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REVIEW

Continuous Production of L-Aspartic Acid

Improvement of Productivity by Both Development of Immobilization Method and Construction of New *Escherichia coli* Strain

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ABSTRACT

For the continuous production of L-aspartic acid from fumaric acid and ammonia by the action of aspartase, the enzyme extracted from *Escherichia coli* or *E. coli* cells having high aspartase activity were immobilized by various methods.

In 1973 we succeeded in the industrial production of L-aspartic acid using *E. coli* cells immobilized with polyacrylamide gel.

For the improvement of this process, we developed a novel technique using κ-carrageenan as the immobilizing matrix for *E. coli* cells. Further, EAPc-7 strain, having higher aspartase activity, was contracted from the parent *E. coli* by continuous cultivation with a definite medium. The aspartase activity was about seven times higher than that of the parent cells. In 1982 we changed from the conventional method to the improved method, using EAPc-7 strain immobilized with κ-carrageenan.

Index Entries: Production, of L-aspartic acid; L-aspartic acid, production of; immobilized aspartase, in production of L-aspartic acid; aspartase, immobilization of; aspartase, with polyacrylamide, production of; immobilized cells, in production of L-aspartic acid; *Escherichia coli* ATCC 11303, immobilization of *E. coli* ATCC 11303;

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polyacrylamide, immobilization of *E. coli* ATCC 11303 with; κ-carrageenan, in production of L-aspartic acid; EAPc-7 a strain having higher aspartase activity; EAPc-7, derived from *E. coli* ATCC 11303 by continuous cultivation; EAPc-7, immobilization of; κ-carrageenan.

INTRODUCTION

L-Aspartic acid is widely used, not only in medicines but also as food additives. Recently, Aspartame®, a dipeptide of L-aspartic acid and L-phenylalanine methylester, was commercialized as a low-calorie, synthetic sweetener. Thus, demand for L-aspartic acid is increasing since it is a raw material for the synthesis of the dipeptide.

The aspartic acid had been industrially produced by fermentative or enzymatic batch process from fumaric acid and ammonia, using the action of aspartase, according to following equation:

$$\underset{\text{Fumaric acid}}{HOOC{-}CH{=}CH{-}COOH} + NH_3 \xrightarrow[\text{aspartase}]{} \underset{\text{L-Aspartic acid}}{HOOC{-}\underset{\displaystyle NH_2}{\underset{|}{CH}}{-}CH_2{-}COOH}$$

Since 1960, L-aspartic acid has been industrially produced by Tanabe Seiyaku Co. Ltd. by a batchwise reaction, using intact *Escherichia coli* having high aspartase activity. However, the procedures have some disadvantages in industrial use because the batch process involves incubation of a mixture containing substrate and enzyme or microbial cells.

On the other hand, since the early 1960s, we have investigated immobilized enzymes with the aim of utilizing them for continuous industrial production. And in 1969, we succeeded in the industrialization of a continuous process for the optical resolution of D,L-amino acids, using immobilized aminoacylase. This is the first industrial application of the immobilized enzyme.

Succeeding this immobilized enzyme process, we studied the continuous production of L-aspartic acid using immobilized biocatalysts, and, in 1973, we commercialized the continuous production of L-aspartic acid by the immobilized microbial cell system.

In this review, we summarize the advances made in the system for the production of L-aspartic acid developed by Tanabe Seiyaku Co. Ltd.

PRODUCTION OF L-ASPARTIC ACID BY IMMOBILIZED ASPARTASE

For continuous production of L-aspartic acid, using immobilized aspartase, we investigated various immobilization methods for aspartase extracted from *E. coli* (*1*), and the results are shown in Table 1.

