Studies on Utilization of Hydrocarbons by Yeasts

Part II. Diterminal Oxidation of Alkanes by Yeasts

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We found that the yeast Y-3 strain reported in the previous paper¹⁾ has a diterminal oxidation system of hydrocarbon.

This yeast capable of growing in mineral-salts solution with hydrocarbons as sole source of carbon produced a series of dioic acid from *n*-undecane. These acids are 1,11-undecane dioic acid, 1,9-nonane dioic acid (azelaic acid), 1,7-heptane dioic acid (pimelic acid) and 1,5-pentane dioic acid (glutaric acid). 1,10-Decane dioic acid (sebacic acid) was also isolated from *n*-decane cultures.

Azelaic acid was partially transformed into pimelic acid and glutaric acid by treating it with resting cells of this yeast.

1,11-Undecane dioic was also transformed into azelaic acid pimelic acid, and glutaric acid by the same treatment as described above.

Microbial oxidation of aliphatic hydrocarbon is believed to occur by a monoterminial oxidation.²⁻⁵⁾

Recently, A. S. Kester and J. W. Foster⁶⁾ showed that hydrocarbons were oxidized by a diterminal oxidation mechanism in corynebacterial organisms besides a monoterminial mechanism.

The present paper deals with diterminal oxidation of hydrocarbons by the yeast Y-3 strain.

MATERIALS AND METHODS

Organism and culture methods

All the experiments were performed with the yeast,

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2) J. E. Stewart, R. E. Kallio, D. P. Stevenson, A. C. Jones and D. O. Schissler, *J. Bacteriol.*, **78**, 441 (1959).

3) E. R. Leadbetter and J. W. Foster, *Nature*, **184**, 1428.

4) M. H. Proctor, *Biochim. Biophys. Acta*, **42**, 559 (1960).

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6) A. S. Kester and J. W. Foster, *J. Bacteriol.*, **85**, 859 (1963).

Y-3 strain, tentatively assigned to *Pichia* sp. in the previous paper.¹⁾

The composition of mineral salts solution used in this work was disodium phosphate 12 g, 2.5 g, monopotassium phosphate 3.5 g, magnesium sulfate 7 g, 1.0 g, sodium chloride 0.5 g, urea 0.85 g, corn steep liquor 0.1 ml and tap water 1 l.

The hydrocarbons used were *n*-undecane of 95% purity and *n*-decane of 98% purity (Tokyo Chemical Industry Co., Ltd.)

Production of various acids in amounts suitable for isolation was carried out in 5 l shaker flask containing 1.5 l of mineral salts solution and 37.5 g of individual hydrocarbon as the sole source of carbon, so the C/N ratio was 30.

From two to five flasks were employed in each experiment. The mineral salts solution was sterilized for 15 min. at 120°C, and hydrocarbon was added just before inoculation. The flasks were shaken on the rotary shaker at 30°C.

Extraction of acids

The kieselguhr was added to the broth and filtered under reduced pressure. The filtrate was acidified to pH 2.0 with H₂SO₄ and extracted two to three

times in a separatory funnel with equivalent volumes of ether.

The combined ether extracts were shaken with 0.5N-NaOH and separated in a funnel. The ether layer was washed by shaking with distilled water. The alkaline solution and washed liquor were combined and, after the acidification, were extracted with ether again.

The ether layer was dried by anhydrous sodium sulfate.

The dried ether extract was concentrated to dryness and crude acid was obtained at the yield of 30 mg/l. broth.

Thin-layer chromatography

For time course of acid formation 50 ml of broth were taken out at intervals of adequate time and extracted as described above. The crude acids mixtures were dissolved into 0.5 ml of ethanol. Twenty μ l of ethanolic acids solution were spotted on kieselgel G plate.

Two different solvent systems were used, in the ascending technique; solvent A, benzene-dioxane-acetic acid (90:25:4), solvent B, benzene-ether-acetic acid (9:2:1).

The plate was dried in an oven at 105°C for 1 hr, and acid spots were developed by spraying with bromocresol green indicator solution.

