HYDROCARBON DEGRADATION BY MICROORGANISMS FROM THE MARINE ENVIRONMENT

A THESIS SUBMITTED TO THE
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BY
SARITA L. SHARMA

DIVISION OF BIOCHEMICAL SCIENCES
NATIONAL CHEMICAL LABORATORY
PUNE-411 008, INDIA

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Dedicated To
My Husband, Vinay

COMPUTERIZED

NATIONAL CHEMICAL LABORATORY
REFERENCE BOOK
(NOT TO BE ISSUED)

Thus grew the tale of Wonderland,

thus slowly, one by one,

Its quaint events were hammered out,

and now the tale is done……

–Lewis Carroll (Alice's Adventures in Wonderland)

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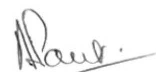
DECLARATION

Certified that the work incorporated in the thesis entitled **"Hydrocarbon Degradation By Microorganisms From The Marine Environment"** submitted by **Sarita L. Sharma** was carried out under my supervision. Such material as has been obtained from other sources has been duly acknowledged in the thesis.



Dr. Aditi Pant

Research Guide

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Sarita Sharma

ABBREVIATIONS

Ara	Arabinose
ATCC	American Type Culture Collection
CMC	Critical micelle concentration
DAB	Diaminobutyric acid
DAP	Diaminopimelic acid
DNA	Deoxyribonucleic acid
DNase	Deoxyribonuclease
DPM	Disintegrations per minute
EDTA	Ethylenediaminetetraacetic acid
FAD	Flavin adenine dinucleotide
FID	Flame ionization detector
FMN	Flavin mononucleotide
G+C content	Guanine + Cytosine content
Gal	Galactose
NAD	Nicotinamide adenine dinucleotide
NADH	Nicotinamide adenine dinucleotide (reduced)
NADP	Nicotinamide adenine dinucleotide phosphate
NADPH	Nicotinamide adenine dinucleotide phosphate (reduced)
NMR	Nuclear magnetic resonance
POPOP	(1,4-Bis (2,5-phenyloxazolyl) benzene
PPO	2,5-Diphenyloxazole
RNA	Ribonucleic acid
RNase	Ribonuclease
SDS	Sodium dodecyl sulfate
Tris	Tris-(hydroxymethyl)-aminomethane
WCSP	Whole cell sugar pattern
Xyl	Xylose

ABSTRACT

Crude oil transport, tanker accidents, industrial runoffs are responsible for contamination by hydrocarbons of the ocean. The serious environmental impact of oil spills such as '*Exxon Valdez*', '*Amoco Cadiz*' have increased global awareness of the problem and over the past 50 years, biodegradation of oil has been extensively studied and a wide variety of microorganisms have been reported to degrade different fractions of crude oil.

The Arabian Sea is a major route for transport of Persian Gulf oil to different countries. Recognizing the importance of tested procedures for clean-up in the event of an oil disaster a Nationally Coordinated Project was initiated in 1991, funded by the Govt. of India, to investigate the presence of oil degrading organisms on the Indian coast and soil and to develop suitable techniques for deployment in case of need. As a part of this project, we have isolated hydrocarbon-degrading microorganisms from marine ecosystems capable of degrading crude oil.

The work presented in this thesis deals with:

1. General introduction
2. Screening, isolation and identification of the crude oil degrading organism
3. Crude oil, kerosene, n-alkanes and pristane degradation by *Rhodococcus* sp. NCIM 5126
4. Production of emulsifiers by *Rhodococcus* sp. NCIM 5126
5. General discussion

Chapter 1
General Introduction

The chapter includes composition of Indian crudes, literature survey on degradation of different hydrocarbons by oil degrading microorganisms, bioremediation, mode of alkane utilization by microorganisms, pathway of alkane degradation and types of emulsifiers produced by oil degrading and non-oil degrading flora have been discussed.

Chapter 2
Screening, Isolation and Identification of the Crude Oil Degrading Organism

Enrichment of environmental samples on 1% (w/v) Assam crude oil resulted in six bacteria and yeasts degrading more than 10% of crude oil. The best degrader showing 30%

degradation by gravimetry was selected for further studies. In terms of both Total Viable Count and oil determined as chloroform-soluble material showed that coastal areas were more contaminated than fresh water habitats.

Fatty acid methyl ester pattern of the whole cells of this orange coloured organism suggested that it was a nocardioform actinomycete. This was confirmed by the presence of *meso*-diaminopimelic acid, arabinose and galactose (cell wall chemotype IV) and mycolic acid in the cell wall of this organism. These results and biochemical tests compared with standard cultures, *Rhodococcus rhodochrous* ATCC 13808 and *Nocardia asteroides* ATCC 19247 helped to assign the isolate to the genus *Rhodococcus*. Further testing showed that our isolate was closest to *R. terrae*. The organism has been deposited in the National Collection of Industrial Microorganisms (NCIM) at National Chemical Laboratory (NCL) as *Rhodococcus* sp. NCIM 5126.

Chapter 3

Crude oil, Kerosene, n-Alkanes and Pristane Degradation by *Rhodococcus* sp. NCIM 5126

Rhodococcus sp. NCIM 5126 degraded aliphatic and aromatic fractions but not the asphaltene fraction of three different crude oils. 72%, 60% and 35% of the aliphatic fraction of Bombay High crude oil, Assam crude oil and Gujarat crude oil respectively were degraded when the isolate was grown in optimized conditions viz. Artificial Sea Water supplemented with 35 mM N as urea, 0.114 mM P as K_2HPO_4, pH 8.0 at 30°C and 150 rpm for 72 hr.