Among the immobilization methods tested, relatively active immobilized aspartase was obtained by the entrapping method, using polyacryl-

TABLE 1
Comparison of Immobilization Method of Aspartase and Its Activity

Immobilization method and carrier	Native apartase used, μmol/h	Immobilized aspartase	
		Activity, μmol/h	Yield of activity, %
Physical adsorption			
Silica gel	8400	10	0.1
Ca phosphate gel	4800	230	4.8
Ionic binding			
DEAE-cellulose	4800	276	5.8
ECTEOLA-cellulose	4800	35	0.7
TEAE-cellulose	4800	336	7.0
DEAE-Sephadex	4800	158	3.3
Covalent binding			
Diazotized PAB-cellulose	700	3	0.4
CM-cellulose azide	700	0	0
Entrapping			
Polyacrylamide gel	1440	417	29.0

amide gel. The stability of the immobilized enzyme column was investigated by operating it continuously for a long period. As the results show, the activity of the column decreased about 50% in the case using 1*M* ammonium fumarate as a substrate solution after operation for about 30 d at 37°C (Fig. 1).

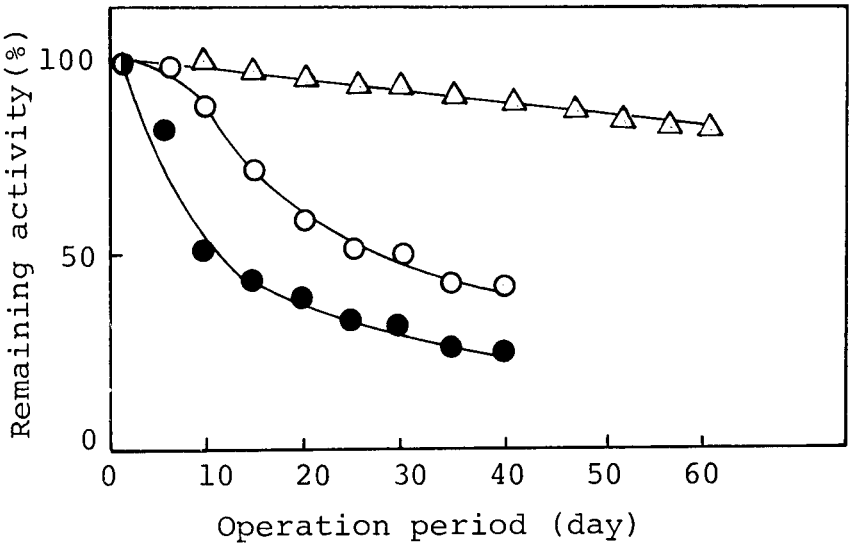


Fig. 1. Operati onal stability of various aspartase preparations: (●), Intact *E. coli* cells (half -life = 10 d); (○), aspartase immobilized with polyacrylamide (half-life = 30 d); and (△), *E. coli* cells immobilized with polyacrylamide (half-life = 120 d).

For application of this method, the enzyme should be extracted from microbial cells before immobilization, because it is intracellular. Further, the activity yield and the stability of the immobilized enzyme were not satisfactory for industrial purposes.

PRODUCTION OF L-ASPARTIC ACID BY IMMOBILIZED MICROBIAL CELLS

Thus, we considered that if microbial cells having aspartase activity could be directly immobilized, the disadvantages in the case using immobilized aspartase might be overcome (2). Because reports on immobilization of whole microbial cells were very scarce at that time, we tried various methods for the immobilization of *E. coli* cells, as follows: (1) entrapping in the polyacrylamide gel matrix; (2) encapsulating in semipermeable polyurea membrane produced from 2,4-toluene diisocyanate and hexamethylenediamine; and (3) crosslinking with a bifunctional reagent, such as glutaraldehyde or 2,4-toluene diisocyanate. Among these methods, the most active immobilized *E. coli* cells were obtained by entrapping them in a polyacrylamide gel lattice.

