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Utilization of Aliphatic Hydrocarbons by Micro-organisms

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I. Introduction

Biological interest in hydrocarbons has expanded to such a degree in the past few years that it is no longer feasible to attempt a review on all phases of microbial hydrocarbon oxidations. In the following pages certain aspects of the oxidation and assimilation of the simple aliphatic alkanes and alk-1-enes, for the most part microbial, will be discussed. The authors have attempted to extend and update those portions of the

excellent reviews by van der Linden and Thijsse (1965) and McKenna and Kallio (1965) concerned with alkanes and alk-1-enes. Since methane represents a somewhat specialized case, it will receive only limited comment in this article.

II. Organisms

No attempt will be made to assemble a list of micro-organisms possessing the ability to oxidize aliphatic hydrocarbons. Information of this type has been tabulated by Beerstecher (1954) and Fühs (1961). From these and other reviews cited previously, the number of bacteria recorded as being "hydrocarbon-oxidizers" far exceeded the number of yeasts and filamentous fungi. The greater propensity for oxidation of aliphatic hydrocarbons by bacteria was more apparent than real since it simply reflected the lack of investigations using yeasts and filamentous fungi.

A cursory attempt will be made to review and update the information on the kinds of yeasts and molds implicated in aliphatic hydrocarbon oxidations. This is being done because later we will be concerned with reviewing recent reports on the catabolism of hydrocarbons by these two groups. This information will be included, along with the more extensive investigations with bacterial systems, in a discussion of microbial oxidation of *n*-alkanes and alk-1-enes.

A. Yeasts

1. n-*Alkanes*

Tausson (1939) first reported the assimilation of alkanes by members of the genera *Debaryomyces*, *Endomyces*, *Hansenula*, *Torulopsis* and *Monilia*. Alkane assimilation by *Candida lipolytica*, *Torulopsis colliculosa* and *Candida tropicalis* was indicated by the work of Just *et al.* (1951). Markovetz and Kallio (1964) presented a hydrocarbon assimilation pattern demonstrating that species belonging to the genera *Candida*, *Debaryomyces*, *Hansenula*, *Rhodotorula* and *Trichosporon* could grow at the expense of certain *n*-alkanes of even-numbered carbon atoms, 10–18. Utilization of *n*-alkanes of even-numbered carbon atoms (10–16) by *C. lipolytica* was indicated by Azoulay *et al.* (1964). Miller *et al.* (1964) demonstrated high yields of cells when *Candida intermedia* was grown on alkanes of 12–18 carbons in mineral salts–hydrocarbon medium. Isolation and screening of 56 strains of yeasts capable of utilizing kerosene were described by Komogata *et al.* (1964); most of the organisms readily assimilated long-chain alkanes from 9–16 carbon atoms and, after taxonomic studies, most of the yeasts were classified as species of the genus *Candida*.

A soil isolate, identified as being a species of *Pichia*, was reported by

Arima *et al.* (1965) to grow on a series of *n*-alkanes from C_9 to C_{13}. *Candida rigida*, *Mycotorula japonica*, *Candida utilis*, *Cryptococcus neoformans*, *Hansenula subpelliculosa*, *Rhodotorula glutinis* and *Saccharomyces chevalieri* were observed to grow in a defined medium at the expense of kerosene. Further, *M. japonica* assimilated *n*-alkanes of 8–16 carbon atoms (Aida and Yamaguchi, 1966). Ten species of the genus *Candida* were found to exhibit varying assimilation patterns when tested on five *n*-alkanes of carbon numbers ranging from 10 through 16 (Otsuka *et al.*, 1966). Some 1200 yeast strains representing 244 species belonging to 10 genera were tested by Scheda and Bos (1966) for assimilation of *n*-decane and *n*-hexadecane. Many strains from the genera *Pichia*, *Debaryomyces*, *Torulopsis* and *Candida* were detected which utilized the two alkanes.

