L-Malic Acid Production from *n*-Paraffins by *Candida brumptii* IFO-0731

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Received December 17, 1976

Candida brumptii IFO-0731, which is known to produce succinic acid from *n*-paraffins, also produced other organic acids. One of them was identified as L-malic acid. Some culture conditions for L-malic acid production from *n*-paraffins were investigated. It was found that urea was the most favorable nitrogen source and 2 g/liter of urea was optimum for L-malic acid production. Under the best condition, 25.4 g/liter of L-malic acid was obtained from 4% (v/v) of super heavy *n*-paraffin and the yield was about 80% (wt./wt.).

There have been many studies on the useful products from *n*-paraffins by microorganisms. As for the fermentation products of the tricarboxylic acid cycle, productions of citric,^{1,2)} isocitric,³⁾ fumaric,^{4,5)} α -ketoglutaric,^{6~8)} and succinic⁹⁾ acids have already been reported. In the previous papers,⁹⁾ we reported that *Candida brumptii* IFO-0731 strain accumulated a large amount of succinic acid in the culture broth and that a small amount of α -ketoglutaric, fumaric and L-malic acids were also detected as by-products on a paper chromatogram. Hitherto, there has been no report on L-malic acid production from *n*-paraffins except for the microbial conversion of fumaric acid to L-malic acid.¹⁰⁾ Accordingly, the authors investigated the L-malic acid production from *n*-paraffins by *Candida brumptii* IFO-0731.

This paper deals with identification of L-malic acid produced from *n*-paraffins and some culture conditions for L-malic acid production.

MATERIALS AND METHODS

Microorganism. *Candida brumptii* IFO-0731 was employed. This strain was originally selected for succinic acid production from *n*-paraffins in our laboratory.

***n*-Paraffins.** A commercial product of super heavy *n*-paraffin (Nikko Petrochemical Co., Ltd.) was used as a carbon source. The composition of this *n*-paraffin was as follows in weight percentage: *n*-C₁₈,

0.1; *n*-C₁₄, 22.7; *n*-C₁₅, 39.4; *n*-C₁₆, 21.0; *n*-C₁₇, 8.2; *n*-C₁₈, 2.6; *n*-C₁₉, 0.9; *n*-C₂₀, 0.4; *n*-C₂₁, 0.2; *n*-C₂₂, 0.1; *n*-C₂₃, 0.1. The purity of *n*-paraffin was 95.7% and specific gravity was 0.775 at 15°C.

Media and culture conditions. The composition of the *n*-paraffin medium for L-malic acid production was as follows: 40 ml of *n*-paraffins; 0.5 g of NH₄Cl; 0.5 g of KH₂PO₄; 0.8 g of MgSO₄·7H₂O; 0.1 g of MnSO₄·4H₂O; 0.4 g of yeast extract; 10 mg of thiamine-HCl; 1 ml of corn steep liquor (Nihon Shokuhin Kako Co., Ltd.); 50 g of CaCO₃ and 960 ml of deionized water. The pH of this medium was adjusted to 6.2 by 2 N NaOH solution. This medium was identical with the succinic acid production medium from *n*-paraffins by this strain except for the concentration of *n*-paraffins.

The medium used for precultivation contained 20 g/liter of malt extract broth dehydrated (Difco Laboratories) and pH was adjusted to 4.7. Throughout these experiments, a 500 ml shaking flask was employed and cultivations were carried out on a reciprocal shaker at 30°C. Seed cultures were carried out in 50 ml of malt extract medium for about 36 hr and 1 ml of this culture broth was inoculated into 30 ml or 50 ml of *n*-paraffin medium for L-malic acid production.

