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Characterization of Bioconversion of Fumarate to Succinate by Alginate Immobilized *Enterococcus faecalis* RKY1

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Abstract

In this study, the immobilization characteristics of *Enterococcus faecalis* RKY1 for succinate production were examined. At first, three natural polymers—agar, κ-carrageenan, and sodium alginate—were tried as immobilizing matrices. Among these, sodium alginate was selected as the best gel for immobilization of *E. faecalis* RKY1. Efficient conditions for immobilization were established to be with a 2% (w/v) sodium alginate solution and 2-mm-diameter bead. The bioconversion characteristics of the immobilized cells at various pH values and temperatures were examined and compared with those of free cells. The optimum pH and temperature of the immobilized cells were the same as for free cells, 7.0 and 38°C respectively, but the conversion ratio was higher by immobilization for all the other pH and temperature conditions tested. When the seed volume of the immobilized cells was adjusted to 10% (v/v), 30 g/L of fumarate was completely converted to succinate (0.973 g/g conversion ratio) after 12 h. In addition, the immobilized cells maintained a conversion ratio of >0.95 g/g during 4 wk of storage at 4°C in a 2% (w/v) $CaCl_2$ solution. In repetitive bioconversion experiments, the activity of the immobilized cells decreased linearly according to the number of times of reuse.

Index Entries: *Enterococcus faecalis* RKY1; succinate; fumarate; immobilized cells; bioconversion.

Introduction

Succinic acid is a C_4-dicarboxylic acid produced as an intermediate of the tricarboxylic acid cycle and also as one of the fermentation products of

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anaerobic metabolism *(1,2)*. It has many industrial applications as a raw material for food, medicine, plastics, cosmetics, textiles, plating, and wastegas scrubbing *(3,4)*. Currently, it is produced commercially by petrochemical processes. Recently, however, great interest has been focused on the enhanced production of succinic acid through an industrial fermentation process *(5)*. Production of succinic acid using a fermentation process represents an alternative synthesis route via the utilization of renewable feedstocks such as corn *(6)*. Microorganisms have the ability to modify chemically a wide variety of organic compounds, referred to as bioconversion. One such example is the previously reported bioconversion of fumarate to succinate by *Enterococcus faecalis* RKY1 *(4,7)*. This strain is able to produce succinic acid at a high yield if cultured anaerobically with glycerol as a hydrogen donor and fumaric acid as a hydrogen acceptor.

Immobilized microorganism technology is increasingly used for producing biochemicals and useful products, such as ethanol and organic acids *(8)*. However, the industrial application of immobilized cells for succinic acid production has rarely been reported. Immobilization is the restriction of cell mobility within a defined space *(9)*. Immobilized cell cultures have the following potential advantages over suspension culture: high cell concentration, high productivity, cell reuse, and reduced cost for cell recovery and recycling *(8,10)*. Despite these advantages, the development of an immobilization technique for succinic acid production has rarely been studied.

Calcium alginate gels are now one of the most widely used supports for the immobilization of whole microbial cells *(11,12)*. Entrapment of cells in alginate is one of the simplest methods of immobilization *(13)*. Alginates are available commercially as water-soluble sodium alginate and have been used for more than 65 yr in the food and pharmaceutical industries as thickening, emulsifying, film forming, and gelling agents *(9)*. Entrapment in insoluble calcium alginate gels is recognized as a rapid, nontoxic, inexpensive, and versatile method for immobilization of cells *(14)*.

In the present study, *E. faecalis* RKY1 was used for bioconversion of fumarate to succinate by cell immobilization. Sodium alginate was used as a supporting material because it forms gels with divalent ions like calcium. We investigated the optimum conditions for whole-cell immobilization of *E. faecalis* RKY1 cells and examined the effect of various culture conditions on succinate production.

