown in Figure 3, L-lysine could be completely degraded after 0.5 h, and the enantiomeric excess of D-lysine reached 99.9% with a yield of 46.5%. The decarboxylation reactions were all performed with the addition of 1 mM PLP. Our results demonstrated that the crude enzyme was the best biocatalyst for enantiopure D-lysine production.

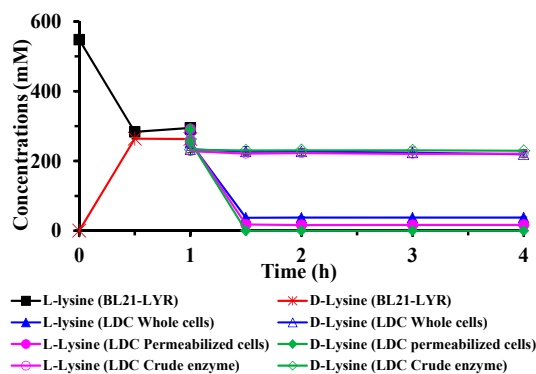


Figure 3. Comparison of the decarboxylation activities of resting whole cells, permeabilized cells, and cell extracts of *E. coli* AST3 expressing lysine decarboxylase. The whole-cell BL21-LYR was first employed to racemizing L-lysine to generate DL-lysine. Subsequently, L-lysine in the DL-lysine mixture was degraded by the biocatalysts of *E. coli* ATS3.

2.3. Optimization of Reaction Conditions for the Two-Step D-Lysine Production Process

2.3.1. Characterization of the Whole-Cell BL21-LYR

To improve the catalytic efficiency of the whole-cell BL21-LYR, it was essential to characterize the effect of the biocatalytic conditions on LYR activity. When the reaction pH ranged from 4.0 to 7.0, it was found that the LYR activity clearly increased with a rise in reaction pH, and reached a maximum at pH 7.0 (Figure 4a). At pH values over 7.0, the LYR activity decreased considerably. To determine the optimal reaction temperature, the bioconversion was carried out at 20, 25, 30, 37, 40, or 45 °C for 10 min. The optimum enzyme activity was exhibited at 37 °C (Figure 4b). Moreover, the addition of metal ions including Ca^{2+} , Co^{2+} , Fe^{2+} , Fe^{3+} , K^+ , Ni^{2+} , Mg^{2+} , Mn^{2+} , Cu^{2+} , and Zn^{2+} (1 mM) had no significant effect on LYR activity (Figure 4c). It has been reported that LYR is a PLP-dependent enzyme [17], and so the effect of PLP concentration was also examined. Our results showed that the addition of PLP could not further improve the specific activity of the whole-cell BL21-LYR (Figure 4d).

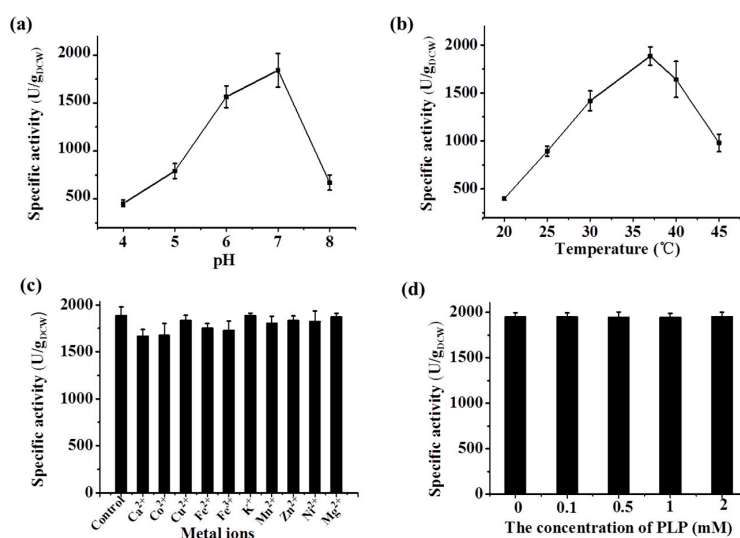


Figure 4. Characterization of the recombinant whole-cell BL21-LYR. (a) Optimal pH of the recombinant BL21-LYR; (b) optimal temperature of the recombinant BL21-LYR; (c) metal ion preference of the recombinant BL21-LYR; (d) the effect of PLP on the activity of the whole-cell BL21-LYR. Data are mean \pm SD for three replicates.

