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Bioconversion of Fumarate to Succinate Using Glycerol as a Carbon Source

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Abstract

In this study, a facultative bacterium that converts fumarate to succinate at a high yield was isolated. The yield of bioconversion was enhanced about 1.2 times by addition of glucose into culture medium at an initial concentration of 6 g/L. When the initial cell density was high (2 g/L), the succinate produced at pH 7.0 for initial fumarate concentrations of 30, 50, 80, and 100 g/L were 29.3, 40.9, 63.6, and 82.5 g/L, respectively, showing an increase with the initial fumarate concentration. The high yield of 96.8%/mole of fumarate in just 4 h was obtained at the initial fumarate concentration of 30 g/L. Comparing these values to those obtained with low cell culture (0.2 g/L), we found that the amount of succinate produced was similar, but the production rate in the high cell culture was about three times higher than was the case in the low cell culture. This strain converted fumarate to succinate at a rate of 3.5 g/L · h under the sparge of CO_2.

Index Entries: Bioconversion; succinate; fumarate; *Enterococcus* sp. RKY1; fumarate reductase.

Introduction

Succinic acid has many industrial applications as a raw material for food, medicine, plastics, cosmetics, textiles, plating, and waste-gas scrubbing. It can be produced either chemically or biologically. Currently, however, it is only manufactured by chemical processes *(1)*. Microorganisms have the ability to modify chemically a wide variety of organic compounds. Such changes are called bioconversion. There have been many reports of the production of industrially useful compounds, such as fumaric acid *(2)*, D-malic acid *(3)*, L-malic acid *(4)*, D-lactic acid *(5)*, L-aspartic acid *(6)*, and

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L-alanine *(7)* by bioconversion. Also, bioconversion of fumarate to succinate by *Escherichia coli (8,9)* and *Aerobacter aerogenes (10)* strains have been reported. Fumarate reductases of these strains were able to accept two electrons from a reduced electron transporter, produced by the oxidation of nicotinamide-adenine dinucleotide (NADH) formed from glucose, presumably as a hydrogen donor for the bioconversion to reduce fumarate to succinate.

Some anaerobically functioning organisms are able to use other external compounds as terminal electron acceptors, instead of oxygen. These organisms contain a respiratory complex that transfers electrons to an alternative terminal electron acceptor. Therefore, when a facultative anaerobic bacterium is grown anaerobically with glycerol as a hydrogen donor and fumarate as a hydrogen acceptor, a very simple electron-transport chain consisting of the anaerobic glycerol-3-phosphate dehydrogenase and fumarate reductase is induced *(11)*.

Glycerol, as a renewable resource, is available as a byproduct after the manufacture of biodiesel fuel from rape oil. Its production cost then greatly depends on the reusage of glycerol. In this study we examined the effects of substrate concentration and other culture conditions on the bioconversion of fumarate to succinate using *Enterococcus* sp. RKY1, a newly isolated bacterium.

Materials and Methods

Microorganism and Medium

Enterococcus sp. RKY1 was isolated from our laboratory culture with respect to its ability to convert fumarate to succinate at a high yield. *E. faecium* KCCM 11968 was used for the comparison of conversion ability. The medium for cell growth contained the following: 10 g glycerol (Yakuri, Osaka, Japan), 30 g sodium fumarate (Wako, Osaka, Japan), 15 g yeast extract (Difco, Detroit), 10 g K_2HPO_4, 1 g NaCl, 0.5 g Na_2CO_3, 0.05 g $MgCl_2 \cdot 6H_2O$, 0.01 g $FeSO_4 \cdot 7H_2O$, 45 g sodium succinate (for succinate adaptation in bioconversion medium), and 1 L distilled water. The medium for bioconversion contained the following: 20 g glycerol (Yakuri), 15 g yeast extract (Difco), 10 g K_2HPO_4, 1 g NaCl, 5 g Na_2CO_3, 0.05 g $MgCl_2 \cdot 6H_2O$, 0.01 g $FeSO_4 \cdot 7H_2O$, various concentrations of fumarate, and 1 L distilled water. The seed culture broth used in this experiment was transferred to a new medium every 12 h for 2 d. The storage stock was maintained in a vial-bottle containing 50% glycerol at –20°C for further preservation.

