Studies on the Utilization of Hydrocarbons by Yeasts

Part IV. On the Dialysis Culture of *Mycotorula japonica* and “Growth-inhibitory Factor” in the Dialyzable Material

By Tokujiro AIDA and Kazuo YAMAGUCHI

Department of Agricultural Chemistry, Ibaraki University

Received November 21, 1968

The growth of *M. japonica* in hexadecane or decane medium was markedly improved by dialysis culture and there existed a “growth-inhibitory factor” in the dialyzable material. This material inhibited the growth in hydrocarbon medium but not in glucose containing medium. Free fatty acids excreted extracellularly by dialysis culture in hexadecane medium were mostly palmitic, myristic and lauric acids in the culturing chamber, but almost exclusively lauric acid was found in the dialyzable material. These acids, assimilated themselves by the organism, inhibited the growth in hexadecane medium but did so only weakly in glucose. It was concluded that lauric acid was at least partly an active principle of “growth-inhibitory factor” in the dialyzable material in hexadecane medium.

Previous studies by Aida and Yamaguchi¹⁾ revealed that dialysis culture improved markedly the assimilation of kerosene by a yeast *Mycotorula japonica* and that in the dialyzable material of dialysis culture there existed a factor which depressed specifically the growth of the organism on kerosene medium but not on glucose. The factor was tentatively designated as “growth-inhibitory factor”. The nature of the active principle of the factor, however, was not known. It was suggested that fatty acids, the metabolic products of hydrocarbon, were responsible, at least partly, for the growth inhibition.

The fatty acids having the same carbon skeleton as the hydrocarbon substrates have been well established as oxidation products from aliphatic alkanes,^{2~6)} and there seems to be

general agreement that β -oxidative breakdown of the alkane chain is involved.^{5,6)} In addition to the significance as the metabolic products of hydrocarbon, antimicrobial activities of long-chain fatty acids were also reported.⁷⁾

Experiments to prove fatty acids as products from hexadecane and their specific inhibitory effects on the growth of *M. japonica* on hydrocarbon medium have been undertaken by the present authors. The results obtained from these studies and from dialysis culture experiments with individual alkanes are presented in this paper.

MATERIALS AND METHODS

Organism and culture methods. *Mycotorula japonica* IAM 4185^{1,8)} was used throughout the experiments. Maintenance and preculture of the organism were

1) T. Aida and K. Yamaguchi, *Nippon Nogeikagaku Kaishi*, **40**, 119 (1966).

2) “Oxygenase”, ed. by O. Hayaishi, Academic Press, Inc., New York, N.Y., 1962, p. 241.

3) J. N. Baptist *et al.*, *Biochim. Biophys. Acta*, **69**, 40 (1963).

4) E. Azoulay *et al.*, *ibid.*, **77**, 554 (1963).

5) G. J. E. Thijsse and A. C. van der Linden, *Antonie van Leeuwenhoek*, **24**, 298 (1958).

6) J. W. Foster, *ibid.*, **28**, 241 (1962).

7) N. P. Willett *et al.*, *J. Bacteriol.*, **91**, 2245 (1966).

8) K. Yamaguchi, *Nippon Nogeikagaku Kaishi*, **19**, 800 (1943).

previously described.¹⁾

Dialysis culture flask, described by Gerhardt *et al.*,⁹⁾ were used. Medium and cultural conditions were also identical with the preceding paper¹⁾ with one exception of carbon source, *i.e.*, hexadecane or decane was used at the concentration of two per cent in place of kerosene.

Dialysis cultures for growth experiments were performed in dialysis culture flasks which consisted of 25-ml top chamber and 65-ml bottom chamber. A sheet of membrane of Visking cellulose (cellulose tubing, size: 5.5 inch, Visking Co.) was interposed between both chambers. The top chamber contained 5 ml of hydrocarbon medium and inoculated with the organism. The bottom chamber was filled with the same medium without hydrocarbon. Two types of control cultures, designated as nondialysis control culture and concentrated nondialysis control culture (abbreviated as nondialysis culture and conc. control, respectively), were also made using dialysis culture flasks, but both types of control flasks had a sheet of gum rubber in place of cellulose membrane between top and bottom chambers. In the nondialysis culture, top chamber had the same volume of the same medium as dialysis culture. Whereas, in the conc. control, top chamber had the same volume of a medium as dialysis culture, but the medium used was concentrated to contain whole quantities of nutrients of both top and bottom chambers in dialysis culture.

