Biodegradation of crude oil and individual hydrocarbons by microorganisms

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BIODEGRADATION OF CRUDE OIL AND INDIVIDUAL HYDROCARBONS BY MICROORGANISMS

by

ASHRAF SAMIR ABDEL-AZIZ IBRAHIM
B.Sc. (Kuwait University)

A DOCTORAL THESIS SUBMITTED IN PARTIAL FULFILMENT OF THE REQUIREMENTS FOR THE AWARD OF

DOCTOR OF PHILOSOPHY
OF LOUGHBOROUGH UNIVERSITY OF TECHNOLOGY

June 1991

SUPERVISORS:  R.J. STRETTON, Ph.D.
               N.A. SORKHOH, Ph.D.
               M.A. GHANNOUM, Ph.D.

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To

MY MOTHER
ACKNOWLEDGMENTS

It is with great pleasure that I sincerely acknowledge the advice, encouragement and guidance given by my supervisors Dr. R.J. Stretton of Loughborough University of Technology and Dr. N.A. Sorkhoh and Prof. M.A. Ghannoum of Kuwait University, throughout this work.

For advice and assistance during my work on lipidology, I gratefully acknowledge Prof. S.S. Radwan of Botany and Microbiology Department at Kuwait University. Thanks are due to Dr. J. Mulder of Botany and Microbiology Department, at Kuwait University for his valuable help in fungal identification.

My thanks go to Mr. J. Greenfield of Chemistry Department of Loughborough University of Technology for his patience and skilful operation of the Mass spectrometer. I am also grateful to all in the electron Microscope Unit at Kuwait University for their technical help and otherwise. The photographic assistance of Mr. S. Rajendra is also greatly appreciated. I would like to thank all my research colleagues for useful discussion, help and for the enjoyable time I had with them.

Finally I would like to express my extreme gratitude to my family for their encouragement and moral support, especially to my brother Mr. Ayman Ibrahim for his computer assistance, and typing this manuscript along with my sister Mrs. Randa Ibrahim.

A.S. Ibrahim
June 1991
SUMMARY

Samples, collected from Kuwait were screened for microorganisms capable of oil degradation. A wide range of bacteria and fungi were able to degrade oil. The bacterial and fungal isolates differed in their ability to degrade crude oil. *Rhodococcus* isolates were more active than fungi in n-alkane biodegradation. Fungi also utilised one or more of the aromatic hydrocarbons studied while bacteria failed to do so.

*Rhodococcus rhodechrous* KUCC 8801 and 8802 were grown in an inorganic medium containing either glucose or dodecane as sole sources of carbon and their total lipids extracted and analysed. Dodecane-grown cells contained more total lipids than glucose-grown cells, these cells had increased levels of sterols, monoacylglycerols, and phosphatidylcholines (PC). Sterols and PC were unequivocally identified in this species.

*Candida albicans* KTCC 89062 grew adequately on n-alkanes with only 12 to 20 carbon chains but not on aromatic hydrocarbons. This isolate grew on glucose better than on any of the alkanes. Alkane-grown cells contained higher proportions of total lipids than glucose-grown cells, and the total lipid content was directly proportional to the alkane chain length. The fatty acid profiles suggested that they are derived through de novo synthesis. The sterol content also increased in alkane-grown cells; the highest level was with dodecane as substrate and progressively lower sterol levels were obtained as the carbon chain length increased. Ergosterol supplementation of the chemically defined medium showed an increase in the uptake of dodecane by cells grown on such medium. The increase in the
concentration of ergosterol supplementation resulted in an increase in dodecane uptake.

Densitometry studies of C. albicans, revealed that phospholipid:sterol ratio decreased when the cells were grown on n-alkanes as compared with glucose-grown cells. The ratio of unsaturated:saturated fatty acids was higher in n-alkane than glucose-grown cells and decreased progressively from C_{12} to C_{20} as substrates.

Growth of R. rhodochrous and C. albicans strains on alkanes but not on glucose was associated with pseudohyphal formation as shown by electron microscopy.
## ABBREVIATIONS

A. Abbreviations Common in Biochemical and Microbiological Research Literature.

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>AMP</td>
<td>Adenosine 5-monophosphate</td>
</tr>
<tr>
<td>ATP</td>
<td>Adenosine 5-triphosphate</td>
</tr>
<tr>
<td>CDM</td>
<td>Chemically defined medium</td>
</tr>
<tr>
<td>CL</td>
<td>Cardiolipin</td>
</tr>
<tr>
<td>COA, COA-SH, acyl-COA</td>
<td>Coenzyme A and its acyl derivative</td>
</tr>
<tr>
<td>Br-C</td>
<td>Branched fatty acid</td>
</tr>
<tr>
<td>DPG</td>
<td>Diphosphatidylglycerol</td>
</tr>
<tr>
<td>EDTA</td>
<td>Ethylenediaminetetra-acetic acid</td>
</tr>
<tr>
<td>FFA</td>
<td>Free fatty acids</td>
</tr>
<tr>
<td>FAD, FADH₂</td>
<td>Flavin adenine dinucleotide and its reduced form</td>
</tr>
<tr>
<td>FMN, FMNH₂</td>
<td>Flavin mononucleotide and its reduced form</td>
</tr>
<tr>
<td>LPC</td>
<td>Lysophosphatidylcholine</td>
</tr>
<tr>
<td>NAD⁺, NADH</td>
<td>Nicotinamide adenine dinucleotide (diphosphopyridine nucleotide) and its reduced form</td>
</tr>
<tr>
<td>NADP⁺, NADPH</td>
<td>Nicotinamide adenine dinucleotide phosphate (triphosphopyridine nucleotide) and its reduced form</td>
</tr>
<tr>
<td>PA</td>
<td>Phosphatidic acid</td>
</tr>
<tr>
<td>PC</td>
<td>Phosphatidylcholine</td>
</tr>
<tr>
<td>PE</td>
<td>Phosphatidylethanolamine</td>
</tr>
<tr>
<td>PG</td>
<td>Phosphatidylglycerol</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
<tr>
<td>--------------</td>
<td>------------------------------------</td>
</tr>
<tr>
<td>PGP</td>
<td>Phosphatidylglycerol-phosphate</td>
</tr>
<tr>
<td>PI</td>
<td>Phosphatidylinositol</td>
</tr>
<tr>
<td>PPI</td>
<td>Inorganic pyrophosphate</td>
</tr>
<tr>
<td>PS</td>
<td>Phosphatidylserine</td>
</tr>
<tr>
<td>TCA cycle</td>
<td>Tricarboxylic acid cycle</td>
</tr>
<tr>
<td>TLC</td>
<td>Thin-layer chromatography</td>
</tr>
<tr>
<td>U.V</td>
<td>Ultraviolet</td>
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B. Unit Abbreviation and Prefixes and Physical Constants.

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<td>A</td>
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<td>cm</td>
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<td>Hour</td>
</tr>
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<td>l</td>
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<tr>
<td>lb</td>
<td>Pound</td>
</tr>
<tr>
<td>μl</td>
<td>Microliter</td>
</tr>
<tr>
<td>μm</td>
<td>Micrometer</td>
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<td>Minute</td>
</tr>
<tr>
<td>ml</td>
<td>Milliliter</td>
</tr>
<tr>
<td>mm</td>
<td>Millimeter</td>
</tr>
<tr>
<td>mol</td>
<td>Mole</td>
</tr>
<tr>
<td>p</td>
<td>Pico-molar</td>
</tr>
<tr>
<td>pH</td>
<td>$-\log[H^+]$; concentration in a solution</td>
</tr>
<tr>
<td>rpm</td>
<td>Revolution per minute</td>
</tr>
<tr>
<td>v/v</td>
<td>Volume/volume</td>
</tr>
<tr>
<td>w/v</td>
<td>Weight/volume</td>
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</table>
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GENERAL INTRODUCTION AND AIM OF THE WORK

The oil industry and various related processes, such as extraction and transport, pose a constant threat to the marine environment as a consequence of the huge influx of petroleum hydrocarbons into this ecosystem. The yearly influx of petroleum pollutants in the sea has been estimated to be between 6 and 10 million tons (Blumer et al., 1971; National Academy of Science, 1975). This problem is more acute in an oil producing area like the Arabian Gulf, where much of the world's oil is produced and large quantities, representing around 60% of the global marine transport of oil (British Petroleum Co., 1980) are transported through this shallow and enclosed sea (Hunter, 1982). Oil pollution in this region arises from local exploration, exploitation, refining and routine handling of petroleum at ports. Recently political unrest and military conflicts aggravated the problem of oil pollution in this area.

El Samra et al. (1986), showed that the north western region of the Arabian Gulf is heavily polluted, compared with other marine environments. These workers showed that the Arabian Gulf had a petroleum hydrocarbon concentration of 1.2-546 µg/ml compared with 0.4-66.8 µg/ml in the Gulf of Mexico (Marchand et al., 1982). Similar findings were reported by Sen Gupta and Kureishy (1981) who stated that the trade and tanker routes across the Arabian Sea were more contaminated than similar routes over the southern Bay of Bengal.

This situation makes the study of different aspects of petroleum degradation a prime concern to this region. However, little attention has so far been directed towards such studies in the Arabian Gulf.
The aim of this investigation was to conduct a survey of Kuwait soil and marine environment for microorganisms capable of oil degradation. It is hoped that a study of such environment, which has been naturally rich in oil since geological time, should lead to the isolation of potent degraders. Identification and characterisation of these isolates would then be performed, and selected isolated strains, efficient in oil degradation, compared for their hydrocarbon degrading abilities with isolates reported to be efficient oil degrades. Biochemical characterisation of promising isolates will be conducted as well.
Petroleum hydrocarbons in soil and sea water represent an important environmental pollution problem. These oil pollutants can occur as a result of massive accidental spills or constant pollution associated with human activities. Hydrocarbon microbiology had its beginnings in 1895 with the observation of Miyoshi (1895) that a common industrial fungus, *Botrytis cinerea*, would attack paraffins. However, until the early 1940's, reports on microbial attack of hydrocarbons were infrequent (Söhngen, 1906, 1913; Takahashi et al., 1965). Table 1 lists some highlights of hydrocarbon microbiology history from 1895 to 1965. Most workers believed that microbial oxidation was a specialised property of a relatively few soil-type organisms. In 1940 Mogilevskii, specifically suggested sampling soil for hydrocarbon-oxidising microorganisms as a means of oil prospecting (Mogilevskii, 1940). At about the same time, work began in earnest to study hydrocarbon-attacking microorganisms in the laboratory. By the early 1950's there was sufficient evidence that hydrocarbon oxidation was a widespread property of microorganisms consequently many workers began studying the mechanism of attack. Since the mid-1950's, a considerable number of papers have appeared on microbial attack of hydrocarbons and related subjects. The ability to break down hydrocarbons has been demonstrated in numerous species. One of the most important tasks of microbiological technology, for increasing the degradation of crude oil, is the isolation of microbial strains capable of degrading crude oil under the unfavourable conditions (Greshnykh et al., 1968; Rambeloarisoa et al., 1984; Berdichevskaya, 1989).
Table 1. Some highlights of hydrocarbon microbiology history (Humphrey, 1967).

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<th>Worker(s)</th>
<th>Comments</th>
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<td>1895</td>
<td>Miyoshi</td>
<td><em>Botrytis cinerea</em> attacks paraffin</td>
</tr>
<tr>
<td>1906</td>
<td>Söhngen</td>
<td>Methane consumption by soil enrichments</td>
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<td>1913</td>
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<td>Observations on hydrocarbon utilisation</td>
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<td>1928</td>
<td>Tausson</td>
<td>Bacterial oxidations of crude oil</td>
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<tr>
<td>1940</td>
<td>Mogilevskii</td>
<td>Use of bacteria for oil prospecting</td>
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<tr>
<td>1942</td>
<td>Johnson <em>et al.</em></td>
<td>Respiration studies on hydrocarbon utilisation</td>
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<td>Blau</td>
<td>Tests for indicating hydrocarbon-degrading microbes</td>
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<tr>
<td>1943</td>
<td>Hassler</td>
<td>1st U.S. Patent on microbial oil prospecting</td>
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<tr>
<td>1943</td>
<td>Strawinski and Stone</td>
<td>Early intensive work on hydrocarbon microbiology</td>
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<tr>
<td>1944</td>
<td>Novelli and ZoBell</td>
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<td>1947</td>
<td>Evans</td>
<td>Early intensive work on aromatic breakdown</td>
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<td>1950</td>
<td>Hayaiski and Hashimoto</td>
<td>Early intensive work on oxygenase</td>
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<td>1952</td>
<td>Davis</td>
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<td>Rapid n-alkane utilisation</td>
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<td>1963</td>
<td>Champagnat</td>
<td>British Patent for edible yeast from crude oil fractions</td>
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<td>1963</td>
<td>Hosler</td>
<td>Production of salicylic acid</td>
</tr>
<tr>
<td>1964</td>
<td>Davis <em>et al.</em></td>
<td>N₂ fixation of methane-oxidising bacteria</td>
</tr>
<tr>
<td>1965</td>
<td>Takahashi <em>et al.</em></td>
<td>Production of L-glutamic acid</td>
</tr>
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</table>
Many microorganisms have been isolated and tested for their ability to degrade crude oil and individual hydrocarbons. The number of bacteria recorded as being "hydrocarbon-oxidisers" far exceeded the number of yeasts and filamentous fungi.

**BACTERIA**

The ability to degrade petroleum hydrocarbons is not restricted to a few microbial genera; a wide variety of microorganisms have been shown to have this ability. ZoBell (1946) in his review noted that more than 100 species, representing 30 microbial genera, had been shown to be capable of utilising hydrocarbons. Since that review, the subject of petroleum microbiology has broadened to include various studies on hydrocarbon dissimilation. More than thirty years later, Bartha and Atlas (1977) listed 22 genera of bacteria, 1 algal genus, and 14 genera of fungi which had been demonstrated to contain members which utilise petroleum hydrocarbons, all of these microorganisms have been isolated from an aquatic environment. Based on frequency of isolation, the most important genera of bacteria for hydrocarbon utilisation in aquatic environments were *Pseudomonas*, *Achromobacter*, *Arthrobacter*, *Micrococcus*, *Acinetobacter*, *Nocardia*, *Corynebacterium*, *Vibrio*, *Brevibacterium*, *Flavobacterium* (Bartha & Atlas, 1977). Bacteria and Yeasts [*Candida*, *Rhodotorula* and *Sporobolomyces*] appear to be the prevalent hydrocarbon degraders in aquatic ecosystems.

Jensen (1975) studied the bacterial flora of soil after application of oily waste and found that the most important species of oil degraders belonged to the genera *Arthrobacter* and *Pseudomonas*. Cundell and Traxler (1974) studied 15 bacterial isolates from an asphaltic flow near a natural
seepage at Cape Simpson, Alaska. The isolates were psychrotrophic and utilised paraffinic, aromatic, and asphaltic petroleum components. The isolates belonged to the bacterial genera *Pseudomonas*, *Brevibacterium*, *Spirillum*, *Xanthomonas*, *Alcaligenes*, and *Arthrobacter*. Mulkins-Phillips and Stewart (1974) reported finding hydrocarbon-utilising bacteria of the genera *Nocardia*, *Pseudomonas*, *Flavobacterium*, *Vibrio* and *Achromobacter* in northwest Atlantic coastal waters and marine sediment.

Walker *et al.* (1976) isolated *Vibrio*, *Pseudomonas*, and *Acinetobacter* species from oil-contaminated sediment and *Pseudomonas* and coryneform species from oil-free sediment. They reported that microorganisms from the oil-free sediment produced greater quantities of polar compounds (asphalts) after degradation, whereas bacterial species isolated from the oil-contaminated sediment provided greater degradation of saturated and aromatic hydrocarbons. In another study, Walker *et al.* examined bacteria from water and sediment for their ability to degrade petroleum. Water samples contained a greater variety of bacterial species capable of degrading petroleum than sediment samples. Cultures from both samples contained *Pseudomonas* and *Acinetobacter* species. Bacteria isolated from water samples showed significantly greater degradation of two-, three-, four-, and five-ring cycloalkanes and mono-, di-, tri-, tetra-, and penta-aromatics compared with bacteria isolated from sediment samples (Walker *et al.*, 1975).

Several investigators have reported that both chemical composition of a crude oil and temperature had a selective influence on the degradability of crude oil and individual hydrocarbon components (Atlas, 1981). Cook and Westlake (1974) isolated, at 4°C, *Achromobacter*, *Alcaligenes*, *Flavobacterium*, and *Cytophaga* on a substrate of Prudhoe Bay crude oil: *Acinetobacter*, *Pseudomonas*, and unidentified
gram-negative cocci on a substrate of Atkinson Point Crude oil, *Flavobacterium, Cytophaga, Pseudomonas,* and *Xanthomonas* with Norman Wells crude oil as substrate; and *Alcaligenes* and *Pseudomonas* on Lost Horse crude oil. At 30°C, the major genera isolated on Prudhoe Bay crude oil were *Achromobacter, Arthrobacter* and *Pseudomonas;* on Atkinson Point crude oil, the major genera were *Achromobacter, Alcaligenes* and *Xanthomonas;* on Norman Wells crude oil, the major genera were *Acinetobacter, Arthrobacter, Xanthomonas,* and other gram-negative rods; and on Lost Horse crude oil, they were *Achromobacter, Acinetobacter,* and *Pseudomonas.*

Several thermophilic hydrocarbon-utilising bacteria have been isolated. Kvasnikov *et al.* (1971) described the physiological properties of gram-positive spore former, *Bacillus circulans* subspecies *thermophilus,* which utilised n-alkanes as sole source of carbon and energy. Klug and Markovetz (1967a) isolated a thermophilic bacillus which utilised n-tetradecane as its carbon source. Other strains belonging to the genus *Bacillus* and capable of growth on n-alkanes (from 12 through 20 carbon atoms) and paraffin at optimum temperatures of 55°C to 60°C have been described (Mateles *et al.,* 1967; Pozomogova, 1971; Pozomogova *et al.,* 1972). A hydrocarbon utilising, obligatory thermophilic bacterium was isolated by Phillips and Perry (1976). They proposed that this organism to be placed in the genus *Thermomicrobiurn* as a new species, to which they gave the name *Thermomicrobiurn fosteri* (Phillips & Perry, 1976). More recently, Loginova *et al.* (1981) have isolated an obligate thermophilic bacteria, *Bacillus stearothermophilus* and *Thermus ruber* from hot water springs of Kunashir Island (USSR) and were able to utilise paraffin. The possible existence of obligate hydrocarbon utilisers is intriguing, since the biochemical degradative pathways indicate hydrocarbon utilisers must also be capable of metabolising fatty acids and alcohols (Atlas, 1981).
Numerical taxonomy has been used to examine petroleum degrading bacteria. Austin et al. (1977) examined 99 strains of petroleum-degrading bacteria, isolated from Chesapeake Bay water and sediment, by numerical taxonomy procedures. Eighty-five percent of the petroleum-degrading bacteria examined in this study were defined at the 80 to 85% similarity level within 14 phenetic groups. The groups were identified as actinomycetes (mycelial forms, 4 clusters), coryneforms, Enterobacteriaceae, Klebsiella aerogenes, Micrococcus spp. (two clusters), Nocardia spp. (two clusters), Pseudomonas spp. (two clusters), and Sphaerotilus natans. These workers concluded that degradation of petroleum was accomplished by a diverse range of bacterial taxa. Of particular note was the finding that some enteric bacteria can utilise petroleum hydrocarbons, the suggestion has been made that some of these enteric bacteria may have acquired this ability through plasmid transfer (Austin et al., 1977).

The species composition of hydrocarbon-oxidising bacteria was studied in carbonaceous oil-bearing strata in Yarino-Kamenolozh and Shemeti deposits in the Perm Cis-Urals by Berdichevskaya (1982). A total of 43 cultures were isolated, which were classified as Mycobacterium, Micrococcus, Brevibacterium, Corynebacterium, Flavobacterium, Achromobacter and Pseudomonas. Metabolic activity was determined in Corynebacterium fascians (two strains), Mycobacterium rubrum (one strain), Pseudomonas mira (one strain) and Flavobacterium peregrinum (one strain) in stratal water varying in salinity. The hydrocarbon-oxidising coryneforms were shown to have a high halotolerance and determined the wide distribution of this group of microorganisms in highly mineralised stratal waters in oil deposits (Berdichevskaya, 1982). Iizuka and Komagata (1964, 1965) isolated the following genera from stratal water from oil deposits in Japan: Arthrobacter,
Brevibacterium, Micrococcus, Sarcina, Alcaligenes, Achromobacter and Pseudomonas. Hydrocarbon-oxidising bacteria from the genera Micrococcus, Brevibacterium, Corynebacterium, Mycobacterium, and Mycococcus, and also hydrocarbon-oxidising yeasts from the genera Candida and Rhodotorula were found in oil samples in the study of oil deposits in Lower Saxony (Wan Hisu-Yüan & Schwartz, 1961). Berdichevskaya et al. (1984) showed that the principle component of the hydrocarbon-oxidising association in the Perm Kama Valley oil fields is bacteria of the genus Rhodococcus. The overwhelming majority of isolated rhodococci belonged to the species Rhodococcus luteus, Rhodococcus maris and fewer belonged to Rhodococcus rhodochrous and Rhodococcus erythropolis.

Norenkova (1966) isolated 19 pure cultures of mycobacteria from subpetroleum water of the oil fields of Sakhalin. They were assigned to the following species: Mycobacterium mucosum (two strains), and one strain of the following: Mycobacterium globiforme, Mycobacterium lacticolum, Mycobacterium rubrum, and Mycobacterium luteum. The mycobacteria were shown to utilise petroleum of varying chemical composition as a carbon source (Norenkova, 1966).

Mycobacterium convolutum has been shown to grow on a wide range of both odd- and even-numbered carbon solid n-alkanes (Hallas & Vestal, 1978; Ascenzi & Vestal, 1979). They reported that growth rates and cell yields of this bacterium decreased as the substrate carbon number increased.

A mixed bacterial population was isolated from foams formed on the surface of a zone chronically polluted with hydrocarbon (Rambeloarisoa et al., 1984). The population was able to degrade crude oil very effectively in the presence of sea water supplemented with nitrogen, phosphorus
and iron. The bacterial strains were identified to genus level only as follows: *Acinetobacter* two strains, *Alcaligenes* two strains, *Flavobacterium* one strain, *Moraxella* one strain, *Micrococcus* one strain, and *Bacillus* one strain (Rambeloarisoa *et al.*, 1984). They reported that the percentage of hydrocarbon degradation by this mixed bacterial population was 81% at 30°C, pH 8, and partial oxygen pressure of 100%. After 12 days incubation, 92 and 83% of saturated and aromatic compounds (mono-, di- and triaromatics) were degraded, respectively, as well as 63% of polar products and 48.5% of asphaltenes.

Also the degradation of chlorinated aliphatic hydrocarbons by *Methylosinus trichosporium* (a methanotrophic bacterium) was studied by Oldenhuis *et al.* (1989).

Degradation of aromatic compounds by bacterial cultures has also received attention. Stucki and Alexander (1987) showed that strains of *Moraxella* sp., *Pseudomonas* sp., and *Flavobacterium* sp. isolated from sewage were able to grow on biphenyl. Another study made by Oldenhuis *et al.* (1989) revealed that several strains of *Pseudomonas* degraded one or more of benzene, toluene, o-, m- and p-xylene, chlorobenzene, o-dichlorobenzene and 1,3,5-trichlorobenzene in soil slurries.

A *Mycobacterium* sp. has been reported to be an efficient polycyclic aromatic hydrocarbon (PAH) degrader. This bacterium has the ability to mineralise, singly and as components in a mixture, of 2-methylnaphthalene, phenanthrene, pyrene, and benzo [OC] pyrene (Heitkamp & Cerniglia, 1989). Weissenfels *et al.* (1990) also isolated a bacterial mixed cultures able to degrade PAH phenanthrene, fluorene and fluoranthene from soil using conventional enrichment techniques. From these mixed cultures, three pure strains were isolated: *Pseudomonas paucimobilis*
degrading phenanthrene, *Pseudomonas vesicularis* degrading fluorene and *Alcaligenes denitrificans* degrading fluoranthene.

Table 2 is a listing of bacteria that can grow on aliphatic and aromatic hydrocarbons and is not necessarily a complete listing.
Table 2. Genera of the bacteria reported to utilise aliphatic and aromatic hydrocarbons.

<table>
<thead>
<tr>
<th>Aliphatic</th>
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<tr>
<td>Achromobacter</td>
<td>Achromobacter</td>
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<tr>
<td>Acinetobacter</td>
<td>Aeromonas</td>
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<tr>
<td>Actinomyces</td>
<td>Alcaligenes</td>
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<tr>
<td>Aeromonas</td>
<td>Arthrobacter</td>
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<tr>
<td>Alcaligenes</td>
<td>Beijerinckia</td>
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<tr>
<td>Arthrobacter</td>
<td>Corynebacterium</td>
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<tr>
<td>Bacillus</td>
<td>Flavobacterium</td>
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<tr>
<td>Beneckea</td>
<td>Moraxella</td>
</tr>
<tr>
<td>Brevibacterium</td>
<td>Mycobacterium</td>
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<tr>
<td>Corynebacterium</td>
<td>Nocardia</td>
</tr>
<tr>
<td>Flavobacterium</td>
<td>Pseudomonas</td>
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<tr>
<td>Methylobacter&lt;sup&gt;a&lt;/sup&gt;</td>
<td></td>
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<tr>
<td>Methylobacterium&lt;sup&gt;a&lt;/sup&gt;</td>
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<tr>
<td>Methylococcus&lt;sup&gt;a&lt;/sup&gt;</td>
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<tr>
<td>Methylocystis&lt;sup&gt;a&lt;/sup&gt;</td>
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<tr>
<td>Methylomonas&lt;sup&gt;a&lt;/sup&gt;</td>
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<tr>
<td>Methylosinus&lt;sup&gt;a&lt;/sup&gt;</td>
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<tr>
<td>Micrococcus</td>
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<tr>
<td>Micromonospora</td>
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<tr>
<td>Mycobacterium</td>
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<tr>
<td>Nocardia</td>
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<tr>
<td>Pseudomonas</td>
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<td>Rhodococcus</td>
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<td>Spirillum</td>
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<tr>
<td>Thermomicrobium</td>
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<td>Vibrio</td>
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<td>Xanthobacter</td>
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<td>Xanthomonas</td>
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</table>

<sup>a</sup> Utilise only methane as a sole carbon source.
YEASTS

Tausson (1939) first reported the assimilation of alkanes by members of the genera Debaryomyces, Endomyces, Hansenula, Torulopsis and Monilia. Just et al. (1951) observed alkane assimilation by Candida lipolytica, Torulopsis colliculosa and Candida tropicalis. In an experiment initiated to select a yeast which would readily utilise long chain n-alkanes and 1-alkenes, some 30 different yeasts were assayed to determine their ability to assimilate hydrocarbons (Markovetz & Kallio, 1964). The two scientists 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, C\textsubscript{10}-C\textsubscript{18}. Utilisation of n-alkanes of even-numbered carbon atoms by Candida lipolytica was indicated by Azoulay et al. (1964). Miller et al. (1964) reported a high yield of cells when Candida intermedia was grown on alkanes of C\textsubscript{12}-C\textsubscript{18} carbons in mineral salt-hydrocarbon medium. They demonstrated that the generation time for this Candida species decreased as the chain length of n-alkane increased from C\textsubscript{12} through C\textsubscript{18}. Isolation and screening of 56 strains of yeasts capable of utilising Kerosene were described by Komagata et al. (1964); most of the organisms readily assimilated long chain alkanes from C\textsubscript{9}-C\textsubscript{16} carbon atoms but not in the range of n-pentane through n-octane, and after taxonomic studies, most of the yeasts were classified as species of the genus Candida.

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 (Aida & 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 \( \text{C}_{10} \) through \( \text{C}_{16} \) (Otsuka et al., 1966). With regard to cell yield, three species gave the greatest response at the expense of n-decane, one on n-undecane, four on n-tetradecane and two on n-hexadecane (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 able to utilise the two alkanes.

Klug and Markovetz (1967b) checked 55 strains of Candida representing 36 species for their ability to utilise n-alkanes of \( \text{C}_{9}-\text{C}_{18} \) carbon atoms. A high percentage of these organisms exhibited the ability to assimilate some members of the hydrocarbon series employed. Lowery et al. (1968) reported that out 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. Miller and Johnson (1966) reported that a mixed culture consisting of Candida intermedia and Candida lipolytica could utilise n-alkanes from gas-oil samples. Pelechová et al. (1971) showed that six yeast strains of Candida, Pichia and Rhodotorula genera were cultivated on a mixture of \( \text{C}_{11}-\text{C}_{23} \) n-alkanes as a sole carbon source. Ahearn and co-workers have examined yeasts that can utilise hydrocarbons and have isolated strains of Candida, Rhodosporidium, Rhodotorula, Saccharomyces, Sporobolomyces and Trichosporon which are capable to degrade hydrocarbons (Ahearn et al., 1971; Cook et al., 1973). Thorpe and Ratledge (1972) showed that Candida 107 and Candida tropicalis grew well on individual n-alkanes from dodecane to hexadecane. Gill and Ratledge (1972) used Candida tropicalis, Saccharomyces carlsbergensis and Candida 107 and showed that the two species of Candida can utilise n-alkanes above \( \text{C}_{8} \) for growth and Saccharomyces
carlsbergensis cannot grow on any hydrocarbon. Like several other yeast strains, Candida maltosa has been reported by Blasig et al. (1988) to utilise long-chain n-alkanes mainly via a mono-terminal degradation pathway. In a growth survey of 30 different yeast on even-numbered C_{10}-C_{18} n-alkanes, it was noted that n-tetradecane was utilised most frequently (Britton, 1984). Oxidation of n-tetradecane by Candida parapsilosis adsorbed on different glass rings has been reported (El-Aassar et al., 1988). Moreover, Candida parapsilosis cells, immobilised on granular clay and aquifer sand, were able to degrade a mixture of n-alkanes (C_{12}-C_{18}) (Omar & Rehm, 1988). In the genus Candida, C_{11} and C_{18} n-alkanes appear to be assimilated most readily. Cell yields also increased with increasing chain length, with n-hexadecane giving the greatest conversion of substrates to cell mass (Britton, 1984). Shennan and Levi (1974) concluded that in yeasts there is an increase in the conversion of n-alkane to biomass and a decrease in the rate of oxidation with increasing chain-length from n-nonane.

Bruyn (1954), Stewart et al. (1960) and Ishikura and Foster (1961) found that 1-hexadecene was assimilated by Candida 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 1-alkenes (C_{10}-C_{18}) (Markovetz & Kallio, 1964). Candida tropicalis was able to assimilate 1-tetradecene, 1-hexadecene and 1-octadecene from a series of 1-alkenes, as reported by Takahashi et al. (1965). Klug and Markovetz (1967a) employed 55 strains representing 36 species of the genus Candida for their growth response to 1-alkenes of 10, 12, 14, 16 and 18 carbon atoms. A high percentage of the organisms exhibited an ability to assimilate some member of the series tested, therefore it appeared that this capacity is not limited to a few isolated species. They concluded that assimilatory patterns of this
type were of marginal taxonomic value (Klug & Markovetz, 1967b). It was also reported that several types of yeasts e.g. Candida lipolytica, Candida maltosa, Candida tropicalis, Candida guilliermondii and Debaryomyces hansenii were able to utilise branched chain and aromatic hydrocarbons as growth substrates (Crow et al., 1980; Cerniglia & Crow, 1981). Table 3 lists the most important genera of yeast that reportedly contain hydrocarbon-oxidising species [for a review of hydrocarbon assimilating yeasts see Shennan and Levi (1974)].

FILAMENTOUS FUNGI

As have mentioned earlier in this chapter, the observation by Miyoshi (1895) that Botrytis cinerea would attack paraffin presumably provided the start of hydrocarbon microbiology. Tausson (1925) showed that Aspergillus niger could utilise paraffin wax as the sole source of carbon for growth, while Hopkins and Chibnall (1932) reported that Aspergillus versicolor grew on hydrocarbons from C_{23} through C_{34} carbons in length.

For the past decade the growth of microorganisms in jet fuel, resulting in the formation of biological sludge and the corrosion of fuel tanks, caused serious concern in aviation industry. It is now generally believed that jet fuel supports growth of the filamentous fungi Cladosporium (Hendey, 1964; Darby et al., 1968; Parbery, 1971; Teh & Lee, 1973) and Hormodendron (Krynitsky & McLaren, 1962; Edmonds & Cooney, 1967).
Table 3. Genera of yeasts reported to utilise aliphatic and aromatic hydrocarbons.

<table>
<thead>
<tr>
<th>Aliphatic</th>
<th>Aromatic</th>
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<td>Candida</td>
<td>Candida</td>
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<tr>
<td>Cryptococcus</td>
<td>Debaryomyces</td>
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<td>Debaryomyces</td>
<td>Saccharomyces</td>
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<td>Endomyces</td>
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<td>Hansenula</td>
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<td>Mycotorula</td>
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<tr>
<td>Pichia</td>
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<tr>
<td>Rhodotorula</td>
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<tr>
<td>Saccharomyces</td>
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<td>Selenotila</td>
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<td>Sporidiobolus</td>
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<tr>
<td>Sporobolomyces</td>
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<tr>
<td>Torulopsis</td>
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<td>Trichosporon</td>
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</table>
Flippin et al. (1964) reported that *Fusarium moniliforme*, isolated from diesel fuel, was found to grow on n-decane and n-dodecane. Koval et al. (1966) found that n-alkanes of diesel fuel were utilised preferentially as the source of carbon for strains of *Mucor*, *Cunninghamamella*, *Penicillium*, *Trichoderma* and *Fusarium*.

Rynearson and Peterson (1965) used a direct soil-baiting method to isolate paraffinolytic fungi by using a paraffin rod. 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 *Cunninghamamella*.

Soil strains of *Fusarium*, as well as *Acremonium*, had been obtained in ethanol and propane enrichment cultures (Dworkin & Foster, 1958; Kester, 1961). A number of molds were assayed by Kester (1961) 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*. Yamada and Torigoe (1966) isolated numerous strains of molds from samples of soil, water, fruit, etc., in a medium containing a 1% mixture of n-alkanes [equal volumes of each alkane from n-nonane through n-octadecane]. Ten of some 450 strains produced organic acids in a medium containing 3% n-alkanes. n-Nonane and n-decane were utilised to the greatest extent, followed by n-undecane and n-dodecane. Apparently only one of these organisms was classified, being placed in the genus *Botrytis* (Yamada & 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.
Aspergillus flavus, Aspergillus niger, Penicillium notatum and Cladosporium resinae were also tested on n-hexane, n-heptane, n-hexadecane and n-octadecane by Tanaka et al. (1968). The latter two n-alkanes supported good growth, whereas the two shorter substrates were not assimilated.

Lowery et al. (1968) surveyed a series of molds on n-alkanes of C₁ through C₁₆ (minus C₁₅). Genera able to assimilate some members of the series were Aspergillus, Cephalosporium, Dematium, Penicillium, Fusarium, Graphium, Mucor, Gliocladium, Paecilomyces, Epicoccum and Trichoderma. One species of Aspergillus had a substrate assimilatory range from n-hexane through n-hexadecane, while Gliocladium catenulatum initiated growth on n-butane and continued growth throughout the series. However, weak responses were recorded in the C₇-C₁₁ range, while growth with the different genera was most frequently found in the range C₁₀ through C₁₆ (Lowery et al., 1968). One Penicillium species utilised the whole series but growth on the shorter chain-length was apparently sparse.

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, Aspergillus, Fusarium and Penicillium. n-Alkanes with less than 10 carbon atoms supported the growth of very few Fusarium, Penicillium and Aspergillus strains and, if growth occurred, it was minimal. Representatives of six other genera (Absidia, Paecilomyces, Cunninghamella, Chloridium, Oidiodendron, and Scolecobasidium) did not assimilate n-alkanes shorter than n-decane (Nyns et al., 1968a,b). Marked differences between strains were noted on n-decane and n-undecane, and n-alkanes from C₁₂ through C₁₆ were the best growth substrates. Markovetz et al. (1968) checked 53
strains of filamentous fungi representing 32 species on even-numbered n-alkanes and l-alkenes of C_{10}-C_{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. These investigators concluded that the 14-carbon member of each series was utilised most frequently, the ability to grow at the expense of an n-alkane did not necessarily allow for the assimilation of the corresponding 1-alkene, and the use of hydrocarbon assimilation tests lacked taxonomic value (Markovetz et al., 1968). Bemmann and Tröger (1975) surveyed a large number of filamentous fungi representing over 160 genera and showed that most of these organisms could grow on a mixture of n-alkanes. Cooney and Proby (1971) showed that Cladosporium resinae grew more rapidly and more extensively on the shorter chain n-alkanes than on the longer chain n-alkanes, except for n-dodecane, which supported less growth than n-tridecane or n-tetradecane. On Jet-A commercial aviation fuel, the organism had an intermediate growth rate but the poorest cell yield (Cooney & Proby, 1971). Perry and Cerniglia (1973) investigated the microbes in marine areas that utilise hydrocarbons as a source of carbon and energy. They isolated several fungi by enrichment culture that grew extensively on crude oils. Three of these isolates, identified as Cunninghamella elegans, Aspergillus versicolor, and a Eupenicillium sp., grew readily on paraffin-base crude oil resulting in a virtually complete disappearance of the oil within 7 days at 26°C. Screening tests demonstrated that Fusarium moniliforme and Fusarium lini could also oxidise hydrocarbons (Zaichenko & Koval, 1966; Koval & Melnik, 1970; Thiele & Rehm, 1979). Davies and Westlake (1979) examined 60 fungal isolates for their ability to grow on n-tetradecane, toluene, naphthalene, and seven crude oils of
various composition. Forty cultures, including 28 soil isolates, could grow on one or more of the crude oils. The genera most frequently isolated from soils were those producing abundant small conidia, e.g. *Penicillium* and *Verticillium* spp. Oil-degrading strains of *Beauvaria bassiana*, *Mortierella* spp., *Phoma* spp., *Scolecobasidium obovatum*, and *Tolypocladium inflatum* were also isolated (Davies & Westlake, 1979).

In an experiment carried out by Lindley *et al.* (1986), several types of filamentous fungi (*Aspergillus versicolor*, *Cladosporium resinae*, *Gliocladium virens*, *Penicillium chrysogenum* and *Penicillium roquefortii*) and yeast (*Candida guilliermondii*, *Candida lipolytica*) showed the ability to attack only the aliphatic fraction of marine diesel fuels. The shorter chain-length n-alkanes were removed first followed by the longer n-alkanes and methyl-alkanes, respectively. *Candida tenuis* metabolised the whole range of fuel hydrocarbons (Lindley *et al.*, 1986).

Kirk and Gordon (1988) examined the ability of arenicolous and other indigenous marine fungi to utilise and transform anthropogenic petroleum compounds. They examined 54 strains and showed that beach-adapted *Corollospora*, *Dendryphiella*, *Lulworthia* and *Varicosporina* species grew using alkanes and alkenes as sole carbon sources. This ability was unrelated to oil pollution at fungal collection sites. No strains of *Arenariomyces trifurcatus* or *Nereiospora cristata* utilised the hydrocarbons (Kirk & Gordon, 1988). Snellman *et al.* (1988) isolated filamentous fungi from tar balls collected at sites in the North Atlantic and North Pacific Oceans and examined their ability to degrade fuel oil (Table 4). Isolates of *Aspergillus*, *Graphium*, *Humicola*, *Lophotrichus*, *Penicillium*, and *Tetracoccusporium* produced extensive growth on no. 2 and 4 fuel oils.
The genera of fungi reported to utilise aliphatic and aromatic hydrocarbons are summarised in Table 5.

Some cyanobacteria and algae have been found to be capable of hydrocarbon degradation. Walker et al. (1975) described a hydrocarbon-utilising achlorophyllous strain of the alga *Prototheca*. Cerniglia et al. (1980) tested nine cyanobacteria, five green algae, one red algae, one brown algae, and two diatoms for their ability to oxidise naphthalene. Their results indicated that the ability to oxidise aromatic hydrocarbons is widely distributed among the cyanobacteria and algae. The species of Cyanobacteria and microalgae capable of oxidising aromatic hydrocarbons are given in Table 6.
Table 4. Fungal isolates from tar balls collected in the North Atlantic and North Pacific oceans (Snellman et al., 1988).

<table>
<thead>
<tr>
<th>Hyphomycetes</th>
<th>Hyphomycetes (contd..)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acremonium</td>
<td>P. chrysogenum</td>
</tr>
<tr>
<td>A. Kiliense</td>
<td>P. citrinum</td>
</tr>
<tr>
<td>A. strictum</td>
<td>P. javanicum</td>
</tr>
<tr>
<td>Alternaria</td>
<td>P. herghei</td>
</tr>
<tr>
<td>Aspergillus</td>
<td>P. oxalicum</td>
</tr>
<tr>
<td>A. chevalieri</td>
<td>Scopulariopsis brevicaulis</td>
</tr>
<tr>
<td>A. ficuum</td>
<td>Tetracoccosporium</td>
</tr>
<tr>
<td>A. fumigatus</td>
<td></td>
</tr>
<tr>
<td>A. heteromorphus</td>
<td></td>
</tr>
<tr>
<td>A. niger</td>
<td></td>
</tr>
<tr>
<td>A. repens</td>
<td></td>
</tr>
<tr>
<td>A. ruber</td>
<td></td>
</tr>
<tr>
<td>A. sydowii</td>
<td></td>
</tr>
<tr>
<td>A. terreus</td>
<td></td>
</tr>
<tr>
<td>A. ustus</td>
<td></td>
</tr>
<tr>
<td>A. versicolor</td>
<td></td>
</tr>
<tr>
<td>Beauvaria</td>
<td></td>
</tr>
<tr>
<td>Cercosporidium</td>
<td></td>
</tr>
<tr>
<td>Cladosporium cladosporioides</td>
<td></td>
</tr>
<tr>
<td>C. resinae</td>
<td></td>
</tr>
<tr>
<td>Epicoccum purpurascens</td>
<td></td>
</tr>
<tr>
<td>Graphium</td>
<td></td>
</tr>
<tr>
<td>Humicola fuscoatra</td>
<td></td>
</tr>
<tr>
<td>Penicillium</td>
<td></td>
</tr>
</tbody>
</table>

| Ascomycetes                          |                                  |
| Chaetomium                            |                                  |
| C. globosum                           |                                  |
| C. murorum                            |                                  |
| C. spirale                            |                                  |
| Lophotrichus ampullus                 |                                  |
| Microasus cinereus                    |                                  |
| Sordaria                              |                                  |
| S. fimicola                           |                                  |

| Zygomycetes                          |                                  |
| Cunninghamella elegans                |                                  |
| Mucor                                 |                                  |
| Syncephalastrum racemosum            |                                  |
Table 5. Genera of filamentous fungi reported to utilise aliphatic and aromatic hydrocarbons.

<table>
<thead>
<tr>
<th>Aliphatic</th>
<th>Aromatic</th>
</tr>
</thead>
<tbody>
<tr>
<td>Absidia</td>
<td>Absidia</td>
</tr>
<tr>
<td>Acremonium</td>
<td>Aspergillus</td>
</tr>
<tr>
<td>Aspergillus</td>
<td>Basidiobolus</td>
</tr>
<tr>
<td>Botrytis</td>
<td>Choanephora</td>
</tr>
<tr>
<td>Cephalosporium</td>
<td>Circinella</td>
</tr>
<tr>
<td>Chaetomium</td>
<td>Cokeromyces</td>
</tr>
<tr>
<td>Chloridium</td>
<td>Conidiobolus</td>
</tr>
<tr>
<td>Cladosporium</td>
<td>Cunninghamella</td>
</tr>
<tr>
<td>Colletotrichum</td>
<td>Curvularia</td>
</tr>
<tr>
<td>Cunninghamella</td>
<td>Epicoccum</td>
</tr>
<tr>
<td>Dematium</td>
<td>Gilbertella</td>
</tr>
<tr>
<td>Epicoccum</td>
<td>Gliocladium</td>
</tr>
<tr>
<td>Fusarium</td>
<td>Helicostylum</td>
</tr>
<tr>
<td>Gliocladium</td>
<td>Hyphochytrium</td>
</tr>
<tr>
<td>Graphium</td>
<td>Mucor</td>
</tr>
<tr>
<td>Helicostylum</td>
<td>Panaeolus</td>
</tr>
<tr>
<td>Helminthosporium</td>
<td>Penicillium</td>
</tr>
<tr>
<td>Monilia</td>
<td>Pestalotia</td>
</tr>
<tr>
<td>Mucor</td>
<td>Phlyctochytrium</td>
</tr>
<tr>
<td>Oidiodendron</td>
<td>Psilocybe</td>
</tr>
<tr>
<td>Paecilomyces</td>
<td>Phycomycetes</td>
</tr>
<tr>
<td>Penicillium</td>
<td>Phytophthora</td>
</tr>
<tr>
<td>Rhizopus</td>
<td>Rhizophyctis</td>
</tr>
<tr>
<td>Scolecobasidium</td>
<td>Rhizopus</td>
</tr>
<tr>
<td>Spicaria</td>
<td>Saprolegnia</td>
</tr>
<tr>
<td>Syncephalastrum</td>
<td>Smittium</td>
</tr>
<tr>
<td>Trichoderma</td>
<td>Syncephalastrum</td>
</tr>
<tr>
<td></td>
<td>Thamnidium</td>
</tr>
<tr>
<td></td>
<td>Thraustochytrium</td>
</tr>
<tr>
<td></td>
<td>Zygorhynchus</td>
</tr>
</tbody>
</table>
Table 6. Different species of Cyanobacteria and microalgae which are able to oxidise aromatic hydrocarbons (Cerniglia, 1984).

<table>
<thead>
<tr>
<th>Cyanobacteria</th>
</tr>
</thead>
<tbody>
<tr>
<td>Oscillatoria sp. (strain JCM)</td>
</tr>
<tr>
<td>Oscillatoria sp. (strain MEV)</td>
</tr>
<tr>
<td>Microrcoleus chthonoplastes (strain BA-1)</td>
</tr>
<tr>
<td>Nostoc sp. (strain MAC)</td>
</tr>
<tr>
<td>Anabaena sp. (strain CA)</td>
</tr>
<tr>
<td>Anabaena sp. (strain 1F)</td>
</tr>
<tr>
<td>Agmenellum quadruplicatum (strain PR-6)</td>
</tr>
<tr>
<td>Coccochloris elabens (strain 17a)</td>
</tr>
<tr>
<td>Aphanocapsa sp. (strain 6714)</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Green algae</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chlorella sorokiniana (strain TX 71105)</td>
</tr>
<tr>
<td>Chlorella autotrophica (strain 580)</td>
</tr>
<tr>
<td>Dunaliella tertiolectra (strain DUN)</td>
</tr>
<tr>
<td>Chlamydomonas angulosa</td>
</tr>
<tr>
<td>Ulva fasciata</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Diatoms</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cylindrotheca sp. (strain N-1)</td>
</tr>
<tr>
<td>Amphora sp. (strain AMB-1)</td>
</tr>
<tr>
<td>Nitzschia sp. (strain K8A)</td>
</tr>
<tr>
<td>Synedra sp. (strain 4D)</td>
</tr>
<tr>
<td>Navicula sp. (strain K1A)</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Red alga</th>
</tr>
</thead>
<tbody>
<tr>
<td>Porphyridium cruentum</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Brown alga</th>
</tr>
</thead>
<tbody>
<tr>
<td>Petalonia fascia</td>
</tr>
</tbody>
</table>
SUBSTRATE SPECIFICITY

The ability of microorganisms to degrade hydrocarbons can be just as specific as the utilisation of other substrates. Therefore, the assimilation of hydrocarbons has been suggested as a possible taxonomic tool (Klug & Markovetz, 1971). The current information about hydrocarbon degradation (Shennan & Levi, 1974; Britton, 1984) generalised four rules for the specificity of hydrocarbon utilisation by microorganisms. These rules can be summarised as follows:

Rule 1. Aliphatic hydrocarbons are readily assimilated as compared to aromatic hydrocarbons.

Rule 2. Long chain n-alkanes are assimilated more readily than short chains.

Rule 3. Saturated compounds are degraded more readily than unsaturated compounds.

Rule 4. Straight chains are degraded more readily than branched chains.

It is clear that the ability to utilise hydrocarbons is widely distributed among diverse microbial populations as well as among different habitats. When natural ecosystems are contaminated with petroleum hydrocarbons, the indigenous microbial communities are likely to contain microbial populations of differing taxonomic relationships which are capable of degrading the contaminating hydrocarbons.
CHAPTER TWO
MECHANISM OF HYDROCARBON UPTAKE

The fact that hydrocarbons have limited solubility in water poses a problem in their transport to the microbial cells which prefer water-soluble substrates. Hydrocarbon-utilising microorganisms must have a mechanism by which they can achieve cell-substrate contact so that a hydrophobic water-insoluble substrates are transported through the cell envelope to the cytosol in order to be degraded, how these microorganisms can achieve that is still completely obscure. However, three different mechanisms for hydrocarbon up-take have been proposed (Singer & Finnerty, 1984; Boulton & Ratledge 1984, 1987).