Determination of cell growth. Cell growth was estimated by measuring optical density (OD) at 660 nm with a spectrophotometer (Shimadzu Spectronic 20). Prior to the measurement, 2 N HCl solution was added to the culture broth to dissolve precipitated compounds and the mixture was diluted appropriately with the Nakahara's solvent.¹¹⁾

Determination of residual *n*-paraffin concentration. Quantitative analysis of *n*-paraffins in the culture broth was carried out by gas-liquid chromatography. The chromatography was run with a Hitachi gas chromatograph model K-53 equipped with a hydrogen flame ionization detector. Measurement conditions were as

follows: column, OV-17; flow rate of N_2 carrier gas, 20 ml/min; column temperature was raised at the rate of $2^\circ\text{C}/\text{min}$ from 110°C to 180°C . $n\text{-C}_{13}$ was added into the cultivated broth as an internal standard material. Subsequently the cultivated broth was mixed with the Nakahara's solvent and was centrifuged at room temperature. The supernatant was directly injected to the gas chromatograph for quantitative analysis.

Determination of organic acids. In this fermentation, four dicarboxylic acids of the tricarboxylic acid cycle were produced. After the cultivation, the culture broth (2~3 ml) was treated with Amberlite IR 120 (H^+ form) to dissolve the precipitated compounds and was centrifuged at 5°C to separate the aqueous layer from floated frozen residual n -paraffins and precipitated cells. The aqueous layer was carefully withdrawn with a hole pipette and was put on the column of Dowex 1×8 (formate form) to adsorb acidic materials. The four organic acids were eluted with $4N$ formic acid solution. The eluate was evaporated to dryness *in vacuo*. For the quantitative analysis by gas-liquid chromatography, methylesters of organic acids were prepared with diazomethane. Esterifications of L-malic, succinic and α -ketoglutaric acids except for fumaric acid were successful in about 100% yield. The quantitative analysis of fumaric acid was carried out by titration with $0.1N$ $KMnO_4$ solution after centrifugation at 5°C . The column temperature of a gas chromatograph was raised at the rate of $5^\circ\text{C}/\text{min}$ from 80°C to 200°C and the other conditions were identical with those used for the determination of n -paraffin concentration. Sebacic acid dimethylester was used as an internal standard material. For the quantitative analysis of L-malic acid, bioassay¹²⁾ with *Lactobacillus arabinosus* was also used.

RESULTS AND DISCUSSION

Identification of L-malic acid

After 7 days of cultivation, the broth was decationized with IR 120 (H^+ form) and organic acids contained in the decationized broth were adsorbed on the column of Dowex 1×8 (formate form) and then eluted selectively with 0.5, 1, 2 and $4N$ formic acid solution.

The eluate containing L-malic acid was collected and L-malic acid was recrystallized from acetone-chloroform solution. The physicochemical properties of the product and authentic L-malic acid are summarized in Table I

TABLE I. COMPARISON OF PHYSICOCHEMICAL PROPERTIES OF THE PRODUCT AND AUTHENTIC L-MALIC ACID

	Product	Authentic L-malic acid
<i>Rf</i> values on paper chromatograms		
Solvent (1) ^{a)}	0.42	0.42
Solvent (2)	0.38	0.38
Melting point ($^\circ\text{C}$)	104~105	104
Elemental analysis (wt. %)		
C	36.0	35.83
H	4.4	4.51
O	59.5	59.66
	(Found)	(Calcd. for)
$[\alpha]_D^{25}$	-2.2°	-1.8°

^{a)} Solvent (1); n -butanol: formic acid: water = 4:1.5:1.

Solvent (2); phenol: water = 72:28.

FIG. 1. Infrared Absorption Spectra of the Product and an Authentic L-Malic Acid in Nujol. Upper curve: product. Lower curve: an authentic L-malic acid.

and IR spectra of both materials are also shown in Fig. 1. From these results, this substance was identified as L-malic acid. Other acids produced were also identified as succinic, fumaric and α -ketoglutaric acids by *R_f* values on paper chromatograms and retention time on a gas-liquid chromatogram.

Effects of inorganic nitrogen sources on L-malic acid production

The effects of nitrogen sources on L-malic acid production were investigated. The composition of the medium used was the same as described in MATERIALS AND METHODS except for the nitrogen source. $(\text{NH}_4)_2\text{SO}_4$, NH_4NO_3 , NH_4Cl , NaNO_3 or $(\text{NH}_2)_2\text{CO}$ was used as a nitrogen source. The concentration of the nitrogen source employed was adjusted to either 150 mg/liter or 300 mg/liter as nitrogen.