Cultures for analysis of extracellular free fatty acids were conducted as follow: Dialysis culture was performed in a dialysis culture flask which consisted of 430-ml top chamber and 850-ml bottom chamber, inserting dialysis membrane (Visking cellulose) between both chambers. The top chamber contained 50 ml of hexadecane medium and inoculated with the organism. The bottom chamber was filled with the medium free from hexadecane. Nondialysis culture was carried out in three 500-ml shaking flasks each containing 100 ml of hexadecane medium. Incubation was carried out at 30°C with shaking for 72 hr in both dialysis and nondialysis cultures. Experiments with washed cell suspension were conducted at 30°C with shaking for 6 hr in the mixture of 1 ml of hexadecane, 5 ml of M/10 phosphate buffer (pH 5.5) and 4 ml of washed cell suspension (approximately 15 mg dry cell weight/ml) harvested from hexadecane medium.

Extraction and esterification of extracellular free fatty acids. Before the extraction of fatty acids, hexadecane was removed by extraction with alkaline ethyl ether to avoid an interference of gas chromatographic analysis of fatty acids by hexadecane. For this purpose, the cell-free media obtained after centrifugation of the cultures were adjusted at pH 8.0 by NaOH and extracted with ethyl ether. The residual aqueous phases were then acidified with HCl to pH 2 and extracted again with ethyl ether in a continuous-extraction apparatus for three days.

Extracts thus obtained were distilled at 40°C and the resulting ether-free residues were esterified by boron trifluoride-methanol according to Metcalfe *et al.*¹⁰⁾ Methyl esters of fatty acids were recovered, dissolved in 1 ml of benzene and analysed by gas-liquid chromatography.

Gas-liquid chromatography. A gas-liquid chromatograph Model GC-1C apparatus (Shimazu Seisakusho Ltd.) with a hydrogen flame ionization detector was used. A column was heated at 190°C and filled with 25% diethylene glycol succinate on Shimalite (Shimazu Seisakusho Ltd., 60 to 80 mesh). Nitrogen was used as the carrier gas (2 kg/cm²). The column temperature was 185°C, detector temperature 210°C, and injection port temperature 250°C.

Measurement of yeast growth. Cultures in hydrocarbon medium containing 0.01% detergent (Emunon 3115, polyethyleneglycol-fatty acid ester, Kao-Atlas Co. Ltd.)¹⁾ were centrifuged. Cells harvested were washed once with the same detergent (0.01%), followed by washing once with distilled water, and finally suspended in distilled water in the original volume. The optical density of the cell suspension was read by a spectrophotometer, Spectronic 20 at 660 mμ.

RESULTS

Dialysis culture of M. japonica in hexadecane or decane medium

Growth of *M. japonica* in dialysis culture flask using hexadecane medium is shown in Fig. 1. Two types of control cultures, nondialysis culture and conc. control, were also made in dialysis culture flask which had a

9) P. Gerhardt and D. M. Gallup, *J. Bacteriol.*, **86**, 919 (1963).

10) L. D. Metcalfe and A. A. Schmitz, *Anal. Chem.*, **33**, 363 (1961).

FIG. 1. Dialysis Culture of *M. japonica* in Hexadecane Medium.

In dialysis culture, 5 ml of hexadecane medium were placed in top chamber and approximately 65 ml of the same medium without hexadecane were filled in bottom chamber of dialysis culture flask. Incubation was carried out at 30°C with shaking.