Klug and Markovetz (1967a) checked 55 strains of *Candida* representing 36 species for their ability to utilize *n*-alkanes of 9–18 carbon atoms. A high percentage of these organisms exhibited the ability to assimilate some member of the hydrocarbon series employed. Lowery *et al.* (1968) found that of 66 yeasts tested only 11 were capable of growing on a liquid medium containing *n*-alkanes as the sole carbon source. These 11 organisms represented three genera—*Candida*, *Debaryomyces* and *Rhodotorula*. Utilization of *n*-alkanes from gas-oil samples inoculated with a mixed culture consisting of *C. intermedia* snd *C. lipolytica* was reported by Miller and Johnson (1966). Dostálek *et al.* (1968), in an extensive study of deparaffination of gas oil by *C. lipolytica*, found that while there was a linear dependence between cell yield and consumption of *n*-alkanes the decrease in the freezing point of gas oil had a distinct course depending on the selective order of utilization of the different chain-length *n*-alkanes.

2. *Alk*-1-*enes*

Bruyn (1954), Stewart *et al.* (1960) and Ishikura and Foster (1961) found that hexadec-1-ene was assimilated by *C. lipolytica*. Representatives of the genera *Candida*, *Debaryomyces*, *Hansenula*, *Rhodotorula* and *Trichosporon* were reported to grow at the expense of at least one member of a series of even-numbered alk-1-enes (10–18) (Markovetz and Kallio, 1964). *Candida tropicalis* was able to assimilate tetradec-1-ene, hexadec-1-ene and octadec-1-ene from a series of alk-1-enes, as reported by Takahashi *et al.* (1965). Klug and Markovetz (1967a) surveyed species of the genus *Candida* for their growth response to alk-1-enes of 10, 12, 14, 16 and 18 carbon atoms. General speaking, over one-half of the organisms were able to utilize one or more alk-1-enes.

Table 1 lists the genera of yeasts reported to assimilate *n*-alkanes and alk-1-enes.

TABLE 1. Genera of Yeasts Reported to Utilize Aliphatic Hydrocarbons for Growth

n-Alkanes	Alk-1-enes
Candida	*Candida*
Cryptococcus	*Debaryomyces*
Endomyces	*Hansenula*
Hansenula	*Rhodotorula*
Mycotorula	
Pichia	
Rhodotorula	
Torulopsis	
Trichosporon	
Saccharomyces	

B. FILAMENTOUS FUNGI

1. n-*Alkanes*

The observation by Miyoshi (1895) that *Botrytis cinerea* would attack paraffin presumably provided the beginning of hydrocarbon microbiology. Tausson (1925) showed that *Aspergillus niger* could utilize paraffin wax as the sole source of carbon for growth, and Hopkins and Chibnall (1932) found that *Aspergillus versicolor* grew on hydrocarbons from 23 through 34 carbons in length.

It should be mentioned, simply because of the organisms concerned, that jet fuel has been reported to support growth of the filamentous fungi *Cladosporium* (Prince, 1961) and *Hormodendron* (Krynitsky and McClaren, 1962; Edmonds and Cooney, 1967). *Fusarium moniliforme* isolated from diesel fuel by Flippin *et al.* (1964) was found to grow on *n*-decane and *n*-dodecane. Koval *et al.* (1966) reported that *n*-alkanes of diesel fuel were utilized preferentially as the source of carbon for strains of *Mucor*, *Cunninghamella*, *Penicillium*, *Trichoderma* and *Fusarium*.

A direct soil-baiting method utilizing a paraffin rod was used successfully by Rynearson and Peterson (1965) to isolate paraffinolytic fungi. Only 20 of the 31 cultures isolated from the rod grew when inoculated into a medium with paraffin as the sole carbon source. These fungi belonged to the genera *Aspergillus*, *Chaetomium*, *Penicillium*, *Syncephalastrum* and *Cunninghamella*. Paraffin utilization was also demonstrated with 3 out of 10 thermophilic fungi (Fergus, 1966). Krause and Lange (1965), studying the effect of addition of various water-insoluble fatty compounds to soil, showed that various *n*-alkanes (C_{11}–C_{20}, C_{22}, C_{23} C_{28}, C_{32}) would support vigorous growth of three species of *Fusarium*.

