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Article

The production of enantiopure D-lysine from L-lysine by a two-strain coupled system

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ABSTRACT

The microbial production of D-lysine to replace chemical approach has gained great interest with the rising concerns over the environment. Here, we employed recombinant *E. coli* strain BL21-LYR with lysine racemase and strain BL-22A-RB-YB with L-lysine monooxygenase and 5-aminovaleramide amidohydrolase to establish a two-strain coupling whole-cell bioconversion system for D-lysine production from L-lysine. To improve the optical purity of D-lysine, the optimal reaction condition for resolution of DL-lysine after the racemization was investigated. The specificity of BL-22A-RB-YB for L-lysine and the effects of reaction condition on bioconversion efficiency of whole-cell were accordingly determined. Under the optimal condition, a maximum 53.5 $g \cdot L^{-1}$ D-lysine and 48.2 $g \cdot L^{-1}$ 5-AVA were obtained with yield of 47.4% and 42.3%, respectively, by the microbial racemization and asymmetric degradation process. The final D-lysine enantiomeric excess was over 99%. Meanwhile, a valuable compound 5-aminovaleric acid was synthesized with the production of D-lysine, indicating the economic feasibility of the two-strain coupling system.

1. Introduction

In the past few years, D-lysine has garnered great attention due to its wide application in the production of valuable pharmaceuticals, such as luteinizing-hormone-releasing hormone analog, or as a drug carrier in the form of poly-lysine [1–3]. Approaches using chemical process to produce D-lysine have achieved initial success [4]. However, the preparation of D-amino acids by chemical racemization is usually carried out under severe experimental conditions, such as strong acid base or high temperature [2,4]. At the same time, chemical resolution of DL-amino acids has a series of problems, such as low resolution efficiency, low purity, high chiral resolution agent and environmental pollution [5]. Liu reported that used chemical racemic coupling enzymes catalyze to synthesis of D-lysine, which greatly improved the efficiency of chiral resolution [4]. However, the disadvantages of chemical racemization cannot be avoided in this method. Therefore, it was desirable to develop an effective method that completely relies on biotechnology to produce D-lysine.

In the previous study, a two-step biological process has been developed for D-lysine production by using L-lysine as raw material, where L-lysine was first racemized to give DL-lysine by the whole-cells expressing lysine racemase, and then L-lysine in the DL-lysine mixture was asymmetrically degraded to generate cadaverine by the crude enzyme solution of L-lysine decarboxylase [6]. D-Lysine yield could reach 48.8% with enantiomeric excess (e.e.) ≥99%. However, the preparation of crude enzymes would increase the cost. Thus, the development of an economic resolution process for enantiopure D-lysine production was highly desired.

Microbial enzyme resolution has been widely used in the production of chiral compound. The advantages of this method are the high catalytic efficiency and specificity, the high optical purity of product, less by-products, and the product easier to separate purification. For example, with the strain *Aspergillus fumigatus* containing L-amino acid oxidase, it could cause the resolution of the three DL-amino acids resulting in the production of optically pure D-alanine (100% resolution), D-phenylalanine (80.2%), and D-tyrosine (84.1%) [7]. L-Lysine-α-oxidase was a flavin-containing enzyme from the wheat fungus Trichoderma viride, which catalyzes the oxidation of L-lysine followed by spontaneous oxidative decarboxylation of the intermediate 6-amino-2-oxocaproic acid to form 5-aminovaleric acid (5-AVA) [8]. The 5-AVA is the precursor of glutarate, an important C5 building block for producing nylon-4,5 (with putrescine), nylon-5,5 (with cadaverine) and other polymers [9–11]. However, the catalytic efficiency of L-lysine-α-oxidase was too low to be used for the resolution of DL-lysine and production of 5-AVA, at the high concentration of L-lysine.

