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Effect of Culture Conditions on the Production of Succinate by *Enterococcus faecalis* RKY1

KANG, KUI-HYUN[1], JONG-SUN YUN[2], AND HWA-WON RYU[3]*

[1]*Department of Chemical Engineering,* [2]*Department of Chemical Technology, and*
[3]*Department of Biochemical Engineering and Institute of Biotechnology, Chonnam National University, Kwangju 500-757, Korea*

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Abstract Bioconversion of fumarate to succinate was anaerobically conducted in a synthetic medium containing glycerol as a hydrogen donor and fumarate as a hydrogen acceptor. We investigated the effects of pH, carbon and nitrogen sources, conversion substrate, and other culture conditions on the production of succinate using a newly isolated *Enterococcus faecalis* RKY1. Addition of a variety of carbonates to the medium significantly increased the rates of production of succinate. The production of succinate and cell growth were relatively satisfactory in the pH range of 7.0–7.6. By using glycerol as a hydrogen donor, high purity succinate was produced with few byproducts. Yeast extract as a sole nitrogen source was the most effective for producing succinate. As a result, the optimum condition of bioconversion was obtained at a medium containing 20 g/l glycerol, 50 g/l fumarate, 15 g/l yeast extract, 10 g/l K_2HPO_4, 1 g/l NaCl, 50 ppm $MgCl_2 \cdot 6H_2O$, 10 ppm $FeSO_4 \cdot 7H_2O$, and 5 g/l Na_2CO_3 at pH 7.0–7.6. Under the optimum condition, a succinate concentration of 153 g/l was produced in 36 h. The total volumetric production rate and the molar yield of succinate were 4.3 g/l/h and 85%, respectively.

Key words: *Enterococcus faecalis* RKY1, bioconversion, fumarate reductase, succinate, fumarate, glycerol

Succinic acid is widely recognized and used as a specialty chemical with its applications in polymers, foods, pharmaceuticals, and cosmetics, along with four-carbon intermediates useful for the production of 1,4-butanediol, tetrahydrofuran, and γ-butyrolactone [20]. Succinic acid is a major end-product of glucose fermentation by anaerobic bacteria such as *Ruminococcus flavefaciens* [6, 7] and *Anaerobiospirillum succiniciproducens* [4, 17, 21] when CO_2 is easily available. However, reported methods of fermentation [13, 14] give a variety of byproducts, such as lactic acid, formic acid, and acetic acid. Furthermore, this procedure must be carried out under oxygen-free culture conditions to maintain strict anaerobiosis and it is required for well-trained operational techniques.

Bioconversion is preferable because of only one specific reaction that is normally catalyzed by an enzyme, resulting in the products of high purity and high yields. Also, it has many advantages such as simplified product recovery, requiring less conversion time, needing only a small amount of equipment, and bearing no environmental hazards [1]. Since bioconversion of fumarate to succinate is conducted with a facultative anaerobe in this study, this procedure is more feasible than the conventional fermentations mentioned above.

Using bioconversion, Goldberg *et al.* [5] reported that amplified *E. coli* JRG 1346 cultures containing 4% fumarate gave molar yields of 41.2% and 125% of succinate after incubation for 1 and 4 days, respectively. Sasaki *et al.* [18] also reported that *E. coli* AHU 1410 showed 43% and 87% of succinate after 3 and 7 days, respectively. Wang *et al.* [19] investigated the production of succinic acid from fumaric acid by a recombinant *E. coli* strain DH5 alpha/pGC1002 containing multicopy fumarate reductase genes, which produced 41 to over 60 g/l of succinate in 48.5 h, starting with 50 to 64 g/l fumaric acid. On the other hand, a wild-type strain in our study has produced succinate from fumarate at significantly higher rates and yields than the recombinant *E. coli* strains used in the above experiments, thus indicating that this alternative method of bioconversion of fumaric acid to succinic acid is more desirable [8, 16].