Previously, soil strains of *Fusarium*, as well as *Acremonium*, had been

obtained in ethane and propane enrichments (Dworkin and Foster, 1958; Kester, 1961). A number of molds were assayed by Kester, as reported by Foster (1962), for their ability to use *n*-tridecane as sole carbon source. The following organisms were found to possess this capacity: *Aspergillus alliaceus*, *Cephalosporium roseum*, *Colletotrichum atramentarium*, *Acremonium patronii*, *Fusarium bulbigenum* and *Monilia bonordenii*. A species of *Botrytis* was able to utilize *n*-nonane and *n*-decane for growth (Yamada and Torigoe, 1966). Tanaka *et al.* (1968) observed that strains of *Aspergillus*, *Penicillium*, *Fusarium* and *Cladosporium* grew at the expense of a hydrocarbon mixture composed of *n*-undecane, *n*-dodecane, *n*-tridecane and *n*-tetradecane. A similar mixture of *n*-alkanes lacking *n*-undecane served as sole carbon source for species of *Helicostylum*, *Rhizopus*, *Aspergillus*, *Penicillium* and *Fusarium* (Ratledge, 1968). Lowery *et al.* (1968) surveyed a series of molds on *n*-alkanes of C_1–C_{14} plus C_{16}. Genera able to assimilate some members of the series were *Aspergillus*, *Cephalosporium*, *Dematium*, *Epicoccum*, *Fusarium*, *Gliocladium*, *Graphium*, *Mucor*, *Paecilomyces*, *Penicillium* and *Trichoderma*. A wide range of fungi were tested by Nyns *et al.* (1968a, b) for their ability to assimilate a series of hydrocarbons which included *n*-alkanes, aromatic hydrocarbons and petroleum fractions. Three genera were particularly endowed with hydrocarbon-assimilating strains—*Fusarium*, *Penicillium* and *Aspergillus*. Markovetz *et al.* (1968) checked 53 strains of filamentous fungi representing 32 species on five even-numbered *n*-alkanes of 10–18 carbon atoms. Species belonging to the genera *Aspergillus*, *Cephalosporium*, *Fusarium*, *Helminthosporium*, and *Spicaria* grew better than the other organisms tested, with the exception of strains of *Cunninghamella* which exhibited profuse growth on all the substrates.

2. *Alk*-1-*enes*

Octadec-1-ene and squalene have been reported to support the growth of three species of *Fusarium* (Krause and Lange, 1965). In a survey of filamentous fungi grown on even-numbered alk-1-enes (C_{10}–C_{18}), Markovetz *et al.* (1968) observed definite growth with the same species which utilized *n*-alkanes (*see n*-Alkanes, p. 3). A tabulation of the genera of fungi reported to utilize aliphatic hydrocarbons is given in Table 2.

C. Bacteria

Probably the only new "type" of bacterium not previously recorded in the annals of hydrocarbon-oxidizing bacteria is the thermophilic bacillus. Klug and Markovetz (1967c) isolated a thermophilic bacillus which utilized *n*-tetradecane as its carbon source. Another thermophilic

TABLE 2. Genera of Filamentous Fungi Reported to Utilize Aliphatic Hydrocarbons for Growth

n-Alkanes	Alk-1-enes
Absidia	*Aspergillus*
Acremonium	*Cephalosporium*
Aspergillus	*Cunninghamella*
Botrytis	*Fusarium*
Cephalosporium	*Helminthosporium*
Chaetomium	*Spicaria*
Chloridium	
Cladosporium	
Colletotrichum	
Cunninghamella	
Dematium	
Epicoccum	
Fusarium	
Gliocladium	
Graphium	
Helicostylum	
Helminthosporium	
Monilia	
Mucor	
Oidiodendron	
Paecilomyces	
Penicillium	
Rhizopus	
Scolecobasidium	
Spicaria	
Syncephalastrum	
Trichoderma	

bacillus was isolated on long chain *n*-alkanes by Mateles *et al.* (1967), and was found to grow on *n*-alkanes from 12 through 20 carbon atoms.

