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Enhancement of Succinate Production by Organic Solvents, Detergents, and Vegetable Oils

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Abstract Bioconversion of fumarate to succinate by *Enterococcus* sp. RKY1 was enhanced when Tween surfactant, organic solvent, and vegetable oil were added to the fermentation medium. The maximum amount of succinate produced was 80.4 g/l after a 24 h incubation when Tween 80 was added to the culture to a final concentration of 0.1 g/l. Triton X-100 was observed to damage the enzymes and inhibit the formation of succinate. The addition of 10 ml/l acetone increased the production of succinate by 110%. Vegetable oils used were found to be effective for succinate production as well as for the cell growth. Similar productivity increases were obtained with corn oil and Tween 80 plus biotin with the total productivity being 3.6 g/l/h, and 3.5 g/l/h, respectively, which was approximately 25% greater than that of the control. Therefore, these results indicate that corn oil can be considered the most appropriate agent for the production of succinate where succinic acid was primarily used in the production of food, medicine, and cosmetics.

Key Words: *Enterococcus* sp. RKY1, bioconversion, succinate, fumarate, Tweens, organic solvent, vegetable oil, corn oil, biotin

Succinic acid (butanedioic acid), $HOOCCH_2CH_2COOH$, is a major metabolic intermediate in most anaerobic microorganisms. Succinic acid is a naturally occurring organic acid that can be produced by fermentation or chemical synthesis. Succinic acid has many industrial applications as raw material for food, medicine, plastics, cosmetics, textiles, plating, and waste-gas scrubbing [10].

The anaerobic fermentation of glycerol by *Streptococci* is dependent upon the presence of external hydrogen acceptor [7]. In these kinds of anaerobes, fumarate reduction to succinate is coupled to electron transport-mediated phosphorylation [3]. In addition to the mutation approach [9], there are alternatives for enhancing the fermentation product [2]. Bioconversion of fumarate by microbes to produce succinate is dependent on various physiological and biochemical parameters.

It has been observed that treatment of microorganisms with organic solvents or surface-active agents can increase the cell membrane permeability [2]. The metabolic products can then be quickly secreted into the culture broth, and thus avoid being degraded inside the cell [1]. Gong *et al.* [6] demonstrated the enhanced L-malic acid production by *Brevibacterium* sp. through the treatment with detergents. Neufeld *et al.* [16] reported the enhanced L-malic acid formation by *Saccharomyces cerevisiae* following the cationic and nonionic surfactant treatment. Goldberg and Stieglitz [5] reported the enhanced yields of fumaric acid with *Rhizopus oryzae* when Tween surfactant or vegetable oil was added to the fermentation medium.

It has been reported that enzymes involved in fumarate reduction, glycerol dehydrogenase and fumarate reductase, are located in the cell membrane [11, 12]. Therefore, seeking an agent for increasing the cell permeability efficiently while not impairing the enzyme system for the succinate production is of great importance. In this study, we investigated the effect of organic solvents, detergents, and vegetable oils on the enhancement of succinate production.

Materials and Methods

Microorganism and Medium

Enterococcus sp. RKY1, a newly isolated strain, was used for the bioconversion of fumarate to succinate [18]. The medium for the bioconversion contained the following: 20 g glycerol (Yakuri, Osaka, Japan), 50 g fumaric acid (Yakuri, Osaka, Japan), 15 g yeast extract (Difco, Detroit, U.S.A.), 10 g K_2HPO_4, 1 g NaCl, 46 g Na_2CO_3, 0.05 g

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$MgCl_2 6H_2O$, 0.01 g $FeSO_4 7H_2O$, and 1 l distilled water, unless otherwise mentioned. Sorbitan monopalmitate, monostearate, monooleate, and trioleate were from the Yakuri Chemical (called Span 40, 60, 80, and 85, respectively). Polyoxyethylene sorbitan monolaurate, monostearate, and monooleate were also from the Yakuri Chemical and called Tween 20, 60, and 80, respectively.

Culture Conditions

Cells were grown anaerobically in 250 ml vials containing 200 ml of medium at 38°C in a rotary shaker at 200 rpm for 12 h. Subculturing in the same medium was carried out for 2 days. Cells were then pelleted (10,000 rpm, 10 min) and transferred to a fermentation medium. Vial cultures were prepared by inoculating 0.6 ml of resting cells into the culture medium (15 ml) in 20 ml vials, followed by incubation at 38 for 24 h on a shaking incubator (Vision Scientific Co., Korea) at 200 rpm. For the fermentor culture, a 2.5 l jar-fermentor (Korea Fermentor Co., Korea) containing 1 l of the optimized medium was inoculated with about 3.0 g/l of resting cells and incubated for 24 h at 38°C, 200 rpm, pH 7.0, with the headspace of the medium replaced with 100% CO_2 gas before inoculation.