TABLE II. EFFECTS OF INORGANIC NITROGEN SOURCES ON L-MALIC ACID PRODUCTION

Cultivations were carried out at 30°C for 10 days in the *n*-paraffin medium containing 8% of (v/v) *n*-paraffins and the indicated nitrogen source. The concentration of nitrogen source was as follows; 1, nitrogen concentration was 150 mg/liter. 2, nitrogen concentration was 300 mg/liter.

Nitrogen source		Final pH	Growth (OD)	L-Malic acid (g/liter)
$(\text{NH}_4)_2\text{SO}_4$	1	6.15	1.00	0
	2	6.00	1.72	3.9
NH_4NO_3	1	6.15	0.74	3.1
	2	6.10	1.48	6.0
$(\text{NH}_2)_2\text{CO}$	1	6.15	0.60	2.8
	2	5.85	0.96	9.1
NaNO_3	1	6.60	0.30	0
	2	6.60	0.34	0
NH_4Cl	1	6.10	0.76	3.7
	2	5.90	0.86	6.7

The results are shown in Table II. It was found that urea was the most favorable nitrogen source for L-malic acid production and the yield was somewhat higher at 300 mg/liter of nitrogen.

Effect of concentration of urea on L-malic acid production

The effect of concentration of urea on L-

FIG. 2. Effect of Urea Concentration on Organic Acids Production.

Cultivations were carried out at 30°C for 7 days in *n*-paraffin medium containing the indicated concentration of urea instead of NH_4Cl .

○—○, growth (OD); □—□, total acids; □—□, L-malic acid; ○---○, succinic acid; △---△, fumaric acid; △—△, α -ketoglutaric acid.

malic acid production was examined. The results are shown in Fig. 2. It was found that L-malic acid increased while fumaric, succinic and α -ketoglutaric acids decreased with an increase in urea concentration. The maximum yield of L-malic acid was gained at 2 g/liter of urea.

Effect of temperature on L-malic acid production

The effect of temperature on L-malic acid production is shown in Table III. The lost volume of the culture broth which ran off in vapour during cultivation was corrected. The cultivation at 30°C was the most suitable for L-malic acid production.

TABLE III. EFFECT OF TEMPERATURE ON L-MALIC ACID PRODUCTION

Cultivations were carried out at the indicated temperature for 7 days in the *n*-paraffin medium containing 2 g/liter of urea instead of NH_4Cl .

Temperature (°C)	Final pH	Growth (OD)	L-Malic acid (g/liter)
26.5	6.55	0.98	9.0
30	6.75	0.84	13.5
37	6.85	1.08	5.9

Effect of aeration on L-malic acid production

The effect of aeration was examined by

TABLE IV. EFFECT OF AERATION ON L-MALIC ACID PRODUCTION

Cultivations were carried out at 30°C for 7 days with a 500 ml shaking flask containing the indicated volume of *n*-paraffin medium. The *n*-paraffin medium contained 2 g/liter of urea instead of NH₄Cl.

Volume of medium in a 500 ml flask (ml)	Final pH	Growth (OD)	L-Malic acid (g/liter)
10	6.40	1.01	6.7
20	6.65	1.09	7.9
30	6.75	0.96	15.6
50	6.85	1.14	16.8
100	6.75	1.24	13.0

changing the volume of medium in a 500 ml shaking flask from 10 ml to 100 ml. The results are shown in Table IV. These data indicated that 30~50 ml of medium in a 500 ml flask was favorable for L-malic acid production.

Effect of concentration of calcium carbonate on L-malic acid production

The effect of CaCO₃ concentration on L-malic acid production is shown in Table V. At a low CaCO₃ concentration, pH of the culture broth decreased and L-malic acid was not detected until the concentration of CaCO₃ increased up to 20 g/liter. The maximum L-malic acid production was obtained at 50 g/liter of CaCO₃.

