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MICROBIAL OXIDATION OF n-PARAFFINIC HYDROCARBONS

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Beerstecher in his book *Petroleum Microbiology* made the following observation: "Petroleum fermentation offers tremendous possiblities for the development of new products. Microorganisms might be used to make many chemicals from petroleum, or they might be used in unit processes in the petrochemical industry. High grade edible fat and protein might also be derived from petroleum through microbial action. In addition, various drugs might be obtained in this manner. Despite the many attractive possibilities in this area, there appears to be at present very little study of the field, and many basic data are needed before the horizons of microbiological petrochemistry can be accurately visualized."

Today, several years later, the use of hydrocarbons as substrates for microorganisms for the production of industrial chemicals is still not realized. Fortunately, however, some basic studies are being done in our universities and elsewhere so that petroleum fermentation may become a reality. With the interest that is being shown in elucidating the mechanism of n-alkane oxidation by microorganisms it seems appropriate to review some of the more recent literature pertaining to methods of isolation, description of microorganisms, and the means of culturing hydrocarbon oxidizers used in these studies. (Leadbetter and Foster, 1960; Stewart *et al.*, 1959; Thijsse and Van Der Linden, 1958).

Webley and De Kock cultured *Nocardia opaca* on glucose for oxidation studies of alkanes because of the difficulty of separating the cells from the hydrocarbon. In a later paper Webley states that this microbe grows well on n-dodecane, n-tetradecane, n-hexadecane, and n-octadecane. He found it grew poorly on heptane, octane, and nonane but could not really assess growth on any of the hydrocarbons tested due to the nature of the substrate. Treccani and Canonica were able to grow a corynebacterium isolated from decomposing organic matter on several aliphatics from C_{11} to C_{28}. Treccani *et al.* isolated three additional microbes, an achromobacter, a nocardia, and a mycobacterium by soil enrichment with pentane and hexane. They obtained pure cultures by plating on nutrient agar. To test growth on the light hydrocarbons up to C_8 the cultures were placed in glass containers and allowed to grow on the vapors. The liquid hydrocarbons were first adsorbed on filter paper and placed in the lid of the Petri dish. All of the cultures tested with the exception of the achromobacter could grow on several n-alkanes from C_3 to C_{28}. The achromobacter utilized C_3 through C_{12}. After Warburg respirometer studies, they concluded that the metabolism of the hydrocarbons was the same (after the initial oxidative step) as the saturated fatty acids, supporting the mechanism of β-oxidation.

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Konovaltschikoff-Mazoyer and Senez found eleven strains of *Pseudomonas* in soil and water, probably contaminated by petroleum products, capable of using C_3 to C_{16} aliphatic paraffins as the sole source of carbon. For their enrichments they used 1 g soil in 1 L mineral-salts medium with 1% hydrocarbon added. They obtained pure cultures by streaking on mineral agar and incubating under heptane vapors. They reported 97 mg of cells of one strain from 1 L of medium containing 1% n-heptane. Later Senez and Konovaltschikoff-Mazoyer grew *Pseudomonas aeruginosa* in 2-L flasks containing 1 L mineral-salts medium and 10 g n-heptane. After six days they reported the formation of 1.08 mM of volatile fatty acids which they identified by paper chromatography. They did not report on the amount of cells produced or the quantity of substrate utilized.

Harris isolated a micrococcus from an asphalt-soil interface which grew well on a number of aliphatic hydrocarbons, but no account was given for method of isolation or determination of growth. Complete oxidation of the hydrocarbons to CO_2 and water was indicated by oxygen uptake and respiratory-quotient studies using glucose-grown cells.

Azoulay and Senez grew cells of *Pseudomonas aeruginosa* on nutrient medium to which they added 5 ml/L of n-heptane. They obtained 2 g of cells from 10 L of medium in 36 hr at 32 C. The cells were used to demonstrate dehydrogenation of several long-chain n-paraffins. They used pyocyanin as an indicator for the dehydrogenation.