The organism grew best in seawater nutrient broth with a protein specific growth rate of 0.09 hr^{-1} and a generation time of 7.7 hr. When supplied with crude oil or specific hydrocarbons, the μ decreased in consonance with the complexity of the supplied hydrocarbon and/or length of the carbon chains. The growth curves showed that as compared to growth in nutrient broth (0.09 hr^{-1}, 7.7 hr), fructose (0.031 hr^{-1}, 22 hr), n-tetradecane (0.018 hr^{-1}, 39 hr), crude oil (0.017 hr^{-1}, 41 hr), the organism was maximally stressed in the presence of pristane (0.016 hr^{-1}, 43 hr). Gas chromatographic analysis of n-alkanes showed that the organism degraded pure alkanes ranging from n-C_{14} to n-C_{18} (n-tetradecane being degraded maximum at 94%), mixed chain alkane (kerosene 92%) and the

branched chain alkane pristane (30%). Cellular fatty acid methyl ester patterns of the organism grown on n-tridecane, n-tetradecane, n-hexadecane, n-heptadecane and pristane showed the predominance of the corresponding fatty acid. GC-MS analysis of the supernatant of the n-tridecane, n-tetradecane, n-hexadecane, n-heptadecane and pristane grown cells showed the presence of both odd numbered fatty acids and dicarboxylic acids indicating presence of α-oxidation and ω-oxidation pathways in addition to the usual β-oxidation pathway for alkane degradation in this organism.

The organism required 0.4 M NaCl concentration similar to that in Artificial Sea Water for growth and degradation although it tolerated upto 1.71 M NaCl, suggesting that the organism is a moderately halophilic organism. Sodium sulfate and sodium chloride were necessary both for growth and crude oil degradation. Sodium sulfate (0.07 M) and sodium chloride (0.085 M) in deionised water entirely replaced all other salts used in Artificial Sea Water. Sodium sulfate was essential for the growth of the organism in non-hydrocarbon medium also. Compared to *E. coli*, there was a higher requirement for sulfur by our organism in terms of sulfur per unit biomass.

Wax coated slow release fertilizers were prepared and their inclusion in the medium increased degradation of the aliphatic fraction of crude oil by about 8%. Cells immobilized on 2% κ-carragennan degraded 10% more crude oil than free cells. The beads were reusable for 7 cycles of 4 days each over a period of one month.

Chapter 4

Production of Emulsifiers by *Rhodococcus* sp. NCIM 5126

Rhodococcus sp. NCIM 5126 produced extracellular emulsifier in the presence of hydrocarbons, during exponential phase of growth. Under optimal conditions, 225 mg/50 ml emulsifier was produced in Artificial Sea Water supplemented with 35 mM N as urea, 0.114 mM P as K_2HPO_4, 1% (w/v) n-tetradecane, pH 8.0 at 30°C and 150 rpm. Only 35 mg/50 ml emulsifier was produced when the organism was grown in deionised water medium. The surface tension of the broth was reduced from 66 dynes/cm to 32 dynes/cm.

Pseudosolubilization of hydrocarbon appears to be the mode of uptake of alkane by this organism. In the presence of the supernatant of n-tetradecane grown *Rhodococcus* sp.,

the solubility of n-tetradecane increased from normal aqueous solubility of $7x10^{-3}$ mg/l to 50 mg/l.

The emulsifier was concentrated by ultrafiltration and isolated by Sepharose-4B gel filtration. It was a glycolipid with about 92% lipid and 5% carbohydrate. Depending on the supplied n-alkane, corresponding fatty acid was incorporated in the lipid fraction of the emulsifier. In all cases, trehalose was the carbohydrate moiety.

Emulsifiers produced on n-tetradecane and pristane were stable from pH 6 to 11 and at temperatures from 10 to 80°C. 100 µg of the emulsifier in 5 ml of total volume of assay, gave 1 unit activity. 1 unit of emulsifier activity is defined as the amount of emulsifier producing an absorbance of 1.0 at 410 nm under the assay conditions. Critical Micelle Concentration (CMC) was 12 mg/l.

Chapter 5
General Discussion

The present work is discussed with respect to the current state of knowledge of actinomycete identification and classification, their role in hydrocarbon degradation and emulsifier production.

CHAPTER 1

General Introduction

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Crude oil pollution occurs in soil (Song *et al.*, 1990), desert (Al-Gounaim *et al.*, 1995), fresh water (Cooney *et al.*, 1985), estuarine (Oudot *et al.*, 1998) and marine ecosystems (Jones *et al*, 1983) though marine environments are often more polluted due to tanker accidents, off shore oil production, coastal oil refineries, pipe line failure, discharge of ballast water, servicing and cleaning of ships, industrial and municipal run off and natural seeps. Large spills can result in significant contamination of ocean and shoreline environment causing public concern. For example, '*Amoco Cadiz*' discharged 2,23,000 tons of oil into the waters along the Brittany coast in 1978; '*Exxon Valdez*' in 1989 released 41,000 tons of oil contaminating 2,090 Km of coastline of Prince William Sound, Alaska. Deliberate release such as 8,20,000 tons of oil in the Persian Gulf, during the Gulf War in 1991, can also be threatening to coastal ecosystems (Swannell *et al.*, 1996). Prince (1993) suggested that accidental release of oil into the ocean is almost equal to that from the natural seeps, for example: of the total contamination by petroleum to the sea, 10% comes from natural seeps and another 10% from catastrophic releases. Even though efforts are being made to minimize such releases, it is unlikely that oil spills can be completely prevented and there will always be a continuing need for eco-friendly and cost effective tools for responding to oil spills (Prince, 1997).

