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Continuous Production of *L*-Malic Acid by Immobilized *Brevibacterium ammoniagenes* Cells*

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Summary. Continuous production of *L*-malic acid from fumaric acid using immobilized microbial cells was investigated. Several microorganisms having fumarase activity were immobilized into a polyacrylamide gel lattice. Among the microorganisms tested, immobilized *Brevibacterium ammoniagenes* IAM 1645 showed the highest enzyme activity, but produced an unwanted by-product, succinic acid. Conditions for suppression of this side reaction were investigated, and bile extract treatment of immobilized cells was found to be effective.

The bile extract treatment of immobilized cells also resulted in a marked increase of reaction rate for *L*-malic acid formation.

No difference was observed between the native enzyme and immobilized cells in optimal pH and temperature of the enzyme reaction.

The effect of temperature on the reaction rate and the stability of fumarase activity of an immobilized cell column were investigated under conditions of continuous enzyme reaction. The decay of enzyme activity during continuous enzyme reaction was expressed by an exponential relationship. Half-life of the fumarase activity of the immobilized cell column at 37°C was calculated to be 52.5 days.

Recently, industrial application of immobilized microbial cells as well as that of immobilized enzymes has been developing (Chibata et al., 1975). In previous papers, we reported that continuous production of *L*-aspartic acid (Chibata et al., 1974; Tosa et al., 1974; Sato et al., 1975), *L*-citrulline (Yamamoto et al., 1974a), urocanic acid (Yamamoto et al., 1974b), and 6-aminopenicillanic acid (Sato et al., 1976) are efficiently carried out by using microbial cells immobilized into a polyacrylamide gel lattice.

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In succession to them, we studied continuous production of *L*-malic acid from fumaric acid using immobilized microbial cells. The results are presented in this paper.

L-Malic acid is an essential compound in cellular metabolism, and is mainly used in the pharmaceutical field. That is, the acid is effective for the treatment of hepatic malfunctioning, especially for hyper-ammonemia (Szam et al., 1972), and is also used as one of the components of amino acid infusion.

MATERIALS AND METHODS

Materials. Acrylamide monomer, deoxycholic acid, and potassium persulfate were obtained from Katayama Chemical Industries, Co., Ltd., Osaka, Japan, and BIS and DMAPN from Tokyo Kasei Kogyo Co., Ltd., Tokyo, Japan. Bile acid was obtained from Difco Laboratories, Michigan, USA, and bile extract from Inolex Pharmaceutical Division, Wilson Pharmaceutical & Chemical Corporation, Illinois, USA. Fumaric acid was obtained from Kawasaki Kasei Kogyo Co., Ltd., Kanagawa, Japan.

Culture of Microorganisms. The medium for culture of the microorganisms having fumarase activity contained 2.0 % glucose, 0.5 % fumaric acid, 1.0 % corn steep liquor, 0.2 % urea, 0.2 % KH_2PO_4, and 0.05 % $MgSO_4 \cdot 7H_2O$ (pH 7.0). A loopful of microorganisms was inoculated in 100 ml of the medium which were distributed to a 500-ml shaking flask, and cultured at 30°C for 24 h with shaking (140 cpm, 8 cm stroke). The cells were collected by centrifugation and subjected to immobilization.

Immobilization of Microbial Cells. Immobilization of microbial cells by polyacrylamide gel was performed according to the procedure of Chibata et al. (1974). The cells (1 g of wet weight) were suspended in 4 ml of 0.9 % saline solution and 750 mg of acrylamide monomer and 40 mg of BIS were added to the suspension. Then 0.5 ml of 5 % DMAPN as an accelerator of polymerization and 0.5 ml of 1 % potassium persulfate as an initiator of polymerization were added. The suspension was allowed to stand at 25°C for 15 min to complete polymerization. The gel formed was made into particles of 9 mesh size, and thoroughly washed with 9 % saline solution.

