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Production of L-Malic Acid from Fumaric Acid by Resting Cells of *Brevibacterium sp.*

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ABSTRACT

The ability of the resting cells of *Brevibacterium sp.* B2D, a flocculent strain, to convert fumaric acid to L-malic acid was investigated under different fermentation conditions. The optimal temperature for the bioconversion is approx 36°C, and the optimal pH is around 6.4. The bacteria do not require special cultural conditions for the development of fumarase. In the presence of relatively high concentrations of fumarate (60 g/L), the bacterial cells exhibited a high rate of L-malic acid production with high yield without the coproduction of unwanted byproducts like succinic acid. A yield of 89.8% of L-malic acid from fumaric acid and an average specific productivity of 0.41 g/g/h was obtained. The presence of surfactant, even at low concentrations like 0.02%, resulted in over a twofold increase in specific malic acid production rate.

Index Entries: Fumaric acid; L-malic acid; *Brevibacterium sp.* B2D; fumarase; effect of surfactant.

INTRODUCTION

L-Malic acid, a naturally occurring four-carbon dicarboxylic acid, is a constituent of almost all plant and animal tissues, and is produced as a normal intermediate in basic metabolism. It is a major acid component in many fruits. Apple juice may contain a total acid concentration of up to 7% L-malic acid *(1)*. Malic acid is commlonly used as a food and beverage ingredient. It has also been used for the treatment of hyperammonemia and liver dysfunction, and as a component for amino acid infusion. Recently, malic acid has been considered as a raw material for the biodegradable polymers *(2)*.

L-Malic acid can be obtained from its natural source. However, this is impractical owing to the small quantities in which it occurs. Large-scale production of malic acid has been achieved through the chemical hydration of chemically deriveld fumaric or maleic acids. It can also be produced through enzymatic hydration of fumaric acid mediated by fumarase (fumarate hydratase, EC 4.2.1.2). Unlike malic

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acid produced naturally, chemically produced malic acid has an equal proportion of the racemic D- and L-forms.

L-Malic acid is often produced extracellularly by microorganisms during their normal metabolism. Some mycelial fungi, such as those belonging to the genus *Aspergillus*, can produce copious quantities of L-malic acid under controlled environmental conditions from simple sugars *(3,4)*. L-Malic acid can also be produced from fumaric acid by fumarase-containing microbial cells. *Brevibacterium ammoniagenes (5)* and *Brevibacterium flavum (6)* are two well-known microorganisms, owing to their high fumarase activities, that have been used in immobilized forms for large-scale production of L-malic acid resulting from their high fumarase activity.

In this work, we investigated the production of L-malic acid from fumaric acid using the resting cells of a flocculent strain of *Brevibacterium*. We examined the optimal conditions, the substrate tolerance, and the effect of surfactants on the bacterium to achieve the optimal conversion.

MATERIALS AND METHODS

Organism and Medium

Brevibacterium sp. strain B2D was used. It was isolated from a laboratory culture with respect to its ability to flocculate during growth. The medium for cell growth contained the following: 3 g yeast extract (Difco, Detroit, MI), 3 g malt extract (Difco), 5 g peptone (Difco), 2 g glucose, and 1 L distilled water. Sterilization was accomplished by autoclaving at 15 lb/in.^{2} for 15 min.

Inoculation and Fermentation Conditions

Bacterial cells were grown aerobically in Erlenmeyer flasks containing the above medium at 25°C on a rotary shaker at 150 rpm for 72 h. Cells were collected aseptically by centrifugation using a clinical centrifuge at 500 rpm. After washing the cell pellets with sterile water, they were pelleted again and transferred to a fermentation substrate. Fermentation was conducted under aerobic conditions using 50-mL Erlenmeyer flasks that contained 10 mL of fermentation medium covered with a milk filter paper followed by aluminum foil. The fermentation medium contained yeast extract-malt extract–peptone (YMP) with ammonium fumarate.

Analytical Methods

Dry bacterial cell mass was determined by using a calibration curve made from the relationship between the packed-cell volume and their dry cell weight. Fumaric acid, L-malic acid, succinic acid, and other organic acids were determined and quantified by high-performance liquid chromatography (HPLC) (Hitachi Instrument, L-6200A) using a Bio-Rad (Hercules, CA) Aminex HPX-87H ion-exclusion column (300 × 7.8 mm) with a refractive index detector (Hitachi Instrument, L-3350 RI). The column was eluted with dilute sulfuric acid (0.005*M*) at a column temperature of 80°C and a flow rate of 0.8 mL/min over a 13-min period. Figure 1A shows the liquid chromatogram of organic acids of interest.

