Mode of Metabolism of 1-Tetradecene by *Candida* Yeasts and Citrates Production[†]

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Metabolic pathways for the oxidation of 1-tetradecene by *Candida lipolytica* were investigated and compared with those for *Candida tropicalis* in order to elucidate the difference in the productivity of citrates reported in the previous paper. No difference was found in the pathways for the initial stage of oxidation of 1-tetradecene between the two strains, indicating that the difference in the productivity of citrates is not attributable to the metabolic pathways. The metabolic rate of 1-tetradecene with *C. lipolytica* was found to be much lower than that with *C. tropicalis*. The production of citrates was much enhanced in the presence of surfactants which were known to be stimulative for microbial metabolism of hydrocarbons and 11~15 g/liters was attained on the 6th day of cultivation.

Liquid α -olefins produced by thermal cracking of paraffin wax, as described in the previous paper,¹⁾ is expected to be a promising novel feed stock for the microbial production of organic acids.

We have investigated the production of citric acids (citric and isocitric acid) from 1-tetradecene by using two *Candida* yeasts and have found, as reported in the previous paper,¹⁾ that *Candida tropicalis* IFO 0589 grows well on 1-tetradecene and accumulates a large amount of citric acids, while *Candida lipolytica* IFO 0746 also grows well on 1-tetradecene but hardly accumulates citric acids at all. These results suggest that the metabolic pathway or the mode of metabolism of 1-tetradecene by *C. lipolytica* is different from those by *C. tropicalis*.

In this paper, therefore, the metabolic pathways in the initial stage of oxidation of 1-tetradecene by these yeasts have been investigated and the Q_{O_2} value on 1-tetradecene and its metabolic intermediates, which represents the rate of metabolism of each substrate,

has also been measured in order to elucidate the difference in the productivity of citric acids between *C. lipolytica* and *C. tropicalis*.

MATERIALS AND METHODS

Microorganisms. *Candida tropicalis* IFO 0589 and *Candida lipolytica* IFO 0746 capable of utilizing liquid paraffins and α -olefins for growth were used throughout the experiments.

Media. A culture medium (medium A) for harvesting cells or cultivating seeds contained NH_4Cl , 6.5 g; $Na_2HPO_4 \cdot 12H_2O$, 1.5 g; KH_2PO_4 , 3.5 g; $MgSO_4 \cdot 7H_2O$, 0.5 g; $FeSO_4 \cdot 7H_2O$, 10 mg; $MnSO_4 \cdot nH_2O$, 0.1 mg; $ZnSO_4 \cdot 7H_2O$, 0.1 mg; $CuSO_4 \cdot 5H_2O$, 5 μ g; biotin, 100 μ g; thiamin-HCl, 100 μ g and yeast extract, 200 mg in 1000 ml of distilled water, and the pH was adjusted to 7.0. Another medium (medium B) for the production of citric acids consisted of NH_4Cl , 2 g; KH_2PO_4 , 0.5 g; $MgSO_4 \cdot 7H_2O$, 0.5 g; $FeSO_4 \cdot 7H_2O$, 10 mg; $MnSO_4 \cdot nH_2O$, 0.1 mg; $ZnSO_4 \cdot 7H_2O$, 0.1 mg; $CuSO_4 \cdot 5H_2O$, 5 μ g; biotin, 100 μ g and thiamin-HCl, 100 μ g in 1000 ml of distilled water. To the above media, an appropriate amount of carbon source was added for supporting cellular growth or citric acids production.

Cultivation. For producing cells or cultivating seeds, 50 ml of medium A supplemented with 2 ml of 1-tetradecene in a 500 ml shake flask were inoculated with five loopfuls of 3~4 day old cells grown on 1-tetradecene-agar slant and incubated at 30°C for 3 days on a reciprocal

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shaker (125 oscills./min, 70 mm stroke). In the course of cellular growth, 0.5 g of sterilized calcium carbonate were added to keep the pH of culture system around 6.0.