The presence of fumarate as a hydrogen acceptor enables facultative anaerobes to grow anaerobically on glycerol or glycerol-3-phosphate (G3P) as a carbon source [9]. Under these conditions, an anaerobic G3P dehydrogenase and fumarate reductase are induced [10] and, consequently, fumarate is reduced to succinate by fumarate reductase [3, 11], whereas glycerol is first dehydrogenated anaerobically, probably forming dihydroxyacetone, which is subsequently phosphorylated and metabolized to acetate. A simple

*Corresponding author
Phone: 82-62-530-1842, Fax: 82-62-530-1849;
E-mail: hwryu@chonnam.ac.kr

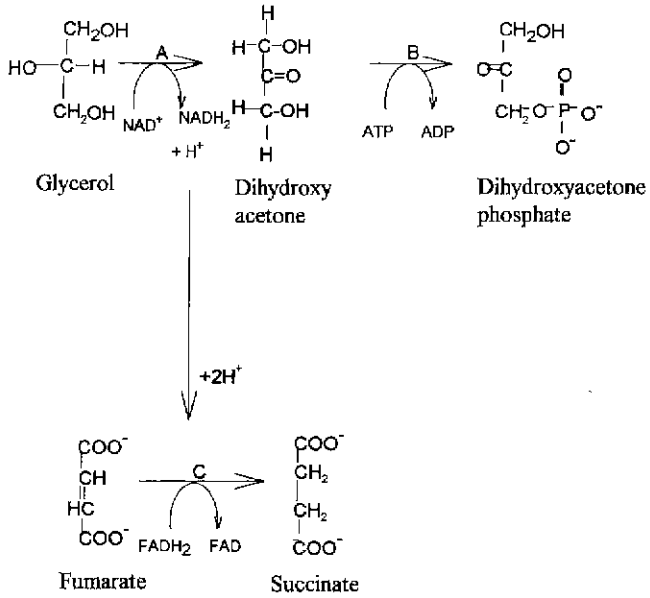

Fig. 1. Pathway of bioconversion of fumarate to succinate using glycerol as a hydrogen donor.
A, Glycerol dehydrogenase, B, dihydroxyacetone kinase, C, fumarate reductase.

pathway depicting the above transhydrogenation is shown in Fig. 1.

In this study, to optimize bioconversion of fumarate to succinate by isolated *Enterococcus faecalis* RKY1, the effects of pHs, carbon sources, nitrogen sources, conversion substrate, and other culture conditions on the production of succinate were investigated.

MATERIALS AND METHODS

Microorganism and Medium

Enterococcus faecalis RKY1 [15] was isolated from the authors' contaminated fermentation culture with an ability of converting fumarate to succinate at a high yield. Based on phylogenetic analysis of the 16S rDNA gene sequence, the strain was identified as a member of the genus *Enterococcus*, which had a high homology (99.6%) to the 16S rDNA gene of *Enterococcus faecalis*. The compositions of the media employed in this study were 20 g/l glycerol, 50 g/l fumarate, 15 g/l yeast extract, 10 g/l K_2HPO_4, 1 g/l NaCl, 5 g/l Na_2CO_3, 50 ppm $MgCl_2 \cdot 6H_2O$, and 10 ppm $FeSO_4 \cdot 7H_2O$. All chemicals used were of the reagent grade. The storage stock was maintained in a vial-bottle containing 50% glycerol at -20°C for further preservation. The seed culture broth was transferred to a new medium every 12 h for 2 days.

Culture Conditions

The isolated strain was anaerobically incubated in 20-ml vials sealed with butyl rubber stoppers, containing 15 ml of culture medium at 38°C for 24 h on a shaking incubator (Vision Scientific Co., Taejon, Korea) at 200 rpm. The vials were vacuum-degassed and CO_2 was added when necessary. For the fed-batch cultivation, a 2.5-l jar-fermentor (KF-2.5L, Korea Fermentor Co., Inchon, Korea) containing 1 l of the medium was inoculated with 40 ml of seed culture [16]. The initial cell concentration of the culture medium was 3.0 g/l.