RESULTS AND DISCUSSION

Brevibacterium sp. B2D showed the ability to convert fumaric acid to L-malic acid when the resting cells were incubated with fumarate. At an initial substrate

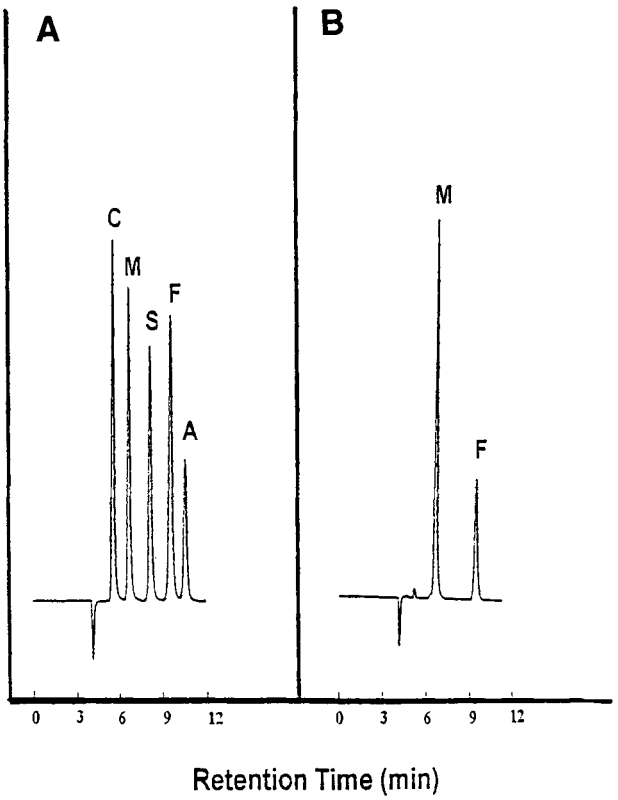

Fig. 1. Liquid chromatograms. **(A)** Retention times for the compounds of interest. C, citric acid (5.98 min); M, L-malic acid (7.19 min); S, succinic acid (8.85 min); F, fumaric acid (10.4 min); A, acetic acid (11.52). **(B)** Bioconversion of fumaric acid to L-malic acid by *Brevibacterium sp.* B2D. Initial fumaric acid concentration, 60 g/L; temperature, 34°C; initial cell density, 2.4 g/L; pH 6.8; incubation time, 48 h. F, fumaric acid (14.4 g/L); M, L-malic acid (47.3 g/L).

concentration of 60 g/L and an initial cell density of 2.4 g/L, fumaric acid was consumed linearly with the production of L-malic acid as the conversion product without apparent lag period (Fig. 2). The results also showed that over 76% (45.6 g/L) of fumaric acid was consumed after 48 h of incubation with an average specific malic acid productivity of 0.41 g/g/h and a concentration of L-malic acid at 47.3 g/L. Cell mass did not increase, and the fumarase activity was maintained for over 100 h of experiments. L-Malic acid yield based on the amount of fumaric acid consumed was 1.037, corresponding to an equivalent of 89.8% of the theoretical value, i.e., 1 g of fumaric acid is converted to produce 1.155 g of L-malic acid. This is based on the following reaction as mediated by fumarase:

$$\text{fumaric acid} + H_2O \rightarrow \text{L-malic acid} \quad (1)$$

No detectable succinic acid was produced, as evidenced by the liquid chromatographic profile shown in Fig. 1B. Succimic acid is the common undesirable byproduct produced by other L-malic acid-producing bacteria, especially when they are immobilized *(5–7)*.