2.3.2. Characterization of the Recombinant Lysine Decarboxylase

The activity of the whole-cell ATS3 containing lysine decarboxylase has been characterized in a previous study [15]. Here, the effects of pH, temperature, metal ion additive, and PLP content on the crude enzyme of ATS3 were investigated. As shown in Figure 5a, the highest specific activity of lysine decarboxylase occurred at pH 6.0, similar to the lysine decarboxylase from *E. coli* MG1655 [18]. We found that lysine decarboxylase was sensitive to pH variance. From pH 5.0 to 7.0, more than 83% of the maximum activity could be retained (Figure 5a). However, enzyme stability decreased rapidly when pH was higher than 7.0. The specific activity sharply decreased to 8.0 U/mg at pH 7.5 (Figure 5a).

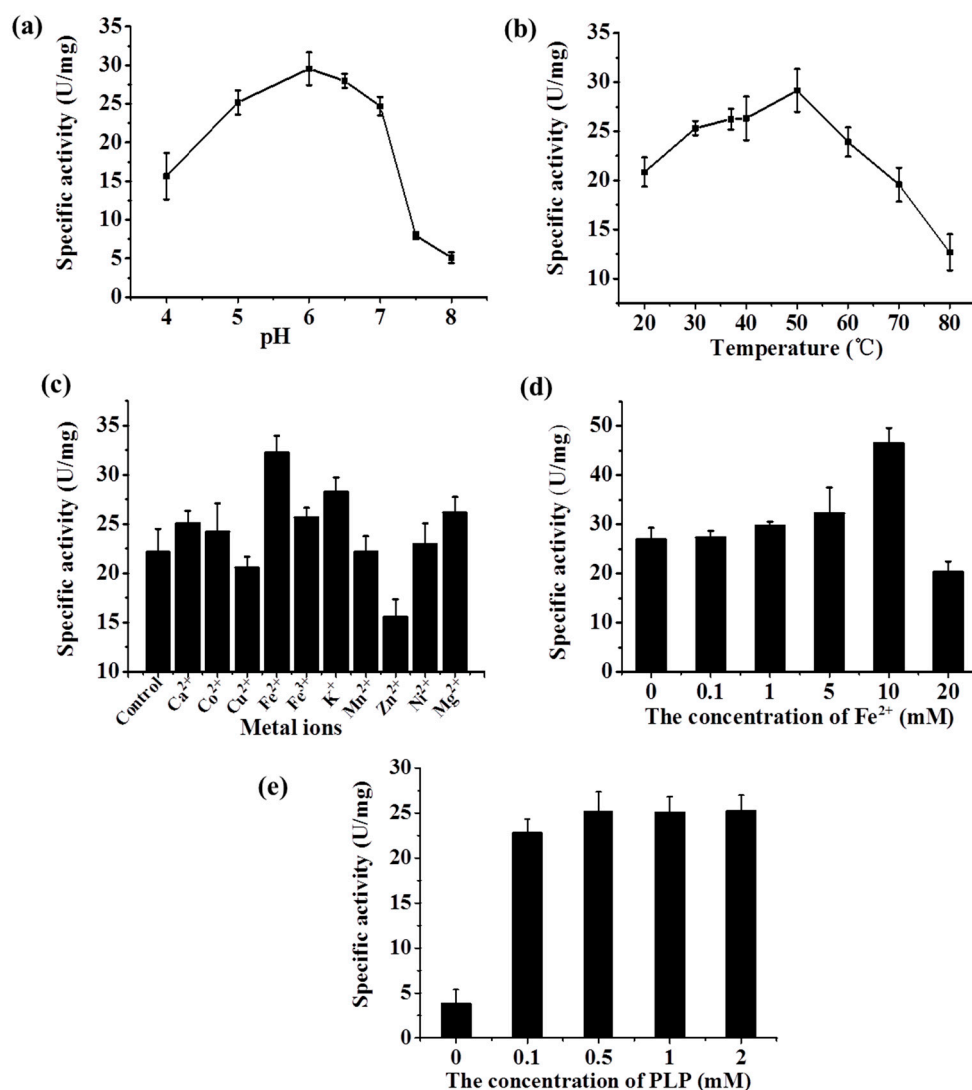


Figure 5. Characterization of the recombinant lysine decarboxylase. (a) Optimal pH of the recombinant lysine decarboxylase; (b) optimal temperature of the recombinant lysine decarboxylase; (c) metal ion preference of the recombinant decarboxylase; (d) optimization of the concentration of Fe²⁺; (e) optimization of the concentration of pyridoxal 5'-phosphate (PLP). Data are mean \pm SD for three replicates.

