# Microbial Production of D-Malate from Maleate

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To produce D-malate from maleate by a microbial reaction, we screened a number of maleate-utilizing microorganisms for enzyme activity by an intact cell system. The strain which showed the best productivity among the 440 strains tested was identified taxonomically as *Arthrobacter* sp. strain MCI2612. The optical purity of the malate produced by this strain was 100% D type. The culture and reaction conditions for the production were studied for this strain. Addition of amino acids such as L-proline, L-histidine, and L-arginine to the culture medium promoted the formation of reaction activity as well as cell growth. Under optimum conditions, 87 g of D-malate per liter was produced in 20 h. The yield was 72 mol%.

D-Malate is very important starting material for the synthesis of various optically active compounds (1, 5). Maleate, which is industrially produced as a raw material for polymers, tetrahydrofuran, fumarate, DL-malate, etc., is an inexpensive chemical. D-Malate is obtained by the separation of DL-malate with expensive compounds such as quinine. Therefore, we aimed to produce D-malate from maleate by a microbial reaction.

Sacks and Jensen (10) reported that an enzyme mixture from corn kernels catalyzed maleate to malate. Thereafter, other publications reported that malease, which catalyzed the hydration of maleate to malate, existed in mammalian kidneys and pseudomonads (3, 4, 8). However, the productivity and the optical purity of malate from maleate have not been characterized. In general, many maleate-assimilating bacteria are known to metabolize maleate to L-malate via fumarate (7). Therefore, microorganisms which have a powerful D-malate-producing enzyme without a maleate isomerase were necessary to produce D-malate.

This report deals with the screening of microorganisms which produce D-malate from maleate, the identification of the strain, and the optimization of the conditions for enzyme formation during microbial cultivation and the microbial reaction for the production of D-malate. This is the first study on microbial production of D-malate stereoselectively from maleate in large amounts.

## MATERIALS AND METHODS

**Chemicals.** $S$-(+)-$\alpha$-Methoxy-$\alpha$-trifluoromethylphenylacetic acid chloride (MTPA-Cl) was purchased from Fluka AG, Buchs, Switzerland. Maleic anhydride was purchased from Wako Pure Chemicals, Osaka, Japan. L-Malate dehydrogenase, aspartate aminotransferase, and D-malate dehydrogenase (decarboxylation) were obtained from Boehringer Mannheim Yamanouchi, Tokyo, Japan. Other chemicals used were commercial products.

**Isolation of microorganisms.** Maleate-utilizing microorganisms were isolated from soil samples with a medium containing 5.0 g of maleate, 2.0 g of $(NH_4)_2SO_4$, 1.4 g of $KH_2PO_4$, 3.1 g of $NaHPO_4 \cdot 12H_2O$, 250 mg of $MgSO_4 \cdot 7H_2O$, 10 mg

of $FeSO_4 \cdot 7H_2O$, 10 mg of $MnSO_4 \cdot 4$–$6H_2O$, and 500 mg of yeast extract in 1,000 ml of tap water (pH 6.5).

**Medium and cultivation.** A basal medium for cultivation consisting of 10 g of glucose, 2 g of maleate, 3 g of $(NH_4)_2SO_4$, 10.5 g of $KH_2PO_4$, 250 mg of $MgSO_4 \cdot 7H_2O$, 10 mg of $FeSO_4 \cdot 7H_2O$, 10 mg of $MnSO_4 \cdot 4$–$6H_2O$, and 500 mg of yeast extract in 1,000 ml of tap water (pH 6.5) was used unless otherwise stated. The isolated strain was incubated in 500-ml shaking flasks containing 50 ml of culture medium at 28°C for 1 day.

**Screening and assay method for D-malate-producing strains.** Cells harvested from 1 ml of the cultured medium were incubated with 20 mg of maleate, 10 μl of toluene, and 25 μmol of phosphate buffer (pH 7.0) in a total volume of 1.0 ml. The reaction was performed at 28°C for 4 h with vigorous shaking.