TABLE V. EFFECT OF CONCENTRATION OF CALCIUM CARBONATE ON L-MALIC ACID PRODUCTION

Cultivations were carried out at 30°C for 7 days in the *n*-paraffin medium containing 2 g/liter of urea instead of NH₄Cl and the indicated concentration of CaCO₃.

CaCO ₃ (g/liter)	Final pH	Growth (OD)	L-Malic acid (g/liter)
0	3.90	2.08	0
5	4.25	1.76	0
10	4.35	1.58	0
20	5.15	1.24	9.3
50	7.65	1.14	13.7
100	7.60	1.00	6.5

Effect of n-paraffin mixture concentration on L-malic acid production

Table VI shows the effect of concentration of *n*-paraffin mixture which was used as a

TABLE VI. EFFECT OF *n*-PARAFFIN MIXTURE CONCENTRATION ON L-MALIC ACID PRODUCTION

Cultivations were carried out at 30°C for 7 days in the *n*-paraffin medium containing the indicated concentration of super heavy *n*-paraffin and 2 g/liter of urea instead of NH₄Cl.

<i>n</i> -Paraffins (% v/v)	Final pH	Growth (OD)	L-Malic acid (g/liter)
1	7.90	0.68	0
2	7.95	0.86	1.6
4	7.65	1.14	13.7
8	5.30	1.12	24.4
12	4.35	1.66	22.8

carbon source. It was thought that at a low concentration, *n*-paraffins were used only for cell growth and that organic acids were not produced. The maximum yield of L-malic acid, 24.4 g/liter was obtained at 8% (v/v) of *n*-paraffins after 7 days of cultivation.

Effect of chain length of n-paraffin on L-malic acid production

n-Paraffin used as a sole carbon source was a mixture of *n*-paraffins from C₁₃ to C₂₃ as described in MATERIALS AND METHODS. From the results summarized in Table VII, the productivity of L-malic acid seemed to depend on the kind of *n*-paraffin used as a carbon source; the longer the carbon number of *n*-paraffin, the higher the yield of L-malic acid. As shown in Table VII, this may possibly be due

TABLE VII. EFFECT OF CHAIN LENGTH OF *n*-PARAFFIN ON L-MALIC ACID PRODUCTION

Cultivations were carried out at 30°C for 7 days in *n*-paraffin medium containing 2 g/liter of urea and *n*-paraffin of the indicated carbon number instead of NH₄Cl and *n*-paraffin mixture.

<i>n</i> -Paraffin (4% v/v)	Final pH	Growth ^{a)} (OD)	Max. growth ^{b)} (OD)	L-Malic acid (g/liter)	Specific growth rate/hr
<i>n</i> -C ₁₄	5.90	1.10	1.10	2.3	0.10
<i>n</i> -C ₁₅	6.20	1.26	1.26	6.7	0.13
<i>n</i> -C ₁₆	6.80	0.98	1.26	14.8	0.16
<i>n</i> -C ₁₇	7.10	0.96	1.80	15.0	0.19
<i>n</i> -C ₁₈	7.15	0.88	1.68	17.4	0.21

^{a)} Final cell concentration at the end of 7 days cultivation.

^{b)} Maximum cell concentration during the cultivation.

to the different specific growth rate of this strain on each *n*-paraffin possessing different carbon number. The maximum cell growth reached after 7 days of cultivation with C₁₄ and C₁₅ but in the cases of C₁₆ and C₁₇, and of C₁₈, the maximum cell growth was obtained after 3 days and 2 days, respectively.

Effects of surface active agents on L-malic acid production

In studies on fermentation products from *n*-paraffins, some surface active agents have been reported to be effective. Then some surface active agents such as Tween 20 (polyoxyethylene sorbitan fatty acid ester type), Noigen EA141 (polyethylene glycol nonyl-phenyl ether), Noigen ES140 (polyethylene glycol oleic acid ester) and Plysurf A210G (polyoxyethylene alkyl ether phosphate type) were added at the concentration of 0.02% or 0.06% (v/v) to both *n*-paraffin medium and glucose medium containing glucose instead of *n*-paraffins as a carbon source. The results

TABLE VIII. EFFECTS OF SURFACE ACTIVE AGENTS ON L-MALIC ACID PRODUCTION

Cultivations were carried out at 30°C for 7 days in either *n*-paraffin or glucose medium containing 2 g/liter of urea instead of NH₄Cl and the indicated surface active agent. The glucose medium was identical with *n*-paraffin medium except for carbon source. The concentration of an agent added was as follows; 1, 0.02%; 2, 0.06% (v/v).