III. Ecological Studies

Ecological aspects were dealt with in a review by Fühs (1961) and certain ecological principles with reference to micro-organisms utilizing hydrocarbons have been applied to the prospecting for petroleum. A review on this topic was compiled by Brisbane and Ladd (1965). Jones and Edington (1968) conducted an ecological survey of the microflora of soil and underlying shale and, in all samples taken, hydrocarbon-oxidizing organisms were found. Perry and Scheld (1968) isolated organisms of numerous genera from soil on non-hydrocarbon substrates and checked

them for their ability to grow on hydrocarbon substrates. Generally, these studies tend to verify that the time-honored practice of using soil as a source of organisms capable of oxidizing hydrocarbons still has merit.

IV. Growth as Indicator of Substrate Specificity

Attempts to explain the significance of substrate specificity of *n*-alkanes and alk-1-enes as related to growth studies may be an exercise relegated to futility. Nonetheless, certain growth studies will be mentioned together with possible explanations of the results.

A. Bacteria

Foster (1962), generalizing on the ability of *n*-alkanes to serve as growth substrates, indicated that *n*-alkanes containing 10–18 carbons were attacked with the greatest frequency and rapidity (for older references *see* Beerstecher, 1966). Reference was made to data obtained by Lukins (1962) for 21 strains of *Mycobacterium* grown on *n*-alkanes of 1 through 18 carbons. Three strains utilized all the substrates from propane through hexadecane. Most of the cultures exhibited a preference for the long chain compounds commencing with 9–11 carbons. *n*-Hexane was found to be toxic to the growth which *Mycobacterium smegmatis* normally attained on other alkanes. This toxicity was proposed as a possible explanation for the refractoriness of intermediate chain length alkanes (C_5–C_9) to support growth. However, it was noted that *n*-hexane was utilized as a growth substrate by other mycobacteria used in the study. Short chain *n*-alkanes were not toxic to a strain of *Corynebacterium* which grew at the expense of a series of C_3—C_{18}, excluding C_{15} and C_{17}. Of the alk-1-enes tested, this *Corynebacterium* strain grew on dodec-1-ene, tetradec-1-ene, hexadec-1-ene and octadec-1-ene. Olefins not supporting growth were ethylene, propylene, *cis*- and *trans*-but-2-ene (Kester and Foster, 1963). Referring to unpublished work of T. Ishikura, Foster (1962) reported that intermediate chain length *n*-alkanes and alkenes (C_5–C_9?) were inhibitory to a number of bacteria, yeasts and fungi growing on non-hydrocarbon media.

Several interesting points appeared in a paper by Finnerty *et al.* (1962) on alkane-oxidizing micrococci. Growth responses to alkanes from methane through *n*-eicosane were checked, and generally growth was absent when alkanes shorter than the C_{10}—C_{12} range served as the carbon source. One strain, S-12.2, isolated from dodec-1-ene, grew only on C_8 through C_{11}. By lowering the growth temperature from 25° to 20°, one strain which utilized C_{11} as the shortest *n*-alkane could now grow at the expense of C_{10}, and the lower limit of growth response with another

strain was extended from C_{12} down to C_{10}. The authors suggested that lower vapor pressures of the *n*-alkanes at the lower temperature indicated that physical characteristics of the hydrocarbon as well as the metabolic potentials of the organism must be considered in assessing the utilizability of hydrocarbons as carbon sources for growth.