Partition column chromatography

For the isolation of various acids, partition column chromatographic technique was used. Crude acids mixtures obtained as described above were dissolved in chloroform and adsorbed on silicic acid column.

Elution was carried out by chloroform followed by chloroform-ethanol mixtures (97% chloroform).

Dioic acids were eluted in the order of chain length.

Crude acids were recrystallized from chloroform and *n*-hexane mixtures.

Gas chromatography

Gas-liquid chromatography was carried out as following conditions.

chromatograph	Shimadzu Model GC-1B
column dimension	150×0.4 cm
solid support	chromosorb W (60/80 mesh)
stationary phase	DEGS-H ₃ PO ₄ (1-1)
temperature	196°C
carrier gas	nitrogen at 30 ml/min
detector	flame ionization detector type HFD-1

Chemical method

Identification of each pure acid was made by co-chromatography on thin-layer, by molecular weight determination (Rast or titration), by elementary analysis and by comparison of the infrared absorption spectrum of the isolated acid with that of an authentic compound.

The mixture of acids was identified by thin-layer chromatography and gas chromatography.

Azelaic acid, pimelic acid, glutaric acid and sebacic acid were obtained from Tokyo Chemical Industry Co., Ltd.

1,11-Undecane dioic acid was synthesized by Willgerodt reaction.⁷⁾

Procedures of resting cell experiments

Yeast cells were collected at the log phase by centrifugation.

Cells were washed four times with the mineral salts solution without the nitrogen source and were suspended in the same salts solution.

The reaction mixtures were prepared as follows: to a Monod test tube were added 7 ml of mineral salts solution with no nitrogen source, 1 ml of 0.01 M-dioic acid solution (neutralized to pH 7.0 by 1 N-NaOH) and 2 ml of cell suspension, the cell concentration of which was five times as much as the concentration in broth at maximum growth in optical density.

The mixtures were incubated overnight at 30°C on Monod shaker

RESULTS

Acid formation

Acid production during growth of Y-3 strain on *n*-undecane was tested. The results are shown in Fig. 1.

This shows that about five spots are developed, and that the acid which appears at early stage and has the largest R_F value disappears at 140 hr cultivation.

The amounts of the two acids which begin to appear at 30 hr after inoculation are gradually increasing with the passage of cultivation time.

These three acids were isolated from *n*-undecane culture and identified. But the

7) D. B. Pattison and M. Carmack, *J. Am. Chem. Soc.*, **68**, 2034 (1946).

FIG. 1. Chromatogram of Organic Acids Produced by Y-3 Strain.

Solvent benzene/dioxane/acetic acid=90/25/4

other two acids which began to appear at 140 hr cultivation were not isolated.

Identification of acids

Chemical properties of organic acids obtained from *n*-undecane and *n*-decane cultures are shown in Table I.

These acids were isolated by column chromatography and recrystallized from adequate solvents.

The acid which had largest R_F value agreed with 1,11-undecane dioic acid synthesized by Willgerodt reaction by cochromatography of thin-layer.

The second acid agreed with azelaic acid and the third also agreed with pimelic acid

by the same method.

The acid isolated from *n*-decane also agreed with sebacic acid. IR-spectra of these acids are shown in Fig. 2.

This shows that the acids isolated from *n*-undecane culture are 1,11-undecane dioic acid, azelaic acid and pimelic acid, respectively.

The acid isolated from *n*-decane culture is the same as sebacic acid.

Degradation of 1,11-undecane dioic acid and azelaic acid by resting cells of Y-3 strain

The acids obtained from resting cell incubation mixture were extracted as described above.

Identification of the extracts was carried out by thin-layer chromatography and gas chromatography.

In thin-layer chromatography two solvent systems were used.

The results are shown in Fig. 3 and 4. This shows that 1,11-undecane dioic acid is transformed into azelaic acid, pimelic acid and glutaric acid, and that azelaic acid is changed into pimelic acid and glutaric acid.

These results are supported by gas chromatography as shown in Fig. 5 and 6.

DISCUSSION

The experiments reported here show that hydrocarbon is oxidized by a diterminal oxidation mechanism in yeast.