Stewart *et al.* and Stewart and Kallio, while studying ester formation by a Gram-negative coccus, set up large scale cultures in 3 L Fernbach flasks containing 1 L mineral-salts medium. Hydrocarbons were added (1 vol. %) and cultures incubated up to 210 hr on a gyratory shaker (230 rpm). They found that decane, dodecane, tetradecane, hexadecane, and octadecane could all be used as substrates for the microorganism, but they reported their yields of cells in ml of packed cells per 6.5 ml of medium. Two and one half grams of ester (calculated as cetyl palmitate) was reported to have been formed from 7.73 g of hexadecane.

Hoerburger in an industrial laboratory grew *Candida tropicalis* in 450 ml mineral salts containing 1.7% of a hydrocarbon "cut" (b.p. 230–290 C). With high aeration (400 L/hr of air) he was able to increase the amount of yeast cells from 1 g to 2.65 g in 22 hr. Because of this high aeration requirement and the variable composition of the cut used, he concluded that the production of protein by this method would not be industrially feasible.

The soil enrichment technique seems by far the most widely used method described in the literature of obtaining isolates from nature. The advantages and limitations of this method have been discussed recently by Leadbetter and Foster for methane-utilizing bacteria and apply equally well to other n-alkane-utilizing microbes. Factors such as pH, temperature, and source of inoculum determine to a great extent the types of microorganisms which are isolated. While the numbers of microbes are greatly enhanced by gas leaks, oil seeps, and contamination by petroleum products, many bacteria, yeasts, and molds can be isolated from soils without such a history. In our laboratory we used the sprinkled-soil plate method described by Davis *et al.* and Leadbetter and Foster and observed several hundred colonies of nocardiae, corynebacteria,

streptomyces and molds on a single plate from 0.1 g garden soil, with hexadecane as the carbon source.

It is apparent that although there are many reports of the utilization of a wide range of n-alkanes by many kinds of microbes there are still little if any data available on the yield and composition of cells and products. It is the purpose of the balance of this paper to discuss experimental results obtained in our laboratory using 4 L fermentors for the oxidation of n-alkanes by a nocardia and by a yeast.

MICROORGANISMS

The nocardia was isolated from an oilfield soil in one of the surveys described by Davis *et al.* A description of this microbe has been given by Raymond and Davis. Figure 1 shows a phase-contrast photograph of a 24-hr culture grown on a mineral agar plate with hexadecane vapors as the only source of carbon. This nocardia is representative of many pigmented strains which we have observed in many different types of soils both on and off oilfields.