In our previous study, a whole-cell biocatalysis process for high-level conversion of L-lysine into 5-AVA was reported in recombinant *Escherichia coli* overexpressing 5-aminovaleramide amidohydrolase (DavA) and L-lysine 2-monooxygenase (DavB) [12]. In this work, after the racemization of L-lysine to generate DL-lysine, the whole cells of 5-AVA producing *E. coli* were employed to investigate its resolution

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activity for racemic mixtures of DL-lysine. After characterizing the optimal conditions, a two-strain coupling whole-cell system for enantiopure D-lysine production from L-lysine was developed by the microbial racemization and asymmetric degradation.

2. Materials and Methods

2.1. Microorganisms and media

The *Escherichia coli* Tans1-T1 and *E. coli* BL21 (DE3) were purchased from TransGen (Beijing, China) and used for gene cloning. The engineered strain *E. coli* BL-22A-RB-YB (the strain could conversion of L-lysine into 5-AVA by overexpressing DavA and DavB) [12] and *E. coli* BL21-LYR (the strain could racemized L-lysine to DL-lysine by overexpressing lysine racemase) were constructed in our laboratory [6]. The strains were routinely cultured in Luria–Bertani (LB) medium and appropriate antibiotics were added at the following concentrations: 100 $mg \cdot L^{-1}$ ampicillin (Amp), 35 $mg \cdot L^{-1}$ chloramphenicol (Cm), and 50 $mg \cdot L^{-1}$ kanamycin (Kan).

2.2. Whole-cell biocatalyst-based bioconversion for 5-AVA production

Pre-culture conditions for the *E. coli* strain were as follows: a single colony was picked from a plate and grown in LB medium. After overnight growth, the seed broth was transferred to 100 ml LB with the 1% of the inoculation amount. Upon reaching an OD600nm of 0.6–0.8, cells were induced by 0.5 $mmol \cdot L^{-1}$ IPTG. After 12 h, 20 °C of incubation, the cells were harvested by centrifugation.

The cells of strain BL21-LYR (OD600 = 5) were re-suspended in 20 ml potassium phosphate buffer solution of 0.2 $mol \cdot L^{-1}$ with a certain concentration of L-lysine [6]. After the reaction at 37 °C for 1 h, the cell pellets of BL21-LYR were removed by centrifugation. Once 1 ml potassium phosphate buffer with cell of BL-22A-RB-YB was mixed with the 19 ml supernatant of the above reaction, the resolution of reaction was started. The effects of reaction temperature, pH, metal ion and surfactants were investigated.

2.3. 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 × 4.6 mm with a pre-column 30 mm × 4.6 mm, 5 μm, Waters, Milford, MA, USA). The column was maintained at 25 °C. One millimolar $CuSO_4 \cdot 5H_2O$ dissolved into water/isopropanol (95:5, v/v) with the flow rate of 0.8 $ml \cdot min^{-1}$ was used as the mobile phase.

5-AVA concentration was analyzed by a HPLC system (Agilent 1100 series, Santa Clara, CA) equipped with an evaporative light scattering detector (ELSD) and a Prevail C18 column (250 × 4.6 mm, 5 μm, Bio-Rad, Hercules, CA, USA). The column temperature was maintained at 28.5 °C. 0.7% (v/v), trifluoroacetic acid aqueous solution was used as the mobile phase at a flow rate of 1.0 $ml \cdot min^{-1}$.

3. Results

3.1. The racemic resolution of DL-lysine by whole cells expressing DavB and DavA

In this work, we attempt to establish a two-strain coupling system for D-lysine production from L-lysine by the microbial racemization and asymmetric degradation with lysine racemase, DavB and DavA as shown in Fig. 1. As the previous study described [6], DL-lysine has been successfully prepared from L-lysine by the whole cells of recombinant *E. coli* expressing L-lysine racemase. However, whether the DavB and DavA could selectively catalyze L-lysine to generate 5-AVA in the mixture of DL-lysine has never been investigated. To identify its feasibility, experiment on specificity of DavB was subsequently performed with the addition of 10 $g \cdot L^{-1}$ L-lysine and 10 $g \cdot L^{-1}$ D-lysine respectively with whole cells of BL-22A-RB-YB. As shown in Fig. 2, the concentration of L-lysine gradually consumed in the reaction mixture and L-lysine was almost completely consumed within 6 h. However, D-lysine concentration had no changes within the same time. Therefore, lysine monooxygenase is a kind of enzyme with specificity for L-lysine.