Partial Purification of Fumarase from B.ammoniagenes. Ten grams of wet cells of *B.ammoniagenes* were suspended in 100 ml of 0.01 M potassium phosphate buffer (pH 7.0) and sonicated at 9 kc for 10 min. The cell debris was removed by centrifugation (12,000 × g, for 10 min), and the supernatant was fractionated with ammonium sulfate. The precipitate between 40 - 70 % saturation was collected by centrifugation and dialyzed overnight against 0.01 M

potassium phosphate buffer (pH 7.0). This preparation had an activity of 8.0 μmoles of *L*-malic acid/min/mg of protein under the standard assay conditions.

Standard Assay of Fumarase Activity. Unless otherwise noted, fumarase activity of immobilized cells was determined as follows. To 7.6 g of immobilized cells, corresponding to 1 g of intact cells, 30 ml of 1 M sodium fumarate (pH 7.5) were added, and the reaction mixture was incubated at 37°C for 1 h with shaking. The reaction was stopped by removing the gel by filtration, and *L*-malic acid formed was determined colorimetrically according to the method of Goodman & Stark (1957) after the remaining fumaric acid was removed as precipitate by acidifying the reaction mixture with an equal volume of 2N-HCl.

In the case of assay for native enzyme, a mixture of 0.05 ml of enzyme solution and 1.95 ml of substrate solution was incubated at 37°C for 1 h. The reaction was stopped by the addition of an equal volume of 2N-HCl, and *L*-malic acid formed was determined in the same manner as for immobilized cells.

Determination of Succinic Acid. After the reaction reached equilibrium, the reaction mixture was acidified by the addition of one-fifth volume of 38 % HCl and the resulting precipitate of fumaric acid was removed by centrifugation, and the content of succinic acid in the supernatant was estimated by paper chromatography (solvent, n-buthanol:acetic acid:H_2O=4:1:1; indicator, 0.1 % bromphenol blue in methanol).

Determination of Fumaric Acid. Concentration of fumaric acid was measured spectrophotometrically at 280 nm according to the method of Bock & Alberty (1953).

RESULTS

Immobilization of Microorganisms Having Fumarase Activity

To select the most active immobilized microorganism, several microorganisms having fumarase activity were immobilized into polyacrylamide gel and the enzyme activities of immobilized cells were determined. As shown in Table 1, *Brevibacterium ammoniagenes* IAM 1645 showed the highest enzyme activity among the microorganisms tested both in the presence and absence in the reaction mixture of CPC, which accelerates the permeability of substrate and/or product through the cell membrane.

Table 1. Fumarase activity of immobilized microbial cells

Microorganism	Formation of *L*-malic acid (mmoles/h/g of cells)	
	None	CPC[a]
Brevibacterium ammoniagenes IAM 1645	0.49	3.37
Corynebacterium equi IAM 1038	0.09	0.42
Escherichia coli ATCC 11303	0.27	0.32
Microbacterium flavum IAM 1642	0.20	0.48
Proteus vulgaris IFO 3045	0.16	0.26

[a]CPC was added in the reaction mixture, at the concentration of 0.02 %.

Suppression of Formation of Succinic Acid

When immobilized cells of *B.ammoniagenes* were used for the production of *L*-malic acid from fumaric acid, unwanted formation of

Table 2
Effect of various treatments on formation of *L*-malic acid and succinic acid

Treatment	Formation of *L*-malic acid (mmoles/h/g of cells)		Formation of succinic acid (mole % of *L*-malic acid)
	a	b	
Control (no treatment)	0.49	4.57	2.5 - 5.0
Autolysis of cells 37°C, 20 h	3.83	4.45	2.5 - 5.0
Freeze-thawing of cells -20°C, 2.5 h	2.57	4.91	2.5 - 5.0
24 h	2.86	5.82	2.5 - 5.0
Heat treatment of cells 55°C, 1 h	3.30	7.04	2.5 - 5.0
Heat treatment of immobilized cells 55°C, 1 h	1.63	4.66	2.5 - 5.0