Based on these data from Kallio's laboratory, it seems reasonable to speculate that many micro-organisms which utilize the long-chain hydrocarbons to the exclusion of the shorter members of a series find that these shorter members are "toxic" because of their greater solubility and therefore their higher concentration. By lowering the temperature, and by extension the solubility of the hydrocarbon, the "toxicity" would be lessened or eliminated. Indeed, Johnson (1964) stated that the number of organisms growing on *n*-hexane increased if the hydrocarbon concentration in the medium was kept below the saturation level. Commenting on the micrococcus mentioned above, which did not grow on alkanes longer than C_{11}, Johnson (1964) broached the subject of solubility. By extrapolation of the solubility data of McAuliffe (1963) for short chain *n*-alkanes, Johnson suggested that the concentration of *n*-decane and higher hydrocarbons in an aqueous medium would be extremely low. This could explain why some organisms do not grow on longer chain hydrocarbons. Another hypothesis to account for growth on longer chain substrates suggested that the micro-organism would attach to a droplet of alkane with the long alkane chain becoming incorporated into the phospholipid micelle of the cell membrane, and that a lyophobic pathway exists from outside the cell membrane to the enzymic site responsible for initiating the attack on the substrate (Johnson, 1964).

More recent data from McAuliffe (1969), Baker (1967), Peake and Hodgson (1966) and Franks (1966) indicate that extrapolation of data from short chain hydrocarbons showing decreased solubility as a function of increased chain length is not valid for longer chain *n*-alkanes ($>C_{10}$). Beginning with C_{11}–C_{12}, *n*-alkanes are "accommodated" in much higher concentration than anticipated from extrapolation of solubility measurements. Apparently the change from a state of true solubility (molecular dispersion) to accommodation (aggregation) begins with C_{11}. McAuliffe's plot (1969) of his data along with the data from the other workers listed above, indicated that C_{12}–C_{18} are "accommodated" in water at approximately the same concentration.

Mention of a paper by Drost-Hansen (1965) dealing with the physical structure of water interfaces seems appropriate at this point. In considering water-hydrocarbon interfaces, he proposes that a considerable "structuring" exists consisting of clusters or "cages" of water molecules which may serve as "binding sites" for the molecules of hydrocarbon at

the interface. Further, his data indicate that the interfacial tension of water and *n*-hexane show a complex behavior in the vicinity of 30°. Therefore it seems reasonable to assume that the inability of an organism to grow on a long-chain hydrocarbon is probably attributable to a metabolic deficiency and not to the lack of "dissolved" or "accommodated" substrate. In the case of short-chain hydrocarbons the concentration of dissolved substrate may be high enough to be "toxic", perhaps as suggested by others, by an effect on the cytoplasmic membrane. However, it seems strange that *n*-hexane, for example, would disrupt the integrity of the cytoplasmic membrane of one mycobacterium while serving as a growth substrate for another mycobacterium, presumably under the same experimental conditions.

Two strains of micrococci were reported to grow at the expense of hexadec-1-ene (Stewart *et al.*, 1960) and Makula and Finnerty (1968) grew *Micrococcus cerificans* on each of the following alk-1-enes as sole carbon source: dodec-1-ene, tetradec-1-ene, pentadec-1-ene, hexadec-1-ene and octadec-1-ene. A micrococcus strain (S-12.2), isolated by enrichment on dodec-1-ene by Finnerty *et al.* (1962), would not grow on the corresponding *n*-alkane, a point which will be discussed (p. 30). For older citations on alkene oxidation, refer to Beerstecher (1954).