Materials and Methods

Microorganism and Medium

E. faecalis RKY1 was isolated from our laboratory culture with respect to its ability to convert fumarate to succinate at a high yield *(15)*. The medium for cell growth contained the following: 10 g of glycerol (Yakuri, Osaka, Japan), 22 g of fumaric acid (Yakuri), 15 g of yeast extract (Difco, Detroit, MI), 10 g of K_2HPO_4 (Yakuri), 1 g of NaCl (Junsei, Tokyo, Japan),

20 g of Na_2CO_3 (Yakuri), 0.05 g of $MgCl_2 \cdot 6H_2O$, 0.01 g of $FeSO_4 \cdot 7H_2O$, and 1 L of distilled water. The medium for bioconversion contained the following: 20 g of glycerol, 22 g of fumaric acid, 15 g of yeast extract, 0.5 g of K_2HPO_4, 1 g of NaCl, 20 g of Na_2CO_3, 0.05 g of $MgCl_2 \cdot 6H_2O$, 0.01 g of $FeSO_4 \cdot 7H_2O$, and 1 L of distilled water. The seed culture broth used in this experiment was transferred to the new media every 6 h or 12 h for 2 d. Cell stocks were made by mixing the culture with sterile glycerol and then stored at –20°C.

Cell Immobilization

Cells were grown at 38°C for 5 to 6 h and then harvested by centrifugation (Vision Scientific, Taejon, Korea) at 18,600*g* for 10 min. After discarding the supernatant, the harvested cells were washed twice with 0.1 *M* Tris-HCl buffer (pH 7.0) and then resuspended to a cell concentration of 2.5 g of dry cell weight/100 mL of buffer solution in 0.1 *M* Tris-HCl buffer. One hundred milliliters of the cell suspension was mixed with 100 mL of a 1–4% (w/v) sodium alginate (Junsei) solution. This mixture of alginate and cells was dropped into a sterile cold 2% (w/v) $CaCl_2$ (Kanto, Tokyo, Japan) solution using a blunt-ended needles connected to a peristaltic pump. Calcium alginate gel beads of approx 2–4 mm in diameter were obtained by using needles of various diameters. After immobilization, the beads were washed with sterilized distilled water, placed into a 2% (w/v) $CaCl_2$ solution, and gently agitated in order to increase the strength of the beads. They were stored in a 2% (w/v) $CaCl_2$ solution at 4°C until use. The agar (Junsei) and κ-carrageenan (Sigma, St. Louis, MO) gels were prepared according to the methods of Han and Chung *(16)*.

Culture Conditions

Batch cultures for cell growth were conducted in 100-mL vials containing 80 mL of growth medium. These cultures were incubated in a shaking incubator (Vision Scientific) at 200 rpm and 38°C. Bioconversion by free cells was prepared by inoculating 0.6 mL of resting cells into the culture medium (15 mL) in 20-mL vials, followed by incubation at 38°C and 200 rpm for 6 h or 12 h in a shaking incubator. For the bioconversion experiments using the immobilized cells, 10% (v/v) of the immobilized cells was inoculated into 20-mL vials containing 15 mL of medium and 100-mL vials containing 80 mL of medium according to experimental condition. These bioconversions were conducted at 38°C and 150 rpm for 12 h in a shaking incubator. Before the cultivations of free and immobilized cells, the inoculated vials were vacuum degassed for 2 min, and high-purity CO_2 gas was charged in these vials for 2 min to incubate the strain anaerobically.

Analytical Methods

Succinic acid and fumaric acid were quantitatively analyzed by high-pressure liquid chromatography with a pump (Waters 510; Millipore), a

Bio-Rad Aminex HPX-87H ion-exclusion column (7.8 × 300 mm; Bio-Rad, Hercules, CA), and an ultraviolet (UV) detector (Waters 486; Millipore). The column was eluted with 5 m*M* sulfuric acid at a flow rate of 0.6 mL/min and column temperature of 35°C. In this study, the amount of succinic acid produced was expressed as sodium succinate (MW 162.14) and residual fumaric acid as sodium fumarate (MW 160.04).

Cell growth of the free cells was measured as the optical density at 660 nm (OD_{660}) with a UV spectrophotometer (UV-160A; Shimadzu, Japan). One optical unit for *E. faecalis* RKY1 is equivalent to 1.118 g of dry cell weight/L. To estimate the effluent cell concentration from immobilized cells, 1 mL of sample was centrifuged at 16,000*g* for 10 min, the supernatant discarded, and the pellet washed twice with deionized water. The pellet was then resuspended in 1 mL of deionized water. This resuspension was used to determine the OD_{660}.