Autolysis, freeze-thawing, or heat-treatment of cells was carried out by suspending 1 g of cells in 4 ml of the supernatant of broth at the indicated conditions. Heat treatment of immobilized cells was carried out by suspending 7.6 g of the immobilized cells in 30 ml of 0.05 M phosphate buffer (pH 7.0) at the indicated conditions. Enzyme activity was determined immediately after immobilization (a) and after treatment of immobilized cells with 0.02 % CPC to obtain high reaction rate (b). CPC treatment was carried out by incubating 7.6 g of immobilized cells with 30 ml of 1 M sodium fumarate (pH 7.5) containing 0.02 % CPC at 37°C for 20 h, and the gel was thoroughly washed with 0.9 % saline solution.

succinic acid was observed. Since it is difficult to remove the by-product from *L*-malic acid, conditions to suppress the side reaction were investigated.

1) Autolysis, Freeze-Thawing, Heat Treatment, and Acetone Treatment. Autolysis, freeze-thawing, and heat treatment of intact cells and heat treatment of immobilized cells were carried out, but these treatments did not successfully suppress the formation of succinic acid (Table 2). Acetone treatment of immobilized cells which were previously treated with CPC was effective under very restricted conditions as shown in Table 3, that is, at 25°C and 50 % concentration of acetone. Treatments with lower concentrations of acetone and lower temperatures, on the contrary, increased the ratio of side reaction (Table 3). Since it is considerably difficult to control temperature and concentration of

Table 3
Effect of acetone treatment on formation of *L*-malic acid and succinic acid

Conditions for treatment			Formation of *L*-malic acid (mmoles/h/g of cells)	Formation of succinic acid (mole % of *L*-malic acid)
Concentration of acetone (%)	Temperature (°C)	Time (min)		
Control (no treatment)			4.57	2.5 - 5.0
0	25	60	5.23	2.5 - 5.0
30	"	"	7.71	> 5.0
38	"	"	7.25	> 5.0
43	"	"	7.45	1.0 - 2.5
50	"	"	7.43	< 0.2
67	"	"	6.02	< 0.2
50	10	60	6.41	> 5.0
"	"	120	6.72	> 5.0
"	20	60	7.37	1.0 - 2.5
"	"	120	7.51	0.2 - 1.0
"	25	30	7.44	0.2 - 1.0
"	"	60	7.43	< 0.2
"	"	120	7.06	< 0.2
"	30	30	5.25	0.2 - 1.0
"	40	"	1.21	< 0.2
"	50	"	1.17	< 0.2

The immobilized cells (7.6 g) pre-treated with CPC were suspended in 30 ml of 0.05 M phosphate buffer (pH 7.0) containing the indicated concentration of acetone. The suspension was shaken at the indicated temperature and for the indicated time. The enzyme activity was determined after washing the gel thoroughly with 0.9 % saline solution.

acetone in such narrow ranges on a large scale, other methods suitable for industrial application were investigated.

2) Detergent Treatments. The results of detergent treatments of immobilized cells are shown in Table 4. Treatments with cationic (CPC) and anionic (SLS) detergents markedly increased the formation of *L*-malic acid, but did not completely suppress the formation of succinic acid. Non-ionic detergent (SL-10) did not affect the formation of *L*-malic acid and succinic acid.

On the other hand, treatments with bile acid, bile extract, and deoxycholic acid, which are ordinarily used as mild solubilizers of membrane or particle-bound enzymes, strikingly decreased the ratio of succinic acid formation to *L*-malic acid formation. However, treatment with Triton X-100, which is used for the same purpose as deoxycholic acid, did not suppress but rather stimulated the formation of succinic acid. For industrial purpose, bile extract treatment is economically most suitable as the extract is readily available. Thus the detailed conditions for bile extract treatment were investigated as follows.

