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Bioconversion of phenylpyruvic acid to L-phenylalanine by mixed-gel immobilization of *Escherichia coli* EP8-10

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ABSTRACT

A mixed-gel of κ-carrageenan and gelatin was used in L-phenylalanine production. The mixed-gel, containing 87.5% κ-carrageenan and 12.5% gelatin [the total gel concentration was 4 wt%], showed the best performance and was selected for further study with *Escherichia coli* EP8-10. The optimum pH and temperature were 8.5 and 37 °C, respectively. The effects of trehalose and Mg^{2+} were studied in the mixed-gel immobilization. Their optimum concentrations were 5×10^{-2} and 2×10^{-3} mol/L, respectively. Under the optimal conditions, 98.3% of the phenylpyruvic acid (PPA) was converted to L-phenylalanine. The activity recovery of the transaminase enzyme in the mixed-gel immobilization was higher than that in single κ-carrageenan immobilization, which was 93.6%. The total PPA conversion rate was over 80% in all 15 batches, suggesting great sustainability in the mixed-gel immobilization. The maximum reaction rate (r_{max}) was calculated to be 4.75×10^{-2} mol/(L g h).

1. Introduction

The aromatic amino acid L-phenylalanine (L-Phe), one of the eight essential amino acids has significant industrial and medical applications, for instance, in the healthcare industry as an amino acid infusion [1,2] and in the food industry as a precursor for manufacturing the low-calorie dipeptide sweetener aspartame [1–4]. In 2006, the world consumption of L-Phe achieved 100 kt [5]. The great market for L-Phe is attracting more interest about research on L-Phe production by biological processes.

L-Phe is produced primarily by chemical synthesis, fermentation and enzymatic preparation [5]. The enzymatic preparation is relatively simple and efficient—a transamination reaction catalyzed by aspartate transaminase with phenylpyruvic acid (PPA) and L-aspartic acid (L-Asp) substrates [2,4,6,7]. However, biotransformation utilizing immobilized cells is more attractive since it is of reduced cost [8]. This technology is being used in the production of ethanol and some organic acids [9,10]. The cell immobilization technique has several advantages, such as the ability to reuse the immobilized cells and utilize high cell densities and the improved system stability [11]. Furthermore, compared to immobilized dissociative enzymes, the operation with immobilized cells is simple and holds high enzyme activity. However, there also are some defects, including toxicity of the mixed-gel for cells and the immobilized particles' low mechanical strength [12]. But the usage of a mixed-gel for immobilization could lead to a lower gel solidifying point, a shorter solidification time and less enzymatic deactivation [13,14]. Calcium alginate–gelatin hydrogels have been used to immobilize α-amylase [15]. To overcome the defects, enhance the reusable ratio of enzyme and reduce the production cost associated with the traditional production route by single gel immobilization, we proposed a mixed-gel immobilization for L-Phe production. The mixed-gel immobilization of *Escherichia coli* EP8-10 was studied. The optimum ratio of κ-carrageenan to gelatin in the mixed-gel was determined, and the effects of pH, temperature, the trehalose and Mg^{2+} addition on the mixed-gel immobilization were investigated. Additionally, the stability and reaction kinetics were discussed.

2. Materials and methods

2.1. Chemicals

L-aspartic acid and L-phenylalanine were purchased from Kangda Amino Acids Co. (Jiangyin, China). The pyridoxal-5′-phosphate (5′-PLP) was purchased from Sigma Chemical Co. (St. Louis, MO). PPA, used as a substrate, was synthesized from the chemical laboratory of Nanjing University of Technology, China. All other materials and reagents were of analytical grade.

2.2. Microorganism and cell cultures

E. coli EP8-10, a transaminase-producing bacterium, had previously been isolated from soil samples [16]. The isolated cells were kept and cultivated on agar

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slants overnight and the fermentations were conducted in a 2-L bioreactors containing 1.5 L buffered media (pH 7.0) containing 2% glucose, 1% beef extract, 2% corn steep liquor, 0.05% $MgSO_4{\cdot}7H_2O$, 0.05% NaCl and 0.05% K_2HPO_4. The pH was adjusted to 7.0 with NH_4OH and the cells were aerobically cultivated at 37 °C for 16 h.

2.3. Cell immobilization

Cells were harvested and centrifuged at 8000 × *g* for 20 min. To entrap the cells in the κ-carrageenan gel, the collected cell pellets of approximately 5 g were re-suspended in a 5-mL saline solution and then mixed with 25 mL 4 wt% κ-carrageenan solution for 5 min at 50 °C using vigorous stirring. The mixtures were cooled and solidified at room temperature and were then cut into 3 mm^3 pieces. These pieces hardened in a sterilized KCl solution (0.3 M) at 4 °C for 4 h [8,17]. The cell entrapment procedure for the gelatin gel was similar to the κ-carrageenan gel, except that the gelatin gel hardened in a 0.05% (w/v) glutaraldehyde solution [18]. The sodium alginate gel was prepared in the way described by Cao and Yongmei [19].

2.4. Analysis

2.4.1. Transaminase assay

The cells were collected and centrifuged at 8000 × *g* for 20 min and then subjected to the enzyme activity assay. One gram wet cells or the immobilized particles from 1 g wet cells were added to a 20-mL reaction solution composed of 0.1 mol/L PPA, 0.11 mol/L L-Asp and 0.1 mmol/L pyridoxal-5′-phosphate (pH 8.5). Reactions were carried out at 37 °C for 30 min with shaking at 150 rpm, which was followed by L-Phe identification. A transaminase activity unit was defined as 1 μmol product formed/min [16].