For testing the production of citric acids from various carbon sources such as 1-tetradecene and its metabolic intermediates, 2 ml of medium B supplemented with 100 mg of carbon source in a large test tube (21 × 200 mm) were inoculated with 0.1 ml of seed culture and incubated at 30°C for 6 days with continuous shaking. In this case, 0.06 g of sterilized calcium carbonate were added to the culture system at the 24th hr of cultivation.

To examine the effect of surfactants on the production of citric acids, cultivations were carried out in a 500 ml shake flask containing 50 ml of medium B supplemented with 3 ml of 1-tetradecene and 25 mg of surfactant for 6 days under the same cultural conditions as above.

Determination of citrates. Fermentation broth was adjusted to pH 2 with 4N-HCl and centrifuged at 5000 × g for 10 min. Citric acid in the supernatant obtained was determined by a spectrophotometrical methods using pentabromacetone,²⁾ and the amount of total citric acids (citric + isocitric acid) was determined after the method of Tabuchi *et al.*³⁾ which modified Saffran's method.

Accumulation of metabolic intermediates of 1-tetradecene. Cells were harvested at the exponential growth phase and washed once with 1/15 M phosphate buffer (pH 7.0). The washed cells were resuspended in 1/15 M buffer to make a reaction mixture which was composed of 20 ml of the cell suspension (containing 5 g of resting cells) and 1 g of substrate and incubated at 30°C for 4 days with continuous shaking.

Isolation and identification of metabolic intermediates. The mixture of oxidation products shown in Fig. 1 was methylated and applied to a silicic acid (Wakogel C-200)

column (19 × 300 mm). The column was eluted with 300 ml of a hexane-ether (80:20) mixture to recover alcoholic and acidic oxidation products. This fraction (tentatively designated as fraction A) was analyzed by means of gas-liquid chromatography and mass spectrometry for the identification of products.

A Shimadzu GC-4C equipped with a flame ionization detector was used for gas-liquid chromatography, and a Hitachi RMU-6E was employed in the mass spectrometric analysis.

The gas-liquid chromatography was performed on a stainless-steel column (3 mm × 2 m) packed with 60/80 mesh Chromosorb W-AW coated with 30% Silicone SE-30. The detector and injection port were maintained at 270°C. The column temperature was programmed at 2°C/min from 160 to 230°C. The carrier gas was N₂ with a flow rate of 20 ml/min. The mass spectra were taken by using 70 eV ionizing energy.

Rate of oxygen uptake. The rates of oxygen uptake on various substrates were determined by using a Warburg constant volume manometer at 30°C. The starved cells employed were prepared by incubating 1-tetradecene-grown cells, which were harvested by the procedure described above, in 1/15 M phosphate buffer at 30°C for 6 hr with continuous shaking.

Chemicals. 1-Tetradecene was purchased from Tokyo Kasei Kogyo Co. and 96.7% grade. The other chemicals used were of the highest grade commercially available.

RESULTS AND DISCUSSION

Metabolic pathways for the oxidation of 1-tetradecene

As to the oxidation of α -olefins by yeasts, two metabolic pathways have been report-

FIG. 1. Procedures for Isolation of Oxidation Products.

ed.^{4,5}) One (pathway I) begins with the oxidation of unsaturated site into 1,2-epoxide followed by the oxidations to form 1,2-diol and α -hydroxycarboxylic acid, which is further decarboxylated into saturated fatty acid. The other (pathway II) begins with the oxidation of saturated site to form primary alcohol, which is further oxidized into unsaturated fatty acid.

Metabolic intermediates of 1-tetradecene accumulated by resting cells of *C. lipolytica* and *C. tropicalis* were firstly examined in order to check the pathways for the oxidation of 1-tetradecene by both strains.