(1) Uptake of dissolved hydrocarbons
(2) Uptake by microbial adherence to hydrocarbon droplet; and
(3) Uptake of emulsified or accommodated hydrocarbons

The first mechanism is of limited importance since it operates with lower molecular weight alkanes (mainly < C_{10}) rather than higher molecular weight alkanes and with solid aliphatic and aromatic compounds. It is believed that such mechanism is not sufficient to support growth of microorganisms on hydrocarbons at high rates. Candida lipolytica grown on liquid medium saturated with undecane and dodecane vapours showed higher growth as compared with the growth of the same yeast on dissolved longer chain alkanes (e.g. tridecane), due to lower solubility of long chain alkanes (Erdtsieck & Rietema, 1969). Similar findings were obtained by Yoshida et al. (1971), who stated that growth rates of Candida tropicalis on alkanes dissolved in the medium from the vapour phase was higher than growth rates using the same alkanes supplied to the medium in the liquid form. Further studies demonstrated that Candida
lipolytica grown on a mixture of n-alkanes (C\textsubscript{11} to C\textsubscript{18}), first utilised shorter chain alkanes followed by the longer ones (Goma et al., 1973).

Aromatic compounds have higher water solubility than long-chain liquid alkanes (Klevens, 1950). Therefore, microorganisms can grow on dissolved aromatic compounds (Wodzinski & Coyle, 1974). Wodzinski and Bertolini (1972) showed that generation time of bacteria growing on naphthalene or dibenzyl was independent of the solid particles added to the medium, suggesting that these aromatic compounds were not utilised in their solid state.

Mechanism (2) and (3) have been proposed for the transport of a liquid hydrocarbons. The former mechanism is proposed for those microorganisms which contact themselves to large hydrocarbon droplets (> 1 \mu m in diameter), whereas the latter one is proposed for smaller accommodated hydrocarbon droplets (< 1 \mu m in diameter) (Singer & Finnerty, 1984). In both mechanisms direct contact between hydrocarbon droplet and the microbial cell seems to be a prerequisite for growth (Kennedy et al., 1975, McLee & Davies, 1971; Cundell et al., 1976). It has been demonstrated that Candida tropicalis have high affinity for alkane, in comparison to glucose-grown cells of Candida tropicalis (Käppeli & Fiechter, 1976). Moreover, Miura et al. (1977) noted that hydrocarbon utilising yeasts were more adherent to hydrocarbon droplets than yeasts which did not grow on hydrocarbons.

Other workers, believe that not all microbial populations growing on hydrocarbon adhere to hydrocarbons. It was found that 40% of the populations of Candida lipolytica did not adhere to hexadecane (Nakahara et al., 1977). The same results were obtained by Gutierrez and Erickson (1977). Although Pseudomonas aeruginosa has the
ability to grow on hydrocarbons, Nakahara et al. (1981) reported that this bacterium did not adhere to these hydrocarbons. On the other hand Rosenberg et al. (1980) demonstrated high adhesion affinity of Acinetobacter calcoaceticus towards nonmetabolisable hydrocarbons such as xylene and octane. This phenomenon could be due to a nonspecific hydrophobic interaction (London van der Waals forces). It has been found that cells which adhere strongly to hydrocarbons can utilise large drops as well as accommodated forms while cells with lower adhesion capabilities grow more effectively on the accommodated form (Miura et al., 1977).

During growth of microbial cells on hydrocarbons, certain changes in the cell envelope seem to be necessary for achieving cell-hydrocarbon contact [for a review see Boulton & Ratledge (1984)]. Hydrocarbon assimilation by yeasts results in the formation of flocs (clumps of cells, hydrocarbon and air bubbles). Floc formation facilitates close contact between cells and hydrocarbon droplets (Mallee & Blanch, 1977). The formation of flocs is presumably caused by the presence of a lipophilic material on the cell walls of hydrocarbon consuming organism (Bos & Boer, 1968). It is proposed that this lipophilic cell-wall components are responsible for adhesion of hydrocarbons. Surface projections were found in alkane-grown Candida sp. (Osumi et al., 1975). These fibrous projections were also present in freeze-fractured alkane-grown Candida tropicalis and they thought to represent a polysaccharide-fatty acid complex responsible for hydrocarbon binding (Kappeli & Fiechter, 1977). This complex is absent in glucose-grown cells and was identified as mannan containing about 4% covalently linked fatty acids (Kappeli et al., 1978). Furthermore, Rosenberg et al. (1982) reported numerous surface fimbriae present on the cell surface of Acinetobacter sp. and suggested that these fimbriae play an important part in
achieving adhesion of cell to the hydrocarbon droplets. Another change in the cell wall include the presence of channels and pores in hydrocarbon assimilating yeasts (Ludvik et al., 1968; Kozlova et al., 1973; Osumi et al., 1975). These large pores which are not observed in electron micrographs of glucose-grown cells (Ludvik et al., 1968) are believed to facilitate the direct entry of hydrocarbons into the cells in a passive diffusion process (Ratledge, 1978; Lindley & Heydemian, 1986).

SOLUBILISATION OF HYDROCARBONS BY PRODUCTION OF BIOSURFACTANTS

Many microorganisms which have the ability to grow on hydrocarbons or any hydrophobic substrates produce surface-active compounds (Kosaric et al., 1987; Wagner 1987; Wagner & Lang, 1988). These bioemulsifiers, which are mainly or entirely extracellular compounds consist of both hydrophilic and hydrophobic moieties (Haferburg et al., 1986; Powalla et al., 1989) (Table 7). The nature of these compounds seems to facilitate the uptake of hydrocarbons into the cells by lowering the surface tension between oil and water interface, thus emulsifying and adhering the hydrocarbon droplet to the cell envelope (Zajic & Mahomedy, 1984). It has been reported by Oberbremer et al. (1990), using a soil population microorganisms, that hydrocarbon degradation rate could be doubled by the addition of sophorose lipids as biosurfactants. Such compounds could be also produced in the absence of water insoluble substrates (Persson & Molin, 1987). However, both types of biosurfactants have a biodegradable nature and are effective at extreme temperatures (Zajic et al., 1977), pH and salinity (Kretschmer et al., 1982).

It should be emphasised that biosurfactant production by microorganisms is of a high commercial value, either in
the field of oleochemical industries [e.g. use of sophorolipid produced by *Candida bombicola* as an additive to lipsticks and other toiletries (Falbe & Schmid, 1986)], or in the field of enhanced oil recovery [e.g. use of emulsan produced by *Acinetobacter calcoaceticus* RAG-1 as an oil cleaning agent (Gutnick & Shabtai, 1987)].

Microorganisms which produce biosurfactants have been classified into three groups with respect to the synthesis of extracellular lipids (Haferburg *et al.*, 1986):

i. Microorganisms which produce bioemulsifiers exclusively when grown on hydrocarbons (e.g. *Corynebacterium* sp., *Arthrobacter* sp. and *Nocardia* sp.).

ii. Microorganisms which produce bioemulsifiers when grown on hydrocarbons as well as water-soluble compounds (e.g. *Pseudomonas aeruginosa*, *Corynebacterium hydrocarboclastus* and *Torulopsis*).

iii. Microorganisms which produce bioemulsifiers exclusively when grown on water-soluble compounds (e.g. *Hansenula cifferi*, *Arthrobacter paraffineus* and *Bacillus subtilis*).

Biosurfactants are also classified according to their chemical structure. They may be relatively simple, low molecular weight glycolipids or complex, high molecular weight molecules of uncertain structures. Table 7; illustrates examples of the diversity of biosurfactant structures.
Table 7. The diversity of biosurfactant structures produced during growth on hydrocarbons or other water insoluble substrates. (Adapted from Ratledge, 1987).

<table>
<thead>
<tr>
<th>Molecular type</th>
<th>Organism</th>
<th>Surfactant Structure</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glycolipids - Bacteria</td>
<td>Arthrobacter sp.</td>
<td>Mono-, di- and tri-corynomycolates of glucose, maltose, maltotriose, etc.</td>
<td>Li et al. (1984)</td>
</tr>
<tr>
<td>Nocardia corynebacteroides SM1</td>
<td></td>
<td>Trehalose-6, 6-dicorynomycolates, and Trehalose-6-monocorynomycolate-Penta(2 α-glucose &amp; 3β-glucose units) lipid</td>
<td>Powalla et al. (1989)</td>
</tr>
<tr>
<td>Pseudomonas aeruginosa</td>
<td>Mono- and di-rhamnolipids</td>
<td>Hisatsuka et al. (1971)</td>
<td></td>
</tr>
<tr>
<td>Rhodococcus H13A</td>
<td>Disaccharide lipid</td>
<td>Bryant (1990)</td>
<td></td>
</tr>
<tr>
<td>R. erythropolis</td>
<td>Trehalose-6, 6-dicorynomycolates</td>
<td>Rapp et al. (1979)</td>
<td></td>
</tr>
<tr>
<td>Fungi</td>
<td>Ustilago maydis</td>
<td>Cellobiose glycolipid</td>
<td>Frantz et al. (1986)</td>
</tr>
<tr>
<td>Yeast</td>
<td>Torulopsis bombicola and T. apicola</td>
<td>Sophorolipids</td>
<td>Cooper &amp; Paddock (1984); Göbbert et al. (1984); Hommel et al. (1987)</td>
</tr>
</tbody>
</table>

- Phospholipids - Bacteria

<p>| Acinetobacter H01-N | Phosphatidylethanolamines | Küppeli &amp; Finnerty (1979) |</p>
<table>
<thead>
<tr>
<th>Molecular type</th>
<th>Organism</th>
<th>Surfactant Structure</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>- Fungi</td>
<td><em>Cladosporium resinae</em></td>
<td>Phosphatidylcholines and Siporin &amp; others</td>
<td>Cooney (1975); Lindley &amp; Heydemann (1986)</td>
</tr>
<tr>
<td>Amino acid-containing lipids (lipopeptides)</td>
<td><em>Bacillus subtilis</em></td>
<td>Surfactin (heptapeptido-3-hydroxy-14-methyl-palmitate)</td>
<td>Lang &amp; Wagner (1987)</td>
</tr>
<tr>
<td>Complex molecules</td>
<td><em>Acinetobacter calcoaceticus</em> (RAG-1)</td>
<td>Emulsan</td>
<td>Zuckerberg et al. (1979), for structure see Gutnick &amp; Shabtai (1987)</td>
</tr>
<tr>
<td></td>
<td><em>A. calcoaceticus</em> (BD4)</td>
<td>Rhamnopolysaccharide</td>
<td>Rosenberg (1986)</td>
</tr>
</tbody>
</table>
UPTAKE OF SOLUBILISED HYDROCARBONS

The exact mechanism by which a solubilised hydrocarbon in conjunction with a surfactant is transferred across the cell envelope to the cytoplasm is not quite clear. Köppeli and Fiechter, (1977) isolated a polysaccharide-fatty acid complex from the cell surface of *Candida tropicalis* growing on alkanes. They suggested that the amphipathic nature of this complex could have a possible role in alkane metabolism. Depending on these findings Ratledge (1987) proposed a hypothesis for the uptake of a solubilised hydrocarbon (Fig. 1).

The hypothesis presumes that the surfactant and the hydrocarbon are in a loose association and together they would enter the lipophilic outer part of the cell envelope. The hydrocarbon would dissociate from the surfactant possibly within an amphipathic receptor/channel molecule as suggested by Köppeli and Fiechter (1977). The hydrocarbon would then partition into the membrane and the surfactant would be returned to the exterior of the cell via the channel. By increasing the concentration of hydrocarbon in the membrane, it would physically move in the only possible direction (i.e. to the cytoplasm). Due to the insoluble nature of the hydrocarbon, it would quickly aggregate to form, first, microdroplets and then into larger agglomerations (hydrocarbon inclusions) readily visible by electron microscopy (Boulton & Ratledge, 1984), keeping the concentration of free hydrocarbon extremely low which ensures the continuity of hydrocarbon uptake.
Figure 1. Hypothesis proposed by Ratledge (1987) for hydrocarbon/surfactant uptake into a microbial cell.
A. General scheme

Outside

Alkane → Surfactant

"Soluble alkane" (loose association of molecules)

Biosynthesis → Dissociation → Inside

Surfactant precursors → Alkane (coalesce into droplets)

B. Receptor/channel concept

Wall

Phospholipid bilayer membrane

Amphipathic receptor/channel

Alkane

Surfactant

Surfactant/alkane
Ratledge (1987) considered the surfactant action specificity [i.e. surfactants appear to promote growth of the producing species on hydrocarbons and may be less effective in stimulating growth of other microorganisms (Ito & Inoue, 1982)] a major support for this hypothesis. This specificity of action require the surfactant/alkane complex to have the correct degree of solubility in the amphipathic receptor or when the complex enters the channel in order for it to dissociate followed by the entrance of hydrocarbon to the cytoplasm across the membrane.

He also considered the model presented by Lindley and Heydeman (1983, 1986) as an indirect support for his hypothesis. Lindley and Heydeman (1986) suggested that the alkane uptake by Cladosporium resinae happens by passive adsorption onto the cell surface. This step is proceeded by an energy-requiring transfer (active transport or pinocytosis) of alkane to the cytoplasm. However, this model requires further testing.

In summary, cells growing on hydrocarbons would undergo certain modifications (these modifications will be discussed in details later in this chapter) to deal with the problem of growing on a water-insoluble substrate causing the adherence of microorganisms to submicron or larger hydrocarbon droplets. Internalisation of the hydrocarbon is accomplished by direct partitioning in the cytoplasmic membrane and/or by solubilisation with inducible surfactants. It has been assumed that transport is by passive diffusion through pores or if solubilising agents are involved, by facilitated diffusion. The presence of hydrocarbon inclusions containing more hydrocarbon than the outside of the cell (Lindley & Heydeman, 1986) imply that an energy-dependent concentration against a gradient has occurred. However, there is no definitive evidence to suggest that active transport of hydrocarbons exists in
microorganisms especially with the fact that the uptake of hydrocarbons is not inhibited by energy poisons (Christensen, 1975). As a result, it is believed that hydrocarbon uptake implies a more complex mechanism than a direct one.

ULTRASTRUCTURAL CHANGES OF HYDROCARBON-UTILISING MICROORGANISMS

There are several reports of ultrastructural dissimilarities between microbial cells which have been grown on hydrocarbon and those grown on non-hydrocarbon substrates. Alterations have been observed in the intracytoplasmic and cellular membrane (Ludvik et al., 1968; Ahearn et al., 1970; Boulton & Ratledge, 1984), cell wall (Ahearn et al., 1970), and storage materials (Kennedy and Finnerty, 1971) of cells grown on hydrocarbon media. Alkane-grown microorganisms generally are characterised by several unusual morphological features, including: (1) presence of intracellular cytoplasmic inclusions, known as hydrocarbon inclusions; (2) presence of intracytoplasmic membranes; and (3) the appearance of numerous microbodies in hydrocarbon-grown yeasts.

1. Hydrocarbon Inclusions

Hexadecane-grown Acinetobacter species H01-N, which has been shown to grow on a variety of alkanes and 1-alkenes greater than 10 carbon in length (chapter 1), is characterised ultrastructurally by the appearance of hydrocarbon inclusions. Kennedy et al. (1975) examined the fine structure of Acinetobacter species after growth on hydrocarbon (paraffinic and olefinic) and non-hydrocarbon substrates and showed that multiple inclusion bodies are
readily apparent and served to characterise an ultrastructural feature associated with only hydrocarbon-grown bacteria. The inclusions appear under electron microscopy as electron-transparent, spherical (0.2 \( \mu \text{m} \) in diameter) cytoplasmic bodies, usually localised at the cell periphery or in close contact with the intracytoplasmic membranes (Kennedy et al., 1975; Scott & Finnerty, 1976a).

Scott and Finnerty (1976b) surveyed a number of alkane-utilising microorganisms and found similar hydrocarbon inclusions in hexadecane-grown Arthrobacter sp. 80, Corynebacterium sp., Mycobacterium album 7E4, Mycobacterium vaccae, Nocardiasp. 72, Nocardia rubra, Candida lipolytica, tetradecane-grown Candida tropicalis, and naphthalene-grown Pseudomonas sp.. These microorganisms do not contain electron transparent inclusions when grown on non-hydrocarbon medium, with the exception of Nocardia rubra, which produces poly-\( \beta \)-hydroxybutyrate (PHB) when grown on nutrient broth. However, this organism produced insignificant amounts of PHB when grown on hexadecane. Electron-dense inclusions were visualised in Nocardia sp., most likely representing lipoidal (wax or glyceride) inclusions.

Phillips and Perry (1976) working with Thermomicrobium fosteri an obligate thermophilic hydrocarbon-utilising bacterium, showed that cells contained an extensive network of intracellular membranes resembling mesosomes, and intracytoplasmic hydrocarbon inclusions during growth on n-heptadecane. Similarly, a hexadecane-grown Penicillium sp. was shown by Cundell et al. (1976) to contain numerous electron-transparent cytoplasmic inclusion bodies resembling hydrocarbon inclusions. Significant quantities of hexadecane were recovered from lysed spheroplasts of solvent-washed Cladosporium resinae (Cooney et al., 1980). Although large vacuoles were observed in electron micrographs of
hexadecane-grown Cladosporium resinae, these were also seen in glucose-grown cells (Smucker & Cooney, 1981). These vacuoles did not resemble typical hydrocarbon inclusions.

Electron-transparent, disc-shaped cytoplasmic vesicles were observed in thin-sections of a hexadecane-grown marine bacterium (Bertrand et al., 1976). These vesicles appear to be membrane-bound in freeze-etched preparations. Although the nature of the material contained in these inclusions was not determined, hexadecane-grown cells contained 20 times more nonsaponifiable lipids than acetate-grown cells, implying that hexadecane was accumulated intracellularly in the vesicles (Bertrand et al., 1976).

Crude oil-grown marine bacteria, Flavobacterium sp. and Brevibacterium sp., are characterised ultrastructurally by the presence of large electron-dense inclusions, which were located predominantly at the cell terminus. However, Flavobacterium sp. had smaller inclusions when grown on marine agar, while inclusion bodies were not found in Brevibacterium sp. grown on the same media. These inclusions were suggested to be lipid storage particles (Atlas & Heintz, 1973).

2. Intracytoplasmic Membranes

The second unique ultrastructural feature of hydrocarbon-grown cells is the presence of an extensive intracytoplasmic membrane system. Kennedy and Finnerty (1975) showed that Acinetobacter species H01-N cultured on hydrocarbons (hexadecane, heptadecane, and 1-hexadecene) resulted in the formation of complex intracytoplasmic membrane systems. The arrangement of the membrane within the cytoplasm appeared as a subperipherally localised structure composed of lipid bilayers organised into sheaths. Single unit bilayer structures appeared to branch off the
main structure and extended toward the cell periphery. The membrane system appeared to be composed of 3-4 "unit membranes" stacked together forming a highly ordered structural complex, often appearing continuous with the cytoplasmic membrane or in physical contact with hydrocarbon inclusions. The most significant observation was that these intracytoplasmic membranes were synthesised only when cells were grown with hydrocarbons as sole source of carbon and energy, suggesting that these membranes are integrally involved in hydrocarbon metabolism (Kennedy & Finnerty, 1975). Furthermore, intracytoplasmic membrane was observed in Acinetobacter sp. grown on hexadecane (Scott & Finnerty, 1976a). The appearance of intracytoplasmic membranes has been associated with the beginning of alkane oxidation (Kennedy & Finnerty, 1975), and with the increase in the cellular phospholipid content of hydrocarbon-grown cells (Makula & Finnerty, 1970; Makula et al., 1975; Ratledge, 1987; Borneleit et al., 1988). Similar intracytoplasmic membrane systems have been observed in a cyclohexane-grown Nocardia sp. (Stirling et al., 1977), and in methylotrophic bacteria (Davies & Whittenbury, 1970).

Chemical characterisation of intracytoplasmic membranes isolated from Acinetobacter sp. grown on hexadecane, resembled the cytoplasmic membrane fraction isolated from the same organism grown on nutrient broth yeast extract qualitatively in phospholipid and neutral lipid composition, polypeptide composition, and enzyme activity (Scott et al., 1976). Nevertheless, the intracytoplasmic membrane fraction was enriched in phospholipids and hexadecane compared with cytoplasmic membrane.

Different potential roles for intracytoplasmic membranes in alkane metabolism in Acinetobacter have been postulated by several workers. The roles that have been suggested for these membranes include the following:
i. The enzymes responsible for initial oxidation of the alkane molecule, and/or for its subsequent oxidation to fatty acid, may be associated with the intracytoplasmic membrane system as integral or peripheral proteins (Kennedy & Finnerty, 1975; Scott & Finnerty, 1976a).

ii. The intracytoplasmic membrane may provide a suitable lipid-rich hydrophobic environment necessary for the oxidation of alkane (Kennedy & Finnerty, 1975).

iii. The membrane may create a continuous channel for alkane transfer from the cytoplasmic membrane to the intracellular site of alkane oxidation or to the hydrocarbon inclusions (Ratledge, 1987) and

iv. The intracytoplasmic membranes may be the site of increased respiratory activity, providing additional membrane for the increased total cytochrome content of hexadecane-grown Acinetobacter (Ensley & Finnerty, 1980; Ensley et al., 1981).

3. Microbodies of Hydrocarbon-Grown Yeasts

Osumi and Fukui (1972) first reported the appearance of numerous microbodies (peroxisomes) in the cytoplasm of alkane-grown Candida species. These microbodies were either absent or only infrequently observed in Candida grown on glucose, ethanol or acetate (Osumi & Fukui, 1972; Osumi et al., 1974, Teranishi et al., 1974).

Osumi et al. (1974) surveyed a number of alkane-utilising yeasts and observed large numbers of microbodies in several species of alkane-grown Candida including C. lipolytica, C. albicans, C. guilliermondii, C. intermedia, C. tropicalis. Tanaka and Iida (1977) reported the
occurrence of microbodies in *Candida rugosa* cells when grown on substrates of hydrocarbons of different chain lengths. These microbodies have been also observed in the hexadecane-grown fungus, *Cladosporium resinae* (Smucker & Cooney, 1981).

The ultrastructure of the microbody in the yeast cell can be defined as a cytoplasmic particulate spherical or oval structure surrounded by a single bilayer membrane with a homogeneous matrix (Osumi & Fukui 1972; Osumi *et al.*, 1974). However, the size, number in one cell, and the mode of occurrence are not definite, depending on what kind of carbon compounds the yeast cells are utilising or what stages they are grown in the culture (Fukui & Osumi, 1973; Davidova *et al.*, 1975, Tanaka & Iida, 1977). One of the remarkable examples is that hydrocarbon-growing cells have numerous microbodies profusely in the cytoplasm (Osumi *et al.*, 1974), while methanol-grown cells have microbodies of relatively large size in a clustered form (Tanaka *et al.*, 1976; Tsubouchi *et al.*, 1976).

Other morphological features have been also reported. Ludvik *et al.* (1968) reported the presence of more abundant endoplasmic reticulum, mitochondria, and fat vacuoles in *Candida lipolytica* cells grown on hydrocarbons. Ratledge (1978) showed that filamentous forms of *Candida tropicalis*, with smooth walls, were produced when grown on n-alkane medium.
CHAPTER THREE
Hydrocarbon metabolism by different microorganisms has been studied intensively during the last thirty years. In general, in most crude oils, paraffinic fraction is the dominant one, this fact is more pronounced in Kuwait crude oil, since paraffinic hydrocarbons constitute up to 72% of this oil (Perry, 1984). It is also more likely that this group of compounds are more susceptible to microbial attack than any other group of hydrocarbons (chapter 1). For this reason and the fact that hydrocarbon compounds are so diverse, this chapter will discuss in details only the metabolism of n-alkanes (not methane which is a specialised case), branched-chain alkanes and n-alkenes. Other groups of compounds such as cycloalkanes and aromatics will be discussed briefly and for a review of both groups see (Perry, 1968, 1984; Trudgill, 1978) and (Dagley, 1975; Gibson, 1977; Cerniglia, 1984; Sleat et al., 1984), respectively. It is beyond the scope of this chapter to cover other hydrocarbon compounds such as methane and other gaseous hydrocarbons (for a review see Sahm, 1977; Vestal, 1984; Hartmans et al., 1989) and halogenated hydrocarbons (for a review see Jones & Howe, 1968a).

OXIDATION OF n-ALKANES

1. Oxidation of Long Chain n-Alkanes (terminal oxidation)

General identification of long chain n-alkanes is that they are those compounds which contain from C_{10} to C_{20} carbon atoms. The degradation of these compounds have been studied thoroughly since most microorganisms prefer to utilise these compounds (Konovalt schikoff-Mazoyer & Senez, 1956; Lukins, 1962; Duncan & Ulrich, 1973). However, other microorganisms
are also able to oxidise alkanes up to \( \text{C}_{44} \) (Haines & Alexander, 1974). It is assumed that the degradation mechanisms for these very long-chain alkanes are similar to those of \( \text{C}_{10}-\text{C}_{20} \) n-alkanes (Rehm & Reiff, 1981).

Microorganisms degrade these long chain alkanes by oxidising the terminal methyl group leading to the production of the corresponding fatty acid by a series of reactions (Boulton & Ratledge, 1984, 1987). This assumption was based on the detection of the homologous fatty acids in the culture media (Senez & Konavaltschifoff-Mazoyer, 1956). Working with \textit{Micrococcus cerificans} stewart et al., (1959) found an incorporation of \(^{18}\text{O} \) into the corresponding fatty acid. The presence of esters, that had an alcohol moiety having a carbon skeleton identical to that of the paraffin from which it was derived (Stewart & Kallio, 1959; Stewart et al., 1959, 1960; Finnerty & Kallio, 1964; Klug & Markovetz, 1967c) indicated that, the terminal methyl group is first oxidised to the corresponding n-alcohol. Three mechanisms were proposed for this initial step in alkane oxidation which is catalysed by a complex hydroxylase:

i. Direct incorporation of oxygen which is catalysed by a mixed function oxidase or monooxygenase (Hydroxylation mechanism).

ii. The formation of alkyl hydroperoxides (hydroperoxidation).

iii. Oxidation via alkenes (dehydrogenation).

The first mechanism is the most supported mechanism of methyl group hydroxylation. The mixed function oxidase or monooxygenase may be linked to one of several different electron carrier systems. To date, two microbial hydroxylation systems have been well characterised. The
rubredoxin-containing \( \omega \)-hydroxylase system found in *Pseudomonas oleovorans* and cytochrome P-450 containing system. The former system was first referred to as a soluble enzyme system that would oxidise n-octane to 1-octanol or fatty acids to \( \omega \)-hydroxy fatty acids (Baptist et al., 1963; Kusunose et al., 1964a). The reaction required reduced NAD, ferrous iron, and molecular oxygen (Gholson et al., 1963; Kusunose et al., 1964b). Further studies made by Peterson et al. (1966, 1967) on the oxidation of n-octane by cell-free extracts of *Pseudomonas oleovorans* showed that the enzyme system is composed of an NADH\( _2 \):rubredoxin oxidoreductase, rubredoxin-like non-haem iron protein and \( \omega \)-hydroxylase. Rubredoxin reductase has been found to be a 55,000 dalton protein with one FAD per molecule (Ueda et al., 1972; Ueda & Coon, 1972). Rubredoxin, which is an electron carrier in the system, had a molecular weight of 19,000 dalton and had no labile sulphide, and was capable of binding 2 atoms of iron per molecule, even though rubredoxin containing only one iron was routinely purified (Peterson & Coon, 1968; Lode & Coon, 1971). The \( \omega \)-hydroxylase which is associated with the cell membrane has a molecular weight of about \( 2 \times 10^6 \) and is an aggregate of 40,800 dalton subunits and was able to catalyse the \( \omega \)-oxidation of fatty acids in vitro (Mckenna & Coon, 1970; Ruettinger et al., 1977) (Fig. 2). Similar rubredoxin systems have been isolated from several hydrocarbon utilising bacteria (Kusunose et al., 1967a,b; Mckenna & Coon, 1970; Van-Eyk & Bartels 1970; Van Ravenswaay Claasen & Van der Linden, 1971; Nieder & Shapiro, 1975; Aurich et al., 1976; Hammer & Liemann, 1976).
Figure 2. Mechanism of n-alkanes oxidation by rubredoxin system to the corresponding alcohol.
NADH\textsuperscript{+}H\textsuperscript{+} → reductase (oxid.) → rubredoxin \( \text{Fe}^{2+} \) → \( \omega \)-Hydroxylase → oxygen \( \text{O}_2 \) → rubredoxin \( \text{Fe}^{3+} \) → \( \text{H}_2\text{O} \) → HOCH\textsubscript{2}\text{-}R → \( \text{H}_3\text{-C-R} \) → NAD\textsuperscript{+} → reductase (red.) → NADH\textsuperscript{+}H\textsuperscript{+}
The other electron carrier system is cytochrome P-450 system. This system is found in both prokaryotic and eukaryotic cells. It was first reported in Corynebacterium 7E1C which was able to oxidise n-octane to l-octanol (Cardini & Jurtshuk, 1968, 1970). The reaction required NADH rather than NADPH which is required in the liver microsomal system (Estabrook et al., 1972), molecular O₂ and a flavoprotein and was sensitive to carbon monoxide. Another study, conducted by Asperger et al. (1981) reported the presence of cytochrome P-450 in Acinetobacter spp. which were capable of growth on hexadecane.

In Candida tropicalis, the alkane hydroxylase system resides within the microsomes and contains cytochrome P-450 and an NADPH: cytochrome c reductase (Gallo et al., 1971), and in association with the complex is a phosphatidylethanolamine (PE) (Duppel et al., 1973). The system was O₂ dependent and NADPH-dependent (Duppel et al., 1973; Lebeault et al., 1971), and the synthesis of cytochrome P-450 was only inducible by long-chain alkanes (≥ 14 carbon atoms), alkenes, secondary alcohols and ketones but not by alkanes of < 14 carbons, polyunsaturated fatty acids, dienes, alkylbenzenes, cholesterol derivatives, or polyaromatic hydrocarbons (Gallo et al., 1973a). In addition to cytochrome P-450 synthesis, growth on alkanes results in induction of NADPH-cytochrome c reductase and cytochrome b₅ (Gallo et al., 1973a,b). The function of NADPH-cytochrome c reductase is believed to be involved in cytochrome P-450 reduction (Kubota et al., 1977; Aoyama et al., 1978). This enzyme was isolated from n-tetradecane-grown Candida tropicalis microsomes and found to have a molecular weight of 67,000 dalton and contains 1 mole each of FMN and FAD/mole enzyme (Bertrand et al., 1979). The purified enzyme did not reduce cytochrome P-450 (presumably because of purification procedures), but still considered as a P-450 reductase since the enzyme levels increased four
fold during growth on tetradecane and that the reductase was similar to P-450 reductase from yeast (Aoyama et al., 1978) and liver microsomes (Pederson et al., 1973). Although NADPH has been said to be the specific co-factor for the cytochrome P-450 system, Rohde et al. (1975) found that NADH and not NADPH was active with the alkane oxidase from Candida lipolytica. Moreover, Iizuka et al. (1968) found that Candida rugosa had a requirement for either NADH or NADPH. This could be due to using only crude enzyme systems which may contain transhydrogenase to interconvert NADH and NADPH or to the presence of impurities in the substrate (Ratledge, 1978). Another explanation was proposed by Britton (1984) who suggested that these findings could be due to variation in the specificity of reduced nucleotides in hydroxylation systems of yeasts (Fig. 3). Candida guilliermondii was also reported to have the cytochrome P-450 during growth on hexadecane (Tittlebach et al., 1976; Schunk et al., 1978; Muller et al., 1979). The cytochrome P-450 was inducible by growth on hexadecane and was strongly influenced by the oxygen concentration in the growth medium (Mauersberger et al., 1980).

Takagi et al. (1980) examined different alkane-utilising yeast strains for cytochrome P-450 content, he found that several Candida tropicalis strains and a Candida pulcherrina strain have an induced P-450 system.

Marchal et al. (1982) showed that Candida lipolytica has the ability in the presence of O₂ and NADPH to hydroxylate fatty acids to ω-hydroxy fatty acid more efficiently as compared to alkane. Such differential activity toward fatty acid and alkane has been routinely reported with no satisfactory explanation.
Figure 3. Mechanism of n-alkanes oxidation by cytochrome P-450 system to the corresponding alcohol.
The overall reaction that is catalysed by the two systems can be summarised by the following equation.

\[
R-\text{CH}_2-\text{CH}_3 + O_2 \xrightarrow{\text{NAD(P)H+H}^+} R-\text{CH}_2-\text{CH}_2\text{OH} + H_2O
\]

Mechanism (ii) the hydroperoxidation, involves the incorporation of molecular \(O_2\) into the alkane by a dioxygenase yielding an n-alkyl hydroperoxide which undergo reduction reaction to produce the primary alcohol. This possible pathway was first suggested by Imelik (1948), who reported that hydroperoxides could be an intermediates in the microbial n-alkane oxidation. Similar findings were reported by Leadbetter and Foster (1960). The results of Stewart et al. (1959) concerning the incorporation of \(^{18}O_2\) into alkanes were consistent with the formation of hydroperoxides, and alkyl hydroperoxides oxidation in alkane-grown Acinetobacter sp. (formerly Micrococcus cerificans) (Finnerty et al., 1962). Cell free extract from the same organism had the ability to degrade alkyl hydroperoxides with some evidence of NADH-dependent hydroperoxide reductase being involved (Mckenna & Kallio, 1965). This mechanism is illustrated as follows:

\[
R-\text{CH}_2-\text{CH}_3 \xrightarrow{O_2} R-\text{CH}_2\text{CH}_2\text{-OOH} \xrightarrow{\text{NAD(P)H+H}^+} R-\text{CH}_2\text{-CH}_2\text{-OH} + H_2O
\]

The third mechanism (dehydrogenation) is an NAD-dependent dehydrogenase, and was first proposed by Senez and Azoulay (1961) and Chouteau et al. (1962) who described an
anaerobic dehydrogenation of n-heptane by crude extracts from *Pseudomonas aeruginosa* in the presence of NAD$^+$, and they also isolated 1-heptene. The mechanism of n-alkane dehydrogenation leading to the corresponding 1-alkene has also been found by others working with different alkanes and different microorganisms (Wagner *et al.*, 1967; Iizuka *et al.*, 1968,1969). Another report by Abbott and Casida (1968) showed that resting cells of *Nocardia salmonicolor* grown on glucose would oxidise hexadecane to a mixture of internal monohexadecenes; the principle product was 7-hexadecene. The most substantial evidence for this mechanism comes from a report by Parekh *et al.* (1977) who managed to isolate and purify an NAD-dependent n-alkane dehydrogenase and an NADPH-dependent alkene hydroxylase, from a *Pseudomonas* sp. grown anaerobically on hexadecane with nitrate as terminal electron acceptor. This organism grew very poorly on n-alkanes under aerobic conditions. The formed alkene was assumed to convert to primary alcohol by two means. Either water would be involved in the hydroxylation process (Iizuka *et al.*, 1968, 1969), or oxygenation of the double bond would lead to a 1,2-epoxide which would be reduced to the primary alcohol (Azoulay *et al.*, 1963).

\[
\begin{align*}
R-\text{CH}_2-\text{CH}_3 & \xrightarrow{\text{NAD}} R-\text{CH}==\text{CH}_2 \\
& \xrightarrow{\text{NADH}+\text{H}^+} R-\text{CH}==\text{CH}_2 \\
& \xrightarrow{\text{H}_2\text{O}} R-\text{CH}_2\text{OH}
\end{align*}
\]

However, in spite of the report from Parekh *et al.* (1977) which can be considered as a special case of anaerobic growth on n-alkanes (poor aerobic growth), there is no substantial evidence which would support this mechanism. Moreover, there are convincing arguments raised by Britton (1984) against this mechanism such as, the energetics of NAD$^+$ reduction by an alkane dehydrogenase are
unfavourable (Mckenna & Kallio, 1965). If primary alcohols are formed from hydration of alkenes, why most microbial systems require O$_2$? (Foster, 1962). If primary alcohols are formed via oxygenation of alkenes, why do n-alkanes and their corresponding 1-alkenes give rise to different oxidative products? (Klug & Markovetz, 1971).

Further oxidation of produced primary alcohols

Although there has been a lot of argument concerning the right mechanism for alkane oxidation to the corresponding alcohol, almost all workers agree that the produced alcohol is oxidised via aldehyde to the corresponding monocarboxylic acid, the reaction is illustrated as follows:

\[
\text{R-CH}_2\text{OH} \rightarrow \text{R-CHO} \rightarrow \text{R-COOH}
\]

This reaction is catalysed by alcohol and aldehyde dehydrogenases which in most cases requires NAD(P)$^+$ as cofactors in bacteria (Tassin & Vandecasteele, 1972), yeast (Liu & Johnson, 1971; Gallo & Azoulay, 1974; Gallo et al., 1974) and fungi (Walker & Cooney, 1973). However, an alcohol dehydrogenase with high affinity for long-chain primary alcohols has been purified from membranes of Pseudomonas aeruginosa, this enzyme did not require NAD$^+$ as cofactor instead it required phenazine methosulphate as an artificial electron acceptor (Tassin et al., 1973).

Subterminal Oxidation of n-Alkanes

Most microorganisms carry on alkane degradation by oxidising one of the two terminal methyl groups. Nevertheless, there have been several reviews regarding subterminal degradation (Britton, 1984; Bühler & Schindler, 1984). This type of reaction involves the oxidation of n-
alkanes at C₂ to form secondary alcohol followed by another oxidation reaction to yield the corresponding ketone (Klug & Markovetz, 1971). This system was first observed in microorganisms degrading short-chain n-alkanes such as propane and butane (Leadbetter & Foster, 1959b). More recently, cell suspensions of methane-utilising bacteria oxidised n-alkane (C₃-C₆) to their corresponding secondary alcohol and methyl ketones (Hou et al., 1979; Patel et al., 1980a,b). The reaction required oxygen and NADH (Patel et al., 1980a) and the formation of secondary alcohol was inhibited by methane suggesting that the methane monooxygenase was responsible for subterminal oxidation of n-alkanes (Patel et al., 1980b).

The further degradation of the ketones has been established in studies with Pseudomonas aeruginosa and is believed to be a Baeyer-Villiger type reaction [an oxygenase reaction which requires O₂ and NAD(P)H] (Britton et al., 1974) leading to the formation of ester intermediate (undecyl acetate) which is split into fatty acid (acetate) and primary alcohol (1-undecanol) (Forney et al., 1967; Forney & Markovetz, 1968, 1969, 1970). The alcohol is further oxidised to the corresponding fatty acid. This pathway received conformation by isolating an oxygenase enzyme which catalysed the conversion of ketones to esters (undecyl acetate) in Pseudomonas cepacia (Britton & Markovetz, 1977). The enzyme contains FAD and requires NADPH. Further conformation of the pathway came from Shum and Markovetz (1974a,b), who isolated an esterase from the same bacterium which is responsible for the cleavage of undecyl acetate. The enzyme was inducible when 2-tridecanone, 2-tridecanol, undecyl acetate were the growth substrate.

Other pathways of subterminal alkanes were reviewed by Rehm and Reiff (1981) and are represented in (Scheme 1).
Scheme 1. Subterminal degradation pathways of long-chain alkanes.
The image contains a chemical reaction diagram. The reactions described include:

1. One Baeyer-Villiger reaction:
   - $\text{H}_3\text{C}$(CH$_2$)$_n$-CH$_2$-(CH$_2$)$_n$-CH$_2$-(CH$_2$)$_n$CH$_3$
   - Reaction with $\text{O}_2$ or $\text{H}_2$
   - Further degradation

2. Two Baeyer-Villiger reactions:
   - $\text{H}_3\text{C}$(CH$_2$)$_n$-O-(CH$_2$)$_n$-O-(CH$_2$)$_n$CH$_3$
   - Reaction with $\text{O}_2$ or $\text{H}_2$
   - Further degradation

The diagram also shows the formation of $\text{R}_1$-$\text{O}$-(CH$_2$)$_n$CH$_3$ and $\text{R}_1$-OH HO-(CH$_2$)$_n$CH$_3$ through different pathways involving $\text{H}_2$O and further degradation of acid and alcohol.
**Metabolism of produced fatty acids**

Fatty acids which are formed from n-alkanes via terminal or subterminal oxidation can be incorporated directly into cellular lipids, therefore, almost all microorganisms which are growing on aliphatic hydrocarbons as a sole carbon source, incorporate a large number of the intermediate fatty acids into their lipids (chapter 4). Also, these fatty acids can be further metabolised by β-oxidation to produce acetyl-CoA which are metabolised via TCA cycle for energy production (Finnerty & Makula, 1975).

The initial reaction before β-oxidation takes place involves the conversion of the acid to the acyl-CoA thioester catalysed by acyl-CoA synthetase (Trust & Millis, 1970, 1971; Duvnjak et al., 1970; Calmes & Deal, 1973).

![Chemical reaction](image)

The role of yeast microbodies produced in some species during incubation with hydrocarbons, is believed to be involved in the metabolism of fatty acids that arise from alkanes oxidation (Kawamoto et al., 1978a; Fukui & Tanaka, 1979a,b, 1981; Numa, 1981). A model that has been revised by Yamada et al. (1980) (Fig. 4), implies that fatty alcohol derived from alkane is oxidised in microbodies, mitochondria as well as in microsomes to the level of fatty acid by dehydrogenase activates. In mitochondria and microsomes, fatty acid is activated to fatty acyl-CoA and is incorporated directly into cellular lipid. In microbodies, fatty acid is oxidised to acetyl-CoA (β-oxidation) which is transferred to the mitochondria to enter the TCA cycle. Furthermore, the produced acetyl-CoA can be further metabolised via glyoxylate cycle (Kawamoto et al., 1978b).
Figure 4. Model suggesting the role of yeast microbodies and Mitochondria in alkane metabolism. (Adapted from Kawamoto et al., 1978,a,b).

Enzymes: 1, cytochrome P-450; 2, NADPH-cytochrome C reductase; 3, alcohol dehydrogenase; 4, aldehyde dehydrogenase; 5, acyl-CoA synthase; 6, catalase; 7 isocitrate lyase; 8, malate synthase; 9, NADP-linked isocitrate dehydrogenase; 10, malate dehydrogenase; 11, citrate synthase; 12, aconitase; 13, NAD-linked isocitrate dehydrogenase; 14, carnitine acetyltransferase. Abbreviations used: Cit, citrate; Gyo, glyoxylate; iCit, isocitrate; Mal, malate; OxAc, oxalacetate; OxGlt, 2-oxoglutarate; Suc, succinate.
Another type of oxidation is the \(\alpha\)-oxidation, in which the fatty acid \(Ca\) is hydroxylated to form an \(\alpha\)-hydroxy fatty acid which will then undergo oxidative decarboxylation to form fatty acid which is shorter than the original one by a \(C_1\) unit (Gurr & James, 1975).

\[
R-(\text{CH}_2)_n-\text{CH}_2-\text{COOH} \rightarrow R-(\text{CH}_2)_n-\text{CH}-\text{COOH} \rightarrow R-(\text{CH}_2)_n-\text{COOH}
\]

The only direct evidence to support this type of oxidation was introduced by Yano and co-workers, who showed that washed-cell suspension of *Arthrobacter simplex* could oxidise palmitic acid to hydroxy palmitic acid (Yano *et al.*, 1969). Cells of the same bacterium grown on pentadecane readily oxidised \(\alpha\)-hydroxy palmitic acid to pentadecanoic acid (Yano *et al.*, 1971).

Third type of fatty acid oxidation was reported in alkane utilising microorganisms is \(\omega\)-oxidation which leads to the formation of either the dicarboxylic acids or to \(\omega\)-hydroxy fatty acids (Ali Khan *et al.*, 1964; Iizuka *et al.*, 1966, Jones & Howe, 1968b; Uchio & Shiio, 1972; Ratledge, 1987). Hill *et al.* (1986) have reported besides the formation of dicarboxylic acids, the formation of small amounts of \(3\)-hydroxy derivatives of the dicarboxylic acids. Shorter chain dicarboxylic acids were also isolated.

\[
\text{CH}_3-\text{CH}_2-(\text{CH}_2)_n-\text{COOH} \quad \text{CH}_3-\text{CH}-(\text{CH}_2)_n-\text{COOH}
\]

\[
\omega\)-hydroxy fatty acid \quad \beta\)-oxidation

\[
\text{HOCH}_2-\text{CH}_2-(\text{CH}_2)_n-\text{COOH} \rightarrow \text{HOOC-CH}_2-(\text{CH}_2)_n-\text{COOH}
\]

dicarboxylic acid
Another partial possible pathway of fatty acids that they undergo chain elongation by C₂ units (Rehm & Reiff, 1981). This pathway could be supported by the findings of high levels of longer-chain fatty acids in the lipids of organisms grown on shorter chain alkanes. Another evidence is the presence of high levels of even-chain fatty acids in the lipids of organisms grown on odd-chain alkanes (chapter 4) (Makula & Finntery, 1968a; Hug & Fiechter, 1973; Cerniglia & Perry, 1974). This could be explained as either an elongation system using propionate took place or an α-oxidation happened to the corresponding fatty acid produced from the odd chain alkane, followed by a C₂ elongation. (Ratledge, 1978, 1987).

Other pathways of fatty acid metabolism have been recognised in certain microorganisms such as the production of glycolipids in *Torulopsis gropengiesseri* (Jones & Howe, 1968b) and production of methyl branched-chain fatty acids from *Corynebacterium simplex* grown on C₁₄-C₁₆ n-alkanes (Yanagawa et al., 1972a). Scheme 2 summarises the metabolism of n-alkanes by microorganisms.

**OXIDATION OF BRANCHED-CHAIN ALIPHATICS**

This group of compounds are less attacked by microorganisms, this may be due to the fact that methyl branched alkanes hinder the uptake into the cell or they are incompatible with the enzymes for β-oxidation (Ratledge, 1978). Therefore, branched-chain aliphatics received relatively little attention in contrast to microbial n-alkane oxidation. The microbiological and biochemical studies related to branched-chain alkanes have been discussed in several reviews (Mckenna, 1972; Pirnik, 1977; Britton, 1984).
Scheme 2. Metabolism of n-alkanes by microorganisms * indicates mainly found as extracellular products.
Sub-terminal oxidations

HO-CH₂-CH₂-(CH₂)ₙ-CH₂-OH

Formation of esters

CH₃-CH₂-(CH₂)ₙ-CH₂-0-C-(CH₂)ₙ-CH₂-CH₃

Wax esters

Formation

CH₃-CH₂-(CH₂)ₙ-CHO

CH₃-CH₂-(CH₂)ₙ-COOH

ω-hydroxylation

Incorporation into cellular lipids, perhaps desaturation or elongation first (e.g. triacylglycerols, phospholipids)

α- and β-oxidation giving 2- and 3-hydroxy fatty acids

β-oxidation system

Glycosidation system

Glycolipids

Acetyl-CoA → TCA cycle → Energy production

Short chain dicarboxylic acids

β-oxidation system

OHC-CH₂-(CH₂)ₙ-COOH

OH

CH₃-CH-(CH₂)ₙ-COOH
Generally, these reviews indicated that 2-methyl-branched alkanes (iso-terminus) were more susceptible to biodegradation than 3-methyl (anteiso terminus) ones, and single branched chain alkanes are usually preferentially oxidised at the furthest end from the branch (Ratledge, 1978).

Early reports by Van der Linden and Thijsse (1965) and Thijsse and Van der Linden (1961, 1963) regarding 2-methylhexane metabolism by *P. aeruginosa* showed that the substrate was oxidised to 5-methylhexanoic acid, 3-methylbutyric acid and 2-methylhexanoic acid. The latter was produced in small amounts and was considered to represent a minor pathway. Further β-oxidation of 2-methylhexanoic acid will lead to the formation of propionic and butyric acids. In case of 5-methylhexanoic acids, β-oxidation can not continue past the formation of 3-methylbutanoyl-CoA.

Multiple branched alkanes such as pristane (2,6,10,14-tetramethylpentadecane) are less degradable compounds than the single branched compounds. Pristane is usually a non-metabolisable compound. However, several organisms have been isolated and found to be able to degrade it (Mckenna and Kallio, 1964; Mckenna, 1972; Pirnik, 1977). The mechanism of oxidation has been elucidated by Mckenna and Kallio (1971) using a *Corynebacterium* sp. and was confirmed by Pirnik et al. (1974) using *Brevibacterium erythrogenes* [re-classified as a member of the *Rhodococcus* group of coryneform bacteria (Pirnik, 1977)]. Scheme 3 summarises the mechanism of pristane degradation by the two organisms. Pristane is first oxidised to the corresponding pristanic acid (i.e 2,6,10,14-tetramethylpentadecanoic acid) which will be further oxidised to either pristandioic acid followed by sequential β-oxidation or 4,8,12-trimethyltridecanoic acid followed by β-oxidation. β-oxidation of the latter compound will lead to the formation
of 2,6,10-trimethylundecanoic acid which is considered as a metabolic dead end, because of the resulting 2-methyl branching pattern that inhibited activation of the fatty acid for an additional cycle of β-oxidation. If by any chance, the β-oxidation of the monoic acid is to proceed, the end product will be iso-buturyl-CoA.