The effect of reaction temperature from 20 to 80 °C on the specific activity of lysine decarboxylase is shown in Figure 5b. The optimum activity was observed when the reaction was carried out at 50 °C. Metal ions (Ca²⁺, Co²⁺, Fe²⁺, Fe³⁺, K⁺, Ni²⁺, Mg²⁺, Mn²⁺, Cu²⁺, and Zn²⁺) (1 mM) were added into the reaction media. It was found that Fe²⁺ was the optimum metal ion additive. The metal ions Ca²⁺,

Co^{2+} , Fe^{2+} , Fe^{3+} , K^+ , Ni^{2+} , and Mg^{2+} also showed positive effects on lysine decarboxylase (Figure 5c). Subsequently, Fe^{2+} concentration in the reaction medium was further optimized. The highest enzyme activity was obtained at a concentration of 10 mM Fe^{2+} (Figure 5d), which was approximately 1.7-fold higher than that without the addition of Fe^{2+} .

Lysine decarboxylase is one of the PLP-dependent enzymes [5–7]. Therefore, the effect of PLP concentration in the reaction medium was examined. The specific activity of lysine decarboxylase was only 3.8 U/mg in the absence of PLP (Figure 5e). However, when 0.1 mM of PLP was added, the specific activity of lysine decarboxylase increased to 23.4 U/mg. With further increase of PLP concentration, only moderate increase was observed in enzyme activity. Taking into account the economic benefits, we determined 0.1 mM PLP to be the optimum concentration.

2.4. Determining the Optimal Condition of the Two-Step D-Lysine Production Process

To determine the optimal conditions of the two-enzyme cascade system, the impact factors on lysine racemase and lysine decarboxylase were compared. As has been described, the optimal pH for the whole-cell BL21-LYR was 7.0, while the specific activity of lysine decarboxylase was the highest at pH 6.0. Considering that lysine decarboxylase was more sensitive to pH variance, the two-enzyme cascade reaction was performed in potassium phosphate buffer with pH 6.0. At this pH, the racemase activity also remained at a high level. The two reactions were all carried out at around 37 °C, where the activities of the two enzymes both reached the optimum (Figures 4b and 5b). For the metal ions, in the first step, none were added into the reaction solution. After the end of the racemization reaction, 10 mM of Fe^{2+} was added to improve the catalytic rate during the lysine decarboxylation process. A supplement of 0.1 mM PLP was also added during the decarboxylation reaction step.

2.5. D-Lysine Production at Different Substrate Concentrations

In this study, L-lysine concentrations ranging from 680 to 1710 mmol/L in the bioconversion mixture were investigated. As illustrated in Figure 6, D-lysine productivity improved with the increasing L-lysine concentration. When the initial L-lysine concentration was 680 mmol/L (Figure 6a), the production rate of D-lysine was 9.6 mmol/L/min, whereas the D-lysine production rate increased to 22.6 mmol/L/min at an L-lysine concentration of 1710 mmol/L (Figure 6d). After bioconversion for 0.5 h, all D-lysine production from different concentrations of L-lysine had nearly reached the equilibrium. After about 1 h, cell extracts of *E. coli* ATS3 were added into the reaction mixture. It was observed that the L-lysine decarboxylation process could be completed within 0.5 h. The L-lysine degradation rate was increased with increasing concentration of the resting L-lysine. Our results indicated that an initial concentration of L-lysine ranging from 680 to 1710 mmol/L appeared to have no influence on either the racemization or decarboxylation processes. As shown in Table 1, a moderate improvement in D-lysine yield was observed with an increase in L-lysine concentration. At a concentration of 680 mmol/L L-lysine, the D-lysine titer was only 287.2 mmol/L, and the molar yield was 46.9%. When the initial L-lysine concentration was increased to 1710 mmol/L, 750.7 mmol/L of D-lysine was produced with a final yield of 48.8%. Meanwhile, 783.4 mmol/L cadaverine was obtained, and the total conversion yield of L-lysine was 99.7%. With the two-enzyme cascade bioconversion process we established, the enantiomeric excess of D-lysine was always higher than 99.0%.

