Studies on the Utilization of Hydrocarbons by Microorganisms

Part XX. Conversion of Fumaric Acid to *l*-Malic Acid by the Association of Two Kinds of Yeasts

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In order to convert fumaric acid to *l*-malic acid continuously by hydrocarbon fermentation, the cultivation of *Candida utilis* IAM 4215 or *Pichia membranaefaciens* IAM 4122 which have a high fumarase activity was combined with fumaric acid fermentation by *Candida hydrocarbofumarica*.

Forty strains of yeasts were tested for their fumarase activities. *P. membranaefaciens* IAM 4122 and *C. utilis* IAM 4215 indicated the high activity. After *C. hydrocarbofumarica* was incubated in *n*-paraffin medium for 5 days, fumaric acid produced was converted to *l*-malic acid by the associated culture with *P. membranaefaciens* IAM 4122 for 4 or 5 days.

Yields of *l*-malic acid based on *n*-paraffin were 72 % and 70 % by *P. membranaefaciens* and *C. utilis*, respectively.

The previous papers^{1,2)} described the results of the taxonomical studies of the microorganism which can produce fumaric acid (FuA) from *n*-paraffin, and the determination of optimal conditions for the acid production by this strain.

This paper concerns with the conversion of FuA produced from *n*-paraffin to *l*-malic acid (*l*-MaA) by the association of another yeast.

In 1965 Sasaki and Takao^{3,4)} had already reported on this idea and method by the association of *Rhizopus* and yeast using glucose as a carbon source. The screening work was carried out to obtain microorganisms which have a high fumarase activity. Two strains of microorganisms, *C. utilis* IAM 4215 and *P.*

membranaefaciens IAM 4122, were selected as the suitable strain.

C. hydrocarbofumarica was cultured in the medium M-1 (shown in Table I) at 30°C for 5 days, and then *C. utilis* IAM 4215 or *P. membranaefaciens* IAM 4122 was inoculated to this fermented broth. FuA in the first broth was converted to *l*-MaA after 4 or 5 days by this mixed cultivation.

MATERIALS AND METHODS

1) *n*-Paraffin. Commercial product of *n*-paraffin mixture was used. The carbon composition of *n*-paraffin was as follows; C₁₀, 0.5 %; C₁₁, 1.6 %; C₁₂, 14.6 %; C₁₃, 42.0 %; C₁₄, 39.7 % and C₁₅, 1.6 %.

2) *Microorganisms*. *C. hydrocarbofumarica*, *C. utilis* IAM 4215 and *P. membranaefaciens* IAM 4122 were used.

3) *Media and cultivation*. The compositions of media were shown in Table I. Medium M-1 was used for *l*-MaA production and medium M-2 was

1) K. Yamada, T. Furukawa and T. Nakahara, *Agr. Biol. Chem.*, **34**, 670 (1970).

2) T. Furukawa, T. Nakahara and K. Yamada, *ibid.*, **34**, 1402 (1970).

3) Y. Sasaki and S. Takao, *Nippon Nogeikagaku Kaishi*, **39**, 436 (1965).

4) Y. Sasaki and S. Takao, *ibid.*, **40**, 190 (1966).

TABLE I. COMPOSITION OF THE MEDIA

<i>n</i> -Paraffin	M-1 60	M-2 (Carbon source <i>n</i> -paraffin 30 g and/or glucose 5~20 g and/or Na-fumarate 5 g)
NH ₄ Cl	3	3
KH ₂ PO ₄	0.5	0.5
K ₂ HPO ₄	0.5	0.5
MgSO ₄ ·7H ₂ O	0.5	0.5
FeSO ₄ ·7H ₂ O	0.01	0.01
ZnSO ₄ ·7H ₂ O	0.005	0.005
MnSO ₄ ·4H ₂ O	0.0005	0.0005
CaCl ₂	0.01	0.01
Yeast extract	0.2	0.2
CaCO ₃	40	—
Distilled water	800 ml	800 ml
Tap water	200 ml	200 ml
(Medium for seed)		
pH was adjusted to 7.0		

used for seed culture for this purpose. Concentration of *n*-paraffin added to the medium M-1 was 6% (w/v). Sterilization of the media was done at 115°C for 10 min.