3.2. Optimal substrate concentration of whole-cell transformation

It is well-known that substrate availability contributes handsomely to the whole-cell biocatalysts [13]. In general, the rate of enzyme reaction would increase as the substrate concentration increased and when the substrate concentration reaches a certain limit, the reaction rate would be stable. But, for some enzyme, when the substrate concentration is too high, the reaction rate would decrease due to the substrate inhibition [14]. Thus, the effect of substrate concentration on 5-AVA production was investigated. As shown in Fig. 3, the productivity of 5-AVA was increased when the substrate concentration was increased from 10 $g \cdot L^{-1}$ to 30 $g \cdot L^{-1}$. However, substrate inhibition was observed when the substrate concentration was higher than 40 $g \cdot L^{-1}$, and the productivity of 5-AVA decreased dramatically. Therefore, when whole cells of BL-22A-RB-YB with OD600 = 10 employed, the optimal substrate concentration was 30 $g \cdot L^{-1}$ and the productivity of 5-AVA was 2.53 $g \cdot L^{-1} \cdot h^{-1}$.

3.3. The effects of reaction temperature and pH on the whole-cell bioconversion efficiency of BL-22A-RB-YB

It is necessary for optimal reaction conditions to improve biocatalytic efficiency. The reaction conditions including cultivation temperature and pH were optimized to study their effects on 5-AVA production and the yield. As shown in Fig. 4(a), the optimal temperature for the 5-AVA was from 37 °C to 40 °C following reaction at 20 °C, 30 °C, 37 °C, 40 °C, or 50 °C as the production of 5-AVA was increased with the increase in temperature between 20 °C and 37 °C. However, when the temperature was over 40 °C, the yield of 5-AVA was obviously decreased.

As shown in Fig. 4(b), a negative effect on the catalytic efficiency was observed under the conditions of strong acid or strong alkaline. When pH was 4.0, the production of 5-AVA was 8.3 $g \cdot L^{-1}$ with a molar yield of 44.3%. The production was increased with the increasing pH and reached the maximum at pH of 7.0, where 18.8 $g \cdot L^{-1}$ 5-AVA was produced with a yield of 97.7%. However, the production of 5-AVA was only 16.7 $g \cdot L^{-1}$, 0.89-fold of the highest production when the pH was 7.5. These results indicated that the optimal pH for the whole-cell bioconversion of strain BL-22A-RB-YB was 7.0.

3.4. Effect of metal ions and surfactant on the whole-cell bioconversion efficiency of BL-22A-RB-YB

The metal ions have been reported to have an effect on the stabilization of the enzyme, and were able to generate the catalytic center of the enzyme [15]. The effects of different metal ions at a concentration of 1 $mmol \cdot L^{-1}$ on whole-cell reaction in strain BL-22A-RB-YB are shown in Fig. 5(a). As shown in Fig. 5, Ca^{2+}, and Mn^{2+} played a positive effect on the whole cells of strain BL-22A-RB-YB, and other metal ions all had negative effects on the 5-AVA production. Among them, when 1 $mmol \cdot L^{-1}$ of Ca^{2+} was added, the production of 5-AVA was 18.9 $g \cdot L^{-1}$ with a yield of 97%. The addition of the same concentration of Mn^{2+} improved 5-AVA production to 19.1 $g \cdot L^{-1}$ and a yield of 98.2% was obtained. Based on the above the results, the optimal metal ions