Analytical Methods

Succinic acid, fumaric acid, lactic acid, and other organic acids were quantitatively analyzed by HPLC with a pump (Millipore Co., Waters 510), a Bio-Rad (Hercules, U.S.A.) Aminex HPX-87H ion-exclusion column (300×7.8 mm), and a UV detector (Millipore Co., Waters 486). The column was eluted with 5 mM sulfuric acid at a column temperature of 35°C and a flow rate of 0.6 ml/min. Cell growth was measured as the absorbance at 660 nm using a UV-spectrophotometer (Shimadzu Co., UV-100A).

Results and Discussion

Detergents Affecting the Bioconversion of Fumarate to Succinate

Nonionic detergents which did not inhibit the biological activity of the cell membrane [1] were used in this study to alter the cell permeability. As shown in Fig. 1, the non-ionic detergents tested were effective at the relatively low concentrations, but at the higher concentrations, they markedly inhibited the production of succinate. The maximum amount of succinate reached 80.4 g/l after 24 h incubation when Tween 80 was added to the culture to the final concentration of 0.1 g/l. An approximately 1.3-fold increase in the productivity of succinate, as compared with the control, was observed. Tween 80 might function by altering the cell permeability, thus facilitating a higher intracellular aspartase concentration [20]. Neufeld *et al.* [16] reported that the reduced activities at the excess levels

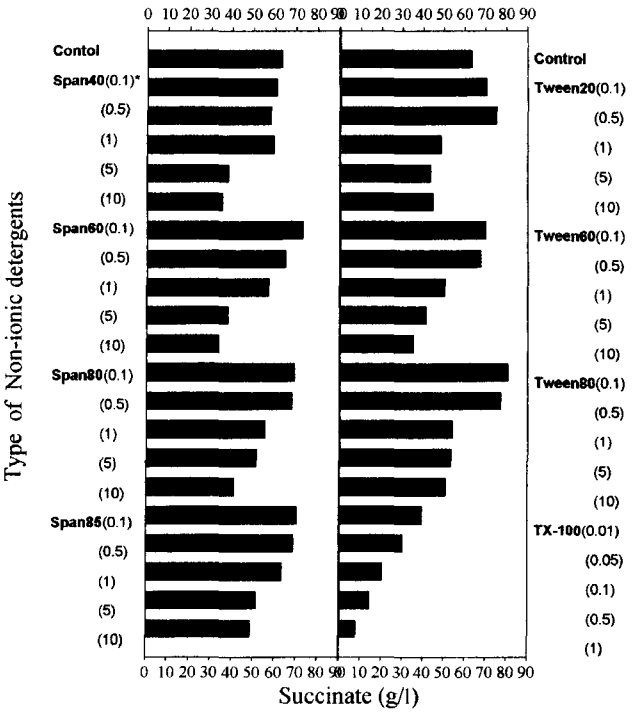

Fig. 1. Effects of various detergents on succinate production.
*Numbers in parentheses indicate the concentration of detergent added to the medium (g/l).

of surfactant might be due to a toxic effect. The results of these experiments agreed with these reports. Takinami *et al.* [19] showed C_{12} to C_{18} chain length acids were more effective for accumulating a large quantity of L-glutamic acid in the medium at low concentration of fatty acid. Similarly, the fatty acids with various chain lengths, such as C_{12} (lauric), C_{16} (palmitic), and C_{18} (stearic), used in this study were also effective for the production of succinate at the relatively low concentrations. This result suggests that for nonionic detergents to accelerate the contact of enzymes in cells and substrates, they are dependent on carbon chain length of fatty acid. The production of succinate by *Enterococcus* sp. RKY1 was greatly inhibited by Triton X-100 with the range of the concentrations of 0.01-1 g/l. Triton X-100, mainly used in extracting fumarate reductase from the cell membrane [13], might damage the conversion enzyme and stop the formation of succinate. It was reported that oleic acid was essential for several lactic acid producing bacteria [4]. It has also been found that the glutamic acid producing bacteria was stimulated to make L-glutamic acid with an oleic acid as well as with biotin [19]. The combinations of the fatty acid ester, Tween 80, and biotin to obtain the succinate formation over 60 g/l were plotted in Fig. 2. The optimum amounts of the two factors for the maximum production of the succinate might be induced to be on the plotted curve. It was found that the ester added increasingly in proportion to the concentration