Cell suspensions precultivated for two days at 30°C were inoculated to 25 ml of the medium in 500 ml shaking flasks, and cultured on a reciprocal shaker (120 strokes/min) at 30°C or 37°C.

4) Methods of analysis

1) *Growth of microorganisms.* Growth of microorganisms was estimated by measuring the optical density at 660 mμ of the diluted culture broth.^{1,5)}

2) *The assay method for FuA and l-MaA.* FuA was assayed by the method of oxidation with 0.1N KMnO₄ soln.⁶⁾ *l*-MaA was assayed by a Warburg's manometer with *Lactobacillus arabinosus* IAM 1041 which was adapted to *l*-MaA.⁷⁾

TABLE II. CONVERSION OF FUMARATE TO MALATE BY YEASTS

Species		FuA (g/liter)	conv. ^{a)} (%)	<i>l</i> -MaA (g/liter)	yield ^{b)} (%)
<i>Candida utilis</i>	IAM 4233	1.62	77.7	5.1	69.9
<i>C. "</i>	IAM 4215	"	"	6.3	87.3
<i>C. mycoderma</i>	" 4564	1.08	85.1	7.0	96.9
<i>C. utilis</i>	" 4277	1.35	81.4	4.9	67.6
<i>C. japonica</i>	" 4185	2.16	70.1	5.0	69.0
<i>C. krusei</i>	" 4801	1.62	77.7	6.0	83.1
<i>C. lipolytica</i>	IFO 0717	2.43	66.5	4.8	66.1
<i>C. tropicalis</i>	Y-11	2.70	62.8	4.5	62.1
<i>C. "</i>	IAM 4924	2.16	70.1	4.7	64.9
<i>C. "</i>	IFO 0006	1.62	77.7	5.6	76.6
<i>C. guilliermondii</i>	IFO 0566	"	"	4.9	67.0
<i>C. "</i>	1598-K1	"	"	5.6	77.3
<i>Pichia membranaefaciens</i>	IAM 4303	1.89	74.0	5.1	70.3
"	" 4122	1.23	83.0	7.3	100.8
"	" 4290	1.89	74.0	7.0	96.5
"	" 4841	1.35	81.4	7.0	96.5
<i>Mycotorula japonica</i>	IAM 4158	1.23	83.1	4.5	62.1
Medium M-2	{ glucose 0.4 % FuNa ₂ 1 % CSL 0.1 %	(FuA; 7.25 g/liter)			

$$^a) \text{ conv. (conversion rate)} = \frac{\text{initial FuA} - \text{final FuA}}{\text{initial FuA}} \times 100$$

$$^b) \text{ yield} = \frac{\text{produced } l\text{-MaA (wt)}}{\text{initial FuA (wt)}} \times 100$$

5) T. Nakahara, K. Hisatsuka and K. Yamada, *J. Fer. Assoc. Japan*, **26**, 413 (1968).

6) "Jikken Kagaku Koza," Vol. 25, ed. by S. Akabori and S. Funabashi, Maruzen Co., Ltd., 1958, p. 57.

7) "Jikken Nogeikagaku," Vol. II, ed. by Department of Agricultural Chemistry, Faculty of Agriculture, The University of Tokyo, Asakura Shoten Co., Ltd., 1960, p. 512.

RESULTS AND DISCUSSION

1) Screening for the microorganisms which have a strong fumarase activity

The selection of microorganisms which have the strong fumarase activity was carried out using many kinds of strains in stock cultures. As shown in Table II, *C. utilis* IAM 4215, *P. membranaefaciens* IAM 4122, *P. membranaefaciens* IAM 4290, *P. membranaefaciens* IAM 4841 and *C. mycoderma* IAM 4564 showed the high activities for the conversion of FuA to *l*-MaA.

2) Identification of the acid

As shown in Table III, elementary analysis and *R_f* value of the product were agreed with those of authentic *l*-MaA. IR spectrum of the product also agreed with that of authentic *l*-MaA as shown in Fig. 1.