Culture Conditions

Vial cultures were prepared by inoculating 0.6 mL of seed cultures into the culture medium (15 mL) in 20-mL vials, followed by incubation at 38°C for 24 h on a shaking incubator (Vision Scientific Co., Taejon, Korea) at 200 rpm. For the fermentor cultures, a 2.5-L jar-fermentor (KF-2.5 L,

Korea Fermentor Co., Taejon, Korea) containing 1 L of the medium was inoculated with 40 mL of seed culture. The experiments ran for 24 h at 38°C, 200 rpm, pH 7.0. In this experiment, the headspace was replaced with 100% CO_2 gas before inoculation. For high cell culture, the cells were harvested from the culture broth by centrifugation at 10,000 rpm for 10 min, were washed once with 0.1-*M* sodium phosphate buffer (pH 7.0), and then were resuspended in the same buffer (40 mL). The initial cell concentration of high cell culture medium was about 2 g/L. The initial cell concentration of the culture medium for low cell culture was about 0.2 g/L.

Analytical Methods

Cell growth was measured as the optical density at 660 nm (OD_{660}) from an ultraviolet (UV)-spectrophotometer (Shimadzu Co., UV-100A). Concentration of glycerol was determined by enzymatic assay (Boehringer Mannheim Co., Mannheim, Germany). Concentration of glucose was determined by using the DNSA method *(12)*. Succinic acid, fumaric acid, lactic acid, and other organic acids were quantitatively analyzed by high-performance liquid chromatography (HPLC) under the following conditions; column, Aminex HPX-87H column, 300 × 7.8 mm (Bio-Rad); column temperature, 35°C; solvent, 0.008 *N* H_2SO_4 (0.6 mL/min); detector, UV 210 nm (Waters 486). In this article, the amount of succinic acid produced was represented as sodium succinate (FW 162.14).

Results and Discussion

Identification of the Bioconversion Rate

Two bacteria that most closely resemble each other in profile of the cellular fatty acid analyzed, *Enterococcus* sp. RKY1, a newly isolated bacterium, and *Enterococcus faecium* KCCM 11968, obtained from the Korean Culture Center of Microorganisms (KCCM), were compared with each other in their bioconversion rate. The time-course of fumarate conversion at high cell density (about 2.0 g/L) is shown in Fig. 1A,B. As shown in Fig. 1A, for *Enterococcus* sp. RKY1 with an initial concentration of 50 g/L, fumarate was completely consumed at the end of 4 h of incubation, the cell mass rapidly increased from an initial concentration of 2 g/L to 7 g/L, and the succinate was rapidly produced to 40.9 g/L, an 81% conversion with a production rate of 10.2 g/L · h. On the other hand, the maximum succinate productivity of *E. faecium* KCCM 11968 reached 16.7 g/L in 2p(h of incubation (Fig. 1B) and the molar conversion yield of succinate on the basis of the amount of substrate supplied was only 38% at the end of the incubation. This strain also produced 14 g/L (29%) of malate, which was not found in Fig. 1A. Consequently, the bioconversion rate of succinate from fumarate by the isolated strain (10.2 g/L · h) was about 15 times higher than that of the same species (0.70 g/L · h). In addition, it was found that the conversion activity of fumarate reductase was significantly high, as compared with KCCM 11968.