Table 4
Effect of detergent treatments on formation of *L*-malic acid and succinic acid

Treatment		Formation of *L*-malic acid (mmoles/h/g of cells	Formation of succinic acid (mole % of *L*-malic acid
Detergent	Concentration (%)		
Control[a]		0.49	—
No addition		0.99	2.5 - 5.0
CPC	0.02	4.57	2.5 - 5.0
"	0.16	3.07	1.0 - 2.5
SLS	0.02	6.05	1.0 - 2.5
SL-10	0.02	1.22	2.5 - 5.0
Triton X-100	0.20	5.36	> 5.0
Bile acid	0.20	6.57	< 0.2
Bile extract	0.20	7.48	< 0.2
Deoxycholic acid	0.20	7.38	< 0.2

[a]Immediately after immobilization.

Immobilized cells (7.6 g) were suspended in 30 ml of 1 M sodium fumarate (pH 7.5) containing one of the detergents shown in the table and stood at 37°C for 20 h. The enzyme activity was determined after washing the gel thoroughly with 0.9 % saline solution.

Conditions for Bile Extract Treatment

1) Concentration of Bile Extract. The concentration of bile extract necessary to suppress the side reaction was tested, and the results are shown in Table 5. The data indicate that a concentration above 0.2 % is necessary under the conditions employed, and 0.3 % is considered to be most suitable.

Table 5
Effect of concentration used for bile extract treatment on formation of *L*-malic acid and succinic acid

Concentration of bile extract (%)	Formation of *L*-malic acid (mmoles/h/g of cells)	Formation of succinic acid (mole % of *L*-malic acid)
None	0.72	2.5 - 5.0
0.05	0.77	2.5 - 5.0
0.10	4.18	1.0 - 2.5
0.20	7.48	< 0.2
0.30	7.71	< 0.2
0.40	7.29	< 0.2

Conditions are given in the legend to Table 4 except for the concentration of bile extract.

2) Temperature. The effect of temperature on the suppression of the side reaction was investigated. As shown in Table 6, a temperature above 30°C was found to be desirable to suppress the side reaction and 37°C is considered to be most favorable.

Table 6
Effect of temperature used for bile extract treatment on formation of *L*-malic acid and succinic acid

Temperature (°C)	Formation of *L*-malic acid (mmoles/h/g of cells)	Formation of succinic acid (mole % of *L*-malic acid)
5	6.46	1.0 - 2.5
20	6.64	0.2 - 1.0
30	6.95	< 0.2
37	7.38	< 0.2
45	6.91	< 0.2

Immobilized cells (7.6 g) were suspended in 30 ml of 1 M sodium fumarate (pH 7.5) containing 0.3 % bile extract and stood at the indicated temperature for 20 h. Other conditions are given in the legend to Table 4.

3) pH. The effect of pH used for bile extract treatment on the formation of *L*-malic acid and succinic acid was investigated (Table 7). The data show that the rates of formation of both acids are almost the same in the ranges of pH 5 - 8, but slightly decreased at pH 9.0.

Table 7
Effect of pH used for bile extract treatment on formation of *L*-malic acid and succinic acid

pH	Formation of *L*-malic acid (mmoles/h/g of cells)	Formation of succinic acid (mole % of *L*-malic acid)
5.0	7.90	< 0.2
6.0	7.87	< 0.2
6.5	7.41	< 0.2
7.0	7.83	< 0.2
7.5	7.63	< 0.2
8.0	7.23	< 0.2
9.0	6.63	< 0.2

Conditions are given in the legend to Table 4 except for pH at the treatment. The buffers employed are 0.03 M acetate (pH 5.0 - 6.0), phosphate (pH 6.5 - 7.5), and borate-phosphate (pH 8.0 - 9.0), respectively.

From these experiments optimal conditions for bile extract treatment were determined to be as follows: Immobilized cells (7.6 g) are suspended in 30 ml of 1 M sodium fumarate (pH 7.5) containing 0.3 % bile extract, and stood at 37°C for 20 h. A summary of fumarase activity of *B.ammoniagenes* cells after bile extract treatment is shown in Figure 1. Compared to intact cells, immobilized cells showed 73 % of the activity after bile extract treatment, though they showed only 5 % of the activity immediately after immobilization.