Figure 2 shows the gas-liquid chromatograms of fraction A recovered from the culture filtrates of both strains by the procedure described above. Two products, P₁ and P₂, as shown in Fig. 2-I, were detected in the fraction from *C. lipolytica*, while only one product, P₁, as shown in Fig. 2-II, was detected in the fraction from *C. tropicalis*. Based on the retention times in gas-liquid chromatography and the mass spectra shown in Fig. 5, P₁ and P₂ were found to be identical with methyl tridecanoate (corresponding to tridecanoic acid as metabolic intermediate) and methyl α -

hydroxymyristate (corresponding to α -hydroxymyristic acid as metabolic intermediate), respectively. This suggests that both *C. lipolytica* and *C. tropicalis* oxidized 1-tetradecene via pathway I. This suggestion is also supported by the results in Table I showing that

TABLE I. OXYGEN UPTAKE ON METABOLIC INTERMEDIATES OF 1-TETRADECENE

In the main compartment of a Warburg flask were placed 1.7 ml of phosphate buffer (pH 7.0) containing substrate 3% (w/v). The center well of the flask contained 0.2 ml of 20% (w/v) KOH, and the side arm contained 0.5 ml of cell suspension containing 3~4 mg cells (dry basis).

Intermediates	Rate of oxygen uptake Q_{O_2} (μ l/mg·hr)	
	<i>C. tropicalis</i>	<i>C. lipolytica</i>
1,2-Epoxy-tetradecane	28.8	20.7
α -Hydroxy-myristic acid	34.0	19.7
Tridecanoic acid	73.9	58.0
Myristoleic acid	70.2	59.1

FIG. 2. Gas-liquid Chromatograms of Oxidation Products Produced from 1-Tetradecene.

I, *Candida lipolytica*; II, *Candida tropicalis*.

Peaks: P₁, methyl tridecanoate; P₂, methyl α -hydroxymyristate. Conditions: A stainless-steel column (3 mm $\times$ 2 m) with 30% Silicone SE-30 on Chromosorb W (AW, 60/80 mesh). Column temperature, 160~230°C (program rate, 2°C/min). Flow rate (N₂), 20 ml/min.

1,2-epoxytetradecane, α -hydroxymyristic and tridecanoic acids belonging to the pathway I are oxidized by both strains at moderate rates. It is notable, however, that myristoleic acid is also oxidized by both strains at fairly high rates, suggesting that the pathway II might also be working for the oxidation of 1-tetradecene, although neither 13-tetradecene-1-ol nor myristoleic acid was detected in the culture filtrates of both strains.

So far as the above results were concerned,

no significant difference was found in the pathway for the initial stage of oxidation of 1-tetradecene between *C. lipolytica* and *C. tropicalis*.

A new alternative pathway for the oxidation of 1-tetradecene

Several metabolic inhibitors were tested for the accumulation of any other intermediates than those detected above in order to compare the metabolic pathways of both strains with

FIG. 3. Gas-liquid Chromatograms of Oxidation Products Produced from 1-Tetradecene in the Presence of Iodoacetamide.

I, *Candida lipolytica*; II, *Candida tropicalis*.

Peaks: P₁, methyl tridecanoate; P₂, methyl α -hydroxymyristate; P₃, 2-methoxytetradecane. Conditions are the same as those in Fig. 2.

FIG. 4. Gas-liquid Chromatograms of Oxidation Products Produced from 2-Tetradecanol.

I, *Candida lipolytica*; II, *Candida tropicalis*.

Peaks: P₄, 2-tetradecanone; P₅, methyl tridecanoate. Conditions are the same as those in Fig. 2.

FIG. 5. Mass Spectra of Products P₁, P₂, P₃ and P₄.

Ionizing energy was 70 eV and accelerating voltage was 1800 V.

TABLE II. CITRATES PRODUCTION AND OXYGEN UPTAKE ON 1-TETRADECENE AND ITS METABOLIC INTERMEDIATES

Substrates	<i>C. tropicalis</i>		<i>C. lipolytica</i>	
	Total ^a citrates (g/liter)	<i>Q</i> _{o₂} ^b (μl/mg·hr)	Total ^a citrates (g/liter)	<i>Q</i> _{o₂} ^b (μl/mg·hr)
<i>n</i> -Tetradecane	45.5	34.6	40.6	28.8
1-Tetradecene	36.6	33.5	3.9	13.8
Tridecanoic acid	21.8	73.9	20.7	58.0
2-Tetradecanol	50.0	49.1	40.1	42.3

^a Cultivations were carried out in large test tubes (21 × 200 mm) containing 2 ml of medium B (described in the text) supplemented with 100 mg of carbon source at 30°C for 6 days with continuous shaking.