The resulted dioic acid continues to oxidise through alternate cleavages of propionyl-CoA and acetyl-CoA units till 2-methylmalonic acid is formed which will be isomerised to form succinyl-CoA. It has been suggested that the dioic acid oxidation was the significant branch of pristane degradative pathway (Pirnik et al., 1974).
Scheme 3. Pathway of pristane oxidation (adapted from Mckenna & Kallio, 1971; Pirnik et al., 1974.)
Pristane

\[ \text{Pristane} \]

\[ \text{Pristanic acid} \]

**\( \omega \)-oxidation**

\[ \text{Pristanoic acid} \]

\[ \text{4,8,12-trimethyltridecanoic acid} \]

\[ \text{C}_{3} \text{ unit} \]

\[ \text{2,6,10-trimethyltridecandioic acid} \]

\[ \text{C}_{2} \text{ unit} \]

**\( \beta \)-oxidation**

\[ \text{C}_{5} \text{ unit} \]

\[ \text{2,6,10-trimethylundecanoic acid} \]

\[ \text{C}_{2} \text{ unit} \]

\[ \text{2,6,10-trimethylundecandioic acid} \]

\[ \text{C}_{3} \text{ unit} \]

\[ \text{2,6-dimethylundecanoic acid} \]

\[ \text{C}_{2} \text{ unit} \]

\[ \text{2,6-dimethylnonadioic acid} \]

\[ \text{C}_{3} \text{ unit} \]

\[ \text{2,6-dimethylheptandioic acid} \]

\[ \text{C}_{2} \text{ unit} \]

\[ \text{2-methylpentadioic acid} \]

\[ \text{C}_{3} \text{ unit} \]

\[ \text{2-methylpentanoic acid} \]

\[ \text{C}_{2} \text{ unit} \]

\[ \text{2-methylmalonic acid} \]

\[ \text{Succinic acid} \]
Another group of branched alkanes are quaternary carbon compounds which are highly resistant to biodegradation. Attempts made by Catelani, et al. (1977) to test Achromobacter sp. for its ability of utilising 2,2-dimethylheptane were unsuccessful, the organism attacked the unhindered terminus and accumulated 2,2-dimethylpropionic acid (pivalic acid). The acid is highly resistant to microbial attack and cannot be used by Achromobacter sp. as a sole source of carbon and energy.

Where an alkyl branch occurs at the anteiso-terminus, the alkyl group will prevent β-oxidation, requiring an additional strategy such as α-oxidation (Beam & Perry, 1974a). Hence, these anteiso-terminus (β-position) compounds are more resistant than iso-terminus compounds (α-position). This fact was confirmed by the work of Schaeffer et al. (1979), who screened 27 known alkane utilising microorganisms for their ability to grow 3-methyl (anteiso) or 2-methyl (ISO) substituted alkanes using 3,6-dimethyloctane (3,6-DMO) and 2,7-dimethyloctane (2,7-DMO). Only 9 were found to oxidise iso-terminus branched-chain alkanes. Anteiso termini prevented biodegradation. They also reported, by using Pseudomonas putida PpG6 and Pseudomonas putida AC4 both of which contain the OCT plasmid, that 2,7-DMO and 3,6-DMO caused a low-level induction of the OCT plasmid to produce alkane hydroxylase enzymes compared with octane and a gratuitous inducer of OCT (Van Eyk & Bartels, 1968; Grund et al., 1975). Another set of experiments with OCT plasmid hydroxylase enzymes being already induced showed that cellular oxidation of branched alkanes is severely limited by iso-termini and is blocked by anteiso-termini (Schaeffer et al., 1979).

The problem of an alkyl branch present at the anteiso-termini can be solved by removing the alkyl group as in isoprenoid alcohol oxidation by Pseudomonas citronellolis.
(Seubert & Fass, 1964). Fall et al. (1979) were able to accomplish a degradability of certain resistant branched hydrocarbons by using *Pseudomonas citronellololis* that contains the citronellol degradative pathway and at the same time has the ability to grow at C$_{12}$ to C$_{16}$ carbon alkanes. A decane-positive mutants (Dec$^+$) (can grow on decane) of this organism showed the ability to degrade 2,6-dimethyl-2-octene, 3,6-DMO and 2,6-dimethyldecane through the citronellol pathway, as determined by conversion of the branched hydrocarbons to citronellol, induction of geranyl-CoA carboxylase (Seubert et al., 1963; Cantwell et al., 1978) and β-decarboxymethylation of the branched hydrocarbon by the cells. Scheme 4 illustrates the mechanism by which certain organisms can degrade 3-methyl branched hydrocarbons, in which the citronellol pathway is combined with an alkane oxidation pathway.

Squalene which is an unsaturated methyl branched hydrocarbon containing 30 carbon atoms was found to be oxidisable by *Arthrobacter* sp. (Yamada et al., 1975, 1977). The degradation products were geranylacetone, which was not metabolised further, and the carboxylic acids geranic, β,β-dimethylacrylic, iso-valeric, and citronelic which all had β-substituted methyl group, therefore making them resistant for further metabolism (Britton, 1984).
1. Terminal Alkenes

Most of the research work on the degradation of olefinic hydrocarbons has been done on the terminal alkenes compounds rather than the internal ones. The initial attack may be at either terminal or at the double bond (Jones & Howe, 1968b; Klug & Markovetz, 1968). Therefore, a variety of products can be formed even in an axenic culture suggesting that microorganisms may use more than one mechanism in degrading alkenes (King & Perry, 1975), e.g. (a) ω-unsaturated alcohols or fatty acids, (b) primary or secondary alcohols or methyl ketones, (C) 1,2-epoxides, and (d) 1,2-diols (Britton, 1984). Britton (1984) illustrated the pathways of 1-alkene degradation (Scheme 5). This scheme does not necessarily represent the oxidation products of every 1-alkene-degrading microorganism.
Scheme 5. Proposed mechanisms for 1-alkene degradation (adapted from Britton, 1984).
I. (major) 
\( CH_3-(CH_2)_n-CH=CH_2 \)  
1-alkene

A. \( OH \) 
\( CH_2(CH_2)_n-CH=CH_2 \)  
\( \omega \)-unsaturated primary alcohol

B. primary saturated alcohol  
\( CH_3(CH_2)_n-CH_2-CH_2 \)  
\( OH \)

C. 1,2-epoxide  
\( CH_3(CH_2)_n-CH=CH_2 \)  
\( OH \)  
\( OH \)

D. secondary saturated alcohol  
\( CH_3(CH_2)_n-C-CH_3 \)  
\( \omega \)-unsaturated secondary alcohol

E. \( \omega \)-unsaturated ketone

\( OH \)  
\( CH_3(CH_2)_n-CH=CH_2 \)

\( \omega \)-unsaturated acid  
\( HOOC(CH_2)_n-CH=CH_2 \)

\( \omega \)-unsaturated fatty acid of the same length of 1-alkene substrate

\( CH_3(CH_2)_n-CH_2-CH_2-COOH \)  
\( CH_3(CH_2)_n-CH_2-COOH \)

\( \omega \)-oxidation

\( C_2 \) unit elongation  
\( \beta \)-oxidation

\( CH_3(CH_2)_n-CH-COOH \)  
\( \alpha \)-hydroxy acid

\( CH_3(CH_2)_n-COOH+CO_2 \)  
saturated fatty acid of chain length one less than the original 1-alkene substrate

\( \beta \)-oxidation  
\( C_2 \) unit elongation  
\( \omega \)-oxidation
It is believed that the primary pathway in alkene oxidation occurs via oxidation of the terminal methyl group at the saturated end of the molecule leading to the formation of $\omega$-unsaturated fatty acid (Stewart et al., 1960) (Scheme 5,A). These findings were confirmed by Van der Linden (1963) and Huybregts and Van der Linden (1964), who reported that in addition to formation of $\omega$-unsaturated acids by Pseudomonas, minor reactions at the double bond lead to the formation of epoxides, diols and $\alpha$-hydroxy acids (Scheme 5,C). May and Abbot (1972, 1973) demonstrated that the alkane-hydroxylating system of Pseudomonas oleovorans also catalysed the hydroxylation of the terminal methyl group at the saturated end as well as the epoxidation of the double bond (Scheme 5,A,C). Abbott and Hou (1973) found that there was no further attack on the epoxide group and further degradation happens by oxidation of the methyl group at the other end of the molecule. The epoxide can be either spontaneously hydrolysed to 1,2-diol without an epoxide hydrase being involved (Huybregtse & Van der Linden, 1964; Van der Linden & Thijsse, 1965), or an enzymatic hydration could be involved e.g. in Pseudomonad (Niehaus & Schroepfer, 1967). In both cases the formed diol will be oxidised to the 2-hydroxy acid which is subsequently degraded by decarboxylation to give the fatty acid of chain length one less than the original alkene (King & Perry, 1975; Jones & Howe, 1968b) (Scheme 5,C).

Evidence for diterminal oxidation have been presented by Jones and Howe (1968c), who isolated $\omega$-unsaturated acids, $\omega$-1-hydroxy acids and $\alpha$, $\omega$-dicarboxylic acids both having the same chain length and one carbon shorter than the substrate from cultures of Torulopsis gropengiesseri grown on 1-alkenes from C$_{14}$ to C$_{18}$. Saturated substrate-length fatty acids have been isolated from Mycobacterium vaccae (King & Perry, 1975) and Corynebacterium simplex (Yanagawa
et al., 1972b) indicating the possible saturation of the double bond at the level of the \( \omega \)-unsaturated fatty acid.

The evidence for pathways B and D (Scheme 5), which include the formation of saturated primary and secondary alcohols, respectively, was first shown in Pseudomonas aeruginosa (Iida & Iizuka, 1971) and Penicillium sp. (Allen and Markovetz, 1970) cultures. The two organisms showed ability to produce 2-tetradecanol and 2-tetradecanone from 1-tetradecene. Klug and Markovetz (1971) stated that methyl ketone as well as aldehydes could be formed by direct hydroxylation of the double bond. These compounds would be reduced to 1- and 2-alkanols. Another proposed mechanism for primary alcohol formation is that they are formed from epoxides by a reductase which cleave and reduce the C-2 side of the oxygen in the epoxide group (Azoulay et al., 1963). Klug and Markovetz (1971) used the same mechanism to justify the formation of secondary alcohols by assuming that the cleavage and reduction of the epoxide would happen on C-1 side of the oxygen. The secondary alcohols are probably further metabolised via the corresponding ketone with a subsequent subterminal oxidation leading to the formation of saturated fatty acid (Kallio, 1969) (Scheme 5,D). Moreover, mass spectral analysis of 1-decanol formed from 1-decene by Candida rugosa demonstrated that the oxygen used in hydroxylation was derived from water, therefore suggesting a hydrase mechanism (Iida & Iizuka, 1971).

The presence of \( \omega \)-unsaturated secondary alcohol in cultures of Candida lipolytica (Klug & Markovetz, 1967c) supports pathway E (Scheme 5). This alcohol could be further metabolised via \( \omega \)-unsaturated methyl ketone followed by subterminal oxidation. A Penicillium sp. also produced \( \omega \)-unsaturated ketones when grown on 1-tetradecene thus providing additional evidence for a subterminal evidence (Allen & Markovetz, 1970).
A good example for almost all the mechanisms proposed by Scheme 5 is *Candida lipolytica* since a diversity of compounds have been isolated from its cultures grown on 1-alkenes substrate (Bruyn, 1954; Klug & Markovetz, 1967c, 1968, 1969).

2. Degradation of Internal Alkenes

Only few reports dealt with internal alkenes degradation. King and Perry (1975) investigated the degradation of 8-heptadecene by *Mycobacterium vaccae*. They found that the major fatty acids produced were a result of conventional terminal oxidation with some shifting of the double bond. They also noticed that these unsaturated fatty acids could be methylated to form the corresponding saturated branched-chain fatty acids.

OXIDATION OF CYCLOPARAFFINS

This group of compounds are considered highly resistant to microbial attack. Early studies showing the inability of microorganisms to attack alicyclic hydrocarbons have been reported (Pelz & Rehm, 1971, 1972; Komagata et al., 1964). Other workers suggested that alicyclic hydrocarbons are completely resistant to microbial attack (Davis, 1967; Tokuyama & Kaneda, 1973). Studies have indicated that aliphatic hydrocarbons are most susceptible to microbial attack, followed by branched aliphatics and the aromatics. The most resistant are the cycloparaffins. As a result one might expect that these cyclic compounds tend to accumulate in nature (Perry, 1984). However, other workers succeeded in isolating several microorganisms which were able to utilise unsubstituted alicyclic hydrocarbons (e.g. Stirling et al., 1977) and alkyl substituted alicyclics (e.g. Tonge & Higgins, 1974).
1. **Metabolism of Unsubstituted Alicyclic Hydrocarbons**

There has been a lot of early reports regarding utilisation of unsaturated alicyclic hydrocarbons especially on cyclohexane (Tausz & Peter, 1919; Fredericks, 1966; Skarzynski & Czekalowski, 1946; Jones & Edington, 1968). These reports were doubtful for some reason or another (e.g. purity of substrates are questionable and contradictory results reported).

The first reliable results were obtained by Stirling *et al.* (1977), who isolated a *Nocardia* sp. from an estuarine mud flat by using methyl cyclohexane as a carbon source. This organism was able to utilise cyclohexane as a growth substrate. Cell-free extracts of the organism contained a cyclohexanol dehydrogenase, cyclohexanone monoxygenase and $\varepsilon$-caprolactone hydrolase. Stirling and Perry (1980) isolated and purified the cyclohexanol dehydrogenase from the same *Nocardia* strain and was found to be NAD-dependant secondary alcohol dehydrogenase. This enzyme showed broad specificity towards cycloalkanols, substituted cycloalkanols (e.g. 2-methylcyclohexanol and cyclohexane diols). The suggested pathway for cyclohexane degradation is shown in Scheme 6. This pathway was found also in *Pseudomonas* sp. which was isolated from ash wood soil by Anderson *et al.* (1980). This *Pseudomonas* had the ability to grow on cyclohexane [it is possible that this organism represents a re-isolation of the same organism which was first reported as *Pseudomonas aeruginosa* by Imelik (1948)], n-hexadecane, benzene, cyclohexane-oxide and methyl cyclohexane. Cell free extract of *Pseudomonas* sp. had a labile cyclohexane hydroxylase activity which was $O_2$ and NADH dependent.
Scheme 6. Proposed pathway for cyclohexane biodegradation by *Nocardia* and *Pseudomonas* isolated from esturaine mud flat and ashwood soil respectively.
2. Metabolism of Alkyl-Substituted Alicyclic Hydrocarbons

In 1969, Arai and Yamada, isolated an ethylcyclohexane-utilising bacteria. They found that a strain of *Alcaligenes faecalis* excreted trans-4-ethylcyclohexanol into the medium during growth on ethylcyclohexane. Similar findings were reported in case of *Pseudomonas aeruginosa* growing on the same substrate (Van Ravenswaay Claasen & Van der Linden, 1971).

Tonge and Higgins (1974) reported a strain of *Nocardia petroleophila* to be efficient in methylcyclohexane utilisation (0.75 g of cells dry weight/g methyl cyclohexane), but growth was slow (generation time 40 hr.). During growth of this organism on methylcyclohexane, 3-methylcyclohexanol and 3-methylcyclohexanone were excreted into the medium suggesting that both are intermediates of methylcyclohexane metabolism.

The metabolism of cyclohexanes substituted with straight-chain alkyl side chains has been reported (Beam & Perry, 1974b). Different species of *Mycobacterium* (*M. vaccae*, *M. rhodochrous* and *M. convolutum*) were found to attack the terminal methyl of the alkyl chain of dodecylcyclohexane causing the formation of the corresponding cyclohexyl fatty acids which is further degraded by β-oxidation as indicated by the accumulation of cyclohexylacetic acid. Cyclohexylacetic acid is converted to cyclohexanecetyl-CoA which is cleaved to acetyl-CoA and cyclohexanone. The latter is catabolised through the pathway illustrated in Scheme 6. Furthermore, the formed cyclohexyl fatty acid could be directly incorporated into the phospholipids without undergoing further degradation. Also, α-oxidation of the cyclohexyl fatty acids could happen as indicated by the presence of cyclohexylpropionate.
and the occurrence of odd-chain fatty acid in the phospholipids following growth on dodecylcyclohexane.

The microbial attack of alkyl substituted cycloparaffins could either happen on the side chain or on the ring. The site of attack will be determined by different factors e.g. nature of the compound and the microbe. Initial microbial attack of the side chain was reported by the early work of Davis and Raymond (1961) and was confirmed subsequently (Beam & Perry, 1974a; Feinberg et al., 1980). Other researchers confirmed the initial ring attack by microorganisms and presented evidence for it (Ooyama & Foster, 1965; Arai & Yamada, 1969; Van Ravenswaay Claasen & Van der Linden, 1971).

There were also reports regarding p-menthane (a substituted cyclohexane) degradation. Tsukamoto et al. (1975, 1977) isolated a Pseudomonas mendocina which can hydroxylate this compound to form cis-p-1-methaneol. The enzyme catalysed the reaction is a monooxygenase.

\[
\begin{align*}
\text{CH}_3 & \quad \text{CH}_3 \\
\text{CH}_3 & \quad \text{CH}_3
\end{align*}
\]

3. Metabolism of Dicyclics

Microbial degradation of dicyclics has been investigated almost thirty years ago, when Colla and Treccani (1960) tested a Flavobacterium and unidentified bacterial strain isolated from soil for their ability to degrade decalin. They identified adipic acid in young cultures by using paper chromatography. They also isolated pimelic acid. Treccani (1965) suggested that the pimelic acid is an artifact, formed in a non-enzymatic reaction.
because decalin-grown cells could not oxidise pimelic, whereas they oxidised adipic acid. However, Blakley (1978) stated that if cyclohexane carboxylic acid occurred as an intermediate in decalin biodegradation, the isolation of pimelic acid would not be unexpected.

\[
\begin{array}{ccc}
\text{Decalin} & \overset{\text{Oxidation}}{\rightarrow} & \text{Pimelic acid} \\
\end{array}
\]

**OXIDATION OF AROMATIC HYDROCARBONS**

Although bacteria and fungi utilise aromatic hydrocarbons, they have different attacking mechanisms. Bacterial oxidation of aromatic hydrocarbons occurs by incorporating two atoms of molecular oxygen into the aromatic ring to form a dihydrodiol with a cis-configuration (Gibson, 1977). The reaction is catalysed by a dioxygenase which is a multicomponent enzyme system, consisting of a flavoprotein, an iron-sulphur protein, and a ferredoxin (Yeh et al., 1977; Crutcher & Geary, 1979). The formed dihydrodiol is further oxidised to form catechols, which undergo ring fission via ortho pathway (i.e. cleavage of the bond between carbon atoms of the two hydroxyl groups of catechol) to yield cis,cis-muconic acid or via meta pathway (i.e. cleavage of the bond between a carbon atom with a hydroxyl group and the adjacent atom) to yield 2-hydroxy muconic semialdehyde (Dagley, 1971) (Scheme 7).

Oxidation of aromatic hydrocarbons by fungi is due to the presence of cytochrome P-450 that catalyse monooxygenase and epoxide hydroxylase reactions to form trans-dihydrodiols (Cerniglia, 1981).
Scheme 7. Pathways of aromatic hydrocarbon by prokaryotic and eukaryotic microorganisms.
CHAPTER FOUR
CELLULAR AND EXTRACELLULAR LIPIDS OF HYDROCARBON-UTILISING MICROORGANISMS

The importance of lipids in the overall structure and function of membrane has been frequently emphasised. The most well-defined function of membrane lipids is that they are involved [e.g. phosphatidylcholine (PC)] in bilayer structure of membranes. However, the presence of many distinct classes of lipids in biomembranes suggests that lipids also play other functional roles (Cullis & De Kruijff, 1979). Lipids are also involved in a variety of cellular functions, for example, in binding of toxins (Donovan et al., 1981, 1982; Gill, 1976), in oxidative phosphorylation (Nelson & Fleischer, 1981), in membrane transport (Milner & Kaback, 1970; Prasad et al., 1975a,b), in membrane-bound enzymes (Sandermann, 1978), and in other general functions related to membrane stability (Bloch, 1979, 1981; Banerji, et al., 1982).

The lipid content of microbial cells can vary significantly both in amount and composition, depending on growth conditions such as temperature, pH, cell age, medium composition, carbon and nitrogen source, additional growth factors and others (Prasad, 1985). Several reviews have dealt with cellular and extracellular lipids as well as fatty acids composition of alkane utilising microorganisms (Rehm & Reiff, 1981; Singer & Finnerty, 1984). This chapter will shed light on the most important work which had been carried out in this field.

BACTERIA

Total fatty acids derived from Acinetobacter strain H01-N (formerly known as Micrococcus cerificans) grown at the expense of odd- and even-carbon n-alkanes have been 81
studied by Makula and Finnerty (1968a). They demonstrated that cultures grown with a variety of nonhydrocarbon substrates serving as sole carbon and energy source yielded only even-carbon fatty acids, whereas even-chain n-alkanes, C\textsubscript{12} through C\textsubscript{18} resulted in even-carbon fatty acids with direct correlation between carbon number of the major fatty acid species and carbon number of the alkane substrate (Makula & Finnerty, 1968a; Patrick & Dugan, 1974). Fatty acid composition resulting from the growth of Acinetobacter H01-N at the expense of odd-carbon n-alkanes, C\textsubscript{11} through C\textsubscript{17} yielded both odd- and even carbon fatty acids, with a transitional shift from even-carbon fatty acids to odd-carbon fatty acids as the carbon number of the alkane substrate increased. Similar results were obtained by Patrick and Dugan (1974) who isolated the polar lipid fraction of an Acinetobacter sp. growing on n-nonane (C\textsubscript{9}). Makula and Finnerty (1968a) found that unsaturated fatty acids (\(\Delta^9\) monounsaturated) comprised a significant percentage of all profiles tested. In a subsequent study, Makula and Finnerty (1972) studied the distribution of cellular fatty acids in defined lipid classes analysed in Acinetobacter H01-N after growth on nutrient broth, tetradecane (C\textsubscript{14}), pentadecane (C\textsubscript{15}), hexadecane (C\textsubscript{16}) and heptadecane (C\textsubscript{17}). They showed that fatty acids obtained from neutral lipid and the phospholipid fractions reflect the alkane chain length used as substrate. An extracellular accumulation of free fatty acid (FFA) was also demonstrated in C\textsubscript{16}-grown cultures that was not apparent in non-hydrocarbon-grown cells. These FFA appeared in the culture broth as direct oxidation products of C\textsubscript{16} metabolism. Based on the above results Makula and Finnerty reported a partial inhibition of de novo long-chain fatty acid biosynthesis from C\textsubscript{2} units due to the preferential incorporation of fatty acids derived from C\textsubscript{16} oxidation.
Patrick and Dugan (1974) reported that polar lipid fractions of an *Acinetobacter* sp. growing on dotriacontane (C₃₂), 1-chlorohexadecane, 1-chlorododecane, 1-chlorodecane, and 1-phenyldodecane contained significant amounts of odd-chain fatty acids in addition to the even ones. Furthermore, cells grown on long-chain alcohols exhibited fatty acid profiles nearly identical to those of cells grown on the corresponding n-alkanes.

Makula and Finnerty (1968b) studied the fatty acid profiles of *Acinetobacter* sp. derived from 1-alkenes. They showed that saturated fatty acids containing even numbers of carbon atoms were produced from 1-dodecene and 1-tetradecene. Growth on 1-pentadecene resulted in the isolation of 14-pentadecenoic acid, indicating methyl-group attack. Growth on 1-hexadecene and 1-octadecene indicated simultaneous methyl group and double-bond attack (chapter 3).

Makula and Finnerty (1970) studied the distribution of phospholipids derived from *Acinetobacter* grown on hexadecane, heptadecane, or acetate serving as the sole carbon source. They showed that major phospholipids were PE, phosphatidylglycerols (PG), and cardiolipin (CL). Minor phospholipids were phosphatidylglycerol phosphate (PGP) and phosphatidylserine (PS). Trace amounts of methylated derivatives of PE were determined by incorporation of ¹⁴C from ¹⁴C-methylmethionine. These workers also reported the presence of phosphatidyl-N-methylethanolamine, phosphatidyl-N,N'-dimethyl-ethanolamine, and PC in trace quantities. Pulse labeling with ¹⁴C-serine demonstrated the direct incorporation of serine into PS followed by decarboxylation to PE (Makula & Finnerty, 1970).

Qualitative and quantitative changes in the lipid composition of hexadecane-grown *Acinetobacter* species H01-N
have been studied by Makula et al. (1975). The cellular lipids obtained from hexadecane-grown cells were characterised by 3- and 18-fold increases in the phospholipid fraction and mono- and diacylglycerol fractions, respectively, over that obtained from nutrient broth-yeast extract-grown cells. The qualitative composition of the cellular lipids were closely similar with the exception that hexadecane comprised 8 to 10% of the total dry cell weight of hexadecane-grown cells. The extracellular lipids obtained from the culture broth of hexadecane-grown cells were comprised of triacylglycerol, mono- and diacylglycerol, FFA, and wax ester. These lipids were either absent or present in minor concentrations in the culture broths of nutrient broth-yeast extract-grown cells. Makula et al. (1975) showed that the exponential growth of Acinetobacter sp. on hexadecane was characterised by the significant accumulation of FFA, monoacylglycerol, and diacylglycerol in the culture medium. However, wax ester did not accumulate extracellularly until the cells entered the stationary phase of growth, thus relating wax ester synthesis to the stationary growth phase.

The increased cellular phospholipid could be explained, in part, by the induction of new membrane synthesis in response to growth on hexadecane and intracellular, membrane-limited, inclusion bodies containing unmodified alkane (chapter 2).

The fatty acid composition of Mycobacterium sp. strain OFS grown on hydrocarbon and non-hydrocarbon substrates was investigated by Dunlap and Perry (1967, 1968). The fatty acids resulting from growth of this species on n-alkanes from C13 to C17 were of the same chain length as the substrate (Dunlap & Perry, 1967). The major fatty acids resulting from growing this organism on different n-alkanes and 1-alkenes, were C16, C16:1, C18:1 and branched
nonadecanoic (Br-C\textsubscript{19}) acids (Dunlap & Perry, 1968). A high concentration of C\textsubscript{15} saturated fatty acid in the cells grown on C\textsubscript{24} and C\textsubscript{28} n-alkanes was reported. n-Alkanes shorter than C\textsubscript{13} or longer than C\textsubscript{17} were not incorporated into cellular fatty acids without some degradation (Dunlap & Perry, 1968). Dunlap and Perry (1968) showed that strain OFS incorporated C\textsubscript{14} to C\textsubscript{17} 1-alkenes into cellular fatty acids as the \(\omega\)-monoenoic fatty acid.

King and Perry (1975) examined the fatty acid pattern in Mycobacterium vaccae strain JOB5 after growth on n-alkanes (C\textsubscript{14}-C\textsubscript{18}), 1-alkenes (C\textsubscript{14}-C\textsubscript{18}), 2- or 3-methyl octadecane, and 8-heptadecene. They showed that moniterminal oxidation of n-alkanes was followed by \(\beta\)-oxidation and that both parent fatty acid and products of \(\beta\)-oxidation were incorporated into cellular lipids. Using \([\textsuperscript{14}C]\) acetate, they demonstrated that there was desaturation of long-chain fatty acids. However, fatty acid chain elongation was not proven. Growth on 1-alkenes results in the incorporation of fatty acids that were products of two primary modes of oxidation: (1) methyl group attack resulting in \(\omega\)-unsaturated fatty acids (chapter 3, scheme 5,A) and (2) double-bond attack resulting in the removal of one carbon from the substrate (chapter 3, scheme 5,C). These workers showed that strain JOB5 grown on 2- and 3-methyl octadecane contained the corresponding iso- or anteiso-fatty acids in significant quantity. Cells cultured on 8-heptadecene contained 8- and 9-heptadecenoic acids, 6- and 7-pentadecenoic acids, 9- and 10-methylheptadecanoic acids, and 7- and 8-methylpentadecanoic acids. Fatty acid composition (C\textsubscript{13} to C\textsubscript{19}) was affected by substrate chain length and was additionally modified by cellular control mechanisms (King & Perry, 1975).

Vestal and Perry (1971) studied the lipid composition of Mycobacterium vaccae strain JOB5 grown on gaseous alkanes.
(C₂–C₄) and showed that it contained considerably more lipid than cells harvested after growth on C₅ to C₇ n-alkanes or on non-hydrocarbon substrates. Propane-grown cells contained more neutral lipid (34.4%) than acetate-grown cells (19.4%) and there was a correspondingly greater percentage of phospholipid in acetate-grown cells. The major phospholipids obtained were CL, PE and PS, and these three components make up 93 to 96% of the total phospholipids (Vestal & Perry, 1971). The major difference between Mycobacterium vaccae strain JOB5 cells grown on hydrocarbon and non-hydrocarbon substrates was in neutral lipid and most markedly in triacylglycerol level which was increased on propane-grown cells. Vestal and Perry (1971) suggested that these glycerides may play a role in the uptake and accumulation of lyophobic hydrocarbon molecules.

In a subsequent study, Hallas and Vestal (1978) studied the lipid composition of Mycobacterium convolutum strain R22 grown on a wide range of both odd- and even-numbered carbon solid n-alkanes and showed that cellular lipid was 2.5 times higher following growth on the hydrocarbon substrates as compared to acetate grown cells. The amount of polar lipid was found to be about half of the cellular lipid in docosane (C₂₂) through octacosane (C₂₈)-grown cells. PE represented about 50% of the polar phospholipid in hydrocarbon- and acetate-grown cells. PS, diphosphatidylglycerol (DPG), and phosphatidic acid (PA) were also detected. The amount of PS was higher (10-14%) in C₂₂ through C₂₈-grown cells and there was a correspondingly smaller amount of DPG compared to acetate-grown cells. These workers suggested that these lipid changes may be associated with the assimilation of the hydrophobic substrates (Hallas & Vestal, 1978). De novo fatty acid synthesis in Mycobacterium convolutum strain was suppressed in solid n-alkane-grown cells, but transport or incorporation of [¹⁴C] acetate into the cells was not affected by solid n-alkanes. Polar lipid fatty acid
analyses indicated there was no direct incorporation of the oxidised substrate. In case of C\textsubscript{19} and C\textsubscript{20}-grown cells, the predominant fatty acids were derived from \(\beta\)-oxidation of the substrate to form C\textsubscript{15} and C\textsubscript{17} fatty acids, and C\textsubscript{16} and C\textsubscript{18} fatty acids, respectively. However, in C\textsubscript{22}-, C\textsubscript{23}-, C\textsubscript{24}-, C\textsubscript{26}-, and C\textsubscript{28}-grown cells, there was an increase in the amount of C\textsubscript{15} fatty acid from 10 to 45\%. Hallas and Vestal (1978) suggested that \(\beta\)-oxidation and a subterminal oxidation cleavage appeared to be the major catabolic routes providing fatty acids, which are then incorporated into lipid.

Raymond and Davis (1960) observed a marked increase in the lipid content of a *Nocardia* sp. grown on liquid n-alkanes such as n-octadecane (C\textsubscript{18}) and n-hexadecane (C\textsubscript{16}). Cells grown on C\textsubscript{16} and C\textsubscript{18} contained about 70\% lipid material. In contrast, cells grown on n-hexane and n-tridecane contained about the same amounts of lipids as glucose-grown cells. The extracted lipid consisted of 60\% triacylglycerols and 40\% aliphatic waxes. Nocardial cells grown on C\textsubscript{16} yielded cetyl palmitate (C\textsubscript{32}) as the principal wax and C\textsubscript{18}-grown cells yielded principally octadecyl stearate (C\textsubscript{36}) (Raymond & Davis, 1960). Davis (1964) grew a *Nocardia* sp. on n-alkanes with 13 to 20 carbon atoms; he found fatty acids of cellular triacylglycerols and aliphatic waxes with carbon skeletons analogous to the carbon skeleton of the n-alkane substrate on which the organism was grown. The alcohol component of waxes produced by the *Nocardia* was invariably of the same chain length as the n-alkane substrate (Davis, 1964). Dunlap and Perry (1967) reported that the predominant fatty acids in propane-grown *Nocardia* species OC2A were C\textsubscript{15}, C\textsubscript{17}, C\textsubscript{17:1}, C\textsubscript{18:1} and Br-C\textsubscript{18}, whereas in acetate-grown cells, the predominant fatty acids were C\textsubscript{16}, C\textsubscript{16:1}, C\textsubscript{18:1}, and Br-C\textsubscript{19}. This strain contained approximately 25\% more lipids after growth on propane than was found in acetate grown cells. Although the cell yield
was significantly lower with propane than observed with acetate.

Romero and Brenner (1966) grew *Pseudomonas aeruginosa* on hexadecane and found significant quantities of saturated fatty acids with 14 to 22 carbons and large quantities of cellular lipids in the cell-free filtrates. The fatty acid composition of triacylglycerols showed that linoleic acid (C_{18:2}) was the major acid present, with palmitic (C_{16:0}) and oleic (C_{18:1}) acids next in concentration. Other saturated and monounsaturated acids were also found (Romero & Brenner, 1966). A major difference was found between the fatty acid composition of neutral and polar lipids. An important difference in the polar lipids was shown by the presence of only traces of C_{18:2} acid and a higher concentration of stearic acid (C_{18:0}). Romero and Brenner showed that hydroxylated fatty acids were principally found in two polar fractions containing rhamnose and glucose, the other polar fraction, containing serine, alanine, ethanolamine, and leucine, was richer in monoenoic fatty acids (Romero & Brenner, 1966).

Lipids were also extracted from cells of *Pseudomonas aeruginosa* grown on a pure hydrocarbon (tridecane), mixed hydrocarbons (JP-4 jet fuel) and on trypticase soya broth (TSB) by Edmonds and Cooney (1969). Growth of *Pseudomonas aeruginosa* on hydrocarbons did not increase the lipid content over that of cells grown on TSB. The average total lipid content of cells (% dry weight) was 7.95, 7.08, and 8.22 of cells from JP-4 jet fuel, tridecane, and TSB, respectively. Free lipids were separated into four fractions termed as "polar, acid, neutral and hydrocarbon fractions". Neutral fraction was the largest fraction. Cells grown on each medium contained saturated and unsaturated C_{14} to C_{20} fatty acids. The unsaturated methylesters were C_{16:1}, C_{18:1} and two unknown components,
while saturated C\textsubscript{16} and C\textsubscript{18} were the major components in cells from each medium (Edmonds & Cooney, 1969). Edmonds and Cooney showed that tridecane-grown cells contained only traces of C\textsubscript{13} and small amounts of C\textsubscript{15} and C\textsubscript{17} acids, and they suggested that the organism's fatty acids were derived from de novo synthesis rather than by direct incorporation of the hydrocarbon.

YEASTS

There are numerous reports on the total lipid contents of various species of yeast (Hunter & Rose, 1972; Rattray \textit{et al.}, 1975; Kaneko \textit{et al.}, 1976; Weete, 1980; Prasad, 1985; Ghannoum \textit{et al.}, 1986) which demonstrated that total lipid levels vary among different strains of different species or similar species. Kaneko \textit{et al.} (1976) compared the lipid composition of 30 species of different yeasts, and it was shown that the level of total lipids varies from as low as 7\% to as high as 32\% of dry cell weight. The predominant component among neutral lipids is triacylglycerol (which was observed to range from 50 to 90\% of the total neutral lipid), whereas the levels of sterol esters, free sterols, and free fatty acids vary between trace amounts to 7-8\%. Yeast contains a typical eukaryotic mixture of phospholipids (Henry \textit{et al.}, 1981). Most yeast species have a phospholipid content of 3 to 7\% of the cell dry weight (Letters, 1968). PC, PS, PE, phosphatidylinositol (PI), and CL constitute the major phospholipids of almost all the yeasts (Prasad, 1985). The minor phospholipids include various lysoderivatives of PG, PGP, DPG, PA, phosphatidylinositol (PI), and the triphosphoinositol (TPI) (Getz, \textit{et al.}, 1970; Jakovcic, \textit{et al.}, 1971; Steiner & Lester, 1972; Weete, 1980). Like higher eukaryotes, most of the yeast phospholipids are characterised by a high level
of unsaturated fatty acid. However, the positional
distribution of fatty acids varies among different
phospholipids of the same or different species (Waechter &
Lester, 1973).

Considerable attention has been focused on the growth
of yeast on by-products of petroleum industry. Although
Saccharomyces cerevisiae and many other yeasts are unable to
utilise various hydrocarbons (Markovetz & Kallio, 1964;
Scheda & Bos, 1966), considerable attention has been focused
on certain genera, especially Candida and Torulopsis (Scheda
& Bos, 1966; Klug & Markovetz, 1971; Bird & Molton, 1972;
Thorpe & Ratledge, 1972; Mishina, et al., 1973; Singh et
al., 1978, 1979a,b). Growth preferentially occurs on n-
alkanes with chain length C_{10} to C_{18}, but most of the
hydrocarbon-utilising yeasts seem unable to metabolise
shorter chain-length alkanes (C_{5} to C_{9}) (Prasad, 1985).

Quantitative rather than qualitative differences have
been found in the cell lipid of several yeast strains grown
on n-alkanes (Pelechova et al., 1971). When Candida
albicans cells were grown with n-alkanes of varying chain
length (C_{10} to C_{18}), growth response was variable, with no
growth on decane (C_{10}) and dodecane (C_{12}), while the growth
rate was slow in smaller chain-length alkanes (C_{13} and C_{14})
as compared to cells grown in longer chain-length (C_{16}
to C_{18}) (Singh et al., 1978, 1979a,b). Singh et al. (1978)
showed that with the increase in chain-length of n-alkanes
(C_{13} to C_{18}), there was a gradual increase in total lipid
content. Furthermore, there was about 9-fold increase in
total lipid content in C_{17} and C_{18} grown cells as compared
with the glucose grown cells (Singh et al., 1978). Mishina
et al. (1977) observed an increase in total lipid contents
when Candida tropicalis and Candida lipolytica cells were
grown on n-alkanes. On the other hand, Thorpe and Ratledge
(1972) reported that when grown on n-alkanes, Candida 107
and Candida tropicalis produced less lipid than when grown on glucose, and they suggested that this may only be a reflection of the method of cultivation.

The composition of lipid obtained after growth of Candida species on individual n-alkanes has been studied (Harries & Ratledge, 1969; Ratledge, 1970; Thorpe & Ratledge, 1972; Singh et al., 1978). It has been shown that increasing alkane chain length generally results in increased amounts of triacylglycerols but decreased quantities of (sterol + partial glyceride) and (steryl esters + wax) (Rattray et al., 1975). Thorpe and Ratledge (1972) showed that Candida 107 and Candida tropicalis contained a significant proportion of triacylglycerols in which the 2-position was occupied by a saturated acid. This was most evident for Candida 107 grown on n-tetradecane and n-pentadecane, where, although the proportion of unsaturated acids was the same as that when glucose had been used as carbon source, 46% and 28%, respectively of the total triacylglycerols contained a saturated acid in the 2-position (Thorpe & Ratledge, 1972). Singh et al. (1978) reported that Candida albicans grown on alkanes of different chain length (C₁₃ to C₁₈), did not show a significant difference in total glyceride content.

An increase in cellular phospholipid was achieved with Candida 107 grown on n-alkanes rather than glucose (Thorpe & Ratledge, 1972). Diatlovickaja et al., (1968) studied the phospholipid derived from Candida tropicalis grown on C₁₄ to C₂₀ n-alkanes, and it was determined as percentage of the total as follows: 41% PC, 37% PE, 8% PS, 5% lysophosphatidylcholine (LPC), 10% fully acylated DPG, and 5% fully acylated PG. A higher level of unsaturated and short-chain fatty acids is associated with phospholipid than with triacylglycerols (Diatlovickaja et al., 1968; Thorpe & Ratledge, 1973). Heptadeca-9,12-dienoic acid occurs
primarily at position 2 of the phospholipid molecule (Diatlovickaja et al., 1968). The increased level of cellular phospholipids in yeasts grown on n-alkanes is associated with the proliferation of the cell plasma membrane and intracytoplasmic elements (chapter 2). Variations in the fatty acid composition of mitochondria obtained from yeast grown on different n-alkanes have been found to influence membrane fluidity and enzyme activity (Skipton et al., 1973, 1974). Furthermore, transport of several compounds via the plasma membrane could be affected by a change in the environment brought about by the altered lipid composition. In this respect, certain amino acid transport in Candida albicans grown on n-alkanes was affected dramatically by altering the phospholipid and ergosterol content of the tested organism (Singh et al., 1978, 1979a). Prasad and his group used various approaches to alter specific lipids of yeast membrane, to explore their effect on amino acid transport and they demonstrated the importance of phospholipids in yeast membrane transport (Trivedi et al., 1982a,b; 1983a,b; Prasad et al., 1983).

The commercial production of lipids by alkane-grown yeast has been considered (Rattray, 1975). The main potential advantage would appear to lie in the biosynthesis of specific fatty acids, e.g. odd-chain acids (Ratledge, 1971). Tanaka et al. (1971), working with mixtures of n-alkanes as substrate, found that the level of ergosterol was approximately 1.0% of the dry weight of Candida tropicalis compared with 0.6% for cells grown on glucose. The ergosterol produced by growing this organism on n-alkane mixture accounted approximately to 5.8 mg/g dry cells after five days cultivation. They suggested that Candida tropicalis pK 233 is a good source of ergosterol for the manufacture of vitamin D.
The fatty acid composition of alkane-grown cells and its membrane fractions depend upon the type of alkane used as the carbon source (Ratledge, 1970; Pelechova et al., 1971; Singh, 1979; Prasad, 1985), the growth phase of the cells (Mishina et al., 1973; Volfova & Pecka, 1973) as well as the temperature and nitrogen source used in the medium (Greshnykh et al., 1968). Shorter chain n-alkanes (<C₁₃) are converted to various fatty acids apparently through the simultaneous operation of a chain elongation pathway and de novo synthesis after β-oxidation of the substrate (Rattray et al., 1975). On the other hand, the longer chain n-alkanes (>C₁₃) yielded primarily fatty acids having the same chain length as the hydrocarbon substrates (Mishina et al., 1973). Unsaturated fatty acid is more efficiently derived from even chain rather than odd chain n-alkanes (Hug & Fiechter, 1973). Mishina et al. (1973) and Thorpe and Ratledge (1972) reported that a particular accumulation of C₁₇:1 acid occurred in Candida lipolytica and Candida tropicalis grown on C₁₁ to C₁₇ odd-chain n-alkanes. Significant conversion of odd-chain n-alkanes to even-chain fatty acid can also occur (Gill & Ratledge, 1973; Hug & Fiechter, 1973; Mishina et al., 1973). In general, the occurrence of C₁₆ and C₁₈ acids has been found to be much lower for yeast grown on hydrocarbon (<C₁₆) compared to glucose or acetate (Ratledge, 1968; Hornei et al., 1972; Hug & Fiechter, 1973).

FILAMENTOUS FUNGI

The utilisation of hydrocarbon substrates by filamentous fungi has received considerable attention (chapter 1). In contrast, the direct incorporation of hydrocarbon substrates into the lipids of filamentous fungi has received little attention.
The fatty acid pattern of Cunninghamella elegans and Penicillium zonatum was determined after growth on acetate, propionate, and n-alkanes (C<sub>13</sub> to C<sub>15</sub>) and 1-alkenes (C<sub>14</sub> to C<sub>18</sub>) by Cerniglia and Perry (1974). They reported that the fatty acid profile of acetate-grown cells showed a predominance of even-carbon fatty acids (C<sub>16</sub>, C<sub>18:1</sub>, C<sub>18:2</sub>), whereas cells grown on propionate showed significantly higher levels of odd-carbon fatty acids (C<sub>15</sub>, C<sub>17</sub>, C<sub>17:1</sub>). Growth on n-alkanes resulted in the incorporation of fatty acids homologous to the growth substrate. The presence, in Cunninghamella elegans, of C<sub>15</sub> (6.2%) and C<sub>17</sub> (3.8%) acids with n-tridecane and C<sub>17</sub> (21.8%) acid with n-pentadecane as substrate led these workers to suggest the addition of 2 or 4 carbons to the fatty acid precursor (Cerniglia & Perry, 1974). They also showed that these fungi contained, predominantly, fatty acids with 18 carbons in length. Consistent level of 18-carbon fatty acids in n-tetradecane- and n-pentadecane-grown fungi (42% in n-tetradecane and 39 and 38% in n-pentadecane-grown cells of Cunninghamella elegans and Penicillium zonatum, respectively) suggests that these fatty acids might be a product of de novo synthesis (Cerniglia & Perry, 1974). Cunninghamella elegans grown on the 1-alkenes from C<sub>14</sub> to C<sub>18</sub> incorporated the unsaturated substrate into cellular fatty acid after oxidation at the saturated end of the molecule (Cerniglia & Perry, 1974).

Fatty acid composition of Cladosporium resinae grown on glucose, Jet-A commercial aviation fuel, and on a series of n-alkanes, C<sub>10</sub> through C<sub>14</sub> was studied by Cooney and Proby (1971). They showed that the predominant fatty acids were C<sub>16:0</sub>, C<sub>18:1</sub>, and C<sub>18:2</sub>, which represented 84 to 92% of the total. In glucose-grown cells odd-carbon fatty acids comprised 2.2% of the total, and 77.8% of the fatty acids present were unsaturated, while in C<sub>10</sub>-grown cells odd-carbon fatty acids represented 1.8%, unsaturated acids comprised 75.9% (Cooney & Proby, 1971). Cells grown on n-
tridecane or n-tetradecane yielded small amounts of acids homologous to the carbon source, but a similar correlation was not noted for n-decane, n-undecane, or n-dodecane. Cells grown on n-undecane or n-tridecane contained more odd-carbon fatty acids (3.2% and 8.4%, respectively) than cells grown on the other substrates. These workers suggested that the fatty acids of *Cladosporium resinae* are derived chiefly from de novo synthesis rather than from direct incorporation of oxidised hydrocarbons (Cooney & Proby, 1971; Kan & Cooney, 1975). They also showed that the extent of direct incorporation increased as the chain length of the hydrocarbon growth substrate was increased (Cooney & Proby, 1971). In a subsequent work, Siporin and Cooney (1975) studied the extracellular lipids of *Cladosporium resinae* grown on glucose, n-dodecane, or n-hexadecane, and showed that neutral lipids were the major lipid fraction and triacylglycerols were the only extracellular neutral lipids detected. Lauric (C_{12:0}) acid was the predominant fatty acid (> 60%) in neutral lipids from all three media, with lesser amounts of myristic (C_{14:0}), palmitic (C_{16:0}) and stearic (C_{18:0}) acids. Extracellular phospholipids identified were PC, PS, PE, and CL or a cardiolipin-like compound (Siporin & Cooney, 1975). Extracellular neutral lipids, phospholipids and FFA contained C_{12:0} acid as their principle fatty acid, and it was the only extracellular FFA detected by Siporin and Cooney (1975). These workers concluded that the fatty acids of extra-cellular lipids do not reflect the chain length of the n-alkane growth substrate (Siporin & Cooney, 1975). According to Kan and Cooney (1975) cellular lipids of *Cladosporium resinae* contain PE and PC as their major phospholipids, with lesser amounts of PS and traces of CL-like compound. Miyazima et al. (1985a) reported that PC, PS, PI, PE and CL were the major phospholipids isolated from an *Aspergillus* sp. grown on glucose or n-alkanes (C_{11}-C_{16}) as a sole carbon and energy sources. Fatty acid analysis of phospholipid fractions
suggested that cellular fatty acids were mainly formed by de novo synthesis. In another study Miyazima et al. (1985b) isolated the non-polar lipids from the same Aspergillus sp. and the major lipids were FFA and triacylglycerols. Other lipids such as sterols, sterol esters, and diacylglycerols were also detected. The results obtained by Miyazima et al. (1985b) indicated that n-alkanes as substrates influenced the incorporation of fatty acids into triacylglycerols.

To summarise, growth of microorganisms on hydrocarbons mainly results in an increase in the cellular lipids, quantitative rather than qualitative changes were observed. Some microorganisms growing on hydrocarbons, oxidise certain n-alkanes or alkenes to the corresponding fatty acids then incorporate them directly into their cellular lipids. Some times direct incorporation of the homologous acid is limited to hydrocarbons with specific chain length. Chain elongation of the formed fatty acid might take place as well. Other microorganisms metabolise the products of initial oxidation via β-oxidation to acetyl-CoA then synthesize cellular fatty acids de novo.
CHAPTER FIVE
MATERIALS AND METHODS

ORGANISMS

Bacteria

Two strains of *Rhodococcus rhodochrous* were used in this study: *R. rhodochrous* KUCC 8801 and KUCC 8802. These were isolated from soil samples contaminated with crude oil from Almagwa oil fields (Al-Ahmadi, Kuwait). The two bacteria as well as other bacterial species were identified by consulting the Bergey's Manual, 9th ed. The identities of the above mentioned *R. rhodochrous* strains were confirmed by the National Collections of Industrial and Marine Bacteria, Scotland. Oil degrading cultures of *Rhodococcus* sp. IS01, *Acinetobacter calcoaceticus* IR07 and *Pseudomonas putida* IR32 were kind gifts from J. Oudot, Museum National d'Histoire Naturelle, Paris, and were used in comparative studies.

Fungi

*Candida albicans* KTCC 89062 was also used throughout this study. This organism was isolated from soil samples taken from a site that was contaminated with crude oil (Almagwa oil field, Al-Ahmadi, Kuwait). The yeast isolate was identified using the criteria of germ-tube formation and the use of API C20 identification system (Montalieu-vercieu, France). Germ-tube formation was performed by using the method of Ghannoum *et al.* (1985). A $10^4$ cells/ml inoculum was added to tubes containing 5 ml of new born calf serum (Gibco, Grand Island, N.Y.) and incubated in a shaking water bath at 37°C for 2-5 h, and examined by phase contrast
microscope (x400) for the formation of germ-tubes. The isolate was considered to have formed a germ-tube, when a narrow tube of at least 2 μm long, extended from the mother cell.