Analytical Methods

Cell concentration was determined by optical density at 660 nm (OD_{660}) using a UV-spectrophotometer (Shimadzu Co., UV-100A, Japan). Fermentation broth containing succinic acid, fumaric acid, lactic acid, and other organic acids were quantitated by high performance liquid chromatography (HPLC; Waters Co., U.S.A.) under the following conditions: 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 paper, the amount of succinic acid produced is expressed as sodium succinate (FW 162.14), and the molar yield (%) of succinic acid was calculated as moles of sodium succinate ($C_4H_4O_4Na_2$) produced per mole of sodium fumarate ($C_4H_2O_4Na_2$) supplied. The results were taken from the maximum values obtained during cultivation.

RESULTS AND DISCUSSION

Environmental Conditions Affecting Succinate Production

The strain used in this study can be grown in both aerobic and anaerobic conditions because of its characteristics of a facultative anaerobe. However, an anaerobic condition is required for the reduction of fumarate to succinate. Figure 2 presents the effect of environmental conditions on succinate production and cell growth. The strain was incubated in an aerobic condition in a 100-ml shaking flask containing 15 ml of culture medium, condition (A). On the other hand, various anaerobic conditions were established by using 20-ml glass vials which were immediately sealed with rubber stoppers (B), adding CO_2 after degassing (C), and adding carbonates such as sodium bicarbonate, calcium carbonate, and magnesium carbonate as a growth factor (D, E, and F, respectively). It was observed that a molar yield of condition (B) on the basis of amount of substrate supplied (58.9%) was about 2-fold higher than that in condition (A) (27.5%), while the highest cell growth was obtained with the cell density of about 4 (OD_{660}) in aerobic condition (A). When various carbonates were added to the medium (D, E, and F), the molar yield increased 1.13, 1.07, and 1.15 times greater than in condition (B), respectively. Therefore, it was highly likely that the succinate production was repressed under aerobic conditions due to anaerobic glycerol dehydrogenase and

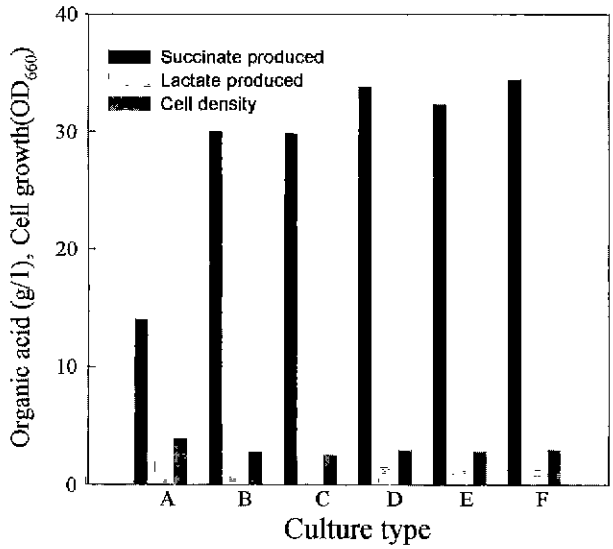

Fig. 2. Comparison of bioconversion of fumarate to succinate, and cell density by aerobic or anaerobic culture.
A, Aeration; B, Sealed, C, CO_2 gas, D, Na_2CO_3; E, $CaCO_3$; F. $MgCO_3$. A: 100-ml flask (15 ml medium), B-F: 20-ml vial (15 ml medium). D-F: charged with CO_2 gas before carbonates were added; initial fumarate, 50 g/l, initial glycerol, 20 g/l; pH 7.0 incubation time, 24 h.

fumarate reductase, which were key enzymes of succinate production in this study, and the added carbonates served to replace carbon dioxide. Although all these carbonates had almost similar results, sodium bicarbonate was used in further experiments, since the addition of calcium and magnesium carbonate to the medium resulted in precipitation because of their low solubility. Thus, the effect of sodium bicarbonate ranging from 0 to 30 g/l on the production of succinate was examined. As shown in Fig. 3, the highest yield of succinate was obtained at an initial concentration of 7 g/l, but the efficiency of succinate production was not satisfactory because lactate was also produced in a small amount. Therefore, the initial sodium bicarbonate concentration of 5 g/l was chosen to be an effective concentration for succinate production with few byproducts.