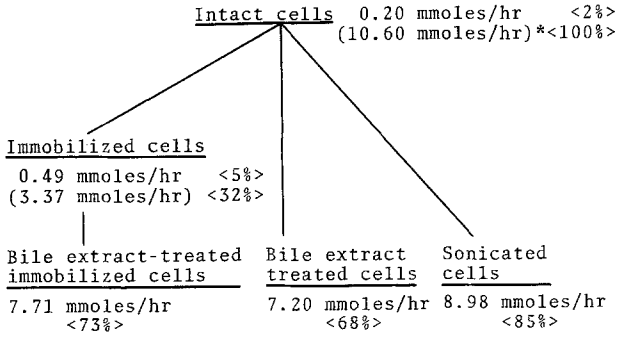

Fig.1
Comparison of fumarase activity of cells after various treatments. The activity indicated is mmoles of *L*-malic acid formed per hour per one gram of cells. Conditions for bile extract treatment of cells are given in the text

Enzymatic Properties of Immobilized Cells

In order to establish the most suitable conditions for production of *L*-malic acid by immobilized *B.ammoniagenes* cells, some enzymatic properties of bile extract-treated immobilized cells were investigated and compared to those of the native fumarase.

1) Effect of pH. Effect of pH on the rate of formation of *L*-malic acid is shown in Figure 2. The optimal pH was 7.0 - 7.5 for both native enzyme and immobilized cells.

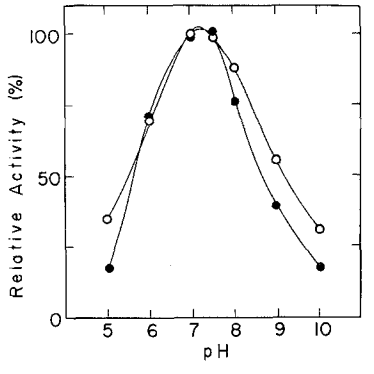

Fig.2
Effect of pH on reaction rate. Conditions are given in the text except for employing 0.5 M substrate in 0.1 M buffer solution. The buffers are acetate (pH 5.0 - 6.0), phosphate (pH 7.0 - 7.5), borate-phosphate (pH 8.0 - 9.0), and borate-carbonate (pH 10.0), respectively. Symbols: ● native enzyme; o immobilized cells

2) Effect of Temperature. The effect of temperature on the formation of *L*-malic acid is shown in Figure 3. The optimal temperature was around 60°C for both native enzyme and immobilized cells. From the data the apparent activation energy was calculated using an Arrhenius plot, and the value for native enzyme was 8,800 cal/mole and that for immobilized cells was 5,801 cal/mole, respectively.

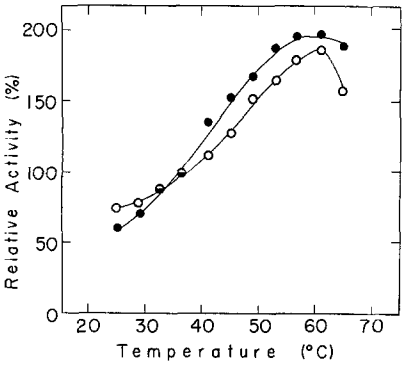

Fig.3
Effect of temperature on reaction rate. Conditions are given in the text except for the reaction temperature. Symbols: ● native enzyme; o immobilized cells

3) Effect of Temperature on Equilibrium Constant. Since there is an equilibrium in the reaction of fumarase, the equilibrium constant, K_{eq}([*L*-malate]/[fumarate]), was determined at indicated temperatures using 1 M sodium fumarate (pH 7.0) as substrate.

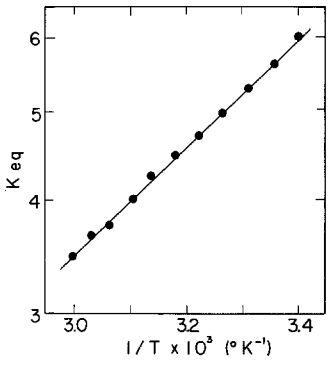

Fig.4
Effect of temperature on equilibrium constant. After the reaction reached equilibrium, concentrations of *L*-malic acid and fumaric acid were determined

The K_{eq} value at 37°C was found to be 4.73. The log plot of K_{eq} against 1/T is shown in Figure 4. A straight line was obtained and the heat of reaction was calculated to be -2,600 cal/mole from the slope of this line.