Other fungal isolates Aspergillus terreus, A. sulphureus, Mucor globosus, Fusarium sp., and Penicillium citrinum were identified by referring to Booth (1971), Raper and Fennel (1977), Pitt (1979) and Domsch et al. (1980).

CULTURAL CONDITIONS

R. rhodochrous KUCC 8801 and KUCC 8802 were maintained on nutrient agar slants (Difco) and on chemically defined medium (CDM) slants supplemented with 1% (v/v) filter sterilised crude oil. C. albicans KTCC 89062 was maintained on the above CDM slants as well as on Sabouraud dextrose agar slants (Difco). Slants were stored at 4°C and sub-cultured every 5 to 6 weeks onto fresh slants.

CRUDE OIL AND HYDROCARBONS

Kuwaiti crude oil from the "Kuwait Oil Company" in Al-Ahmadi, Kuwait, was used in these studies. Crude oil was weathered by sparging it with nitrogen gas (flow rate 50 cm³/l/min) for 24 h at 25°C to remove gaseous hydrocarbons which might be toxic to microorganisms (Britton, 1984). The oil was subsequently heated in a water bath at 60°C for 1 h to decrease viscosity then sterilised by membrane filtration, pore size 0.45 μm (Whatman's Maidstone, U.K.).
Hydrocarbons used during these studies were: n-alkanes [hexane, heptane, nonane, decane, dodecane, tetradecane, hexadecane, octadecane, and eicosane (Fluka AG)], 1-alkene [tridecene (Fluka AG)], branched chain [iso-octane (Fluka AG)] and aromatic hydrocarbons [benzene (J.T. Baker chemical Co.), phenanthrene (Winlab Limited, U.K.), anthracene and naphthalene (Sigma Chemical Company, St. Louis, Missouri)]. Liquid hydrocarbons were sterilised by membrane filtration, pore size 0.45 μm.

MEDIA AND BUFFER SOLUTIONS

A chemically defined medium (CDM) was used as the basal medium for all studies (unless otherwise specified). This medium has the following composition:

A - Salts (g/l)

<table>
<thead>
<tr>
<th>Compound</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sodium nitrate (NaNO₃)</td>
<td>0.85</td>
</tr>
<tr>
<td>Potassium dihydrogen orthophosphate (KH₂PO₄)</td>
<td>0.56</td>
</tr>
<tr>
<td>Disodium hydrogen phosphate (Na₂HPO₄)</td>
<td>0.86</td>
</tr>
<tr>
<td>Potassium sulphate (K₂SO₄)</td>
<td>0.17</td>
</tr>
<tr>
<td>*Magnesium sulphate (MgSO₄·7H₂O)</td>
<td>0.37</td>
</tr>
<tr>
<td>Calcium chloride (CaCl₂·2H₂O)</td>
<td>0.007</td>
</tr>
<tr>
<td>Fe (III).EDTA</td>
<td>0.004</td>
</tr>
</tbody>
</table>
* Prepared as a stock solution (4 g/100 ml), sterilised by autoclaving at 121°C for 15 min, then added to the medium.

Before sterilisation, the above medium was supplemented with 2.5 ml trace element solution consisting of:

**B - Trace Element Solutions (g/l)**

<table>
<thead>
<tr>
<th>Component</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>Zinc sulphate (ZnSO₄·7H₂O)</td>
<td>2.32</td>
</tr>
<tr>
<td>Manganese sulphate (MnSO₄·4H₂O)</td>
<td>1.78</td>
</tr>
<tr>
<td>Boric acid (H₃BO₃)</td>
<td>0.56</td>
</tr>
<tr>
<td>Cupric sulphate (CuSO₄·5H₂O)</td>
<td>1.00</td>
</tr>
<tr>
<td>Sodium molybdate (Na₂MoO₄·2H₂O)</td>
<td>0.39</td>
</tr>
<tr>
<td>Cobalt chloride (CoCl₂·6H₂O)</td>
<td>0.42</td>
</tr>
<tr>
<td>Potassium iodide (KI)</td>
<td>0.66</td>
</tr>
<tr>
<td>EDTA</td>
<td>1.00</td>
</tr>
<tr>
<td>Ferrous sulphate (FeSO₄·7H₂O)</td>
<td>0.40</td>
</tr>
<tr>
<td>Nickel chloride (NiCl₂·6H₂O)</td>
<td>0.004</td>
</tr>
</tbody>
</table>

\[ \text{pH} = 6.8 \pm 0.2 \]

For sea water and salt marsh samples 30 g/l sodium chloride (NaCl) was added to the medium. The above medium was sterilised by autoclaving at 121°C and 15 lb/in² for 15 min.

For isolating and enumerating fungi, (CDM) was supplemented with 10 ml/l vitamin solution composed of (mg/l):

101
Vitamin Solution

Biotin 0.01
Pyridoxin 2.0
Nicotinic acid 2.0
Thiamin 2.0
Calcium pantothenate 2.0
Meso-inositol 10.0

Sterilised by membrane filtration (0.45 μm).

The pH of media was adjusted to 7 or 5.6 by using $\text{Na}_2\text{HPO}_4/\text{NaH}_2\text{PO}_4$ buffer system to favour bacteria and fungi, respectively. For solid media, 20 g/l agar (Difco) was added to the CDM.

Oil Agar Plates

The crude oil can be introduced onto the chemically defined medium plates by spreading 0.1 ml filter sterilised weathered crude oil (1:1 v/v with CCl$_4$) over the agar surface with a spreader. The agar was allowed to dry out for several days at room temperature to ensure the adsorption of the mixture. The plates were then dried to allow evaporation of all the CCl$_4$. This technique ensured even dispersal of oil on the medium.

ENUMERATION OF OIL UTILISING MICROORGANISMS

Desert and garden soil samples, as well as samples from oil fields and salt marshes were collected from different localities in Kuwait (Fig. 5). The samples were taken
aseptically (using sterile universal bottles) after removing the 5 cm surface layers. Water and marine sediment samples were collected 20 m offshore from the Arabian Gulf at about 10 km intervals; CDM supplemented with 10 g/l weathered crude oil, as a sole source of carbon, was used for counting microorganisms. The pH was adjusted to 7 and 5 to favour bacteria and fungi, respectively. The microbial numbers were monitored using spread plate count technique (Koch, 1981). In which dilutions for soil and sea sediment samples were prepared by using 0.9% (w/v) NaCl (saline). One gram soil or sea sediment samples were added to 10 ml sterile saline, serial dilutions were carried out by transferring, each time, 1 ml to a fresh 9 ml sterile saline. Triplicate plates from two separate serial dilutions were incubated for 7 and 14 days before counting. Mean values were calculated and the number of organisms was recorded as number per gram dry weight sample. For sea water analysis 100 ml water samples were filtered by using sterilised membrane filters (pore size 0.45 μm). The membrane filters were then aseptically transferred to Petri dishes containing oil agar media and incubated for similar periods. Triplicate plates were prepared, the mean values were calculated and the number of organisms was recorded as number per 100 ml of sample. The incubation temperature was 25°C and 30°C for fungi and bacteria, respectively.
Figure 5. The map of Kuwait showing sites of collection

- Oil field
- Garden soil
- Oil polluted ports
- Swimming beach
In addition to the methods mentioned above two methods were used to isolate oil utilising microorganisms:

1. In situ technique using Golf balls (Gilbert & Higgins, 1978).

This technique was used to isolate oil utilisers from the marine environment. A 47 mm diameter cellulose acetate filter disc (8 μm pore size) was impregnated with weathered crude oil and inserted through a slit made in a perforated plastic practice golf ball to form an equatorial diaphragm. After rescaling the ball with a hot knife, it was placed into a perforated container which was suspended in sea water for 15 days. The filter disc was then cut to 15 mm circles using a sterilised cork borer and shaken vigorously in sea water with 2 mm glass beads for 5 min to release the microorganisms which were plated on oil agar plates and purified by subsequent culturing.

2. Laboratory enrichments technique.

The technique was used to isolate microorganisms from soil, sea sediment and salt marsh samples. A 2 g sample, collected from areas of oil contamination was added to flasks containing 50 ml CDM supplemented with 50 μl weathered crude oil. The flasks were incubated at 30°C in an orbital shaker (160 rpm). After 48 h, 2 ml suspensions were removed from the old cultures and transferred to fresh flasks containing 50 ml CDM supplemented with 500 μm weathered crude oil, which were incubated at 30°C in the orbital shaker (160) rpm
for a further 48 h. After the second transfer 10 ml suspensions were removed and centrifuged at 3000 rpm for 20 min. This step produced a cell pellet and an oily pellicle which were streaked onto oil agar plates. Pure colonies were obtained by further subculturing.

**GROWTH STUDIES**

**Growth of Selected Organisms on Pure Hydrocarbons**

In qualitative studies, two methods were used to investigate the growth of selected microorganisms on pure hydrocarbons.

1. Vapour phase technique:

   This technique was used for liquid hydrocarbons. Bacteria and fungi were grown in nutrient broth and potato dextrose broth (Difco), respectively. Cells were harvested by centrifugation at 4000 (rpm), washed three times with sterile saline then suspended in saline. Bacteria and fungi were inoculated onto CDM, without oil in Petri dishes. Sterile filter papers, placed in the lids, were impregnated, each with 0.5 ml of filter sterilised hydrocarbons. The bases of the plates were then replaced, kept inverted and the plates sealed with masking tape to minimise loss of vapour.

2. Droplet dispersion technique:

   This technique was used for higher molecular weight hydrocarbons, which are solid at room temperature. Hydrocarbons were first dissolved in
diethylether to give 0.1% solutions (w/v) and 1 ml of each solution was sprayed in a fume cupboard as a thin even film over dried uninoculated CDM plates. After the diethyl ether had evaporated the test microorganisms were streaked and the plates sealed.

Plates obtained by both methods as well as control plates (treated similarly except that hydrocarbons were not introduced) were incubated at 30°C and 25°C for 7 and 14 days to favour the growth of bacteria and fungi, respectively. Plates were examined for colony development, which was accompanied, in case of the droplet dispersion technique plates, by a clearing zone around the opaque solid hydrocarbon.

For quantitative studies, 0.2 ml of cells suspensions (10^7 cells/ml) were inoculated into 50 ml CDM containing 10 g/l of each hydrocarbon. The cultures were incubated in an orbital shaker (160 rpm) for 7 days in case of bacterial growth and 14 days in case of fungal growth. The biomass was collected by centrifugation for bacteria and filtration for fungi, quickly washed with hexane to remove attached hydrocarbons (Cerniglia & Perry, 1974), then dried overnight at 105°C after which the cells were weighed.

**Measuring Growth Rate of C. albicans KTCC 89062 by Viable Cell Count**

0.2 ml of cells suspensions (10^7 cells/ml) were inoculated into 50 ml CDM containing either 1% (w/v) glucose or 1% (v/v) of the tested hydrocarbon. These flasks were incubated in an orbital shaker (160 rpm) at 37°C. One ml samples were withdrawn from the flasks at 60 min intervals and introduced to test tubes containing 9 ml sterile saline.
One ml samples of appropriate dilutions were then plated in triplicate in 6 mm Petri dishes in which 6 ml Sabouraud dextrose agar was introduced after cooling to about 45°C. The plates were left to dry at room temperature and incubated at 37°C. Colonies were counted after 48 h using colony counter (Gallenkamp colony counter, U.K.) and the results were expressed as log viable counts per ml. Since filamentation was observed under the microscope, it was necessary to estimate the effect of hydrocarbons on growth using other dry weight determinations.

**Measuring Growth Rate of Microorganisms by Dry Weight Determination**

Hydrocarbon utilising organisms were grown overnight in 50 ml conventional media (i.e nutrient broth for *R. rhodochrous* spp. and Sabouraud dextrose broth (Difco) for *C. albicans* KTCC 89062. These cultures were added to 450 ml of the same fresh conventional media which were incubated overnight at 37°C to get a high yield of cells. Cells were harvested by centrifugation (4000 rpm) washed three times with CDM then suspended in the same medium (1 l) without hydrocarbon, and 20 ml samples were dispensed in sterile conical flasks. To each flask 0.2 ml of hydrocarbon was added. Cultures were incubated at 37°C in orbital shaker (160 rpm). At different time intervals triplicate samples were removed, either centrifuged (in case of *C. albicans*) at 4000 rpm for 15 min or filtered (in case of *R. rhodochrous*) using membrane filters (pore size 0.45 μm). Cells were washed with hexane to remove attached hydrocarbons (El-Aassar, 1988), then dried overnight at 105°C in an oven (Memmert, W. Germany). The biomass was determined by weighing (Mettler, AE 163, Switzerland).
EFFECT OF HYDROCARBONS ON CELL MORPHOLOGY

Scanning Electron Microscopy (SEM)

*R. rhodochrous* KUCC 8801, 8802 and *C. albicans* KTCC 89062 were grown in CDM containing either 1% (w/v) glucose or 1% (v/v) pure hydrocarbons for 72 and 24 h, respectively at 37°C in an orbital shaker. Cells were collected by centrifugation (4000 rpm, 15 min) washed with (0.9%, w/v) saline and suspended in glutaraldehyde (1.5%, w/v TAAB Laboratories, Reading, U.K.) for 2 min. Cells were re-centrifuged and resuspended in a fresh sample (about 2 ml) of the same fixative for 16 h at 4°C. Fixed cells were prepared for SEM as described earlier by Ghannoum and Al-Khars (1984). Cells were collected by centrifugation (4000 rpm, 15 min), washed three times in buffer, dehydrated by applying a series of ethanol (50% to 100%) and suspended in 100% ethanol to give the required density. A drop of the suspension was transferred to a microscope cover slip and left to air-dry in a desiccator containing anhydrous CaCl₂. The samples were then coated with gold-palladium (60:40) in a high vacuum unit (Hummer X sputter Coater, Technics Inc., Alexandra, VA) to obtain a coating of approximately 2 nm thickness. These samples were examined in a stereoscan electron microscope (Novascan 30, W. Germany) at an angle of 45°, and operated at 15 kV with a resolution of 10 Å. Photographs were obtained using polaroid film (Polaroid, 4 x 5 Land film, image 9 x 11.5 cm, Type 55, U.S.A.).

Transmission Electron Microscopy

Microorganisms were grown in CDM containing either 1% (w/v) glucose or 1% (v/v) pure hydrocarbons for 24 h at 37°C in an orbital shaker. Cells were collected by centrifugation
(4000 rpm, 15 min), washed three times with (0.9%, w/v) saline and fixed in 2.5% (v/v) glutaraldehyde in 0.1 M sodium cacodylate buffer, pH 7.0, at 4°C for 2 h. Cells were then placed in freshly made 2% (w/v) KMnO₄ solution at 4°C for 2 h. The cells were centrifuged at 4200 rpm for 15 min and placed in a fresh solution of KMnO₄ for 2 h. The cells were washed five times with 0.9% (w/v) saline by repeated centrifugation. They were then placed in a solution containing 1% (w/v) potassium dichromate and 1% uranyl acetate for 2 h at 4°C. These were washed several times with distilled water and embedded in 2% (w/v) Bacto-agar (Difco), left to set and cut into small cubes (0.5-1.0 mm³) which were dehydrated through an ethanol series [30, 50, 70, 90 & 100% (v/v)]. The 100% ethanol was replaced with propylene oxide (TAAB Laboratories, Reading) twice for 20 min and samples were embedded in Epon (TAAB Laboratories, Reading) by graded impregnation. TAAB 812 permix kit hard was used: Hardener (1) Dodecenyl succinic anhydride (D.D.S.A.) 100 ml quantity, Hardener (2) Methylendomethyleneephthalic anhydride (M.N.A.) 50 ml quantity, and Accelerator tri-dimethylaminemethylphenol (D.M.P.-30) in 5 ml quantity. 50 ml of M.N.A. was added to 100 ml of D.D.S.A. and the contents of the ampoules of accelerator (5 ml) was added. This was mixed thoroughly, and approximately 100 g of resin was ready for use. The mixture can be stored for about 3 months.

All samples were embedded in beam capsules (TAAB Laboratories, Reading) and ultrathin sections prepared using Reichert-Jung Ultramicrotome (W. Germany) with a glass knives (angle 55°) and collected in a water trough using New 200 Athene grids. Sections were post-stained with uranyl acetate (dissolved in 70% ethanol) for 20 min and lead citrate (TAAB Laboratories, Reading), (0.04%) in solution.
with sodium hydroxide, 0.1 N, carbonate free for 5 min. Extensive surface washing was done, first with sodium hydroxide (carbonate free) and then with distilled water. These were dried using millipore filters. Electron micrographs were recorded with JOEL 100 CX electron microscopy operated at 60 or 80 kV accelerating voltage.

ANALYTICAL TECHNIQUES

Crude Oil Samples

The biodegradation of crude oil was studied by gas liquid chromatography (GLC). 0.2 ml cells suspended in sterile saline (10^7 cells/ml) were used to inoculate 50 ml (CDM) supplemented with 1% (v/v) crude oil. Cultures were incubated at 30°C for 7 days in case of bacterial cultures and 25°C for 14 days when fungi were tested for oil degradation efficiency. The residual oil was recovered from the supernatant liquid of bacterial and fungal cultures by extracting three times with 10 ml hexane. The organic layer containing residual crude oil, was collected each time, combined together and Na₂SO₄ (anhydrous) was added to absorb any water remaining. The constituent hydrocarbons were resolved by GLC using a Pye-Unicam 204 instrument equipped with an SP-2100 column with a temperature programme 60-250°C, raising the temperature 16°C/min. Individual hydrocarbons were identified by comparing their retention times with those of standard samples. The identities of individual components were further confirmed by comparison of retention times of the peaks with those of reference standards using 3% OV-1 as a second column. The GLC profiles were compared with the profile of the same amount of crude oil treated similarly but without inoculation with
microorganisms (control). Oil consumption was estimated quantitatively by comparing the total peak area in the GLC-profile of residual oil to that in the GLC-profile of the same amount of oil recovered from control samples. To minimise quantitative errors the external standardisation method was used employing a precision syringe to ensure that reproducible sample sizes could be injected onto the column (Lee et al., 1984). Triplicate analysis of the same sample were reproducible to within ± 5%.

**Individual Hydrocarbon Samples**

For measuring the uptake of individual hydrocarbons by microorganisms, two techniques involving the use of infrared spectrophotometry (I.R) and GLC were applied:

1. Measuring the uptake of hydrocarbons using IR spectrophotometer.

*R. rhodochrous* KUCC 8801 and KUCC 8802 were quantitatively investigated for their ability to utilise pure hydrocarbons by using IR spectrophotometer (Willard et al., 1981). Different concentrations of filter sterilised dodecane [5, 10, 15 and 2.0%, (v/v)] were added to 50 ml of CDM in 250 ml Erlenmeyer flasks. These flasks were inoculated with 0.1 ml suspension (10^7 cells/ml) of an overnight culture of bacterial cells in phosphate buffer. Uninoculated sterile controls were also prepared by adding 10 mg of HgCl₂ per flask. Both test and control flasks were incubated in a rotary shaker at 30°C. Flasks were removed at different time intervals (0, 5, 10 and 15 days) and contents of each flask were sonicated for 5 min at 20-40 KHz and 140 W using MSE Sonicator. The samples were
acidified with 1 ml 1 M HCl and 3 g NaCl. The contents were then transferred into a separatory funnel and extracted (3 times) with 15 ml CCl₄ (spectra grade). A sample of the CCl₄ was withdrawn and transferred into a cell of 10 mm path length where the IR-spectra were recorded using a Perkin-Elmer 398 IR spectrophotometer. CCl₄ was routinely incorporated as a reference. The sum of peak heights above the base line at 3.50, 3.42 and 3.38 μm, representing -CH stretching frequencies of -CH₃ and -CH₂ was taken as a quantitative measurement of the amount of dodecane. The amount of dodecane was determined with reference to a standard curve constructed using a range of dodecane concentrations in CCl₄ (prepared in volumetric flasks) to correspond with the amount used in the flasks.


Post-growth utilisation of dodecane by R. rhodochrous KUCC 8801, was estimated quantitatively and compared with the activity of certain bacteria known to be efficient oil degraders (Oudot, personal communication). Cells were grown overnight in 50 ml nutrient broth (Difco). This culture was used to inoculate 450 ml of nutrient broth which was incubated overnight at 37°C. Cells were harvested by centrifugation, washed three times with CDM then suspended in CDM without dodecane to end up with a cell number = 10⁷ cells/ml and 20 ml samples were dispensed in sterile conical flasks. To each flask 0.2 ml of dodecane was added. Cultures were incubated at 37°C in orbital shaker (160 rpm). At different time intervals samples were removed, and one only was supplied with
0.2 ml of dodecane to serve as a control. The cells were harvested by centrifugation and the supernatant retained. Cells were subsequently washed 3 times with boiling water to remove adhered dodecane, centrifuged and the resulting supernatant retained and added to the previous supernatant. Dodecane was then recovered by extracting 3 times with 10 ml hexane or heptane using a separatory funnel and the organic layer was collected after drying using anhydrous Na₂SO₄. The uptake of hydrocarbon was estimated by GLC analysis using SP-2100 column and 3% OV-1 as a second column. Triplicate samples were eluted at 140°C with helium as a carrier gas (45 ml/min) and the hydrocarbons detected with flame ionisation detector. The peak areas were compared with those of zero time and the percent uptake was calculated.

Lipid Extraction and Analysis

The total lipids were extracted from samples of the tested organisms grown for stationary phase on CDM supplemented with either 1% (w/v) glucose, or 1% (v/v) n-alkanes with different chain lengths as sole sources of carbon and energy. Cells were harvested by centrifugation (4000 rpm, 15 min), washed 3 times with boiling water and resuspended in propan-2-ol and incubated at 70°C for 45 min, this step was included in order to inactivate degradative enzymes such as phospholipases (Hitchcock et al., 1986). The suspension was cooled to 25°C, re-centrifuged and the supernatant retained. Cells were then extracted with chloroform/methanol (2:1, v/v) three times and purified using established procedures (Folch et al., 1957). Crude lipids were dissolved in 100 ml chloroform/methanol (2:1, v/v) and washed twice with 30 ml water saturated with
chloroform (30 ml water + 5 ml CHCl₃) using separatory funnel. The lower layer in both extractions was collected, combined and dried using rotary evaporator. The pure lipids were kept for 30 min in a desiccator containing CaCl₂ anhydrous then weighed using analytical balance. Since relatively large amounts of the tested hydrocarbon absorbed by the cells usually interfered with analysis of the cells lipids, the hydrocarbon fractions were eliminated from all extracts. This was achieved by preparative thin layer chromatography on plates of silica gel G [Merck Kieselgel G, type 60, (0.5 mm thickness)] using petroleum ether (40-60°C) as a solvent (Vachon et al., 1982). After discarding the front zone of the sorbent carrying the hydrocarbons, total lipids were recovered from the sorbent by eluting five times with chloroform:diethylether:ethanol (1:1:1, v/v/v) (Kates, 1972). The extract was analysed by thin layer chromatography (TLC) on plates of silica gel G [Merck Kieselgel G, type 60, (0.25 mm thickness)]. Apolar classes were fractionated using hexane:diethylether:acetic acid (90:10:1 or 75:25:1, v/v/v) (Mangold & Malins, 1960). Ionic and other polar lipids were resolved by two-dimensional chromatography using chloroform:methanol:7N ammonium hydroxide (65:25:4, v/v/v) in the first direction and chloroform:methanol:acetic acid:water (170:25:25:4, v/v/v/v) in the second direction (Nichols, 1964). The lipid fractions were detected with iodine vapour or by charring at 220°C after spraying the chromatoplates with 50% H₂SO₄. Charred fractions were measured densitometrically using Bechman R-112 Densitometer. Individual classes were identified by comparing their chromatographic behaviour with that of authentic samples and by using specific spray reagents (Dittmer & Lester, 1964; Siakotos & Rouser, 1965; Stahl, 1962; Vioque, 1984). The following spray reagents were used:
1. Dragendorff’s reagent used for phosphatidylcholine (PC) identification.

   (a) 40 g potassium iodide dissolved in 100 ml distilled water

   (b) 1.7 g bismuth subnitrate dissolved in 100 ml of 20% acetic acid

5 ml of solution (a) was mixed with 20 ml of solution (b) then diluted to 75 ml to give the desired spray reagent. The TLC plate was sprayed with the reagent and PC appeared as orange-red spot.

2. Lifschutz reagent used for sterols identification.

   The plate was sprayed with Lifschutz reagent [concentrated sulphuric acid:glacial acetic acid (1:1, v/v)] then heated in an oven at 120°C for 5 min. Sterols appeared as red or purple spots.

3. α-Naphthol reagent used for glycolipids identification.

   The plate was left to dry after running the TLC, then sprayed with α-naphthol reagent till it is wet, and left to dry. The plate was sprayed with 50% sulphuric acid and heated in an oven at 220°C for 20 min. Glycolipids appeared as purple spots.

The identity of phospholipid fraction isolated from R. rhodochrous spp. by TLC was confirmed by the analysis of their partial degradation products, after mild hydrolysis, using two dimensional paper chromatography (Dawson 1984).
The IR spectra of fractions isolated by preparative TLC were recorded using a Perkin-Elmer 398 IR-spectrophotometer, the UV spectra using a Pye-Unicam SP 8000 equipment and the 1H-NMR spectra using a Varian analytical instrument. The results were compared with spectra of authentic samples. Mass spectrometric analysis was done using an equipment of Kratos (MS80/DS90, double focusing reverse near-Johnson geometry). Total lipids and individual lipid classes resolved by two-dimensional TLC (Radwan, 1978) were subjected to methanolation using the following method (Chalvardjian, 1964):

For each 2 mg total lipids, 4 ml of 1% sulphuric acid in absolute methanol was added and the mixture heated under nitrogen gas at 90°C for 90 min. The reaction mixture was cooled to room temperature and the resulting methyl esters were extracted five times with diethyl ether. The resulting methyl esters were purified by TLC and analysed by gas chromatography [Pye-Unicam 204 fitted with a flame ionisation detector, an integrator (SP 4290) and a glass column, 2 m x 4 mm, i.d., packed with 15% DEGS on Anakrom D, 100-120 mesh at a temperature of 180°C, with nitrogen as a carrier gas. Each analysis was repeated using the non-polar column 10% Silar CP on Gas-Chrom Q].

For extracellular lipids extraction, the supernatant resulted from harvesting the cells for lipid extraction is further tested using the method of Bligh and Dyer. The supernatant filtered using membrane filters (pore size 0.45 μm). Chloroform and methanol were added to the supernatant to achieve the following proportionalities, chloroform: methanol:supernatant (1:2:0.8, v/v/v). Chloroform and supernatant were then added with mixing to arrive at the final proportions, chloroform:methanol:supernatant (2:2:1.8, v/v/v) (Stein & Smith, 1982). This separated into two phases if left for an hour, the lower layer contained the
lipids. The extraction was completed by rotary evaporation under vacuum at 50°C. The upper phase was extracted as a check that all the lipids was in the lower phase. The extracts were purified from excess alkane by following the method of Vachon et al. (1982) and the lipids were recovered as described earlier. Lipids were resolved by TLC using chloroform:methanol:acetic acid (85:20:1, v/v/v). In order to determine the emulsifier activity of extracellular lipids, different amounts of the crude extracellular lipids were dissolved in 0.1 ml of 2-methyl naphthalene:hexadecane mixture (1:1, w/v). The resulting solution is completed to 6.5 ml using Tris-buffer (0.02 M, pH 7.2) then sonicated for 2 seconds at 20-40 KHz and 140 W using MSE sonicator. The produced emulsion is allowed to stand for 30 min, and the absorbance was measured at 540 nm. The critical micelle concentration (i.e. the dilution at which an emulsion was not formed) can be determined by plotting the optical density against the range of crude extract dilutions.

Sterol Extraction and Analysis

Cells were grown overnight in CDM supplemented with 10 g/l glucose. These were harvested at early stationary phase by centrifugation, washed three times with 0.9% saline and suspended in saline to give a cell number of 2 x 10^8/ml. Subsequently, 1 ml portions were used as inoculum for sterol analysis studies in 250 ml CDM supplemented with 1% (w/v) of either glucose or 1% (v/v) n-alkanes and incubated for 24 h. Cells were then harvested by centrifugation (4000 rpm, 15 min) washed with boiling water, 3 times, then extracted for sterols by a modification of the method of Fryberg et al. (1974). KOH (1.5 g) in 2 ml of distilled water was added to 0.2 g wet weight of intact cells and the volume of the suspension adjusted to 10 ml by the addition of ethanol.
The solution was refluxed under nitrogen for 3 h, diluted with an equal amount of water and extracted with four volumes of heptane. The extract was washed with water, dried over anhydrous Na₂SO₄ and evaporated in vacuo to give the total sterols.

The sterols were separated by TLC using silica gel G plates [Merck Kieselgel G, type 60, (0.25 mm thickness)] and 40-60°C petroleum ether:diethyl ether (3:1, v/v) as developing solvent. The lifschutz reagent [concentrated sulphuric acid:glacial acetic acid (1:1, v/v)] was used to detect sterols as red or purple spots. Fractions separated by TLC were identified by comparison of their Rf values with commercially available standards. Sterols were analysed with Pye-unicam SP8-400 UV/VIS spectrophotometer (Cambridge, U.K.). Preparative TLC was used to separate individual sterols ready for identification using different spectroscopic methods.

Gas chromatography of total sterols was done by the preparation of the silylated sterols derivatives according to Vandenheuvel and Court (1968). Up to 1 mg of sterols extract were reacted in a glass-stoppered flask with 50 µl of hexamethyl disilazane and 50 µl of 10% trimethyl chlorosilane in chloroform (v/v), the reagents being added in that order. Brief mixing by stirring or vibration was applied after each addition. The reaction mixture was left at room temperature for at least 4 h. Excess solvent and reagents were removed using the method of Vandenheuvel et al. (1965). 50 µl of CS₂ was then added to the flask to dissolve the reaction mixture. 0.5 µl portions were loaded on SP-2100 column (3% on 100/120 gaschrome Q) in a Pye-Unicam gas chromatograph (series 204). Comparison of retention times of the peaks with those of reference
standards using polydimethylsiloxane (JXR) as a second column. The samples were eluted at 230°C with nitrogen as a carrier gas (60 ml/min) and the non-saponifiable lipids detected with flame ionisation detector. Individual components were identified by a comparison of the retention times relative to ergosterol (Rerg) with those of commercially available standards.

Effect of Ergosterol Supplementation on Hydrocarbon Uptake

*C. albicans* KTCC 89062 was grown overnight in 50 ml Sabouraud dextrose broth (Difco). This culture was used to inoculate 450 ml of Sabouraud dextrose broth which was incubated overnight at 37°C. Cells were harvested by centrifugation (4000 rpm, 15 min), washed 3 times with CDM then suspended in 1 l of CDM without hydrocarbon, and 20 ml samples were dispensed in sterile conical flasks. To each flask 0.2 ml of dodecane was added. These flasks served as control. Other flasks treated in the same manner were supplemented with 5, 10, 15, or 20 μg/ml ergosterol from a stock solution of 4 mg/ml in 95% ethanol. The same volume of 95% ethanol was added to control flasks. To study the effect of viability of *C. albicans* on the enhancement of dodecane uptake by ergosterol, gentle heat killing of the yeast was carried out by heating at 60°C for 30 min (Lee & King, 1983).

Cultures were incubated at 37°C in an orbital shaker (160 rpm). At different time intervals samples were removed, and one only was supplied with 0.2 ml of dodecane to serve as control. The cells were harvested by centrifugation and the supernatant retained. The same procedure followed in post growth experiment was then applied.
CHAPTER SIX
RESULTS

ENUMERATION AND ISOLATION OF OIL UTILISING MICROORGANISMS

Tables 8 and 9 summarise the bacteria and fungal counts recovered on oil agar medium in soil and marine samples (Fig. 6). Fungi occurred mainly as mycelia rather than other surviving propagules, as confirmed by the conventional buried slide technique.

The fungal flora consisted of Candida albicans, Aspergillus spp., A. terreus, A. sulphureus, Mucor globosus, Fusarium sp., Penicillium spp. and P. citrinum, arranged in descending predominance. Bacterial isolates included Bacillus spp., Enterobacteriaceae, Pseudomonas spp., Nocardia spp., Streptomyces spp., and Rhodococcus spp. Among these, Rhodococcus strains were relatively the most abundant. The numbers of Rhodococcus cells in water samples showed great variation from one locality to another. They were especially high in oil polluted localities, but low in non-polluted water samples (Table 9). In all localities Rhodococcus spp. were readily isolated by the golf ball technique. These isolates were highly pleomorphic, consisting of filaments or coryneform Gram-positive non-motile rods which sub-divided in old cultures into cocci (Fig. 7). The chemotaxonomic analysis of R. rhodochrous KUCC 8801 revealed that cell wall contained diamino acid which was identified as diaminopimelic acid and that the cell wall total lipids contained tuberculostearic acid in addition to major amounts of palmitic (C₁₆:0), palmitoleic (C₁₆:1) and stearic (C₁₈:0) acids. This isolate was catalase positive and oxidase negative. It decomposed tyrosine and Tween 80 but not adenine and urea. It grew on
glucose, maltose, mannitol, sorbitol, sodium adipate, sodium benzoate, sodium citrate, sodium lactate, testosterone, L-tyrosine, glycerol and p-hydroxybenzoic acid as sole sources of carbon, but not on inositol, or trehalose.
Table 8. Soil bacteria and fungi utilising crude oil as a sole source of carbon and energy.

<table>
<thead>
<tr>
<th>Soil sample</th>
<th>Bacterial colony forming units</th>
<th>Fungi colonies</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>7d</td>
<td>14d</td>
</tr>
<tr>
<td>Oil field 1</td>
<td>(6.5±0.4)×10^7</td>
<td>(6.7±0.1)×10^7</td>
</tr>
<tr>
<td>Oil field 2</td>
<td>(1.2±0.01)×10^8</td>
<td>(1.3±0.08)×10^8</td>
</tr>
<tr>
<td>Oil field 3</td>
<td>(1.6±0.03)×10^9</td>
<td>(1.7±0.06)×10^9</td>
</tr>
<tr>
<td>Oil field 4</td>
<td>(1.9±0.1)×10^9</td>
<td>(2.0±0.1)×10^9</td>
</tr>
<tr>
<td>Garden 1</td>
<td>(4.27±0.3)×10^7</td>
<td>(4.3±0.4)×10^7</td>
</tr>
<tr>
<td>Garden 2</td>
<td>(3.3±0.07)×10^7</td>
<td>(3.35±0.6)×10^7</td>
</tr>
</tbody>
</table>

Each value is the mean ± SD (cells/g) of three determinations. Values varied by < 10%.
Table 9. Marine bacteria and fungi utilising oil as a sole source of carbon and energy.

<table>
<thead>
<tr>
<th>Site</th>
<th>No. of bacterial colony forming unit in sea sediment/1g</th>
<th>No. of bacterial colony forming unit in sea water/100 ml</th>
<th>No. of fungal colonies in sea water/100 ml</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>No.</td>
<td>7d</td>
<td>14d</td>
</tr>
<tr>
<td>1</td>
<td>(10.5±0.9)×10^3</td>
<td>(30±0.1)×10^3</td>
<td>(0.6±0.05)×10</td>
</tr>
<tr>
<td>2</td>
<td>(15±1.2)×10^3</td>
<td>(37±0.8)×10^3</td>
<td>(2.9±0.1)×10</td>
</tr>
<tr>
<td>3</td>
<td>(26.1±0.5)×10^3</td>
<td>(42±1.6)×10^3</td>
<td>(4.0±0.2)×10</td>
</tr>
<tr>
<td>4</td>
<td>(57.3±1.6)×10^3</td>
<td>(65±0.9)×10^3</td>
<td>(6.0±0.2)×10</td>
</tr>
<tr>
<td>5</td>
<td>(156±3.8)×10^3</td>
<td>(305±1.8)×10^3</td>
<td>(1.9±0.1)×10^2</td>
</tr>
</tbody>
</table>

* Oil polluted sites.

N.D. Not determined

Each value is the mean ± SD of three determinations. Values varied by < 10%.
Figure 6. Oil degrading microorganisms isolated on oil agar plate:

(a) control plate (without inoculation).
(b, c, d, e, and f) test plates.
Figure 7. Light and scanning electron micrographs of *R. rhodochrous* KUCC 8801.

Note that this organism at various stages of growth shows striking similarity to *Arthrobacter*, *Corynebacterium* (b & c) and *Nocardia* (a & c). a = 24 h; b = 48 h; c = 72 h. (a & c, x 2500 & 3000 respectively).
GROWTH STUDIES

Growth of Selected Organisms on Pure Hydrocarbons

The results of screening of representative predominant bacteria and fungi for growth on media provided with 10 g/litre each of a pure hydrocarbon are presented in Table 10. Short chain alkanes were not utilised by R. rhodochrous but straight chain alkanes with 12 to 20 carbon atoms and the alkene tridecene were readily utilised. The biomasses were directly proportional to the hydrocarbon chain length. The branched chain iso-octane and the aromatic hydrocarbons were not attacked by these bacteria. Similar results were obtained for C. albicans KTCC 89062. On the other hand the fungal isolates tested could utilise one or more of the aromatic hydrocarbons studied in addition to the medium chain n-alkanes (Table 10).

Measuring Growth Rate of Selected Microorganisms by Dry Weight Determination

The growth curves of R. rhodochrous KUCC 8801 and 8802 on glucose and dodecane (C_{12}) are shown in Figure 8. It is apparent that 12 h samples were in the exponential phase of growth whereas the 30 h cultures were in the stationary phase. The results obtained from these curves indicate that both Rhodococcus strains grew better on glucose than on dodecane and R. rhodochrous KUCC 8801 produced more dry weight yield as compared with R. rhodochrous KUCC 8802 when grown on both substrates.

Figure 9 represents the growth of C. albicans KTCC 89062 on glucose and different chain length of n-alkanes. It is apparent that samples obtained after 15 h were in the
exponential phase growth whereas 24 h samples were in the early stationary phase. In the case of the utilisable n-alkanes, there was an increase in cellular yield as the chain length was increased but it was always significantly lower than CDM supplemented with glucose. It is also clear that the bacterial cellular yield was higher than yeast cellular yield in all substrates tested.

Measuring Growth Rate of *C. albicans* KTCC 89062 by Viable Cell Count

The growth of *C. albicans* KTCC 89062 on CDM supplemented with either glucose or n-alkanes was further examined in Figure 10. It is apparent that this technique is not a good one for measuring growth rate of hydrocarbon utilising microorganisms since definite results can not be obtained from Figure 10. This could be due to the nature of *C. albicans* KTCC 89062 growth on hydrocarbons (i.e. filamentation production and cells are highly adhered to hydrocarbon droplets).
Table 10. Screening of selected isolates for growth on individual hydrocarbons.

<table>
<thead>
<tr>
<th>Hydrocarbons</th>
<th>Bacteria</th>
<th>Fungi</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td><em>R. rhodochrous</em></td>
<td><em>A. terreus</em></td>
</tr>
<tr>
<td></td>
<td>KUCC 8801</td>
<td>KUCC 8802</td>
</tr>
<tr>
<td>Hexane</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Cyclohexane</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>Heptane</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Iso-octane</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Nonane</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Decane</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Tridecane</td>
<td>123±0.8</td>
<td>41±0.6</td>
</tr>
<tr>
<td>Tetradecane</td>
<td>245±1.7</td>
<td>234±2.3</td>
</tr>
<tr>
<td>Hexadecane</td>
<td>317±0.8</td>
<td>290±1.4</td>
</tr>
<tr>
<td>Octadecane</td>
<td>394±2.5</td>
<td>387±0.7</td>
</tr>
<tr>
<td>Eicosane</td>
<td>505±1.6</td>
<td>409±0.5</td>
</tr>
<tr>
<td>Benzene</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Anthracene</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Phenanthrene</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Naphthalene</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

Values indicate the mean ± SD (dry biomasses in mg/50 ml medium) of three determinations.

N.D. Not determined,  - No growth.
Figure 8. Growth curves of \textit{R. rhodochrous} KUCC 8801 and \textit{R. rhodochrous} KUCC 8802 on CDM supplemented with; ---, glucose. \( \Delta \), C\textsubscript{12}.
Figure 9. Growth curves of *C. albicans* KTCC 89062 on CDM supplemented with: —, glucose. +, C_{12}, \bigtriangleup, C_{14}, \Delta, C_{16}, \times, C_{18}, \nabla, C_{20}.
Incubation Period (h)

Biomass (g/l)
Figure 10. Growth curves of *C. albicans* RTCC 89062 on CDM supplemented with: —, glucose. 

+ C_{12}, \big< C_{14}, \bigtriangleup, C_{16}, \times, C_{18}, \nabla, C_{20}. 
EFFECT OF HYDROCARBONS ON CELL MORPHOLOGY

Scanning Electron Microscopy (SEM)

Scanning electron micrographs of *R. rhodochrous* strains showed that this organism tend to form filaments when grown in CDM supplemented with dodecane. 72 h cultures grown in CDM supplemented with glucose were small rod shaped cells compared to dodecane-grown cultures (Fig. 11).

*C. albicans* KTCC 89062 cells grown in CDM supplemented with glucose were generally smooth-walled bodies, spherical to elongated in shape, typical of the yeast form of *C. albicans* (Fig. 12a). Growth in the presence of n-alkanes led to the formation of significant numbers of pseudohyphae (Fig. 12b,c).

Transmission Electron Microscopy

The election micrographic study showed that *R. rhodochrous* cells grown on CDM supplemented with dodecane always contained 2-3 rarely more, hydrocarbon inclusions per cell with a mean diameter of about 0.2 μm.

Similar results were obtained for *C. albicans* KTCC 89062, electron micrographs showed that the yeast cells grown on n-alkanes contain large vacuoles in the cytoplasm compared to glucose-grown cells.
Figure 11. SEM of *R. rhodochrous* grown as a shake culture in CDM supplemented with glucose (a), and $C_{12}$ (b & c). x 2,500.
Figure 12. SEM of *C. albicans* KTCC 89062 grown as a shake culture in CDM supplemented with glucose (a), and *n*-alkanes (b & c). x 5,000.
Figure 13. Transmission electron micrographs of glucose (a) and C\textsubscript{12} (b) grown cells of \textit{R. rhodochrous}. \textit{a,b} x 20,000.

H, hydrocarbon inclusion
Figure 14. Transmission electron micrographs of glucose (a) and n-alkane (b & c) grown cells of *C. albicans* KTCC 89062. x 4,800.

N, nucleus
M, mitochondria
H, hydrocarbon inclusion
Biodegradation of Crude Oil

Bacterial and fungal isolates, grown in shaken cultures in a medium containing weathered crude oil as a sole source of carbon and energy, differed in their biodegradation activity. Active isolates brought about visible physical changes in the medium after only 24 h, whereas less active isolates took up to 1 week to achieve this. Three distinct types of changes brought about by bacterial isolates were equally frequent: 1. formation of homogeneous oil emulsions in the aqueous medium; 2. disintegration of oil into 1-2 mm granular particles suspended in the aqueous phase and 3. development in the aqueous medium of large clumps (Fig. 15).

Gas chromatography of the residual oil recovered after microbial growth (Fig. 16 I,II) showed that Rhodococcus isolates were more active than fungi in n-alkane biodegradation as indicated by the intensities of the peak areas of the residual hydrocarbons. The spectrum of the initial degradation of oil by bacteria was identical.

Figure 16,II clearly indicates that a difference exists in the ability of various fungal isolates to degrade crude oil. M. globosus was the most active oil degrader among the fungi. The weathered crude oil was degraded by C. albicans up to 34.1 ± 2.3% after 15 days and the C_{14} to C_{18} fractions were apparently utilised preferentially with chains containing both odd and even numbers of carbon atoms being degraded (Fig. 16,III).
The results of comparative analysis between *R. rhodochrous* KUCC 8801 and other bacterial cultures known to be efficient oil degraders revealed that *R. rhodochrous* KUCC 8801 was more efficient in oil degradation. These results are presented qualitatively in Figure 17 and quantitatively in Table 11.
Figure 15. Physical changes brought about by bacterial isolates in CDM supplemented with crude oil.

From left to right: (a) control (crude oil in the medium without bacteria); (b) production of crude oil emulsion in the liquid medium; (c) disintegration of crude oil into small granules; (d) production of a clump adsorbing the oil.
Figure 16. GLC profiles of crude oil recovered from microbial cultures.

I - Bacterial cultures grown for 5 days at 30°C. (a) control crude oil; (b) oil recovered from *R. rhodochrous* KUCC 8801 culture; (c) oil recovered from *R. rhodochrous* KUCC 8802. II - Fungal cultures grown for 15 days at 25°C. (a) control crude oil; (b) oil recovered from *Fusarium* sp. culture; (c) oil recovered from *A. terreus* culture; (d) oil recovered from *A. sulphureus* culture; (e) oil recovered from *M. globosus* culture. III - *C. albicans* culture grown for 15 days at 30°C (a) control crude oil; (b) oil recovered from *C. albicans* KTCC 89062.
Figure 17. GLC profiles of crude oil recovered from microbial cultures.

I - 3 day cultures. II - 5 day cultures. (a) control crude oil; (b) oil recovered from *R. rhodochrous* KUCC 8801 cultures; (c) oil recovered *Rhodococcus* sp. IS01 cultures; (d) oil recovered from *A. cacoaceticus* IR07 cultures; (e) oil recovered from *P. Putida* IR32 cultures.
Table 11. Crude oil utilisation by different bacterial isolates.

Data are expressed in percent of total peak areas in gas chromatograms based on the total peak area of crude oil chromatograms.

<table>
<thead>
<tr>
<th>Organisms</th>
<th>[%] Hydrocarbons consumed</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>3 d</td>
</tr>
<tr>
<td><strong>R. rhodochrous KUCC 8801</strong></td>
<td>85.0</td>
</tr>
<tr>
<td><strong>Rhodococcus sp. IS01</strong></td>
<td>32.9</td>
</tr>
<tr>
<td><strong>Acinetobacter calcoaceticus IR07</strong></td>
<td>54.4</td>
</tr>
<tr>
<td><strong>Pseudomonas putida IR32</strong></td>
<td>18.8</td>
</tr>
</tbody>
</table>

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Dodecane Utilisation by R. rhodochrous

Dodecane was selected with the objective of improving its utilisation by controlling cultural conditions by two of R. rhodochrous, namely KUCC 8801 (pink colonies) and KUCC 8802 (orange colonies). In contrast to strain KUCC 8802, strain KUCC 8801, could liquefy gelatin and ferment sucrose and glucose. Its growth was lighter in colour and resulted in the disintegration of an emulsion. The two isolates grew best at pH 8. The pH values of soil and water samples studied ranged between 7.7 and 8. The optimum temperature for growth was around 40°C, but the cells of both isolates tolerated 50°C. Both isolates grew best without any added NaCl, but could tolerate up to 5% (w/v) of this salt. Table 12 presents results of the kinetics of dodecane utilisation by the two test isolates. It is apparent that, after 10 and 15 days, R. rhodochrous KUCC 8801 utilised, much more dodecane than R. rhodochrous KUCC 8802. Figure 18 is the standard curve of different concentrations of dodecane.

Post Growth Experiments

The measurement of post-growth utilisation of dodecane by R. rhodochrous KUCC 8801, in comparison to the non Kuwaiti isolates, revealed that this organism could decompose more of the available hydrocarbon than the other isolates (Fig. 19). Calculation by peak triangulation showed that the proportions of dodecane consumed by R. rhodochrous KUCC 8801, Rhodococcus sp. ISO1 and A. calcoaceticus IR07 after 48 h were 76.9, 43.2 and 36.6%, respectively.
Table 12. Dodecane utilisation by *Rhodococcus rhodochrous*.

<table>
<thead>
<tr>
<th>Dodecane (g/l)</th>
<th><em>R. rhodochrous</em> KUCC 8801</th>
<th><em>R. rhodochrous</em> KUCC 8802</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>5d</td>
<td>10d</td>
</tr>
<tr>
<td>5</td>
<td>0.3±0.02</td>
<td>4.8±0.04</td>
</tr>
<tr>
<td>10</td>
<td>1.5±0.06</td>
<td>8.4±0.15</td>
</tr>
<tr>
<td>15</td>
<td>1.2±0.18</td>
<td>11.6±0.39</td>
</tr>
<tr>
<td>20</td>
<td>0.5±0.06</td>
<td>14.7±0.6</td>
</tr>
</tbody>
</table>

Data are expressed in g/l dodecane consumed after the given incubation periods and are means of three determinations.