Optimum Initial pHs. The effect of pH on succinate production was investigated at pH values between 5.8 and 8.0 with 0.1 M potassium phosphate buffer. As shown in Fig. 4, succinate production and cell growth increased with increase of the pH and reached its optimum pH at 7.0. The bioconversion of fumarate to succinate was relatively constant in the pH range of 7.0-7.6 with the succinate production in the range of 40-43 g/l. Thus, it was found that a bioconversion for succinate production was feasible when pH was controlled at the range of 7.0-7.6.

Optimization of Nutrient Compositions Carbon Sources. The effects of various carbon sources on succinate production were carefully investigated. Monosaccharides such as glucose, fructose, and galactose; disaccharides such as sucrose and lactose; sugar alcohol such as glycerol; and renewable resources such as corn steep liquor and whey were used as carbon sources with the initial concentration of 20 g/l each. The same initial concentration of 30 g/l of fumarate was used at this time. As shown in Fig. 5, among the carbon sources used, glycerol was particularly found to be the most effective for succinate production, resulting in a succinate concentration of 24.5 g/l with a molar yield of 81%. This result correlates with the findings of Kistler and Lin [9] in that the facultative anaerobes required glycerol as a hydrogen donor for the bioconversion of fumarate to succinate, and that the anaerobic utilization of glycerol is characteristic of

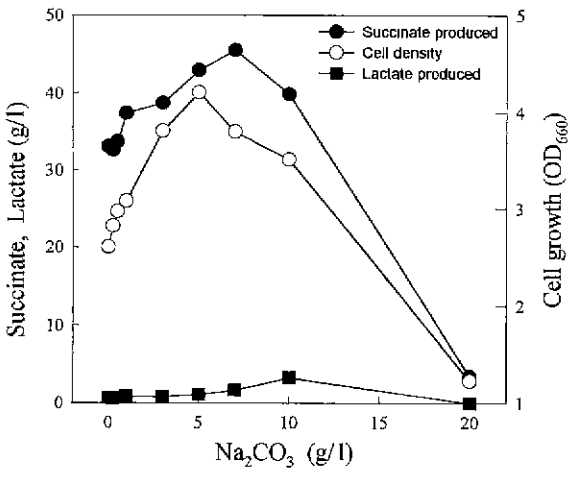

Fig. 3. Effect of Na_2CO_3 concentration on succinate, lactate production, and cell growth.
Twenty-ml vial (15 ml medium), initial fumarate, 50 g/l; initial glycerol 20 g/l; initial pH, 7.0; incubation time, 24 h.

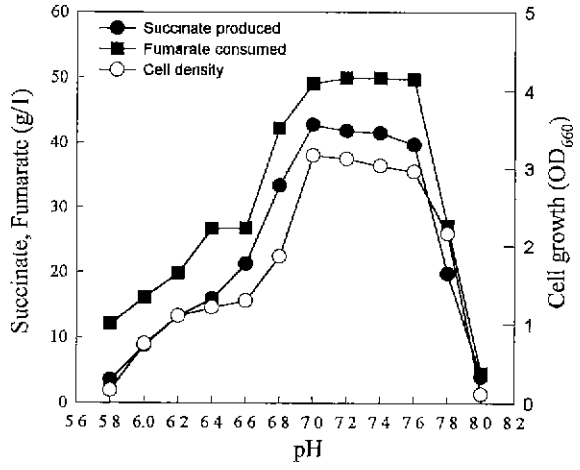

Fig. 4. Effect of pH on succinate production, fumarate consumption, and cell growth (0.1 M potassium phosphate buffer at 25°C).
Twenty-ml vial (15 ml medium); initial fumarate, 50 g/l; initial glycerol, 20 g/l, incubation time, 24 h