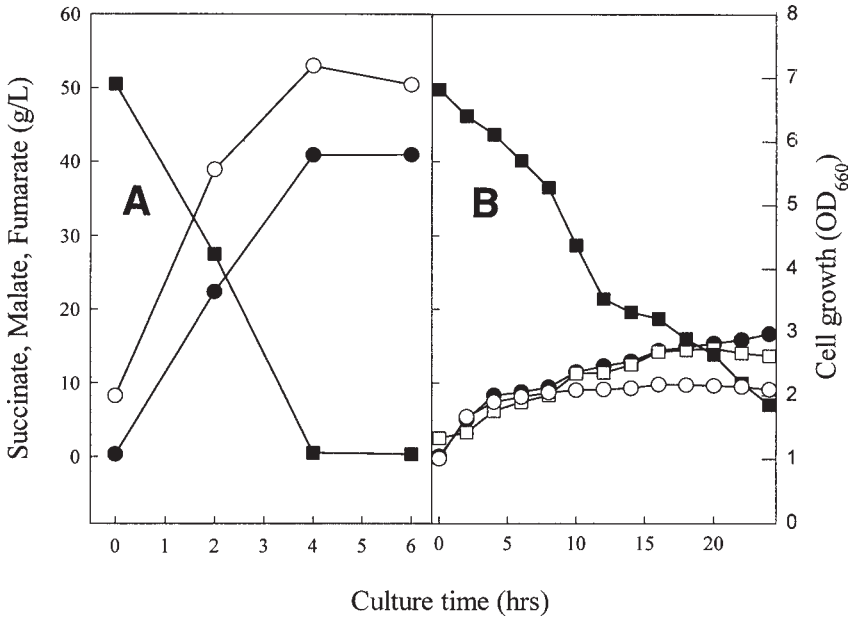

Fig. 1. Time-course of succinate, malate production, residual fumarate, and cell growth by high cells of **(A)** *Enterococcus* sp. RKY1 and **(B)** *E. faecium* KCCM 11968. 2.5-L fermentor (1 L medium); initial fumarate concentration, 50 g/L; initial glycerol concentration, 20 g/L; pH 7.0; 38°C; 200 rpm; pH neutralizer 2 *M* Na_2CO_3. —●—, succinate produced; —□—, malate produced; —■—, residual fumarate; —○—, cell density.

Effect of pH

From the preliminary experiments, which were conducted at various pH values between 5.8 and 8.0 with 0.1 *M* potassium phosphate buffer, we found that conversion of fumarate to succinate is feasible if pH is controlled at the optimum range of 7.0–7.6. Therefore, the strain was incubated under no pH control, pH 7.0, or pH 8.0 with the initial fumarate concentration of 80 g/L. The pH in the fermentor was maintained by the addition of 2 *M* Na_2CO_3. The effect of pH change on the production was not significant among these conditions; however, the maximum succinate of 65.9 g/L (molar yield of 82%) was obtained at pH 7.0 (Fig. 2). At pH 8.0, succinate concentration gradually decreased after 16 h of incubation, from maximum value of 58.7 to 51.8 g/L at the end of the 24-h incubation. Under this condition, the highest production rate of *Enterococcus* sp. RKY1 reached a maximum value (3.7 g/L · h) at 16-h incubation.

Effect of Glucose

We examined the effect of glucose as a supplemented carbon source on the conversion of fumarate to succinate. The initial glucose concentration added into the medium containing 20 g/L of glycerol was varied, ranging