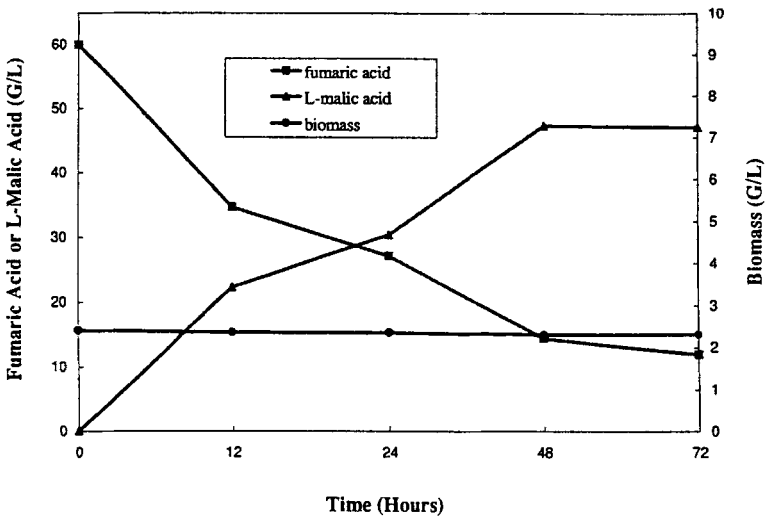

Fig. 2. Bioconversion of fumaric acid to L-malic acid by *Brevibacterium sp.* B2D. ■, Fumaric acid; ▲, L-malic acid; ●, cell density; temperature, 34°C, pH 6.8.

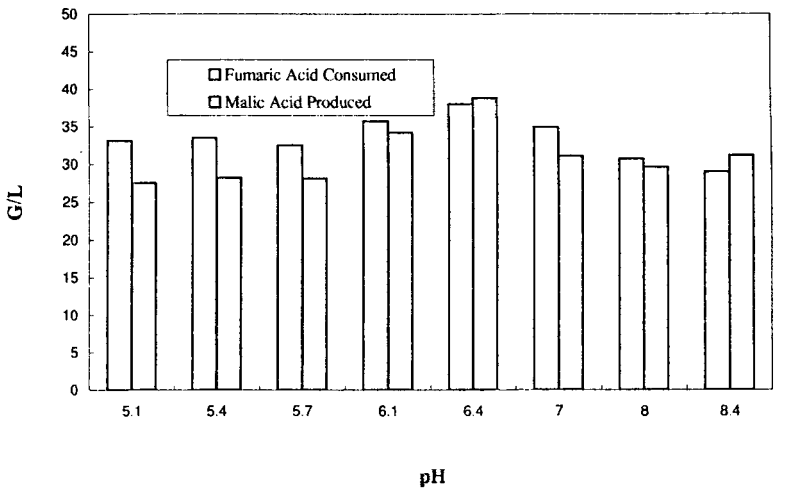

Fig. 3. Consumption of fumaric acid and the production of L-malic acid by *Brevibacterium sp.* B2D at different initial pHs. Initial cell density, 2.4 g/L; initial fumaric acid concentration, 60 g/L; temperature, 34°C; incubation time, 48 h.

Effect of pH and Temperature

The effect of pH was studied in the range of 5.1–8.4. After 36 h of incubation at 34°C with an initial substrate concentration of 50 g/L, over 50% of the fumaric acid was converted to L-malic acid with an effective yield of 88% at pH 6.4. L-malic acid production increased with increasing pH value and reached its optimal at 6.4. At a higher pH value, the rate of L-malic acid production declined slightly (Fig. 3). Similarly, incubation temperature had no significant effect over a range of 25–40°C. The initial conversion rate of fumaric acid to L-malic acid is relatively constant over the temperature range of 32–38°C with the specific L-malic acid produc-

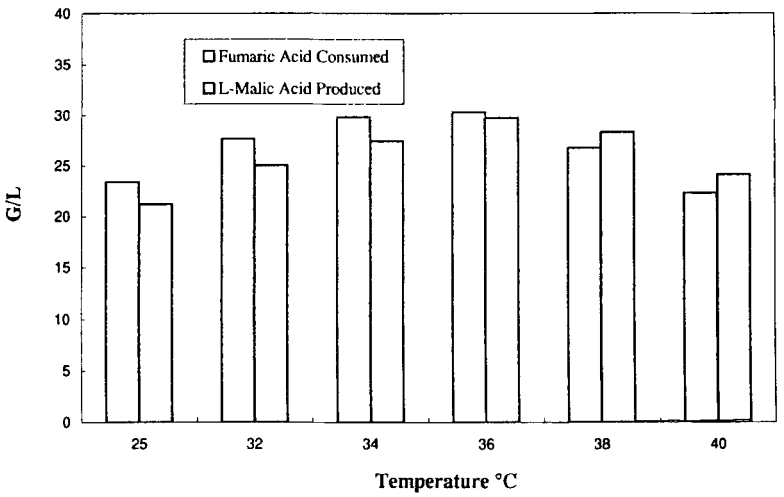

Fig. 4. Consumption of fumaric acid and the production of L-malic acid by *Brevibacterium sp.* B2D at different temperature. Initial cell density, 2.4 g/L; initial furmaric acid concentration, 60 g/L; initial pH 6.8; incubation time, 48 h.

tivity (g malic acid/g cell/h) in the range of 0.4–0.45. The specific productivity was slightly lower at 25 and 40°C (Fig. 4).

