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Note

Stabilization of L-Aspartate β-Decarboxylase Activity of *Pseudomonas dacunhae* Immobilized with Carrageenan

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To improve L-alanine production from L-aspartic acid by immobilized *Pseudomonas dacunhae* cells, the stabilization of L-aspartate β-decarboxylase activity of the immobilized cells was investigated.

When *P. dacunhae* cells from the stationary growth-phase were used for immobilization, the operational stability of the enzyme activity of the cells immobilized with κ-carrageenan and treated with glutaraldehyde was found to be highest. When immobilized cells were treated with 0.4 M glutaraldehyde in the presence of 0.4—0.5 M L-lysine, a very stable immobilized preparation was obtained, the enzyme activity having a half-life of about 100 days at 37°C. Further, the activity at alkaline pH was improved by one-fourth over that at the optimum pH.

The application of immobilized microbial cells for transformation of organic compounds is a subject of increasing interest.[1] We are industrially producing L-aspartic acid and L-malic acid by using immobilized *Escherichia coli*[2-5] and immobilized *Brevibacterium flavum*,[6] respectively.

In the previous paper,[7] we described the continuous production of L-alanine from L-aspartic acid by using the L-aspartate β-decarboxylase (EC 4.1.1.12) activity of *Pseudomonas dacunhae* cells immobilized with κ-carrageenan.

To further improve L-alanine production, we investigated methods to stabilize the enzyme activity and to increase enzyme activity at alkaline pH.

Materials and Methods

Materials κ-Carrageenan was obtained from Sansho Co. Ltd., Osaka. Meast (extract from beer yeast) was obtained from Ebiosu Pharmaceuticals Co. Ltd.

Culture of *P. dacunhae* For culture of *Pseudomonas dacunhae* IAM 1152, the medium (pH 7.3) contained 3.2% sodium L-glutamate, 0.5% Meast, 0.05% KH_2PO_4, and 0.01% $MgSO_4 \cdot 7H_2O$. The microorganism was inoculated in 70 ml of medium in a 500-ml shaking flask, and shake-cultured at 30°C for 24—40 hr. Cells were collected by centrifugation and suspended in cell free broth to a 50% (w/v) concentration for immobilization.

Immobilization of *P. dacunhae* cells Portions of 68 ml of 5% (w/v) κ-carrageenan solution in 0.9% NaCl and 32 ml of the cell suspension were mixed thoroughly at 45°C, and the mixture was cooled to 5°C to form a gel. To increase the gel-strength, the gel was soaked in 500 ml of 2% KCl solution at 5°C for 16 hr. The resulting rigid gel was cut into cubes of 3 mm and thoroughly washed with 2% KCl solution.

Glutaraldehyde treatment of immobilized cells and activation Immobilized cells (6.3 g) were suspended in 20 ml of 0.2 M buffer solution containing KCl and hexamethylenediamine or L-lysine, a fixed volume of 2.5 M glutaraldehyde solution was added to a final concentration of 0.025—1.0 M, and the mixture was shaken for 10—60 min at 10°C. Then

the immobilized cells were collected by filtration and thoroughly washed with 2% KCl solution. To activate the immobilized cells, they were incubated at 37°C for 44 hr in 30 ml of substrate solution, which contained 1 M ammonium L-aspartate and 0.1 mM pyridoxal phosphate (pH 5.5).

Assay of L-aspartate β-decarboxylase activity Intact cells: To 0.02 g of intact cells was added 3 ml of the substrate solution, and the mixture was shaken at 37°C for 1 hr, then heated at 100°C for 5 min. L-Alanine formed was determined by bioassay with *Leuconostoc citrovorum* ATCC 8081.[8]

Immobilized cells: Immobilized cells (6.3 g, corresponding to 1 g of intact cells) were incubated with 30 ml of the substrate solution under shaking at 37°C for 1 hr. After removal of the gel by filtration, L-alanine formed was determined.

One unit of enzyme is defined as the amount of enzyme that forms 1 μmole of L-alanine per minute.

Continuous enzyme reaction using column Immobilized cells (6.3 g) were packed into a column (diameter, 1.2 cm; length, 9 cm). A solution of 2 M ammonium L-aspartate (pH 6.2) containing 0.1 mM pyridoxal phosphate was continuously passed upward at a retention time of 3.3 hr at 37°C. To determine the enzyme activity of the column, the effluent was collected at a retention time of 0.5 hr, and L-alanine produced in the effluent was measured.

Results and Discussion

Effect of cultivation time on enzyme activity and operational stability To apply the immobilized enzymes and immobilized microbial cells to industrial production of useful organic compounds requires high enzyme activity and good operational stability of the activity.

In the case of the fumarase activity of *B. flavum* immobilized with κ-carrageenan, the most stable preparation was obtained by immobilization of cells in the stationary growth-phase.[6] Therefore, we investigated the effect of cultivation time of *P. dacunhae* on the L-aspartate β-decarboxylase activity and operational stability of the immobilized preparation. Table 1 shows that the enzyme activities of intact cells cultivated for 24 hr or more and of immobilized preparations of these cells varied little with cultivation time. However, the immobilized cells cultivated for 24—28 hr, which corresponds to the stationary growth-phase, showed higher operational stability. In subsequent immobilization experiments, cells of 24-hr cultivation were used.