The amounts of malate produced and maleate consumed were determined by high-pressure liquid chromatography (HPLC) with a refractive index detector. The column used was Shodex sugar SH1821 (8 by 30 mm; Showa Denko, Tokyo, Japan). For screening, the proportion of D-malate among malate produced was determined for convenience by the method of Lowry and Passonneau (6) and Wolfgang and Radler (11). L-Malate was assayed by the reduction of $NAD^+$ with L-malate dehydrogenase and aspartate aminotransferase. D-Malate was assayed by the reduction of $NAD^+$ with D-malate dehydrogenase (decarboxylation). Optically active malate was assayed in detail by the method reported by Dale et al. (2). The supernatant was acidified with 5 N $H_2SO_4$ to pH 1.0 and saturated with $(NH_4)_2SO_4$. To this solution was added ethyl acetate, and the mixture was vigorously shaken. The malate formed was recovered from the upper layer by evaporating the ethyl acetate. The malate was purified by silica gel chromatography with $n$-hexane–ethyl acetate (3:7, vol/vol). Dimethyl malate was prepared with HCl-methanol. The dimethyl malate and MTPA-Cl were mixed with carbon tetrachloride (5 drops) and dry pyridine (5 drops) and allowed to stand in a stoppered flask for 12 h. Water (1 ml) was added, and the reaction mixture was transferred to a separatory funnel with ether (10 ml). After being washed successively with dilute hydrochloric acid, saturated sodium carbonate solution, and water, the ether solution was dried over $MgSO_4$ and then filtered. The organic phase was evaporated, and the residue was dried in vacuo for several minutes. The MTPA esters of malate were analyzed with a Shimadzu GC-7A gas-liquid chromatograph

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equipped with a flame ionization detector. The capillary column used was a Rascot Silar-5CP (0.25 mm by 50 m; Nihon Chromato Works, Ltd., Tokyo, Japan). Cell concentration was determined from the optical density at 660 nm.

## RESULTS

**Screening of D-malate-producing strains.** From 440 strains of maleate-assimilating bacteria isolated from soil, 82 strains that could produce more than 5.0 g of malate per liter in 24 h were obtained. The proportion of D-malate among total malate produced by the 82 malate producers was measured by the enzymatic method described in Materials and Methods. Only one strain produced L-malate stereospecifically, and the products of 32 strains were mixtures of D- and L-malate. Fifty strains which specifically produce D-malate were obtained, and the optical purity of their products was above 90%. Among these strains, the best D-malate producer, 2612, was selected and used in further experiments.

**Identification of microorganisms.** Strain 2612 is a non-spore-forming, gram-positive bacterium and shows a rod-coccus growth cycle. Surface colonies on nutrient agar were 2 to 3 mm in diameter after a week, convex with entire margin. The optimum growth temperature was 25 to 30°C; growth occurred at 9°C but not above 38°C. This strain grew at pH 5 to 10. It was positive for aerobic catalase and negative for oxidase and urease. No growth occurred anaerobically. Acids were not formed from glucose and other sugars. The strain was methyl red negative. Gelatin and esculin were hydrolyzed. Starch and Tween 80 were not hydrolyzed. Indole and hydrogen sulfide were not produced. The G+C content of the DNA was 64.4%. This strain contains lysine as a diamino acid in the cell wall. The acyl type of the cell wall was acetyl. The major menaquinone was MK-9($H_2$). Thus, this strain was identified as an *Arthrobacter* sp. The cell wall contains rhamnose and galactose. This strain has the peptidoglycan Lys-Ser-Thr-Ala. Among the strains classified under the genus *Arthrobacter* (9), no species shows the same characteristics as those of strain 2612.

**Identification of the reaction product.** The reaction product of *Arthrobacter* sp. strain MCI2612 isolated by the procedure described in Materials and Methods and authentic D-malate had the same retention time (12.7 min) in the HPLC analysis. It was identified by infrared and $^1H$ nuclear magnetic resonance: (270 MHz, $D_2O$) δ 2.87 (2H, dd), 4.60 (1H, t); IR (KBr) $\nu_{max}$ cm$^{-1}$ 1733, 1635, 1398, 1265, 1190, 1101. Figure 1 shows the gas chromatogram of the MTPA dimethyl esters of the product and DL-malate. It shows that the optical purity of the produced malate is 100% D type.

**Culture conditions for D-malate production by *Arthrobacter* sp. strain MCI2612.** The effect of various substances added to the basal medium on the D-malate-producing activity in intact cells was investigated.