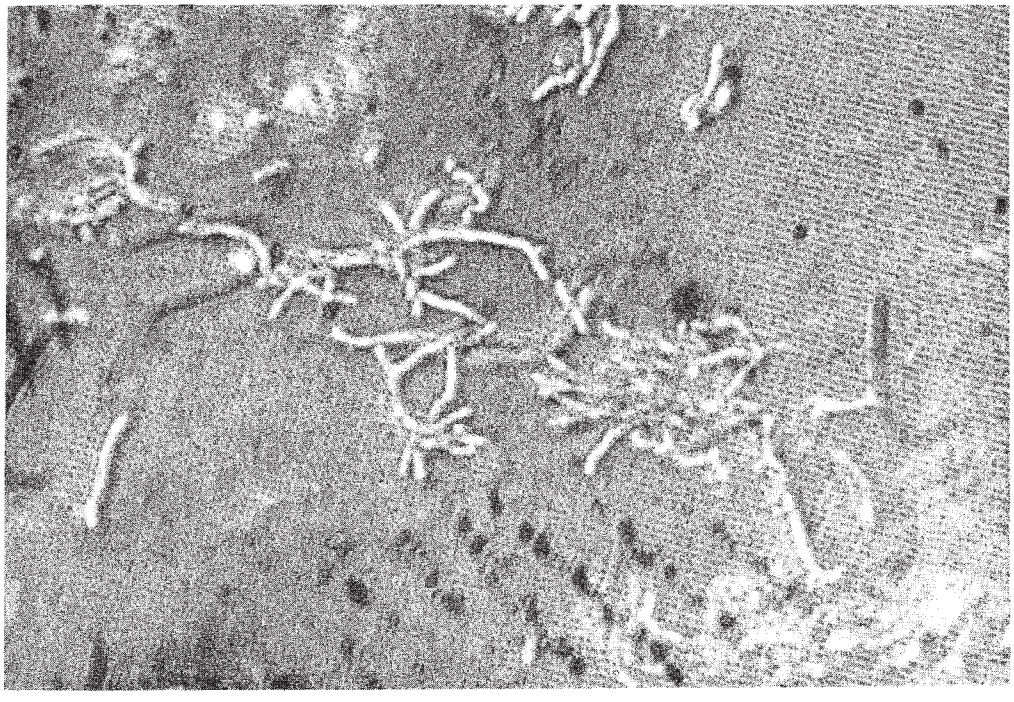

Fig. 1. Cells of a *Nocardia.* sp. (24 hr) grown on hexadecane vapors. Phase-contrast photomicrograph.

The yeast was isolated from topsoil near Dallas, Texas by the enrichment technique. One gram of soil was placed in 50 ml of Czapek's mineral medium in which 5% n-octadecane was substituted for sugar. The pH was maintained around 4.0 and the system incubated at 30 C on a gyratory shaker. After several days, microscopic examination revealed small yeast-like organisms. These were isolated on glucose – peptone – yeast extract agar and transferred to the modified Czapek's medium using octadecane as the only source of carbon. Young cultures

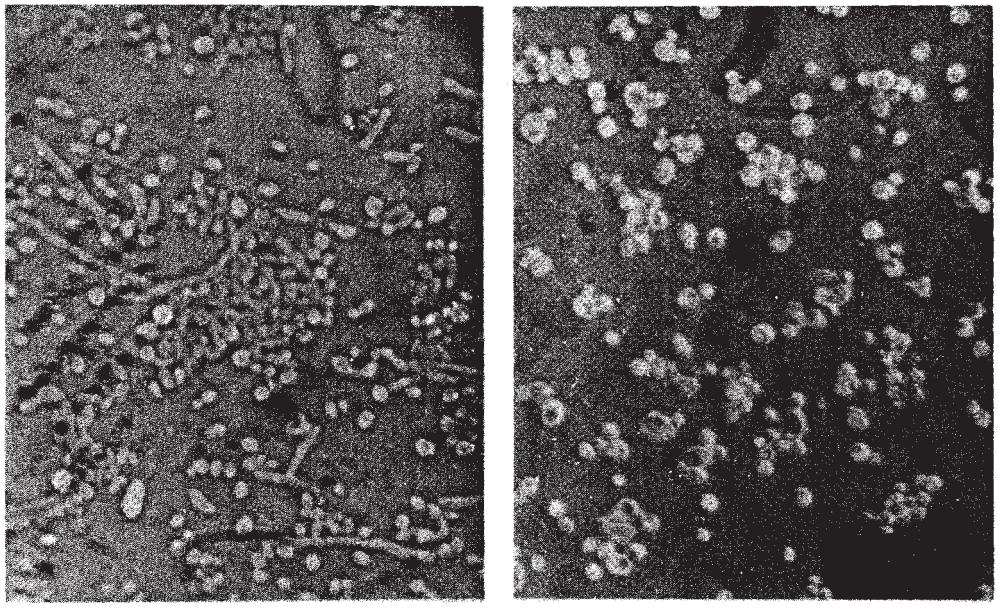

Fig. 2. Yeast cells grown on n-hexadecane. Phase-contrast photomicrographs.

of this microbe form long filaments or a pseudomycelium in stationary nutrient and n-alkane media. In shake systems or fermentors it looks like a budding yeast. Figure 2 is a phase-contrast photograph of both forms. In the fermentors the cells are about 2.0 × 4.0 μ. The colonies are smooth, convex, soft, and cream-colored on nutrient agar plates. Growth on n-octadecane is much better with nitrate than ammonium sulfate as a nitrogen source. This microbe has been tentatively identified as belonging to the genus *Candida*.