E. coli *ATCC 11303 Immobilized with Polyacrylamide Gel*

To prepare the most effective immobilized *E. coli* by the polyacrylamide method, the type and concentration of bifunctional reagents for crosslinking and the concentration of acrylamide monomer were investigated systematically (2). As bifunctional reagents, *N,N'*-methylenebisacrylamide, *N,N'*-propylenebisacrylamide, diacrylamide dimethylether, 1,2-diacrylamide ethyleneglycol, *N,N'*-diallyltartardiamide, ethyleneurea bisacrylamide, and 1,3,5-triacryloyl hexahydro-s-triazine were used. As a result, the activities of the immobilized *E. coli* were almost the same, except for the cases of ethyleneurea bisacrylamide and 1,3,5-triacryloyl hexahydro-*s*-triazine. For industrial purposes, we chose *N,N'*-methylenebisacrylamide, because it was commercially available at low cost. The concentrations of acrylamide monomer and *N,N'*-methylenebisacrylamide and the amounts of cells to be entrapped were also investigated, and optimum conditions were established for the immobilization of *E. coli* as follows.

The *E. coli* cells (1 kg, wet weight) collected from cultured broth were resuspended in 2 L of the broth and cooled to 8°C. Acrylamide monomer (750 g) and *N,N'*-methylenebisacrylamide (40 g) were dissolved in 2.4 L of water and the monomer solution cooled to 8°C. The *E. coli* cell suspension and monomer solution were mixed at 8°C. To this mixture, 100 mL of 25% (v/v) β-dimethylaminopropionitrile (as an accelerator of polymerization) and 500 mL of 1% potassium persulfate (as an initiator of polymerization) were added and the reaction mixture allowed to stand at 20–25°C. The polymerization reaction started after about 5 min, and the

temperature of the reaction mixture increased as polymerization proceeded. When the temperature reached 30°C, the resulting stiff gel was rapidly cooled with ice-cold water and allowed to stand for 15–20 min to complete the polymerization. The gel was made into granules, about 3–4 mm in diameter, for industrial use and washed with water.

An interesting phenomenon was observed with the immobilized *E. coli* cells. When freshly prepared immobilized cells were suspended at 37°C for 24–48 h in a substrate solution, their activity increased about ten times. Since this phenomenon is advantageous for continuous production of L-aspartic acid, the activation mechanism was investigated in detail. As a result, the apparent enzyme activity was found to be elevated by an increase of membrane permeability for substrate and/or product resulting from autolysis of the cells in the gel.

By using a column packed with immobilized *E. coli* cells, the operational stability was investigated. As shown in Fig. 1, it was found that the immobilized cell column was very stable, and its half-life was 120 d at 37°C (*3,4*).

For industrial application of this technique, we carried out kinetic and engineering analyses of continuous enzyme reaction, using a column packed with immobilized *E. coli* cells (*4*), and the aspartase reactor system was designed. Since this aspartase reaction is exothermic, the column reactor used for industrial production of L-aspartic acid was designed as a multistage system with cooling, as shown in Fig. 2 (*5*).

This immobilized cell system has been used industrially since 1973 by Tanabe Seiyaku Co. Ltd., Japan. The overall production costs of this system were reduced to about 60% of that of the conventional batchwise reaction using intact cells because of the marked increase of productivity of L-aspartic acid per unit of cells, reduction of labor costs resulting from automation, and an increase in the yield of L-aspartic acid. Furthermore, the procedure employing immobilized cells is advantageous from the standpoint of waste treatment. This is the first industrial application of immobilized microbial cells as a solid catalyst.

E. coli *ATCC 11303 Immobilized with κ-Carrageenan*

As stated above, the polyacrylamide gel method is advantageous for immobilization of microbial cells and for industrial application. However, there are some limitations of this method. That is, some enzymes are inactivated during the immobilization procedure by the action of the acrylamide monomer β-dimethylaminopropionitril or by potassium persulfate. Therefore, this method is not always satisfactory for immobilization of enzymes and microbial cells.

Thus, in order to find out a more general immobilization technique and to improve the productivities of the immobilized microbial cell system, we screened various synthetic and natural polymers (*6*).