(i) **Inorganic nitrogen sources.** Supplementation with various inorganic nitrogens such as $(NH_4)_2SO_4$, $NaNO_3$, $NH_4Cl$, $NH_4NO_3$, and urea was investigated. The activity was the highest when $NH_4NO_3$ was used as the inorganic nitrogen source. The optimal concentration of $NH_4NO_3$ was 0.6%.

(ii) **Organic nitrogen sources.** Supplementation with various organic nitrogens was investigated. Yeast extract, malt extract, NZ-amine, soybean hydrolysate, and corn steep liquor were favorable for the formation of the activity. Five grams of yeast extract per liter was the most effective for the activity.

(iii) **Carbon sources.** The effect of various carbon sources on the formation of reaction activity was investigated. Organic acids such as succinate, acetate, fumarate, and citrate were
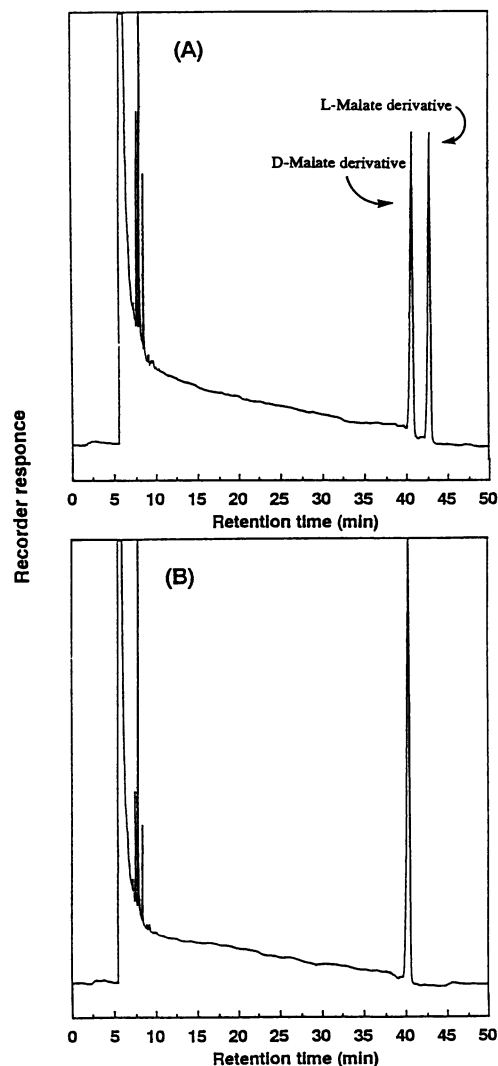


FIG. 1. Gas chromatographic analysis of dimethyl (+)-MTPA esters of authentic DL-malate (A) and the product (B). Conditions for analysis: column, Rascot Silar-5CP; temperature, 220°C; helium, 1.0 ml/min.

unfavorable for cell growth. Monosaccharides such as glucose and fructose, disaccharides such as sucrose and maltose, and glycitols such as glycerol, mannitol, and sorbitol were suitable as the carbon source. Sorbitol was effective for cell growth and for the activity in growing cells. The optimum concentration of sorbitol was 10 g/liter. More than 15 g of a carbon source per liter repressed the formation of the reaction activity.

(iv) **Amino acids.** The effect of various amino acids on the formation of reaction activity was investigated. As shown in Table 1, L-proline, L-histidine, L-arginine, and L-serine increased this activity. However, the addition of L-cystine and L-valine inhibited the growth of *Arthrobacter* sp. strain MCI2612. Moreover, when the mixture containing these effective amino acids was added to the medium, the D-malate-producing activity was not increased when compared to that induced by the addition of L-proline (2 g/liter).