OXIDATION SYSTEMS

The fermentors used have been described by Raymond and Davis. The size of the baffles, impeller, and other dimensions were designed very close to those recommended by Finn for maximum turbulence. Figure 3 shows a bank of these systems used to study oxidation of hydrocarbons. The speed of agitation has a large effect on the growth rate of cultures on hexadecane and octadecane. It was found that an optimal rate could be obtained at 1600 to 1750 rpm. At this speed with 2 L of media in each system the substrate was well emulsified into very small globules with very little tendency to float to the surface. This agitation effect was demonstrated with the nocardia culture, where under similar conditions except for rate of agitation, 6.4 and 8.3 g of cells were produced in the same length of time at 1150 and 1725 rpm, respectively. This vigorous agitation has the added effect of reducing the amount of air that had to be provided through the sparger. A flow of 100 to 200 ml/min of air was used to provide a slight positive pressure inside the fermentors to reduce the chances of contamination.

It was observed that several factors affected growth and composition of nocardia and yeast cells in the fermentors. First the concentration of substrate relative to the amount of inoculum was very critical for the nocardia. If the

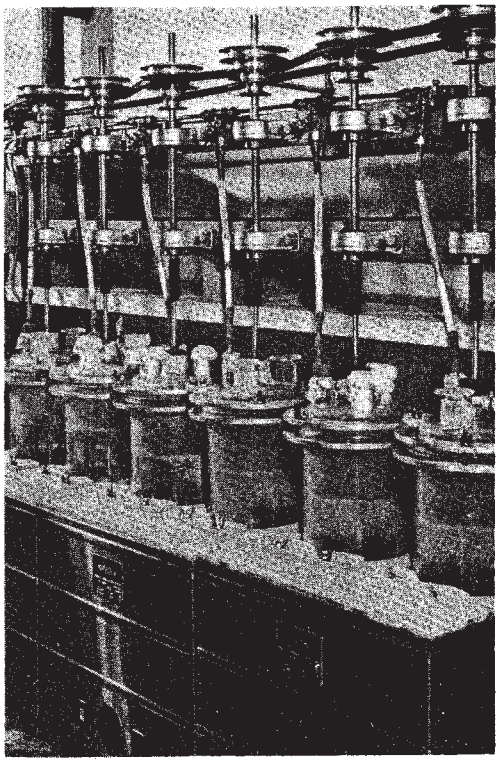

Fig. 3. 4-L fermentors used to study oxidation of n-alkanes by microorganisms.