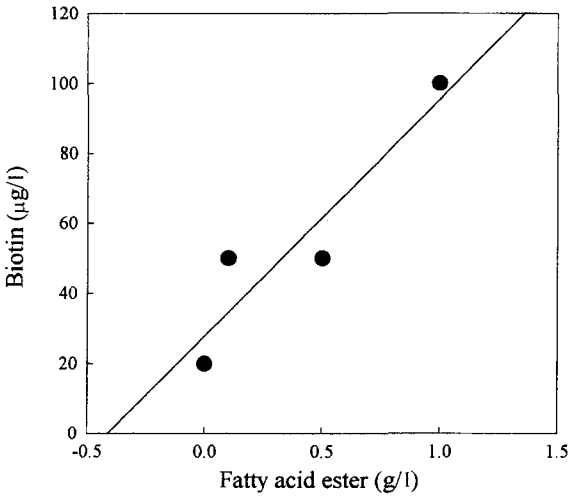

Fig. 2. Interrelation of fatty acid ester, Tween 80, to biotin.
The amounts of Tween 80 and biotin, when succinate over 60 g/l was accumulated, are plotted.

of biotin played an important part in accumulating succinate.

Organic Solvents Affecting the Bioconversion of Fumarate to Succinate

Treatment with organic solvent has been used to increase the production of organic acids and amino acids by resting cell suspensions of bacteria and yeasts. Therefore, the enhancement of succinate production with various organic solvents such as benzene, toluene, xylene, chloroform, acetone, and n-butanol was investigated. As shown in Fig. 3, the addition of 10 ml/l acetone to the *Enterococcus* sp.

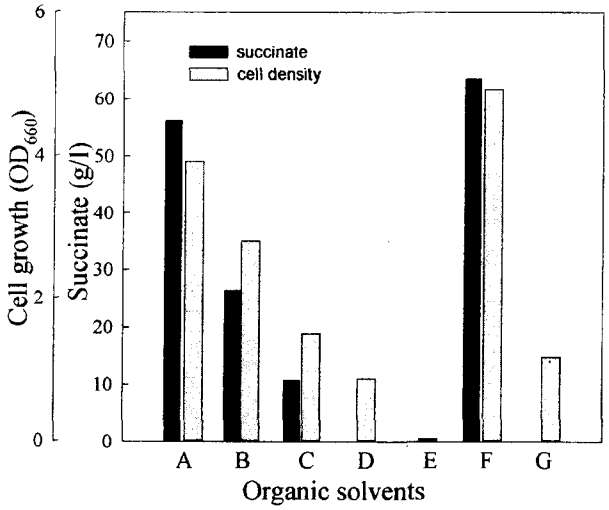

Fig. 3. Effect of organic solvents on succinate production and cell growth.
A. Control; B. Benzene; C. Toluene; D. Xylene; E. Chloroform; F. Acetone; G. *n*-Butanol. 200 ml vial (15 ml medium); initial fumaric acid, 60 g/l; initial glycerol, 20 g/l; initial organic solvent, 10 ml/l; time, 12 h.

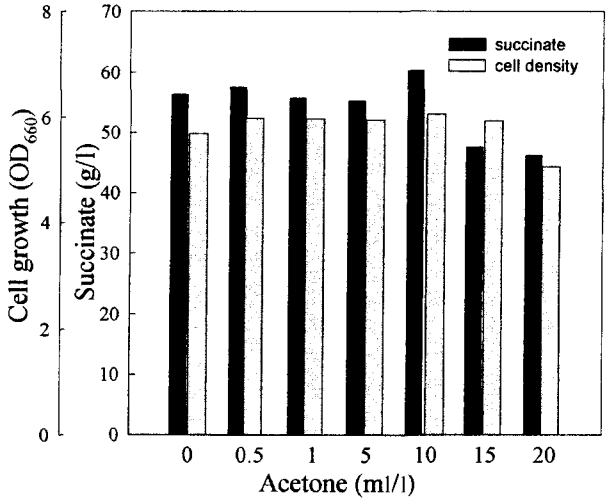

Fig. 4. Effects of acetone concentration on succinate production and cell growth.
20 ml vial (15 ml medium); initial fumaric acid, 60 g/l; initial glycerol, 20 g/l; incubation time, 12 h.

RKY1 cell suspension increased the production of succinate from fumarate to 110%. The other organic solvents tested, compared to acetone, showed an unfavorable effect on both succinate production and the cell viability. Specifically, xylene, which has been reported to increase yields of L-sorbosone by *Gluconobacter melanogenus* IFO 3293 [14] and of D-malic acid by *Ustilago sphaerogena* [15], completely inhibited the conversion of fumarate to succinate.