Crude oil (petroleum) is a naturally occurring, brown to black flammable liquid principally associated with sedimentary rock beneath the earth's surface. It is believed to be derived from marine plant and animal debris subject to high temperature and pressure. Regardless of origin, all crude oils are composed of saturated or aliphatic hydrocarbons containing n-alkanes, branched and cyclic alkanes, aromatics like naphthalene, phenanthrene or anthracene or substituted aromatics chlorobenzene or nitrobenzene and asphaltenes containing phenols, fatty acids, ketones, esters, porphyrins and resins with variable amounts of sulfur, nitrogen and oxygen compounds and trace amounts of metals. The composition of crude oil varies from one oil field to another. It also varies during the life of a single oil field (Speight, 1999). Depending on the relative ratio of the hydrocarbon classes that predominate in the mixture, crude oil is classified as paraffinic, naphthenic and asphaltic (Matar and Hatch, 1994). Table 1.1 represents the composition of main Indian crude oils, as determined by column chromatography.

Table 1.1 Composition of main Indian crude oils

Crude oil	Aliphatic fraction (%)	Aromatic fraction (%)	Asphaltene fraction (%)
Bombay High	82	17	1
Assam Crude	69	22	9
Gujarat Crude	55	37	8

Bombay High crude oil contained the highest proportion of saturates followed by Assam crude oil. Gujarat crude oil contained a relatively high proportion of aromatics and asphaltenes.

That microorganisms degrade hydrocarbons is well known. After an early report by Bushnell and Hass (1941), Zobell (1946) reported more than 100 species representing 30 microbial genera capable of utilizing hydrocarbon emphasizing their wide distribution in nature. Almost all components of crude oil are biodegradable, although they differ in their susceptibility to microbial attack. Saturates or alkanes are most readily and widely utilized whereas branched chain alkanes are less used growth substrates. Cycloalkanes are highly toxic to microorganisms as compared to low molecular weight aromatics (Bartha and Atlas, 1977). Asphaltenes resist microbial attack on their own but their co-oxidation with n-alkanes has been reported (Bertrand *et al.*, 1983; Rontani *et al.*, 1985). Fedorak and Westlake (1981) reported preferential utilization of aromatics to n-alkanes whereas Foght *et al.* (1990) suggested that specific organisms degrade specific classes of hydrocarbon, microbes which utilize n-hexadecane are different from those degrading phenanthrene. After an oil spill, wind and wave action rapidly form a thin slick of oil over the surface of ocean. Low molecular weight components of crude oil evaporate, some of the components dissolve and some emulsify. These changes coupled with microbial and photochemical oxidation of crude oil components form water-in-oil emulsions also known as "Chocolate mousse". The heavy residues form the tar balls. Both mousse and tar balls present a small surface area compared to their volume and hence resist microbial attack (Clark, 1986; Leahy and Colwell, 1990).

Some petroleum fractions can be mutagenic or carcinogenic and even in small quantities, are able to destroy microalgae and juvenile forms of many marine microorganisms. Besides environmental hazards, oil pollution, particularly in coastal

waters, presents a serious problem to recreation, commercial fisheries and public health (Gutnick and Rosenberg, 1977).

A combination of physical, chemical and biological processes need to be implemented for effective removal of spilled oil. Mopping up of floating oil using slick lickers or floating booms is helpful in sheltered harbours, oil can also be absorbed into powdered cork, peat or straw. Use of gelling agents and chemical dispersants is not cost effective and has proved harmful for natural flora whereas burning and sinking of oil is difficult in practice (Clark, 1986) therefore bioremediation is the only environment-friendly, viable alternative to disaster management.

Bioremediation is the process of encouraging the natural process of biodegradation of oil by application of special conditions. As oil degradation is a highly oxidative process, providing air and therefore oxygen under pressure (Dinnen *et al.*, 1990) or injecting aqueous solution of hydrogen peroxide (Raymond *et al.*, 1986) is useful. Alternative electron acceptors such as nitrate have also been employed (Al-Bashir *et al.*, 1990). Addition of extra carbon sources such as molasses and proteins along with inorganic nutrients also lead to enhanced biodegradation of heavy oil (Ying *et al.*, 1990). All these treatments are used in 'land-farming' for the destruction of oily waste (Bartha and Bossert, 1984). Soluble inorganic salts of nitrogen and phosphorus get diluted and may not be available selectively to the degrading population. Oleophilic fertilizers such as paraffinized urea, paraffin supported magnesium ammonium phosphate, ferric octoate, octylphosphate have been used by Olivieri *et al.* (1976) for the treatment of the Sarir crude oil spill. These fertilizers float at the oil/water interface and are selectively available to oil-degrading flora. Oleophilic fertilizers as well as hydrogen peroxide enhanced bioremediation of the '*Exxon Valdez*' oil spill (Atlas, 1991; Bragg *et al.*, 1994). Inipol EAP22® which is a microemulsion of a saturated solution of urea in oleic acid containing tri (laureth-4)-phosphate and butoxy ethanol, adhered to oil and provided fertilizer at the oil/water interface (Ladousse and Trammier, 1991). Customblen® 28-8-0, a slow release encapsulated formulation of ammonium nitrate and ammonium phosphate, released nutrients with every tidal immersion and stimulated the natural rate of biodegradation two to five times with no detectable adverse environmental effect (Bragg *et al.*, 1994; Prince, 1992). Lindstrom *et al.* (1991) and Pritchard and Costa (1991)