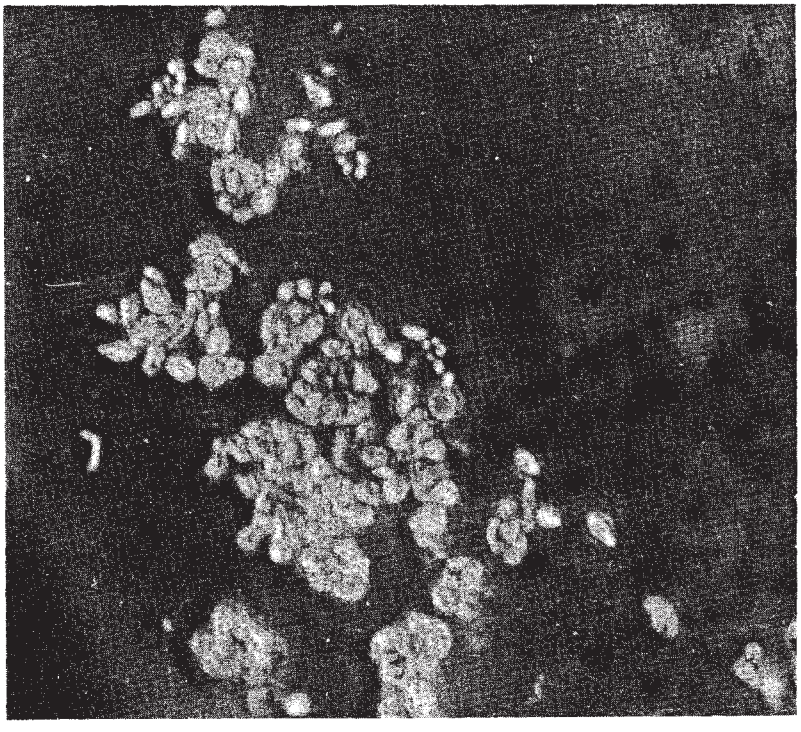

Fig. 4. Cells of a *Nocardia* sp. containing 78% lipid. Grown on n-octadecane with asparagine as N source. Phase-contrast photomicrograph.

ratio of n-octadecane to cells was 20:1 or greater no growth occurred. Under similar circumstances a ratio of 7:1 yielded excellent growth. Thus it was necessary when starting with a small inoculum to add small quantities of substrate early in the fermentation. Although this point was not specifically tested for the yeast in the fermentors, tests in 250-ml indented shake systems indicated that 5% octadecane had no adverse effects. The probable cause of this difference can be observed microscopically. The nocardia cells become so firmly adsorbed to the substrate that cells are not visible under phase contrast unless a small amount of some dye such as rose bengal is first added to the suspension. The yeast cells are readily seen without such treatment and do not appear to be adsorbed very firmly to the substrate. Evidently in the instance of high substrate-to-cell ratio the nocardia cells are so completely surrounded by substrate that other nutrients are not available. This adsorption difference between the nocardia and yeast is also observed when the cells are centrifuged and excess substrate remains. Most of the yeast cells are thrown down while the nocardia cells remain trapped in the floating oil layer and have to be recovered by washing with petroleum ether. A schedule of addition of substrate which was quite successful for the nocardia and yeast are shown in Tables I and II.

Table 1. Oxidation of n-Octadecane by a Nocardia in 4-L Fermentors

Substrate added (g)*	80.00
Substrate remaining (g)	28.00
Cells produced (g)	49.80
Lipid (%)	59.8
Polymer (g)	2.8

*Urea and n-octadecane were added to 2 L of mineral-salt medium (0.6% Na_2HPO_4, 0.4% KH_2PO_4, 0.08% $MgSO_4$) in the following manner: initial, 0.6 g urea and 8.0 g n-octadecane; 17 hr, 0.3 and 8.0; 40 hr, 0.6 and 16.0; 48 hr, 1.5 and 48.0. Length of incubation 112 hr, temperature 30 C, impeller 1600 rpm, and air 200 ml/min.

Varying the type and concentration of nitrogen affected both the cell yields and lipid production. The results are similar to those observed for sugars for maximum lipid production by other microorganisms, i.e., high C:N ratios favor lipid synthesis, and low C:N ratios increase protein synthesis. The nocardia has been studied more thoroughly than the yeast and these effects are demonstrated for this microbe in Tables III and IV. Figure 4 is a photograph of the nocardia cells containing 78% lipid as the result of growing on n-octadecane and 0.1% asparagine.