Results and Discussion

Determination of Gel Matrix

Immobilized cells entrapped in three natural polymers typically used for immobilization of enzymes and cells—agar, κ-carrageenan, and sodium alginate *(9)*—were compared for their conversion ratio and effluent cells. Table 1 gives the effect of each immobilizing carrier on succinate production and effluent cell concentration. The conversion ratio (grams/gram) term is expressed as the value of succinate produced per initial fumarate. It was shown that with an initial fumarate concentration of 30 g/L, about 0.96 g/g (28.9 g/L) was converted to succinate after a 12-h incubation with the alginate-entrapped cells, whereas when the cells were entrapped in the agar or κ-carrageenan gels, only about 0.60 g/g (18.7 and 18.0 g/L, respectively) of fumarate was converted to succinate after the 12-h incubation. Effluent cells from agar, Ca-alginate, and κ-carrageenan gels were 2.3, 0.29, and 0.31 g/L, respectively. Since the agar and κ-carrageenan gels were formed by lowering the temperature, the strength of these gels was somewhat lower and, therefore, the effluent cell concentrations from these gels were higher. Furthermore, these gels have disadvantages in that the preparation of beads with these types of gels is quite difficult and that many of the microbial cells that were immobilized were killed or damaged owing to the high temperature necessary to keep the gel molten. By contrast, Ca-alginate gels have a higher rigidity and the preparation of their beads is quite easy. Thus, sodium alginate was selected for the immobilization of *E. faecalis* RKY1.

Concentration of Sodium Alginate

The matrix for Ca-alginate bead was prepared using a solution of sodium alginate between 1 and 4% (w/v). The beads of 1% (w/v) sodium alginate were not rigid enough and, therefore, were not spherical. The 4% (w/v) sodium alginate solution was difficult to work with because of

Table 1
Effect of Gel Matrix Used for Cell Immobilization on Bioconversion of Fumarate to Succinate and Effluent Cell Concentration[a]

Type of matrix	Succinate concentration (g/L)	Residual fumarate (g/L)	Conversion ratio (g/g)	Effluent cell concentration (g/L)
Free cell	29.5	0.5	0.983	4.5
Ca-alginate[b]	28.9	1.1	0.965	0.29
Agar[c]	18.7	11.3	0.622	2.3
κ-Carrageenan[c]	18.0	12.0	0.599	0.31

[a]Bioconversion was conducted in a 20-mL vial with 15 mL of medium at 38°C for 12 h. The seed volume of immobilized cells was 10% (v/v). The initial fumarate concentration was 30 g/L. The conversion ratio (g/g) is expressed as the value of succinate produced per initial fumarate.
[b]Bead-type gel of 3 mm in diameter.
[c]Cubic-type gel 3 × 3 × 3 mm.

Table 2
Effect of Sodium Alginate Concentration on Bioconversion of Fumarate to Succinate and Effluent Cell Concentration[a]

Sodium alginate concentration (% [w/v])	Succinate concentration (g/L)	Residual fumarate (g/L)	Conversion ratio (g/g)	Effluent cell concentration (g/L)
1.0	29.5	0.5	0.983	0.30
2.0	29.2	0.8	0.973	0.27
3.0	28.7	1.3	0.958	0.17
4.0	28.6	1.4	0.954	0.17

[a]Bioconversion was conducted in a 20-mL vial with 15 mL of medium at 38°C for 12 h. The seed volume of immobilized cells was 10% (v/v). The initial fumarate concentration was 30 g/L. The conversion ratio (g/g) is expressed as the value of succinate produced per initial fumarate.

its high viscosity. As shown in Table 2, the 2% (w/v) sodium alginate concentration was determined to be the optimum concentration for efficient bioconversion. When the concentration of the sodium alginate solution was 2% (w/v), about 0.97 g/g of the initial fumarate, 30 g/L, was converted to succinate and the effluent cell concentration was 0.27 g/L. Since an increase in the sodium alginate concentration would reduce the pore size of the Ca-alginate beads, both the conversion ratio and effluent cell concentration would also decrease. Klein et al. *(11)* also reported that as the pore size of Ca-alginate beads decreased, the mass transfer rate within the beads was decreased accordingly.