TABLE III. COMPARISON BETWEEN PRODUCT AND AUTHENTIC *l*-MaA

Elementary analysis		Product C; 36.1 % H; 4.5	Authentic <i>l</i> -MaA C; 35.8 % H; 4.5
Paper chromatography <i>R_f</i>	Solvent		
	<i>n</i> -butanol	4)	
	{acetic acid	1)	0.49
	{water	1)	0.49
	{phenol	4)	
	{water	1)	0.41

3) Determination of the optimal conditions

l-MaA production from *n*-paraffin was carried out as shown in Fig. 2 to determine the optimal conditions.

1) Effect of period for preculture on the production of *l*-MaA. As shown in Table IV,

FIG. 2. Culture for Production of MaA.

TABLE IV. EFFECT OF PERIOD FOR PRECULTURE ON THE PRODUCTION OF *l*-MaA

Strain	Yield of <i>l</i> -MaA (g/liter)		
	Period for preculture 0 4 6(day)		
<i>P. membranaefaciens</i> IAM 4122	20.7	26.4	33.3
<i>C. utilis</i> IAM 4215	9.8	12.9	14.8

Total period of culture; 14 days (30°C).

FIG. 1. Infrared Absorption Spectra of the Product and Authentic MaA in Nujol.

suitable period for preculture was 6 days, but considering of the total period of culture, 5 days preculture was selected.

2) *Effect of seed medium on the production of l-MaA.* Effect of carbon source in the seed media was studied. As shown in Table V, the seed medium containing sodium fumarate was good for the l-MaA production. Effect of period for the seed culture was also examined, and it was found that two days seed culture was suitable as shown in Table VI.

3) *Effect of addition of NH_4Cl and K_2HPO_4 on the production of l-MaA.* As shown in Table

VII, addition of NH_4Cl was effective for both strains on the production of l-MaA. This result seems to owe to the pooriness of nitrogen source in the first fermented broth for the growth of *C. utilis* IAM 4215 or *P. membranaefaciens* IAM 4122. Addition of K_2HPO_4 was also effective on the production of l-MaA as shown in Table VIII. Therefore, the following experiments were carried out by enforcing NH_4Cl and K_2HPO_4 in the first fermented broth.

4) *Effect of temperature on the production of l-MaA.* In order to promote the rate of production of l-MaA, effect of temperature for mixed culture was examined. As shown

TABLE V. EFFECT OF SEED MEDIUM ON THE PRODUCTION OF l-MaA

Seed media ^{a)} (carbon source)	Fermented broth		
	pH	Growth (O. D.)	l-MaA (g/liter)
Glucose (2 %)	7.6	3.01	19.5
Glucose (0.5 %) + Na-Fumarate (0.5 %)	7.8	3.12	30.4
n-Paraffin (3 %)	7.6	3.28	23.6
n-Paraffin (3 %) + Na-Fumarate (1 %)	7.4	2.75	30.2

^{a)} Basal medium M-2
Cultural conditions;
Preculture: 30°C for 5 days
Mixed culture: 30°C for 5 days

TABLE VI. EFFECT OF PERIOD FOR SEED CULTURE ON THE PRODUCTION OF l-MaA

Period (day)	Fermented broth	
	Growth (O. D.)	l-MaA (g/liter)
2	3.05	31.2
4	3.10	28.0

Medium for seed: M-2 (glucose 0.5 %, fumarate 0.5 %)

Cultural conditions;
Preculture: 30°C for 5 days
Mixed culture: 30°C for 5 days

TABLE VII. EFFECT OF ADDITION OF NH_4Cl ON THE PRODUCTION OF l-MaA

Addition of NH_4Cl	Yield of l-MaA (g/liter)	
	<i>C. utilis</i> IAM 4215	<i>P. membranaefaciens</i> IAM 4122
0 (g/liter)	34.8	35.9
1.5	36.5	41.9

Medium for seed; M-2 (glucose 0.5 %, fumarate 0.5 %)

Cultural conditions;
Preculture: 30°C for 5 days
Mixed culture: 30°C for 5 days (for *Candida*)
37°C for 5 days (for *Pichia*)

TABLE VIII. EFFECT OF ADDITION OF K_2HPO_4 ON THE PRODUCTION OF l-MaA

Addition of K_2HPO_4	Fermented broth			
	pH	Growth (O. D.)	l-MaA (g/liter)	FuA (g/liter)
0 (g/liter)	7.6	2.99	28.3	4.55
0.5	7.8	3.25	32.5	1.51