(v) **Inducers.** Various inducers were investigated. As shown in Table 2, D-malate-producing activity is considered not to be an inducible enzyme, but the addition of maleate,

TABLE 1. Effect of amino acids on the formation of D-malate-producing activity[a]

| Amino acid (g/liter) | Cultivation | | Initial rate of D-malate production (mg/ml/h) |
| --- | --- | --- | --- |
| | Final pH | Growth (OD$_{660}$)[b] | |
| None | 5.3 | 16.9 | 1.31 |
| L-Methionine (2) | 5.6 | 16.7 | 1.44 |
| DL-Alanine (2) | 5.9 | 19.6 | 1.68 |
| L-Proline | | | |
| (1) | 5.6 | 17.9 | 1.93 |
| (2) | 5.8 | 19.2 | 2.34 |
| (3) | 6.0 | 18.9 | 2.35 |
| L-Histidine (2) | 5.7 | 17.1 | 1.90 |
| L-Isoleucine (2) | 5.5 | 20.2 | 1.57 |
| L-Arginine (2) | 5.9 | 17.8 | 2.11 |
| L-Aspartic acid (2) | 6.0 | 17.9 | 1.85 |
| L-Asparagine (2) | 6.0 | 17.3 | 1.79 |
| Glycine (2) | 6.0 | 17.8 | 1.74 |
| L-Lysine (2) | 5.3 | 18.4 | 1.70 |
| L-Tyrosine (2) | 5.9 | 14.1 | 1.04 |
| L-Cystine (2) | 6.2 | 7.90 | 0.18 |
| L-Serine (2) | 5.9 | 18.6 | 1.93 |
| L-Threonine (2) | 5.7 | 19.0 | 1.84 |
| L-Valine (2) | 6.1 | 9.88 | 0.78 |
| L-Glutamine (2) | 6.1 | 19.4 | 1.80 |
| L-Glutamic acid (2) | 6.2 | 16.9 | 1.49 |

[a] The composition of the culture medium was 10 g of sorbitol, 2 g of maleate, 2 g of $NH_4NO_3$, 3 g of $KH_2PO_4$, 10.5 g of $K_2HPO_4$, 250 mg of $MgSO_4 \cdot 7H_2O$, 10 mg of $FeSO_4 \cdot 7H_2O$, 10 mg of $MnSO_4 \cdot 4-6H_2O$, 5 g of yeast extract, and amino acids in 1,000 ml of tap water. Mean values from three experiments are shown.

[b] OD$_{660}$, optical density at 660 nm.

TABLE 2. Effect of inducers on the formation of D-malate-producing activity[a]

| Inducer (g/liter) | Cultivation | | Initial rate of D-malate production (mg/ml/h) |
| --- | --- | --- | --- |
| | Final pH | Growth (OD$_{660}$) | |
| None | 5.8 | 18.3 | 1.09 |
| Maleic acid | | | |
| (1) | 5.7 | 18.3 | 1.73 |
| (2) | 5.9 | 18.4 | 2.19 |
| (3) | 5.9 | 18.9 | 2.39 |
| (4) | 6.0 | 19.6 | 2.22 |
| (5) | 5.8 | 19.3 | 2.26 |
| Fumaric acid (2) | 6.6 | 19.4 | 0.31 |
| D-Malic acid (2) | 6.5 | 22.0 | 1.60 |
| L-Malic acid (2) | 6.5 | 20.2 | 0.66 |
| Citraconic acid (2) | 6.6 | 20.0 | 1.47 |
| Mesaconic acid (2) | 6.5 | 20.0 | 0.59 |
| DL-cis-Epoxysuccinic acid (2) | 5.8 | 19.0 | 0.85 |
| DL-trans-Epoxysuccinic acid (2) | 6.0 | 19.1 | 0.31 |
| L-(+)-Tartaric acid (2) | 7.0 | 19.7 | 0.27 |
| Nicotinic acid (1) | 6.7 | 19.5 | 0.71 |
| 2,5-Dihydroxybenzoic acid (2) | 6.7 | 20.7 | 0.75 |
| Crotonic acid (0.5) | 6.6 | 9.58 | 0.12 |
| L-Ascorbic acid (2) | 6.1 | 18.3 | 0.18 |
| o-Phthalic acid (2) | 5.9 | 19.6 | 0.58 |

[a] The composition of the culture medium was the same as in Table 1 except that indicated chemicals replaced the maleate and 2 g of L-proline per liter was present. Mean values from three experiments are shown.

[b] OD$_{660}$, optical density at 660 nm.

D-malate, and citraconate to the medium increased the activity. Maleate was suitable as an inducer for the formation of D-malate-producing activity. The optimum concentration of maleate was 3 g/liter.