Table 1. The production of D-lysine from different concentration of L-lysine.

L-Lysine (mM)	D-Lysine (mM)	Cadaverine (mM)	D-Lysine Yield	Enantiomeric Excess
680	287.2	323.5	46.9%	99.5%
1030	446.0	479	48.1%	99.5%
1370	601.0	628.5	48.8%	99.4%
1710	750.7	783.4	48.8%	99.3%

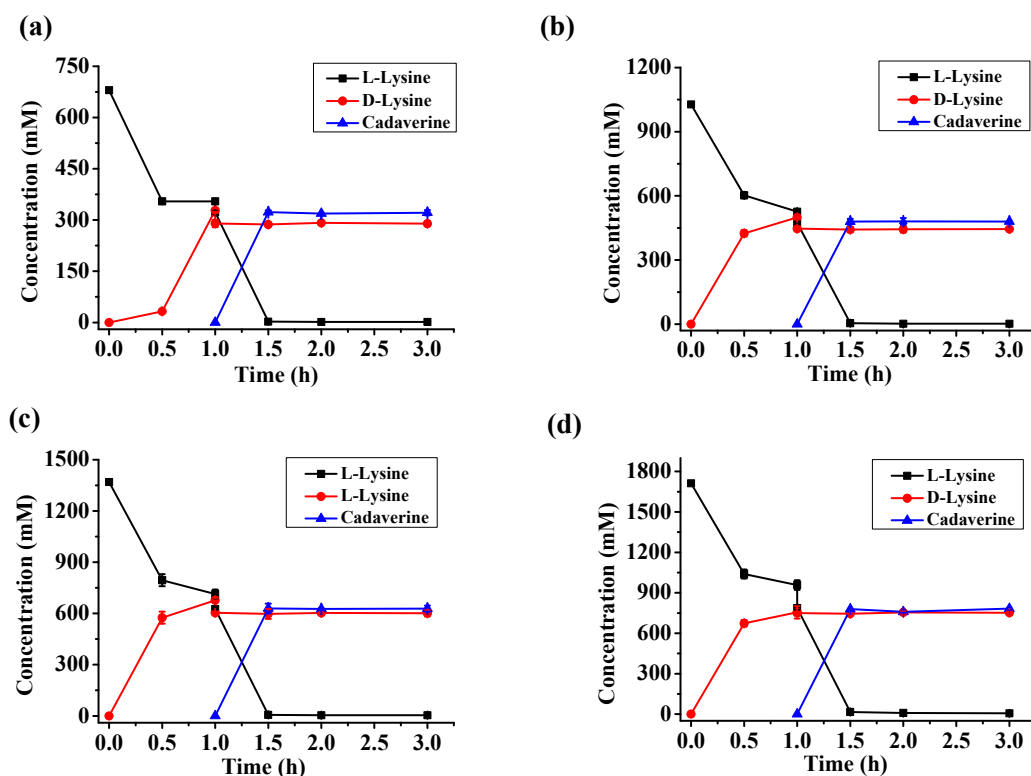


Figure 6. The production of D-lysine from different concentration of L-lysine. The initial L-lysine was 680 mmol/L (a); 1030 mmol/L (b); 1370 mmol/L (c); and 1710 mmol/L (d), respectively.

3. Discussion

D-Amino acids have been proved to be naturally present in many bacteria, plants, and animals [19–21], which exhibited widespread applications in industry, such as pharmaceuticals, food, and cosmetics [5]. For example, D-amino acids can be used to synthesize the peptides which are very potent in the inhibition of HIV [22]. Thus, an efficient process for the large-scale production of D-amino acids is highly desirable. As one of the important D-amino acids, the widespread application of D-lysine in industry has also been reported [2,3]. In our study, we established a two-step process for D-lysine production from L-lysine by the microbial racemization and asymmetric degradation with lysine racemase and decarboxylase.