2.4.2. Determination of substrates and products

The amount of L-Phe and PPA was determined by capillary electrophoresis and Fe^{3+} chromatography, respectively [20,21].

The bioconversion rate of L-Phe production was determined for both immobilized particles and free cells by calculating the quantity of L-Phe produced per hour, per gram cells and per liter substrate solution.

2.4.3. Mechanical strength of immobilized particles

The mechanical strength of the immobilized particles (3 mm^3 pieces) was measured with a set of table balances, weights and glass sticks in a 1-cm^2 section area. The peak value at the strip rupture moment was recorded in g/cm^2 [22].

3. Results and discussion

3.1. Overall performance of three carriers

The three natural polymers, κ-carrageenan, gelatin and calcium alginate, are typically used for single-gel immobilization of *E. coli* EP8-10, and the aspartate transaminase activities and mechanical strengths of the immobilized particles in these matrices were compared (Table 1). The aspartate transaminase recovery activities were 75.6%, 51.9% and 65.5%, respectively.

κ-Carrageenan appeared to be more efficient and stable [8], but has a low mechanical strength and high solidifying point (around 50 °C [13]), which may potentially cause instability and low enzymatic activity recovery. In contrast, the gelatin-based carrier exhibited great mechanical strength but the lowest aspartate transaminase activity recoveries. Gelatin and κ-carrageenan were selected for further study with the aim to combine them to yield high recovery and mechanical strength.

Table 1
Effect of Gel matrix used for cell immobilization on bioconversion of phenylpyruvic acid to L-phenylalanine.

Type of matrix	Recovery of aspartate transaminase activity	Mechanical intensity (g/cm^2)
Gelatin[a]	51.9%	694
Ca-alginate[b]	65.5%	286
κ-Carrageenan[a]	75.6%	227

[a] Cubic particles (3 mm in length).
[b] Spherical particles (3 mm diameter).

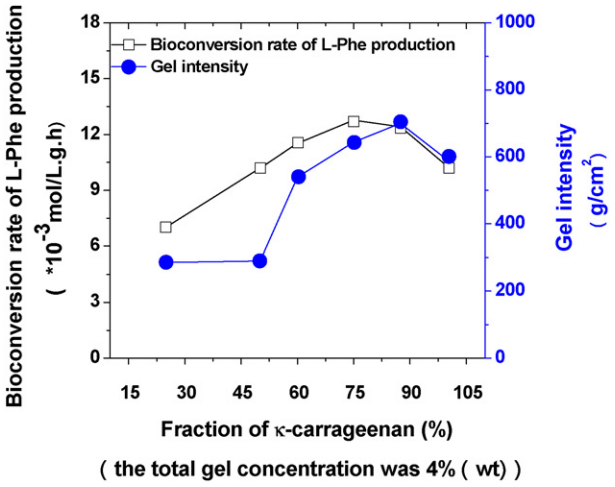

Fig. 1. Effect of the κ-carrageenan fraction in the mixed-gel on the bioconversion rate of L-Phe production and its gel intensity.

3.2. Concentration of κ-carrageenan and gelatin of the mixed-gel immobilization

When a certain gelatin was added into a κ-carrageenan solution, the gel solidifying point fell, the solidification time decreased, and the enzymatic deactivation was reduced [13]. Mixed-gels with κ-carrageenan in the range from 25% (1:3, κ-carrageenan to gelatin) to 100% (single κ-carrageenan gel) were used to immobilize *E. coli* EP8-10. The total gel concentration was kept at 4 wt% in all experiments. When κ-carrageenan was 87.5% (7:1, κ-carrageenan to gelatin), the highest gel intensity (700 g/cm^2) was achieved (Fig. 1). The highest bioconversion rate of L-Phe production was observed at 75% κ-carrageenan and 25% gelatin. Therefore, a range from 75% to 87.5% κ-carrageenan in 4 wt% of the mixed-gel and 87.5% κ-carrageenan were often selected for follow-up studies. At this composition, the bioconversion rate of L-Phe production was 1.24×10^{-2} mol/(L g h). Compared with the single κ-carrageenan gel immobilization, the gel solidifying point at this ratio dropped from 50 to 42 °C, and the solidification time shortened from 5 to 3 min.

3.3. Effect of pH and temperature

The activity of the enzyme was related to pH and temperature; the enzymatic reactions in L-Phe production were conducted at a pH range of 7.0–10.0 and a temperature range of 30–50 °C. A pH value of 8.5, which is often used in the bioconversion [6], was selected for future studies. The optimum temperature for free or immobilized cells was 37 °C (Fig. 2).

3.4. Effect of trehalose on the mixed-gel

The impact of some common protecting agents, trehalose, saccharose and glucose, on the bioconversion rate was studied. Previous studies have shown that trehalose can protect the cellular membrane and proteins. In this study, the bioconversion rate of PPA to L-Phe in the presence of 5×10^{-2} mol/L trehalose in the mixed-gel immobilized particles was higher than that in the presence of 5×10^{-2} mol/L saccharose or glucose (Fig. 3). A possible explanation is that trehalose can protect live cells and bioactive substances from deactivation [23].

Furthermore, the effects of different concentrations of trehalose were studied in the mixed-gel system. When the concentration of trehalose was 5×10^{-2} mol/L, the bioconversion rate of L-Phe production reached a maximum of 1.36×10^{-2} mol/(L g h)