^b Experimental conditions were the same as those in Table I.

each other in detail, and one more intermediate, in addition to P₁ and P₂, was found to be accumulated by both strains by the presence of iodoacetamide, an inhibitor to isocitrate dehydrogenase.⁶⁾ This product, P₃, was detected in fraction A as shown in gas-liquid chromatograms in Fig. 3 and was identified with 2-methoxytetradecane (corresponding to 2-tetradecanol as metabolic intermediate) by analyzing mass spectrum shown in Fig. 5-P₃.

Two oxidation products were further accumulated, when 2-tetradecanol was subjected to the oxidation by resting cells of both strains, and detected in fraction A as shown by peaks P₄ and P₅ in gas-liquid chromatograms in Fig.

4. Peak P₅ was supposed to be identical with methyl tridecanoate (corresponding to tridecanoic acid as an oxidation product), since the retention time of P₅ in Fig. 4 was the same as that of peak P₁ in Fig. 2. P₄ was identified with 2-tetradecanone by analyzing mass spectrum shown in Fig. 5-P₄. In addition, as shown in Table II, 2-tetradecanol was oxidized at fairly high rates and utilized by both strains for the production of citrates as well as for the cellular growth.

Above results suggest that a new alternative pathway, in which 1-tetradecene is oxidized *via* 2-tetradecanol and 2-tetradecanone, may be present in both *C. lipolytica* and *C. tropi-*

calis, although further enzymatic studies are required to make the presence of this new pathway sure.

On the other hand, the fact that 1-tetradecene was oxidized by both strains in the same alternative way in the presence of such a metabolic inhibitor as iodoacetamide may also support the conclusion that there is no difference, by which the difference in the productivity of citrates is elucidated, in the metabolic pathway for the initial stage of oxidation of 1-tetradecene between *C. lipolytica* and *C. tropicalis*.

Citrates production and oxygen uptake on 1-tetradecene and its metabolic intermediates

After coming to the above conclusion, we next examined the productivity of citrates to see whether it was related to the rate of the metabolism of the substrates, since the Q_{O_2} value of *C. lipolytica* on each of the metabolic intermediates of 1-tetradecene, as shown in Table I, was found to be somewhat lower than that of *C. tropicalis* on the same substrate. Table II shows the amount of citrates produced and the rate of oxygen uptake on *n*-tetradecane, 1-tetradecene, tridecanoic acid and 2-tetradecanol. The results in this table indicate that the ratio of the productivity of citrates by *C. lipolytica* to that by *C. tropicalis* on each substrate is roughly equal to the ratio of the Q_{O_2} value of *C. lipolytica* to that of *C. tropicalis* on that substrate. Above all, it is notable that the Q_{O_2} value of *C. lipolytica* on 1-tetradecene is much lower than that of *C. tropicalis* on 1-tetradecene and *C. lipolytica* on *n*-tetradecane, and the reason why 1-tetradecene is utilized by *C. lipolytica* for the growth but not for the production of citrates seems to be closely related to the low Q_{O_2} value indicating that the rate of metabolism of 1-tetradecene is relatively low.

Effects of surfactants on citrates production

The above assumption may be confirmed if the production of citrates from 1-tetradecene by *C. lipolytica* is stimulated by increasing the

TABLE III. EFFECT OF SURFACTANTS ON CITRATES PRODUCTION FROM 1-TETRADECENE BY *C. lipolytica*

Cultivations were carried out in 500 ml shake flasks containing 50 ml of medium B (described in the text) supplemented with 3 ml of 1-tetradecene and 25 mg of surfactant (or 1 ml of RL concentrate*) at 30°C for 6 days on a reciprocal shaker (125 oscill./min, 70 mm stroke).