$CaCl_2$ Concentration

$CaCl_2$ must be added to the medium in order to harden the Ca-alginate beads during cultivation *(12)*. Therefore, the effect of the amount of $CaCl_2$

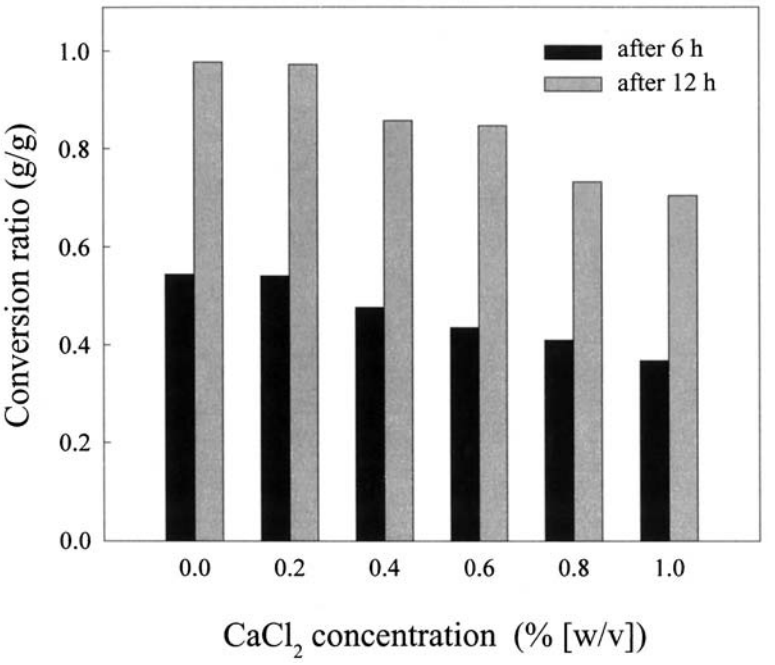

Fig. 1. Effect of $CaCl_2$ concentration added to the medium on bioconversion of fumarate to succinate by alginate-entrapped *E. faecalis* RKY1. Bioconversion was conducted in a 20-mL vial with 15 mL of medium at 38°C for 6 h and 12 h. The seed volume of immobilized cells was 10% (v/v), and the initial fumarate concentration was 30 g/L.

added to the medium on the conversion ratio was investigated. The $CaCl_2$ concentration used was between 0.2 and 1.0% (w/v). As shown in Fig. 1, the conversion ratio decreased slightly as the $CaCl_2$ concentration added in the medium increased after both 6 and 12 h of incubation with the conversion ratios for 0.2% $CaCl_2$ being 0.54 and 0.97 g/g, respectively. As the $CaCl_2$ concentration increased above 0.25% (w/v), however, the conversion ratio gradually decreased further.

Seed Volume of Immobilized Cells

To investigate the effect of the seed volume of the immobilized cells, 5, 10, 20, 30, 40, and 50% (v/v) samples were examined at 38°C for 12 h in 20-mL vials with 15 mL of medium; Figure 2 presents the results. The optimum seed volume of immobilized cells was found to be 10% (v/v) with a conversion ratio of 0.97 g/g. As the seed volume of the immobilized cells was increased further, above 10% (v/v), the conversion ratio did not change much during the first 6 h of incubation but significantly decreased after 12 h. These results suggest that the immobilized cells at the higher concentrations consumed more substrate for cellular maintenance and that adjustment of the seed volume is essential for efficient bioconversion. According to these results, the most appropriate seed volume of the immobilized cells was 10% (v/v).