In our work, L-lysine was used as the starting material and racemized to generate DL-lysine under the biocatalysis of amino acid racemases. Currently, a variety of amino acid racemases, such as those for alanine, glutamate, serine, aspartate, and arginine, have been discovered in bacteria, archaea, and eukaryotes [23–25]. However, only a handful of amino acid racemases that are active with lysine have been isolated. In our study, the racemases from strain *P. mirabilis* (LYR) and *L. paracasei* (AAR) were expressed in *E. coli*. The LYR protein was highly soluble in *E. coli*, and the whole-cell BL21-LYR showed higher lysine racemization activity with an approximate D-lysine yield of 48%. The biochemical characterization study revealed that the racemase activity of the whole-cell BL21-LYR was most active at 37 °C and pH 7.0, while the characterization of purified enzyme LYR in the previous study revealed that the optimal pH was between 8.0 and 9.0, and the optimum temperature was 50 °C [8]. The high temperature and pH might affect the survival of the whole cells. Therefore, the activity of whole cell showed a difference with the purified enzyme. Meanwhile, it was found that the addition of metal ions did not enhance the activity of BL21-LYR, indicating that the racemization activity of LYR was independent of metal ions, which was in accordance with the conclusion from the previous studies on the purified enzyme LYR [8].

After the racemization reaction, the microbial asymmetric degradation of L-lysine in the DL-lysine mixture solution was performed to generate the enantiopure D-lysine. L-Lysine decarboxylase is one of the PLP fold type I enzymes, which selectively catalyze the decarboxylation of L-lysine to generate cadaverine [15,18]. The engineered *E. coli* ATS3 used in our previous study was constructed by overexpressing endogenous *cadA* gene encoding lysine decarboxylase [15,18]. Meanwhile, the ribose 5-phosphate-dependent pathway genes *pdxS* and *pdxT* from *Bacillus subtilis* were introduced into the engineered *E. coli* for de novo PLP biosynthesis [15]. As shown in Figure 3, the crude enzyme of ATS3 exhibited the highest L-lysine decarboxylation activities, and was identified as the best biocatalyst. We also determined that the lysine decarboxylase we used in this study was specific for L-lysine (Figure S1). Furthermore, the characterization of lysine decarboxylase revealed that decarboxylation activity was dependent on Fe^{2+} ion and PLP additive. Bagni et al. also reported that lysine decarboxylase needs Fe^{2+} as a cofactor [26]. However, the activation mechanism of Fe^{2+} on decarboxylase was still unclear. For the PLP-dependent decarboxylation, it referred to the transfer of the proton during the elimination of the CO_2 [27]. Fe^{2+} has been reported to be a good metal as redox center, which might be associated with its role in the activation of lysine decarboxylase [28].

According to the characteristics of lysine racemase and lysine decarboxylase, a two-step process for efficient D-lysine production from L-lysine was established through a two-enzyme cascade system. When L-lysine at different concentrations was used as the starting material, D-lysine with enantiomeric excess $\geq 99\%$ could always be synthesized efficiently. For example, after 1 h of racemization reaction and 0.5 h of decarboxylation reaction, 750.7 mmol/L D-lysine could be obtained from 1710 mmol/L L-lysine with a yield of 48.8%. Meanwhile, with the increase of L-lysine, the production rate of D-lysine was increased, which suggested that no substrate inhibition on the activity of whole-cell BL21-LYR was observed when the initial L-lysine concentration ranges from 680 to 1710 mmol/L. The synthesis of cadaverine was also desirable for D-lysine separation due to their large difference in the polarity. In conclusion, the two-enzyme cascade for D-lysine production described in our work shows promise for the large-scale synthesis of D-amino acids.

4. Materials and Methods

4.1. Chemicals and Enzymes

Isopropyl- β -D-1-thiogalactopyranoside (IPTG), L-lysine (>98%), and D-lysine (>98%), were purchased from Sigma Aldrich (St. Louis, MO, USA). The restriction enzymes, T4 DNA ligase and agarose gel DNA purification kit were supplied by TaKaRa Biotechnology (Otsu, Japan).