Strain; *Candida utilis* IAM 4215

Medium for seed; M-2 (glucose 0.5 %, fumarate 0.5 %)

Cultural conditions;
Preculture: 30°C for 5 days
Mixed culture: 30°C for 5 days

TABLE IX. EFFECT OF TEMPERATURE ON THE PRODUCTION OF *l*-MaA

Temp. (mixed culture)	Yield of <i>l</i> -MaA (g/liter)	
	<i>C. utilis</i> IAM 4215	<i>P. membranaefaciens</i> IAM 4122
30°C	35.3	24.2
37°C	34.5	35.9

Medium for seed; M-2 (glucose 0.5 %, fumarate 0.5 %)

Cultural conditions;

Preculture: 30°C for 5 days

Mixed culture: 5 days for *Candida*
6 days for *Pichia*

TABLE X. EFFECT OF pH ON PRODUCTION OF *l*-MaA

pH	Fermented broth I			II		
	final pH	growth (O. D.)	<i>l</i> -MaA (g/liter)	final pH	growth (O. D.)	<i>l</i> -MaA (g/liter)
6	6.8	2.22	28.7	6.8	2.15	29.1
7	7.4	2.58	23.5	7.8	1.90	30.1

Strain; *Candida utilis* IAM 4215

Medium for seed; M-2 (glucose 0.5 %, fumarate 0.5 %)

Cultural conditions;

Preculture: 30°C for 5 days

Mixed culture: 30°C for 5 days

TABLE XI. COMPARISON IN SEVERAL CULTURAL CONDITIONS

Strains	Medium (ml)	Seed (ml)	NH ₄ Cl and salts soln. (ml)	<i>l</i> -MaA (g/liter)	Fermented volume (ml)	Broth yield %
<i>C. utilis</i> IAM 4215	25	10	0	31.4	33	65
	25	5	10 ^{a)}	29.1	37.5	70.7
	25	5	1 ^{b)}	35.0	30	68.2
<i>P. membranaefaciens</i> IAM 4122	25	10	0	24.0	36.5	56.9
	25	5	10 ^{a)}	31.2	35.5	72.0

Medium for seed; M-2 (glucose 0.5 %, fumarate 0.5 %)

^{a)} NH₄Cl 30 mg, K₂HPO₄ 5 mg, KH₂PO₄ 5 mg, MgSO₄·7H₂O 5 mg

^{b)} NH₄Cl 37.5 mg, K₂HPO₄ 12.5 mg

Cultural conditions;

Preculture: 30°C for 5 days

Mixed culture: 30°C for 5 days (*Candida*)
37°C for 4 days (*Pichia*)

in Table IX, in the case of *C. utilis* IAM 4215 30°C was suitable for the *l*-MaA production, on the other hand 37°C was preferred for *P. membranaefaciens* IAM 4122.

5) *Effect of pH on the production of l-MaA.* Effect of pH on the production of *l*-MaA was examined. Table X shows that there was no difference between pH 6 and 7.

6) *Comparison in several conditions.* In order to increase the yield of *l*-MaA, several conditions that is to say, the effects of the quantities of the seed broth, NH₄Cl and some

salts, were examined.

The preculture was carried out with *Candida hydrocarbofumarica* in 25 ml of the medium M-1 at 30°C for 5 days.

As shown in Table XI, using *C. utilis* IAM 4215 as *l*-MaA producer from FuA, the effects of seed broth, NH₄Cl and salts were not clear. On the other hand, in the case of *P. membranaefaciens* IAM 4122, those effects were considerable. Taking account of the results shown in Table VII, the effect of NH₄Cl may be dominant on the production of MaA.

The yield was calculated as follows.

Yield

$$= \frac{l\text{-MaA in broth (wt)}}{(n\text{-p in feed}) + \{(\text{FuA} + \text{glucose}) \text{ in seed}\} (\text{wt})} \times 100$$

In the optimal conditions obtained, the yields of *l*-MaA by *C. utilis* IAM 4215 and *P. membranaefaciens* IAM 4122 were 70% and 72%, respectively.