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Short communication

Continuous production of L-phenylalanine from phenylpyruvic acid and L-aspartic acid by immobilized recombinant *Escherichia coli* SW0209-52

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Abstract

A recombinant strain *Escherichia coli* SW0209-52 was immobilized with κ-carrageenan for the production of L-phenylalanine via phenylpyruvic acid (PPA) and L-aspartic acid (L-Asp). To enhance operational stability, the immobilized cells were treated with 0.05% (w/v) glutaraldehyde and the relative activity was 122.8% compared with that without treatment. The conversion yield could stay above 88% in 10 batch cycles, indicating the stability of the immobilized cells. Continuous L-phenylalanine production with PBR was studied under different feeding PPA concentrations (15–40 g/L) and residence time (2.2–10 h). Considering both conversion yield and volumetric productivity, studies on the operational stability of the reactor were conducted under the condition of 30 g/L PPA feeding concentration and 10 h residence time. The system could be operated for 40 days without significant decline and the average productivity was 1.85 g/L/h and the conversion yield was above 85% throughout the 40 days.

Keywords: L-Phenylalanine; Phenylpyruvic acid; Glutaraldehyde; *Escherichia coli*; Immobilization; Packed bed reactor

1. Introduction

L-phenylalanine (L-Phe) is an important amino acid and widely used in pharmaceutical and food industries. Its production has been stimulated by the great demand on the diet sweetener aspartame, a peptide made up of L-phenylalanine and L-aspartic acid (L-Asp) [1,2]. L-Phe can be produced by several chemical and biochemical processes [3]. The process for production of L-Phe via bioconversion of phenylpyruvic acid (PPA) and L-aspartic acid by transaminase has been demonstrated very promising, because of high conversion yield [4–6]. Although high cost of phenylpyruvic acid and difficulties in downstream process were the main disadvantages of the process, the cost of phenylpyruvic acid was decreased because of the efficient production with hydantoin [7] and the problems in separation of L-Phe could be resolved by adsorption with resin or extraction with hollow fiber membrane or nanofiltration membrane [8–10].

A recombinant strain *Escherichia coli* SW0209-52 containing high transaminase activity was successfully applied for the preparation of L-Phe with PPA and L-Asp [11]. In order to allow the cell reutilization and fulfill the continuous operation, different methods of immobilization have been tried [12]. The process conditions for conversion were optimized under batch mode [13]. In order to enhance the stability of immobilized cells, different methods using glutaraldehyde were tested in this study. The effects of operational conditions and the possibility of continuous L-phenylalanine production by immobilized cells in a packed bed reactor (PBR) were also described.

2. Material and methods

2.1. Materials

PPA was provided by Nanjing University of Chemical Technology. L-Asp was obtained from Sizhou Amino Acid Co. (Wuxi, China). Pyridoxal 5′-phosphate (5′-PLP) was purchased from Sigma Chemical Co. (St. Louis, MO, USA). All other chemicals were from commercial sources and of analytical grade.

2.2. Microorganism and cultivation

E. coli SW0209-52, a recombinant strain with high transaminase activity, which was preserved in our lab, was used in the study. Strain SW0209-52 was constructed by transformation of plasmid pKB7 containing *E. coli* tyrB gene to

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E. coli JM107 [14]. The culture medium contained: 3.0% sodium glutamine, 1.0% beef extract, 1.0% corn steep liquor, 0.05% $MgSO_4 \cdot 7H_2O$ and 0.05% K_2HPO_4, pH 7.0. After sterilization, 200 μg/mL ampicillin was added. The culture was carried out in a 500 mL shaking flask containing 50 mL medium at 30 °C and 200 rpm for 10 h, and then culture temperature was shifted to 37 °C for another 12 h cultivation.

2.3. Immobilization

After the cultivation, the broth was centrifuged at 8000 rpm for 10 min to recover the cells. The pellets were washed and suspended in sterile saline. The cell suspension was mixed with κ-carrageenan aqueous solution at 45 °C so that the final slurry was 2.5% (w/v) with respect to κ-carrageenan and 7.5% (w/v) with respect to the cell wet weight. The mixture was quickly poured into a plate to harden. The gel was then immersed in 0.2 M KCl for 4 h at 4 °C to enhance strength. The gel was cut into cubes of 1 cm^3 before use [15].