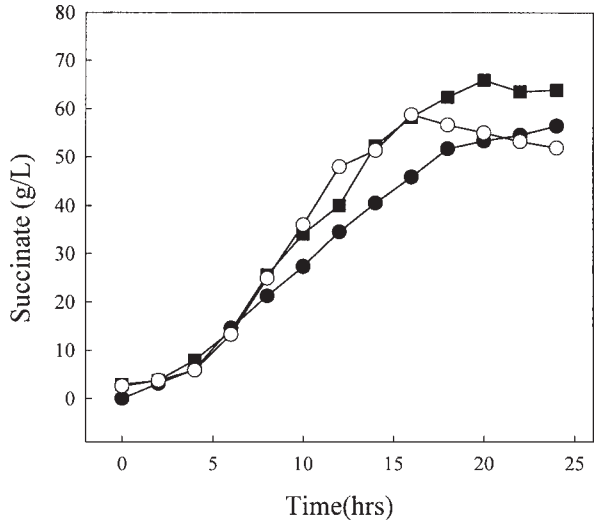

Fig. 2. Effect of pH on succinate production by *Enterococcus* sp. RKY1. 2.5-L fermentor (1 L medium); initial cell density, 0.2 g/L; initial fumarate concentration, 80 g/L; initial glycerol concentration, 20 g/L; 38°C; 200 rpm; pH neutralizer 2 *M* Na_2CO_3. —●—, no pH control; —■—, pH 7.0; —○—, pH 8.0.

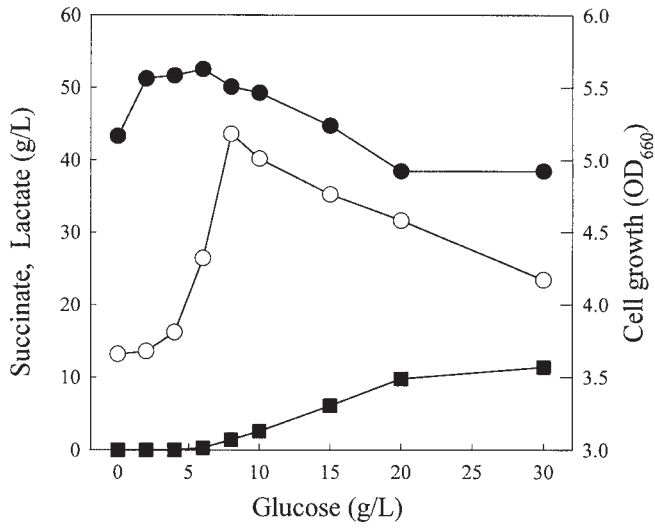

Fig. 3. Effect of glucose concentration on succinate, lactate production, and cell growth. 20-mL vial (15 mL medium); initial fumarate concentration, 50 g/L; initial glycerol concentration, 20 g/L; pH 7.0; incubation time, 24 h. —●—, succinate produced; —○—, cell density; —■—, lactate produced.

from 0 to 30 g/L at the initial fumarate concentration of 50 g/L. As shown in Fig. 3, the optimum glucose concentration was 6 g/L, resulting a succinate concentration of 52.5 g/L with a molar yield of 103.8%. However, further addition of glucose (up to 30 g/L) inhibited succinate production.

On the other hand, the addition of glucose as an energy source resulted in abundant growth. It is postulated that fumarate reacted with the reduced nicotinamide adenine (NAD) generated in the oxidation of glyceraldehyde-3-phosphate, and thus altered the role of pyruvate in the regeneration of oxidized NAD. The metabolism of pyruvate via the dismutation and phosphoroclastic pathways would then account for the increased cell growth as well as lactate, acetate, and carbon dioxide production. Also it can be found that if the level of glucose is more than that needed for effective biosynthesis of cell matter, the electron derived from degradation of formate formed from pyruvate dismutation is used for reduction of an intermediate product, pyruvate, to lactate as a fermentation product. One can assume that succinate production was inhibited by lactate accumulation. As a result, a 1.2 times increase in molar yield was obtained when glucose was added into the medium.

Effect of Initial Substrate Concentration at Low Cell Culture

The effect of the initial fumarate concentration on the production of succinate was investigated with the low initial cell density (about 0.2 g/L). As shown in Fig. 4, the amounts of succinate produced at pH 7.0 with the initial fumarate concentration of 30, 50, 80, and 100 g/L were 26.4, 47.9, 65.9, and 70.7 g/L, respectively, increasing with the initial concentration. The maximum amount of succinate was obtained at the initial substrate concentration of 100 g/L, but with a slower production rate (1.9 g/L · h) compared with the results (2.2, 3.4, and 2.7 g/L · h, respectively) of the other initial substrate concentrations of 30, 50, and 80 g/L. Namely, at the concentration of 100 g/L, the incubation time was extended to more than six times as compared with 30 g/L of initial fumarate concentration. On the other hand, the maximum yield of 95% per mole of fumarate was obtained at the initial fumarate concentration of 50 g/L in 14 h.