Comparison of Stability of Fumarase Activities of Intact Cells and Immobilized Cells

To clarify the superiority of immobilized cells over intact cells under conditions of continuous enzyme reaction, the stability of the fumarase activity of each of them was investigated. Both intact cells and immobilized cells were treated with bile extract and were stood in 1 M sodium fumarate (pH 7.0) at 37°C. At the indicated intervals the remaining activities of the cells and immobilized cells were determined. The results are shown in Figure 5. It is clear that stability of immobilized cells is much higher than that of intact cells.

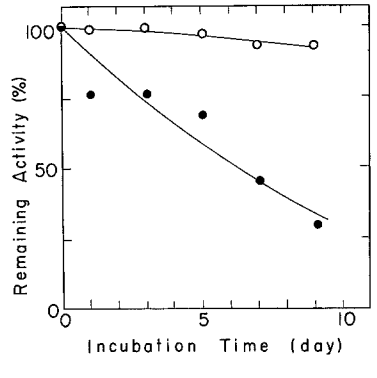

Fig.5
Comparison of stability of fumarase activity of cells and immobilized cells. Bile extract treatment of *B.ammoniagenes* cells was carried out as follows. One gram of intact cells was suspended in 5 ml of 1 M sodium fumarate (pH 7.5) containing 0.3 % bile extract and stood at 37°C for 20 h. Then, the suspension was dialyzed at 20°C overnight against 1 M sodium fumarate (pH 7.0). Other conditions are given in the text. Symbols: ● cells; o immobilized cells

Continuous Production of *L*-Malic Acid Using a Column Packed with Immobilized Cells

Conditions for continuous production of *L*-malic acid were investigated using a column packed with bile extract-treated im-

mobilized *B.ammoniagenes* cells and 1 M sodium fumarate (pH 7.0) as substrate.

1) Effect of Temperature and Flow Rate of Substrate Solution on Formation of L-Malic Acid. The relation between flow rate of substrate solution and formation of *L*-malic acid at various temperatures is shown in Figure 6. At 37°C the reaction reached equilibrium when flow rates of substrate solution were below SV = 0.23 or above 4.3 h of retention time.

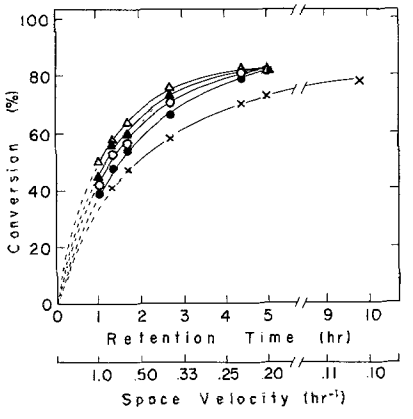

Fig.6
Effect of temperature and flow rate of substrate solution on formation of *L*-malic acid. Conditions are given in the text. Column size is 1.6 × 17.5 cm. Symbols: Δ 45°C; ▲ 41°C; o 37°C; ● 33°C; x 29°C

2) Effect of Temperature on Stability of Immobilized Cells. The stability of the fumarase activity of the immobilized cell column was investigated by continuously flowing substrate solution (SV = 0.71) at various temperatures. From a graph of log plots of reaction rate against operation period, the decay of enzyme activity was found to be well expressed by an exponential relationship, $\Phi = \exp(-K_d \cdot \theta)$, where Φ is the ratio of decay of activity, K_d is a deactivation rate constant, and θ is time of operation. The k_d value of each temperature was estimated by the least square regression method, and the relationship between log K_d and 1/T is shown in Figure 7. A straight line was obtained, indicating that K_d is expressed by an Arrhenius type equation, $K_d = A_d \cdot \exp(-E_d/RT)$, where A_d is a frequency factor, R is the gas constant, and E_d is a deactivation energy, respectively. From the least square regression method the values of A_d and E_d were calculated to be 3,788/day and 7,741 cal/mole, respectively. The deactiviation rate constant of indicated temperature was calculated from the equation, $K_d = 3{,}788 \cdot \exp(-7{,}741/RT)$, and the calculated decay line of each temperature was drawn in Figure 8. The experimental data points were well coincident with the calculated line at each temperature. The half-life of fumarase activity of the immobilized cell column operated at 37°C was calculated to be 52.5 days under the conditions employed.

