Basic Studies for Continuous Production of L-Aspartic Acid by Immobilized Escherichia coli Cells

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By using a column packed with immobilized Escherichia coli cells entrapped in a polyacrylamide gel lattice, conditions for continuous production of L-aspartic acid from ammonium fumarate were investigated. When a solution of 1 M ammonium fumarate (pH 8.5) containing 1 mM Mg²⁺ was passed through the immobilized cell column at a flow rate of space velocity (SV) = 0.8 at 37 C, the highest rate of reaction was attained. From the column effluents, L-aspartic acid was obtained in good yield. The immobilized cell column was very stable.

In the accompanying paper (1), we revealed details of the immobilization of Escherichia coli cells having high aspartase activity and the enzymatic properties of such immobilized cells. In this paper we present a method for continuous production of L-aspartic acid from ammonium fumarate by using the aspartase activity of immobilized E. coli cells.

MATERIALS AND METHODS

Materials. Acrylamide monomer and potassium persulfate were obtained from Katayama Chemical Industries Co., Ltd. (Osaka, Japan). N,N'-methylenebisacrylamide and β-dimethylaminopropionitrile were obtained from Tokyo Kasei Kogyo Co., Ltd. (Tokyo, Japan).

Preparation of immobilized E. coli cells. E. coli ATCC 11303 was cultured under aerobic conditions at 37 C for 24 h in 1 liter of medium (pH 7.0) containing ammonium fumarate (30 g), K₂HPO₄ (2 g), MgSO₄.7H₂O (0.5 g), CaCO₃ (0.5 g), and corn steep liquor (40 g). The cells were collected by centrifugation and washed with physiological saline. One gram of the packed cells was suspended in 4 ml of physiological saline. To the cell suspension, acrylamide monomer (0.75 g), N,N'-methylenebisacrylamide (40 mg), 5% β-dimethylaminopropionitrile (0.5 ml), and 2.5% K₂S₂O₃ (0.5 ml) were added, and the mixture was stored at 37 C for 30 min. The resulting gel was blended with a Waring blender. The immobilized cells were activated by incubation with 1 M ammonium fumarate (pH 8.5, 1 mM Mg²⁺) at 37 C for 48 h.

Estimation of L-aspartic acid in eluate. The L-aspartic acid produced in the eluate was measured by bioassay using Leuconostoc mesenteroides P-60 (2).

RESULTS

The conditions for continuous production of L-aspartic acid were investigated in detail by using an immobilized E. coli cell column.

Effect of pH. The effect of pH on the formation of L-aspartic acid by the immobilized cell column was investigated. The optimal pH value was found to be 8.5 (Fig. 1).

Effect of temperature. The effect of temperature on the formation of L-aspartic acid by the immobilized cell column was investigated. The optimal temperature was found to be 50 C (Fig. 2).

Stability at various temperatures. The stability of the immobilized cell column was investigated at various temperatures. The immobilized cell column was inactivated at 45 or 50 C (Fig. 3). However, the column was very stable at 37 C.

Protective effect of metal ions. As described in the accompanying paper (1), metal ions such as Mg²⁺, Mn²⁺, and Ca²⁺ protect immobilized cells from inactivation by heat. The protective effects of these metal ions were investigated in the continuous formation of L-aspartic acid by the column process. These metal ions showed stabilizing effects during the continuous enzyme reaction (Fig. 4).

Relationship of flow rate and production of L-aspartic acid from ammonium fumarate. A solution of 1 M ammonium fumarate (pH 8.5, 1 mM Mg²⁺) was passed through the immobilized cell column at various flow rates, and the rate of formation of L-aspartic acid was measured (Fig. 5). The data show that the flow rate of space velocity (SV) = 0.8 was the maximal flow rate possible for the complete conversion of ammonium fumarate to L-aspartic acid.

Stability of the immobilized cell column. The stability of the immobilized cell column was investigated by continuously passing
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FIG. 1. Effect of pH on the reaction rate of the immobilized cell column. A 0.5-M fumaric acid-0.7 M NH₄Cl solution (adjusted to indicated pH with 5 N NaOH) containing 1 mM Mg²⁺ was applied to the immobilized cell columns (1.6 by 12.5 cm) at 37 C at the flow rate of SV = 2.0. The L-aspartic acid produced in the effluent was measured.