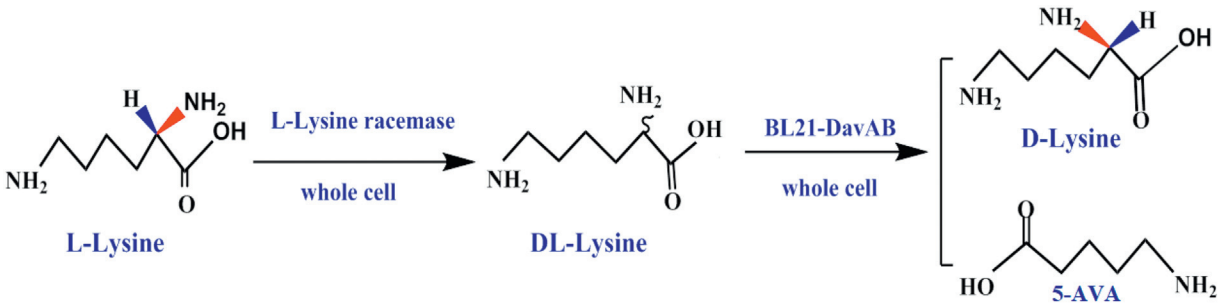

Fig. 1. The pathway of synthesizing D-lysine from L-lysine catalyzed by two-strain coupling whole-cell system.

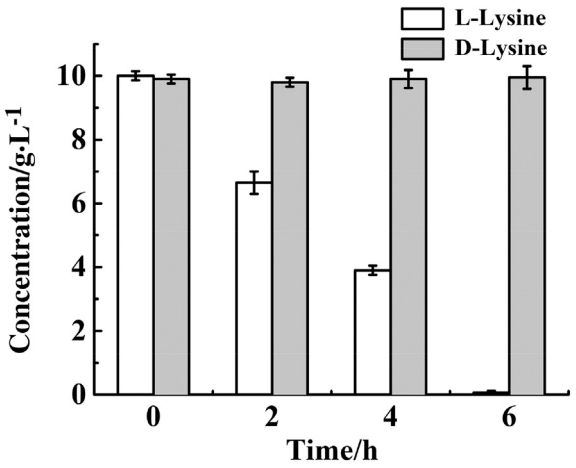

Fig. 2. The identification of the specificity of lysine monooxygenase.

were Mn^{2+} in improving the whole-cell catalytic efficiency for strain BL-22A-RB-YB.

Surfactant is a kind of metabolites with hydrophilic and lipophilic group. In the whole-cell bioconversion system, the addition of surfactant could increase the permeability of cell membrane to improve the transport efficiency of the substrates [15,16]. As shown in Fig. 5(b), the addition of 0.5 $g \cdot L^{-1}$ SDS had no obvious influence on the 5-AVA production. However, when other surfactants including X-100, Tween-80 and betaine were added, the 5-AVA production was all improved. Especially, the addition of 0.5 $g \cdot L^{-1}$ betaine resulted in the highest 5-AVA production, which was increased to 9.82 $g \cdot L^{-1}$ with a molar yield of 98.9%. Thus, the surfactant betaine was added for the resolution process in the following experiment.

3.5. The production of enantiopure D-lysine with different concentrations of L-lysine

After optimizing the bioconversion condition of the whole-cell BL-22A-RB-YB to improve the resolution efficiency of DL-lysine, we attempted to identify the feasibility of synthesizing enantiopure D-lysine by using the two-strain coupling system. The L-lysine concentration ranging from 60 $g \cdot L^{-1}$ and 120 $g \cdot L^{-1}$ was performed to explore their influences on D-lysine optical purity. As shown the results in Fig. 6(a), when the initial L-lysine concentration was 60 $g \cdot L^{-1}$, the production rate of D-lysine was 2.4 $g \cdot L^{-1} \cdot min^{-1}$. With the increasing L-lysine concentration, D-lysine production rate was gradually increased (Fig. 6). When the concentration of L-lysine was 120 $g \cdot L^{-1}$, D-lysine production rate reached 3.8 $g \cdot L^{-1} \cdot min^{-1}$ (Fig. 6(d)). Meanwhile, the titer of D-lysine and 5-AVA was also gradually increased with the increasing substrate concentration. At the condition of 120 $g \cdot L^{-1}$ L-lysine, 53.5 $g \cdot L^{-1}$ D-lysine was obtained with a yield of 47.6%, and 5-AVA titer was accumulated to 48.2 $g \cdot L^{-1}$. More importantly, enantiomeric excess (e.e.) was all over 99% at the different substrate concentration (Table 1), indicating the high efficiency of whole cells of BL-22A-RB-YB in asymmetric degradation of L-lysine. Based on these results, a two-strain coupling whole-cell system for enantiopure D-lysine production from L-lysine was established.