As a result, we found that several polymers formed a gel lattice suitable for entrapping microbial cells (*6*). Among the polymers tested,

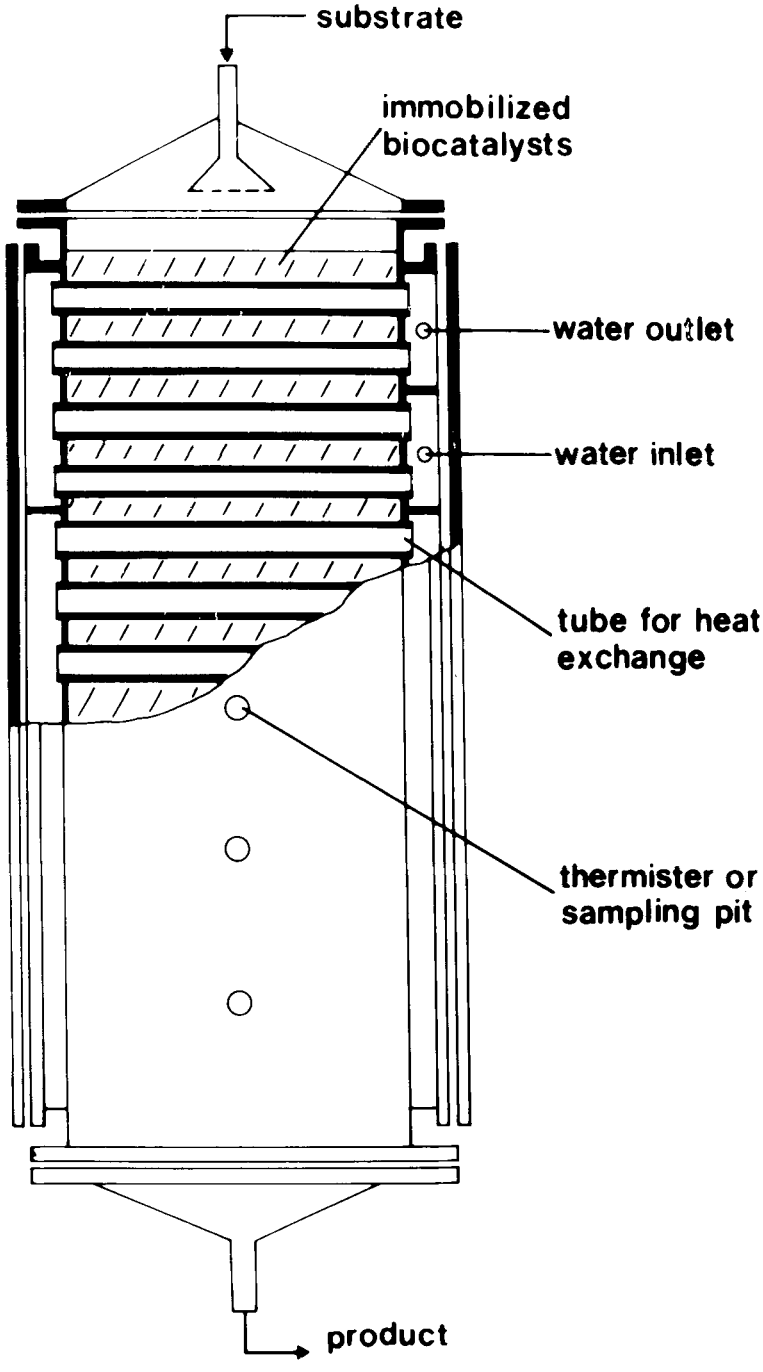


Fig. 2. Schematic diagram of heat-exchange type column for production of L-aspartic acid (5).

κ-carrageenan was the most suitable for immobilization of microbial cells.

κ-Carrageenan is a naturally occurring hydrocolloid, consisting of a high-molecular-weight, linear-sulfated polysaccharide, and is mainly