FIG. 2. Effect of temperature on the reaction rate of the immobilized cell column. A solution of 1 M ammonium fumarate (pH 8.5, 1 mM Mg²⁺) was applied to the immobilized cell columns (2.2 by 9 cm) at the indicated temperature at the flow rate of SV = 1.5. The L-aspartic acid produced in the effluent was measured.

FIG. 3. Stability of the immobilized cell column at various temperatures. A solution of 1 M ammonium fumarate (pH 8.5, 1 mM Mg²⁺) was applied to immobilized cell columns (2.2 by 9 cm) at the indicated temperature at the flow rate of SV = 1.5. The L-aspartic acid produced in the effluent was measured.

through it a substrate solution of 1 M concentration at various flow rates at 37 C. The immobilized cell column was very stable (Fig. 6). If the substrate solution was passed through at SV = 0.5, a decrease in the activity of the immobilized cells was not observed and complete conversion of 1 M ammonium fumarate to L-aspartic acid was maintained for over 1 month.

Continuous production of L-aspartic acid.
A solution of 1 M ammonium fumarate (pH 8.5, 1 mM Mg²⁺) was passed through the immobilized cell column (10 by 100 cm) at a flow rate of 6 liters/h at 37 C. Six liters of the effluent was adjusted to pH 2.8 with 60% H₂SO₄ at 90 C and then incubated at 15 C for 2 h. The L-aspartic acid which crystallized out was collected by filtration and washed with water. The yield was 762 g (95% of theoretical); [α]₀°₂₅⁺ = +25.5 (c = 8 in 6 N HCl).

DISCUSSION
L-Aspartic acid is used for medicinals and food additives and is industrially produced by fermentation and enzymatic methods using the action of aspartase on ammonium fumarate
(3-5). These batch procedures employing soluble aspartase or intact cells have some disadvantages for industrial purposes, however. To overcome these disadvantages, we have studied the continuous production of L-aspartic acid by using a column packed with immobilized aspartase (6). However, in that case, it was necessary to extract the aspartase from E. coli cells before the immobilization process, and the enzyme activity of the immobilized aspartase was only about 25% after 20 days of operation. Thus, we concluded that the immobilized enzyme was not satisfactory for the industrial production of L-aspartic acid. We felt that if the microbial cells could be directly immobilized in a stable form, these disadvantages could be overcome. We extensively investigated the immobilization of E. coli cells (1), and the conditions for continuous production of L-aspartic acid from ammonium fumarate by the action of immobilized E. coli cells are presented in this paper.

The immobilized cell column is very stable, and the process can be automatically controlled, indicating that the labor cost will be dramatically reduced (Fig. 6). This new technique is very efficient and superior to the

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**Fig. 4.** Effect of metal ions on the continuous reaction by the immobilized cell column. A solution of 1 M ammonium fumarate (pH 8.5) containing 1 mM metal ions was applied to the immobilized cell columns (2 by 9.5 cm) at 37 C at the flow rate of SV = 0.8. The L-aspartic acid produced in the effluent was measured.

**Fig. 5.** Relationship of flow rate to the production of L-aspartic acid from ammonium fumarate. A solution of 1 M ammonium fumarate (pH 8.5, 1 mM Mg2+) was applied to the immobilized cell column (1.6 by 20 cm) at 37 C at the indicated flow rates. The L-aspartic acid produced in the effluent was measured.

**Fig. 6.** Stability of the immobilized cell column. A solution of 1 M ammonium fumarate (pH 8.5, 1 mM Mg2+) was applied to the immobilized cell columns (1.6 by 25 cm) at 37 C for 36 days at the indicated flow rates. Formation of L-aspartic acid from ammonium fumarate was determined by microbioassay.
continuous fermentation method. It will be the subject of increased interest in the fermentation industry.

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LITERATURE CITED