reported that addition of fertilizer resulted in increased mineralization potentials of n-hexadecane and phenanthrene in soils contaminated with crude oil. Mohan *et al.* (1979) patented the use of microorganisms *Micrococcus* ATCC 14987 and ATCC 21909, *Corynebacterium* ATCC 21235, *Candida* ATCC 9226, *Achromobacter* ATCC 21990 and *Arthrobacter* ATCC 21908 in combination with dispersing agents Span 80 and Tween 80 to disperse oil slicks. *Pseudomonas aeruginosa* and *Pseudomonas putida* were engineered to have multiple compatible plasmids for degradation of camphor, octane, salicylate and naphthalene (Chakrabarty, 1981). Bioaugmentation (Prince, 1992) is thus a useful addition of microorganisms for the bioremediation of oil spills and slicks.

Hydrocarbons are sparingly soluble in water. The solubility of n-C_6, n-C_{10}, n-C_{14} and n-C_{19} in water at 25°C is $1.1x10^{-4}$, $5.2x10^{-5}$, $7x10^{-6}$ and $6x10^{-6}$ g/l respectively (Einsele and Fiechter, 1971). Erdtsieck and Reitema (1969) demonstrated the growth of *Candida lipolytica* on liquid medium saturated with vapours of n-undecane and n-dodecane. On n-tridecane and longer chain alkanes limited growth occurred presumably because of the lower solubility of these alkanes. However, Aiba *et al.* (1969) and Mimura *et al.* (1971) observed that extremely low amounts of dissolved longer chain alkanes did not support good growth of degrader. The constraint on uptake by solubility may be overcome by direct contact of cells with large hydrocarbon droplets. These flocs contain clumps of cells, alkane and air bubbles. The strong hydrophobic outer membrane possessed by most alkane-utilizers enhances adhesion to the surface of the oil droplet and substrate uptake takes place through diffusion or active transport (Miura *et al.*, 1977). Kappeli and Fiechter (1976) observed that hydrophobicity of alkane-grown cells was greater than glucose-grown cells due to the presence of cell wall associated fatty acid-mannan-complex in the former.

A third mechanism of alkane uptake has been proposed where the cell releases a hydrocarbon solubilizing factor which enhances the solubility of long chain alkanes. Goswami and Singh (1991) isolated and characterized such a factor from *Pseudomonas* N1. They suggested that both pseudosolubilizing factor and emulsifying factor were necessary for n-hexadecane uptake. Their solubilizing factor increased the solubility of hexadecane from $6.3 \ x10^{-3}$ mg/l to 19 mg/l.

For the transport of hydrocarbon into the cells, microbes may rearrange the cell wall structures by the formation of canals (Dmitriev *et al.*, 1980), ultramicroscopic pores or peroxisomes (Osumi *et al.*, 1974) in case of a yeast or by production of fimbriae as in *Acinetobacter calcoaceticus* (Rosenberg *et al.*, 1982). n-Hexadecane grown *Acinetobacter* sp. HO1-N is characterized ultrastructurally by an extensive intracytoplasmic membrane system which extends across the length of the cell as multilaminar sheaths of bilayer membranes continuous with the cytoplasmic membrane. Such membranes have not been observed in *Acinetobacter* grown on non-hydrocarbon substrates, suggesting that they are involved in hydrocarbon metabolism (Kennedy *et al.*, 1975).

When hydrocarbon is transported inside the cell it is oxidized in the periplasmic space. The first reaction is the formation of corresponding n-alcohol, which is catalyzed by a complex hydroxylase that may be linked to one of several different electron-carrier systems. Of these, Ratledge (1978) has described 'Cytochrome P–450' system and 'Rubredoxin' system. Cytochrome P–450 system, which requires NADH rather than NADPH, was first described in *Corynebacterium* sp. strain 7E1C for the hydroxylation of n-octane. In *Candida tropicalis*, this enzyme is located within the microsomes besides NADPH-cytochrome *c* reductase. Other closely associated components are phospholipid and phosphatidylethanolamine. Only n-alkanes with more than 14 carbon atoms are the inducers of these systems. Involvement of rubredoxin system has been demonstrated in cell free extracts of n-octane grown *P. putida*. It consists of three protein components, a rubredoxin-like non-haem iron protein, NADH-rubredoxin reductase and ω-hydroxylase. The possibility of free radical mechanism producing a hydroperoxide (R-CH_2-CH_2OOH) and subsequently primary alcohol has also been raised. However, as hydroperoxides have never been isolated, this possibility seems unlikely. Both rubredoxin system and cytochrome P–450 system are mixed function oxidases and the end product is a primary alcohol in both the cases.

n-Alkanes can be dehydrogenated to alkenes by an NAD dependent dehydrogenase, the reaction involves formation of an epoxide and addition of water to form the corresponding primary alcohol (Fig.1.1).