4.2. Bacterial Strains and Growth Conditions

The *Escherichia coli* Tans1-T1 was purchased from TransGen (Beijing, China) and used for gene cloning. The *E. coli* BL21 (DE3; TransGen) and pET-28a vector (Novagen, San Diego, CA, USA) were employed as a host for gene expression. The engineered strain *E. coli* AST3 with lysine decarboxylase (EC 4.1.1.18) activity was preserved in our laboratory [15]. The *E. coli* strains were routinely cultured in modified Luria–Bertani (LB) medium (10 g/L tryptone, 5 g/L yeast extract and 10 g/L sodium chloride) containing 50 mg/mL kanamycin (Kan).

4.3. Construction of Plasmids

The lysine racemase (LYR, EC 5.1.1.5) from strain *P. mirabilis* BCRC10725 and the amino acid racemase (AAR, EC 5.1.1.10) from strain *L. paracasei* ATCC 334 were reported to be capable of racemizing lysine [8,16]. The two genes were codon-optimized for *E. coli* expression system [29], and synthesized by Genewiz Company (Jiangsu, China). The two gene fragments were cloned into NcoI/XhoI sites of plasmid pET-28a to generate the plasmid pET-28a-LYR and pET-28a-AAR, respectively (Figure 2a). The two recombinant plasmids were then transformed into *E. coli* BL21

(DE3) competent cell to obtain the recombinant strain BL21/pET-28a-LYR, and BL21/pET-28a-AAR. *E. coli* BL21 (DE3) harboring empty plasmid pET-28a was used as the control.

4.4. Expression of Recombinant Proteins in *E. coli* BL21(DE3)

The recombinant strain BL21/pET-28a-LYR, and BL21/pET-28a-AAR were inoculated from a freshly transformed single colony to 10 mL of LB medium. After cultivation for 8 h at 37 °C, the recombinant strain BL21/pET-28a-LYR and BL21/pET-28a-AAR were then seeded into 100 mL of LB medium with 50 mg/L kanamycin at an inoculation volume of 1%. Cell densities were monitored by measuring the optical density at 600 nm (OD_{600}). Upon reaching OD_{600} of 0.6–0.8, cells were induced by IPTG addition at 1.0 mM. After incubation for 12 h at 30 °C, cells were harvested by centrifugation ($6000\times g$, 5 min, 4 °C), and resuspended in 200 mM potassium phosphate buffer (pH 6.0). The recombinant strain AST3 was then seeded into 100 mL of culture medium [15] with 100 mg/L ampicillin and 34 mg/L chloramphenicol at an inoculation volume of 2%. After incubation for 12 h at 37 °C, cells were harvested by centrifugation ($6000\times g$, 5 min, 4 °C), and resuspended in 200 mM potassium phosphate buffer (pH 6.0). To prepare the crude enzyme, the cell paste was sonicated for 15 min (2 s worktime with 2 s interval). Cell extracts were then centrifuged for 15 min at $10,000\times g$ to remove cell debris. The supernatant was analyzed using sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) analysis (12% acrylamide).

4.5. Racemase and Decarboxylase Activity Characterization

In the present study, one unit of racemase activity was defined as the amount of dry cell weight (DCW) that produced 1 μ M of amino acid enantiomer from its corresponding enantiomer per minute. The impact factors associated with racemase activity were investigated in a reaction solution containing resting cells ($OD_{600} = 5$), 820 mmol/L L-lysine, and 200 mM sodium phosphate buffer (pH = 7.0) in a total volume of 10 mL. The optimal pH for racemase activity was confirmed in the 200 mM sodium phosphate buffer pH 4.0–8.0. The effect of temperature on racemase activity was determined by measuring the enzyme activity between 20 and 45 °C. To determine the metal ions preference of racemase, the effects of K^+ , Fe^{2+} , Fe^{3+} , Mg^{2+} , Mn^{2+} , Cu^{2+} , Zn^{2+} , Ca^{2+} , Co^{2+} , and Ni^{2+} additives were investigated.