Lowering the oxygen tension in the fermentors did not result in any changes in product formation by nocardia but it markedly changed the composition and yield of cells. The results of this study are shown in Table V. It should be noted that the yield of cells decreases as the oxygen concentration is lowered. It is possible that there was an increase in a water-soluble polymer, later shown to be produced by this microorganism when growing on hydrocarbons. This polymer

Table 2. Oxidation of n-Octadecane by a Yeast in 4-L Fermentors

	0.2% NH_4NO_3	0.1% NH_4NO_3	0.1% NH_4NO_3
Substrate added (g)*	28.0	12.8	9.6
Substrate remaining (g)	10.1	1.4	0.4
Cells produced (g)	12.2	8.5	7.5
Lipid (%)	6.7	-	18.2
Liquor analyses			
Ether extract (g)	0.54	0.45	0.40
Volatile acids (meq)	2.5	0.0	0.0

*n-Octadecane added to 2 L of mineral-salts medium (0.01% Na_2CO_3, 0.001% $CaCl_2$, 0.002% $MnSO_4$, 0.001% $FeSO_4$, 0.3% Na_2HPO , 0.02% $MgSO_4$, 0.2% KH_2PO_4) in the following manner:

initial	4.0 g	-	1.6 g
2 hr	-	0.8 g	-
7	-	4.0	-
16	8.0	-	-
20	16.0	-	4.0
24	-	8.0	-
26	-	-	4.0
Length of incubation (hr)	96	96	41

Table 3. Effect of Nitrogen Sources on Nocardial Cell Yield and Lipid Formation

(Substrate, 824 mg n-octadecane)

Nitrogen Source	N in cpd. in %	n-Octadecane utilized in mg	Dried cell wt. in mg	Conversion to cells in %	Cell lipid in %	Final* pH
$(NH_4)_2SO_4$ (0.1%)	21	606	445	74	72	6.0
$(NH_4)_2SO_4$ (0.3%)	21	293	224	83	43	5.0
NH_4NO_3 (0.3%)	35	293	207	70	46	5.0
KNO_3 (0.1%)	14	394	252	64	70	8.0
KNO_3 (0.3%)	14	795	530	67	65	8.1
Urea (0.1%)	47	808	603	75	64	7.0
Urea (0.3%)	47	772	522	68	27	7.2
Asparagine (0.1%)	21	512	369	72	78	6.9
Asparagine (0.3%)	21	791	586	74	69	6.0

*The initial pH was buffered at neutrality with the 0.5% phosphate salts in the regular mineral-salts medium. Incubation, 144 hr at 30° C in 100 ml medium in 250 ml bottles agitated on gyratory shaker.

has not been identified but is produced in good yields under some circumstances. This was not known at the time of these experiments on oxygen concentration.

Table 4. Effect of Nitrogen Concentration on Oxidation of n-Octadecane by Nocardia in 4-L Fermentors*

Octadecane added (g)	32.000	80.000	100.000
Octadecane recovered (g)	11.620	29.181	33.030
Cells recovered (g)	13.800	43.286	57.394
Lipid in cells (%)	65.2	55.9	50.1
Liquor analyses			
Ether extract (g)	0.188	0.310	0.614
Carbon analysis (g)	-	3.290†	3.510†
Volatile acids (meq)	0.0	3.7	7.9
Nitrogen added (g)	0.42 $(NH_4)_2SO_4$	2.2 $(NH_4)SO_4$	3.5 Urea
Residual nitrogen	-	0.437	0.475

*Impeller speed 1725 rpm, pH 7.1, temperature 30 C, air flow 60 to 150 ml/min. Mineral medium had the following composition: 0.01% Na_2CO_3, 0.001% $CaCl_2$, 0.002% $MnSO_4$, 0.02% $MgSO_4$, 0.001% $FeSO_4$, 0.3% Na_2HPO_4, 0.2% KH_2PO_4. Incubation time 120 hr.
†Capsular slime or polymer.