Surface active agent		Final pH	Growth (OD)	L-Malic acid (g/liter)
<i>n</i> -Paraffin medium		6.80	1.36	16.8
+ Noigen EA	1	6.50	1.72	8.2
141	2	6.65	1.80	10.2
+ Tween 20	1	6.10	0.68	1.3
	2	7.25	0.15	0.8
+ Noigen ES	1	7.50	1.72	10.6
140	2	7.35	1.72	—
+ Plysurf	1	7.00	1.38	9.0
A210G	2	6.70	1.36	10.3
Glucose medium		6.65	2.48	1.7
+ Noigen EA	1	6.65	2.64	1.5
141	2	6.75	2.60	1.9
+ Tween 20	1	6.65	2.52	2.9
	2	6.65	2.36	1.9
+ Plysurf	1	6.50	2.72	2.8
A210G	2	7.10	2.64	1.7

are shown in Table VIII. There was no effect of surface active agents in glucose medium but in *n*-paraffin medium, Tween 20 was inhibitory to both cell growth and L-malic acid production. The other agents caused decrease in L-malic acid production to some degree.

Effect of addition of glucose or sorbitol to n-paraffin medium

This strain produced a large amount of L-malic acid in *n*-paraffin medium but small in glucose medium. The effect of glucose or sorbitol added to *n*-paraffin medium was examined. The carbon concentration of 3.9 g of glucose or 4 g of sorbitol in 30 ml of medium corresponds to that of 8% (v/v) of *n*-paraffins. As shown in Table IX, the addition of glucose gave rather inhibitory effect on L-malic acid production, while the addition of sorbitol increased its yield. Although it was interesting to make clear the role of sorbitol on L-malic acid production, its role has not yet been known.

TABLE IX. EFFECT OF ADDITION OF GLUCOSE OR SORBITOL TO *n*-PARAFFIN MEDIUM

Cultivations were carried out at 30°C for 7 days. The indicated amounts of carbon sources were added to the *n*-paraffin medium containing 2 g/liter of urea instead of NH₄Cl. Glucose, sorbitol and *n*-paraffin medium are abbreviated as G, S and *n*-P medium. Glucose or sorbitol was also used as a sole carbon source instead of *n*-paraffins and the media with these modifications are shown as G medium and S medium, respectively.

Carbon sources in 30 ml medium	Final pH	Growth (OD)	L-Malic acid (g/liter)
<i>n</i> -P medium			
+ G 1 g	6.60	2.16	13.7
+ G 3 g	5.95	3.04	7.3
+ G 5 g	5.95	2.80	9.3
+ S 0.1 g	7.30	1.32	17.6
+ S 0.5 g	7.20	1.60	16.6
+ S 1 g	6.45	1.68	21.3
+ S 3 g	6.45	1.76	17.6
+ S 5 g	6.40	1.80	15.0
<i>n</i> -P medium	7.65	1.14	13.7
G medium 3.9 g	5.95	3.04	2.6
S medium 2 g	6.90	1.74	trace
S medium 4 g	6.70	2.08	trace

Effects of corn steep liquor (CSL) concentration and CSL components on L-malic acid production

The effect of CSL concentration on L-malic acid production is shown in Table X. The addition of 0.2 ml/liter of CSL gave the best productivity of 25.4 g/liter L-malic acid and its yield was about 80% (wt./wt.). To elucidate the effects of minor components contained in CSL, *i.e.*, vitamins and metals, on acid production and cell growth, following experiments were carried out. To investigate the effects of metals, 1 ml of CSL was placed in a crucible and burned to ashes on a gas burner. One ml of deionized water was added to this preparation and it was used as crude metals. The mixture of 4 mg of each of nicotinic acid, pyridoxine-HCl, Ca-pantothenate and *p*-amino benzoic acid and 1 mg of thiamine-HCl and 0.4 mg of each of biotin and cyanocobalamin per liter was used as a vitamin mixture.