± = Standard deviation.
Figure 18. Standard curve constructed using a range of $C_{12}$ concentrations in CCL$_4$.

a. Control  
b. After 5 days incubation  
c. After 10 days incubation  
d. After 15 days incubation

Initial $C_{12}$ concentration = 1.5% (w/v).
Figure 19. Post-growth utilisation of dodecane by *R. rhodochrous* KUCC 8801 (a), *Rhodococcus* sp. ISO1 (b), and *A. calcoaceticus* IRO7 (c).
Lipid and Sterol Composition

1. *R. rhodochrous* strains

The total lipid and total sterol contents of dodecane-grown cells were higher than those of glucose-grown cells (Table 13). The relative yield of lipids and sterols for dodecane-grown cells, for isolate KUCC 8801, was 3.7 and 2.1 fold higher than cells grown on glucose, respectively. Similar results were obtained for isolate KUCC 8802. The quantitative measurements showed that all extracts contained more apolar than polar lipids (Table 14). Apolar lipid classes in various extracts were tri-, di- and monoacylglycerols as well as free sterols (Fig. 20). Dodecane-grown cells contained more of monoacylglycerols and sterols than glucose-grown cells.

The identity of the sterol fraction was confirmed as follows: On the chromatoplates, this fraction gave positive reactions with sterol-detecting spray reagents (Stahl, 1962). The retention times of cell sterols in GLC were identical with those of authentic samples of lanosterol and squalene. A lanosterol fraction separated by preparative TLC showed an IR spectrum identical with that of standard lanosterol (Fig. 21). The NMR spectrum (Fig. 22) was identical with that of standard lanosterol with $^1$H nmr 1(CDC$_3$): 4.18-5.45 [1 H, m, CH=C (CH$_3$)$_2$], 3.50 (1 H, S, -OH), 3.05-3.32 (1 H, dd, -CH OH-), 1.55-2.23 (6 H, m, allylic protons), 0.64-1.55 (40 H, m, saturated aliphatic protons). Mass spectrometry gave a molecular ion of 426 m/e (Fig. 23) corresponding to that of standard lanosterol. The ionic and other polar lipid classes all consisted mainly of phospholipids viz phosphatidylethanolamines (PE), phosphatidymethylethanolamines, phosphatidylglycerols.
(PG) and phosphatidylcholines (PC). The extracts also contained an unknown polar acyl glycolipid (positive reaction with α-naphthol reagent), which migrated on TLC plates close to the apolar fraction (Fig. 24, 25).

The identity of the class of PC in the total lipids was confirmed as follows: It showed a chromatographic (unidimensional TLC) behaviour similar to that of standard compound with six different solvents comprising neutral, alkaline and acidic systems (Radwan 1984). It gave positive reactions for phosphorus and choline (Dragendorff's reagent). It revealed an IR spectrum identical with that of standard PC (Fig. 26). On mild hydrolysis, it was identical with the standard sample in producing glycerophosphocholine which was identified by two paper chromatography (Dawson 1984). Dodecane-grown cells contained more of PC and the unknown polar glycolipid than glucose-grown cells. The supernatant of dodecane-grown cells contained measurable amounts of glycolipids (0.033 g/300 ml), which again migrated on TLC plates close to the apolar fraction (Fig. 27a). The supernatant of glucose-grown cells also contained this glycolipid fraction but with smaller quantities (0.009 g/300 ml). Figure 27b shows the results of an experiment where a range of dilutions of the crude glycolipid (isolated from the supernatant of dodecane-grown cells) were tested for emulsifier activity. The critical micelle concentration (i.e. the dilution at which an emulsion was not formed) had a value of 1.8 mg/ml at 25°C.
Table 13. Total lipid and sterol contents of two strains of *Rhodococcus rhodochrous*

<table>
<thead>
<tr>
<th></th>
<th>Strain KUCC 8801</th>
<th>Strain KUCC 8802</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Glucose</td>
<td>Dodecane</td>
</tr>
<tr>
<td>Total lipids</td>
<td>1.1±0.1</td>
<td>4.1±0.4</td>
</tr>
<tr>
<td>Total sterols</td>
<td>0.28±0.06</td>
<td>0.58±0.04</td>
</tr>
</tbody>
</table>

Values are expressed in % of dry biomass. Each value is the mean of two determinations in two different experiments.

± = Standard deviation.
Table 14. Lipid composition of two strains of *Rhodococcus rhodochrous*

<table>
<thead>
<tr>
<th></th>
<th>Strain KUCC 8801</th>
<th>Strain KUCC 8802</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Glucose</td>
<td>Dodecane</td>
</tr>
<tr>
<td>1] Apolar lipid classes:</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Triacylglycerols</td>
<td>48.7±1.6</td>
<td>28.8±1.3</td>
</tr>
<tr>
<td>Diacylglycerols</td>
<td>9.1±0.3</td>
<td>8.5±0.5</td>
</tr>
<tr>
<td>Sterols</td>
<td>3.3±0.09</td>
<td>9.2±0.4</td>
</tr>
<tr>
<td>Monoacylglycerols</td>
<td>5.3±0.3</td>
<td>14.9±0.8</td>
</tr>
<tr>
<td>2] Polar lipid classes:</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Phosphatidylcholines</td>
<td>4.4±0.1</td>
<td>8.8±0.5</td>
</tr>
<tr>
<td>Phosphatidylethanolamines</td>
<td>8.2±0.8</td>
<td>7.8±0.5</td>
</tr>
<tr>
<td>Phosphatidylglycerols</td>
<td>5.3±0.1</td>
<td>4.6±0.3</td>
</tr>
<tr>
<td>Phosphatidylmethylethanolamines</td>
<td>5.1±0.2</td>
<td>4.7±0.2</td>
</tr>
<tr>
<td>Unknown glycolipid</td>
<td>10.6±0.5</td>
<td>12.7±0.8</td>
</tr>
</tbody>
</table>

Values are expressed in % of total lipids. Each value is the mean ± standard deviation of two determinations in two different experiments.

Trace = less than 0.1%. Values varied by < 10%.
Figure 20. Typical TLC plate showing apolar lipids of *R. rhodochrous* strains.

The sorbent was Silica Gel G; the solvent was hexane/diethyl ether/acetic acid (75:25:1, by vol.). The lipids were visualised by charring. (1) lanosterol standard, (2) fatty acid standard, (3) *R. rhodochrous* KUCC 8802 grown on C$_{12}$, (4) *R. rhodochrous* KUCC 8802 grown on glucose, (5) *R. rhodochrous* KUCC 8801 grown on C$_{12}$, (6) *R. rhodochrous* KUCC 8801 grown on glucose, (7) triacylglycerols standard. TG, triacylglycerols; DG, diacylglycerols; S, sterols; MG, monoacylglycerols; PL, polar lipids.
Figure 21. IR-spectra of lanosterol isolated from lipids of *R. rhodochrous* cells grown in CDM supplemented with dodecane (upper), and a lanosterol standard (lower).
Figure 22. H-NMR spectrum of lanosterol isolated from lipids of *R. rhodochrous* cells grown in CDM supplemented with dodecane.
Figure 23. Mass spectrum of lanosterol isolated from lipids of *R. rhodochrous* cells grown in CDM supplemented with dodecane.
Figure 24. Typical TLC plate of polar lipids of *R. rhodochrous* strains.

The sorbent was Silica Gel G; the solvent was chloroform/methanol/7M-ammonium hydroxide (65:25:4, by vol.). The lipids were visualised by charring. (1,6) phospholipid standard, (2) *R. rhodochrous* KUCC 8802 grown on C$_{12}$, (3) *R. rhodochrous* KUCC 8802 grown on glucose, (4) *R. rhodochrous* KUCC 8801 grown on C$_{12}$, (5) *R. rhodochrous* KUCC 8801 grown on glucose. N, neutral lipids; X, unknown glycolipids; PE, phosphatidylethanolamines; PC, phosphatidylcholines.
Figure 25. Typical two dimensional TLC plates of polar lipids from *R. rhodochrous* KUCC 8801 grown on (a) glucose, and (b) C_{12}.

The sorbent was Silica Gel G; the solvents were (I) chloroform/methanol/7M-ammonium hydroxide (65:30:4, by vol.) and (II) chloroform/methanol/acetic acid/water (170:25:25:4, by vol.). The lipids were visualised by charring. S, start; PI, phosphatidylinositols; PS, phosphatidylserines; PC, phosphatidylcholines; PE, phosphatidylethanolamines; PME, phosphatidylmethyl-ethanolamines; PG, phosphatidylglycerols; X, unknown glycolipids; CL, cardiolipins; X_{1}, Glycolipids; X_{2}, tentatively identified as lysophosphatidylethanolamines.
Figure 26. IR-spectra of phosphatidylcholines isolated from lipids of *R. rhodochrous* (upper) and egg phospholipids (lower).
Figure 27a. Typical TLC plate showing the supernatant lipids isolated from *R. rhodochrous* strains.

The sorbent was Silica Gel G; the solvent was chloroform/methanol/acetic acid (85:20:1 by vol.). The lipids were visualised by α-naphthol reagent followed by charring. (1) *R. rhodochrous* KUCC 8802 grown on glucose, (2) *R. rhodochrous* KUCC 8802 grown on C\textsubscript{12}, (3) glycolipid standard, (4) *R. rhodochrous* KUCC 8801 grown on C\textsubscript{12}, (5) *R. rhodochrous* KUCC 8801 grown on glucose. X, unknown glycolipids.

Figure 27b. Evaluation of emulsifying activity of extracellular glycolipids extracted from the supernatant of *R. rhodochrous* KUCC 8802 grown on dodecane.

C.M.C., critical micelle concentration
Fatty Acid Patterns

The fatty acid patterns of total lipids from glucose- and dodecane-grown bacteria are shown in Table 15. The predominant acyl moieties in all extracts were palmitic (C₁₆:₀) and oleic (C₁₈:₁) acids. Traces of tuberculostearic acid (C₁₈:₀,Me) were also present. Relatively large proportions of palmitoleic acid (C₁₆:₁) were present in extracts of glucose but not dodecane-grown cells of both *R. rhodochrous* strains studied. Lipids from dodecane-grown cells of both strains contained, relatively, more lauric (C₁₂:₀), myristic (C₁₄:₀), palmitic (C₁₆:₀) and linolenic (C₁₈:₃) acids than lipids from glucose-grown cells.

The data in Table 16 show the fatty acid patterns of individual glycerophospholipids from glucose- and dodecane-grown cells of *R. rhodochrous*. The phospholipid classes of glucose-grown cells of *R. rhodochrous* KUCC 8801 contained appreciable proportions of linoleic acid (C₁₈:₂) which decreased when the cells were grown on dodecane. Palmitoleic acid occurred only in PG of this strain, but was distributed among all other phospholipid classes of *R. rhodochrous* KUCC 8802. In PC of both strains, relatively high linolenic acid concentrations were associated with cell growth on dodecane. Phospholipid classes from dodecane-grown cells of both strains contained higher proportions of lauric and myristic acids than the same classes from glucose-grown cells. The highest concentrations of lauric and myristic acids were detected in PC and the unknown glycolipid. Tuberculostearic acid was confined to PG and the unknown glycolipid.
Table 15. Constituent fatty acids of total lipids from two strains of *Rhodococcus rhodochrous*

<table>
<thead>
<tr>
<th>Fatty acids</th>
<th>Strain KUCC 8801</th>
<th></th>
<th>Strain KUCC 8802</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Glucose</td>
<td>Dodecane</td>
<td>Glucose</td>
<td>Dodecane</td>
</tr>
<tr>
<td>12 : 0</td>
<td>tr</td>
<td>2.1±0.2</td>
<td>tr</td>
<td>12.4±0.4</td>
</tr>
<tr>
<td>14 : 0</td>
<td>tr</td>
<td>16.7±1.5</td>
<td>tr</td>
<td>10.1±0.8</td>
</tr>
<tr>
<td>16 : 0</td>
<td>34.0±1.9</td>
<td>48.5±2.5</td>
<td>23.0±0.8</td>
<td>36.6±0.8</td>
</tr>
<tr>
<td>16 : 1</td>
<td>20.0±1.8</td>
<td>1.5±0.1</td>
<td>18.4±0.5</td>
<td>1.8±0.09</td>
</tr>
<tr>
<td>18 : 0</td>
<td>tr</td>
<td>tr</td>
<td>tr</td>
<td>tr</td>
</tr>
<tr>
<td>18 : 0(Me)*</td>
<td>tr</td>
<td>tr</td>
<td>tr</td>
<td>tr</td>
</tr>
<tr>
<td>18 : 1</td>
<td>44.0±3.0</td>
<td>26.8±2.3</td>
<td>52.0±3.2</td>
<td>35.7±2.8</td>
</tr>
<tr>
<td>18 : 2</td>
<td>0.8±0.01</td>
<td>tr</td>
<td>4.6±0.2</td>
<td>tr</td>
</tr>
<tr>
<td>18 : 3</td>
<td>1.2±0.09</td>
<td>4.4±0.3</td>
<td>2.0±0.1</td>
<td>3.4±0.2</td>
</tr>
</tbody>
</table>

Values are expressed in % of total fatty acids. Each value is the mean of two determinations in two separate experiments.

± = standard deviation
tr = trace, less than 0.1%

* Tuberculostearic acid
Table 16. Constituent fatty acids of individual phospholipids from strains of *Rhodococcus rhodochrous*

<table>
<thead>
<tr>
<th>Fatty acids</th>
<th>Strain KUCC 8801</th>
<th>Strain KUCC 8802</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Glucose</td>
<td>Dodecane</td>
</tr>
<tr>
<td>Phosphatidylcholines</td>
<td></td>
<td></td>
</tr>
<tr>
<td>12 : 0</td>
<td>tr</td>
<td>12.2±0.4</td>
</tr>
<tr>
<td>14 : 0</td>
<td>tr</td>
<td>33.3±2.0</td>
</tr>
<tr>
<td>16 : 0</td>
<td>43.0±2.1</td>
<td>10.0±0.8</td>
</tr>
<tr>
<td>16 : 1</td>
<td>tr</td>
<td>tr</td>
</tr>
<tr>
<td>18 : 0</td>
<td>1.4±0.1</td>
<td>13.3±1.0</td>
</tr>
<tr>
<td>18 : 1</td>
<td>28.6±0.9</td>
<td>20.6±1.9</td>
</tr>
<tr>
<td>18 : 2</td>
<td>26.0±1.6</td>
<td>tr</td>
</tr>
<tr>
<td>18 : 3</td>
<td>1.0±0.03</td>
<td>10.6±0.8</td>
</tr>
<tr>
<td>Phosphatidylethanolamines</td>
<td></td>
<td></td>
</tr>
<tr>
<td>12 : 0</td>
<td>tr</td>
<td>2.7±0.2</td>
</tr>
<tr>
<td>14 : 0</td>
<td>tr</td>
<td>8.9±0.4</td>
</tr>
<tr>
<td>16 : 0</td>
<td>44.8±2.9</td>
<td>54.3±3.0</td>
</tr>
<tr>
<td>16 : 1</td>
<td>tr</td>
<td>tr</td>
</tr>
<tr>
<td>18 : 0</td>
<td>0.8±0.05</td>
<td>1.7±0.09</td>
</tr>
<tr>
<td>18 : 1</td>
<td>34.2±1.8</td>
<td>31.4±1.6</td>
</tr>
<tr>
<td>18 : 2</td>
<td>18.1±1.7</td>
<td>0.5±0.03</td>
</tr>
<tr>
<td>18 : 3</td>
<td>2.1±0.1</td>
<td>0.5±0.01</td>
</tr>
</tbody>
</table>
Table 16. (contd..)

<table>
<thead>
<tr>
<th>Fatty acids</th>
<th>Strain KUCC 8801</th>
<th>Strain KUCC 8802</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Glucose</td>
<td>Dodecane</td>
</tr>
<tr>
<td>Phosphatidylglycerols</td>
<td></td>
<td></td>
</tr>
<tr>
<td>12 : 0</td>
<td>tr</td>
<td>4.1±0.2</td>
</tr>
<tr>
<td>14 : 0</td>
<td>tr</td>
<td>5.0±0.2</td>
</tr>
<tr>
<td>16 : 0</td>
<td>31.7±1.7</td>
<td>30.9±1.3</td>
</tr>
<tr>
<td>16 : 1</td>
<td>14.3±1.2</td>
<td>20.0±0.8</td>
</tr>
<tr>
<td>18 : 0</td>
<td>1.9±0.05</td>
<td>3.8±0.07</td>
</tr>
<tr>
<td>18 : 0(Me)*</td>
<td>2.2±0.3</td>
<td>3.3±0.1</td>
</tr>
<tr>
<td>18 : 1</td>
<td>35.1±0.8</td>
<td>30.0±1.7</td>
</tr>
<tr>
<td>18 : 2</td>
<td>10.7±1.1</td>
<td>tr</td>
</tr>
<tr>
<td>18 : 3</td>
<td>4.1±0.3</td>
<td>2.9±0.1</td>
</tr>
</tbody>
</table>

Unknown glycolipid

|              |         |           |         |           |
| 12 : 0       | tr      | 14.1±0.7  | -       | 9.9±0.1   |
| 14 : 0       | tr      | 28.1±0.5  | -       | 23.2±1.2  |
| 16 : 0       | 30.1±0.9| 19.0±1.3  | -       | 7.8±0.9   |
| 16 : 1       | 10.0±0.6| 16.5±1.1  | -       | 11.0±0.8  |
| 18 : 0       | 0.4±0.01| tr        | -       | 7.8±0.3   |
| 18 : 0(Me)*  | 40.4±2.2| 20.8±1.8  | -       | 27.3±0.5  |
| 18 : 1       | 14.6±1.0| tr        | -       | tr        |
| 18 : 2       | 2.0±0.1 | 0.7±0.03  | -       | 12.5±1.4  |
| 18 : 3       | 2.5±0.2 | 0.8±0.03  | -       | 0.5±0.0   |

Values are expressed in % of total fatty acids of each class. Each value is the mean ± SD of two determinations in two separate experiments.

tr = trace, less than 0.1%.
- = Not Determined
* Tuberculostearic acid
2. *C. albicans* KTCC 89062

The lipid composition of *C. albicans* was affected by growth in the presence of n-alkanes when compared to cells grown with glucose. The lipid fraction was consistently greater, as a percentage of dry weight, in all the samples grown on n-alkanes as carbon source, rather than glucose. The sterol fraction was also raised significantly above the level found when glucose was the carbon source. There was a gradual decline in the sterol fraction as the chain length increased from C₁₂ to C₂₀ (a decline from 1.33 to 0.59% of the dry weight) (Table 17).

When the lipid fraction was subjected to further analysis it was found that the apolar lipids predominated over the polar lipids, irrespective of the carbon source used to supplement CDM (Table 18). The levels of free sterols, monoacylglycerols and steryl esters were significantly higher while diacylglycerols and triacylglycerols levels were significantly reduced in cells grown in n-alkane supplemented CDM when compared with CDM supplemented with glucose (Table 18 & Fig. 28).

When the polar lipids were examined there was an increase in the levels of steryl glycosides, PE and PC with a decrease in the levels of ceramide monohexosides and phosphatidic acid when *C. albicans* was grown in CDM supplemented with n-alkanes as carbon source in comparison with glucose containing CDM (Table 18 & Fig. 29, 30).
Table 17. Yield and lipid content of a Candida albicans KTCC 89062 grown on individual n-alkanes or glucose for 1 day at 37°C in batch culture shaken at 160 rpm.

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Yield of biomass (g dry wt/l)</th>
<th>Total lipid (% dry weight)</th>
<th>Sterols (% dry weight)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dodecane</td>
<td>1.01±0.05</td>
<td>4.96±0.03</td>
<td>1.33±0.08</td>
</tr>
<tr>
<td>Tetradecane</td>
<td>1.27±0.09</td>
<td>4.22±0.05</td>
<td>1.28±0.07</td>
</tr>
<tr>
<td>Hexadecane</td>
<td>1.83±0.009</td>
<td>6.11±0.09</td>
<td>1.09±0.001</td>
</tr>
<tr>
<td>Octadecane</td>
<td>1.86±0.06</td>
<td>6.10±0.02</td>
<td>1.19±0.003</td>
</tr>
<tr>
<td>Eisocane</td>
<td>2.08±0.05</td>
<td>3.41±0.06</td>
<td>0.59±0.001</td>
</tr>
<tr>
<td>Glucose</td>
<td>5.48±0.1</td>
<td>2.25±0.07</td>
<td>0.26±0.009</td>
</tr>
</tbody>
</table>

Values are the mean ± SD of three separate experiments.
Table 18. Comparison of lipids from *C. albicans* KTCC 89062 grown on glucose or n-alkanes of different chain length.*

<table>
<thead>
<tr>
<th></th>
<th>Glucose</th>
<th>C12</th>
<th>C14</th>
<th>C16</th>
<th>C18</th>
<th>C20</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>1) Apolar lipids</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Steryl esters</td>
<td>8.8±0.6</td>
<td>9.2±0.3</td>
<td>8.9±0.4</td>
<td>10.2±0.8</td>
<td>13.6±1.1</td>
<td>11.9±0.8</td>
</tr>
<tr>
<td>Alkyl esters</td>
<td>9.2±0.8</td>
<td>12.0±1.2</td>
<td>9.9±0.7</td>
<td>11.2±1.1</td>
<td>4.5±1.3</td>
<td>Tr</td>
</tr>
<tr>
<td>Triacylglycerols</td>
<td>13.1±1.1</td>
<td>10.0±1.2</td>
<td>9.1±0.3</td>
<td>6.5±0.3</td>
<td>4.0±0.8</td>
<td>5.7±1.2</td>
</tr>
<tr>
<td>Fatty acids</td>
<td>7.6±0.4</td>
<td>9.7±1.3</td>
<td>8.3±0.7</td>
<td>9.1±0.3</td>
<td>10.8±1.2</td>
<td>11.1±0.5</td>
</tr>
<tr>
<td>Diacylglycerols</td>
<td>18.5±1.1</td>
<td>0.9±0.01</td>
<td>7.2±0.2</td>
<td>6.4±0.5</td>
<td>6.5±0.7</td>
<td>6.3±0.7</td>
</tr>
<tr>
<td>Sterols</td>
<td>9.8±0.3</td>
<td>18.4±1.6</td>
<td>16.7±1.2</td>
<td>22.0±1.4</td>
<td>15.4±0.9</td>
<td>16.3±0.8</td>
</tr>
<tr>
<td>Monoacylglycerols</td>
<td>2.8±0.05</td>
<td>7.5±1.1</td>
<td>6.8±0.9</td>
<td>9.8±0.9</td>
<td>6.8±1.3</td>
<td>7.3±1.2</td>
</tr>
<tr>
<td><strong>2) Polar lipids</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Unknown glycolipid</td>
<td>Tr</td>
<td>4.8±0.6</td>
<td>3.6±0.4</td>
<td>Tr</td>
<td>3.5±0.02</td>
<td>4.1±0.8</td>
</tr>
<tr>
<td>Ceramide mono-</td>
<td>5.4±0.2</td>
<td>3.4±0.1</td>
<td>3.5±0.01</td>
<td>2.5±0.1</td>
<td>3.1±0.3</td>
<td>3.0±0.1</td>
</tr>
<tr>
<td>hexosides</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Steryl glycosides</td>
<td>1.1±0.01</td>
<td>3.5±0.3</td>
<td>3.9±0.05</td>
<td>2.4±0.0</td>
<td>3.0±0.1</td>
<td>6.9±0.5</td>
</tr>
<tr>
<td>Phosphatidyl-</td>
<td>1.4±0.1</td>
<td>1.2±0.01</td>
<td>2.7±0.2</td>
<td>ND</td>
<td>6.3±0.6</td>
<td>6.2±0.8</td>
</tr>
<tr>
<td>glycerols</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Phosphatidyl-</td>
<td>6.2±0.2</td>
<td>8.6±0.04</td>
<td>7.2±0.7</td>
<td>7.9±0.6</td>
<td>7.7±0.6</td>
<td>7.3±0.7</td>
</tr>
<tr>
<td>ethanolamines</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Phosphatidyl-</td>
<td>7.4±0.3</td>
<td>10.8±1.1</td>
<td>7.5±0.9</td>
<td>10.6±0.9</td>
<td>11.8±0.8</td>
<td>10.8±0.7</td>
</tr>
<tr>
<td>cholines</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Phosphatidyl-</td>
<td>3.0±0.1</td>
<td>ND</td>
<td>1.1±0.1</td>
<td>ND</td>
<td>1.4±0.3</td>
<td>Tr</td>
</tr>
<tr>
<td>inositolos</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Phosphatidyl-</td>
<td>3.4±0.2</td>
<td>ND</td>
<td>1.3±0.2</td>
<td>ND</td>
<td>1.6±0.2</td>
<td>Tr</td>
</tr>
<tr>
<td>serines</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cardiolipins</td>
<td>ND</td>
<td>Tr</td>
<td>2.3±0.4</td>
<td>1.4±0.2</td>
<td>ND</td>
<td>3.1±0.06</td>
</tr>
<tr>
<td>Phosphatidic acids</td>
<td>2.3±0.05</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>Tr</td>
</tr>
</tbody>
</table>

* Values are expressed as the percentage (w/w) of the total amount of lipid and are mean ± SD of three determinations.

Tr, trace; ND, not detected
Figure 28. Typical TLC plate showing apolar lipids of *C. albicans* KTCC 89062 grown on glucose or different chain length of n-alkanes.

The sorbent was Silica Gel G; the solvent was hexane/diethyl ether/acetic acid (90:10:1, by vol.). The lipids were visualised by charring. (1) lanosterol standard, (2) fatty acid standard, (3) C\textsubscript{20}, (4) C\textsubscript{18}, (5) C\textsubscript{16}, (6) C\textsubscript{14}, (7) C\textsubscript{12}, (8) glucose, (9) triacylglycerol standard. SE, steryl esters; AE, alkyl esters; TG, triacylglycerols; FA, fatty acids; DG, diacylglycerols; S, sterols; MG, monoacylglycerols; PL, polar lipids.

Figure 29. Typical TLC plate of polar lipids of *C. albicans* KTCC 89062 grown on glucose or different chain length of n-alkanes.

The sorbent was Silica Gel G; the solvent was chloroform/methanol/7M-ammonium hydroxide (65:25:4, by vol.). The lipids were visualised by charring. (1) C\textsubscript{20}, (2) C\textsubscript{18}, (3) C\textsubscript{16}, (4) C\textsubscript{14}, (5) C\textsubscript{12}, (6) glucose, (7) phospholipid standard. N, neutral lipids; X, unknown glycolipids; CMH, ceramide monohexosides; SG, steryl glycosides; PG, phosphatidylglycerols; PE, phosphatidylethanolamines; PC, phosphatidylcholines.

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Figure 30. Typical two dimensional TLC plates of polar lipids isolated from C. albicans KTCC 89062 grown on glucose or different chain length of n-alkanes.

The sorbent was Silica Gel G; the solvents were (I) chloroform/methanol/7M-ammonium hydroxide (65:30:4, by vol.) and (II) chloroform/methanol/acetic acid/water (170:25:25:4, by vol.). The lipids were visualised by charring. (a) glucose, (b) C_{12}, (c) C_{14}, (d) C_{16}, (e) C_{18}, (f) C_{20}. S, start; PI, phosphatidylinositols; PS, phosphatidylserines; PA, phosphatidic acids; PC, phosphatidylcholines; PE, phosphatidylethanolamines; PG, phosphatidylglycerols; X, unknown glycolipids; CMH, ceramide monohexosides; SG, steryl glycosides; ESG, esterified steryl glycosides; CL, cardiolipins; FA, fatty acids; NL, neutral lipids.
The fatty acids, found in the total lipid fraction, of *C. albicans* had a predominance of C16 and C18 carbon atoms, irrespective of the carbon source (Table 19). Palmitic acid (C16:0) was the major saturated fatty acid present and oleic acid (C18:1) was the principal unsaturated acid. When *C. albicans* was grown in the presence of n-alkanes rather than glucose there was an increase in the ratio of unsaturated:saturated fatty acids. This was brought about by a decrease in the level of both palmitic and stearic (C18:0) acids with an increase in oleic (C18:1), linoleic (C18:2) and C>18:3 acids. However, the levels of linolenic acid (C18:3) were also decreased. No significant effect on the level of palmitoleic acid (C16:1) was observed (Table 19). These changes in specific fatty acid levels found in the total lipid fraction are mirrored in the fatty acids found in the major lipid classes (Table 20).
Table 19. Constituent fatty acids of total lipids from *C. albicans* KTCC89062 grown on glucose or n-alkanes of different chain length.*

<table>
<thead>
<tr>
<th>Fatty acid</th>
<th>Glucose</th>
<th>C₁₂</th>
<th>C₁₄</th>
<th>C₁₆</th>
<th>C₁₈</th>
<th>C₂₀</th>
</tr>
</thead>
<tbody>
<tr>
<td>16:0</td>
<td>28.4±1.4</td>
<td>17.5±1.3</td>
<td>16.4±0.7</td>
<td>21.3±1.1</td>
<td>20.4±1.2</td>
<td>10.2±1.8</td>
</tr>
<tr>
<td>16:1</td>
<td>10.9±0.5</td>
<td>10.9±0.8</td>
<td>10.3±0.4</td>
<td>11.4±0.8</td>
<td>12.1±0.6</td>
<td>8.2±0.4</td>
</tr>
<tr>
<td>18:0</td>
<td>10.2±0.3</td>
<td>5.0±1.0</td>
<td>5.3±0.2</td>
<td>6.7±0.4</td>
<td>5.8±0.3</td>
<td>8.5±0.6</td>
</tr>
<tr>
<td>18:1</td>
<td>35.5±2.1</td>
<td>42.6±2.8</td>
<td>38.3±3.1</td>
<td>34.7±2.1</td>
<td>36.4±3.1</td>
<td>31.3±1.9</td>
</tr>
<tr>
<td>18:2</td>
<td>11.4±1.2</td>
<td>19.3±1.3</td>
<td>24.7±2.1</td>
<td>20.0±1.3</td>
<td>18.2±1.2</td>
<td>20.8±2.1</td>
</tr>
<tr>
<td>18:3</td>
<td>1.1±0.01</td>
<td>Tr</td>
<td>Tr</td>
<td>Tr</td>
<td>Tr</td>
<td>Tr</td>
</tr>
<tr>
<td>&gt; 18:3</td>
<td>2.5±0.3</td>
<td>4.7±0.1</td>
<td>5.0±0.6</td>
<td>5.9±0.7</td>
<td>7.1±0.8</td>
<td>11.0±0.9</td>
</tr>
</tbody>
</table>

**Ratio**  
unsaturated:saturated  
without > 18:3

* Values are expressed as percentages (w/w); each value is the mean ± SD of three determinations.

Tr, trace

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Table 20. Fatty acid patterns of major lipid classes from *C. albicans* ATCC 89062 grown on glucose or n-alkanes of different chain length.†

<table>
<thead>
<tr>
<th>Fatty acid</th>
<th>Glucose</th>
<th>C12</th>
<th>C14</th>
<th>C16</th>
<th>C18</th>
<th>C20</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Phosphatidylethanolamines</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>16:0</td>
<td>24.8±1.8</td>
<td>16.2±0.7</td>
<td>14.3±1.2</td>
<td>19.8±1.8</td>
<td>18.4±0.5</td>
<td>14.4±0.6</td>
</tr>
<tr>
<td>16:1</td>
<td>11.7±0.9</td>
<td>9.8±1.3</td>
<td>14.0±1.0</td>
<td>11.5±1.2</td>
<td>8.4±1.0</td>
<td>10.4±0.6</td>
</tr>
<tr>
<td>18:0</td>
<td>5.3±0.3</td>
<td>3.1±0.5</td>
<td>3.5±0.3</td>
<td>3.2±0.8</td>
<td>3.8±0.2</td>
<td>3.9±0.8</td>
</tr>
<tr>
<td>18:1</td>
<td>32.9±2.1</td>
<td>39.6±0.7</td>
<td>37.8±0.9</td>
<td>32.0±0.5</td>
<td>32.1±0.4</td>
<td>29.5±1.2</td>
</tr>
<tr>
<td>18:2</td>
<td>20.9±1.9</td>
<td>25.9±1.1</td>
<td>24.1±0.3</td>
<td>24.6±0.6</td>
<td>26.7±0.5</td>
<td>27.4±1.3</td>
</tr>
<tr>
<td>18:3</td>
<td>2.3±0.2</td>
<td>1.5±0.3</td>
<td>0.6±0.09</td>
<td>2.3±0.3</td>
<td>1.7±0.2</td>
<td>1.9±0.1</td>
</tr>
<tr>
<td>&gt; 18:3</td>
<td>2.1±0.8</td>
<td>3.9±0.2</td>
<td>5.7±0.4</td>
<td>6.6±0.1</td>
<td>8.9±0.8</td>
<td>12.5±0.6</td>
</tr>
<tr>
<td><strong>Phosphatidylcholines</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>16:0</td>
<td>23.6±1.7</td>
<td>8.7±0.8</td>
<td>7.9±0.2</td>
<td>12.9±0.8</td>
<td>13.3±0.9</td>
<td>12.8±0.9</td>
</tr>
<tr>
<td>16:1</td>
<td>15.1±0.3</td>
<td>14.6±0.4</td>
<td>15.8±0.5</td>
<td>15.0±1.1</td>
<td>16.1±1.2</td>
<td>7.6±0.6</td>
</tr>
<tr>
<td>18:0</td>
<td>2.8±0.3</td>
<td>2.6±0.1</td>
<td>1.6±0.3</td>
<td>1.2±0.2</td>
<td>1.5±0.9</td>
<td>5.9±0.8</td>
</tr>
<tr>
<td>18:1</td>
<td>36.0±1.1</td>
<td>44.8±1.4</td>
<td>39.4±1.2</td>
<td>36.6±0.5</td>
<td>39.7±0.2</td>
<td>32.7±0.8</td>
</tr>
<tr>
<td>18:2</td>
<td>15.2±0.9</td>
<td>22.7±0.4</td>
<td>30.2±1.3</td>
<td>25.6±0.4</td>
<td>21.3±0.5</td>
<td>27.8±1.4</td>
</tr>
<tr>
<td>18:3</td>
<td>1.3±0.01</td>
<td>0.4±0.05</td>
<td>1.0±0.1</td>
<td>1.1±0.05</td>
<td>0.3±0.01</td>
<td>1.4±0.05</td>
</tr>
<tr>
<td>&gt; 18:3</td>
<td>6.0±0.4</td>
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<td>4.1±1.1</td>
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<td>7.8±0.2</td>
<td>11.8±0.7</td>
</tr>
<tr>
<td><strong>Triacylglycerols</strong></td>
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<td>16:0</td>
<td>30.7±1.4</td>
<td>17.1±0.3</td>
<td>14.8±0.5</td>
<td>17.8±0.8</td>
<td>19.9±0.8</td>
<td>22.7±1.2</td>
</tr>
<tr>
<td>16:1</td>
<td>9.8±1.0</td>
<td>9.3±0.6</td>
<td>8.8±0.8</td>
<td>9.3±0.4</td>
<td>11.6±0.9</td>
<td>7.5±1.1</td>
</tr>
<tr>
<td>18:0</td>
<td>17.6±0.9</td>
<td>5.2±0.8</td>
<td>4.8±0.6</td>
<td>5.2±0.1</td>
<td>5.9±0.5</td>
<td>8.7±0.9</td>
</tr>
<tr>
<td>18:1</td>
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<td>41.5±0.8</td>
<td>38.6±1.2</td>
<td>38.9±0.9</td>
<td>35.5±1.2</td>
<td>39.7±0.2</td>
</tr>
<tr>
<td>18:2</td>
<td>7.1±0.9</td>
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<td>23.2±0.4</td>
<td>23.3±0.8</td>
<td>18.6±1.0</td>
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<td>Tr</td>
<td>Tr</td>
<td>0.5±0.01</td>
</tr>
<tr>
<td>&gt; 18:3</td>
<td>2.1±0.3</td>
<td>8.0±0.4</td>
<td>8.5±0.5</td>
<td>5.5±0.1</td>
<td>8.5±0.9</td>
<td>9.5±0.3</td>
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<td><strong>Fatty acids</strong></td>
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</tr>
<tr>
<td>16:0</td>
<td>30.4±0.6</td>
<td>23.0±0.8</td>
<td>22.1±1.0</td>
<td>21.4±0.7</td>
<td>25.0±0.7</td>
<td>23.8±0.9</td>
</tr>
<tr>
<td>16:1</td>
<td>9.1±0.6</td>
<td>8.6±0.3</td>
<td>8.5±0.9</td>
<td>7.6±0.2</td>
<td>6.9±0.8</td>
<td>6.1±0.8</td>
</tr>
<tr>
<td>18:0</td>
<td>15.2±0.7</td>
<td>13.1±0.4</td>
<td>14.3±1.1</td>
<td>13.4±0.4</td>
<td>13.5±1.2</td>
<td>13.7±1.0</td>
</tr>
<tr>
<td>18:1</td>
<td>32.4±1.5</td>
<td>39.6±0.9</td>
<td>34.4±1.2</td>
<td>37.6±0.9</td>
<td>32.9±0.9</td>
<td>33.1±0.5</td>
</tr>
<tr>
<td>18:2</td>
<td>9.8±0.3</td>
<td>12.2±0.5</td>
<td>15.8±0.3</td>
<td>14.7±0.9</td>
<td>12.6±0.6</td>
<td>13.0±0.4</td>
</tr>
<tr>
<td>18:3</td>
<td>1.3±0.1</td>
<td>0.8±0.02</td>
<td>0.5±0.01</td>
<td>Tr</td>
<td>0.3±0.05</td>
<td>0.7±0.1</td>
</tr>
<tr>
<td>&gt; 18:3</td>
<td>1.9±0.5</td>
<td>2.7±0.1</td>
<td>4.4±0.8</td>
<td>5.3±0.5</td>
<td>8.4±0.6</td>
<td>9.6±0.5</td>
</tr>
</tbody>
</table>

* Values are expressed in % (w/w) and are the means of ± SD of three determinations.

Tr, trace
Effect on Sterols

The examination of the sterol fraction of the isolate of *C. albicans* KTCC 89062 showed that there were quantitative differences apparent between cells grown on various carbon sources, as shown by TLC analysis (Fig. 31). Two major components were found in cells grown on glucose: 4,4-dimethyl sterols (such as lanosterol) and 4-methyl sterols. These were significantly reduced in cells grown on n-alkanes. There was an increase in 4-desmethyl sterols (such as ergosterol) in the later cells.

The UV absorption spectra of sterol fraction of *C. albicans* grown in the presence of glucose or individual n-alkanes showed that the spectra had peaks at 271, 282 and 293 nm, identical with the peaks of standard ergosterol (Fieser & Fieser, 1959). Quantitative estimation of the sterol fractions by UV measurements confirmed the higher levels of sterols in cells grown on n-alkanes as compared to glucose-grown cells (Fig. 32).
Figure 31. Typical TLC of sterols extracted from C. albicans KTCC 89062 cultures grown on glucose or different chain length of n-alkanes.

The sorbent was Silica Gel G; the solvent was pet. ether (40-60°C)/diethyl ether (3:1, by vol.). The sterols were visualised by charling. (1) C$_{20}$, (2) C$_{18}$, (3) C$_{16}$, (4) glucose, (5) C$_{12}$, (6) C$_{14}$. Identities of the spots: SQ, squalene; SE, stereryl esters; L, 4, 4-dimethyl sterol (lanosterol); ME, 4-methyl sterol; E, 4, 4-desmethyl sterol (ergosterol); DG, diacylglycerols.
Figure 32. Ultraviolet spectra of sterol extracted from *C. albicans* KTCC 89062 cultures grown on glucose or n-alkanes as a sole carbon source.
Quantitative examination, by GLC, showed that the level of ergosterol was increased significantly in all the cells grown on n-alkanes (Table 21). The levels were increased by approximately 50% in C. albicans grown on n-alkanes with chain lengths of C₁₂ and C₁₄ and approximately 40% on n-alkanes with chain lengths C₁₆ to C₂₀. Zymosterol was reduced to trace amounts in the n-alkane series. The most significant reduction was in the level of lanosterol by nearly 40%. On most substrates there was a fall in the level of squalene.

Effect of Ergosterol Supplementation on Hydrocarbon Uptake

Table 22 summarises the effect of ergosterol supplementation on the uptake of dodecane. When C. albicans was grown on ergosterol-supplemented CDM it showed an increase in the uptake of dodecane, as measured by the post-growth studies, compared to the control. This was particularly noticeable at the initial stages of incubation where the uptake of dodecane was much higher than in the control. The increased hydrocarbon uptake in response to ergosterol supplementation was concentration dependent (Table 22) which was true at various incubation periods. The stimulation of dodecane uptake by ergosterol was observed with viable cells but not with non-viable yeast cells (Table 23).
Table 21. Comparison of Sterols from *C. albicans* KTCC 89062 grown on glucose or n-alkanes of different chain length.

<table>
<thead>
<tr>
<th>Sterols</th>
<th>Rerg</th>
<th>Glucose</th>
<th>C₁₂</th>
<th>C₁₄</th>
<th>C₁₆</th>
<th>C₁₈</th>
<th>C₂₀</th>
</tr>
</thead>
<tbody>
<tr>
<td>Squalene</td>
<td>0.3</td>
<td>7.5±0.3</td>
<td>2.7±0.1</td>
<td>3.3±0.08</td>
<td>5.0±0.4</td>
<td>8.7±0.4</td>
<td>3.7±0.09</td>
</tr>
<tr>
<td>Calciferol</td>
<td>0.8</td>
<td>1.0±0.06</td>
<td>3.9±0.1</td>
<td>1.0±0.3</td>
<td>5.2±0.2</td>
<td>7.3±0.05</td>
<td>4.4±0.2</td>
</tr>
<tr>
<td>24-methylcholesterol</td>
<td>0.9</td>
<td>2.4±0.09</td>
<td>Tr</td>
<td>Tr</td>
<td>Tr</td>
<td>Tr</td>
<td>Tr</td>
</tr>
<tr>
<td>Ergosterol</td>
<td>1.0</td>
<td>49.4±0.7</td>
<td>78.0±0.9</td>
<td>76.8±1.2</td>
<td>83.0±0.4</td>
<td>70.2±0.8</td>
<td>69.2±0.6</td>
</tr>
<tr>
<td>4,14-dimethylcholesterol</td>
<td>1.1</td>
<td>5.7±0.4</td>
<td>Tr</td>
<td>3.7±0.4</td>
<td>3.7±0.3</td>
<td>Tr</td>
<td>4.3±0.1</td>
</tr>
<tr>
<td>Obtusifoliol</td>
<td>1.2</td>
<td>15.6±0.3</td>
<td>11.8±0.4</td>
<td>12.1±0.8</td>
<td>12.0±0.3</td>
<td>7.5±0.3</td>
<td>13.6±0.7</td>
</tr>
<tr>
<td>Lanosterol</td>
<td>1.3</td>
<td>15.6±0.08</td>
<td>3.0±0.1</td>
<td>1.5±0.2</td>
<td>5.1±0.1</td>
<td>6.3±0.4</td>
<td>4.8±0.6</td>
</tr>
<tr>
<td>24-methyl dihydrosta-</td>
<td>1.4</td>
<td>1.8±0.2</td>
<td>0.6±0.07</td>
<td>1.6±0.1</td>
<td>Tr</td>
<td>Tr</td>
<td>Tr</td>
</tr>
<tr>
<td>lanosterol</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Values are expressed as the percentage (w/w) of the total amount of sterols and are means ± SD of three determinations.

Tr, trace.
Rerg, retention time relative to ergosterol.
Table 22. Effect of ergosterol concentration on the uptake of dodecane by *C. albicans* KTCC 89062.

<table>
<thead>
<tr>
<th>Time (h)</th>
<th>Control</th>
<th>Supplementation with ergosterol (µg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>5</td>
</tr>
<tr>
<td>2</td>
<td>1.9±0.02</td>
<td>2.4±0.06</td>
</tr>
<tr>
<td>4</td>
<td>8.6±1.0</td>
<td>12.8±1.1</td>
</tr>
<tr>
<td>8</td>
<td>28.5±0.9</td>
<td>34.7±0.2</td>
</tr>
<tr>
<td>24</td>
<td>37.9±3.1</td>
<td>44.6±1.0</td>
</tr>
</tbody>
</table>

Data are expressed as percentage of dodecane consumed by *C. albicans* KTCC 89062 and are means ± SD of three determinations.
Table 23. Effect of ergosterol on dodecane uptake by viable and non-viable cells of *C. albicans* KTCC 89062.

<table>
<thead>
<tr>
<th>Time (h)</th>
<th>Control</th>
<th>Control</th>
<th>Control</th>
</tr>
</thead>
<tbody>
<tr>
<td>2</td>
<td>9.0±1.5</td>
<td>20.3±0.7</td>
<td>7.2±1.8</td>
</tr>
<tr>
<td>4</td>
<td>23.7±0.9</td>
<td>36.1±0.6</td>
<td>30.9±0.5</td>
</tr>
<tr>
<td>8</td>
<td>41.7±0.6</td>
<td>50.3±0.9</td>
<td>35.3±0.6</td>
</tr>
<tr>
<td>24</td>
<td>49.0±1.1</td>
<td>74.9±0.7</td>
<td>40.5±1.2</td>
</tr>
</tbody>
</table>

Data are expressed as percentage of dodecane consumed by *C. albicans* KTCC 89062 and are means ± SD of three determinations.
CHAPTER SEVEN
ENUMERATION AND ISOLATION OF OIL UTILISING MICROORGANISMS

Oil-utilising bacteria and fungi were isolated from both soil and marine environments in Kuwait. In this study *Rhodococcus* species were relatively the most abundant. Berdichevskaya *et al.* (1984, 1989) isolated several strains of *Rhodococcus* spp. from Perm Kama Valley oil fields. Among these *Rhodococci*, which were capable of hydrocarbon utilisation, *R. rhodochrous* was isolated. Other workers also reported the decomposition of hydrocarbons by representatives of the genus *Rhodococcus* (Rapp *et al*., 1979; Kretschmer *et al*., 1982; Kretschmer & Wagner, 1983). *R. erythropolis* was isolated from an oil-containing soil samples and had the ability to grow on a mixture of n-alkanes (C<sub>12</sub>-C<sub>16</sub>). Previously, this bacterium had been tentatively identified as *Nocardia rhodochrous* (Rapp *et al*., 1979).

The bacterial species most frequently associated with oil degradation include *Pseudomonas* spp. (Fuhs, 1961; Lebel & Mironov, 1973), *Nocardia* and Coryneforms (Polyakova, 1962; Cundell & Traxler, 1973; Mulkins-Phillips & Stewart, 1974), Arthrobacter (Cook & Westlake, 1974). Austin *et al.* (1977) examined 99 strains of oil degrading bacteria isolated from sediment and marine samples collected from oil polluted and unpolluted areas of Chesapeake Bay and found that *Pseudomonas* and *Nocardia* were the most frequently isolated genera. Oudot *et al.* (1986) isolated Corynebacterium and *Nocardia* species from agricultural soil contaminated with crude oil. Furthermore, Arthrobacter sp. was isolated from oil-contaminated tundra soil collected from Cape Simpson oil.
seep in Northern Alaska (Cundell & Traxler, 1974). This bacterium was tentatively identified by determining Gram reaction, cell morphology and pigmentation in addition to a few biochemical tests. Other species of bacteria have also been reported by a number of workers to be capable of degrading petroleum (Chapter 1).

Figure 7 shows that the isolated *Rhodococcus*, at certain stages of its life cycle, shows striking similarity in morphology to *Arthrobacter*, *Nocardia* and *Corynebacterium*. The cells acquire the coryneforms with irregularly stained walls typical of *Corynebacterium*. Very young cultures consist of filaments which fragment into rods as in *Nocardia*, and the rods, in turn, subdivide into cocci as in *Arthrobacter*. In view of that, it is not improbable that *Rhodococcus* spp. is a widely spread hydrocarbon utiliser in nature, and that it has sometimes been misidentified on account of its striking similarity to the above mentioned genera due to inadequate identification systems (e.g. Cundell & Traxler, 1974: Rapp et al., 1979).

Another possibility for the difference from the findings of others may be primarily due to the ecological nature of this region (arid) compared with temperate regions where most of the publications in literature came from. In this respect, Mulkins-Phillips and Stewart (1974) suggested that certain petroleum-degrading bacteria may be specific to given ecological niches. Therefore, it can be assumed that the extreme environment of Kuwait may be selective for certain microorganisms which can tolerate the harsh conditions of such an environment. *Rhodococcus rhodochrous* strains have an optimum temperature for growth at about 40°C and both tolerated 50°C. Furthermore, *R. rhodochrous* strains grew best at pH 8. The pH values of soil and water samples...
studied ranged between 7.7 and 8. It is also possible that the domination of Rhodococcus spp. observed in this study may be due to the composition of the Kuwait crude oil. The chemical composition of crude oils has been shown to be a factor in the determination of the types of bacteria which grow on oils (Westlake et al., 1974). In comparison with other crude oils, Kuwait crude oil has a high percentage of paraffins (alkanes). These hydrocarbons constitute up to 72% of the crude oil (the remaining hydrocarbons are 20% cycloparaffins and 8% aromatics), whereas in most of other crude oils the percentage of the paraffinic fraction ranges between 27-56%, except for Pennsylvania crude oil which has a paraffinic fraction percentage of 70% resembling in this respect Kuwait crude oil (Perry, 1984). The results obtained from Table 10 showed that the two bacterial isolates utilised only aliphatic hydrocarbons.