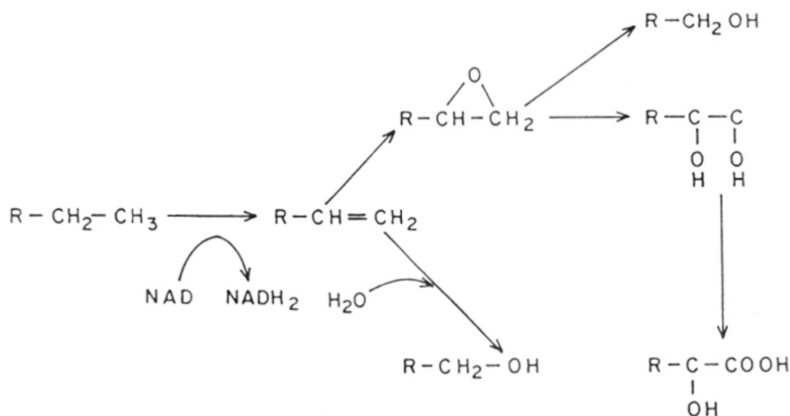

Figure 1.1 Dehydrogenation of alkanes to alkenes and subsequent reactions (Rehm and Reiff, 1981)

The next reaction is the formation of the corresponding fatty aldehyde and fatty acid, which requires NAD and NADP dependent alcohol and aldehyde dehydrogenase respectively. Formation of these intermediates can take place via terminal oxidation pathways where one or both the terminal methyl groups are oxidized. Klug and Markovetz (1971) demonstrated a monoterminal oxidation pathway with the formation of the corresponding fatty acids in bacteria, yeasts and fungi (Fig. 1.2).

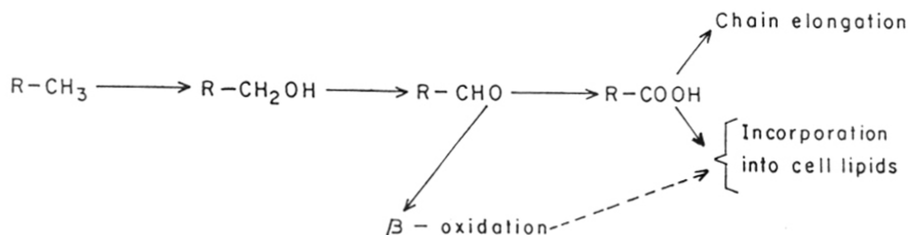

Figure 1.2 Monoterminal oxidation of long chain alkanes (Klug and Markovetz, 1971)

Some n-alkane-grown bacteria and yeasts produce extracellular products, which suggest a pathway of diterminal alkane oxidation (Fig. 1.3).

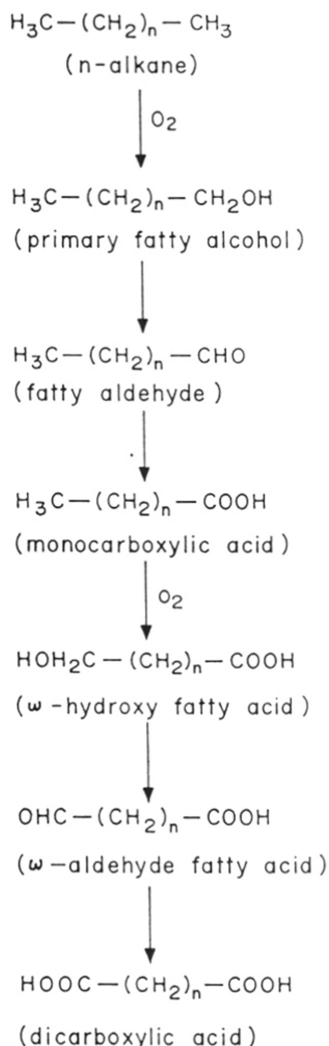

Figure 1.3 Diterminal oxidation of long chain alkanes (Singer and Finnerty, 1984)

In the first step one methyl group of n-alkane is oxidized by the monoterminal route to the corresponding fatty acid, which is subsequently oxidized to ω-hydroxy fatty acid and then ω-methyl group is oxidized to form a dicarboxylic acid. The most important criterion for the detection of diterminal pathway is the isolation of diterminal degradation products, especially dioic acids excreted into the medium (Rehm and Reiff, 1981).

In the subterminal oxidation pathway, the first step involves the formation of a secondary alcohol, which is subsequently oxidized to a ketone. As shown in Fig. 1.4, the further degradation of ketone in the 2-position, yields undecyl acetate which then splits into 1-undecanol and acetate.