4. Discussion

In the recent years, the bio-based production of chemicals has attracted much interest in replacement of traditionally chemical approach with the increasing concerns about environmental problems and fossil fuel resource availability [17]. D-lysine was one of the important D-amino acids, and was widely used in pharmaceuticals industry [18]. In our study, we employed two recombinant *E. coli* strains expressing lysine racemase, and DavB and DavA respectively for the microbial racemization and asymmetric degradation of L-lysine to obtain the enantiopure D-lysine. As the optimum racemization condition of recombinant lysine racemase has been determined in our previous study [12], the resolution process catalyzed by the whole cells of BL-22A-RB-YB was focused on to investigate the optimal reaction condition for asymmetric degradation of L-lysine in DL-lysine mixture to generate the enantiopure D-lysine.

DavB could catalyze the oxygenative decarboxylation of L-lysine and produces 5-aminovaleramide, which was then converted to give 5-AVA by DavA [19]. The whole-cell BL-22A-RB-YB with the expression of DavB and DavA was constructed for the efficient production of 5-AVA with L-lysine as a starting material in our previous study. To identify its feasibility in the resolution of DL-lysine, specificity of DavB for L-lysine was first determined. Furthermore, the optimum reaction pH and temperature for microbial resolution process by whole cells of BL-22A-RB-YB were

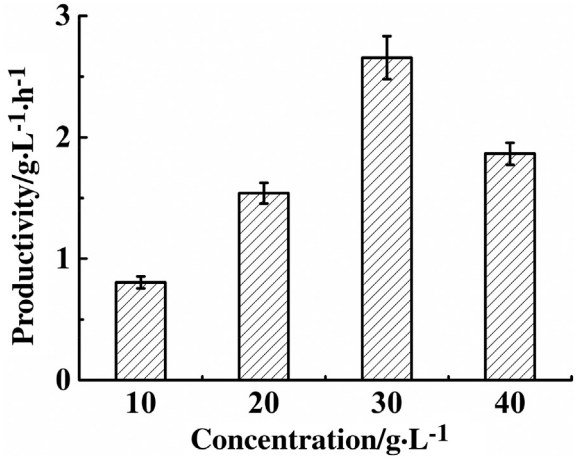

Fig. 3. Effects of substrate concentrations on the productivity of 5-aminovalerate.