The lysine decarboxylase assay was carried out in a 50 mL centrifuge tube with 5 mL reaction broth containing 270 mmol/L L-lysine, 200 mmol/L sodium phosphate buffer (pH = 7.0), 1.0 mmol/L PLP, and 0.2 g/L crude enzyme. One unit of lysine decarboxylase activity was defined as the amount of enzyme that transformed 1 μ M of L-lysine to cadaverine per minute. For pH optimization, the reaction was performed in 200 mmol/L sodium phosphate buffer (pH 4.0–8.0). To optimize temperature, the decarboxylase activity was measured in the condition with temperatures varying between 20 and 45 °C. To determine the effect of metal ions on enzyme activity, we measured decarboxylase activity in the reaction broth containing K^+ , Fe^{2+} , Fe^{3+} , Mg^{2+} , Mn^{2+} , Cu^{2+} , Zn^{2+} , Ca^{2+} , Co^{2+} , and Ni^{2+} . The PLP content in the reaction mixture was also optimized.

4.6. The Microbial Production of Enantiopure D-Lysine and Cadaverine

In this study, L-lysine was first biologically racemized to give DL-lysine under the biocatalysis of lysine racemase. The recombinant strain BL21/pET-28a-LYR was resuspended into 20 mL of reaction mixture containing 550 mmol/L, 680 mmol/L, 1030 mmol/L, 1370 mmol/L, or 1710 mmol/L L-lysine and 200 mmol/L sodium phosphate buffer (pH 6.0) with an OD_{600} of 5. The whole-cell biocatalyst was carried out in a 50 mL flask at 37 °C. Samples were taken at the specific intervals to measure the concentration of L-lysine and D-lysine.

When the reaction reached the equilibrium, the BL21/pET-28a-LYR cells were removed by centrifugation. The supernatant was maintained at 80 °C for 10 min. Then the whole cell (5.0 of OD_{600}) or crude enzyme (0.2 g/L) of the strain AT33 expressing L-lysine decarboxylase was added into the supernatant to selectively catabolize L-lysine to generate cadaverine and CO_2 . The reaction mixture

also contained 200 mmol/L sodium phosphate buffer (pH 6.0), 10 mmol/L Fe^{2+} , and 0.1 mmol/L PLP. To identify the best biocatalyst for the enantiopure D-lysine production, the activity of resting whole cells, permeabilized cells, and crude enzyme was comparatively investigated under the condition of 550 mmol/L L-lysine.

4.7. Analysis Methods

The D-lysine and L-lysine in the reaction mixture were analyzed via a high-performance liquid chromatography (HPLC) system (Agilent 1290 series; Agilent, Palo Alto, CA, USA) equipped with a UV-vis detector (wavelength = 254 nm) and a chirex chiral column (Chirex 3126 (D)-penicillamine 150 mm \times 4.6 mm with a pre-column 30 mm \times 4.6 mm, 5 μm , Waters, Milford, MA, USA). The column was maintained at 25 $^{\circ}\text{C}$. One millimolar $\text{CuSO}_4 \cdot 5 \text{H}_2\text{O}$ dissolved into water/isopropanol (95:5, *v/v*) with the flow rate of 0.8 mL/min was used as the mobile phase.

The cadaverine concentrations were determined by reverse-phase high performance liquid chromatography (HPLC) using an Agilent 1290 Infinity System equipped with a fluorescence detector (FLD, G1321B, Agilent, Palo Alto, CA, USA). The cadaverine concentration was determined by precolumn dansyl chloride derivatization following a previously described procedure [16].

5. Conclusions

In the current work, we have developed a two-step microbial process for high-level conversion of L-lysine to D-lysine through a lysine racemase and decarboxylase cascade system. The recombinant strain BL21-LYR, used as the biocatalyst of a racemization reaction to generate DL-lysine, was constructed by expressing lysine racemase from the strain *P. mirabilis* (LYR). Subsequently, lysine decarboxylase was employed for the asymmetric degradation of L-lysine to generate cadaverine. To produce D-lysine with high enantiomeric excess, the decarboxylation activities of resting whole cells, permeabilized cells, and crude enzyme were compared, and the crude enzyme was identified as the best biocatalyst. After characterization of the lysine racemase and decarboxylase, the two-step reaction was performed under optimal condition, and 750.7 mmol/L D-lysine could be obtained from 1710 mmol/L L-lysine. The enantiomeric excess of D-lysine was higher than 99%.

Supplementary Materials: The following are available online at www.mdpi.com/2073-4344/6/11/168/s1, Figure S1: Determining the substrate specificity of the lysine decarboxylase.

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