- Dialysis culture.
- Conc. control.
- △-·-△ Nondialysis culture.

sheet of gum rubber instead of cellulose membrane. In the nondialysis culture, supply of nutrients contained in bottom chamber into top chamber through the membrane and diffusion of any inhibitor from top to bottom chamber are prevented. On the other hand, the conc. control eliminates diffusion of inhibitor, but the medium placed in top chamber is supplemented initially with the whole quantities of nutrients in bottom chamber. As shown in Fig. 1, growth in dialysis culture was more abundant than not only that in nondialysis culture but also that in conc. control. This is the case with decane medium. The results obtained in the similar experiments performed in decane medium are presented in Fig. 2, exhibiting more greatly enhanced growth in dialysis culture than both nondialysis culture and conc. control. These results suggest that in dialysis culture, a growth-inhibi-

FIG. 2. Dialysis Culture of *M. japonica* in Decane Medium.

Cultural conditions were similar to the description in Fig. 1 with an exception of decane instead of hexadecane.

- Dialysis culture.
- Conc. control.
- △-·-△ Nondialysis culture.

tory factor is diffused through the membrane from top to bottom chamber besides supply of nutrients from bottom chamber.

Inhibition of growth of M. japonica by dialyzable material of dialysis culture

M. japonica was cultured in dialysis culture for 72 hr in hexadecane or decane medium. The outer solutions of dialysis (850 ml) were adjusted at pH 7.5 and concentrated by distillation *in vacuo* into 50 ml. Centrifuged supernatants of the concentrates were then filtered through a Seitz filter, added aseptically into the respective hydrocarbon and glucose media and tested their effects on growth of the organism. The results are presented in Table I. It was noticed that dialyzable material obtained from dialysis culture in hexadecane medium inhibited the growth in hexadecane medium (62.1% inhibition) but did not inhibit significantly in glucose medium. In the case of decane medium, the similar selective inhibitory effect on the growth of the organism was also observed; the dialyzable

TABLE I. INHIBITION OF GROWTH OF *M. japonica* BY DIALYZABLE MATERIAL OF DIALYSIS CULTURE

Dialyzable material from medium of	Concentration (%)	Inhibition (%)	
		Hydrocarbon medium	Glucose medium
Hexadecane	0.1	27.6	10.3
"	0.5	62.1	2.3
Decane	1.0	28.1	4.1
"	2.5	65.6	2.8

Preparation of dialyzable material was described in the text. Growth inhibition tests were carried out at 30°C with shaking for 48 hr in glucose and for 72 hr in the same hydrocarbon medium as those used in dialysis culture.

FIG. 3. Inhibition of Growth in Decane Medium by Dialyzable Material.

Experimental conditions were similar to the description in Table I.

○—○ Without dialyzable material.
●---● With dialyzable material at the concentration of 2.5% (v/v).

material from decane medium exhibited 65.6% inhibition in decane medium whereas only 4.1% in glucose medium.

Time course of growth under the presence of the dialyzable material was followed in decane medium (Fig. 3). It was shown that the inhibition of the growth by the dialyzable material in the hydrocarbon medium was not a lengthening of lag period but depression of maximal growth level.

Extracellular free fatty acids produced by *M. japonica*

From the results described above, it might be suspected that "growth-inhibitory factor" was produced in culturing chamber, and diffused through the membrane into the outer solution, resulting in dilution of it and improvement of growth in culturing chamber, and that it could be closely related with hydrocarbon metabolism, because of its specific inhibition of hydrocarbon assimilation. Then, fatty acids excreted into the medium and diffused into the outer solution by dialysis culture were examined by gas chromatographic analysis. Experiments were also conducted for fatty acids produced by washed cell suspension and by nondialysis culture.

Washed cell suspension grown on hexadecane was incubated with the same substrate at 30°C for 6 hr with shaking, and the supernatant was examined for free fatty acid. The results obtained were compared with those obtained in nondialysis culture which was performed at 30°C for 72 hr. As shown in Fig. 4A, the organism produced mainly palmitic and myristic acids together with minor components of two unidentified substances, X_1 and X_2 , by incubation of cell suspension. Whereas, in nondialysis culture, lauric and myristic acids appeared as the main peaks but palmitic acid as a minor one (Fig. 4B).