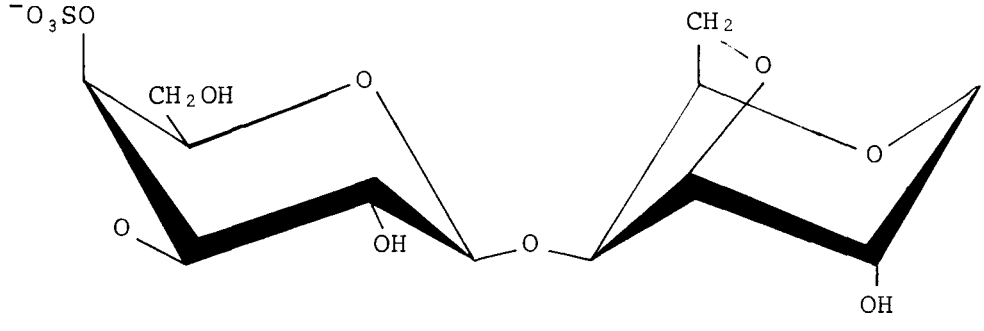
composed of D-galactose and 3,6-anhydro-D-galactose and their ester sulfate derivatives. Its molecular structure is shown in Fig. 3. It is commercialized by extraction from red algae seaweeds and is widely used in the food and cosmetic industries as a gelling, thickening, and stabilizing agent.

κ-Carrageenan becomes a gel by cooling, as in the case of agar. Its gelation occurs also by contacting with a solution containing potassium ions. The rigidity of κ-carrageenan gel in the presence of potassium ions increases with increasing potassium concentration.

Taking into consideration these characteristics of κ-carrageenan, we extensively investigated conditions suitable for immobilization of microbial cells using κ-carrageenan, and optimum conditions were established for the immobilization of *E. coli* (*7–9*) as follows.

One kilogram of whole cells of *E. coli* (wet weight) was suspended in 1 L of physiological saline at 40°C, and 0.184 kg of κ-carrageenan was dissolved in 3.75 L of the physiological saline at 45°C. The two solutions were mixed together and the mixture cooled at around 10°C for 30 min. In order to increase gel strength, the obtained gel is soaked in a cold 0.3*M* potassium chloride solution. After this treatment, the resulting stiff gel was formed into a cubic gel of 3 × 3 × 3 mm with a knife (*9*).

The aspartase activity and the operational stability of *E. coli* cells immobilized with κ-carrageenan were compared with those immobilized with polyacrylamide. As a result, it was found that the enzyme activity of immobilized cells prepared with κ-carrageenan was much higher, and operational stability was increased by hardening treatment with glutaraldehyde and hexamethylenediamine. Its half-life was 680 d or almost two yr. Table 2 is a comparison of the productivities of *E. coli* cells immobilized with polyacrylamide and with κ-carrageenan for production of L-aspartic acid. When the productivity of immobilized preparation with polyacrylamide was taken as 100, that of cells immobilized with



D-galactose-4-sulfate 3,6-anhydro-D-galactose

Fig. 3. Molecular structure of κ-carrageenan.

κ-carrageenan and hardened with glutaraldehyde and hexamethylenediame was 1500, i.e., 15 times higher. Because this carrageenan method is clearly more advantageous than the polyacrylamide method, we changed from the conventional polyacrylamide method to the new carrageenan method for industrial production of L-aspartic acid in 1978. When a 1000-L column is used, theoretical yield of L-aspartic acid is 3.4 t/d and 100 t/mo.

Aspartase-Hyperproducing Mutant of E. coli *ATCC 11303 Immobilized with κ-Carrageenan*

In our laboratory, the EAPc-7 strain having higher aspartase activity was constructed from *E. coli* ATCC 11303 by continuous cultivation with a definite medium (*10*). Namely, cells of *E. coli* ATCC 11303 were mutagenized with *N*-methyl-*N'*-nitrosoguanidine by the modified method of Adelberg et al. (*11*). Mutagenized cells (2×10^{10}) were transferred into a 100-mL chemostat vessel containing 50 mL of Asp-*N* medium (a medium containing L-aspartic acid as the sole nitrogen source) and incubated at 30°C. Cells grown fast in Asp-*N* medium were enriched by continuous cultivation and spread on Asp-*N* agar plates. After 1–2 d of incubation at 30°C, any large colonies found were purified by single-colony isolation, and the EAPc-7 strain having higher aspartase activity was isolated. The aspartase activity was about seven times higher than that of the parent *E. coli* ATCC 11303. However, this strain also had fumarase activity, which converts fumaric acid to L-malic acid. This is disadvantageous for the production of L-aspartic acid from fumaric acid and ammonia. Thus, in order to employ this strain for industrial production of L-aspartic acid, it was necessary to eliminate the fumarase activity.