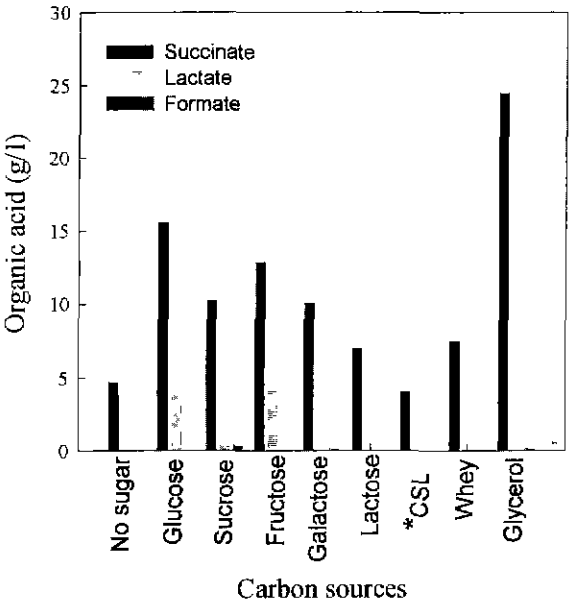

Fig. 5. Effect of carbon sources on succinate, lactate, and formate production.
Twenty-ml vial (15 ml medium); initial fumarate, 30 g/l, initial pH, 7.0; incubation time, 24 h; *CSL: Corn Steep Liquor.

Enterococcus faecalis [2]. In addition, by using glycerol as the carbon source, succinate of high purity (Fig. 6) was

RT (min)	Organic acid	Initial conc. (g/l)	Final conc. (g/l)
10.74	Succinate	1.8	24.56
12.40	Formate	0.0	0.11
13.56	Fumarate	30.0	0.04

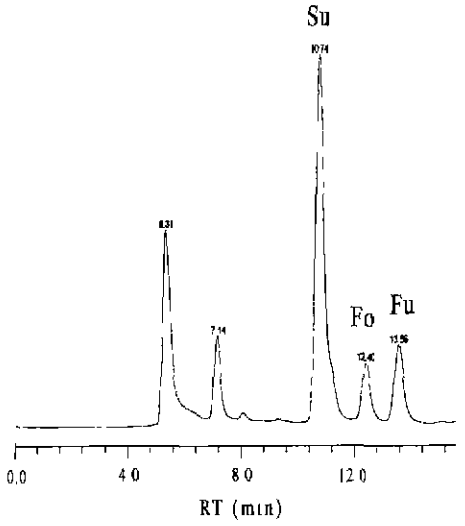

Fig. 6. HPLC chromatogram of bioconversion of fumarate to succinate at 24 h using glycerol as a carbon source.
Culture conditions: 20 ml vial (15 ml medium), initial fumarate, 30 g/l; initial pH, 7.0; incubation time, 24 h. HPLC conditions: Aminex HPX-8H, 300×1.7 mm (Bio-rad), 0.008 N H_2SO_4, 0.6 ml/min, UV 210 nm, 35°C. Su, succinate; Fo, formate; Fu, fumarate.

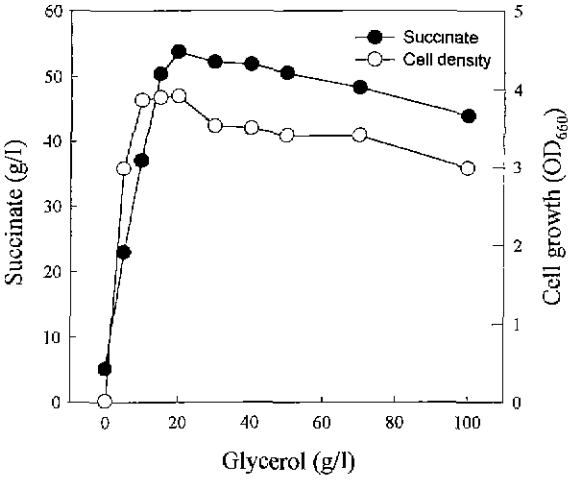

Fig. 7. *Effect of glycerol concentration on succinate production* and cell growth.
Twenty-ml vial (15 ml medium), initial fumarate, 60 g/l; initial pH, 7.0, incubation time 24 h.