Results obtained in the culture fluid and in the outer solution of dialysis culture are presented in Fig. 5, indicating that in the culturing chamber, lauric, myristic and palmitic acids occurred as the main peaks besides an additional unidentified peak, presumably identical with X_1 , whereas the outer solution gave almost exclusively lauric acid.

The unidentified peaks observed, X_1 and X_2 , were not examined further because they not appeared as major peaks under the experimental conditions.

The facts that dialysis culture gave lauric acid but not palmitic and myristic acids in the outer solution in spite of the occurrence

FIG. 4. Gas-liquid Chromatograms of Methyl Esters of Fatty Acids Excreted into the Medium by (A) Incubation with Cell Suspension and (B) Nondialysis Culture.

I: Lauric acid.
 II: Myristic acid.
 III: Palmitic acid, X₁ and X₂: Unidentified.

FIG. 5. Gas-liquid Chromatograms of Methyl Esters of Extracellular Free Fatty Acids Appeared in (A) the Culture Fluid and (B) the Outer Solution of Dialysis Culture.

I: Lauric acid.
 II: Myristic acid.
 III: Palmitic acid, X₁: Unidentified.

TABLE II. FATTY ACID DIFFUSION THROUGH CELLULOSE MEMBRANE

Fatty acid	Fatty acid diffused μg/hr
Palmitic acid	4.6
Myristic acid	6.6
Lauric acid	17.6

A mixture of each 30 mg of acids in 100 ml of 0.1M phosphate buffer was placed in 430-ml top chamber and the same buffer filled in 850-ml bottom chamber of dialysis culture flask having Visking cellulose as dialysis membrane.

of all these three acids in the culturing chamber, suggest a possibility that the membrane used may permit exclusively lauric acid to permeate into the outer solution. In order to confirm this possibility, a mixture of the same quantities of authentic samples of palmitic, myristic and lauric acids was shaken with phosphate buffer in top chamber of dialysis culture flask at 30°C for 6 hr, and after the period of time, the acids were extracted from the bottom chamber by acidic ethyl ether. Results of gas-liquid chromatographic analysis of the recovered ether extract are presented in Table II. Lauric acid diffused through the membrane relatively fast as compared with myristic and palmitic acids.

Inhibition of hexadecane assimilation by fatty acids

Inhibitory effects on the growth of the organism by these fatty acids including lauric acid occurring in the outer solution of dialysis were investigated. The results are shown in Table III. Growth inhibitions ranged from 73.5 to 83.8 per cent in hexadecane medium by these three fatty acids tested, whereas the corresponding values in glucose medium were 28.5 to 33.3 per cent. It may be therefore noticed that these fatty acids exhibit potent inhibition on the growth in the hexadecane medium but relatively little effect in the glucose medium, similarly with the dialyzable material though degrees of inhibitions are somewhat differed. Accordingly, from these

TABLE III. INHIBITION OF GROWTH OF *M. japonica* BY FATTY ACID

Fatty acid	Concentration (mg/ml)	Inhibition (%)	
		Hexadecane medium	Glucose medium
Palmitic	1	50.0	30.7
"	3	73.5	33.3
Myristic	1	83.8	28.5
Lauric	1	82.5	30.9

Experimental conditions were similar to the description in Table I.

TABLE IV. GROWTH OF *M. japonica* ON FATTY ACID MEDIUM

Carbon source*	Concentration (%)	Growth O.D.**
None		0.07
Hexadecane	2.0	0.48
Palmitic acid	0.1	0.58
"	0.3	0.61
"	0.5	0.45
Myristic acid	0.1	0.63
"	0.5	0.54
Lauric acid	0.1	0.59
"	0.3	0.51
"	0.5	0.44

* Basal medium was hexadecane medium without hexadecane.

** Shaking culture at 30°C for 72 hr.

facts and the evidence that lauric acid exists in the outer solution of dialysis, it may be concluded that an inhibitory substance in the dialyzable material is, at least partly, lauric acid.

Finally, assimilation of these fatty acids by the organism was examined. The results are presented in Table IV. These palmitic, myristic and lauric acids supported the growth of the organism in the similar extents to that in the case of hexadecane as the growth substrate, indicating that these acids were metabolized and assimilated by the organism.