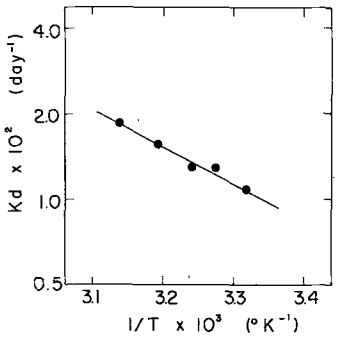

Fig.7

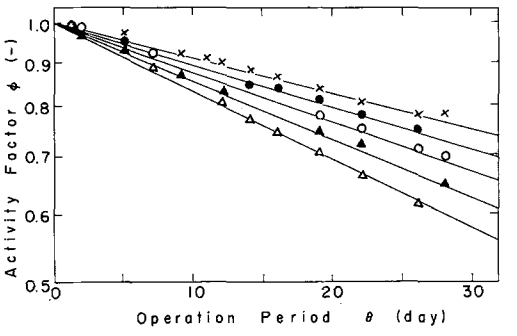

Fig.8

Fig.7
Relationship between temperature and deactivation rate constant. Conditions are given in the text

Fig.8
Effect of temperature on stability of fumarase activity of immobilized cell column. Conditions are given in the text. Symbols: x 29°C; ● 33°C; o 37°C; ▲ 41°C; Δ 45°C

Since it has been found that divalent metal ions such Ca^{2+}, Mg^{2+}, and Mn^{2+} stabilize the enzyme activity of immobilized cells (Chibata et al., 1974; Tosa et al., 1974; Yamamoto et al., 1974b), the effect of these metal ions on the stability of fumarase activity of immobilized *B.ammoniagenes* cells was investigated. These metal ions, however, did not improve the stability of the immobilized cell column.

3) Preparation of L-Malic Acid. A solution of 1 M sodium fumarate (pH 7.0) was passed through the immobilized cell column (1.6 × 17.5 cm) at 37°C at flow rate of SV = 0.23. To 1 l of the effluent of the column, 200 ml of 38 % HCl were added and the fumaric acid precipitated was recovered by filtration. About 70 g of $Ca(OH)_2$ were added to the filtrate until pH 6 - 7. After standing for 16 h at 5°C, the resulting crystals of calcium *L*-malate dihydrate were collected by filtration, and dried at 50°C. Yield was 150 g (90 % based on effluent content). The calcium *L*-malate dihydrate obtained was suspended in 1 l of H_2O, and 70 ml of 60 % (v/v) H_2SO_4 were added. The resulting precipitate of $CaSO_4$ was removed by filtration and the filtrate was passed through a column (150 ml) of Amberlite IR-120 (H^+ form) and then through a column (150 ml) of Amberlite IRA-93 (OH^- form). The columns were washed with 500 ml of H_2O. The effluent was concentrated at 60°C in vacuo. To the syrup of *L*-malic acid 700 ml of isopropyl alcohol were added, and then concentrated in vacuo. The resulting crystals of *L*-malic acid were collected by filtration and dried at 50°C. Yield was 53 g

(55 % based on calcium *L*-malate dihydrate); $[\alpha]^{2}_{D}{}^{o}$ = - 2.2 (c = 4.0 in H_2O), mp = 100°C. The mother liquor can be used for another lot, or *L*-malic acid in the mother liquor can be recovered as the calcium salt in a good yield. The total yield of *L*-malic acid from fumaric acid was about 70 %.