produced with few unwanted organic acids, compared to other carbon sources used. The optimum concentration of glycerol ranging from 0 to 100 g/l was examined with the initial fumarate concentration of 60 g/l, since glycerol was the most suitable source on the succinate production. Figure 7 shows that the succinate production and cell growth were enhanced with increasing amounts of glycerol of up to 20 g/l, but cell growth was inhibited when it was above 20 g/l of glycerol. It was likely that substrate inhibition occurred due to excessive substrate concentration. The highest amount of succinate at 53.8 g/l (molar yield of 89%) was obtained at an initial glycerol concentration of 20 g/l. On the other hand, it should be noted that about 5.0 g/l succinate was produced with no addition of glycerol to the culture medium. This is not in agreement with the results of Miki and Lin [12], in which neither glycerol nor fumarate alone stimulated the production of succinate. However, it is possible that any organic acids, such as formate, pyruvate, lactate etc., produced in the metabolic pathway resulted in small amounts of succinate production.

Nitrogen Sources. To examine the effect of various nitrogen sources on succinate production, we used complex nitrogens such as polypeptone, yeast extract, bactopeptone, corn steep liquor, and whey with an initial concentration of 15 g/l, as well as inorganic nitrogen such as $(NH_4)_2SO_4$, sodium-L-glutamate, NH_4NO_3, and KNO_3 with an initial concentration of 5 g/l each, with the initial fumarate concentration of 30 g/l. Figure 8 illustrates that the presence of yeast extract in the medium stimulated succinate production (27.1 g/l) by more than 4 times those of other nitrogen sources. On the other hand, the mixtures of other nitrogen sources with yeast extract did not enhance succinate production as expected. Furthermore,

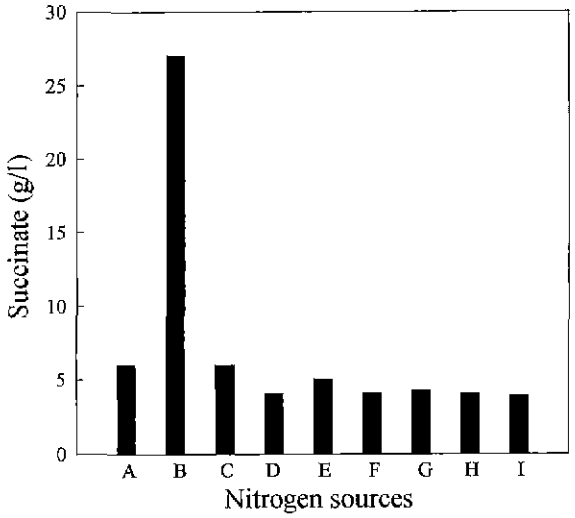

Fig. 8. Effect of nitrogen sources on succinate production.
A, Polypeptone; B, yeast extract; C, bactopeptone; D, CSL; E, whey; F, $(NH_4)_2SO_4$; G, sodium-L-glutamate; H, NH_4NO_3; I, KNO_3. Twenty-ml vial (15 ml medium); initial fumarate, 30 g/l, initial glycerol 20 g/l; initial complex nitrogen conc., 15 g/l; initial inorganic nitrogen conc., 5 g/l; pH 7.0; incubation time, 24 h

under these conditions, lactate was produced in large amounts as an unwanted byproduct (data not shown). Figure 9 shows the effect of yeast extract in the range of 0 to 50 g/l on succinate production. The amount of succinate produced seemed to be higher with the increasing concentration of yeast extract even at lower levels, but at more than 15 g/l of yeast extract, no additional succinate was produced, while the cell growth increased gradually up to 50 g/l.

Fumarate Concentrations. To investigate the effect of fumarate concentration on succinate production, the initial fumarate concentrations were adjusted to 0-150 g/l. As

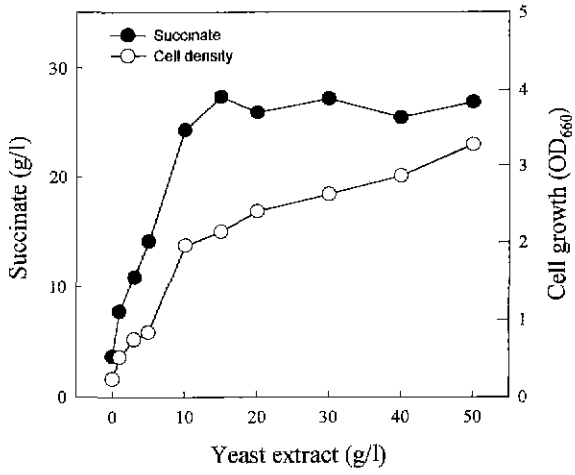

Fig. 9. Effect of yeast extract concentration on succinate production and cell growth.
Twenty-ml vial (15 ml medium); initial fumarate, 30 g/l, initial glycerol 20 g/l; initial pH, 7.0; incubation time, 24 h