DISCUSSION

Enhancement of assimilation of hydrocarbon by dialysis culture and specific growth inhibi-

tion by its dialyzable material noticed previously in kerosene medium¹¹ were again demonstrated also in the case of individual *n*-alkane, i.e., hexadecane or decane which were used as the representatives of the most and less assimilable alkane substrates for *M. japonica*, respectively.¹¹ These findings seem to indicate that the effect of dialysis culture on alkane assimilation is fairly general phenomenon, at least, inasmuch as the organism is concerned.

From the evidence presented for the growth inhibition by the dialyzable substance, it is likely that the improvement of hydrocarbon assimilation by dialysis culture may be attributed to the presence of "growth-inhibitory factor" in the dialyzable material. On the other hand, Yamaguchi *et al.*¹¹ reported that hydrocarbon assimilation by the organism was enhanced by amino acids, and in the case of carbohydrate medium enhanced growth in dialysis culture was explained by supply of nutrients from reservoir.^{9,12} However, much higher level of growth in dialysis culture than conc. control indicates that improvement of growth by dialysis culture is due to diffusion of "growth-inhibitory factor" into the outer solution of dialysis besides supply of nutrients such as amino acids from reservoir.

Fatty acids of cellular lipids of microbial cells assimilating *n*-alkanes have been extensively investigated,¹³⁻¹⁶ and it was reported that the major fatty acid was of the same chain length as the substrate¹⁵ and there existed β -oxidation of fatty acids formed.¹⁴ In the present work using hexadecane medium,

11) K. Yamaguchi and T. Aida, *Sci. Rep. Fac. Agr. Ibaraki Univ.*, **16**, 111 (1968).

12) S. Tanaka *et al.*, *Bull. Nat. Inst. Anim. Hlth.*, **48**, 39 (1964).

13) E. M. Romero and R. R. Brenner, *J. Bacteriol.*, **91**, 183 (1966).

14) M. Mizuno *et al.*, *Agr. Biol. Chem.*, **30**, 506 (1966).

15) K. R. Dunlap and J. J. Perry, *J. Bacteriol.*, **94**, 1919 (1967).

16) R. Makula and W. R. Finnerty, *ibid.*, **95**, 2102 (1968).

it was also noticed that palmitic acid was the major acid and myristic and lauric acids also occurred in most experiments, although these acids were excreted into the medium in the free forms.

However, it is of interest to point out that the fatty acids accumulated in the medium were changed by the cultural conditions. In nondialysis culture, palmitic acid which occurred as a major acid in the incubation with cell suspension was less emerged, accompanied with accumulation of lauric and myristic acids. When dialysis culture was made, palmitic acid again appeared in the culturing chamber, attended with diffusion of lauric acid into the outer solution of dialysis. This may suggest that accumulated lauric acid in nondialysis culture inhibits the oxidation of hexadecane to palmitic acid and the inhibition may be released in dialysis culture by diffusion of lauric acid from the culturing chamber into the outer solution of dialysis.

Evidence obtained that lauric acid exists in the dialyzable material and it inhibits the growth in hexadecane medium indicates that

at least in part, lauric acid is the "growth-inhibitory factor" in the dialyzable material. Sinclair *et al.*¹⁷⁾ described the presence of some inhibitory substances, probably organic acids, from their "recycling experiments" although not identified. Furthermore, in *Lactobacillus acidophilus*, enhanced growth and accumulation of lactic acid by dialysis culture in glucose medium were reported.⁸⁾ It may be pointed out that lauric acid is an intermediate product of hexadecane catabolism and metabolized further by the organism, in contrast with end-product nature of lactic acid in *L. acidophilus*. From the view of this catabolite nature of lauric acid, it may be suggested that the inhibition of hexadecane assimilation by the acid may be attributed to a catabolite inhibition or repression on hexadecane-oxidizing system of the organism. Further investigations are under way to elucidate the mechanism of lauric acid inhibition.

17) N. A. Sinclair and J. L. Stokes, *J. Bacteriol.*, **83**, 1147 (1962).