Table 1. Effect of cultivation time on the activity and the operational stability of L-aspartate β-decarboxylase of immobilized *P. dacunhae* cells.

Cultivation time (hr)	Cell wet weight (mg/ml)	Specific activity (units/g wet cell)		Half-life** at 37°C (day)
		Intact cells	Immobilized cells*	
20	18.5	177	98	31
24	21.5	225	114	46
28	21.4	220	106	44
32	20.8	225	111	30
40	20.0	217	118	30

* *P. dacunhae* cells were immobilized as described in the text and treated with 0.1 M glutaraldehyde in 0.2 M acetate buffer (pH 6) containing 2% KCl at 10°C for 10 min.

**Estimated by assuming exponential decay of the activity vs. time.

Effect of hexamethylenediamine for glutaraldehyde treatment on enzyme activity and operational stability When *E. coli* immobilized with κ-carrageenan was treated with glutaraldehyde in the presence of hexamethylenediamine, the operational stability of the aspartase activity of the immobilized preparation was doubled.[5] In the case of the immobilized *P. dacunhae*, all immobilized preparations treated with glutaraldehyde in the presence of hexamethylenediamine at various pHs showed similar enzyme activity but higher operational stability (Table 2) in comparison with non-treated preparations. In particular, the operational stability of the gels was increased by about 50% by the glutaraldehyde treatment at pH 7.

The effect of the glutaraldehyde concentration and treatment time on the enzyme activity and the operational stability of the immobilized preparation was investigated (Table 3). On treatment with glutaraldehyde at concentrations of 0.05 M and less in the presence of 0.1 M hexamethylenediamine, high activities were retained and the operational stabilities were similar to that on treatment with 0.1 M glutaraldehyde in the

Table 2. Effect of pH in hexamethylenediamine-glutaraldehyde treatment on activity and operational stability of L-aspartate β-decarboxylase of immobilized *P. dacunhae* cells.

pH*	Specific activity (units/g wet cell)	Half-life (day)
5	120	48
6	111	59
7	128	72
8	124	60
9	140	59

* The immobilized preparations were treated with 0.1 M glutaraldehyde in 0.2 M buffer solution (pH 5, 6: acetate, 7: phosphate, 8, 9: phosphate-borate) containing 0.1 M hexamethylenediamine and 2% KCl at 10°C for 10 min.

absence of the diamine (data not shown).

On treatment with 0.1 M glutaraldehyde in the presence of 0.1 M hexamethylenediamine, enzyme activities decreased while operational stabilities increased. The glutaraldehyde treatment for 30 min gave the maximum value of half-life, i.e. 81 days, together with a small decrease of the enzyme activity. The effect of hexamethylenediamine in glutaraldehyde treatment is thought to be tighten the cross-linkage of the cell wall and κ-carrageenan matrix, thus preventing leakage of the enzyme from the cells and the gel matrix. However, treatment with 0.2 M glutaraldehyde in the presence of 0.1 M hexamethylenediamine caused a sharp fall in

Table 3. Effect of glutaraldehyde treatment in the presence of hexamethylenediamine or L-lysine on activity and operational stability of L-aspartate β-decarboxylase of immobilized *P. dacunhae* cells.

Concentration (M)		Specific activity	Half-life
Hexamethylenediamine or L-lysine	Glutaraldehyde[a]	(units/g wet cell)	(day)
0	0	167	14
Hexamethylenediamine			
0.1	0.025	148	40
	0.05	159	41
	0.1[b]	128	72
	0.1	106	81
	0.1[c]	102	53
	0.2	25	—[d]
0.4	0.1	135	40
	0.2	108	45
	0.4	50	—[d]
L-Lysine			
0.2	0.2	105	44
	0.3	108	42
0.4	0.2	111	46
	0.3	116	67
	0.4	120	103
	0.5	112	102
	1.0	58	—[d]
1.0	0.5	120	75
	1.0	108	78

[a] The immobilized preparations were treated with glutaraldehyde for 30 min in 0.2 M phosphate buffer (pH 7) containing 2% KCl at 10°C.
[b] Treatment time, 10 min.
[c] Treatment time, 60 min.
[d] Half-life was not determined.

the enzyme activity. Treatment with 0.1—0.2 M glutaraldehyde in the presence of 0.4 M hexamethylenediamine gave similar enzyme activities to those obtained with 0.1 M glutaraldehyde in the presence of 0.1 M hexamethylenediamine, but lower operational stabilities. And treatment with 0.4 M glutaraldehyde in the presence of 0.4 M hexamethylenediamine also caused a sharp drop in the enzyme activity.