On the other hand, dissimilatory fumarate reduction proceeds via an electron transport system involving specific dehydrogenases, electron carriers, and fumarate reductase, and the components of the system are all cell membrane bound. In the case of chloroform, the cell growth was also completely inhibited. This could be explained by assuming that the membrane-bound enzyme system responsible for succinate production was severely damaged by the organic solvents. This result agreed with the observation of Harrop *et al.* [8] on organic solvent toxicity to bacteria. On the other hand, acetone concentrations from 0.5 to 10 ml/l did not significantly inhibit succinate formation with more than 15 ml of acetone per liter repressing the formation of succinate (Fig. 4). In contrast, the presence of acetone rarely shows a loss of cell density. It appeared that the enzyme system for succinate production was more vulnerable to the organic solvents than to the cell viability.

Vegetable Oils Affecting the Bioconversion of Fumarate to Succinate

Figure 5 shows the effects of various vegetable oils on succinate production and cell growth. It was known that Tween surfactants and vegetable oils increased the rate of organic acid accumulation [5]. In general, the vegetable oil

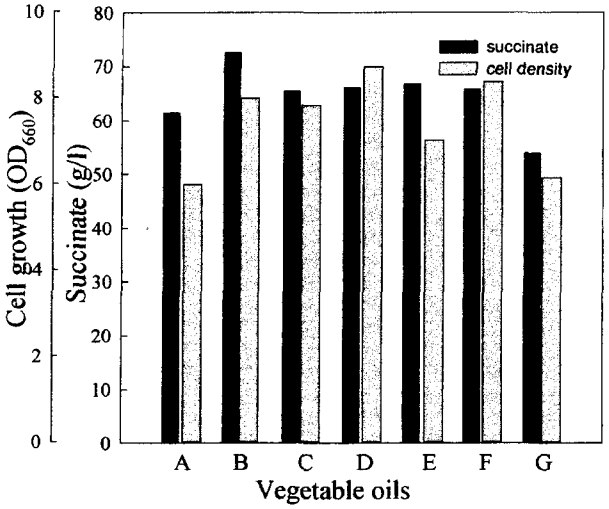

Fig. 5. Effects of vegetable oils on succinate production and cell growth.
A. Control; B. Corn oil; C. Soybean oil; D. Cottonseed oil; E. Coconut oil; F. Olive oil; G. Mineral oil. 20 ml vial (15 ml medium); initial fumaric acid, 60 g/l; initial vegetable oil, 1 g/l; incubation time, 12 h.

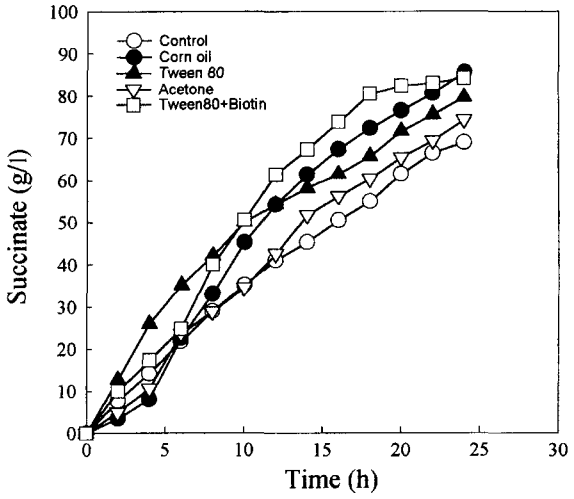

Fig. 6. Effect of control, Tween 80, acetone, corn oil and Tween 80 + biotin on succinate production by *Enterococcus* sp. RKY1.
2.5-liter fermentor (1-liter medium); initial fumaric acid, 70 g/l; initial glycerol, 20 g/l; added glucose, 5 g/l; pH 7.0; initial cell density, 2.7~3.0; 30°C; 200 rpm; pH neutralizer, 2 M Na_2CO_3.

used was found to be effective for succinate production as well as for the cell growth. The enhanced cell growth of 8.7 (OD_{660}) from cottonseed oil was remarkedly higher than that from others after 12 h incubation. It was likely that the added fatty acid ester served as a carbon source. Also, corn oil was the most suitable agent for the efficient succinate production by *Enterococcus* sp. RKY1. The conversion was enhanced about 1.2-fold by addition of corn oil to the final concentration of 1 g/l to the reaction mixture and the amount of succinate production was 72.5 g/l at this condition. This might be due to a favorable ratio of 2.5 for unsaturated and saturated fatty acids, especially, oleic and palmitic acids, which were contained in corn oil [17]. The optimal concentration of corn oil in the reaction mixture for succinate production was examined and determined to be 1 g/l with the succinate amount of 75 g/l (data not shown).