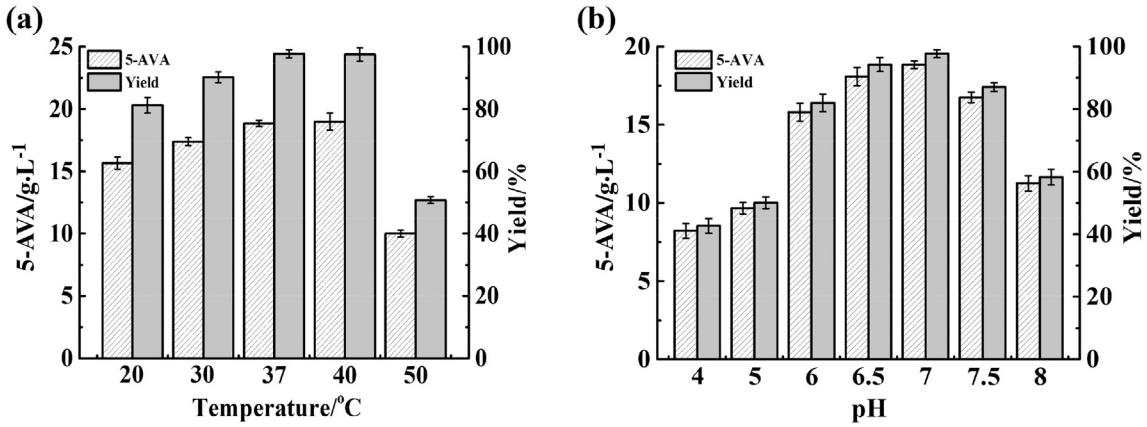

Fig. 4. The effects of reaction temperature (a) and reaction pH (b) on the whole-cell bioconversion efficiency of BL-22A-RB-YB.

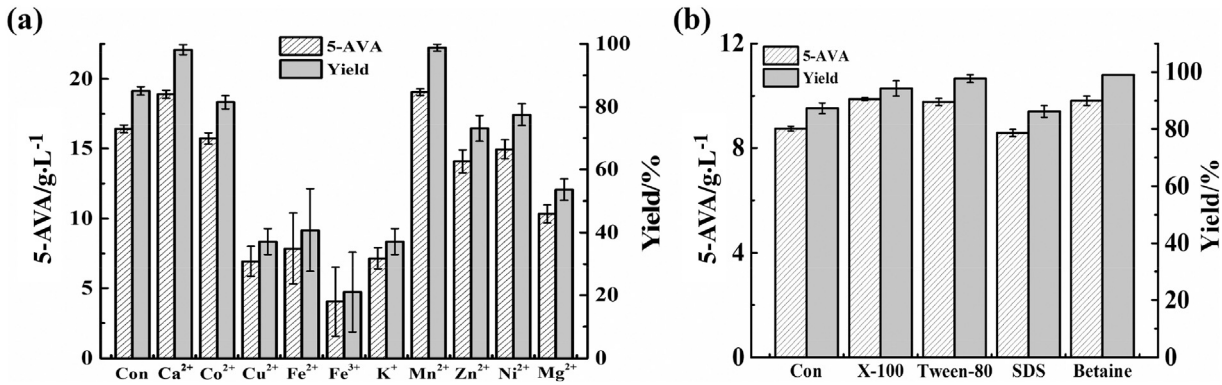

Fig. 5. The effects of metal ions (a) and surfactant (b) on the whole-cell bioconversion efficiency of BL-22A-RB-YB.