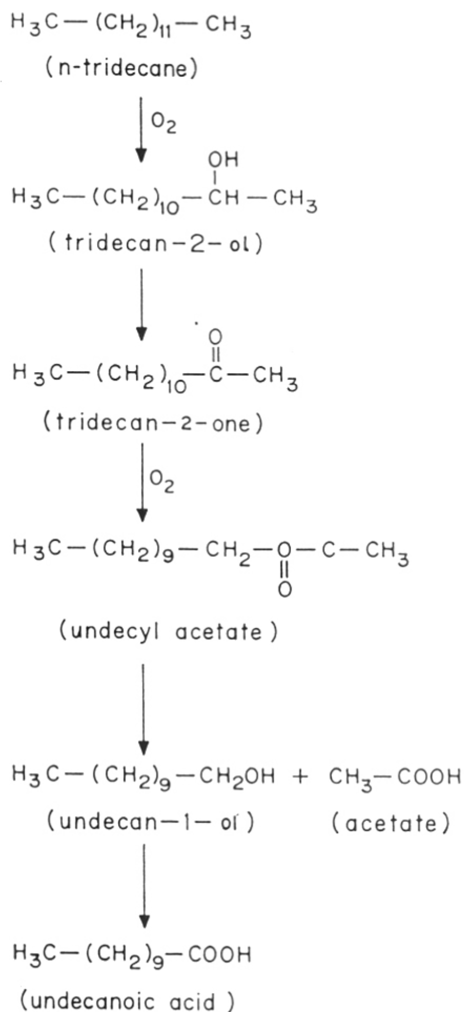

Figure 1.4 Subterminal oxidation pathway of long chain alkanes (Singer and Finnerty, 1984)

The Bayer-Villiger type of reaction takes place by incorporation of molecular oxygen in the presence of NADPH. In bacteria that utilize n-alkanes as sole carbon

source, subterminal oxidation may be a minor, alternative pathway to the monoterminal oxidation pathway. In contrast, for those bacteria that 'co-oxidize' n-alkane, as in case of *Bacillus* sp., *Streptomyces* sp., *Arthrobacter* sp. products indicative of subterminal alkane oxidation appear to be primary (Rehm and Reiff, 1981).

Whatever may be the pathway used by the organism, in every case fatty acids are the predominant product of n-alkane degradation. They may be incorporated into more complex lipids without further modification also called as 'direct/intact incorporation mechanism', which results in the increase in the lipid content of the microorganism (Hug and Fiechter, 1973). Microorganisms grown on even number carbon alkanes possess high amounts of the even number carbon fatty acids whereas organisms grown on odd number carbon alkanes contain high amounts of the odd number carbon fatty acids in their lipids.

The fatty acids may be further metabolized by a variety of metabolic routes. Oxidation via the usual β-oxidation pathway yields acetyl-CoA, or propionyl-CoA. The C_2 units formed are subsequently oxidized through the Kreb's cycle or glyoxylate pathway. α-Oxidation results in a fatty acid one carbon less than the original n-alkane and even number carbon n-alkanes give rise to odd number carbon fatty acid and vice versa. ω-Oxidation leads to the formation of dioic acids. Fatty acids may be subject to chain elongation, desaturation or hydroxylation. Sometimes further degradation of fatty acids may be hindered by the formation of a glycolipid as in case of *Torulopsis gropengiesseri* grown on n-alkane (Jones and Howe, 1968). The fate of the fatty acid will depend on the organism. However, unlike hydrocarbons, fatty acids are transported into bacterial cells in the form of their coenzyme A esters. This mechanism ensures that the potential toxicity of free fatty acids to the cell is eliminated (Ratledge, 1978; Boulton and Ratledge, 1987).

There are fewer microorganisms able to utilize branched chain hydrocarbons. Single branched alkanes are preferentially oxidized at the terminus most distant from the branching point. The resulting fatty acid is incorporated into the cell lipids or further metabolized (King and Perry, 1975). Subsequent degradation via β-oxidation will give rise to α or β-methyl butyric acid or ω-oxidation will produce β-methyl succinic acid as determined for *Brevibacterium erythrogens* (Pirnik *et al.*, 1974).

For a multiple branched alkane such as pristane (2,6,10,14-tetramethylpentadecane), the mechanism of oxidation has been elucidated using *Corynebacterium* sp. (McKenna and Kallio, 1971) and *Brevibacterium erythrogens* (Pirnik *et al.*, 1974). Both produce 4,8,12-trimethyltridecanoic acid and 2-methylglutaric acid either through β-oxidation pathway or by ω-oxidation followed by β-oxidation. The pathway is summarized in Fig. 1.5.