Effect of L-lysine in glutaraldehyde treatment on enzyme activity and operational stability In the batchwise decarboxylation reaction catalyzed by immobilized cells, the pH of the reaction mixture increased from 5.5 to 9.0 due to the liberation of carbon dioxide. Consequently, to produce L-alanine efficiently, an immobilized preparation with high activity at alkaline pH is required. When an enzyme is bound to a polyanionic carrier, the optimum pH for the enzyme reaction generally shifts to the alkaline side.[9] Therefore, we fixed L-lysine into the gel matrix, since L-lysine has two amino groups, which can interact with glutaraldehyde similarly to those of hexamethylenediamine, and one carboxyl group, which may make the microenvironmental pH within the preparation more acidic. Then we investigated the effect of glutaraldehyde treatment in the presence of L-lysine on the enzyme activity of the immobilized *P. dacunhae* and its operational stability.

Glutaraldehyde treatment at 0.2—0.3 M in the presence of 0.2 M L-lysine afforded operational stabilities similar to that obtained with 0.1 M glutaraldehyde in the absence of L-lysine (see Table 1). However, treatment with 0.4—0.5 M glutaraldehyde in the presence of 0.4 M L-lysine increased the half-life to 100 days. Higher concentrations of 0.5—1.0 M glutaraldehyde in the presence of 1 M L-lysine did not further enhance the operational stability.

Effect of pH on enzyme activity Figure 1 shows the effect of pH on the enzyme activity of various immobilized *P. dacunhae* preparations.

The pH activity curve of the preparation treated with 0.1 M glutaraldehyde in the presence of 0.1 M hexamethylenediamine was of similar profile to that of the nontreated preparation. On the other hand, the preparations treated with glutaraldehyde in the presence of L-lysine showed higher activity at alkaline pH. Furthermore, the optimum pH for enzyme reaction of the preparation treated with 1 M glutaraldehyde in the presence of 1 M L-lysine shifted from 6.0 to 6.5. This shift of pH activity curve may be attributable to the carboxyl group of L-lysine fixed within the immobilized preparation. Consequently, the immobilized preparation treated with glutaraldehyde in the presence of L-lysine was superior with regard to enzyme activity and operational stability to that treated with glutaraldehyde in the presence of hexamethylenediamine.

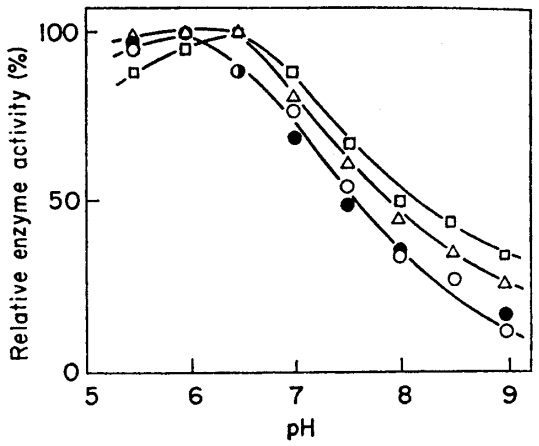

Fig. 1. Effect of pH on L-aspartate β-decarboxylase activity of various immobilized *P. dacunhae* preparations.
The enzyme activity of the immobilized cells was determined with 1.0 g of immobilized cells in 10 ml of 1 M phosphate buffer (pH 5.5~9.0) containing 0.4 M ammonium L-aspartate and 0.1 mM pyridoxal phosphate.
Symbols: ●, treated with 0.1 M glutaraldehyde in the presence of 0.1 M hexamethylenediamine at pH 7 for 30 min; △, treated with 0.4 M glutaraldehyde in the presence of 0.4 M L-lysine at pH 7 for 30 min; □, treated with 1 M glutaraldehyde in the presence of 1 M L-lysine at pH 7 for 30 min; ○, not treated.

We are now investigating a reactor system for continuous production of L-alanine from L-aspartic acid by immobilized *P. dacunhae* cells treated with 0.4 M glutaraldehyde in the presence of 0.4 M L-lysine, which is superior to those treated with 1 M glutaraldehyde in

the presence of 1 M L-lysine with regard to activity and operational stability.

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References

1) Chibata, I., Tosa, T.: *Adv. Appl. Microbiol.*, **22,** 1 (1977).
2) Chibata, I., Tosa, T., Sato, T.: *Appl. Microbiol.*, **27,** 878 (1974).
3) Tosa, T., Sato, T., Mori, T., Chibata, I.: *Appl. Microbiol.*, **27,** 886 (1974).
4) Sato, T., Mori, T., Tosa, T., Chibata, I., Furui, M., Yamashita, K., Sumi, A.: *Biotechnol. Bioeng.*, **17,** 1797 (1975).
5) Nishida, Y., Sato, T., Tosa, T., Chibata, I.: *Enzyme Microbial Technol.*, **1,** 95 (1979).
6) Takata, I., Yamamoto, K., Tosa, T., Chibata, I.: *Eur. J. Appl. Microbiol.*, **7,** 161 (1979).
7) Yamamoto, K., Tosa, T., Chibata, I.: *Biotechnol. Bioeng.*, **22,** 2045 (1980).
8) Snell, E. E.: *Methods in Enzymology*, (Colowick, S. P., Kaplan, N. O.), p. 477, Academic, New York (1957).
9) Goldstein, L., Levin, Y., Katchalski, E.: *Biochemistry*, **3,** 1913 (1964).

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