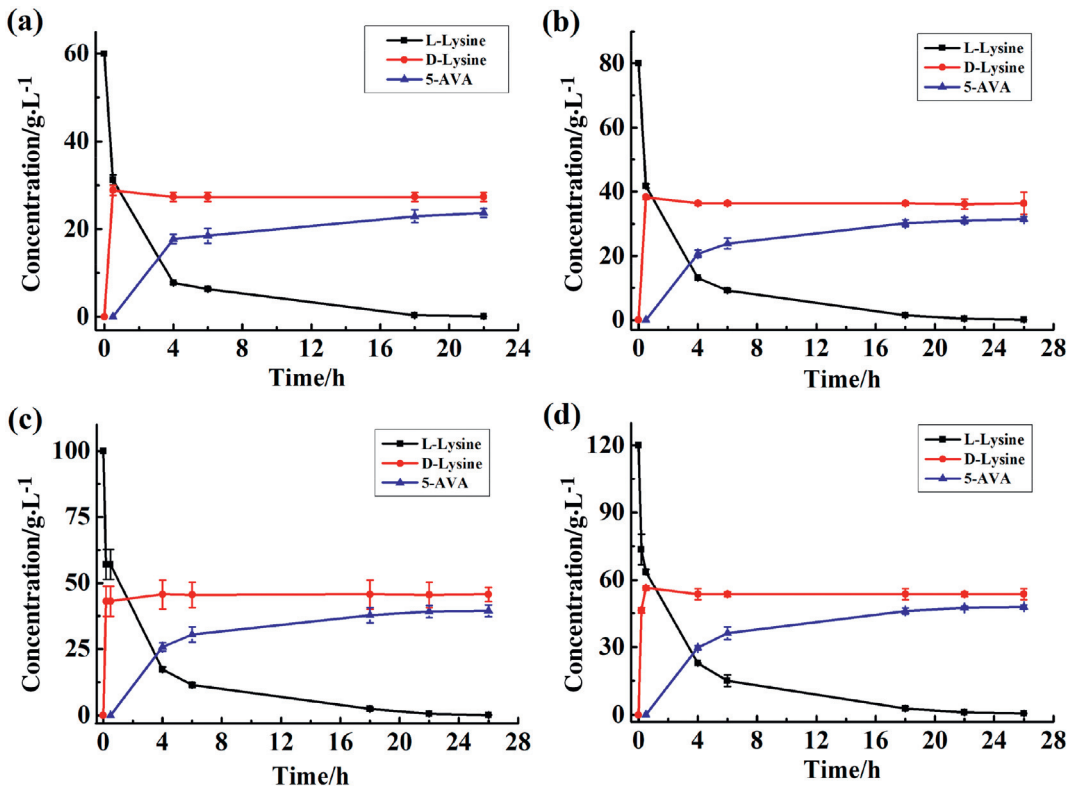

Fig. 6. D-lysine production from different concentration of L-lysine.

Table 1
Effects of initial substrate concentration on optical purity of D-lysine

Initial L-lys/g·L^{-1}	D-Lys/g·L^{-1}	5-AVA/g·L^{-1}	D-Lys yield	e.e. value/%
60	27.3	23.7	47.8%	99.1
80	36.4	36.4	47.9%	99.3
100	45.6	40.4	48%	99.4
120	53.5	48.2	47.6%	99.4

investigated, the results agreed with a previous study showing that the optimal temperature and pH for purified DavA and DavB catalyzed 5-aminovalerate synthesis was 37 °C and 7, respectively [20]. Meanwhile, to improve process efficiency and increase the optical purity of D-lysine, the effects of metal ion and surfactant were examined. As a result, the addition of Ca^{2+} and Mn^{2+}, good metals for substrate activation and electrostatic stabilization [10], was found capable of increasing the bioconversion efficiency. Meanwhile, the addition of surfactants, such as X-100, Tween-80 and betaine for increased cell permeabilization, could also improve the 5-AVA production. Similarly, permeabilization of *E. coli* O44 K74 cells with TritonX-100 has a positive effect on crotonobetaine synthesis [21], and treatment of *C. lini* ST-1 with Tween-80 enhances DHEA synthesis 7α, 15α-diOH-DHEA [22]. The identification of these positive factors could significantly optimize the resolution efficiency and be helpful for the enantiopure D-lysine production.

After the optimization of process reaction condition, D-lysine with enantiomeric excess ≥99% was synthesized efficiently with an approximate yield of 47.6% at the different concentration of L-lysine. Meanwhile, another compound 5-AVA was synthesized with the production of D-lysine. As we knew, 5-AVA was an important C5 platform chemical and could be used as the precursor for synthesizing 1,5-pentanediol, glutarate, and 5-hydroxyvalerate [10]. These results indicated the economic feasibility of the two-strain coupling system we established in the large-scale production of enantiopure D-lysine.

5. Conclusions

In this work, a two-strain coupling whole-cell bioconversion system for D-lysine production from L-lysine was established by using recombinant *E. coli* strains. The racemization of L-lysine to generate 5-AVA and D-lysine through *E. coli* strain BL-22A-RB-YB. To produce D-lysine with high enantiomeric excess, the reaction condition for resolution of DL-lysine after the racemization was optimized, and 53.5 g·L^{-1} D-lysine could be obtained from 120 g·L^{-1} L-lysine with a yield of 47.6%. The final enantiomeric excess of D-lysine was higher than 99%.

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