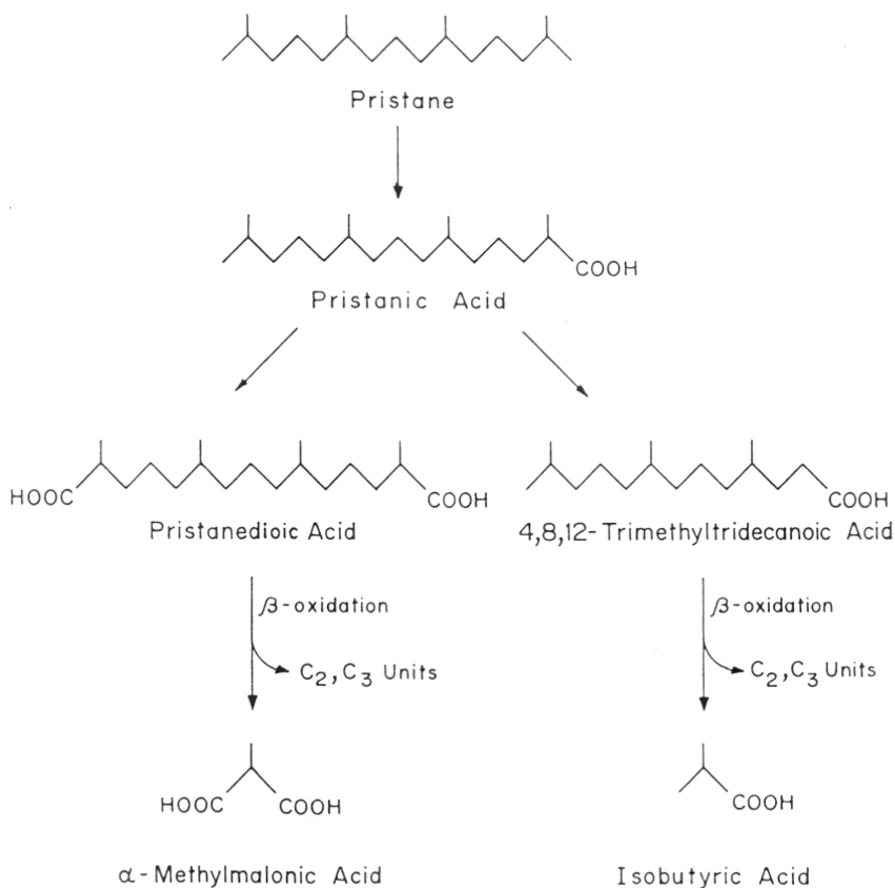

Figure 1.5 Pathway of oxidation of pristane (McKenna and Kallio, 1971; Pirnik *et al.*, 1974)

Nakajima *et al.* (1985 a, b) reported *Rhodococcus* sp. BPM 1613 capable of degrading pristane, 1-pristene, farnesane and hexadecanene. Cox *et al.* (1974) studied the oxidation of pristane by *Mycobacterium fortuitum*. Isolation of various (R) and (S) isomers of 2,6,10,14-tetramethylpentadecan-1-ol, 2,6,10,14-tetramethylpentadecanoic acid and 4,8,12-trimethyltridecanoic acid indicate that methyl group oxidation is stereospecific and the chiral centres at positions 6 and 10 are important in determining the oxidizability of such molecules.

Microbial oxidation of n-alkanes results in the production of many useful compounds. Abbot and Gledhill (1971) and Fukui and Tanaka (1980) reviewed compounds produced by microbes and classified them into three groups. The **Group 1** involves *compounds which can also be synthesized from carbohydrates* such as amino acids, organic acids, polysaccharides, nucleic acids, nucleotides, nucleosides, vitamins and coenzymes, antibiotics, enzymes and cell mass. The **Group 2** contains *products related to physiological and metabolic features of alkane assimilation* such as trehalose lipids, lipopolysachharides, rhamnolipids, riboflavin, FMN, FAD, cytochrome c, coenzyme A, ergosterol, catalase, lipoproteins and biotin-vitamer. The **Group 3** consists of *products which have structures closely related to n-alkane substrates* such as monocarboxylic acids, dicarboxylic acids, ω-hydroxy fatty acids, fatty alcohols, wax esters and enzymes participating in metabolism of n-alkanes.

Patents have been filed for the production of dioic acid from long chain alkanes. Nippon Mining Co. Ltd. (1982) patented a process for the preparation of long chain dicarboxylic acids by *Candida tropicalis* on straight chain alkanes. Tetradecane-1,14-dicarboxylic acid is a valuable raw material for the preparation of perfumes (Fukui and Tanaka, 1980). Mobil Oil Corporation (1975) patented a procedure for the preparation of aldehydes from long chain paraffins using *Pichia* sp. whereas Philips Petroleum Co. (1976) filed a patent for the production of ketones from n-paraffin oxidation by *Torulopsis bombicola*. Exxon (1980) patented the production of methyl ketones from *Methylosinus, Methylobacter* and *Methylococcus*. KAO Chemical Co. (1984) used biosurfactants for stabilization of coal slurries thereby improving the transportation of coal. A surfactant produced by *Candida* sp. is patented for use in textile, pharmaceutical and cosmetics industry by Research Institute of Synthetic Fibres in USSR (1984).

Biosurfactants or bioemulsifiers are of interest in recent years due to their possible application in enhanced oil recovery, crude oil spill clean up, in paper and pulp industry for microbial pulping and treatment of lignosulfonates, in food industry for meat processing and as thickening agents, in mining for microbial leaching of minerals and recovery of minerals and in pharmaceuticals as nutritional additives, coagulation factors and immunosuppressing agents (Kosaric *et al.*, 1987). Harvey *et al.* (1990) demonstrated the removal of '*Exxon Valdez'* spilled oil from Alaskan gravel by a rhamnolipid emulsifier produced by *P. aeruginosa*. Use of emulsifier produced by bacterial isolate Pet 1006, a proprietary strain patented by Petrogen Inc. (USA), for clean up of oil storage tank was successfully carried out by Banat *et al.* (1991) and more than 90% of the hydrocarbon trapped in the sludge was recovered. Zajic and Knettig (1976) patented a mucopolysaccharide emulsifier produced by *Corynebacterium hydrocarboclastus* for dispersion of oil spills whereas Wagner *et al.* (1980) patented glycolipid produced by yeast for oil spill clean up from soil. However 'Emulsan', a *N*- and *O*-lipoacylated heteropolysaccharide consisting of D-galactosamine, aminouronates and fatty acid esters obtained from *Acinetobacter* ATCC 31012 and patented by Gutnick *et al.* (1980) is the only bioemulsifier till date which is produced industrially and used for cleaning of oil contaminated containers, recovery of oil from oil wells and removal of oil spills. Bioemulsifier have an edge over chemical surfactants because they may be produced on renewable substrates, they may be modified to meet specific requirements, they are effective at higher temperatures, pH and salinity and, most important, because they are biodegradable.