Table 5. Oxidation of n-Octadecane by Nocardia Under Varying Oxygen Concentrations in 4-L Fermentors*

	20% O_2	10% O_2	2% O_2
Substrate added (g)	10.400	10.400	10.400
Substrate recovered (g)	0.293	0.363	4.707
Cells (g)	8.519	6.612	3.073
Lipid in cells (%)	56.0	73.0	21.0
Acetone-insoluble lipid in cells (%)	21.6	7.9	2.8
Liquor analyses			
Ether extract (g)	0.198	0.289	0.093
Volatile acids	0	0	0
Air Flow (ml/min)			
0 to 24 hr	100	100	100
24 to 48 hr	200	180†	260‡

*Physical conditions: 3 L of mineral medium of following composition: 0.01% Na_2CO_3, 0.001% $CaCl_2$, 0.002% $MnSO_4$, 0.02% $MgSO_4$, 0.001% $FeSO_4$, 0.075% Na_2HPO_4, 0.05% KH_2PO_4, 0.1% $(NH_4)_2SO_4$. Impeller speed 1725 rpm, temperature 30 C, pH 7.1.
†10% O_2 + 90% N_2.
‡2% O_2 + 98% N_2.

When the nocardia cells were extracted with chloroform-methanol (2 : 1) they were found to be very high in lipid in those systems containing hexadecane or octadecane as the substrate. This effect of length of the hydrocarbon chain on the lipid is shown in Table IV and VI. Glucose-grown nocardia contained from 16-28% lipid. This high lipid content peculiar to hexadecane- and octadecane-grown cells was due largely to waxes which make up to 40% of the lipid. The percentage of the total cell weight of these waxes is listed as the acetone-

Table 6. Oxidation of n-Hexane, n-Tridecane and n-Hexadecane by Nocardia in 4-L Fermentors*

	n-Hexane	n-Tridecane	n-Hexadecane
Substrate added (g)	55.000	21.000	40.196
Substrate remaining †	-	-	-
Cell (g)	4.399	7.52	17.800
Lipid in cells (%)	27.8	25.8	47.7
Acetone-insoluble lipid in cells (%)	2.8	3.4	18.2
Liquor analyses			
Ether extract (g)	0.177	- ‡	-
Volatile acids (meq)	1.585	-	-
Nitrogen source [$(NH_4)_2SO_4$] (%)	0.1	0.2	0.3
Length of incubation (hr)	120	72	72

*Physical conditions same as those given in Table IV.
†Due to losses from volatility it was not possible to determine this value.
‡Not determined.

insoluble fraction of the lipid. The isolation and identification of this fraction has been described by Raymond and Davis and Stewart *et al.* The nocardia produced cetyl myristate (14%) and cetyl palmitate (86%) from hexadecane; octadecyl palmitate (20%) and octadecyl stearate (80%) from octadecane. The identity of the alcohol portion of the wax from octadecane-grown cells was established by saponification and isolation of octadecanol. No waxes were found in the lipid of nocardia grown on glucose, hexane, or tridecane or in the yeast lipid from cells grown on octadecane. The function of these waxes other than as products of cellular storage is unkown, but they presumably pile up as a diversionary process to further oxidation of terminally oxidized intermediates. In the nocardia this pile-up evidently was restricted to C_{16} and C_{18} alkanes because the C_{16} and C_{18} fatty acids are more common to its lipid metabolism.

SUMMARY

Data have been presented which demonstrate the conversion of several n-alkanes into cells of a nocardia and a *Candida* species. The weight yield is very high because of the incorporation of oxygen. Products other than cells were not produced in any significant quantities. A capsular slime polymer was isolated from the nocardia cultures but was not identified.

The composition of the nocardia cells was affected by concentration of nitrogen compounds, oxygen, and the substrate.

As a result of this study it seems reasonable to conclude that the technology necessary for production of cells fron n-alkanes is not too different from that used today for nonhydrocarbon substrates. Further, the utilization of n-alkanes proceeds just as rapidly and with higher yields than are ordinarily realized with nonhydrocarbon substrates. The relatively inexpensive nature of some n-alkanes makes them attractive sources for industrial microbiological processes.

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