TABLE X. EFFECT OF CORN STEEP LIQUOR (CSL) CONCENTRATION ON L-MALIC ACID PRODUCTION

Cultivations were carried out at 30°C for 7 days in the *n*-paraffin medium containing 2 g/liter of urea instead of NH₄Cl and the indicated concentration of CSL.

CSL (ml/liter)	Final pH	Growth (OD)	L-Malic acid (g/liter)
0.1	6.0	1.11	24.4
0.2	6.75	1.16	25.4
0.5	7.0	1.30	17.8
1.0	7.2	1.53	17.2
2.0	7.5	1.80	11.4

The cells harvested by centrifugation of precultivation in 50 ml of malt extract medium were washed once with 0.1 M phosphate buffer, pH 6.4, and resuspended in 50 ml of deionized water. This suspension was inoculated for L-malic acid production in *n*-paraffin medium shown below. The medium lacking CSL, yeast extract and thiamine-HCl from basal medium was prepared and effects of CSL ashes and a vitamin mixture were tested. To see the individual effect of vitamins, one of the vitamins was omitted from the synthetic

TABLE XI. EFFECTS OF CSL ASHES AND VITAMINS ON L-MALIC ACID PRODUCTION

Cultivations were carried out at 30°C for 7 days in the basal medium supplemented with the indicated additives. For the basal medium, the medium lacking CSL, yeast extract and thiamine-HCl from *n*-paraffin medium was used. *n*-Paraffin medium was used as control. Vitamin mixture consisted of 4 mg of each of nicotinic acid, pyridoxine-HCl, Ca-pantothenate and *p*-aminobenzoic acid and 1 mg of thiamine-HCl and 0.4 mg of each of biotin and cyanocobalamin per liter.

Addition (+) or omission (—)	Final pH	Growth (OD)	L-Malic acid (g/liter)
Control	7.4	1.56	11.8
+ CSL ashes	6.6	0.22	0.5
+ Vitamin mixture	7.3	0.01	0.4
+ CSL ashes and vitamin mixture	6.45	1.82	8.6
+ CSL ashes and vitamin mixture			
— Nicotinic acid	6.9	1.94	6.7
— Pyridoxine-HCl	6.75	1.88	5.2
— Ca-Pantothenate	7.05	1.82	7.2
— <i>p</i> -Aminobenzoic acid	6.5	1.94	8.8
— Thiamine-HCl	6.2	0.36	0.6
— Biotin	6.8	0.14	0.2
— Cyanocobalamin	6.7	1.92	8.7

medium. The results are shown in Table XI. Both CSL ashes and a vitamin mixture were essential for cell growth, and thiamine-HCl

FIG. 3. Time Course of L-Malic Acid Production. Cultivation was carried out at 30°C in a 500 ml shaking flask in *n*-paraffin medium containing 2 g/liter of urea instead of NH₄Cl. Δ---Δ, pH; ●---●, growth (OD); □---□, concentration of *n*-paraffin; ○---○, L-malic acid; ●---●, succinic acid; Δ---Δ, fumaric acid; ○---○, α-ketoglutaric acid.

and biotin could substitute for a vitamin mixture.

Time course of L-malic acid production

Typical time courses of cell growth and organic acids productions in a shaking flask cultivation are shown in Fig. 3. L-Malic acid began to appear at the end of exponential growth phase and the maximum yield was obtained after 6 to 7 days of cultivation. The similar result was shown in productions of succinic, fumaric and α -ketoglutaric acids.

Acknowledgement. The authors wish to thank Dr. Mikio Sato of Central Research Laboratory, Idemitsu Kosan Co., Ltd. for his useful advice.

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