In order to increase the stabilization of the immobilized cells, glutaraldehyde was used to treat cells and immobilized cells in two different methods, which were to treat the free cells with glutaraldehyde before immobilization and to treat the immobilized cells with glutaraldehyde. In the first method, whole cells were suspended in sterile saline with two different glutaraldehyde concentrations (0.025%, 0.05%, w/v). The suspensions were incubated for 1 h, and then the pellets collected by centrifugation (8000 rpm, 10 min) were immobilized as described above. In the second method, the immobilized cells obtained by the method described above were maintained under 4 °C, and stirred with 0.2 M KCl solution containing two different glutaraldehyde concentrations (0.05%, 0.25%, w/v) for 15 min. After treatment, the immobilized cells were washed with cold 0.2 M KCl solution.

2.4. Enzymatic reaction in shaking flask

Immobilized cells (total biomass loading of 0.5 g free cells) were suspended in 10 mL substrate solution containing 400 mg PPA, 1000 mg L-Asp, 2 μmol PLP, 20 μmol $MgSO_4$. The mixture was incubated at 37 °C for 8 h under mild shaking condition. Then the mixture was filtered and the supernatant was measured by HPLC for analysis. Enzymatic kinetic parameters were determined by changing the concentration of PPA from 0.06 to 0.49 M and the concentration of L-Asp from 0.07 to 0.6 M. The initial rate was defined as the yield of L-Phe produced by immobilized cells or free cells of the same biomass in 1 h. Experiments of repeated batch operation with the immobilized cells were carried out by washing the immobilized cells with cold 0.2 M KCl solution at the end of each cycle, and then the immobilized cells were continuously reused for the next batch cycle.

2.5. Continuous production of L-phenylalanine in PBR

Continuous L-phenylalanine production was carried out in a packed bed reactor (Ø 50 mm × 200 mm, 200 mL working volume) filled with immobilized cells. The reactor was operated at 37 °C in a temperature-controlled incubator. A substrate solution was continuously fed into the reactor from the top of the column and the effluent was collected at the bottom. Substrate (PPA) with different concentration (15, 20, 30, and 40 g/L) was used. For each concentration, the reactor was operated at different residence time ranging from 2.2 to 10 h.

2.6. Analytical methods

The concentration of PPA and L-Phe were determined by high-pressure liquid chromatography (HPLC, Hewlett Packard 1100, USA) using a column of LiChrospher 100Rp-18 (Agilent, USA) with methanol and 50 mM KH_2PO_4 (30:70, v/v; 0.7 mL min^{-1}) as mobile phase and eluted products were detected at 210 nm.

3. Result and discussion

3.1. Effect of glutaraldehyde on immobilized cells

For long-term continuous production of L-phenylalanine with an immobilized cell reactor, the enzyme in immobilized cells must be stable under operational conditions. As shown in Table 1, treatment of immobilized cells with 0.05% (w/v) glutaraldehyde enhanced both enzyme activity and operational stability. The relative activity was 122.8% (compared with that without glutaraldehyde treatment), and the activity after four cycles retained at 91.5% of the initial level. This fact could be attributed to the decrease of enzyme leakage by cross-linking functions among the chemical reagent, cell wall and parts of cells proteins.

3.2. Kinetic parameters of catalytic reaction with immobilized cells

The effect of substrate concentration on the initial reaction rate with free and immobilized cells was investigated respectively, within the PPA concentration range of 0.06–0.49 M and the L-Asp concentration range of 0.07–0.60 M. Kinetics parameters of the Ping Pong reaction model were determined [16]. As shown in Table 2, the $K_{m,app}^{PPA}$ and the $K_{m,app}^{Asp}$ for immobilized cells were both higher than that of free cells, while the $V_{max,app}$ for the immobilized cells was lower than that of free cells. The changes of K_m and V_{max} between free cells and immobilized cells were mainly caused by the characters of carrier and mass transfer limitation.

3.3. Repeated batch operation

Repeated batch operation was designed to examine the reusability of the immobilized cells. Fig. 1 showed that the conversion yield of the reaction could stay above 88% for at

Table 1
The effect of glutaraldehyde on immobilized cells

Methods	Relative activity (%)	Remained activity (%)		
		Second use	Third use	Fourth use
Untreated	100.0	91.8 ± 2.7	84.8 ± 3.9	80.3 ± 2.8
Treated with 0.025% glutaraldehyde before immobilization	107.7 ± 3.6	92.2 ± 2.1	88.7 ± 3.5	84.9 ± 4.3
Treated with 0.05% glutaraldehyde before immobilization	88.3 ± 2.6	90.1 ± 1.9	87.5 ± 2.6	85.2 ± 2.4
Treated with 0.05% glutaraldehyde after immobilization	122.8 ± 3.1	96.4 ± 2.2	94.7 ± 1.7	91.5 ± 2.5
Treated with 0.25% glutaraldehyde after immobilization	96.4 ± 3.0	95.3 ± 2.6	92.6 ± 1.5	90.6 ± 2.1