In the case of fungi, the most frequently isolated species were Candida albicans, Aspergillus terreus, A. sulphureus, Mucor globosus, Fusarium sp. and Penicillium citrinum. M. globosus was the most active in oil degradation (Fig. 16,II). Davies and Westlake (1979), screening oil-polluted and unpolluted soils collected from northern Canadian oil-producing areas, reported Penicillium and Verticillium spp. as the most frequently isolated genera. Fedorak et al. (1984) isolated 224 fungi from marine water and sediment samples taken from the strait of Juan de Fuca and northern Puget sound (state of Washington). Slightly over 70% of the fungi capable of growth on Prudhoe Bay crude oil belonged to the genus Penicillium. On the other hand, Oudot et al. (1986) found that most of the active fungi (isolated from oil contaminated agricultural soil) in oil degradation were Aspergillus and Penicillium spp. C. albicans was also reported to be a hydrocarbon
degrader (Osumi et al., 1974; Shennan & Levi, 1974; Singh et al., 1978).

Bacterial and fungal counts obtained from the present study (Table 8,9) showed high numbers of colony forming units when compared with other counts conducted by other researchers elsewhere (Tagger et al., 1983; Edlund et al., 1986). For an example, the number of bacteria that were able to grow on oil agar plates, isolated from sea sediments, were between 10 to $10^7$ times higher than the numbers of bacteria isolated by Mulkins-Phillips and Stewart (1974) using samples collected from North western Atlantic temperate to arctic marine environments. This could be due to the fact that the Arabian Gulf is a heavily polluted sea (Sen Gupta & Kureishy, 1981; El Samra et al., 1986) and it may be assumed that microbial strains active in oil degradation naturally occur in habitats containing petroleum since geological ages.

GROWTH AND COMPARATIVE STUDIES AS WELL AS CRUDE OIL ANALYSIS

The results obtained in Table 10 and Figure 16 showed that Rhodococcus isolates were more efficient in the consumption of aliphatic hydrocarbons than fungi. The two Rhodococcus strains were active in utilising $> C_{10}$ n-alkanes and failed to grow on shorter chain length of n-alkanes. However, Woods and Murrell (1989) isolated from soil a strain of R. rhodochrous capable of utilising only propane ($C_3$) from the n-alkanes tested ($C_1$-$C_8$) and was not capable of growth on alkenes. They found with their strain of R. rhodochrous a unique oxygenase enzyme (in terms of its inhibitor profile) which was not previously reported. The isolate of C. albicans used was able to grow adequately on only a narrow range ($C_{12}$ to $C_{20}$) of n-alkanes. In this it
differed from other strains of this organism (Prasad, 1985; Klug & Markovetz, 1967b) where n-alkanes C_9 to C_{11} were also utilised. This may be explained in terms of the high toxicity of these short-chain alkanes towards these isolates due to their greater solubility in the aqueous phase (Gill & Ratledge, 1972; Britton, 1984). Also the specificity of enzymes, involved in hydrocarbon degradation, towards their substrates could play an important role in determining the type of substrates to be degraded (Woods & Murrell, 1989).

Aromatic hydrocarbons were not degraded at all by any of the bacterial strains or C. albicans isolated in this study (Table 10), again they could be toxic to these organisms due to their greater solubility in the aqueous phase compared with long-chain liquid alkanes (Kleven, 1950). ZoBell (1969) suggested that some components of crude oil may be bacteriostatic or bactericidal. Although fungal isolates were able to utilise both aliphatic and aromatic hydrocarbons, they were more efficient in the degradation of the former. A salient point is that aliphatic hydrocarbons constitute up to 72% of Kuwait crude oil (Perry, 1984). It was also reported that aliphatic hydrocarbons are more susceptible to microbial attack than aromatic hydrocarbons (Chapter 1).

The metabolism of n-alkanes by bacteria and fungi present in samples of weathered Kuwaiti crude oil (crude oil free from volatile hydrocarbons) (Fig 16) was in agreement with the range of individual n-alkanes metabolised. Generally, in the crude oil the n-alkanes with chain lengths of C_{12} to C_{18} were preferentially metabolised, irrespective of whether an odd or even number of carbon atoms was present in the chain. Atlas et al. (1981) also found that when crude oil was degraded by a mixed microbial population the
n-alkanes were preferentially degraded. However, Fedorak and Westlake (1981a,b) observed, with mixed microbial populations, that the aromatic compounds present in crude oil were attacked first and then the n-alkanes.

In this study, *Rhodococcus* spp. were the most efficient oil degrader. This is particularly true of *R. rhodochrous* KUCC 8801 which was shown by IR studies to be more efficient than *R. rhodochrous* KUCC 8802 in dodecane utilisation. After 15 days incubation *R. rhodochrous* KUCC 8801 was able to degrade 77.5% of the 2% (v/v) dodecane tested, whereas *R. rhodochrous* KUCC 8802 degraded only 35% of the same hydrocarbon. Furthermore, *R. rhodochrous* KUCC 8801 was more efficient in crude oil utilisation than other isolates known to be active oil degraders as shown by GLC and post-growth studies. After three days, *R. rhodochrous* KUCC 8801 was about 2.5 fold more active in crude oil degradation than *Rhodococcus* sp. IS01, and about 4.5 times more active than *Pseudomonas putida* IR 32. This higher efficiency was maintained, albeit to a lesser extent, after five days incubation (Table 11). Post-growth results give further confirmation of the superior activity of *R. rhodochrous* KUCC 8801 (Fig. 19). After 48 hours, *R. rhodochrous* KUCC 8801 was about 2 fold more active in dodecane degradation than *Rhodococcus* sp. IS01 and *Acinetobacter calcoaceticus* IR07. Cane et al. (1983) investigated the degradation of Kuwait crude oil by an unidentified bacterium (Gram-negative motile rod which grew well on n-alkanes C_{10} - C_{18} but not on C_{9} or aromatic hydrocarbons) isolated from a sample of marine mud from Penarth, South Wales. The bacterium was able to degrade almost 80% of the crude oil within 3 days up to the concentration of 0.4-0.5% (v/v). On the other hand, *R. rhodochrous* degraded 85% of 1.0% (v/v) crude oil within 3
days. In this respect, Tagger et al. (1983) confirmed the advantage of natural bacterial communities in crude oil degradation over the introduced ones. Moreover, earlier investigators (e.g. Davies & Westlake, 1979; Fedorak & Westlake, 1981a,b; Foght & Westlake, 1982) used enrichment cultures in order to isolate oil utilisers. In the present study large numbers of oil utilising bacteria, with a majority of Rhodococcus spp., were directly counted in soil and sea water samples without enrichment. This indicates that this genus is actually propagating and metabolising oil actively in its habitat.

There was consistently a lower yield of C. albicans cells in CDM supplemented with n-alkanes when a comparison was made with CDM supplemented with glucose as the carbon source (Fig. 9). Growth yield of C. albicans cells grown on CDM supplemented with n-alkanes was two to five times lower than the yield obtained from cells grown on CDM supplemented with glucose (Table 17). A similar reduction in the yield of cell mass for C. albicans NCL 3100 grown on n-alkanes was observed by Singh et al. (1978) who found that alkane-grown cells were five to six times lower in cell yield compared to glucose-grown cells. Growth rate of R. rhodochrous strains grown on CDM supplemented with dodecane was slightly slower as compared with glucose-grown cells (Fig. 8).

The results obtained from Figure 10 indicate that measuring growth rate of hydrocarbon utilising microorganisms using the total viable count method is not a good technique to use especially if the microorganism tend to form filaments in the medium and adhere to the hydrocarbon droplets.
LIPID AND STEROL COMPOSITION

Lipids of microorganisms growing in batch cultures are dramatically altered by the different stages of growth (Castelli et al., 1969a,b,c). Due to this reason and the fact that, a slow growth rate, seen with C. albicans growing on n-alkanes, does encourage the accumulation of lipid in yeasts (Ratledge, 1978), determinations were made at the same point in the growth cycle, for each batch culture, so that valid comparisons could be made. The total lipid and total sterol contents of R. rhodochrous strains and C. albicans of hydrocarbon-grown cells were higher than those of glucose-grown cells. These results are in agreement with other findings (Chapter 4). The increase in the cellular lipids of hydrocarbon-grown cells is a common feature among hydrocarbon degraders. This increase in cellular lipids is due to the direct oxidation of n-alkanes to the corresponding fatty acids (Chapter 3). Hug et al. (1974) reported that a high cellular lipid concentration is necessary for hydrocarbon uptake, and not merely a reflection of the lipophilic nature of the substrate.

In the case of bacteria, the results show that the lipids of R. rhodochrous grown on glucose consist of a mixture of phospholipids and tri-, di- and mono-acylglycerols, with smaller proportions of sterols. The same composition is maintained when the bacteria are grown on dodecane as sole source of carbon and energy, except that these cells accumulate more sterols, monoacylglycerols, phosphatidylcholines (PC) and unknown glycolipid (Table 14). In this respect, Makula et al. (1975) reported an increase in the phospholipid, mono- and diacylglycerol fractions of Acinetobacter sp. grown on hexadecane as compared with nutrient broth- and yeast extract-grown cells. Moreover,
Vachon et al. (1982) studied the cellular and extracellular lipid composition of *Acinetobacter lwoffi* during diauxic growth on ethanol and hexadecane and found a 2.5-fold increase in cellular phospholipid content as a result of growth on hexadecane. Additional cellular lipids detected in this bacterium were free fatty acids, tri-, di-, and monoacylglycerols. Wax esters and fatty alcohols were present in trace amounts.

The phospholipid composition of *R. rhodochrous* is similar to that of other Gram-positive bacteria and differs mainly in the occurrence of PC. This phospholipid has been recorded in some Gram-negative bacteria (e.g. *Pseudomonas aeruginosa* and *Sarcina lutea*) but not Gram-positive bacteria or actinomycetes [for review see Lechevalier (1982)]. In view of that, polar lipids occur primarily in association with the cell membranes, the question arises as to whether PC, sterols and/or the unknown glycolipid in *R. rhodochrous* may be involved in the uptake and/or metabolism of hydrocarbon. The results obtained in Table 15 and 16 provide some evidence in favour of the role played by these lipid classes in hydrocarbon uptake. PC and the unknown polar lipid from dodecane-grown cells contained the highest concentrations of lauric (C\textsubscript{12}:0) and myristic (C\textsubscript{14}:0) acids. Lauric acid is known to arise from dodecane during the initial phase of its utilisation (Thorpe & Ratledge, 1972); myristic acid may have been formed from lauric acid by subsequent chain elongation (Chapter 3).

The occurrence of sterols in the hydrocarbon-utiliser *R. rhodochrous* is also quite unusual. Most procaryotes are known to lack sterols; exception are for example, methane-oxidising bacteria, in which squalene and a 4-methyl sterols are present (Harwood & Russell, 1984), in this respect *R.
rhodochrous strains resemble these methylotrophs. Due to their hydrophobic nature, it may be assumed that sterols in the *Rhodococcus* strains form hydrophobic sites in the cell membrane through which close contact between cells and hydrocarbon droplets is being facilitated (Chapter 2). This hypothesis is supported by the fact that dodecane-grown cells, especially of strain KUCC 8801, contain more sterols than glucose-grown cells. The hypothesis introduced by Ratledge (1987) suggested that a hydrocarbon droplet (perhaps in association with a biosurfactant) would enter the cell envelope through the receptor/channel molecule reported by Kappeli and Fiechter (1977) (Chapter 2). It is possible that sterols isolated from *R. rhodochrous* represent an alternative molecules for the polysaccharide-fatty acid complex described by Kappeli and Fiechter (1977).

The unknown glycolipid may also play a similar role as a biosurfactant (Rapp et al., 1979; Bryant, 1990) (Table 7) in alkane-uptake by strain KUCC 8802, which contains more of this glycolipid in dodecane than in glucose-grown cells (Table 14, Fig. 25). Considerable amounts of this glycolipid are also present in the growth medium of dodecane-grown cells (0.033 g/300 ml). Figure 27b gives further confirmation of the emulsifying property possessed by the unknown glycolipid. The extracellular glycolipid had a critical micelle concentration value of 1.8 mg/ml. Rapp et al. (1979) suggested a role for an acyl-glycolipid, trehalose-6,6-dicorynomycolates, in alkane uptake by *R. erythropolis*. It is of interest that the unknown glycolipid in *R. rhodochrous* strains is the richest lipid class in tuberculostearic acid (Table 16) which is a branched fatty acid, like the acyl moiety of the biosurfactant from *R. erythropolis*. It appears that in dodecane-uptake, strain KUCC 8801 may depend primarily on hydrophobic sterol sites...
in the cell membrane, while strain KUCC 8802 relies primarily on glycolipids as a biosurfactant, with the alternate mechanism being complementary in both strains. Presence of extracellular glycolipid with emulsifying activity, suggests that uptake of hydrocarbon is probably accomplished by facilitated diffusion rather than a passive one (Chapter 2).

The increase in glycolipids of dodecane-grown cells could be correlated with the decrease in di- and triacylglycerols. It is known that diacylglycerols act as a precursor in glycolipids and triacylglycerols synthesis (Harwood & Russell, 1984). The decrease in triacylglycerols suggests that the synthetic pathway of this neutral lipid from diacylglycerols has been suppressed, probably due to the conversion of the latter glyceride to glycolipids (Fig. 33).
Figure 33. Synthesis of galactosylglycerides (glycolipids) from diacylglycerols (adapted from Harwood & Russell, 1984).
ACP: Acyl carrier protein
UDP: Uridine diphosphate
Unlike the majority of bacteria, *R. rhodochrous* contains tuberculostearic acid [esterified only in phosphatidylglycerols (PG) and the unknown glycolipid], resembling in this respect *Corynebacterium simplex* that was described by Yanagawa et al. (1972a). The *C. simplex* had the ability to synthesise tuberculostearic acid (10-methyl fatty acids) when grown on n-alkanes mixture (C$_{14}$-C$_{16}$). They also showed that when n-octadecane was used as the growth substrate, a large amount of tuberculostearic acid was synthesized. However, the tuberculostearic acid found in their bacterium was esterified in phosphatidylinositol mannoside (PIMS). A pathway of methyl-branched fatty acids synthesis in *C. simplex* was proposed by Yanagawa et al. (1972a) and presented in scheme 8. The total lipid extracted of this bacterium corresponded to about 13% of the dry cell weight and consisted of almost equal amount of neutral and polar lipids, comprised PIMS and CL as the major components, and PG and PE as the minor components. In this respect, *R. rhodochrous* cells have PC and PE as their major phospholipids. These changes observed in the level of lipid classes could be due to genus differences.
Scheme 8. Synthesis of branched-chain fatty acids from C_{14-16} n-alkanes in Corynebacterium simplex (Yanagawa et al., 1972a).
de novo synthesis
chain elongation

$\text{CH}_3-(\text{CH}_2)_n-\text{CH}_3$

$\text{CH}_3-(\text{CH}_2)_n-\text{COOH}$

$\text{CH}_3-(\text{CH}_2)_{n-9}-\text{CH}=$

$\text{CH}=$

$\text{CH}-(\text{CH}_2)_7-\text{COOH}$

$\text{CH}_3-(\text{CH}_2)_{n-9}-\text{CH}=$

$\text{CH}=$

$\text{CH}-(\text{CH}_2)_7-\text{COO}-(\text{X}-\text{P})$ (Phospholipid)

\[ \text{SAM} \rightarrow \text{Methionine} \]

\[ \text{SAM} \rightarrow \text{B}_{12}^+ \text{enzyme} \rightarrow \text{FAH}_4 \]

\[ \text{SAH} \rightarrow \text{Homocysteine} \]

\[ \text{SAM: } S\text{-Adenosylmethionine} \]

\[ \text{SAH: } S\text{-Adenosylhomocysteine} \]

\[ \text{FAH}_4: \text{Tetrahydrofolate} \]
Although polyunsaturated linoleic (C\textsubscript{18}:2) and linolenic (C\textsubscript{18}:3) acids are distributed among various phospholipids, more linolenic acid is esterified in PC when the cells utilise dodecane than when they grow on glucose. Makula and Finnerty (1972) reported an increase in the unsaturated fatty acids isolated from phospholipids of Acinetobacter grown on > C\textsubscript{15} alkanes, accompanied by a decrease in oleic (C\textsubscript{18}:1) acid, as the chain length of n-alkanes increased. Ratledge (1987) reported that desaturation of the saturated fatty acids derived from n-alkanes must occur as cells require their phospholipids to be mobile and this would not occur if they were composed entirely of saturated acids. It is known that increased polyunsaturated fatty acid levels result in increased fluidity of the membranes (Hakomori, 1986), probably facilitating the passage of the hydrocarbons by becoming less of a barrier against the hydrocarbon droplets.

Ascenzi and Vestal (1979) showed that when Mycobacterium convolutum R 22 was grown on the n-alkanes C\textsubscript{13} through C\textsubscript{16} the predominant fatty acids were of the same chain length as the growth substrate. De novo fatty acid synthesis was inhibited by hexadecane, hexadecanoic acid, and hexadecanoyl coenzyme A (CoA). Cells that were grown on C\textsubscript{14} through C\textsubscript{16} n-alkanes had about 25 times less acetyl-CoA carboxylase activity than did cells grown on acetate or propane. These workers suggested that the regulation of de novo synthesis of fatty acids in M. convolutum can occur by inhibition and/or repression of the acetyl-CoA carboxylase. The results obtained in this study confirm earlier findings of Ascenzi and Vestal (1979), it was found that hydrocarbon-grown cells accumulate considerable amounts of fatty acids in their lipids with chain lengths equivalent to those of the hydrocarbons. It is known that an initial step of
hydrocarbon utilisation involves their oxidation to the corresponding fatty acids (Ascenzi & Vestal, 1979). It is interesting, therefore, that lauric and myristic acids become esterified mainly in PC. In eucaryotic systems, exogenous fatty acids appear intracellularly mainly in this class of phospholipids (Radwan & Mangold, 1980). Nevertheless, Yanagawa et al. (1972a,b) studied the utilisation of n-alkanes and 1-alkenes by C. simplex and indicated that the synthetic pathways of the cellular fatty acids of cells grown on various n-alkanes or 1-alkenes changed markedly according to the chain lengths of the substrates. From shorter chain hydrocarbons (C\textsubscript{12}, C\textsubscript{14}) the fatty acids were found to be synthesized mainly via de novo synthetic pathway in a similar manner to those from glucose-grown cells, while chain elongation and intact incorporation occurred to a very small extent. On the other hand, an intact incorporation mechanism was preferential in cells grown on longer chain hydrocarbons (C\textsubscript{16}, C\textsubscript{18}). When n-pentadecane or 1-pentadecene was used as the substrate, Yanagawa et al. (1972b) suggested that these three mechanisms operate simultaneously.

Concerning C. albicans, the composition of the lipid classes of this isolate did not differ from what is usually found in different species of yeasts (Chapter 4). However, the differences between the lipid contents of glucose- and n-alkane-grown cells were significant. The increase in total lipid content correlated with a decrease in cell yield. The level of sterol was correlated more closely with the chain length of the n-alkane substrate, i.e the highest level was detected with dodecane as substrate and fell progressively to eicosane (Table 17). Detailed analysis has shown that the increase in sterols was due to an increase in ergosterol which is the main sterol found in C. albicans
cells. Jeong et al. (1975) and Sica et al. (1984) found that ergosterol was the major sterol isolated from Pichia spp. and C. lipolytica grown on n-alkanes, respectively. The increase in ergosterol levels was most pronounced on CDM supplemented with dodecane and tetradecane. A significant increase was also observed in hexadecane to eicosane supplementation over the level found in CDM supplemented with glucose as sole carbon source.

Contradictory results were obtained by Singh and co-workers regarding ergosterol levels in C. albicans. Cultivation of C. albicans strain 3100 cells on pentadecane, hexadecane, heptadecane, and octadecane brought about 30-70% drop in total ergosterol level and it did not change significantly when grown on tridecane and tetradecane as compared with glucose-grown cells (Singh et al., 1978). In a subsequent study, Singh et al. (1979b) observed that more ergosterol was produced by C. albicans on n-alkanes of chain length less than heptadecane. However, they found, with their strain of C. albicans, that there was no significant change in ergosterol levels if heptadecane or octadecane was used. Our study showed that the increased ergosterol levels are accompanied by a decrease in the level of lanosterol, zymosterol and squalene (Table 21), suggesting that ergosterol biosynthesis from squalene proceeds at a higher rate in the presence of hydrocarbons as compared with cells grown on glucose (Van den Bossche et al., 1978). It is possible that n-alkanes are being used by C. albicans to synthesize ergosterol via the conversion of acetate (formed by β-oxidation of the fatty acids produced from n-alkanes oxidation) to mevalonate followed by the formation of squalene (Weete, 1980).
The changes in the non-sterol fraction of the lipids were most pronounced in the alteration of the ratio of unsaturated:saturated fatty acids. There was a pronounced fall in the levels of palmitic (C\textsubscript{16}:0) and stearic (C\textsubscript{18}:0) acids with a corresponding rise in oleic (C\textsubscript{18}:1) and linoleic acids (Table 19, 20). This may be correlated with the increase in sterol levels as well as in PE and PC levels which are the major phospholipids in order to maintain membrane fluidity and integrity. An increase in the phospholipid content has been reported in \textit{C. albicans} cells grown on n-alkanes as compared with glucose-grown cells (Singh \textit{et al.}, 1978). Singh \textit{et al.} found that PC and PE, which are the major phospholipids in \textit{C. albicans}, were about 2- to 3-fold more in tridecane-, tetradecane-, and pentadecane-grown cells. However, with their strain of \textit{C. albicans} they found that the total contents of PS and PI were higher in C\textsubscript{13} to C\textsubscript{16}-grown cells, and they were not detected in the phospholipids of C\textsubscript{17}-grown cells. CL was about 0.8% of the total phospholipids in glucose-grown cells and it could not be detected in any of the alkane-grown cells (Singh \textit{et al.}, 1978). In \textit{C. albicans} KTCC 89062 PI and PS, in general, decreased when n-alkanes were used as a growth substrate. In addition, CL was not detected in glucose-grown cells, while appearing in n-alkanes-grown cells (Table 18). These contradictory results could be due to the fact that Singh \textit{et al.} carried out the lipid analysis of their strain of \textit{C. albicans} at the exponential growth phase and at 30°C, whereas in this study the analysis was made at the stationary growth phase and at 37°C. It is known that different growth phases as well as different temperatures affect the composition of cellular lipids (Prasad, 1985).
Two dimensional TLC of *R. rhodochrous* and *C. albicans* (Fig. 25,30) and the results presented in Table (14,18) suggested that the synthesis of PC and PE in cells growing on n-alkanes proceeds at a higher rate compared to glucose-grown cells, this was evident by the disappearance of PA which is the first phospholipid to be formed in the synthetic pathway (Harwood & Russell, 1984). It was suggested by Thorpe and Ratledge (1972) that high concentrations of phospholipid are required to maintain the integrity of the enlarged and deeply invaginated cytoplasmic membranes of hydrocarbon assimilating cells. Electron micrographs of *C. albicans* KTCC 89062 as well as *R. rhodochrous* showed an increase in the surface area of the cytoplasm of n-alkane cells due to the pseudohyphal formation.

Presence of unknown glycolipids in *C. albicans* KTCC 89062 cells, which increased in n-alkane grown cells, may suggest that this type of polar lipid may play a role in the hydrocarbon uptake as a biosurfactant. Several yeasts have been reported for their ability to produce glycolipids as biosurfactants (Cooper & Paddock, 1984; Göbbert et al., 1984; Hommel et al., 1987). However, this assumption needs further testing in order to be confirmed. Again the increase in the unknown glycolipids was correlated with a decrease in di- and triacylglycerols with an increase in monoacylglycerols fraction. Singh et al. (1978) showed that there was no significant difference in total glyceride content of *C. albicans* when grown on glucose or n-alkanes. However, Thorpe and Ratledge (1972) reported a decrease in triacylglycerols of *Candida 107* and *C. tropicalis* when grown on various n-alkanes.

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It is known that membrane lipids are involved in several cellular functions including solute transport (Prasad & Rose, 1986). The role exerted by any individual lipid component in solute transport is not known and attempts at assessing such roles are difficult, particularly in view of the fact that alterations in membrane permeability are largely dependent upon the presence, nature and amounts of lipids in the surface layers of the yeast (Gale et al., 1975). In addition, the phospholipid:sterol ratio seems to exert an important role in membrane function (Gottlieb & Show, 1967). In this respect, this study showed that the phospholipid:sterol ratio decreased when R. rhodochrous and C. albicans cells were grown in the presence of n-alkanes as compared with glucose-grown cells. Thus, growth in the presence of hydrocarbons results in a complex dynamic situation, rather than a simple one, in response to the environment, and consequently the relative importance of any one component in the transport of hydrocarbons is difficult to speculate. Nevertheless, the function of lipids, in particular the sterols, could be to create a lipophilic region, in an otherwise hydrophilic environment to allow direct contact between the cell and the hydrocarbon and therefore causing diffusion and accumulation of the alkane substrate. This assumption receives support from the results indicating that supplementing cultures of C. albicans with ergosterol resulted in an increase of hydrocarbon uptake (Table 22). After two hours, C. albicans cells supplemented with 20 µg/ml ergosterol were about 22.5 times more active in dodecane uptake than unsupplemented cells. The higher activity was maintained, though to a lesser extent (2.5 times), after 24 hours. This is due to an increase in the uptake activity of the unsupplemented cells caused by a possible high rate of ergosterol biosynthesis from n-alkanes (Table 21). However, as
indicated by the viability tests [stimulation of dodecane uptake by ergosterol was observed only with viable cells (Table 23)], this process is clearly not a physical phenomenon, but a biological one and is probably controlled by specific enzyme system/s. The enzymes involved in the first step of alkane oxidation is thought to be located in or near the cytoplasmic membrane (Hug et al., 1974).

The fatty acids composition isolated from C. albicans KTCC 89062 grown on different chain length of n-alkanes suggested that they are derived through simultaneous operation of a chain elongation pathway and de novo synthesis after β-oxidation of the substrate (Chapter 4). This assumption receives support by the fact that eicosane-grown cells did not contain any C20 fatty acids. However, direct incorporation of fatty acids, after desaturation, could happen in case of using n-octadecane as a growth substrate (Table 19,20).

EFFECT OF HYDROCARBONS ON CELL MORPHOLOGY

The alteration in the levels of lipids extracted from alkane-grown cells reflects the levels in the cell membrane (Prasad, 1985) and these are possibly correlated with the altered permeability requirements of cells growing on hydrocarbons (Hug et al., 1974). These changes in membrane lipid composition can affect cellular morphology, e.g. sterol and phospholipid composition of the membrane is involved in the yeast-mycelium and white-opaque phenotypic variations in C. albicans (Ghannoum et al., 1986, 1989).

S.E.M. of R. rhodochrous showed that this organism tends to form filaments during growth on dodecane as compared with glucose-grown cells (Fig. 11). Further
investigation by using transmission electron microscopy techniques, confirms earlier observations (Kennedy et al., 1975; Scott & Finnerty, 1976a) in that bacterial cells grown on hydrocarbons contain vacuoles of submicron size, termed "hydrocarbon inclusions". It is believed that these hydrocarbon inclusions are formed due to the fact that hydrocarbons are water insoluble compounds, as a result they would quickly aggregate to form such inclusions (Ratledge, 1987).

Hydrocarbon inclusions were first identified by Kennedy et al. (1975) in Acinetobacter sp. growing on n-hexadecane, as the unmodified hydrocarbon growth substrate by using X-ray diffraction and gas chromatography analysis, and later by chemical characterisation of isolated inclusions (Scott & Finnerty, 1976a). It has been reported that the isolated hydrocarbon inclusions contained 71.5% hydrocarbon, 15.8% protein, 9.5% phospholipid, and 3.2% neutral lipid (Singer & Finnerty, 1984). Scott and Finnerty (1976a) provided an electron micrographs as an evidence for the presence of smooth-surfaced, monolayer inclusion membrane by using freeze-etch technique. The chemical analysis of inclusion membranes obtained by using this technique showed that these membranes closely resemble the cytoplasmic and intracytoplasmic membranes in phospholipid composition [PE is the major phospholipid], with the exception of having a higher proportion of PG. Compared with other membranes, Scott and Finnerty (1976a) showed that the hydrocarbon inclusions contained a high value of lipid phosphorous to protein (1.0 µmol/mg), a high value of hydrocarbon to protein (20 µmol/mg), and a small amount of 2-keto-3-deoxyoctulosonic acid to protein (95.0 pmol/mg) ratios. The major neutral lipid class associated with the hydrocarbon inclusions was wax ester (57% of the total neutral lipid),

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whereas in other membranes, fatty alcohol was the major neutral lipid class. The polypeptide composition of the inclusion membrane is dissimilar to any other cellular membrane fraction of hydrocarbon-grown *Acinetobacter* and contained 14 polypeptide species (Scott *et al.*, 1976). Kappeli and Finnerty (1979) showed that the enhanced solubility of hexadecane in the growth medium of hexadecane-grown *Acinetobacter* HOI-N was related to the accumulation of an extracellular vesicular component. The vesicle was characterised as lipopolysaccharide-rich, phospholipid-rich particle (88.6% of the total phospholipids is PE+PG), resembling in this respect the inclusion membrane. Borneleit *et al.* (1988) reported similar results by working with *Acinetobacter calcoaceticus* 69V.

Intracytoplasmic membrane systems (ICMS) which have been reported by a few researchers to be present in hydrocarbon-grown cells (Chapter 2), have not been detected in *R. rhodochrous* spp. In this respect the *R. rhodochrous* strains resemble other bacterial isolates which were reported to lack such ICMS (Atlas & Heintz, 1973; Kormendy & Wayman, 1974; Kennedy *et al.* 1975).

Filamentous forms of *C. tropicalis*, with smooth walls, were produced when it was grown on media containing n-alkanes (Ratledge, 1978). Similarly, growth of *C. albicans* in media containing n-alkanes resulted, in this study, in pseudohyphal formation (Fig. 12).

Ludvik *et al.* (1968) showed that hydrocarbons penetrate through the cell wall of *C. lipolytica* and accumulate at the surface of the cytoplasmic membrane bringing about numerous morphological changes of the cell including thickening of the cytoplasmic membrane and development of deep
invaginations and digital projections of this structure. In this respect, Yamaguchi et al. (1974) showed that filamentous cells of *C. albicans* are characterised by the extensive development of ICMS and endoplasmic reticulum. Such ICMS form whorled structures composed of multiple layers of unit membrane. Thus, it is proposed that the development of pseudohyphae observed in this study by *R. rhodochrous* and *C. albicans* in response to growth in n-alkanes is to increase the uptake of hydrocarbons by increasing the surface area of the cytoplasmic membrane.

The transmission electron micrographs of *C. albicans* KTCC 89062 grown on n-alkanes, revealed the presence of hydrocarbon inclusions. These findings are in agreement with the results of Scott and Finnerty (1976b) who found that hexadecane-grown cells of *Candida lipolytica* and tetradecane-grown cells of *C. tropicalis* contain electron transparent inclusions. Furthermore, Kormendy and Wayman (1974) found that *Candida utilis* and *Arthrobacter* sp. have such hydrocarbon inclusions when grown on butane or butanol.

To summarise, Kuwait soil and marine environments were found to be rich in oil-degrading microorganisms. Identification and oil degradation activity of these microorganisms had been carried out and it was found that such organisms, in particular *R. rhodochrous* strains, were potent in crude oil degradation. Biochemical analysis of lipids and sterols of *R. rhodochrous* and *C. albicans* suggested that sterols and lipids (especially phospholipids as well as glycolipids) could play a role in hydrocarbon uptake.
CONCLUSION AND FUTURE WORK

More investigation should be carried out regarding hydrocarbon uptake, little information is known about the mechanism by which a microorganism achieves hydrocarbon uptake and the role different lipid classes exert on this process. Emphasis should be put on the role of sterols and individual phospholipids being the major lipid components in the cell envelope. Results obtained from this study indicated the important role played by these two lipid classes, especially sterol, in hydrocarbon uptake. Specific compounds known to interfere with the biosynthesis of certain lipid classes could be used to alter the levels of lipids in the tested cells. Then these cells should be further tested for their ability to uptake hydrocarbons by applying post growth experiments (chapter 5). The compounds which affect cellular lipids include, hydroquinone and ascorbic acid known to affect sterol synthesis (Singh et al., 1979a,b), hydroxylamine known to affect phospholipid synthesis (Prasad, 1986), cerulenin affecting fatty acid synthesis (Hoberg et al., 1968), and tunicamycin affecting glycoprotein synthesis (Douglas & McCourtie, 1983; McCourtie & Douglas, 1985). Sterol deficient mutants can also be used in studying the role of sterols in hydrocarbon uptake.

Future work should also take into consideration, the high efficiency of *R. rhodochrous* in oil degradation and the possibility of using these isolates in controlling oil pollution. It is believed that *Rhodococcus* strains present in Kuwaiti environment could play an important role in controlling the recent Gulf oil spill, since these bacteria are adapted to the extreme environmental conditions of this region (Radwan, 1991).
It is also important to assess the biotechnological capabilities of *R. rhodochrous* strains and *C. albicans* versus biosurfactant and the production of intermediates, especially in ergosterol production by *C. albicans* since the results obtained during this study suggest hydrocarbon grown *C. albicans* as a commercial source of ergosterol.


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APPENDIX I
Figure 1. I.R. spectrum illustrates the dodecane (0.5%, w/v) consumption by *R. rhodochrous* KUCC 8801.

a - control
b - after 5 days incubation
c - after 10 days incubation
d - after 15 days incubation
Figure 2. I.R. spectrum of different sterols isolated from *C. albicans* KTCC 89062.

a - ergosterol  
b - lanosterol  
c - calciferol  
d - squalene
Figure 3. Gas-liquid chromatograms on 15% DEGS of methyl esters of fatty acids isolated from, (a) *R. rhodochrous* grown on glucose, (b) *R. rhodochrous* grown on dodecane, (c) *C. albicans* KTCC 89062 grown on glucose, (d) *C. albicans* grown on n-alkane. Column temperature was 180°C.

Key to the methyl esters of fatty acids: 1, lauric (C_{12:0}); 2, myristic (C_{14:0}); 3, palmitic (C_{16:0}); 4, palmitoleic (C_{16:1}); 5, stearic (C_{18:0}); 6, tuberculostearic; 7, oleic (C_{18:1}); 8, linoleic (C_{18:2}); 9, linolenic (C_{18:3}); 10, C_{>18:3} acids.
Figure 4. Gas-liquid chromatograms on 3% SP-2100 of trimethylsilyl (TMS) derivatives of total sterols from (a) *R. rhodochrous* grown on glucose, (b) *R. rhodochrous* grown on dodecane, (c & d) *C. albicans* KTCC 89062 grown on glucose, (e & f) *C. albicans* KTCC 89062 grown on n-alkane.

Key to gas-liquid chromatograms: 1, squalene; 2 & 3, breakdown products of ergosterol; 4, calciferol; 5, zymosterol; 6, ergosterol; 7, 4,14-dimethylzymosterol; 8, obtusifoliol; 9, lanosterol; 10, 24-methylidihydrolanosterol.
1 - The Mean

\[ \bar{x} = \frac{\sum x}{n} \]

2 - Standard Deviation

\[ SD = \sqrt{\frac{\sum (x - \bar{x})^2}{n - 1}} \]

\( \bar{x} \) signifies the mean.

\( x \) signifies each of the values observed.

\( n \) signifies number of terms.
APPENDIX III

(PUBLICATIONS)
LIST OF PUBLICATIONS


Crude Oil and Hydrocarbon-Degrading Strains of *Rhodococcus rhodochrous* Isolated from Soil and Marine Environments in Kuwait

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ABSTRACT

Soil and marine samples collected from different localities in Kuwait were screened for microorganisms capable of oil degradation. Both fungi and bacteria were isolated. The fungal flora consisted of *Aspergillus terreus*, *A. sulphureus*, *Mucor globosus*, *Fusarium* sp., and *Penicillium citrinum*. *Mucor globosus* was the most active oil-degrading fungus isolated. Bacterial isolates included *Bacillus* sp., *Enterobacteriaceae*, *Pseudomonas* spp., *Nocardia* spp., *Streptomyces* spp. and *Rhodococcus* spp. Among these *Rhodococcus* strains were the most efficient in oil degradation and, relatively speaking, the most abundant. Bacterial and fungal isolates differed in their ability to degrade crude oil, with *Rhodococcus* isolates being more active than fungi in n-alkane biodegradation, particularly in the case of *R. rhodochrous*. In addition to medium chain n-alkanes, fungi utilized one or more of the aromatic hydrocarbons studied, while bacteria failed to do so. *R. rhodochrous* KUCC 8801 was shown by GLC and post-growth studies to be more efficient in oil degradation than isolates known to be active oil degraders.

INTRODUCTION

The oil industry and various related processes, such as extraction and transport, pose a constant threat to the marine environment as...
consequence of the huge influx of petroleum hydrocarbons into this ecosystem. The yearly influx of petroleum pollutants in the sea has been estimated to be between 6 and 10 million tons (Blumer et al., 1971; National Academy of Science, 1975). This problem is more acute in an oil producing area like the Arabian Gulf, where much of the world's oil is produced and large quantities, representing around 60% of the global marine transport of oil (British Petroleum Co., 1980), are transported through this shallow and enclosed sea (Hunter, 1982). Oil pollution in this region arises from local exploration, exploitation, refining and routine handling of petroleum at ports.

El Samra et al. (1986) showed that the northwestern region of the Arabian Gulf is heavily polluted, compared with other marine environments. These workers showed that the Arabian Gulf had a petroleum hydrocarbon concentration of 1.2-546 μg ml⁻¹, compared with 0.4 to 66.8 μg ml⁻¹ in the Gulf of Mexico (Marchand et al., 1982). Similar findings were reported by Sen Gupta & Kureishy (1981) who stated that the trade and tanker routes across the Arabian Sea were more contaminated than similar routes over the southern Bay of Bengal.

This situation makes the study of different aspects of petroleum degradation a prime concern to this region. However, little attention has so far been directed towards such studies. To fill this gap, we have recently embarked on a research programme to try to address certain aspects of biodegradation of crude oil and some individual hydrocarbons.

The initial aim of this investigation was to conduct a survey of the Kuwaiti soil and marine environment for microorganisms capable of oil degradation. It is hoped that a study of such environment, which has been naturally rich in oil since geological time, should lead to the isolation of potent degraders. Identification and characterisation of these isolates would then be performed, and selected isolated strains, efficient in oil degradation, compared for their hydrocarbon degrading abilities with isolates reported to be efficient oil degraders.

MATERIALS AND METHODS

Samples and cultures

Desert and garden soil samples, as well as samples from oil fields and salt marshes, were collected from different localities in Kuwait (Fig. 1). The samples were taken aseptically after removing the 5 cm surface layers. Samples of water and marine sediment were collected 20 m offshore from the Arabian Gulf at about 10 km intervals. Kuwaiti crude oil obtained
Crude oil hydrocarbons into this pollutants in the sea has been (Blumer et al., 1971; National more acute in an oil producing more than the global marine transport of was reported through this shallow and in this region arises from local oil handling of petroleum at the northwestern region of the Arabian oil producing marine environments. These had a petroleum hydrocarbon had with 0.4 to 66.8 µg ml⁻¹ in the similar findings were reported by that the trade and tanker routes different than similar routes over the different aspects of petroleum However, little attention has to fill this gap, we have recently to address certain aspects of individual hydrocarbons. to conduct a survey of the microorganisms capable of oil environment, which has been should lead to the isolation of characterisation of these isolates would results, efficient in oil degradation, abilities with isolates reported from the ‘Kuwait Oil Company’ in Al-Ahmadi, Kuwait, was used in these studies. An inorganic medium, supplemented with 10 g litre⁻¹ weathered crude oil, as a sole source of carbon, was used for counting bacteria. Crude oil was weathered by sparging it with nitrogen gas (flow rate 50 cm³ litre⁻¹ min⁻¹) for 24 h at 25°C. The oil was subsequently heated in a water bath at 60°C for 1 h to decrease viscosity, and then was sterilised by membrane filtration, pore size 0.45 µm. The inorganic medium had the following composition (g litre⁻¹): 0.85 NaNO₃, 0.56 KH₂PO₄, 0.86 Na₂HPO₄, 0.17 K₂SO₄, 0.37 MgSO₄·7H₂O, 0.007 CaCl₂·2H₂O, 0.004 Fe (III) EDTA, 2.5 ml of a trace element solution consisting of (g litre⁻¹): 2.32 ZnSO₄·7H₂O, 1.78 MnSO₄·4H₂O, 0.56 H₃BO₃, 0.039 Na₂MoO₄·2H₂O, 0.42 COCl₂·6H₂O, 0.66 KI, 10 EDTA, 0.4 FeSO₄·7H₂O, 0.004 NiCl₂·6H₂O. Solid media were prepared by adding 20 g litre⁻¹ of agar. For sea water and salt marsh samples 30 g litre⁻¹ NaCl was added. For enumerating fungi, the above medium was supplemented with 10 ml litre⁻¹ vitamin solution...
composed of (mg litre\(^{-1}\)): 0.01 biotin, 2.0 pyridoxin, 2.0 nicotinic acid, 2.0 thiamin and 10.0 mesoinositol. The pH was adjusted to 7 and 5 to favour bacteria and fungi, respectively. The microbial numbers were monitored using a spread plate count technique (Koch, 1981). Filter-sterilised, weathered crude oil (0.1 ml) (1:1 v/v with CCl\(_4\)) is spread over the agar surface with a spreader. If the agar is allowed to dry out for several days at room temperature, the mixture is quickly adsorbed. The plates are then dried to allow evaporation of all the CCl\(_4\). This technique ensured even dispersal of oil in the medium. Dilutions for soil and sea sediment samples were prepared by using 0.9% w/v NaCl. Triplicate plates from two separate serial dilutions were incubated for 7 and 14 days before counting. Mean values were calculated and the number of organisms was recorded as number per gram dry weight sample. For sea water analysis 100 ml water samples were filtered by using sterilised membrane filters (pore size 0.45 \(\mu\)m). The membrane filters were then aseptically transferred to Petri dishes containing oil agar media and incubated for similar periods. Triplicate plates were prepared, the mean values were calculated, and the number of organisms was recorded as number per 100 ml of sample. The incubation temperature was 25 and 30°C for fungi and bacteria, respectively.

The golf ball technique (Gilbert & Higgins, 1978) was also used to isolate oil utilisers from the marine environment. A 47 mm diameter cellulose acetate filter disc (8 \(\mu\)m pore size) was impregnated with weathered crude oil and inserted through a slit made in a perforated plastic practice golf ball to form an equatorial diaphragm. After rescaling the ball with a hot knife, it was placed into a perforated container, which was suspended in sea water for 15 days. The filter disc was then cut and shaken vigorously in sea water with 2 mm glass beads for 5 min to release the microorganisms which were then plated on the above media. Conventional enrichment techniques, using the above media, were used to isolate oil utilisers from sea sediments.

Bacterial isolates were identified by consulting the Bergey's Manual, 9th ed. and fungi by referring to Booth (1971), Raper & Fennell (1977), Pitt (1979) and Domsch et al. (1980). The identities of certain bacterial isolates were confirmed by the National Collections of Industrial and Marine Bacteria, Scotland. Oil-degrading cultures of Rhodococcus sp. ISO1, Acinetobacter calcoaceticus IR07 and Pseudomonas putida IR32 were kind gifts from J. Oudot, Museum National d'Histoire Naturelle, Paris, France, and were used in comparative studies.

**Electron microscopy**

Cells were suspended in glutaraldehyde (1.5% w/v) for 2 min, collected by centrifugation and re-suspended in a fresh sample of the same fixative for
Crude oil and hydrocarbon-degrading strains of R. rhodochrous

16 h at 4°C. Fixed cells were prepared for scanning electron microscop; following the method of Ghannoum and Al Khars (1984). These sample were examined in a stereoscan electron microscope (Novascan 30) at an angle of 45°.

Growth on pure hydrocarbons

The aliphatic hydrocarbons used were hexane, heptane, iso-octane, nonane, decane, dodecane, tridecane, tetradecane, hexadecane, octadecane and eicosane. The aromatic hydrocarbons were benzene, anthracene, phenanthrene and naphthalene. In qualitative studies, the vapour phase technique was used for liquid hydrocarbons. Bacteria and fungi were inoculated onto the inorganic medium, mentioned above, without oil in Petri dishes. Sterile filter papers placed in the lids were impregnated, each with 0.5 ml of filter sterilised hydrocarbon. The bases of the plates were then replaced, kept inverted and the plates sealed with masking tape. Solid hydrocarbons were first dissolved in diethyl ether to give 0.1% solutions (w/v) and 1 ml of each solution was sprayed as a thin even film on an agar plate. After the solvent had evaporated, the test microorganisms were streaked and the plates sealed. For quantification, microorganisms were inoculated into liquid medium containing 10 g litre⁻¹ of each hydrocarbon. The cultures were incubated on an orbital shaker and the biomass was collected by centrifugation for bacteria and filtration for fungi, quickly washed with hexane to remove attached hydrocarbons, then dried and weighed.

Analytical techniques

The biodegradation of crude oil was studied by gas chromatography (GLC). The residual oil was recovered from the supernatant of bacterial and fungal cultures by extracting three times with hexane. The constituent hydrocarbons were resolved by GLC using a Pye-Unicam 204 instrument equipped with an SP-2100 column with a temperature programme 60-250°C, raising the temperature 16°C min⁻¹. Individual hydrocarbons were identified by comparing their retention times with those of standard samples. The identities of individual components were further confirmed by comparison of retention times of the peaks with those of reference standards, using 3% OV-1 as a second column. The GLC-profiles were compared with the profile of the same amount of crude oil treated similarly, but without inoculation with microorganisms (control). Oil consumption was estimated quantitatively by comparing the total peak area in the GLC-profile of residual oil to that in the GLC-profile of the same amount of oil recovered from control samples. To minimise quantitative errors, the
external standardisation method was used, employing a precision syringe to ensure that reproducible sample sizes could be injected onto the column (Lee et al., 1984). Triplicate analyses of the same sample were reproducible to within ±5%. The same method was also employed for the post-growth studies.

Selected bacterial isolates were characterised and evaluated with regard to their oil degrading potential. Utilisation of pure hydrocarbons by these bacterial isolates was studied quantitatively in infra-red (IR) spectrophotometry (Willard et al., 1981). A sample of filter-sterilised dodecane (250 µl) was added to 50 ml of the inorganic medium in 250 ml Erlenmeyer flasks. These flasks were inoculated with 0.1 ml suspension of an overnight culture of bacterial cells in phosphate buffer. Uninoculated sterile controls were also prepared by adding 10 mg of HgCl₂ per flask. Both test and control flasks were incubated in a rotary shaker at 30°C. Flasks were removed at different time intervals (0, 5, 10 and 15 days) and contents were analysed for residual dodecane as follows: the entire contents of each flask were sonicated for 5 min at 20-40 kHz and 140 W, and acidified with 1 ml 1 M HCl and 3 g NaCl. The contents were then transferred into a separatory funnel and extracted (three times) with 15 ml CCl₄ (spectra grade). A sample of the CCl₄ phase was withdrawn and transferred into a cell of 10 mm path length where the IR-spectra were recorded using a Perkin-Elmer 398 IR spectrophotometer. CCl₄ was routinely incorporated as a reference. The sum of peak heights above the base line at 3.50, 3.42 and 1.38 µm, representing —CH₃, —CH₂ and —CH stretching frequencies, was taken as a quantitative measurement of the amount of dodecane. The amount of dodecane was determined with reference to a standard curve constructed using a range of dodecane concentrations in CCl₄ to correspond with the amount used in the flasks.

**TABLE I**

<table>
<thead>
<tr>
<th>Soil sample</th>
<th>Bacterial colony forming units</th>
<th>Fungal colonies</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>7 days</td>
<td>14 days</td>
</tr>
<tr>
<td>Oil field 1</td>
<td>(6.5 ± 0.4) x 10⁷ (6.7 ± 0.1) x 10⁷</td>
<td>(6.8 ± 0.1) x 10⁷ (6.2 ± 0.06) x 10⁷</td>
</tr>
<tr>
<td>Oil field 2</td>
<td>(1.2 ± 0.01) x 10⁸ (1.3 ± 0.08) x 10⁸</td>
<td>(4.7 ± 0.3) x 10⁵ (9.3 ± 0.8) x 10⁵</td>
</tr>
<tr>
<td>Oil field 3</td>
<td>(1.65 ± 0.03) x 10⁸ (1.7 ± 0.06) x 10⁸</td>
<td>(8.1 ± 0.5) x 10⁵ (1.3 ± 0.08) x 10⁶</td>
</tr>
<tr>
<td>Oil field 4</td>
<td>(1.9 ± 0.1) x 10⁸ (2.0 ± 0.1) x 10⁸</td>
<td>(3.3 ± 0.3) x 10⁵ (7.2 ± 0.3) x 10⁵</td>
</tr>
<tr>
<td>Garden 1</td>
<td>(4.27 ± 0.3) x 10⁷ (4.3 ± 0.4) x 10⁷</td>
<td>(3.0 ± 0.08) x 10⁵ (4.2 ± 0.06) x 10⁵</td>
</tr>
<tr>
<td>Garden 2</td>
<td>(3.1 ± 0.07) x 10⁷ (3.15 ± 0.6) x 10⁷</td>
<td>(3.0 ± 0.2) x 10³ (9.5 ± 0.3) x 10²</td>
</tr>
</tbody>
</table>

Each value is the mean ± SD (± g⁻¹) of three determinations. Values varied by < 10%.
employing a precision syringe and evaluated with regard to pure hydrocarbons by these in infra-red (IR) spectrophotometer. Oil samples were sonicated with 1 ml HCl and into a separatory funnel and 1 grade). A sample of the CCl₄ 10 mm path length where (Oudot, pers. comm.). Cells were collected and contents were analysed for of each flask were sonicated differed with 1 ml HCl and as a quantitative reference. The sum of peak 3-38 μm, representing —CH₃, was taken as a quantitative the amount of dodecane was constructed using a range of d with the amount used in the

<table>
<thead>
<tr>
<th>Source of Carbon and Energy</th>
<th>Fungal colonies</th>
</tr>
</thead>
<tbody>
<tr>
<td>7 days</td>
<td>14 days</td>
</tr>
<tr>
<td>±0.1 x 10⁴</td>
<td>(6.2 ± 0.06) x 10⁴</td>
</tr>
<tr>
<td>±0.3 x 10⁴</td>
<td>(9.3 ± 0.08) x 10⁴</td>
</tr>
<tr>
<td>±0.5 x 10⁴</td>
<td>(1.3 ± 0.08) x 10⁴</td>
</tr>
<tr>
<td>±0.2 x 10⁴</td>
<td>(7.2 ± 0.3) x 10⁴</td>
</tr>
<tr>
<td>±0.08 x 10⁴</td>
<td>(4.2 ± 0.06) x 10⁴</td>
</tr>
<tr>
<td>±0.2 x 10⁴</td>
<td>(9.5 ± 0.5) x 10⁴</td>
</tr>
</tbody>
</table>

mum. Values varied by <10%.