Agents Increasing Conversion of Fumarate to Succinate by *Enterococcus* sp. RKY1 Cell Suspension

Cell growth and conversion of strain RKY1 cultured in media containing either detergent, organic solvents, or vegetable oil were optimized and compared. The conversion reactions were carried out in a 2.5 l-fermentor containing 1 l of the respective medium for 24 h. As indicated in Fig. 6 and Table 1, Tween 80, corn oil, and Tween 80 plus biotin exhibited a favorable enhancement effect on the molar yield and volumetric succinate productivity at a low concentration of 0.1 g/l, 1 g/l, and 1 g/l plus 100 μg/l, respectively, as compared with control. The similar productivity and molar conversion yield on the basis of the amount of fumarate consumed were obtained with corn oil

Table 1. Effect of various agents on yield and productivity of succinate[a]

Agent[b]	Time (h)	Final cell con. (g/l)	Fumarate used (g/l)	Succinate produced (g/l)	$Y_{P/S}$ %[c]	SP g/g/h[d]	VP g/l/h[e]
None	24	5.4	77.8	69.0	87.6	0.53	2.9
Tween 80	24	7.5	86.3	79.6	91.0	0.44	3.3
Acetone	24	6.7	83.6	74.4	87.9	0.46	3.1
Corn oil	24	8.7	91.3	85.5	92.5	0.41	3.6
Tween 80 + biotin	24	7.2	89.2	84.3	93.3	0.49	3.5

[a]Initial cell density, 3 g/l; Temperature, 38°C; Agitation, 200 rpm; pH, 7.0.
[b]Surfactant con:Tween 80, 0.1 g/l; Acetone, 10 ml/l; Corn oil, 1 g/l; Tween 80 + biotin, 1.0 g/l+100 μg/l.
[c]$Y_{P/S}$, Maximum mol of succinate/mol of fumarate consumed (mol%).
[d]SP, Average specific productivity of succinate (g succinate/g cells/h).
[e]VP, Average volumetric productivity of succinate (g succinate/l/h).

and Tween 80 plus biotin, amounting to 3.6 g/l/h and 92.5% and 3.5 g/l/h and 93.3%, respectively. Corn oil increased the succinate amount from 69.0 g/l to 85.5 g/l, i.e. an increase of 24%. Therefore, the results indicated that corn oil could be considered the most proper solvent in the production of succinate since succinic acid was mostly used in the production of food, medicine, and cosmetics. Figure 7 shows the effect of agents on the cell growth. The cell growth was greatly stimulated, up to 160%, by the addition of corn oil. When Tween 80 or Tween 80 plus biotin was added, the similar cell growth was obtained. The succinate production in the case of Tween 80 (79.6 g/l) was lower than that in the case of Tween 80 plus biotin (84.3 g/l). It was presumed that the added biotin acted as a cofactor of dehydrognases, leading to the improvement in all the bioconversion parameters.

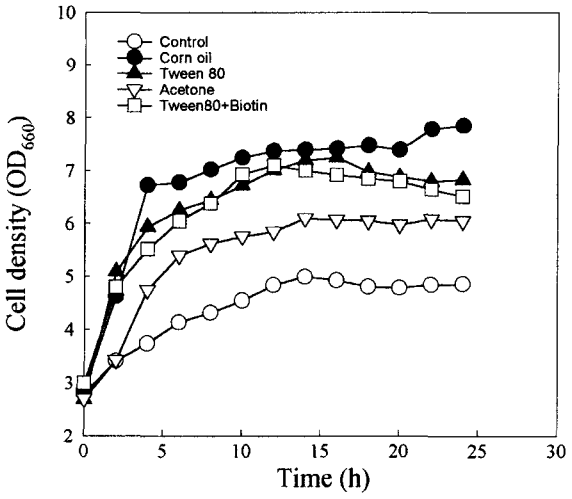

Fig. 7. Effect of control, Tween 80, acetone, corn oil and Tween 80+biotin on cell growth by *Enterococcus* sp. RKY1.
2.5 l fermentor (1-liter medium); initial fumaric acid, 70 g/l; initial glycerol, 20 g/l; added glucose, 5 g/l; pH 7.0; initial cell density, 2.7-3.0; 30°C; 200 rpm; pH neutralizer, 2 M Na_2CO_3.

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