Effect of Substrate Concentration

The effect of initial substrate concentration was examined in the range of 20–100 g/L to determine the optimal condition for L-malic acid production. An increase in initial substrate concentration not only led to the increase in accumulation of L-malic acid, but also led to the increase in the average specific L-malic acid productivity (qp). The effects of initial substrate concentration on various parameters are shown in Table 1. The highest value in qp was obtained when the highest S_0 (100 g/L) was used, resulting in a product yield of approx 1, corresponding to 86% of the theoretical. The lower yield of L-malic acid from low substrate concentration is probably the result of the consumption of substrate or product during fermentation.

Effect of Surfactants

Surfactants are known to enhance the rates of conversion of fumaric acid to L-malic acid. In an immobilized system using *Saccharomyces cerevisiae*, such surfactants as Triton X-100 increased the specific malic acid productivity from 0.34 (0.046) to 0.45 mmol/h/g cell (0.06 g/g/h), an increase of 32% *(8)*. Similarly, in immobilized *B. flavum*, the cells were pretreated with surfactant to increase the productivity *(6)*. The most dramatic example of the effect of surfactant is the ability of a strain of *S. cerevisiae* amplified for fumarase to convert fumaric acid to malic acid. In the presence of surfactants, such as anionic deoxycholate, the rate of conversion escalated from zero to as high as 50 mmol/g cell/h or 6.75 g/g/h *(9)*.

We examined the effect of some surfactants on the conversion rate of fumaric acid to malic acid. Triton X- 100, Nonidet p-40, and deoxycholate exhibited a strong enhancement effect on the specific malic acid productivity even at a low

Table 1
Effect of Substrate Concentration[a]

S_o, (g/L)	Fumaric acid used, g/L	L-malic acid produced, g/L	Y(p/s), g/g	qp, g/g/h[b]	QP, g/L/h[c]
20	17.4	14.2	0.810	0.123	0.296
40	33.6	31.4	0.934	0.273	0.654
50	40.2	39.6	0.985	0.344	0.825
60	45.6	47.3	1.037	0.410	0.985
80	48.0	48.0	1.000	0.417	1.000
100	54.3	53.9	0.993	0.468	1.123

[a]Initial cell density, 2.4 g/L; temperature, 34°C; pH 6.8; incubation time, 48 h.
[b]qp, Average specific productivity of L-malic acid (g malic acid/g cells/h).
[c]QP, Average volumetric productivity of L-malic acid (g malic acid/L/h).

Table 2
The Effect of Surfactant on Bioconversion[a]

Surfactant, %	qp, g/g/h[b]
Control	0.67
Triton X-100	
0.02	1.31
0.05	1.34
0.10	1.40
Nonidet p-40	
0.02	1.38
0.05	1.39
0.10	1.39
Deoxycholate	
0.02	1.27
0.05	1.21
0.10	1.21
Tween-80	
0.1	0.85
0.2	0.85
SLS	
0.1	0.00

[a]Initial cell density, 2.4 g/L; temperature, 25°C; pH 6.8; incubation time, 48 h; fumaric acid concentration, 60 g/L.
[b]qp, Average specific productivity of L-malic acid (g malic acid/g cells/h).

concentration of 0.02% (Table 2). A twofold increase in specific productivity was obtained when the aforementioned surfactants were present. At higher concentrations, the effect was similar to that at lower concentrations. Tween-80 exhibited slight enhancement of bioconversion, whereas sodium lauryl sulfate (SLS) exhibited a detrimental effect. Fumarase activity in the present of SLS was not detected nor can the activity be restored after the removal of SLS, indicating the denaturation of fumarase by SLS.

CONCLUSION

From the data presented, *Brevibacterium* sp. B2D is characterized by its strong fumarase activity and has the potential for the large-scale conversion of fumaric acid to L-malic acid. The specific activity is generally higher than those reported for immobilized-cell systems. There is no special pretreatment needed to carry out conversion. Furthermore, the resting cell system exhibited relatively high yield, and in particular, no byproduct, such as succinic acid was detected. The bacterial culture grows well and can be collected by simple centrifugation, since this strain of bacterium flocculates easily. Compared to the chemical method, the biological conversion produces only L-isomer that can be used in food systems for human consumption. With its flocculating characteristics, this bacterial strain can easily be used without immobilization by recycling through centrifugation or by using cell-settling recycling techniques that can be easily adapted for commercial operation.

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