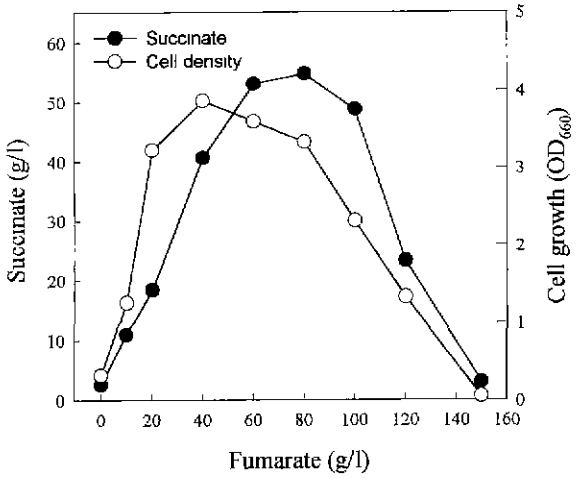

Fig. 10. Effect of fumarate concentration on succinate production and cell growth.
Twenty ml vial (15 ml medium), initial glycerol conc. 20 g/l; initial pH, 7.0, incubation time, 24 h

shown in Fig. 10, the maximum amount of succinate (54.8 g/l) was obtained at an initial fumarate concentration of 80 g/l. The presence of more than 80 g/l fumarate inhibited the succinate production. It is not yet certain whether this inhibition was caused by the high fumarate concentration in the medium, or by the high amount of succinate produced during the bioconversion process. On the other hand, the molar conversion yields on the basis of initial fumarate concentrations of 10, 20, 40, 60, 80, 100, 120, and 150 g/l were 18, 30, 100, 88, 68, 48, 38, and 5 %, respectively. From these results, we concluded that the optimum fumarate concentration as a conversion substrate was about 40 g/l.

Inorganic and Metal Salts. Figure 11 presents the effect of dibasic potassium phosphate concentrations (ranging from 0 to 20 g/l) on succinate production and cell growth. As the concentration level of potassium phosphate increased, the production of succinate also increased up to 43 g/l. It did not increase more at higher than 10 g/l K_2HPO_4 concentration, while the level of cell growth was almost proportional to the initial potassium phosphate concentrations, resulting in the highest cell density of 4.5 (OD_{660}) at 20 g/l K_2HPO_4. The significance of this result is that large amounts of potassium ions are necessary for the efficient cell growth of the strain. Furthermore, the effect of sodium chloride concentrations on succinate production was investigated by using it in the range of 0 to 10 g/l, and the optimum concentration was found to be 1 g/l (data not shown). The effect of various trace metals on succinate production with an initial concentration of 50 ppm in a 20-ml vial culture was examined and the results are shown in Fig. 12. Magnesium ion was the most effective for producing succinate, which was about 1.14-fold greater than the case with no metal ions added. Therefore, the

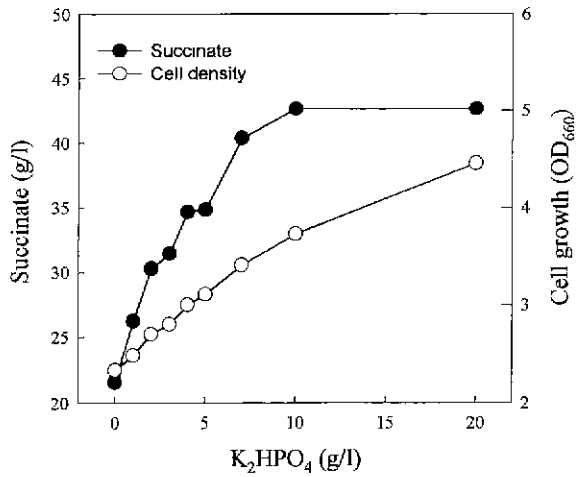

Fig. 11. Effect of K_2HPO_4 concentration on succinate production and cell growth.
Twenty-ml vial (15 ml medium), initial fumarate 50 g/l; initial glycerol 20 g/l; pH, 7.0; incubation time, 24 h.