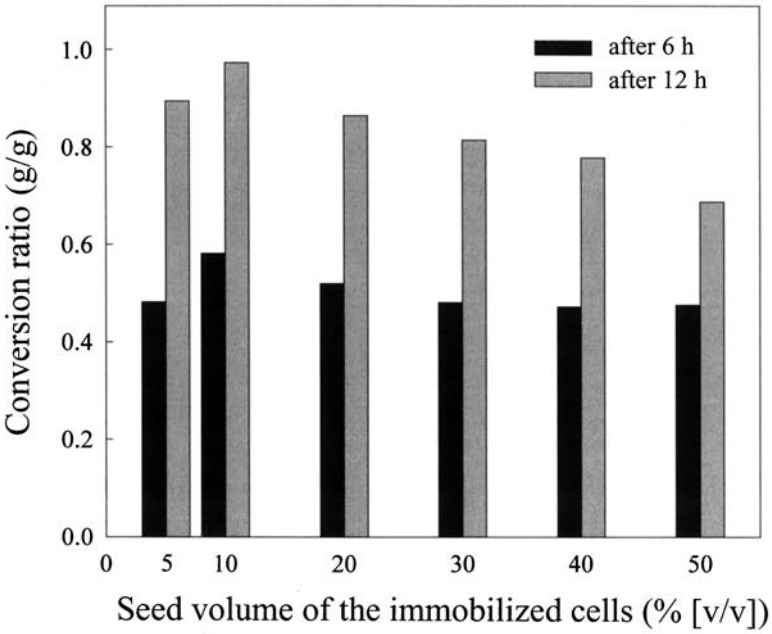

Fig. 2. Effect of seed volume of immobilized cells on bioconversion of fumarate to succinate by alginate-entrapped *E. faecalis* RKY1. Bioconversion was conducted in a 100-mL vial with 80 mL of medium at 38°C for 6 and 12 h. The initial fumarate concentration was 30 g/L.

Immobilized Bead Size

The effect of the immobilized bead diameter on succinate production and fumarate consumption was examined. The bead diameter used in this experiment was 2, 3, and 4 mm; Figure 3 presents the results. The succinate produced using beads with a 2 and 3 mm diameter was 29.2 and 28.9 g/L (a 0.973 and 0.965 g/g conversion ratio), respectively, after a 12-h incubation. When the immobilized bead diameter was 4 mm, the succinate concentration and conversion ratio after a 12-h incubation were 26.7 g/L and 0.89 g/g, respectively, showing that the conversion ratio of the 4-mm bead was lower than that of the 2- and 3-mm beads. Aksu and Bülbül *(8)* reported that when *Pseudomonas putida* cells were entrapped in Ca-alginate gels for the degradation of phenol, as the diameter of the immobilized bead was increased the degradation rate of phenol decreased owing to the diffusion limitation of substrates and products. According to our study, the optimum diameter for the Ca-alginate bead was found to be 2 mm.

Initial pH and Temperature

The effect of the initial pH on the conversion ratio with free and immobilized cells was examined. Bioconversion was conducted at 38°C for 12 h under anaerobic conditions and with a seed volume of 10% (v/v). The conversion ratios of the free-cell culture in the pH range of 5.0–8.5 were