**Reaction conditions for D-malate production with Arthrobacter sp. strain MCI2612.** The effect of pH on the activity was investigated. For a 30-min reaction, pH 6.5 to 8.5 seemed to be preferable. The optimum pH was about 7.0. The effect of reaction temperature was examined. At a temperature of around 50°C, the activity decreased rapidly and the reaction did not proceed for a long time. The optimal temperature for the production of D-malate was 36°C.

The effect of adding organic solvents and surfactants on D-malate production was examined. The concentration of detergents and solvents was 1% of the reaction mixture. Triton X-100, Triton N-101, and tergitol NPX remarkably increased the activity; the initial reaction rate was about ten times that with no added detergents. Triton N-101 showed the optimum result for the production of D-malate.

**Production of D-malate by intact cells.** Production of D-malate by intact cells of Arthrobacter sp. strain MCI2612 grown on optimum medium was determined. Since concentrations of maleate greater than 5% inhibited D-malate production, the reaction was done at 37°C for 20 h by keeping the concentration of maleate below 5% through successive feeding of maleate. The time course for D-malate synthesis is shown in Fig. 2. A total of 87 g of D-malate per liter was produced after 20 h of reaction. The molar yield of D-malate was 72%.

## DISCUSSION

Arthrobacter sp. strain MCI2612, newly isolated from soil, showed high activity for the conversion of maleate to

D-malate. Many maleate-assimilating bacteria metabolize maleate to L-malate via fumarate, as Perry and Edward (7) reported; however, the malate produced by Arthrobacter sp. strain MCI2612 was 100% D type. The result suggests that only maleate hydratase, not maleate isomerase, was responsible for the maleate metabolism.

We investigated the enzymatic production of D-malate from maleate with intact cells of Arthrobacter sp. strain MCI2612. Since intact cells were directly used to provide the enzyme in this reaction, it was considered necessary to
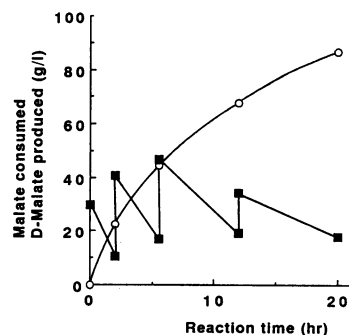


FIG. 2. Time course of conversion of maleate to D-malate. Cells were grown at 28°C on the culture medium, which was the same as in Table 1 except that it contained 2 g of L-proline per liter and 3 g of maleate per liter. Triton N = 101 (1%) was added to the reaction mixture instead of toluene. The reaction was done at 37°C. Symbols: ○, D-malate produced; ■, residual maleate. After 2, 6, and 12 h of incubation, maleate · 2Na was fed to the reaction mixture. Mean values from three experiments are shown.

establish culture conditions under which cells grew well and enzyme was sufficiently accumulated in the cells. Sorbitol, yeast extract, and $NH_4NO_3$ gave good results for cell growth and the formation of enzyme activity. The addition of amino acids such as L-proline, L-histidine, and L-arginine to the culture medium promoted enzyme formation. Inducer was not essential for the formation of enzyme, but the addition of maleate, D-malate, and citraconate increased the enzyme activity. This effect suggested that the reaction substrate, the product, and the related compound accelerate the induction of this enzyme.

The reaction conditions for the production of D-malate are as follows. The optimum pH for this reaction was 7.0, and the optimum temperature was 36°C. The reaction activity decreased rapidly when the reaction was done at high temperature. Addition of toluene, Triton X-100, Triton N-101, and tergitol NPX to the reaction mixture was effective. Triton N = 101 most remarkably enhanced the activity. Finally, the activity of the culture medium was about 22 times higher than the unoptimized conditions for the culture and reaction conditions. As already described, 87 g of D-malate per liter was produced in 20 h. The conversion ratio and the molar yield of D-malate were 86.5 and 72%, respectively, with only a small percentage of by-products such as pyruvate and oxaloacetate. D-Malate dehydrogenase (decarboxylation) is known as a D-malate-decomposing enzyme (11). Mutants which lack the enzyme or the addition of the inhibitor to the reaction mixture would make it possible to attain a higher yield. We are the first to isolate an *Arthrobacter* sp. as a good producer of D-malate in a strict stereoselective manner from maleate in a high concentration.

Our interest is focused on the maleate hydratase of this strain. We are now proceeding with the purification and characterization of the maleate hydratase.

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