Effect of Initial Substrate Concentration at High Cell Culture

The effect of the initial fumarate concentration on the production of succinate at high cell culture (2 g/L) is shown in Fig. 5. The succinate productions at pH 7.0 for initial fumarate concentration of 30, 50, 80, and 100 g/L were 29.3, 40.9, 63.6, and 82.5 g/L, respectively, and the dependence of succinate production on fumarate was very similar to the low cell culture (Fig. 3). At the initial fumarate concentration of 100 g/L, the reaction rate also showed a tendency to become slow. It is obvious that the amount of succinate produced at high cell culture was close to that at low cell culture, but the culture time was only one-third of that in low cell culture. Goldberg et al. *(8)* reported that amplified *Escherichia coli* JRG 1346 cultures containing 4% fumarate gave succinate molar yields of 41.2 and 125 % after incubation for 1 and 4 d, respectively. It has also been reported by Sasaki et al. *(9)* that *E. coli* AHU 1410 showed succinic acid yields of 43 and 87% (based on 4% fumaric acid) after 3 and 7 d, respectively. On the

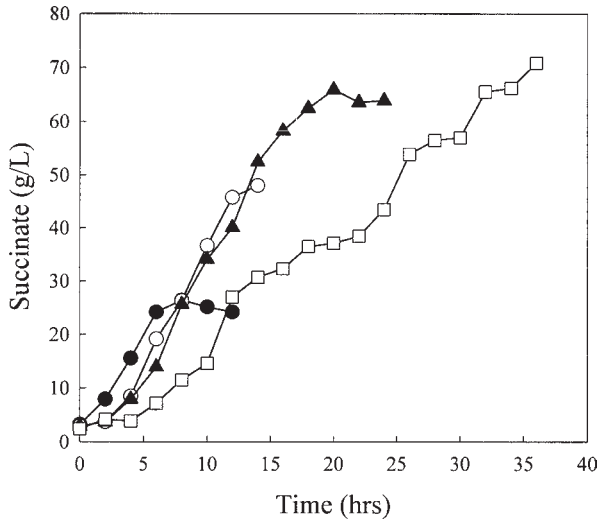

Fig. 4. Effect of initial fumarate concentration on succinate production for low cell concentration of *Enterococcus* sp. RKY1. 2.5-L fermentor (1 L medium); initial cell density, 0.2 g/L; initial glycerol concentration, 20 g/L; pH 7.0; 38°C; 200 rpm; pH neutralizer 2 *M* Na_2CO_3. —●—, Fumarate 30 g/L; —○—, fumarate 50 g/L; —▲—, fumarate 80 g/L; —□—, fumarate 100 g/L.

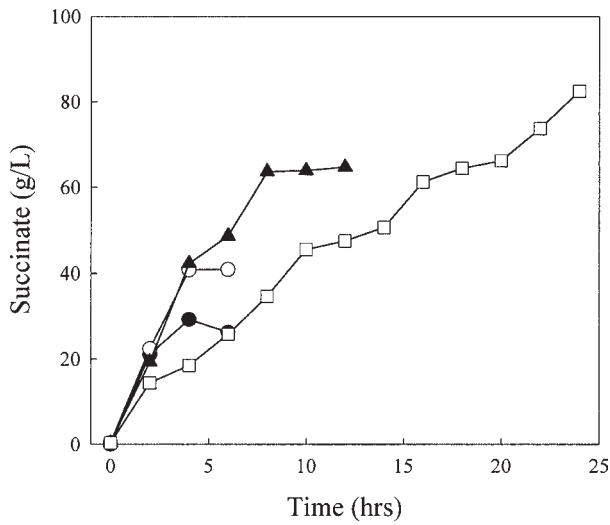

Fig. 5. Effect of initial fumarate concentration on succinate produced for high cell concentration of *Enterococcus* sp. RKY1. 2.5-L fermentor (1 L medium); initial cell density, 2.0 g/L; initial glycerol concentration, 20 g/L; pH 7.0; 38°C; 200 rpm; pH neutralizer 2 *M* Na_2CO_3. —●—, Fumarate 30 g/L; —○—, fumarate 50 g/L; —▲—, fumarate 80 g/L; —□—, fumarate 100 g/L.