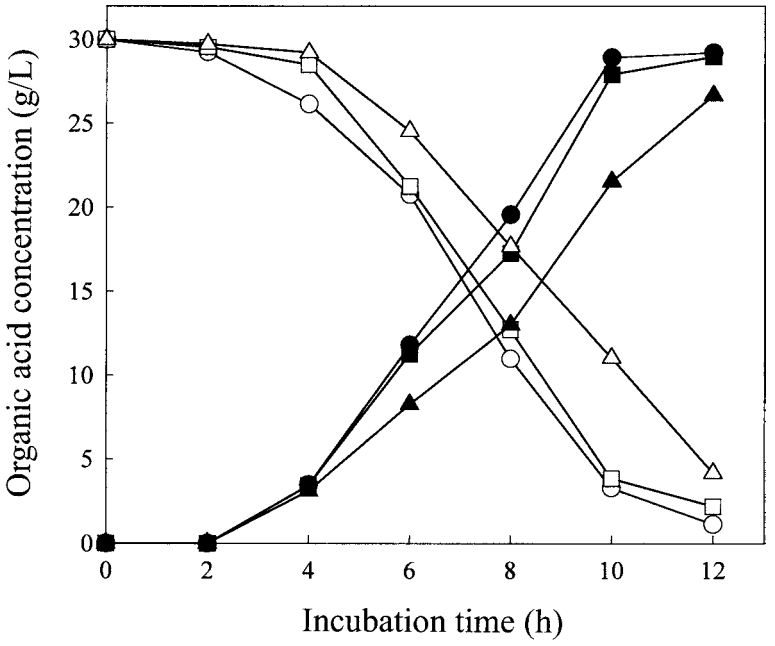

Fig. 3. Effect of immobilized bead size on succinate production and fumarate consumption. Bioconversion was conducted in a 100-mL vial with 80 mL of medium at 38°C for 12 h. The seed volume of immobilized cells was 10% (v/v), and the initial fumarate concentration was 30 g/L. Solid symbols indicate the succinate produced and open symbols the residual fumarate (—●—, —○—, 2 mm; —■—, —□—, 3 mm; —▲—, —△—, 4 mm).

similar to those of immobilized cell culture with the optimum pH for both the free and immobilized bioconversions being 7.0. However, the conversion ratio for the immobilized cells was higher than that of the free cells for all pH values tested.

To determine the effect of temperature on succinate production, experiments were conducted at various temperatures for 12 h under anaerobic conditions with a 10% seed volume. The best succinate conversion after a 12-h incubation was seen at 38°C for both the free and immobilized cells (0.983 g/g for the free cells and 0.973 g/g for the immobilized cells). Above 40°C, however, the immobilized cells were able to produce succinate more efficiently than the free cells. When the cells were incubated at 50°C, the conversion ratio of the free and immobilized cells were 0.121 and 0.38 g/g, respectively. These results suggest that the Ca-alginate gel provides a protective barrier against heat transfer, as was reported elsewhere *(12)*. Krisch and Szajani *(17)* reported that when *Acetobacter aceti* cells were immobilized in Ca-alginate gels, the immobilized cells produced more acetic acid than free cells at higher temperatures.

Storage Stability of Immobilized Cells

The immobilized cells were stored in a 2% (w/v) $CaCl_2$ solution at 4°C for 10 wk to investigate their storage stability. As shown in Fig. 4, the

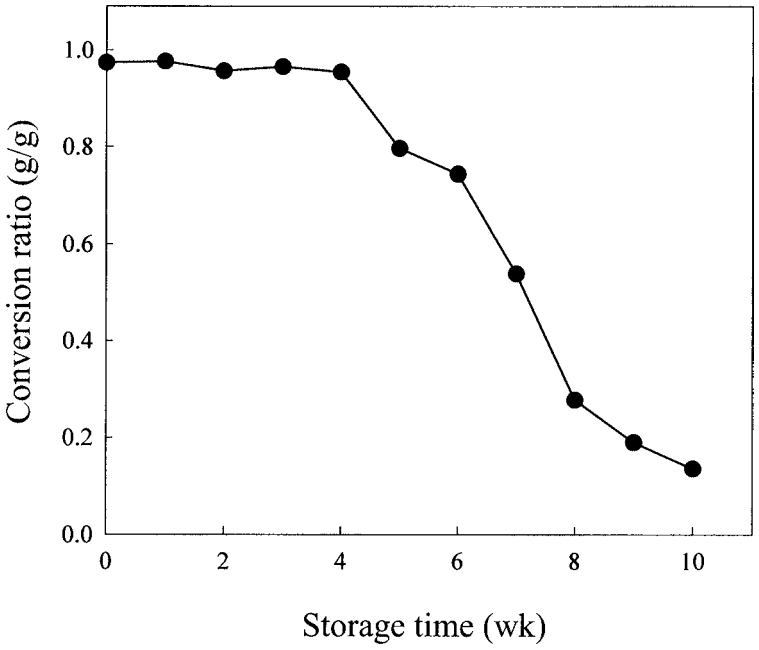

Fig. 4. Storage stability of immobilized cultures of *E. faecalis* RKY1. The immobilized cells were stored in a 2% (w/v) $CaCl_2$ solution until used. Bioconversion was conducted in a 20-mL vial with 15 mL of medium at 38°C for 12 h per each batch. The seed volume of immobilized cells was 10% (v/v), and the initial fumarate concentration was 30 g/L.

conversion ratio for a 12-h incubation after storage for 4 wk was >0.95 g/g. When the immobilized cells were stored for 5 or 6 wk, the conversion ratio decreased by about 0.20 g/g of its initial value. When stored for 10 wk, the conversion ratio of immobilized cells decreased to only 0.14 g/g. Therefore, *E. faecalis* RKY1 cells entrapped in Ca-alginate gels can be stored for up to 4 wk without noticeable loss of cellular activity.