Before leaving the bacteria some mention should be made of the growth responses of pseudomonads to hydrocarbons since members of the genus *Pseudomonas* have been used extensively in studies concerned with the catabolism of aliphatic hydrocarbons. Konovaltschikoff-Mazoyer and Senez (1956) found 11 strains of *Pseudomonas* capable of growth at the expense of *n*-alkanes (C_7–C_{16}). Thijsse and Zwilling-de Vries (1959) in a comparative study of branched and straight-chain alkanes reported that *n*-pentane through *n*-hexadecane were used for growth by a pseudomonad. Although no growth survey related to alk-1-enes has been published, *Pseudomonas aeruginosa* is known to utilize oct-1-ene (Huybregtse and van der Linden, 1964) and tetradec-1-ene (Markovetz *et al.*, 1967) as growth substrates.

B. Yeasts

From statements in the literature one obtains the impression, which as it turns out may be correct, that yeasts and filamentous fungi more readily utilize long-chain rather than short-chain hydrocarbons. Only in the past several years have studies appeared on substrate specificity as related to growth, and only a few of these investigations employed a comprehensive series of substrates.

In an experiment initiated to select a yeast which would readily utilize long chain *n*-alkanes and alk-1-enes, and thereby presumably be

a good organism for a study of the catabolic degradation of these substrates, some 30 different yeasts were assayed to determine their ability to assimilate hydrocarbons (Markovetz and Kallio, 1964). Genera, from which representatives were found to assimilate some member of the series tested, are noted in the section on ORGANISMS (p. 4). Substrates used were even-numbered *n*-alkanes and alk-1-enes of 10 through 18 carbons. The 14-carbon member of each series was utilized most frequently. As a group, the alk-1-enes were assimilated to a somewhat lesser degree. It was suggested that hydrocarbon assimilation tests may have potential value in delineation of species in certain genera. It was also noted that the hydrocarbon–air interface in agar slants frequently acted as a growth demarcator in that growth might occur above or below the surface of the hydrocarbons, sometimes depending on whether the substrate was an alkane or an alk-1-ene. The physicochemical and biochemical implications of cells growing essentially in an atmosphere containing substrate as opposed to cells actually immersed in the substrate were not pursued.

Species of the genus *Candida* were used in a number of growth studies. *C. lipolytica* was unable to utilize shorter *n*-alkanes (C_5–C_9) but it did assimilate *n*-decane, *n*-dodecane, *n*-tetradecane and *n*-hexadecane. Cell yields increased with chain length (Azoulay *et al.*, 1964). Of the *Candida* species checked on *n*-alkanes by Komogata *et al.* (1964), most of the organisms which grew utilized *n*-alkanes in the carbon range of 9 through 16, but not in the range of *n*-pentane through *n*-octane. *n*-Decane and *n*-tetradecane appeared to elicit the strongest assimilatory responses. Miller *et al.* (1964) demonstrated that the generation time for *C. intermedia* decreased as the chain length of the *n*-alkane increased from C_{12} through C_{18} (minus *n*-tridecane). Takahashi *et al.* (1965) checked *C. tropicalis* against a series of *n*-alkanes and alk-1-enes. The *n*-alkane series ranged from *n*-pentane through *n*-eicosane and growth was observed in the C_{12}–C_{20} range with the best cell yields arising in the C_{15}–C_{19} range. Even-numbered alk-1-enes from hex-1-ene through octadec-1-ene and including hept-1-ene were also employed. Alk-1-enes of 14, 16, and 18 carbons supported growth to approximately the same degree. Cell yields obtained from these alk-1-enes were approximately the same as those obtained from the corresponding *n*-alkane of the same chain length. *n*-Nonane through *n*-octadecane were utilized by *C. petrophillum* with cell yields increasing with increased chain length to a maximum which leveled off in the range of C_{14}–C_{17}, with a drop in yield occurring on *n*-octadecane (Mizuno *et al.*, 1966). Ten species of *Candida* exhibited varying assimilation patterns on *n*-decane through *n*-hexadecane (even carbons only) as reported by Otsuka *et al.* (1966). In regard to cell yield, three species gave the greatest response at the expense of