Surfactants	Total citrates (g/liter)
Span 80	13.2
Tween 80	11.2
Tween 20	12.3
RL concentrate*	14.4
No addition	2.6

* Culture filtrate of *P. aeruginosa* grown on *n*-tetradecane.

rate of metabolism of the substrate. Therefore, three surfactants, Tween 20, Tween 80 and Span 80, which had been reported to be effective in increasing the rate of microbial metabolism of hydrocarbons,^{7,8)} were tested for the stimulation of citrates production from 1-tetradecene. A concentrate (tentatively designated as RL concentrate) of culture filtrate from *Pseudomonas aeruginosa* grown on *n*-tetradecane was also tested, since it had been reported that a rhamnolipid contained in the filtrate stimulated the microbial metabolism of hydrocarbons.^{9,10)}

The results obtained are shown in Table III. All of the four surfactants tested strongly stimulated the citrates production, and the accumulation of citrates attained to 11~15 g/liter on the 6th day of cultivation in the presence of one of these surfactants, while only 2~3 g/liter of citrates were produced in the absence of surfactants. In Fig. 6, a time course of citrates production from 1-tetradecene by *C. lipolytica* in the presence of RL concentrate is compared to that in its absence. In the initial stage of the cultivation, as shown in the figure, 1-tetradecene was consumed more rapidly in the presence of RL concentrate than in its absence, and the stimulation of the consumption of 1-tetradecene in this phase of growth seemed to have a signifi-

FIG. 6. Effect of RL concentrate* on Citrates Production from 1-Tetradecene by *Candida lipolytica*.

The cultures were incubated at 30°C in 500 ml shake flasks containing 50 ml of medium B (described in the text) supplemented with 3 ml of 1-tetradecene and 1 ml of RL concentrate* on a reciprocal shaker (125 oscills./min, 70 mm stroke).

—, RL concentrate 2%; ---, control; ○, total citrates; □, residual 1-tetradecene; △, growth (U.O.D. at 660 nm).

* Culture filtrate of *Pseudomonas aeruginosa* grown on *n*-tetradecane.

cant effect on the production of citrates in the later stage, although no citrates were accumulated in the initial stage. About 30% of 1-tetradecene consumed in the middle and final stages of the cultivation were converted into citrates in the presence of RL concentrate, and 15 g/liter of citrates were accumulated on the 6th day of cultivation, while only 3 g/liter of citrates were obtained in the absence of RL concentrate.

Based on the above results, the reason why there was no large accumulation of citrates by *C. lipolytica* at the expense of 1-tetradecene may partly be attributed to the slow metabolic rate of 1-tetradecene. However, the difference in the productivity of citrates between *C. lipolytica* and *C. tropicalis* may not be fully elucidated only by the difference in the rate of metabolism of 1-tetradecene, since 40 g/liter of citrates were accumulated by *C. tropicalis* at the expense of 40 g of 1-tetradecene, while 15 g/liter of citrates were produced from the same amount of 1-tetradecene by *C. lipolytica* even

in the presence of surfactants. Therefore, further studies are still required to discover another factor to which the difference in the productivity of citrates could also be attributed.

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REFERENCES

- 1) M. Terasawa and J. Takahashi, *Nippon Nôgeikagaku Kaishi*, **53**, 227 (1979).
- 2) M. Kusunose, *Protein, Nucleic Acid and Enzyme*, **2**, 50 (1957).
- 3) Y. Tanaka, Y. Tawara, T. Tabuchi and M. Abe, *Nippon Nôgeikagaku Kaishi*, **44**, 499 (1970).
- 4) M. J. Klug and A. J. Markovetz, *J. Bacteriol.*, **96**, 1115 (1968).
- 5) M. J. Klug and A. J. Markovetz, *Biotechnol. Bioeng.*, **11**, 427 (1969).
- 6) R. F. Colman, *J. Biol. Chem.*, **243**, 2454 (1968).
- 7) A. Tanaka, H. Maki and S. Fukui, *J. Ferment.*

- Technol.*, **45**, 1156 (1967).
- 8) T. Nakahara, K. Hisatsuka, M. Karaki, N. Sano and K. Yamada, Abstracts of Papers, Annual Meeting of Soc. Ferment. Technol. Japan, Osaka, November, 1969, p. 58.
- 9) K. Hisatsuka, T. Nakahara and K. Yamada, *Agric. Biol. Chem.*, **35**, 686 (1971).
- 10) S. Itoh, H. Honda and F. Tomita, *J. Antibiotics*, **23**, 855 (1971).
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