Repetitive Batch Operation

The operational stability of the immobilized cells was examined by repetitive batch bioconversions. Each batch reaction was conducted at 38°C in a 100-mL vial containing 80 mL of medium and under anaerobic conditions. After one batch was finished, the Ca-alginate beads were removed and washed using the sterilized mesh and water and then inoculated into fresh medium. The initial concentration of fumarate used was 30 g/L, and the time for one batch was set at 12 h; Figure 5 presents the results. Immobilized cells were repeatedly used for five batches, or about 60 h. Until the third batch bioconversion, the conversion ratio was above 0.80 g/g. However, it decreased to 0.77 and 0.57 g/g by the fourth and fifth batches, respectively. These results suggest that as the batch bioconversion was

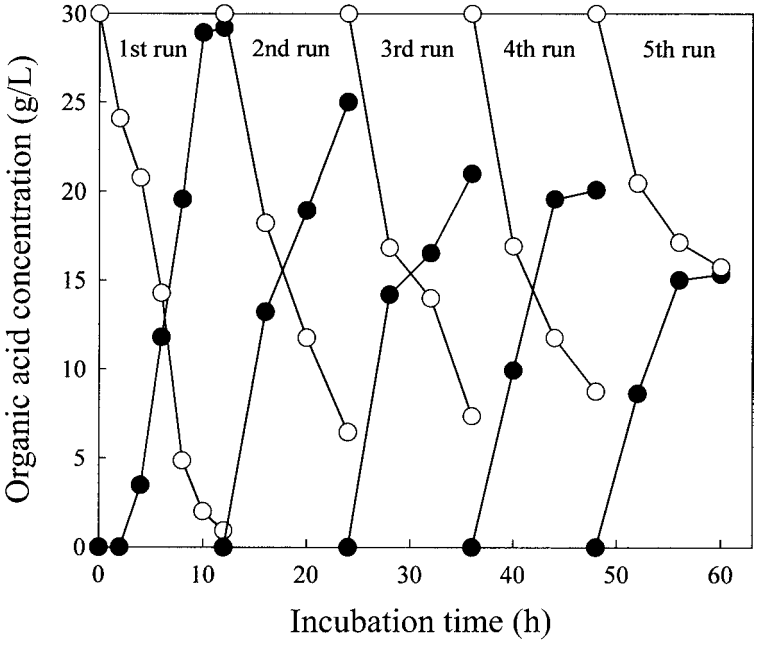

Fig. 5. Repetitive batch bioconversion profiles for cultures of immobilized *E. faecalis* RKY1. Repetitive bioconversion was carried out in a 100-mL vial with 80 mL of medium at 38°C for 12 h per each batch. The seed volume of immobilized cells was 10% (v/v), and the initial fumarate concentration was 30 g/L. (—●—), Succinate produced; (—○—), residual fumarate.

repeated, the *E. faecalis* RKY1 cells entrapped in the Ca-alginate gels seemed to be damaged by the low pH values in the matrix at the end of each batch.

Conclusion

E. faecalis RKY1 cells, a newly isolated strain, were immobilized in various gelling media in order to characterize the bioconversion of fumarate to succinate through the use of immobilized cells. Among the various natural polymers tested—agar, κ-carrageenan, and sodium alginate—sodium alginate was the best material for whole-cell immobilization of *E. faecalis* RKY1. An enhanced production of succinate was achieved when the sodium alginate concentration and the diameter of the calcium alginate beads were adjusted to 2% (w/v) and 2 mm, respectively. The results with *E. faecalis* RKY1 showed that immobilization can also enhance the stability of cells and protect them from the effects of alterations in pH and temperature. The efficient bioconversion of fumarate to succinate was demonstrated through immobilization of *E. faecalis* RKY1 in alginate.

Acknowledgment

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