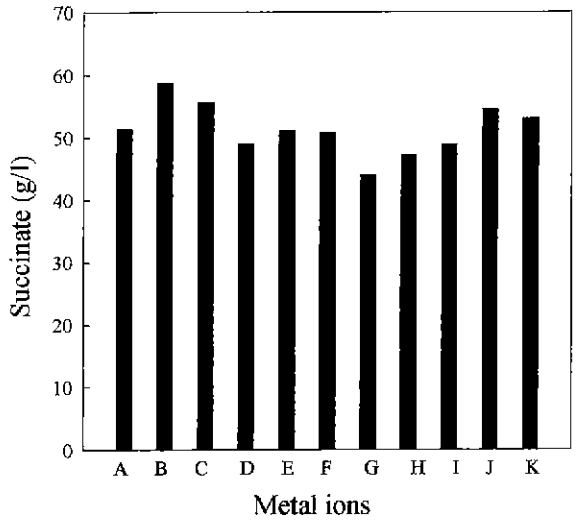

Fig. 12. Effect of metal ions on succinate production.
A. No metal. B. $MgCl_2$; C, $MgSO_4$; D. $CaCl_2$, E, $FeSO_4$; F, $ZnSO_4$, G, $MnCl_2$; H. $MnSO_4$; I, $CuSO_4$; J. $CoCl_2$; K, $BaCl_2$. Twenty-ml vial (15 ml medium), initial fumarate 60 g/l; initial pH 7.0. incubation time, 24 h.

presence of magnesium ions such as magnesium chloride or magnesium sulfate were found to be favorable for succinate production, while calcium or manganese ions in the medium inhibited the production.

Production of Succinate by *Enterococcus faecalis* Strain RKY1

The production of succinate by wild-type *Enterococcus faecalis* RKY1 grown on optimum medium was determined. Fed-batch operation was initiated prior to complete fumarate exhaustion in the medium (i.e. a detected residual fumarate concentration of approximately 1.0 g/l). Since concentrations of fumarate greater than 8% inhibited succinate production,

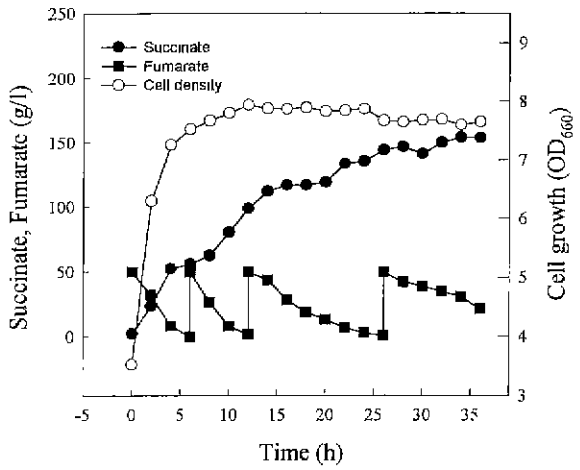

Fig. 13. Time course of bioconversion of fumarate to succinate in fed-batch cultivation.
2.5-l fermentor, CO_2 replacement, total fumarate conc., 200 g/l; initial glycerol conc., 20 g/l (plus 10 g/l fed); initial cell density, 3 g/l; pH 7.0 38°C, 200 rpm; pH neutralizer, 2 M Na_2CO_3.

the reaction was carried out at 38°C for 36 h by keeping the fumarate concentration below 5% through successive feeding of fumarate and glycerol. The time course for succinate production is shown in Fig. 13. A total of 153 g of succinate per liter was produced after 36 h of the reaction. The total volumetric production rate and the molar yield of succinate were 4.3 g/l/h and 85%, respectively.

Acknowledgments

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