other hand, at a similar initial fumarate concentration (3% fumarate) and after 4 h of incubation, the newly isolated strain showed a succinate molar yield of 96.8% in this study. The effects of initial substrate concentration on

Table 1
Effect of Substrate Concentration on Yield and Productivity of Succinate[a]

S_o, g/L	Time, h[b]	Fumarate used, g/L	Succinate produced, g/L	$Y_{P/S}$, mol%	SP, g/g/h[c]	VP, g/L/h[d]
30	4	30.0	29.3	96.8	1.0	7.3
50	4	50.0	40.9	80.7	1.3	10.2
80	8	79.2	63.6	79.3	1.0	7.9
100	24	92.9	82.5	87.7	0.6	3.4

[a]Initial cell density, 2 g/L; temperature 38°C; agitation 200 rpm; pH 7.0.
[b]Time, Time of maximum succinate production (h).
[c]SP, Average specific productivity of succinate (g succinate/g cells/h).
[d]VP, Average volumetric productivity of succinate (g succinate/L/h).

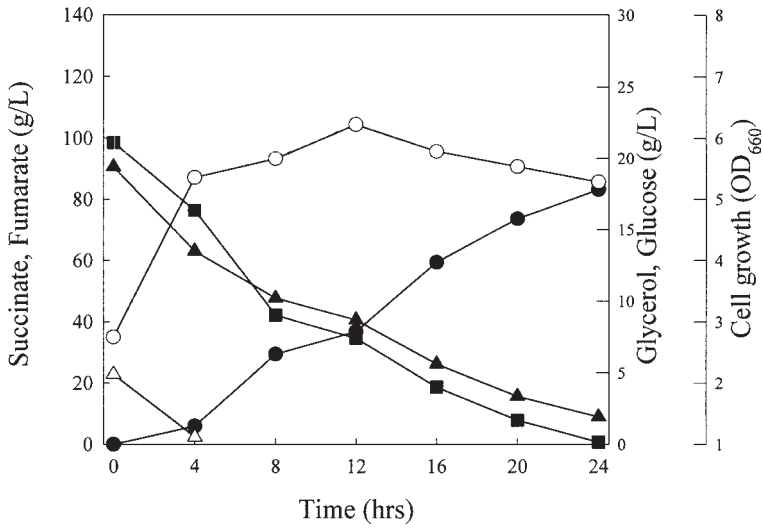

Fig. 6. Time-course of bioconversion of fumarate to succinate by *Enterococcus* sp. RKY1. 2.5-L fermentor (1 L medium); 0.1 vvm CO_2; initial fumarate concentration, 100 g/L; initial glycerol concentration, 20 g/L; glucose added, 5 g/L; initial cell density, 3 g/L; pH 7.0; 38°C; 200 rpm; pH neutralizer 2 *M* Na_2CO_3. —●—, Succinate produced; —■—, residual fumarate; —○—, cell density; —▲—, glycerol; —△—, glucose.

yield and productivity of succinate are shown in Table 1. When the initial fumarate concentration was 50 g/L, the highest volumetric productivity under high cell culture was 10.2 g/L · h, which was about three times higher than that under low cell culture (3.4 g/L · h).