Several treatments for specifically eliminating fumarase activity from the strain were tested, and it was found that when the strain was treated in a culture broth (pH 4.9), containing 50 m*M* L-aspartic acid, at 45°C for 1 h, fumarase activity was almost completely eliminated, without inactivating aspartase (*12*).

The aspartase activity of treated EAPc-7 cells immobilized with κ-carrageenan was about four times higher than that of the parent *E. coli* cells immobilized with κ-carrageenan. The immobilized preparation was very stable in comparison with the preparations of untreated EAPc-7 cells and the parent *E. coli* cells, and its half-life was 126 d. The reason for its increased stability is thought to be the inactivation of some proteases in the intact EAPc-7 cells because, when the intact cells were treated at pH 4.9 and 45°C for 1 h, their caseinolytic activity was reduced to about 10% of its initial level.

The concentration of L-aspartic acid, fumaric acid, and L-malic acid in the effluent from columns packed with untreated and treated EAPc-7 cells immobilized with κ-carrageenan was measured, and the results are

TABLE 2
Comparison of Productivities of *E. coil* Immobilized with Polyacrylamide and κ-Carrageenan for Production of L-Aspartic Acid

Immobilization method	Aspartase activity, U/g cells	Stability at 37°C, half-life, d	Relative productivity,[a] %
Polyacrylamide	18,850	120	100
Carrageenan	56,340	70	174
Carrageenan (GA)[b]	37,460	240	397
Carrageenan (GA + HMDA)[b]	49,400	680	1498

[a]Productivity = $\int_0^t E_0 \exp(-kdt)dt$: E_0 = initial activity; kd = decay constant; t = operation period.
[b]GA: glutaraldehyde, HMDA: hexamethylenediamine

TABLE 3
Concentration of Acids in the Effluent from Immobilized Cell Columns[a](12)

	Concentration of acids in effluent, m*M*					
	Untreated cell column			Treated cell column		
Operation period, d	L-Aspartic acid	Fumaric acid	L-Malic acid	L-Aspartic acid	Fumaric acid	L-Malic acid
1	956.3	12.1	31.6	987.4	10.7	1.9
10	954.1	17.4	28.5	987.7	10.4	1.9
20	953.2	12.1	34.7	988.1	9.9	2.0

[a]A solution of 1*M* ammonium fumarate (pH 8.5) containing 1 m*M* Mg^{2+} was applied to the column packed with immobilized cell preparations at the flow rate of space velocity = 1 h^{-1} at 37°C.

TABLE 4
Comparison of Productivities of Various Immobilized *E. coli* Cells for Production of L-Aspartic Acid (12)

Immobilized preparation	Aspartase activity, μmol/h/g cells	Half-life at 37°C, d	Relative productivity, %[a]
Parent cells	56,300	70	100
Untreated EAPc-7 cells	203,060	70	361
Treated EAPc-7 cells	192,140	126	614

[a]Productivity of immobilized parent cells was taken as 100%. Productivity = $\int_0^t E_0 \exp(-kdt)dt$: E_0 = initial activity; kd = decay constant; t = operation period.

shown in Table 3. In the case of treated cells, formation of L-malic acid was below 2 m*M* and formation of L-aspartic acid was about 30 m*M* higher than in the case of the untreated cells.

EAPc-7 cells immobilized with κ-carrageenan for production of L-aspartic

The efficiency of the parent *E. coli* cells and untreated and treated acid, was compared. The results show that when the productivity of the parent *E. coli* cell preparation was taken as 100, the treated EAPc-7 cell preparation exhibited the highest productivity (Table 4), i.e., almost six times that of the parent cell preparation.

In 1982, we changed from the conventional method for production of L-aspartic acid to the improved method, using treated EAPc-7 cells having higher aspartase activity. This improved method gives us very satisfactory results in industrial production of L-aspartic acid.

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