Crude oil and hydrocarbon-degrading strains of R. rhodochrous

Post-growth utilisation of dodecane by a selected bacterial isolate was estimated quantitatively and compared with the activity of certain bacteria known to be efficient oil degraders (Oudot, pers. comm.). Cells were collected after growth in dodecane-containing medium by centrifugation, washed suspended in the liquid medium without dodecane, and 10 ml samples were dispensed in sterile bottles. To each bottle 0.1 ml of dodecane was added and the bottles were incubated in a shaking water bath at 30°C. At different time intervals, two bottles were removed, and one only was supplied with 0.1 ml of dodecane to serve as a control. The hydrocarbons in the two bottles were then immediately recovered by extracting twice with 5 ml heptane, followed by GLC analysis, after which their peak areas were compared.

RESULTS

Oil utilising microorganisms

Tables 1 and 2 summarise the bacterial and fungal counts recovered on oil agar medium in soil and marine samples. Fungi occurred mainly as mycelial rather than other surviving propagules, as confirmed by the conventional burial slide technique.

The fungal flora consisted of Aspergillus spp., A. terreus, A. sulphureus, Mucor glabosus, Fusarium sp., Penicillium spp. and P. citrinum, arranged in descending predominance. Bacterial isolates included Bacillus spp., Enterobacteriaceae, Pseudomonas spp., Nocardia spp., Streptomyces spp. and Rhodococcus spp. Among these, Rhodococcus strains were relatively the most abundant. The numbers of Rhodococcus cells in water samples showed great variation from one locality to another. They were especially high in oil polluted localities, but low in non-polluted water samples (Table 2). In all localities, Rhodococcus spp. were readily isolated by the gold ball technique. These isolates were highly pleomorphic, consisting of filaments or coryneform Gram-positive non-motile rods which sub-divided in old cultures into cocci (Fig. 2). The chemotaxonomic analysis of R. rhodochrous KUCC 8801 revealed that the cell wall diamino acid was diaminopimelic acid and that its total lipids contained tuberculostearic acid, in addition to major amounts of palmitic, palmitoleic and stearic acids. This isolate was catalase positive and oxidase negative. It decomposed tyrosine and Tween 80 but not adenine and urea. It grew on glucose, maltose, mannitol, sorbitol, sodium adipate, sodium benzoate, sodium citrate, sodium lactate, testosterone, L-tyrosine, glycerol and p-hydroxybenzoic acid as sole sources of carbon, but not on inositol, or trehalose.
### TABLE 2

Marine Bacteria and Fungi Utilizing Oil as a Sole Source of Carbon and Energy

<table>
<thead>
<tr>
<th>Site no.</th>
<th>Number of bacterial colonies forming units in sea sediment [$\times 10^3$]</th>
<th>Number of bacterial colonies forming units in sea water [$\times 10^3$]</th>
<th>Number of fungal colonies in sea water [$\times 10^3$]</th>
</tr>
</thead>
<tbody>
<tr>
<td>7 days</td>
<td>14 days</td>
<td>7 days</td>
<td>14 days</td>
</tr>
<tr>
<td>1</td>
<td>$(10.5 \pm 0.9) \times 10^3$</td>
<td>$(30 \pm 0.4) \times 10^3$</td>
<td>$(0.8 \pm 0.04) \times 10^3$</td>
</tr>
<tr>
<td>2</td>
<td>$(15 \pm 1.2) \times 10^3$</td>
<td>$(37 \pm 0.2) \times 10^3$</td>
<td>$(3.2 \pm 0.1) \times 10^3$</td>
</tr>
<tr>
<td>3</td>
<td>$(26 \pm 0.5) \times 10^3$</td>
<td>$(42 \pm 0.4) \times 10^3$</td>
<td>$(7.0 \pm 0.6) \times 10^3$</td>
</tr>
<tr>
<td>4</td>
<td>$(57 \pm 1.6) \times 10^3$</td>
<td>$(65 \pm 0.9) \times 10^3$</td>
<td>$(7.0 \pm 0.1) \times 10^3$</td>
</tr>
<tr>
<td>5</td>
<td>$(156 \pm 3.5) \times 10^3$</td>
<td>$(30 \pm 1.8) \times 10^3$</td>
<td>$(1.9 \pm 0.1) \times 10^3$</td>
</tr>
</tbody>
</table>

* Oil polluted sites.
ND Not determined.

Each value is the mean ± SD of three determinations. Values varied by <10%.
Table 1: Cell counts of R. rhodochrous in various treatments.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Count (cell/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>(156 ± 3.8) × 10³</td>
</tr>
<tr>
<td>2</td>
<td>(305 ± 1.8) × 10³</td>
</tr>
<tr>
<td>3</td>
<td>(108 ± 0.1) × 10³</td>
</tr>
<tr>
<td>4</td>
<td>(2.1 ± 0.2) × 10³</td>
</tr>
<tr>
<td>5</td>
<td>(1.1 ± 0.1) × 10³</td>
</tr>
</tbody>
</table>

* Oil polluted sites.
ND Not determined.
Each value is the mean ± SD of three determinations. Values varied by <10%.

Fig. 2: Light and scanning electron micrographs of R. rhodochrous, KCC 8801. Note that this organism is present at various stages of growth. (a) Conidiation and hyphae; (b) and (c) hyphae and hyphae, respectively. Micrographs a, b, and c = 3000x magnification.
Biodegradation of crude oil

Bacterial and fungal isolates, grown in shaken cultures in a medium containing weathered crude oil as a sole source of carbon and energy, differed in their biodegradation activity. Active isolates brought about visible physical changes in the medium after only 24 h, whereas less active isolates took up to 1 week. Three distinct types of changes brought about by bacterial isolates were equally frequent: 1. formation of homogeneous oil emulsions in the aqueous medium; 2. disintegration of oil into 1–2 mm granular particles suspended in the aqueous phase; and 3. development in the aqueous medium of large clumps.

Utilisation of individual hydrocarbons

The results of screening of representative predominant bacteria and fungi for growth on media provided with 10 g litre\(^{-1}\) each of a pure hydrocarbon are presented in Table 3. Short chain alkanes were not utilised by *Rhodococcus*, but straight chain alkanes with 12 to 20 carbon atoms and the alkene tridecene were readily utilised. The biomasses were directly proportional to the hydrocarbon chain length. The branched chain isooctane and the aromatic hydrocarbons were not attacked by these bacteria. On the other hand, the fungal isolates tested could utilise one or more of the aromatic hydrocarbons studied in addition to the medium chain \(n\)-alkanes (Table 3).

Gas chromatography of the residual oil recovered after microbial growth (Fig. 3) showed that *Rhodococcus* isolates were more active than fungi in \(n\)-alkane biodegradation, as indicated by the intensities of the peak areas of the residual hydrocarbons.

Figure 3 clearly indicates that a difference exists in the ability of various fungal isolates to degrade crude oil. *Mucor globose* was the most active oil degrader among the fungi. The spectrum of the initial degradation of oil by bacteria was identical.

Dodecane utilisation by *Rhodococcus rhodochrous*

Dodecane was selected to study its utilisation under controlled cultural conditions by two isolates of *Rhodococcus*, namely KUCC 8801 and KUCC 8802. In contrast to strain KUCC 8802, strain KUCC 8801 could liquify gelatin and ferment sucrose and glucose. Its growth was lighter in colour and resulted in the disintegration of crude oil into small granules, whereas KUCC 8802 caused the production of an emulsion. The two isolates grew best at pH 8. The pH values of soil and water samples studied
isolates brought about only 24 h, whereas less active changes brought about by formation of homogeneous oil droplets and migration of oil into 1–2 mm thick phase; and 3. development in

<table>
<thead>
<tr>
<th>Bacteria</th>
<th>Hydrocarbons</th>
<th>A. rhodochrous</th>
<th>A. subterraneus</th>
<th>M. globosum</th>
<th>Fusarium sp.</th>
</tr>
</thead>
<tbody>
<tr>
<td>R. rhodochrous KUCC 8801</td>
<td>Hexane</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td></td>
<td>Cyclohexane</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td></td>
<td>Heptane</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td></td>
<td>Nonane</td>
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<td>ND</td>
<td>ND</td>
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<tr>
<td></td>
<td>Decane</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td></td>
<td>Dodecane</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td></td>
<td>Tetradecane</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
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<tr>
<td></td>
<td>Hexitane</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
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<tr>
<td></td>
<td>Octadecane</td>
<td>ND</td>
<td>ND</td>
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<td>ND</td>
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<tr>
<td></td>
<td>Eicosane</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
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<tr>
<td></td>
<td>Anthracene</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td></td>
<td>Perhydroanthracene</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td></td>
<td>Naphthalene</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
</tbody>
</table>

Values indicate dry biomasses in mg/50ml medium.
ND: Not determined. — No growth.
Data represent the means of three determinations.
The optimum temperature for growth was around 40°C, but the cells of both isolates tolerated 50°C. Both isolates grew best without any added N, but could tolerate up to 5% (w/v) of this salt. Table 4 presents results of the kinetics of dodecane utilisation by the two test isolates. It is apparent that, after 10 and 15 days, *R. rhodochrous* KUCC 8801 utilised much more dodecane than *R. rhodochrous* KUCC 8802.

The results of the comparative analysis are presented qualitatively in Fig. 4 and quantitatively in Table 5, and show that *R. rhodochrous* KUCC 8801 was more efficient in oil degradation than three isolates from other habitats.
obial cultures: (I) bacterial cultures overgrown from \textit{R. rhodochrous} KUCC 8802; (II) fungal cultures grown from \textit{Fusarium} sp. culture; (c) oil from \textit{A. sulphiuricus} culture; (d) oil from a control culture.

Temperatures for growth was set at 50°C. Both isolates grew up to 5% (w/v) of this salt. Using two tests, \textit{R. rhodochrous} KUCC 8801 and KUCC 8802.

Presented qualitatively in that \textit{R. rhodochrous} KUCC in three isolates from other

\begin{table}[h]
\centering
\caption{Dodecane Utilization by \textit{Rhodococcus rhodochrous}}
\begin{tabular}{|c|c|c|c|c|c|c|}
\hline
\textbf{Dodecane (g litre}^{-1}) & \textbf{R. rhodochrous KUCC 8801} & & & \textbf{R. rhodochrous KUCC 8802} & & \\
\hline
 & 5 days & 10 days & 15 days & 5 days & 10 days & 15 days \\
\hline
5 & 0.3 ± 0.02 & 4.8 ± 0.04 & 5.0 ± 0.01 & 1.1 ± 0.3 & 4.2 ± 0.04 & 4.4 ± 0.03 \\
10 & 1.5 ± 0.06 & 8.4 ± 0.15 & 8.5 ± 0.05 & 1.7 ± 0.09 & 6.4 ± 0.27 & 7.4 ± 0.01 \\
15 & 1.2 ± 0.18 & 11.6 ± 0.39 & 11.7 ± 0.26 & 1.5 ± 0.11 & 2.6 ± 0.57 & 6.2 ± 0.04 \\
20 & 0.5 ± 0.06 & 14.7 ± 0.6 & 14.9 ± 0.58 & 1.3 ± 0.22 & 2.3 ± 0.28 & 6.7 ± 0.10 \\
\hline
\end{tabular}
\begin{flushleft}
Data are expressed in g litre}^{-1} dodecane consumed after the given incubation periods and are means of three determinations.
\end{flushleft}
\end{table}

![Fig. 4. GLC profiles of crude oil recovered from microbial cultures. (I) 3 day cultures. (II) 5 day cultures. (a) Control crude oil; (b) oil recovered from \textit{R. rhodochrous} KUCC 8801 cultures; (c) oil recovered from \textit{Rhodococcus sp.} ISO1 cultures; (d) oil recovered from \textit{A. caldarietius} IR07 cultures; (e) oil recovered from \textit{P. putida} IR32 cultures.](image)
Crude Oil Utilisation by Different Bacterial Isolates

<table>
<thead>
<tr>
<th>Organisms</th>
<th>3 days</th>
<th>5 days</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Rhodococcus</em> KUCC 8801</td>
<td>85.0</td>
<td>93.1</td>
</tr>
<tr>
<td><em>Rhodococcus</em> sp. ISOI</td>
<td>329</td>
<td>81.1</td>
</tr>
<tr>
<td><em>Acinetobacter calcoaceticus</em> ISO7</td>
<td>544</td>
<td>91.2</td>
</tr>
<tr>
<td><em>Pseudomonas putida</em> IR32</td>
<td>188</td>
<td>47.6</td>
</tr>
</tbody>
</table>

Data are expressed in per cent of total peak areas in gas chromatograms based on the total peak area of crude oil chromatograms.

The measurement of post-growth utilisation of dodecane by *Rhodococcus* KUCC 8801 in comparison to the non-Kuwaiti isolates revealed that this organism could decompose more of the available hydrocarbon than the other isolates (Fig. 5). Calculation by peak triangulation showed that the proportions of dodecane consumed by *Rhodococcus* KUCC 8801, *Rhodococcus* sp. ISOI and *A. calcoaceticus* ISO7 after 48 h were, respectively, 76.9, 43.2 and 36.6%.

DISCUSSION

Oil-utilising bacteria and fungi were isolated from both soil and marine environments in Kuwait. In this study bacterial isolates were more efficient in the consumption of aliphatic hydrocarbons than fungi. Although fungal isolates were able to utilise both aliphatic and aromatic hydrocarbons, they were more efficient in the degradation of the former. A salient point is that aliphatic hydrocarbons constitute up to 72% of Kuwait crude oil (Perry, 1984).

The most efficient of the bacterial isolates in both oil degradation and relative abundance, were *Rhodococcus* spp. In the case of fungi, the most frequently isolated species were *Aspergillus terreus*, *A. sulphureus*, *Mucor globosus*, *Fusarium* sp. and *Penicillium citrinum*. *M. globosus* was the most active in oil degradation. Davies and Westlake (1979), screening oil-polluted and unpolluted soils, reported *Penicillium* and *Verticillium* spp. as the most frequently isolated genera. Fedorak *et al.* (1984) showed that slightly over 70% of the fungi capable of growth on oil belonged to the genus *Penicillium*, whilst Oudot *et al.* (1986) found that most of the active fungi in oil degradation were *Aspergillus* and *Penicillium* spp.
Bacterial species most frequently associated with oil degradation include Pseudomonas spp. (Fuhs, 1961; Lebel & Mironov, 1973; Austin et al., 1977), Nocardia and Coryneforms (Polyakova, 1962; Cundell & Traxler, 1973; Mulkins-Phillips & Stewart, 1974; Oudot et al., 1986), but other species of bacteria have also been reported by a number of workers to be capable of degrading petroleum (Fuhs, 1961; Polyakova, 1962; Cundell & Traxler, 1973; Austin et al., 1977; Oudot et al., 1986). In this study, Rhodococcus spp. were the most commonly isolated bacteria. Furthermore, this species was found to be the most efficient oil degrader. This is particularly true of R. rhodochrous KUCC 8801 which was shown by GLC and post-growth studies to be more efficient in crude oil utilisation than other isolates known to be active oil degraders. After 3 days, R. rhodochrous KUCC 8801 was about 2.5 fold more active in crude oil degradation than Rhodococcus sp. ISO1, and about 4.5 times more active than Pseudomonas putida IR32. This higher efficiency was maintained, albeit to a lesser extent, after 5 days' incubation. Post-growth results give further confirmation of the superior activity of R. rhodochrous KUCC 8801 (Fig. 5).

This difference from the findings of others may be due to the ecological nature of this region. In this respect, Mulkins-Phillips and Stewart (1974) suggested that certain petroleum-degrading bacteria may be specific to given ecological niches. It is also possible that the domination of Rhodococcus spp. observed in this study may be due to the composition of the Kuwait crude oil (Perry, 1984).

![Fig. 5. Post-growth utilization of dodecane by (a) R. rhodochrous KUCC 8801 (b) Rhodococcus sp. ISO1 and (c) A. calcoaceticus IRO7.](image-url)
It is hoped that this study will lead to more detailed work on the biotechnological capabilities of these isolates versus pollution control, biosurfactant and the production of intermediates.

ACKNOWLEDGEMENTS

This investigation was supported by Kuwait University, Research Council, grant no. S0033 and Kuwait Foundation for the Advancement of Science, grant no. 87-01-04. Thanks are due to Dr J. Mulder for his valuable help in fungal identification.

REFERENCES


to more detailed work on the plate versus pollution control, mediates.

MENTS

it University, Research Council, or the Advancement of Science, Mulder for his valuable help in


Sterols and diacylglycerophosphocholines in the lipids of the hydrocarbon-utilizing prokaryote Rhodococcus rhodochrous

N.A. Sorkhoh, M.A. Ghannoum, A.S. Ibrahim, R.J. Stretton* & S.S. Radwan Department of Botany and Microbiology, Faculty of Science, Kuwait University, P.O. Box 5969, Safat 13060, Kuwait and *Department of Chemistry, University of Technology, Loughborough LE11 3TU, U.K

Accepted 14 May 1990

Sterols and diacylglycerophosphocholines in the lipids of the hydrocarbon-utilizing prokaryote Rhodococcus rhodochrous were extracted from two strains of teriology glucose·

There have been several studies on the lipids of hydrocarbon-utilizing bacteria such as Micrococcus cerificans (Makula & Finnerty 1968a,b, 1970, 1972) and Acinetobacter species (Makula et al. 1975; Yachon et al. 1982; Singer & Finnerty 1984). In such studies, emphasis was put on comparing the fatty acid compositions of hydrocarbon-utilizing bacteria contain considerable amounts of fatty acids with the same carbon chain as the hydrocarbon substrate (Davis 1964; Dunlap & Perry 1967; Makula & Finnerty 1968a,b, 1972; Patrick & Dugan 1974; King & Perry 1975; Ascenzi & Vestal 1979). This is expected in view of the fact that an initial step of alkane assimilation involves its oxidation to the corresponding fatty acid (Ascenzi & Vestal 1979). The latter is subsequently oxidized to acetyl-CoA which is then used by the bacteria to produce cell material and/or energy (for reviews see Ratledge 1979; Britton 1984). Some studies deal with the lipid class composition of alkane-utilizing bacteria. According to Hallas & Vestal (1978) cellular lipids of Mycobacterium concotulum contain diacylglycerophosphoethanolamines as the major phospholipid in both hydrocarbon and non-hydrocarbon-grown cells. Diacylglycerophosphoserines, diacylglycerophosphoglycerols, phosphatidic acids and diphosphatidyglycerols were also found. In addition, Makula & Fin­nerty (1970) reported the presence of cardiolipin in M. cerificans grown on hydrocarbons. Vestal & Perry (1971) stated that the lipid class that increases most by growth of M. raccace on propane is that of triacylglycerols.

Bacteria characteristically lack sterols in their membranes (Wee­te 1980), the notable exceptions being some methylotrophs, in which squalene and 4-methyl sterols are present (Harwood & Russell 1984). Also lacking in prokaryotes
Lipids in Rhodococcus rhodochrous

Stretton* & J.

In the lipids of the
Rhodococcus rhodochrous

Materials and Methods

O R G A N I S M S  A N D C U L T U R E  C O N D I T I O N S

Two strains of Rhodococcus rhodochrous, KUCC 8801 and KUCC 8802, were isolated from oil-saturated soil samples of an oil field in Kuwait. Soil samples were taken from the edges of pools which were routinely used to dispose of excess extracted crude oil. The samples were taken aseptically after removing the 5 cm surface layers. A diluted soil suspension in sterile water was plated on an inorganic medium supplemented with 10 g/l weathered crude oil (by sparging it with nitrogen gas for 24 h) as a sole source of carbon and energy. The inorganic medium had the following composition (g/l): NaNO₃, 0·9; KH₂PO₄, 0·6; Na₂HPO₄, 0·9; K₂SO₄, 0·2; MgSO₄·7H₂O, 0·4; CaCl₂, 2H₂O, 0·007; Fe (III) EDTA, 0·0043; 2·5 ml of a trace element solution consisting of (g/l): ZnSO₄·7H₂O, 2·3; MnSO₄·4H₂O, 1·8; H₃BO₃, 0·6; CuSO₄·5H₂O, 1·0; Na₂MoO₄·2H₂O, 0·4; CoCl₂·6H₂O, 0·4; KI, 0·7; EDTA, 1·0; FeSO₄·7H₂O, 0·4; NiCl₂·6H₂O, 0·004. The pH of the medium was adjusted to 7·2. The strains isolated were purified by replating on agar medium and identified according to Goodfellow (1968). The identification was confirmed by the National Collection of Industrial and Marine Bacteria, Scotland. The two strains of R. rhodochrous were grown in 50 ml amounts of the above inorganic medium dispensed in 250 ml flasks. The medium was supplemented with 10 g/l either of dodecane or glucose. Each flask received 1 ml of a 36 h culture containing 10⁸ cells. Cultures were incubated on an orbital shaker at 120 rev/min, at 30°C for 5 d. This incubation period yielded cultures all of which were in the stationary phase of growth. The cells were harvested by centrifugation and washed twice with boiling water under oxygen-free nitrogen to remove adhering dodecane and deactivate lipases (Kates 1972; Radwan 1984). Preliminary experiments had revealed that cells washed with boiling water remained intact and that the washing water did not contain any lipid classes other than hydrocarbons, as shown by thin layer chromatography (TLC).

L I P I D  E X T R A C T I O N  A N D  A N A L Y S I S

Total lipids were extracted from the bacteria with chloroform: methanol (2:1, v/v) three times and purified according to the method of Folch et al. (1957). Boiling solvents were used in the first extraction only. Since the relatively large amounts of the dodecane absorbed by the cells usually interfered with the analysis of lipid classes, the hydrocarbon fractions were eliminated from all extracts. This was achieved by preparative TLC on silica gel with petroleum ether (40-60) as a solvent (Vachon et al. 1982). After discarding the front zone of the sorbent carrying the hydrocarbons, total lipids were recovered from the sorbent by eluting five times with chloroform : diethyl ether : ethanol (1:1:1, v/v/v) (Kates 1972). Total sterols were extracted by a modification of the method of Fryberg et al. (1974). KOH (1·5 g) in 2 ml of distilled water was added to 0·2 g fresh cells and the volume of the suspension completed to 10 ml with ethanol (equivalent to 150 g KOH/l). The solution was refluxed under nitrogen for 3 h, diluted with an equal amount of water and extracted with four volumes of heptane. The extract was washed with water, dried over anhydrous Na₂SO₄ and evaporated in vacuo to give the total sterol extract. The lipid extracts were analysed by TLC on plates of silica gel. Non-polar lipid classes were fractionated with hexane : diethyl ether : acetic acid (90:10:1 and 75:25:1, v/v/v) (Mangold & Malins 1960). Hexane : diethyl ether : acetic acid (60:40:1, v/v/v) was used to resolve individual sterols. Ionic and other polar lipids were resolved by two-dimensional chromatography using chloroform : methanol : 7 mol/l ammonium hydroxide (65:25:4, v/v/v) in the first direction and chloroform : methanol : acetic acid : water.
The soil samples studied contained $10^6 - 10^7$ Rhodococcus rhodochrous cells per g dry wt., as determined by the standard dilution method with crude oil as a sole source of carbon and energy. In addition, much lower numbers ($10^2 - 10^3$ cells/g soil) of other oil-utilizing bacteria such as Bacillus spp., Pseudomonas spp. and Nocardia spp. were recorded. The chemotaxonomic analysis of R. rhodochrous KUCC 8801 revealed that the cell wall diamino acid was diaminopimelic acid and that its total lipids contained tuberculostearic acid in addition to major amounts of palmitic, palmitoleic and stearic acids. This strain was catalase positive and oxidase negative. It decomposed tyrosine and Tween 80 but not adenine and urea. It grew on glucose, maltose, mannitol, sorbitol, sodium adipate, sodium benzoate, sodium citrate, sodium lactate, tasterone, L-tyrosine, glycerol and p-hydroxybenzoic acid as sole sources of carbon, but not on inositol or trehalose. The original culture of strain KUCC 8801 was pink whereas that of KUCC 8802 was orange in colour. In addition, the former produced biofilms in oil-containing media in which the oil and cells were concentrated, whereas the latter grew evenly dispersed in the culture. Both strains grew better on glucose than dodecane.

**Table 1. Total lipid and sterol contents of two strains of Rhodococcus rhodochrous**

<table>
<thead>
<tr>
<th>Strain KUCC 8801</th>
<th>Strain KUCC 8802</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glucose</td>
<td>Glucose</td>
</tr>
<tr>
<td>Dodecane</td>
<td>Dodecane</td>
</tr>
<tr>
<td>Total lipids</td>
<td>1.4 ± 0.1</td>
</tr>
<tr>
<td></td>
<td>4.1 ± 0.4</td>
</tr>
<tr>
<td>Total sterols</td>
<td>0.28 ± 0.06</td>
</tr>
<tr>
<td></td>
<td>0.58 ± 0.04</td>
</tr>
</tbody>
</table>

Values are expressed in % of dry biomass. Each value is the mean of two determinations in two different experiments. ± standard deviation

$170:25:25:4$ (v/v/v/v) in the second direction (Nichols 1964). The lipid fractions were detected with iodine vapour or by charring after spraying the chromatoplates with $50\%$ H$_2$SO$_4$. Charred fractions were measured densitometrically with a Beckman R-112 Densitometer. Individual classes were identified by their chromatographic behaviour with standards and specific spray reagents (Dittmer & Lester 1964; Siakotos & Rouse, 1965; Stahl 1967; Vioque 1984). The identity of phospholipid fractions isolated by preparative TLC was confirmed by the analysis of their partial degradation products, after mild hydrolysis, with two-dimensional paper chromatography (Dawson 1984). The i.r. spectra of fractions isolated by preparative TLC were recorded on a Perkin-Elmer 398 IR-spectrophotometer, the u.v. spectra, with a Pye-Unicam SP 8000 spectrophotometer and the $^1$H-NMR spectra using a Varian analytical instrument. The results were compared with spectra of authentic samples. Total lipids and individual lipid classes resolved by two-dimensional TLC (Radwan 1978) were subjected to methanolysis (Chalvardjian 1964). The resulting methyl esters were purified by TLC and analysed by gas chromatography (GC) (Pye-Unicam 204 equipped with a flame ionization detector, an integrator and a glass column, 2 m x 4 mm, i.d., packed with 15% DEGS on Anakrom D, 100-120 mesh at a temperature of 180°C, with nitrogen carrier gas. Each analysis was repeated using a non-polar column 10% Silar CP on Gas-Chrom Q.) Derivatization of sterols for GLC was done by hexamethyldisilazane, followed by trimethylsilylchlorosilane, as described elsewhere (Ghannoum et al. 1990). The steroid derivatives were analysed by GC with a glass column packed with 3% SP 2100 on Chromosorb W-HP, 100-120 mesh or 3% JXR. Retention times were compared with those of standard fatty acid and steroid derivatives (Sigma). The GC results were quantified by peak triangulation using the integrator attached to the equipment.

**Results**

**Bacterial Strains**

The total lipid and sterol contents of dodecane-grown cells were higher than those of glucose-grown cells (Table 1). The relative yield of lipids and sterols for dodecane-grown cells, for strain...
Lipids in Rhodococcus rhodochrous

Table 2. Lipid composition of two strains of Rhodococcus rhodochrous

<table>
<thead>
<tr>
<th>Lipid class</th>
<th>Strain KUCC 8801 Glucose</th>
<th>Dodecane</th>
<th>Strain KUCC 8802 Glucose</th>
<th>Dodecane</th>
</tr>
</thead>
<tbody>
<tr>
<td>Apolar lipid classes:</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Triacylglycerols</td>
<td>48.7 ± 1.6</td>
<td>28.8 ± 1.3</td>
<td>12.1 ± 0.9</td>
<td>7.8 ± 0.2</td>
</tr>
<tr>
<td>Diacylglycerols</td>
<td>9.1 ± 0.3</td>
<td>8.5 ± 0.5</td>
<td>3.5 ± 0.1</td>
<td>2.9 ± 0.1</td>
</tr>
<tr>
<td>Sterols</td>
<td>3.3 ± 0.09</td>
<td>9.2 ± 0.4</td>
<td>21.3 ± 1.1</td>
<td>23.6 ± 0.8</td>
</tr>
<tr>
<td>Monoacylglycerols</td>
<td>5.3 ± 0.3</td>
<td>14.9 ± 0.8</td>
<td>17.3 ± 0.7</td>
<td>26.4 ± 1.7</td>
</tr>
<tr>
<td>Polar lipid classes:</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Diacylglycerophosphocholines</td>
<td>4.4 ± 0.1</td>
<td>8.8 ± 0.5</td>
<td>tr</td>
<td>3.8 ± 0.2</td>
</tr>
<tr>
<td>Diacylglycerophosphoethanolamines</td>
<td>8.2 ± 0.8</td>
<td>7.8 ± 0.5</td>
<td>15.1 ± 0.1</td>
<td>5.0 ± 0.3</td>
</tr>
<tr>
<td>Diacylglycerophosphoglycerols</td>
<td>5.3 ± 0.1</td>
<td>4.6 ± 0.3</td>
<td>6.9 ± 0.6</td>
<td>2.5 ± 0.1</td>
</tr>
<tr>
<td>Diacylglycerophosphomethylethanolamines</td>
<td>5.1 ± 0.2</td>
<td>4.7 ± 0.2</td>
<td>7.1 ± 0.2</td>
<td>1.4 ± 0.09</td>
</tr>
<tr>
<td>Unknown glycolipid</td>
<td>10.6 ± 0.5</td>
<td>12.7 ± 0.8</td>
<td>15.7 ± 1.1</td>
<td>25.8 ± 1.9</td>
</tr>
</tbody>
</table>

Values are expressed in % of total lipids. Each value is the mean ± standard deviation of two determinations in two different experiments.

KUCC 8801, was 3.7 and 2.1 fold higher than cells grown on glucose, respectively. Similar results were obtained for strain KUCC 8802.

The quantitative measurements showed that all extracts contained more apolar than polar lipids (Table 2). Apolar lipid classes in various extracts were tri-, di- and monoacylglycerols as well as free sterols. Dodecane-grown cells contained more monoacylglycerols and sterols than glucose grown cells.

The identity of the sterol fraction was confirmed as follows: on the chromatoplates this fraction gave positive reactions with sterol-detecting spray reagents (Stahl 1967). The retention times of cell sterols in GC were identical with those of authentic samples. A lanosterol fraction separated by preparative TLC showed an NMR spectrum identical with that of standard lanosterol. The ionic and other polar lipid classes all consisted mainly of phospholipids viz. diacylglycerophosphoethanolamines, diacylglycerophosphomethylthanolamines and diacylglycerophosphocholines. The extracts also contained an unknown polar acyl glycolipid (positive reaction with 2-naphthol reagent), which migrated on TLC plates close to the apolar fraction (fatty acid composition shown in Table 4).

Table 3. Constituent fatty acids of total lipids from two strains of Rhodococcus rhodochrous

<table>
<thead>
<tr>
<th>Fatty acids</th>
<th>Strain KUCC 8801 Glucose</th>
<th>Dodecane</th>
<th>Strain KUCC 8802 Glucose</th>
<th>Dodecane</th>
</tr>
</thead>
<tbody>
<tr>
<td>12:0</td>
<td>tr</td>
<td>2.1 ± 0.2</td>
<td>12.4 ± 0.4</td>
<td>14:0</td>
</tr>
<tr>
<td>14:0</td>
<td>16.7 ± 1.5</td>
<td>tr</td>
<td>10.1 ± 0.8</td>
<td>16:0</td>
</tr>
<tr>
<td>16:0</td>
<td>34.0 ± 1.6</td>
<td>48.5 ± 0.3</td>
<td>23.0 ± 0.6</td>
<td>36.6 ± 0.8</td>
</tr>
<tr>
<td>16:1</td>
<td>20.0 ± 1.8</td>
<td>1.5 ± 0.1</td>
<td>18.4 ± 0.5</td>
<td>16.0</td>
</tr>
<tr>
<td>18:0</td>
<td>tr</td>
<td>tr</td>
<td>tr</td>
<td>18:0</td>
</tr>
<tr>
<td>18:0:Me*</td>
<td>tr</td>
<td>tr</td>
<td>tr</td>
<td>18:1</td>
</tr>
<tr>
<td>18:1</td>
<td>44.0 ± 3.0</td>
<td>26.8 ± 2.3</td>
<td>52.0 ± 3.2</td>
<td>35.7 ± 2.8</td>
</tr>
<tr>
<td>18:2</td>
<td>0.8 ± 0.01</td>
<td>46.0 ± 2.2</td>
<td>15.0 ± 0.2</td>
<td>18:3</td>
</tr>
<tr>
<td>18:3</td>
<td>1.2 ± 0.09</td>
<td>44.0 ± 0.3</td>
<td>20.0 ± 0.1</td>
<td>3.4 ± 0.2</td>
</tr>
</tbody>
</table>

Values are expressed in % of total fatty acids. Each value is the mean ± standard deviation of two determinations in two separate experiments.

tr, trace, less than 0.1%.

* Tuberculostearic acid.
Table 4. Constituent fatty acids of individual phospholipids from two strains of Rhodococcus rhodochrous

<table>
<thead>
<tr>
<th>Fatty acids</th>
<th>Strain KUCC 8801</th>
<th>Strain KUCC 8802</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Glucose</td>
<td>Dodecane</td>
</tr>
<tr>
<td>Diacylglycerophosphocholines:</td>
<td></td>
<td></td>
</tr>
<tr>
<td>12:0</td>
<td>tr</td>
<td>12.2 ± 0.4</td>
</tr>
<tr>
<td>14:0</td>
<td>tr</td>
<td>23.3 ± 2.0</td>
</tr>
<tr>
<td>16:0</td>
<td>43.0 ± 2.1</td>
<td>10.0 ± 0.8</td>
</tr>
<tr>
<td>18:1</td>
<td>tr</td>
<td>24.7 ± 0.8</td>
</tr>
<tr>
<td>18:2</td>
<td>14.0 ± 0.1</td>
<td>13.3 ± 1.0</td>
</tr>
<tr>
<td>18:3</td>
<td>28.6 ± 0.9</td>
<td>20.6 ± 1.9</td>
</tr>
<tr>
<td>18:5</td>
<td>26.0 ± 1.6</td>
<td>18.0 ± 0.8</td>
</tr>
<tr>
<td>20:3</td>
<td>10.0 ± 0.03</td>
<td>10.6 ± 0.8</td>
</tr>
<tr>
<td>Diacylglycerophosphocholasilanes:</td>
<td></td>
<td></td>
</tr>
<tr>
<td>12:0</td>
<td>tr</td>
<td>2.7 ± 0.2</td>
</tr>
<tr>
<td>14:0</td>
<td>tr</td>
<td>6.9 ± 0.4</td>
</tr>
<tr>
<td>16:0</td>
<td>44.8 ± 2.9</td>
<td>54.3 ± 1.0</td>
</tr>
<tr>
<td>16:1</td>
<td>tr</td>
<td>21.2 ± 1.3</td>
</tr>
<tr>
<td>18:0</td>
<td>0.8 ± 0.05</td>
<td>1.7 ± 0.09</td>
</tr>
<tr>
<td>18:1</td>
<td>34.2 ± 1.8</td>
<td>31.4 ± 1.6</td>
</tr>
<tr>
<td>18:2</td>
<td>18.1 ± 1.7</td>
<td>0.5 ± 0.03</td>
</tr>
<tr>
<td>18:3</td>
<td>21.0 ± 0.1</td>
<td>0.5 ± 0.01</td>
</tr>
<tr>
<td>Diacylglycerophosphoglycerols:</td>
<td></td>
<td></td>
</tr>
<tr>
<td>12:0</td>
<td>tr</td>
<td>4.4 ± 0.2</td>
</tr>
<tr>
<td>14:0</td>
<td>tr</td>
<td>5.0 ± 0.2</td>
</tr>
<tr>
<td>16:0</td>
<td>31.7 ± 1.7</td>
<td>33.9 ± 1.3</td>
</tr>
<tr>
<td>16:1</td>
<td>14.3 ± 1.2</td>
<td>20.0 ± 0.8</td>
</tr>
<tr>
<td>18:0</td>
<td>1.9 ± 0.05</td>
<td>3.8 ± 0.07</td>
</tr>
<tr>
<td>18:0Me*</td>
<td>2.2 ± 0.3</td>
<td>3.3 ± 0.1</td>
</tr>
<tr>
<td>18:1</td>
<td>35.1 ± 0.8</td>
<td>33.0 ± 1.7</td>
</tr>
<tr>
<td>18:2</td>
<td>10.7 ± 1.1</td>
<td>tr</td>
</tr>
<tr>
<td>18:3</td>
<td>4.1 ± 0.3</td>
<td>2.9 ± 0.01</td>
</tr>
<tr>
<td>Unknown glycolipid:</td>
<td></td>
<td></td>
</tr>
<tr>
<td>12:0</td>
<td>tr</td>
<td>14.0 ± 0.7</td>
</tr>
<tr>
<td>14:0</td>
<td>tr</td>
<td>28.1 ± 0.5</td>
</tr>
<tr>
<td>16:0</td>
<td>30.1 ± 0.9</td>
<td>19.0 ± 1.3</td>
</tr>
<tr>
<td>16:1</td>
<td>10.0 ± 0.6</td>
<td>16.5 ± 1.1</td>
</tr>
<tr>
<td>18:0</td>
<td>0.4 ± 0.01</td>
<td>tr</td>
</tr>
<tr>
<td>18:0Me*</td>
<td>40.4 ± 2.2</td>
<td>20.8 ± 1.8</td>
</tr>
<tr>
<td>18:1</td>
<td>14.6 ± 1.0</td>
<td>tr</td>
</tr>
<tr>
<td>18:2</td>
<td>2.0 ± 0.1</td>
<td>0.7 ± 0.03</td>
</tr>
<tr>
<td>18:3</td>
<td>2.5 ± 0.2</td>
<td>0.8 ± 0.03</td>
</tr>
</tbody>
</table>

Values are expressed in % of total fatty acids of each class. Each value is the mean ± standard deviation of two determinations in two separate experiments.

tr, trace, less than 0.1%.

- not determined.

* Tuberculostearic acid.

acidic systems (Radwan 1984). It gave positive reactions for phosphorus and choline (Dra­
gendorff's reagent). It revealed an i.r. spec­
trum identical with that of standard di­
acylglycerophosphocholines. On mild bydrolysis
it was identical with the standard sample in
producing glycerophosphocholine which was
identified by 2-dimensional paper chromato­
graphy (Dawson 1984). Dodecane-grown cells
contained more of diacylglycerophospho­
cholines and the unknown polar glycolipid than
glucose-grown cells. The culture filtrates espe­
cially of dodecane-grown cells were found to
contain considerable amounts of the unknown
glycolipid.

FATTY ACID PATTERNS

The fatty acid patterns of total lipids from
glucose and dodecane-grown bacteria are
Lipids in Rhodococcus rhodochrous

shown in Table 3. The predominant acyl moieties in all extracts were palmitic (16:0) and octadecenoic (18:1) acids. Traces of tuberculostearic acid (18:0, Me) were also present. Relatively large proportions of palmitoleic acid (16:1) were present in extracts of glucose but not dodecane-grown cells of both R. rhodochrous strains. Lipids from dodecane-grown cells of both strains contained relatively more lauric (12:0), myristic (14:0), palmitic (16:0) and linolenic (18:3) acids than lipids from glucose-grown cells.

Palmitoleic acid was distributed among all other phospholipid classes of both strains. The phospholipid classes of glucose-grown cells of R. rhodochrous KUCC 8801 contained appreciable proportions of linoleic acid (18:2) which decreased when the cells were grown on dodecane. Palmitoleic acid occurred only in diacylglycerophosphoglycerols of this strain, but was distributed among all other phospholipid classes of R. rhodochrous KUCC 8802. In diacylglycerophosphocholines of both strains, relatively high linolenic acid concentrations were associated with growth on dodecane. Phospholipid classes from dodecane-grown cells of both strains contained higher proportions of lauric and myristic acids than the same classes from glucose-grown cells. The highest concentrations of lauric and myristic acids were detected in diacylglycerophosphocholines and the unknown glycolipid. Tuberculostearic acid was confined to diacylglycerophosphoglycerols and the unknown glycolipid.

Discussion

Our results show that the lipids of R. rhodochrous grown on glucose consist of a mixture of glycerophospholipids and tri-, di- and monoacylglycerols, with smaller proportions of sterols. The same composition is maintained when the bacteria are grown on dodecane as sole source of carbon and energy, except that these cells accumulate more sterols, monoacylglycerols, diacylglycerophosphocholines and an unknown glycolipid. The phospholipid composition of R. rhodochrous is similar to that of other Gram-positive bacteria and differs mainly in the occurrence of diacylglycerophosphocholines. This latter phospholipid has been recorded in some Gram-negative bacteria but not in Gram-positive bacteria or actinomycetes (for review see Lechevalier 1982). In view of the fact that polar lipids occur primarily in association with the cell membranes, the question arises as to whether acylglycerophosphocholines, sterols and/or the unknown compound in R. rhodochrous may be involved in the uptake and/or metabolism of hydrocarbons. Our results provide some evidence in favour of this hypothesis. Diacylglycerophosphocholines and the unknown polar lipid from dodecane-grown cells contained the highest concentrations of lauric and myristic acids. Lauric acid is known to arise from dodecane during the initial phase of its utilization (Thorpe & Ratledge 1972). Myristic acid may have been formed from lauric acid by subsequent chain elongation.

The occurrence of sterols in the hydrocarbon-utilizer R. rhodochrous is also quite unusual. Most prokaryotes are known to lack sterols; exceptions are, for example, methane-oxidizing bacteria (Harwood & Russell 1984). It may be assumed that sterols in our Rhodococcus strains form hydrophobic sites in the cell membrane through which the alkane is transported. This hypothesis is supported by the fact that dodecane-grown cells, especially of the strain KUCC 8801, contain more sterols than glucose-grown cells. The unknown glycolipid may also play a similar role as a biosurfactant in alkane-uptake by strain R. rhodochrous KUCC 8802, which contains more of this glycolipid in dodecane than in glucose-grown cells. Considerable amounts of this glycolipid are also present in the growth medium. Rapp et al. (1979) suggested a role for an acylglycolipid, trehalose-6,6'-dicorynomycocales, in alkane uptake by R. erythropolis. It is of interest that the unknown glycolipid in our strains is the richest lipid class in tuberculostearic acid (Table 4) which is a branched fatty acid, like the acyl moiety of the biosurfactant from R. erythropolis. It appears that in dodecane-uptake, strain KUCC 8801 may depend primarily on hydrophobic sterol sites in the cell membrane, while strain KUCC 8802 relies primarily on glycolipids as biosurfactants, with the alternate mechanism being complementary in both strains.

Unlike the majority of bacteria, R. rhodochrous contains tuberculostearic acid (esterified only in diacylglycerophosphoglycerols and the unknown polar lipid), in addition to the polyunsaturated linoleic and linolenic acids.
distributed among various phospholipids. More
linolenic acid is esterified in diacylgllycerophosphocholines when the cells utilize dodecane
than when they grow on glucose. It is known
that increased polyunsaturated fatty acid levels
result in increased fluidity of the membranes
(Hakomori 1986), probably facilitating the
passage of the hydrocarbons. In confirmation
of earlier reports (Makula & Fivestal 1968a,b,
1970, 1972; Makula et al. 1975), we have found
that hydrocarbon-grown cells accumulate consi-
derable amounts of fatty acids in their lipids
with chain lengths equivalent to those of the
hydrocarbons. It is known that an initial step of
hydrocarbon utilization involves their oxidation
to the corresponding fatty acids (Ascenzi &
Vestal 1979). It is interesting therefore that
lauric and myristic acids become esterified
mainly in diacylglycerophosphocholines. In
eukaryotic systems, exogenous fatty acids
appear intracellularly mainly in this class of
phospholipids (for review see Radwan &
Mangold 1980).

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and Kuwait Foundation for the Advancement
of Science, Grant No. 87-01-04.

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Lipids in Rhodococcus rhodochrous


Growth of *Candida albicans* on hydrocarbons: influence on lipids and sterols

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**Abstract**

*Candida albicans* KTCC 88062, an isolate from a crude oil polluted soil sample in Kuwait, grew adequately on n-alkanes with only 12 to 20 carbon chains but not on aromatic hydrocarbons. This isolate grew on glucose better than on any of the alkanes. Alkane-grown cells contained higher proportions of total lipids than glucose-grown cells, and the total lipid content was directly proportional to the alkane chain length. The sterol content also increased in alkane-grown cells; the highest level was with C18 as substrate and progressively lower sterol levels were obtained as the carbon chain length increased. The phospholipid:sterol ratio decreased when the cells were grown on alkanes as compared with glucose grown cells. The ratio of unsaturated:saturated fatty acids was higher in alkane than glucose grown cells and decreased progressively from C18 to C10 as substrates. Growth on alkanes but not on glucose was associated with pseudohyphal formation.

**Introduction**

Microbial growth on crude oil as a sole source of carbon has been studied for the purpose of either producing single cell protein (Gounelle de Poncetel, 1972; Hamer and Hamdan, 1979) or examining the metabolites of oil released into the environment (Fedorak and Westlake, 1981a,b; Atlas et al., 1981). There have also been studies on the ability of a range of organisms to grow on aromatic hydrocarbons (Crow et al., 1980; Certiglia and Crow, 1981), n-alkanes (Ratledge, 1978; Wlodarczyk, 1981) or on hydrocarbon fuels (Lindley et al., 1986) as carbon sources.

The effect of growth in n-alkanes, on cell composition, has been undertaken in respect of protein, carbohydrate, nucleic acid and lipid (Wlodarczyk, 1981). A significant change in the composition of a micro-organism growing on n-alkanes is in the relative proportions of the various fatty acids found in the lipid (Ratledge, 1978). There may also be an increase in the total lipid content of such cells (Petechova et al., 1971; Singh et al., 1978). The other changes occurring in cell composition tend to be much less marked and so have not merited detailed investigation.

This paper reports on the utilisation of Kuwaiti crude oil, n-alkanes and aromatic hydrocarbons by a strain of *Candida albicans*, isolated from soil, and the influence of these substrates on the composition of cellular lipid and cell morphology.
Materials and methods

Organism
The strain of *Candida albicans* KTCC 89062 used throughout this study was isolated from soil samples taken from a site which was contaminated with crude oil. The samples were taken specifically after the removal of the 5 cm surface layers. The isolate was identified using the criteria of germ-tube formation and API20C (Ghannoum et al., 1985).

Chemicals
Kuwaiti crude oil, obtained from the Kuwait Oil Company, Al-Ahmadi, Kuwait, was used throughout this work. Samples were weathered by sparging with nitrogen gas (50 cm² min⁻¹). This was used as the sole source of carbon in the isolation of the organism.

The hydrocarbons used were: *n*-alkanes [hexane, heptane, isooctane, nonane, decane. tetradecane, hexadecane. octadecane and eicosane (Fluka AG)] and aromatic hydrocarbons [benzene (J. T. Baker Chemical Co.), phenanthrene (Winlab Limited, England), anthracene and naphthalene (Sigma St Louis, U.S.A.)].

Media
A chemically defined medium (CDM) was used as the basal medium throughout. It contained (g 1⁻¹): 0.85 NaNO₃, 0.56 KH₂PO₄, 0.86 Na₂HPO₄, 0.17 K₂SO₄, 0.37 MgSO₄.7H₂O, 0.007 CaCl₂.2H₂O, 0.004 Fe (III) EDTA, 2.5 ml of a trace element solution consisting of (g 1⁻¹): 2.32 ZnSO₄.7H₂O, 1.78 MnSO₄.4H₂O, 0.56 H₂BO₃, 1.0 CuSO₄.5H₂O, 0.39 Na₂MoO₄.2H₂O, 0.42 CoCl₂.6H₂O, 0.66 KI, 1.0 EDTA, 0.4 FeSO₄.7H₂O, 0.004 NiCl₂.6H₂O. The above medium was supplemented with 10 ml 1⁻¹ vitamin solution composed of (mg 1⁻¹): 0.01 biotin, 2.0 pyridoxin, 2.0 nicotinic acid, 2.0 thiamin and 10.0 meso-inositol. The pH was adjusted to 5.6 using Na₂HPO₄/NaH₂PO₄ buffer system and the incubation temperature was 30°C. For solid media 20 g 1⁻¹ agar was added to the above medium.