DISCUSSION

Industrial production of *L*-malic acid from fumaric acid has been performed by enzymatic batch processing using the broth of *Lactobacillus brevis* (Kitahara et al., 1960). From an industrial standpoint a continuous enzyme reaction system using immobilized enzyme is considered to be more advantageous than a batch process using soluble enzyme from a microbial broth (Messing, 1975). Recently, Marconi et al. (1975) reported that fumarase can be efficiently immobilized into cellulose triacetate, and that the immobilized fumarase makes it possible to develop an economically attractive method to produce *L*-malic acid.

In our previous papers we revealed that microbial cells immobilized into polyacrylamide gel are very advantageous for industrial production of useful compounds such as *L*-aspartic acid (Chibata et al., 1974; Tosa et al., 1974; Sato et al., 1975), *L*-citrulline (Yamamoto et al., 1974a), urocanic acid (Yamamoto et al., 1974b), and 6-aminopenicillanic acid (Sato et al., 1976). The merits of using immobilized cells instead of immobilized enzymes for continuous enzyme reaction systems are as follows: (1) Processes for extraction and purification of enzymes are not necessary. (2) Yield of enzyme activity on immobilization is high. (3) Operational stability is generally high. (4) Cost of enzyme is low. However, the demerit of using immobilized cells is that one or more side reactions may occur.

In the present case unwanted formation of succinic acid occurs. So the suppression of this side reaction was the key point for application of immobilized *B.ammoniagenes* cells to industrial production of *L*-malic acid. Conditions suppressing the side reaction were investigated. As the results show, acetone treatment (Table 3) and bile acid, bile extract, or deoxycholic acid treatment (Table 4) were found to be effective. Among these treatments bile extract treatment of immobilized cells was considered to be most effective and easily applicable, and the best conditions for bile extract treatment were determined (Tables 5 - 7). The effect of acetone or bile extract on suppression of succinic acid formation may possibly be explained as follows: (1) Stabilities of fumarase and enzymes related to succinic acid formation respond differently to these treatments. (2) Co-factors necessary for succinic acid formation are leaked out from the cells, or the regenerating systems of the co-factors are inactivated by these treatments. In addition to the

suppression of succinic acid formation, the membrane barrier for substrate and/or product transport is destroyed by these treatments, resulting in the increase of reaction rate.

Optimal pH and temperature of the reaction were not different for native fumarase and bile extract-treated immobilized cells, but apparent activation energy for immobilized cells was considerably lower than that for native enzyme (Fig.3).

Stability of fumarase activity was much increased by immobilization of cells (Fig.5) as in the cases of aspartase of immobilized *Escherichia coli* (Chibata et al., 1974) and *L*-arginine deiminase of immobilized *Pseudomonas putida* (Yamamoto et al., 1974a). This is one of the merits of using immobilized microbial cells.

The decay of fumarase activity of the immobilized cell column was well expressed by an exponential relationship involving operation period at any temperature, $\Phi = \exp[-3{,}788\ \exp(-7{,}741/RT)\cdot\theta]$ (Fig.8). Further, the relationship between temperature and reaction rate, r, can be expressed by an Arrhenius equation, $r = 71.2\cdot\exp(-5{,}801/RT)$ (Fig.3). From these equations the decrease of reaction rate at any temperature after a certain period of operation can be estimated and compensation can be made by raising reaction temperature to overcome gradual inactivation. That is, the temperature policy can be determined to maintain the productivity of the immobilized cell column at a constant level.

Theoretically, 15.4 metric tons of *L*-malic acid can be produced in one month using a 1,000-l volume column fed at a flow rate of 200 l/h of 1 M sodium fumarate (pH 7.0). We have been operating this immobilized *B.ammoniagenes* cell column for the industrial production of *L*-malic acid since 1974, and we are satisfied with this system from the viewpoints of product quality and economics.

ABBREVIATIONS

BIS = N,N'-methylenebisacrylamide
CPC = cetylpyridinium chloride
DMAPN = β-dimethylaminopropionitrile
SL-10 = sorbitan monolaurate
SLS = sodium lauryl sulfate

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