Bioconversion of Fumarate to Succinate

Figure 6 shows a bioconversion of fumarate to succinate by RKY1 under 100 mL/min CO_2 sparge (99.99% of CO_2). Initial fumarate concentration of 100 g/L was gradually consumed to about 0.7 g/L after 24-h incubation, and about 17.5 g/L of glycerol was utilized from the initial

concentration of 20 g/L. Also, a cell mass of 6.9 g/L was quickly reached in 12 h, and then it was slightly decreased to 5.8 g/L at the end of the 24-h incubation. The succinate production reached 83.1 g/L with the molar conversion ratio of succinate of 82% after the 24-h incubation. Also, it is interesting that the decreasing rate of glycerol is proportional to that of fumarate. It is known that the glycerol is phosphorylated by glycerol kinase, then the resulting glycerol-3-phosphate (G3P) is oxidized to dihydroxyacetone phosphate *(13)*. It is believed that the hydrogen released by this oxidization is transferred to fumarate, even though the exact route remains unclear. Therefore, the parallel decrease of these two substrates can be explained by the transfer of hydrogen from G3P to fumarate, which is reduced to succinate.

Conclusions

The bioconversion of fumarate to succinate using glycerol as a hydrogen donor was performed in this study. Among the pH conditions tested, the maximum concentration of 65.9 g/L succinate was obtained at pH 7.0. The conversion of fumarate to succinate by *Enterococcus* sp. RKY1 was enhanced about 1.2 times by the addition of glucose as a supplemented carbon source with an initial concentration of 6 g/L in the culture medium. At high cell culture (2.0 g/L), the amount of succinate produced was similar to that at low cell culture (0.2 g/L), except that culture time was only one-third of that in low cell culture. At the initial fumarate concentration of 100 g/L, the maximum concentration of 82.5 g/L succinate was reached within 24 h. The strain showed a succinate molar yield of 96.8% after 4 h of incubation at the initial fumarate concentration of 30 g/L. These results indicate that *Enterococcus* sp. RKY1 strain can produce succinate from fumarate at a significantly higher yield and higher rate than any amplified *E. coli* or *A. aerogenes* strains that have been reported previously. Since glycerol, a renewable resource, derived from the manufacture of biodiesel fuel can be used as starting material, the advantages of using glycerol as a substrate include that succinate can be produced with a cheaper raw material in high concentration with high purity and high yield, permitting economic recovery of succinic acid.

Acknowledgment

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References

1. Datta, R. (1992), US Patent, No 5,143,833 A.
2. Nakajima-Kambe, T., Nozue, T., Mukouyama, M., and Nakahara, T. (1997), *J. Ferment. Bioeng.* **84(2),** 165–168.
3. Asano, Y., Ueda, M., and Yamada, H. (1993), *Appl. Environ. Microbiol.* **59(4),** 1110–1113.

4. Wang, X., Gong, C. S., and Tsao, G. T. (1996), *Biotechnol Lett.* **18(12),** 1441–1446.
5. Shigeno, T. and Nakahara, T. (1991), *Biotechnol Lett.* **13(6),** 427–432.
6. Suzuki, Y., Yasui, T., Mino, Y., and Abe, S. (1980), *Eur. J. Appl. Microbiol. Biotechnol.* **11,** 23–27.
7. Takamatsu, S., Umemura, I., Yamamoto, K., Sato, T., Tosa, T., and Chibata, I. (1982), *Eur. J. Appl. Microbiol. Biotechnol.* **15,** 147–152.
8. Goldberg, I., Lonberg-Holm, K., Bagley, E. A., and Stieglitz, B. (1983), *Appl. Environ. Microbiol.* **45(6),** 1838–1847.
9. Sasaki, Y., Takao, S., and Hotta, K. (1970), *J. Ferment. Technol.* **48,** 782–786.
10. Takao, S. and Hotta, K. (1973), *J. Ferment. Technol.* **51,** 19–25.
11. Miki, K. and Lin, E. C. C. (1973), *J. Bacteriol.* **114(2),** 767–771.
12. Miller, G. L. (1959), *Anal. Chem*. **31(3),** 426–428.
13. Lehninger, A. L., Nelson, D. L., and Cox, M. M. (1993), *Principles of Biochemistry*, 2nd ed., Worth, New York.