Cultural conditions
*Candida albicans* KTCC 89062 was maintained on Sabouraud dextrose agar (Difco) until required, then it was subcultured into CDM containing glucose (10 g 1⁻¹). Cells were harvested by centrifugation and washed three times with 0.9% saline and then suspended in saline to give a cell number of 2 x 10⁸ ml⁻¹. Subsequently, 1 ml portions were used as inoculum for lipid analysis studies in CDM supplemented with (10 g 1⁻¹) either glucose or *n*-alkanes for 24 h. Cells were then harvested by centrifugation. This incubation period gave cultures which were at the same stage of growth.

Growth on pure hydrocarbons
In qualitative studies the vapour phase technique was used for liquid hydrocarbons. *C. albicans* was inoculated onto CDM, without oil, in Petri dishes. Sterile filter papers placed in the lids were impregnated, each with 0.5 ml of filter sterilised hydrocarbon. The bases of the plates were then replaced, kept inverted and the plates sealed with a masking tape. Solid hydrocarbons were
first dissolved in diethyl ether to give 0.1% (w/v) solutions and 1 ml of each solution was sprayed as a thin even film on an agar plate. After the solvent had evaporated C. albicans was streaked out and the plates sealed.

Analytical techniques

Crude oil samples

The biodegradation of crude oil was studied by gas liquid chromatography (GLC). The residual oil was recovered from the supernatant of cultures of C. albicans by extracting three times with hexane. The constituent hydrocarbons were resolved by GLC using a Pye-Unicam 204 instrument equipped with an SP-2100 column with a temperature programme 60-250°C, raising the temperature 16°C min⁻¹. Individual hydrocarbons were identified by comparing their retention times with those of standard samples. The identities of individual components were further confirmed by comparison of retention times of the peaks with those of reference standards using 3% OV-1 as a second column. The GLC-profiles were compared with the profile of the same amount of crude oil treated similarly but without inoculation with micro-organisms (control). Oil consumption was estimated quantitatively by comparing the total peak area in the GLC-profile of residual oil to that in the GLC-profile of the same amount of oil recovered from control samples. To minimise quantitative errors the external standardisation method was used employing a precision syringe to ensure that reproducible sample sizes could be injected onto the column (Lee et al. 1984). Triplicate analyses of the same sample were reproducible to within ±5%.

Lipid extraction and analysis

Total lipids were extracted from samples of C. albicans grown on glucose and n-alkanes with different chain lengths as sole sources of carbon and energy. The cell pellets were resuspended in propan-2-ol and incubated at 70°C for 45 min; this step was included in order to inactivate degradative enzymes such as phospholipases (Hitchcock et al., 1986). The suspension was cooled to 25°C, re-centrifuged and the supernatant retained. Cells were then extracted with chloroform/methanol (2:1, v/v) and purified using established procedures (Folch et al., 1957).

Lipids were separated from residual hydrocarbons by preparative thin layer chromatography using petroleum ether (40-60) as a solvent (Vachon et al., 1982). The extract was analysed by thin layer chromatography (TLC) on plates of silica gel G (0.25 mm thickness). Apolar compounds were resolved by the solvent system hexane/diethyl ether/acetic acid (75:25:1 by vol) (Mangold and Malins, 1960). Polar lipids were analysed by two-dimensional chromatography using chloroform/methanol/7 M-ammonium hydroxide (65:25:4, by vol) in the first direction and chloroform/methanol/acetic acid/water (170:25:25:1, by vol) in the second direction (Nichols, 1964). The spots were visualized with iodine vapour or by charring at 220°C after spraying the plates with 50% H₂SO₄. Individual classes were identified by comparing their chromatographic behaviour with that of authentic samples and by using specific spray reagents (Stahl, 1962; Dittmer and Lester, 1964; Siakotos and Rouser, 1965).

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The identity of the phospholipid fractions isolated by preparative TLC was confirmed by the analysis of their partial degradation products (Dawson, 1984). The IR spectra of fractions were recorded using a Perkin-Elmer 398 IR spectrophotometer and compared with the spectra of authentic samples. Lipid fractions resolved by TLC and subjected to charring were quantified densitometrically using a Beckman R-112 densitometer.

Samples of apolar and polar lipid fractions separated by preparative TLC were subjected to methanolation (Chalvardian, 1964). The resulting methyl esters were purified by TLC and analysed by gas liquid chromatography (GLC) using a Pye Unicam model 204 gas chromatograph fitted with a glass column (1.83 m x 4 mm i.d.) packed with 15% DEGS on Anakrom D, 100—120 mesh.

**Sterol extraction and analysis**

Sterols were extracted by a modification of the method of Fryberg et al. (1974). KOH (1.5 g) in 2 ml of distilled water was added to 0.2 g wet weight of cells and the volume of the suspension adjusted to 10 ml by the addition of ethanol. The solution was refluxed under nitrogen for 3 h, diluted with an equal amount of water and extracted with four volumes of heptane. The extract was washed with water, dried over anhydrous Na₂SO₄, and evaporated in vacuo to give the sterol extract. The extracted sterols were examined by GLC (Ghannoum et al. 1989).

**Electron microscopy**

Cells were suspended in glutaraldehyde (1.5%, w/v) for 2 min, collected by centrifugation and re-suspended in a fresh sample of the same fixative for 16 h at 4°C. Fixed cells were prepared for scanning electron microscopy as described earlier (Ghannoum and Al Khars, 1984). These samples were examined in a stereoscan electron microscope (Novascan 30) at an angle of 45°.

**Results**

The isolate of *C. albicans* grew in CDM with added weathered crude oil, though not as abundantly as when the medium was supplemented with glucose. The weathered crude oil was degraded by *C. albicans* up to 34.1 ± 2.3% after 14 d and the C₁₄ to C₁₈ fractions were apparently utilized preferentially with chains containing both odd and even numbers of carbon atoms being degraded (Figure 1).

When CDM was supplemented with n-alkanes only chain lengths C₁₂ to C₂₀ were assimilated and gave measurable growth. The short chain alkanes and aromatic hydrocarbons were not utilised by this isolate of *C. albicans*. Maximum cell mass was obtained following growth in CDM supplemented with glucose. In the case of the utilisable n-alkanes there was an increase in cellular yield (dry weight) as the chain length was increased but it was always significantly lower than CDM supplemented with glucose (Table 1).

**Effect on lipid composition**

The lipid composition of *C. albicans* was affected by growth in the presence of n-alkanes when compared with cells grown in CDM supplemented with...
Candida albicans and hydrocarbons
Table 1  Yield and lipid content of a C. albicans KTCC 89062
grown on individual n-alkanes or glucose for 1 day at 37°C in batch culture shaken at 160 rpm

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Yield of biomass (g dry wt/l)</th>
<th>Total lipid (% dry wt)</th>
<th>Sterols (% dry wt)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dodecane</td>
<td>1.01 ± 0.05</td>
<td>4.96 ± 0.03</td>
<td>1.33 ± 0.08</td>
</tr>
<tr>
<td>Tetradecane</td>
<td>1.27 ± 0.09</td>
<td>4.22 ± 0.05</td>
<td>1.28 ± 0.07</td>
</tr>
<tr>
<td>Hexadecane</td>
<td>1.83 ± 0.009</td>
<td>6.11 ± 0.09</td>
<td>1.09 ± 0.001</td>
</tr>
<tr>
<td>Octadecane</td>
<td>1.86 ± 0.08</td>
<td>6.10 ± 0.02</td>
<td>1.19 ± 0.003</td>
</tr>
<tr>
<td>Eicosane</td>
<td>2.08 ± 0.05</td>
<td>3.41 ± 0.06</td>
<td>0.59 ± 0.001</td>
</tr>
<tr>
<td>Glucose</td>
<td>5.48 ± 0.1</td>
<td>2.25 ± 0.07</td>
<td>0.26 ± 0.009</td>
</tr>
</tbody>
</table>

Values are the mean ± SE of three separate experiments.

When the lipid fraction was subjected to further analysis it was found that the apolar lipids predominated over the polar lipids, irrespective of the carbon source used to supplement CDM (Table 2). The levels of free sterols, monoglycerides, steryl esters and triglycerides were significantly higher while diglyceride levels were significantly reduced in cells grown in n-alkane supplemented CDM when compared with CDM supplemented with glucose (Table 2).

When the polar lipids were examined there was an increase in the levels of steryl glycosides, phosphatidylethanolamines and phosphatidylcholines with a decrease in the levels of ceramide monohexosides and phosphatidic acid when C. albicans was grown in CDM supplemented with n-alkanes as carbon source in comparison with glucose containing CDM (Table 2).

The fatty acids, found in the total lipid fraction, of C. albicans had a predominance of C₁₆ and C₁₈ carbon atoms, irrespective of the carbon source (Table 3). Palmitic acid (16:0) was the major saturated fatty acid present and oleic acid (18:1) was the principal unsaturated acid. When C. albicans was grown in the presence of n-alkanes rather than glucose there was an increase in the ratio of unsaturated:saturated fatty acids. This was brought about by a decrease in the level of both C₁₂:0 and C₁₆:0 with an increase in C₁₈:1, C₁₈:2 and C₁₈:3. However, the levels of C₁₈:3 were also decreased. No significant effect on the level of C₁₈:1 was observed (Table 3). These changes in specific fatty acid levels found in the total lipid fraction are mirrored in the fatty acids found in the phospholipids (Table 4).

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Table 2 Comparison of lipids from *C. albicans* KTCC 89062 grown on glucose or n-alkanes of different chain length.

<table>
<thead>
<tr>
<th>Lipids</th>
<th>Glucose</th>
<th>C₁₁</th>
<th>C₁₄</th>
<th>C₁₆</th>
<th>C₁₈</th>
<th>C₂₀</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Apolar</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Steryl esters</td>
<td>8.8 ± 0.6</td>
<td>9.2 ± 0.3</td>
<td>8.9 ± 0.4</td>
<td>10.2 ± 0.8</td>
<td>13.6 ± 1.1</td>
<td>11.5 ± 0.8</td>
</tr>
<tr>
<td>Alkyl esters</td>
<td>9.2 ± 0.8</td>
<td>12.0 ± 1.2</td>
<td>9.9 ± 0.7</td>
<td>11.2 ± 1.1</td>
<td>4.5 ± 1.3</td>
<td>Tr</td>
</tr>
<tr>
<td>Triacylglycerols</td>
<td>13.1 ± 1.1</td>
<td>10.0 ± 1.2</td>
<td>9.1 ± 0.3</td>
<td>6.5 ± 0.3</td>
<td>40 ± 0.8</td>
<td>57 ± 12</td>
</tr>
<tr>
<td>Fatty acids</td>
<td>7.6 ± 0.4</td>
<td>9.7 ± 1.3</td>
<td>8.3 ± 0.7</td>
<td>9.1 ± 0.3</td>
<td>10.8 ± 1.2</td>
<td>11.1 ± 0.5</td>
</tr>
<tr>
<td>Diacylglycerols</td>
<td>18.5 ± 1.1</td>
<td>0.9 ± 0.01</td>
<td>7.2 ± 0.2</td>
<td>6.4 ± 0.5</td>
<td>0.5 ± 0.7</td>
<td>6.3 ± 0.7</td>
</tr>
<tr>
<td>Sterols</td>
<td>9.8 ± 0.3</td>
<td>18.4 ± 1.6</td>
<td>16.7 ± 1.2</td>
<td>22.0 ± 1.4</td>
<td>1.4 ± 0.9</td>
<td>16.2 ± 0.8</td>
</tr>
<tr>
<td>Monoacylglycerols</td>
<td>2.8 ± 0.05</td>
<td>7.5 ± 1.1</td>
<td>6.8 ± 0.9</td>
<td>9.8 ± 0.9</td>
<td>6.8 ± 1.3</td>
<td>7.2 ± 1.2</td>
</tr>
<tr>
<td><strong>Polar</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Unknown glycolipid</td>
<td>Tr</td>
<td>4.8 ± 0.6</td>
<td>3.6 ± 0.4</td>
<td>Tr</td>
<td>3.5 ± 0.02</td>
<td>4.1 ± 0.8</td>
</tr>
<tr>
<td>Caramide monohexosides</td>
<td>5.4 ± 0.2</td>
<td>3.4 ± 0.1</td>
<td>3.5 ± 0.01</td>
<td>2.5 ± 0.1</td>
<td>3.1 ± 0.3</td>
<td>3.0 ± 0.1</td>
</tr>
<tr>
<td>Steryl glycosides</td>
<td>1.1 ± 0.01</td>
<td>3.5 ± 0.3</td>
<td>3.9 ± 0.08</td>
<td>2.4 ± 0.09</td>
<td>3.1 ± 0.1</td>
<td>0.5 ± 0.5</td>
</tr>
<tr>
<td>Diacylglycerophosphoglycerols</td>
<td>1.4 ± 0.1</td>
<td>1.2 ± 0.01</td>
<td>2.1 ± 0.2</td>
<td>ND</td>
<td>0.3 ± 0.6</td>
<td>6.2 ± 0.8</td>
</tr>
<tr>
<td>Diacylglycerophosphoethanolamines</td>
<td>6.2 ± 0.2</td>
<td>8.6 ± 0.04</td>
<td>7.2 ± 0.7</td>
<td>7.9 ± 0.6</td>
<td>7.7 ± 0.6</td>
<td>7.3 ± 0.7</td>
</tr>
<tr>
<td>Diacylglycerophosphocholines</td>
<td>7.4 ± 0.3</td>
<td>10.8 ± 1.1</td>
<td>7.5 ± 0.9</td>
<td>10.6 ± 0.9</td>
<td>11.8 ± 0.8</td>
<td>10.5 ± 0.7</td>
</tr>
<tr>
<td>Diacylglycerophosphoinositols</td>
<td>3.0 ± 0.1</td>
<td>ND</td>
<td>1.1 ± 0.1</td>
<td>ND</td>
<td>14 ± 0.3</td>
<td>Tr</td>
</tr>
<tr>
<td>Diacylglycerophosphoserines</td>
<td>3.4 ± 0.2</td>
<td>ND</td>
<td>1.3 ± 0.2</td>
<td>ND</td>
<td>16 ± 0.2</td>
<td>Tr</td>
</tr>
<tr>
<td>Cardiolipins</td>
<td>ND</td>
<td>Tr</td>
<td>2.3 ± 0.4</td>
<td>1.4 ± 0.2</td>
<td>ND</td>
<td>3.1 ± 0.6</td>
</tr>
<tr>
<td>Phosphatidic acids</td>
<td>2.3 ± 0.05</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>Tr</td>
</tr>
</tbody>
</table>

*Values are expressed as the percentage (w/w) of the total amount of lipid and are mean ± SD of three determinations.

Tr, trace; ND, not detected.
Table 3 Constituent fatty acids of total lipids from C. albicans KTCC 89062 grown on glucose or 
n-alkanes of different chain length

<table>
<thead>
<tr>
<th>Fatty acid</th>
<th>Glucose</th>
<th>C₁₂</th>
<th>C₁₄</th>
<th>C₁₆</th>
<th>C₁₈</th>
<th>C₂₀</th>
</tr>
</thead>
<tbody>
<tr>
<td>16:0</td>
<td>28.4 ± 1.4</td>
<td>17.5 ± 1.3</td>
<td>18.4 ± 0.7</td>
<td>21.3 ± 1.1</td>
<td>20.4 ± 1.2</td>
<td>20.2 ± 1.8</td>
</tr>
<tr>
<td>16:1</td>
<td>10.9 ± 0.5</td>
<td>10.9 ± 0.8</td>
<td>10.3 ± 0.4</td>
<td>11.4 ± 0.9</td>
<td>12.1 ± 0.6</td>
<td>8.2 ± 0.4</td>
</tr>
<tr>
<td>18:0</td>
<td>10.2 ± 0.3</td>
<td>5.0 ± 1.0</td>
<td>5.1 ± 0.2</td>
<td>6.7 ± 0.4</td>
<td>5.8 ± 0.3</td>
<td>8.5 ± 0.6</td>
</tr>
<tr>
<td>18:1</td>
<td>35.5 ± 2.1</td>
<td>42.5 ± 2.8</td>
<td>38.3 ± 3.1</td>
<td>34.7 ± 2.1</td>
<td>36.4 ± 3.1</td>
<td>31.3 ± 1.9</td>
</tr>
<tr>
<td>18:2</td>
<td>11.4 ± 1.2</td>
<td>19.3 ± 1.3</td>
<td>24.7 ± 2.1</td>
<td>20.0 ± 1.3</td>
<td>18.2 ± 1.2</td>
<td>20.8 ± 2.1</td>
</tr>
<tr>
<td>18:3</td>
<td>1 ± 0.01</td>
<td>Tr</td>
<td>Tr</td>
<td>Tr</td>
<td>Tr</td>
<td>Tr</td>
</tr>
<tr>
<td>&gt;18:3</td>
<td>2.5 ± 0.3</td>
<td>4.7 ± 0.1</td>
<td>5.0 ± 0.6</td>
<td>5.9 ± 0.7</td>
<td>7.1 ± 0.8</td>
<td>11.0 ± 0.9</td>
</tr>
<tr>
<td>Ratio**</td>
<td>1</td>
<td>1.2</td>
<td>1.2</td>
<td>1.3</td>
<td>1.5</td>
<td>2.4</td>
</tr>
</tbody>
</table>

* Values are expressed as percentages (w/w); each value is the mean ± SD of three determinations.
** Ratio unsaturated:saturated without fatty acid > 18:3.
Tr: trace.

Effect on morphology
Cells grown in CDM supplemented with glucose were generally smooth-walled bodies, spherical to elongated in shape, typical of the yeast form of C. albicans (Figure 2a). Growth in the presence of n-alkanes led to the formation of significant numbers of pseudohyphae (Figures 2b and 2c).

Discussion
The isolate of C. albicans used was able to grow adequately on only a narrow range (C₁₂ to C₁₈) of n-alkanes. In this it differed from other strains of this organism (Prasad, 1985; Klug and Markovetz, 1967) when n-alkanes C₆ to C₁₂ were also utilised. It is possible that the lower chain lengths studied were actually toxic (Gill and Ratledge, 1972) to this isolate, rather than not being utilisable. Overall there was consistently a lower yield of cells in CDM supplemented with n-alkanes when a comparison was made with CDM supplemented with glucose as the carbon source. A similar reduction in the yield of cell mass for C. albicans NCL 3100 grown on n-alkanes was observed by Singh et al. (1978).

The metabolism of the n-alkanes present in samples of weathered Kuwaiti crude oil was in agreement with the range of separate n-alkanes metabolised. In the crude oil the n-alkanes with chain lengths of C₁₄ to C₁₈ were preferentially metabolised, irrespective of whether an odd or even number of carbon atom was present in the chain. Atlas et al. (1981) also found that when crude oil was degraded by a mixed microbial population the n-alkanes were preferentially degraded. However, Fedorak and Westlake (1981a,b) observed, with mixed
Table 4  Fatty acid patterns of major lipid classes from *Candida albicans* KTCC 89062 grown on glucose or *n*-alkanes of different chain length*  

<table>
<thead>
<tr>
<th>Fatty Acid</th>
<th>Glucose</th>
<th>C₁₂</th>
<th>C₁₄</th>
<th>C₁₆</th>
<th>C₁₈</th>
<th>C₂₀</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Diacylglycerophosphatidylcholines</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>18:0</td>
<td>24.8 ± 0.8</td>
<td>16.2 ± 0.7</td>
<td>14.3 ± 1.2</td>
<td>19.8 ± 1.8</td>
<td>18.4 ± 0.5</td>
<td>14.4 ± 0.6</td>
</tr>
<tr>
<td>16:1</td>
<td>11.7 ± 0.9</td>
<td>9.8 ± 1.3</td>
<td>14.0 ± 1.0</td>
<td>11.5 ± 1.2</td>
<td>9.4 ± 1.0</td>
<td>10.4 ± 0.6</td>
</tr>
<tr>
<td>18:0</td>
<td>5.3 ± 0.1</td>
<td>3.1 ± 0.5</td>
<td>3.5 ± 0.3</td>
<td>3.2 ± 0.8</td>
<td>3.8 ± 0.2</td>
<td>3.9 ± 0.8</td>
</tr>
<tr>
<td>18:1</td>
<td>32.9 ± 2.1</td>
<td>39.6 ± 0.7</td>
<td>37.8 ± 0.9</td>
<td>32.0 ± 0.5</td>
<td>32.1 ± 0.4</td>
<td>29.5 ± 1.2</td>
</tr>
<tr>
<td>18:2</td>
<td>20.9 ± 1.9</td>
<td>25.9 ± 1.1</td>
<td>24.1 ± 0.3</td>
<td>24.6 ± 0.6</td>
<td>28.7 ± 0.5</td>
<td>27.4 ± 1.3</td>
</tr>
<tr>
<td>18:3</td>
<td>2.3 ± 0.6</td>
<td>1.5 ± 0.3</td>
<td>1.6 ± 0.9</td>
<td>2.3 ± 0.3</td>
<td>1.7 ± 0.2</td>
<td>1.9 ± 0.1</td>
</tr>
<tr>
<td>&gt;18:3</td>
<td>2.1 ± 0.8</td>
<td>3.9 ± 0.2</td>
<td>5.7 ± 0.4</td>
<td>6.6 ± 0.7</td>
<td>3.9 ± 0.3</td>
<td>2.5 ± 0.6</td>
</tr>
<tr>
<td><strong>Diacylglycerophosphatidylcholines</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>16:0</td>
<td>33.6 ± 1.7</td>
<td>3.7 ± 0.3</td>
<td>7.9 ± 0.2</td>
<td>12.3 ± 0.8</td>
<td>12.2 ± 0.8</td>
<td>12.8 ± 0.9</td>
</tr>
<tr>
<td>16:1</td>
<td>15.1 ± 0.3</td>
<td>14.6 ± 0.4</td>
<td>15.8 ± 0.5</td>
<td>15.0 ± 1.1</td>
<td>16.1 ± 1.2</td>
<td>7.6 ± 0.6</td>
</tr>
<tr>
<td>18:0</td>
<td>2.8 ± 0.3</td>
<td>2.6 ± 0.1</td>
<td>1.6 ± 0.3</td>
<td>1.2 ± 0.2</td>
<td>1.5 ± 0.6</td>
<td>5.9 ± 0.8</td>
</tr>
<tr>
<td>18:1</td>
<td>36.0 ± 1.1</td>
<td>44.8 ± 1.4</td>
<td>39.4 ± 1.2</td>
<td>36.8 ± 0.5</td>
<td>39.7 ± 0.2</td>
<td>32.7 ± 0.8</td>
</tr>
<tr>
<td>19:2</td>
<td>15.2 ± 0.9</td>
<td>22.7 ± 0.4</td>
<td>30.2 ± 1.3</td>
<td>25.6 ± 0.4</td>
<td>21.3 ± 0.5</td>
<td>27.8 ± 1.4</td>
</tr>
<tr>
<td>&gt;18:3</td>
<td>1.3 ± 0.01</td>
<td>0.4 ± 0.05</td>
<td>1.0 ± 0.1</td>
<td>1.1 ± 0.05</td>
<td>0.3 ± 0.01</td>
<td>1.4 ± 0.05</td>
</tr>
<tr>
<td><strong>Triglycerides</strong></td>
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</tr>
<tr>
<td>16:0</td>
<td>36.7 ± 1.4</td>
<td>17.1 ± 0.3</td>
<td>14.8 ± 0.5</td>
<td>17.8 ± 0.8</td>
<td>19.9 ± 0.8</td>
<td>22.7 ± 1.2</td>
</tr>
<tr>
<td>16:1</td>
<td>9.8 ± 1.0</td>
<td>9.3 ± 0.6</td>
<td>8.8 ± 0.8</td>
<td>9.3 ± 0.4</td>
<td>11.6 ± 0.9</td>
<td>7.5 ± 1.1</td>
</tr>
<tr>
<td>18:0</td>
<td>17.6 ± 0.9</td>
<td>5.2 ± 0.9</td>
<td>4.8 ± 0.6</td>
<td>5.2 ± 0.1</td>
<td>5.3 ± 0.5</td>
<td>5.7 ± 0.9</td>
</tr>
<tr>
<td>18:1</td>
<td>32.3 ± 1.3</td>
<td>41.5 ± 0.8</td>
<td>38.5 ± 1.2</td>
<td>38.9 ± 0.9</td>
<td>35.5 ± 1.2</td>
<td>39.7 ± 0.2</td>
</tr>
<tr>
<td>18:2</td>
<td>7.1 ± 0.9</td>
<td>18.9 ± 1.0</td>
<td>23.2 ± 0.4</td>
<td>23.3 ± 0.8</td>
<td>18.6 ± 1.0</td>
<td>11.4 ± 0.3</td>
</tr>
<tr>
<td>&gt;18:3</td>
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<td>Tr</td>
<td>1.3 ± 0.1</td>
<td>Tr</td>
<td>Tr</td>
<td>0.5 ± 0.01</td>
</tr>
<tr>
<td><strong>Fatty acids</strong></td>
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<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>16:0</td>
<td>30.4 ± 0.6</td>
<td>23.0 ± 0.8</td>
<td>22.1 ± 1.0</td>
<td>21.4 ± 0.7</td>
<td>25.0 ± 0.7</td>
<td>23.8 ± 0.9</td>
</tr>
<tr>
<td>16:1</td>
<td>9.1 ± 0.6</td>
<td>8.6 ± 0.3</td>
<td>8.5 ± 0.9</td>
<td>7.6 ± 0.2</td>
<td>6.9 ± 0.8</td>
<td>5.1 ± 0.8</td>
</tr>
<tr>
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<td>15.2 ± 0.7</td>
<td>13.1 ± 0.4</td>
<td>14.3 ± 1.1</td>
<td>13.4 ± 0.4</td>
<td>13.5 ± 1.2</td>
<td>13.7 ± 1.0</td>
</tr>
<tr>
<td>18:1</td>
<td>32.4 ± 1.5</td>
<td>38.6 ± 0.9</td>
<td>34.4 ± 1.2</td>
<td>37.6 ± 0.8</td>
<td>32.9 ± 0.8</td>
<td>33.1 ± 0.5</td>
</tr>
<tr>
<td>18:2</td>
<td>9.8 ± 0.3</td>
<td>12.2 ± 0.5</td>
<td>15.8 ± 0.3</td>
<td>14.7 ± 0.8</td>
<td>12.6 ± 0.6</td>
<td>13.0 ± 0.4</td>
</tr>
<tr>
<td>&gt;18:3</td>
<td>1.2 ± 0.1</td>
<td>0.8 ± 0.02</td>
<td>0.5 ± 0.01</td>
<td>Tr</td>
<td>0.3 ± 0.05</td>
<td>0.7 ± 0.1</td>
</tr>
</tbody>
</table>

* Values are expressed in % (w/w) and are the means of ± SD of three determinations.

Tr. trace.

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Figure 2  SEM of C. albicans grown as a shake culture in CDM supplemented with glucose (a), and n-alkanes (b and c). x5,000.
microbial populations, that the aromatic compounds present in crude oil were attacked first and then the n-alkanes. In the case of C. albicans, in monoculture, the aromatic hydrocarbons were not degraded at all, although again they could be toxic to this strain.

A slow growth rate, seen with C. albicans growing on n-alkanes, does encourage the accumulation of lipid in yeasts (Ratledge, 1978). For this reason, determinations were made at the same point in the growth cycle, for each batch culture, so that valid comparisons could be made. The differences between the lipid contents of cells grown on glucose and n-alkane supplemented CDM were sufficiently large for them to be meaningful. The increase in total lipid content correlated with a decreased cell yield. The level of sterol was correlated more closely with the chain length of the n-alkane substrate, i.e. the highest level was detected with C12 as substrate and fell progressively to C20 as substrate.

The changes in the non-sterol fraction of the lipids were most pronounced in the alteration of the ratio of unsaturated:saturated fatty acids. There was a pronounced fall in the levels of C18:0 and C18:1 with a corresponding rise in C18:1 and C18:3. This may be correlated with the increase in sterol levels in order to maintain membrane fluidity and integrity. This has, also, apparently affected the balance of the phospholipids. An increase in the phospholipid content has been reported for Candida 107 (Gill and Ratledge, 1972) under carbon limited growth conditions and also in substrate excess (Thorpe and Ratledge, 1972).

It is known that membrane lipids are involved in several cellular functions including solute transport (Prasad and Rose, 1986). The role exerted by any individual lipid component in solute transport is not known and attempts at assessing such roles are difficult, particularly in view of the fact that alterations in membrane permeability are largely dependent upon the presence, nature and amounts of lipids in the surface layers of the yeast (Gale et al., 1975). In addition, the phospholipids:sterol ratio seems to exert an important role in membrane function (Gottlieb and Shaw, 1967). In this respect, our study showed that the phospholipids:sterol ratio decreased when C. albicans cells were grown in the presence of n-alkanes as compared with glucose grown cells. Thus, growth in the presence of hydrocarbons results in a complex dynamic situation, rather than a simple one, in response to the environment, and consequently the relative importance of any one component in the transport of hydrocarbons is difficult to speculate.

The alteration in the levels of lipids extracted from alkane grown cells reflects the levels in the cell membrane (Prasad, 1985) and these are possibly correlated with the altered permeability requirements of cells growing on hydrocarbons (Hug et al., 1974). These changes in membrane composition can affect cellular morphology, because the sterol and phospholipid composition of the membrane is involved in the yeast-mycelium and white-opaque phenotypic variations in C. albicans (Ghannoum et al., 1986, 1989). Filamentous forms of Candida tropicalis, with smooth walls, were produced when it was grown on media containing n-alkanes (Ratledge, 1978). Similarly, growth of C. albicans in media containing n-alkanes resulted, in this study, in pseudohyphal formation.
Ludvik et al. (1963) showed that hydrocarbons penetrate through the cell wall of C. lipolytica and accumulate at the surface of the cytoplasmic membrane bringing about numerous morphological changes of the cell including thickening of the cytoplasmic membrane and development of deep invaginations and digit projections of this structure. In this respect, Yamaguchi et al. (1974) showed that filamentous cells of C. albicans are characterized by the extensive development of intracytoplasmic membrane systems (ICMS) and endoplasmic reticulum. Such ICMS form whorled structures composed of multiple layers of unit membrane. Thus, we propose that the development of pseudohyphae observed in this study in response to growth in n-alkanes is to increase the uptake of hydrocarbons by increasing the surface area of the cytoplasmic membrane.

Acknowledgements
This work was supported by Kuwait University Research Council, grant number SO 033, SO 042, and Kuwait Foundation for the Advancement of Science, grant number 87-01-04. The assistance of Miss R. Yassin in bibliographic research is appreciated.

References
Candida albicans

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Accepted 10 May 1990
**Growth of *Candida albicans* in the presence of hydrocarbons: a correlation between sterol concentration and hydrocarbon uptake**

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**Summary.** *Candida albicans* KTCC 89062 grown on n-alkanes showed higher levels of sterol content as compared to glucose-grown cells. Certain sterols, such as lanosterol, were significantly reduced in cells grown on n-alkanes, while others, such as ergosterol, increased in these cells. Sterol fractions declined as the chain length of the n-alkanes increased. Ergosterol supplementation of the chemical-defined medium showed an increase in the uptake of dodecane (C₁₂) by cells grown on such medium. Increase in the concentration of ergosterol supplementation resulted in an increase in C₁₂ uptake. The uptake of C₁₂ was not stimulated by ergosterol supplementation in the case of non-viable yeast cells.

**Introduction**

The effects of growth of *Candida* spp. utilizing n-alkanes on lipid content, particularly the fatty acid levels, have been widely studied (Mishina et al. 1977; Miyazima et al. 1985; Pelechova et al. 1971; Singh et al. 1978; Thorpe and Ratledge 1972). There have been relatively few investigations of the effects of similar growth conditions on the nature and levels of the sterols present in fungi. Tanaka et al. (1971), working with mixtures of n-alkanes as substrates, found that the level of ergosterol was approximately 1.0% of the dry weight of *C. tropicalis* compared with 0.6% for cells grown on glucose. In *C. lipolytica* growing on a substrate of unspecified n-alkanes, ergosterol accounted for 81% of the sterol fraction (Sica et al. 1984). Ergosterol was also the major sterol component in *Pichia* spp. (Jeong et al. 1975). Mishina et al. (1977) reported that there was no significant change in total ergosterol in *C. albicans* grown on C₁₇ and C₂₀ alkanes; however, it was 20–40% more in cells grown on alkanes of shorter chain length.

The role of the increase in sterol levels in fungi growing on n-alkanes is not understood. Prasad and Rose (1986) consider that there is little evidence to implicate sterols in amino acid transport. The aim of this investigation was to study the effect of growth of *C. albicans* on hydrocarbons as a sole source of carbon on the level and nature of sterols and to study the possible role of sterols in the uptake of hydrocarbons by this yeast.

**Materials and methods**

**Microorganism.** The strain of *C. albicans* KTCC (Kuwait Type Culture Collection) 89062 used throughout this study was isolated from soil samples taken from a site that was contaminated with crude oil. The samples were taken aseptically after the removal of the 5-cm surface layers. The isolate was identified using the criteria of germ-tube formation and API 20C (Dermount 1979; Ghannoun et al. 1985). Stock cultures were maintained on slopes of modified Sabouraud agar medium (Difco, Detroit, Mich., USA), stored at 4°C. These were subcultured onto fresh medium every 4–5 weeks.

**Chemicals.** The hydrocarbons used were: n-alkanes [hexane, heptane, isooctane, nonane, decane, dodecane, tetradecane, hexadecane, octadecane and eicosane (Fluka, Neu-Ulm, FRG) and aromatic hydrocarbons (benzene (J. T. Baker Chemical Co., Deventer, The Netherlands), phenanthrene (Winlab Limited, London, UK), anthracene and naphthalene (Sigma Chemical Co., St. Louis, Mo., USA)].

**Cultivation.** *C. albicans* KTCC 89062 was grown on a rotary shaker (160 rpm) at 37°C in a chemically defined medium (CDM) of the following composition (g/l): 0.82 NaNO₃, 0.56 KH₂PO₄, 0.86 Na₂HPO₄, 0.17 K₂SO₄, 0.37 MgSO₄·7H₂O, 0.007 CaCl₂·2H₂O, 0.004 Fe(II) EDTA, 2.5 ml of a trace element solution consisting of (g/l): 2.32 ZnSO₄·7H₂O, 1.78 MnSO₄·4H₂O, 0.56 H₂BO₃, 1.0 CuSO₄·5H₂O, 0.39 Na₂MoO₄·2H₂O, 0.42 CoCl₂·6H₂O, 0.66 KI, 1.0 EDTA, 0.4 FeSO₄·7H₂O, 0.004 NiCl₂·6H₂O. The above medium was supplemented with 10 ml/l vitamin solution composed of (mg/l): 0.01 biotin, 2.0 pyridoxine, 2.0 nicotinic acid, 2.0 thiamine and 10.0 L-methionine. The pH was adjusted to 5.6 using Na₂HPO₄/NaH₂PO₄ buffer solution. Cells were grown overnight in CDM supplemented with 10 g/l glucose. These were harvested at early stationary phase by centrifugation, washed three times with 0.9% saline and suspended in saline to give a cell number of 2 x 10⁸/ml. Subsequently, 1-ml portions were used as inoculum for sterol analysis studies in 250 ml CDM.
supplemented with 10 g/l of either glucose, or n-alkanes for 1 day. Cells were then harvested by centrifugation. This incubation period gave cultures that were at the same stage of growth.

Analytical methods. Sterols were extracted by a modification of the method of Fryberg et al. (1974): KOH (1.5 g) in 2 ml of distilled water was added to 0.2 g wet weight of intact cells and the volume of the suspension adjusted to 10 ml by the addition of ethanol. The solution was refluxed under nitrogen for 3 h, diluted with an equal amount of water and extracted with four volumes of heptane. The extract was washed with water, dried over anhydrous Na2SO4, and evaporated in vacuo to give the total sterols.

The sterols were fractionated by thin layer chromatography (TLC) using silica gel G plates (0.25 mm) and 40-60 petroleum ether:diethyl ether (3:1, v/v) as a developing solvent. Lifschutz reagent (concentrated sulphuric acid:glacial acetic acid (1:1, v/v)) was used to detect sterols as red or purple spots. Fractions separated by TLC were identified by comparison of their Rf values with commercially available standards. Sterols were analysed with an SP-2400 UV/VIS spectrophotometer (Pye, Cambridge, UK). Preparative TLC was used to separate the individual sterols ready for identification using different spectroscopic methods.

Silylated sterols were prepared according to Vandenheuvel and Court (1968). Up to 1 mg sterol extract was reacted in a glass-stoppered flask with 50 μl hexamethyl disilazane and 50 μl of 10% trimethyl chlorosilane in chloroform (v/v), the reagents being added in that order. Brief mixing by stirring or vibration was applied after each addition. The reaction mixture was left at room temperature for at least 4 h. Excess solvent and reagents were removed using the method of Vandenheuvel et al. (1965). Carbon disulphide (CS2; 50 μl) was then added to the flask to dissolve the reaction mixture. Portions (0.5 μl) were loaded on an SP2100 column (3% on 100/120 gaschrom Q) in a Pye Unicam (Cambridge, UK) gas chromatograph (series 204). Comparison of retention times of the peaks with those of reference standards using polydimethylsiloxane (JXR®) as a second column. The samples were eluted at 230°C with nitrogen as carrier gas (60 ml/min) and the non-saponifiable lipid detected with a flame ionization detector (FID). Individual components were identified by a comparison of the retention times relative to ergosterol (Rf) with those of commercially available standards.

Effect of ergosterol supplementation on hydrocarbon uptake. C. albicans KTCC 89062 was grown overnight in 50 ml Sabouraud Dextrose broth (SDB; Difco). This culture was used to inoculate 450 ml SDB, then incubated overnight at 37°C. Cells were harvested by centrifugation, washed three times with CDM then suspended in the same medium (1 l) without hydrocarbon, and 20-ml samples were dispensed in sterile conical flasks. Each flask was then divided into 0.2 ml dodecane. These flasks served as a control. Other flasks treated in the same manner were supplemented with 5, 10, 15 or 20 μg/ml ergosterol from a stock solution of 4 mg/ml in 95% ethanol. The same volume of 95% ethanol was added to control flasks. To study the effect of viability of C. albicans on the enhancement of dodecane uptake by ergosterol, gentle heat killing of the yeast was carried out by heating at 60°C for 30 min (Lee and King 1983).

Cultures were incubated at 37°C in an orbital shaker (160 rpm). At different time intervals samples were removed, and only one was supplied with 0.2 ml dodecane to serve as a control. The cells were harvested by centrifugation and the supernatant retained. Cells were subsequently washed three times with boiling water, centrifuged and the resulting supernatant retained and added to the previous supernatant. Dodecane was then recovered by extracting three times with 10 ml hexane. The uptake of hydrocarbon was estimated by GLC analysis using an SP2100 column and 3% OV-1 as a second column (Sorkhoh et al. 1990).

Triplicate samples were added at 140°C with helium as a carrier gas (45 ml/min) and the hydrocarbons detected by FID. The peak areas were compared with those of zero time and the percentage uptake was calculated.

Results

The sterol content of n-alkane-grown cells was consistently higher, as a percentage of dry weight, in all the samples grown on n-alkanes, rather than glucose. There was, however, a gradual decline in the sterol fractions as the chain length increased from C12 to C20 (Table 1).

The examination of the sterol fraction of the isolate C. albicans KTCC 89062 showed that there were quantitative differences apparent between cells grown on the various carbon sources, as shown by TLC analysis (Fig. 1). Two major components found in the cells grown on glucose, 4,4-dimethyl sterols (such as lanosterol) and 4-methyl sterols, were significantly reduced in cells grown on n-alkanes. There was an increase in 4-desmethyl sterols (such as ergosterol) in the latter cells.

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Yield of biomass (g dry wt/l)</th>
<th>Sterols (% dry weight)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dodecane</td>
<td>1.01</td>
<td>1.33</td>
</tr>
<tr>
<td>Tetradecane</td>
<td>1.27</td>
<td>1.28</td>
</tr>
<tr>
<td>Hexadecane</td>
<td>1.82</td>
<td>1.09</td>
</tr>
<tr>
<td>Octadecane</td>
<td>1.86</td>
<td>1.19</td>
</tr>
<tr>
<td>Eicosane</td>
<td>2.08</td>
<td>0.59</td>
</tr>
<tr>
<td>Glucose</td>
<td>5.48</td>
<td>0.26</td>
</tr>
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</table>

Fig. 1. Typical thin layer chromatogram of sterols extracted from Candida albicans KTCC 89062 cultures grown on glucose (G) or n-alkanes of different chain length (Cn-C20) as sole carbon source: SQ, squalene; SE, sterol esters; L, 4,4-dimethyl sterol(lanosterol); ME, 4-methyl sterol; E, 4-desmethyl sterol (ergosterol); DG, diglycerides
Table 2. Comparison of sterols from *C. albicans* KTCC 89062 grown on glucose or n-alkanes of different chain length

<table>
<thead>
<tr>
<th>Sterols</th>
<th>Chain length</th>
<th>R&lt;sub&gt;avg&lt;/sub&gt; Glucose</th>
<th>Chain length</th>
<th>Glucose</th>
<th>Chain length</th>
<th>Glucose</th>
<th>Chain length</th>
<th>Glucose</th>
<th>Chain length</th>
<th>Glucose</th>
<th>Chain length</th>
<th>Glucose</th>
</tr>
</thead>
<tbody>
<tr>
<td>Squalene</td>
<td>C&lt;sub&gt;20&lt;/sub&gt;</td>
<td>0.3 7.5</td>
<td>C&lt;sub&gt;12&lt;/sub&gt;</td>
<td>2.7 3.3</td>
<td>C&lt;sub&gt;16&lt;/sub&gt;</td>
<td>5.0 8.7</td>
<td>C&lt;sub&gt;18&lt;/sub&gt;</td>
<td>3.7</td>
<td>C&lt;sub&gt;20&lt;/sub&gt;</td>
<td>7.3 4.4</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Calzf erol</td>
<td>C&lt;sub&gt;20&lt;/sub&gt;</td>
<td>0.8 1.0</td>
<td>C&lt;sub&gt;12&lt;/sub&gt;</td>
<td>3.9 1.0</td>
<td>C&lt;sub&gt;16&lt;/sub&gt;</td>
<td>5.2</td>
<td>C&lt;sub&gt;18&lt;/sub&gt;</td>
<td>7.3</td>
<td>C&lt;sub&gt;20&lt;/sub&gt;</td>
<td>7.3 4.4</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Zymosterol</td>
<td>C&lt;sub&gt;20&lt;/sub&gt;</td>
<td>0.9 2.4</td>
<td>C&lt;sub&gt;12&lt;/sub&gt;</td>
<td>Tr Tr</td>
<td>C&lt;sub&gt;16&lt;/sub&gt;</td>
<td>Tr Tr</td>
<td>C&lt;sub&gt;18&lt;/sub&gt;</td>
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<td>C&lt;sub&gt;20&lt;/sub&gt;</td>
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<tr>
<td>Ergosterol</td>
<td>C&lt;sub&gt;20&lt;/sub&gt;</td>
<td>1.0 49.4</td>
<td>C&lt;sub&gt;12&lt;/sub&gt;</td>
<td>78.0 76.8</td>
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<td>69.2</td>
<td></td>
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<tr>
<td>4,14-Dimethylzymosterol</td>
<td>C&lt;sub&gt;20&lt;/sub&gt;</td>
<td>1.1 5.7</td>
<td>C&lt;sub&gt;12&lt;/sub&gt;</td>
<td>Tr Tr</td>
<td>C&lt;sub&gt;16&lt;/sub&gt;</td>
<td>3.7 3.7</td>
<td>C&lt;sub&gt;18&lt;/sub&gt;</td>
<td>4.3</td>
<td>C&lt;sub&gt;20&lt;/sub&gt;</td>
<td>4.3</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Obtusifoliol</td>
<td>C&lt;sub&gt;20&lt;/sub&gt;</td>
<td>1.2 16.6</td>
<td>C&lt;sub&gt;12&lt;/sub&gt;</td>
<td>11.8 12.1</td>
<td>C&lt;sub&gt;16&lt;/sub&gt;</td>
<td>12.0 7.5</td>
<td>C&lt;sub&gt;18&lt;/sub&gt;</td>
<td>13.6</td>
<td>C&lt;sub&gt;20&lt;/sub&gt;</td>
<td>13.6</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Lanosterol</td>
<td>C&lt;sub&gt;20&lt;/sub&gt;</td>
<td>1.3 15.6</td>
<td>C&lt;sub&gt;12&lt;/sub&gt;</td>
<td>3.0 1.5</td>
<td>C&lt;sub&gt;16&lt;/sub&gt;</td>
<td>5.1</td>
<td>C&lt;sub&gt;18&lt;/sub&gt;</td>
<td>4.8</td>
<td>C&lt;sub&gt;20&lt;/sub&gt;</td>
<td>4.8</td>
<td></td>
<td></td>
</tr>
<tr>
<td>24-Methyl 23,24-dihydro-lanosterol</td>
<td>C&lt;sub&gt;20&lt;/sub&gt;</td>
<td>1.4 1.8</td>
<td>C&lt;sub&gt;12&lt;/sub&gt;</td>
<td>0.6 1.6</td>
<td>C&lt;sub&gt;16&lt;/sub&gt;</td>
<td>Tr Tr</td>
<td>C&lt;sub&gt;18&lt;/sub&gt;</td>
<td>Tr Tr</td>
<td>C&lt;sub&gt;20&lt;/sub&gt;</td>
<td>Tr Tr</td>
<td></td>
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</tbody>
</table>

Values are expressed as the percentage (w/w) of the total amount of sterols. R<sub>avg</sub>, retention time relative to ergosterol.

The UV absorption spectra of the sterol fraction of *C. albicans* grown in the presence of glucose or individual n-alkanes showed that the spectra had peaks at 271, 282 and 293 nm, identical with the peaks of standard ergosterol (Fieser and Fieser 1959). Quantitative estimation of the sterol fractions by UV measurements confirmed the higher levels of sterols in cells grown on n-alkanes as compared to glucose-grown cells.

Quantitative examination by GLC showed that the level of ergosterol was significantly increased in all the cells grown on n-alkanes (Table 2). The levels were increased by approximately 50% in *C. albicans* grown on n-alkanes with chain lengths of C<sub>12</sub> and C<sub>14</sub> and approximately 40% on n-alkanes with chain lengths C<sub>16</sub> to C<sub>20</sub>. Zymosterol was reduced to trace amounts in the n-alkane series. The most significant reduction was in the level of lanosterol by nearly 40%; on most substrates there was a fall in the level of squalene.

Table 3 summarises the effect of ergosterol supplementation on the uptake of dodecane. When *C. albicans* was grown on ergosterol-supplemented CDM it showed an increase in the uptake of dodecane, as measured by post-growth studies, compared to the control. This was particularly noticeable at the initial stages of incubation where the uptake of dodecane was much higher than in the control. The increased hydrocarbon uptake in response to ergosterol supplementation was concentration-dependent (Table 3). This was true at various incubation periods.

The stimulation of dodecane uptake by ergosterol was observed with viable cells but not with non-viable yeast cells (Table 4).

### Discussion

When *C. albicans* was grown on n-alkanes there was an increase in the level of sterols and detailed analysis has shown this increase to be due mainly to an increase in ergosterol. This effect was most pronounced on CDM supplemented with C<sub>12</sub> and C<sub>14</sub> n-alkanes. A significant increase was also observed with C<sub>16</sub> to C<sub>20</sub> supplementation over the level found in CDM supplemented with glucose as sole carbon source. Mishina et al. (1977) observed that more ergosterol was produced by *C. albicans* on n-alkanes of chain lengths less than C<sub>20</sub>. However, they found with their strain of *C. albicans* that there was no increase in ergosterol levels if C<sub>20</sub> was used. This study showed that the increased ergosterol levels are accompanied by a decrease in the level of lanosterol, zymosterol and squalene, suggesting that ergosterol biosynthesis from squalene proceeds at a higher rate in the presence of hydrocarbons as compared with cells grown on glucose (van den Bosch et al. 1978).

The changes in sterols observed in this study suggest that a particular type of membrane is required for growth on n-alkanes. The enzymes involved in the first step of alkane oxidation is thought to be located in or near the cytoplasmic membrane (Hug et al. 1974). The function of the lipids, in particular the sterols, could be to create a lipophilic region in an otherwise hydrophilic environment, to allow diffusion and accumulation of the alkane substrate. This assumption receives support from the results indicating that supplemented ergosterol resulted in increased hydrocarbon uptake by this yeast. Nevertheless, as indicated by the viability test, this process is clearly not a physical phenomenon, but a biological one, and is probably controlled by specific enzyme systems. Changes in sterols and lipids may be
reflected in ultrastructural modifications of the cell. Ludvik et al. (1968) showed that the cytoplasmic membranes of *C. lipolytica* grown on hydrocarbons are thicker and contain deep projections.

The results of the present study could have a practical value, since they suggest hydrocarbon-grown *C. albicans* as a commercial source of ergosterol.

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