TABLE I. CHEMICAL PROPERTIES OF ORGANIC ACIDS PRODUCED BY Y-3 STRAIN

	Sample	Melting point		Molecular wt.		Elementary analysis	
		Authentic acid	Mixed	Found	Theoretical	Found	Theoretical
1,11-Undecane dioic acid	111.0~111.5	112.0	111.0	°220	216.3	{C: 61.13 H: 9.29	C: 61.06 H: 9.32
Azelaic acid	105.5~106.0	106.5	105.5	*195	188.2	{C: 57.10 H: 8.51	C: 57.43 H: 8.57
Pimelic acid	104.0~105.0	104.5	104.0	°162	160.2	{C: 52.39 H: 7.59	C: 52.49 H: 7.55
Sebacic acid	133.5~134.5	134.5	133.0	—	202.0	{C: 59.29 H: 8.89	C: 59.38 H: 8.97

Rast Method; ° Titration

FIG. 2a. Infrared Spectrum of Acid Produced from *n*-Undecane by Y-3 Strain.

Solid line: authentic 1,11-undecane dioic acid
 Broken line: microbial product (nujol)

FIG. 2b. Infrared Spectrum of Acid Produced from *n*-Undecane by Y-3 Strain.

Solid line: authentic azelaic acid
 Broken line: microbial product (nujol)

FIG. 2c. Infrared Spectrum of Acid Produced from *n*-Undecane by Y-3 Strain.

Solid line: authentic pimelic acid
 Broken line: microbial product (nujol)

FIG. 2d. Infrared Spectrum of Acid Produced from *n*-Decane by Y-3 Strain.

Solid line: authentic sebacic acid
Broken line: microbial product

(nujol)

FIG. 3. Thin-layer Chromatogram from Resting Cell Experiments.

Solvent: benzene-dioxane-acetic acid (90:25:4)
Cont.: no substrate 11D: 1,11-undecane dioic acid
9D: azelaic acid 8D: suberic acid
7D: pimelic acid 6D: Adipic acid 5D: glutaric acid
R.C.: treated with resting cells.

The dioic acids produced by this yeast are metabolized by the resting cells in such a way as other dioic acids the carbon skeletons of which are shorter by two atoms than that of the substrate, are produced.

We believe that this degradation is probably carried out by β -oxidation mechanism.

In bacteria, Kester and Foster⁶¹ obtained decane and dodecane dioic acids etc., but shorter chain-length dioic acids than the substrate alkanes of their series were not demonstrated.

We could isolate azelaic and pimelic acid

FIG. 4. Thin-layer Chromatogram from Resting Cell Experiments.

Solvent: benzene-ether-acetic acid (9:2:1)
Cont.: no substrate 11D: 1,11-Undecane dioic acid
9D: azelaic acid, 8D: suberic acid
7D: pimelic acid, 6D: adipic acid 5D: glutaric acid
R.C.: treated with resting cells.

from *n*-undecane culture besides 1,11-undecane dioic acid.

Dioic acids pathway reported here has not yet been demonstrated hitherto in yeast.

These observations show that diterminal oxidation of alkanes may be widely distributed in microorganisms.

We could not isolate the monoic acids such as undecanoic acid, decanoic acid and nonanoic acid etc. from either cultural filtrate or cells.

FIG. 5. Gas Chromatogram of Acids Extracted from Incubation Mixtures.
Azelaic acid was treated with resting cells.

FIG. 6. Gas chromatograms of Acids Extracted from Incubation Mixtures.
1,11-undecane dioic acid was treated with resting cells.

This may merely mean that these acids do not accumulate in detectable amounts.

After hydrolysis of extracts from cells, the acid mixtures, the carbon-chain length of which was longer than C_{13} , were isolated.

Anyhow, there may be a monoic acid path-

way, although we could not isolate them.

There are at least two kinds of degradation pathway of alkanes in microorganisms: monoic acid pathway and dioic acid pathway.

The scheme of these pathways is shown in Fig. 7.

FIG. 7. Scheme of Degradation Pathway.