Microbial emulsifiers have been extensively reviewed (Desai, 1987; Fiechter, 1992; Desai *et al.*, 1994; Desai and Banat, 1997). Haferburg *et al.* (1986) divided microorganisms into 3 groups depending on the nature of carbon source used by them for the production of emulsifier. The organisms which produce emulsifier exclusively during growth on alkanes, the organisms which produce emulsifier on both alkanes and water-soluble carbon sources and the organisms which produce emulsifier exclusively during growth on water soluble compounds. *Corynebacterium* (Cooper *et al.*, 1978), *Rhodococcus* (Rapp *et al.*, 1979), *Candida* (Pareilleux, 1979) are representative of the first group. *Pseudomonas* producing rhamnolopid using glycerol or hexadecane (Hisatsuka *et al.*, 1977) and *Torulopsis* which produce sophorose lipid when grown on

either glucose or alkane (Tulloch *et al.*, 1968) are representative of the second group. *Arthrobacter* which produces trehalose lipid using sucrose and fructose (Itoh and Suzuki, 1974) and *Rhodotorula* producing pentitolesters of β-D hydroxypalimitic acid and β-D hydroxystearic acid when grown on glucose (Tulloch and Spencer, 1964) are representative of the third group.

Some part of the fatty acids synthesized by oxidation of hydrocarbons is incorporated into emulsifiers. However, for the synthesis of a carbohydrate moiety, other pathways have to be employed. Gluconeogenesis or reversal of glycosis reactions synthesizes polysaccharide precursors such as glucose-6-phosphate giving rise to trehalose, sophorose, rhamnose or mannose depending on the organism (Boulton and Ratledge, 1987).

Bioemulsifiers can be produced by microorganisms in the exponential phase of growth (Espuny *et al.*, 1996) or in the stationary phase (Singer and Finnerty, 1990) and also by resting cells (Gobbert *et al.*, 1984). The nature of carbon source and chain length of hydrocarbon substrate influence bioemulsifier production, *Corynebacterium hydrocarboclastus* and *Rhodococcus erythropolis* both produce surface active agents on intermediate chain length n-alkanes only (Rapp *et al.*, 1977). The nature of nitrogen source and its availability also plays an important role in emulsifier production. Zinjarde (1996) stated that *Yarrowia lipolytica* released extracellular emulsifier in the stationary phase of growth when ammonium ions were limiting in the medium. The addition of multivalent cations to the culture medium can have a positive effect on emulsifier production as observed in case of surfactin formation by *Bacillus subtilis*, where either Fe^{++} or Mn^{++} salts stimulated production (Cooper *et al.*, 1981). On the other hand, limitation of Fe^{++}, Mg^{++} or Ca^{++} caused overproduction of rhamnolipids by *Pseudomonas* sp. DSM 2874 (Guerra-Santos *et al.*, 1986). Whether N-limitation or limitation of multivalent cations affects the biochemical pathway of production of emulsifier directly or indirectly by change of cell physiology is not clear, however pH, temperature, availability of oxygen are definitely important in emulsifier production (Syldatk and Wagner, 1987). Temperature dependent yield of bioemulsifier has been reported for *Arthrobacter paraffineus* ATCC 19558. Increased rate of agitation as well as oxygenation also increased the surfactant production for the same organism (Duvnjak *et*

al., 1982) whereas sophorose lipid formation by *Torulopsis bombicola* was both pH and temperature dependent (Gobbert *et al*., 1984).

Hydrocarbon degrading and emulsifier producing organisms have been reported from polluted soils and waters. Chronic addition of hydrocarbons to an ecosystem results in selective increase in the degrader population. Mulkins-Philip and Stewert (1974) demonstrated the presence of hydrocarbon utilizers in coastal sediments and water from the North Atlantic and showed that the total heterotrophic population of hydrocarbon degraders ranged from 10–100%. Buckley *et al*. (1976) analyzed samples from estuarine waters of North Carolina and observed that at all but 2 sites, the majority of the species were able to grow on hydrocarbons whereas Leahy *et al*. (1990) observed that lack of enrichment of microbial communities on hydrocarbon resulted in poor degradation of hydrocarbons. Austin *et al*. (1977) identified total of 99 petroleum degrading bacteria belonging to 14 phenetic groups like *Nocardia, Pseudomonas, Micrococcus, Klebsiella* in samples from oil polluted regions of Chesapeake Bay. They observed that after *Pseudomonas*, actinomycetes were the dominant hydrocarbon-degrading group. Buhler and Schindler (1984) and Atlas (1981) have reported oil-degrading organisms. Radwan and Sorkhoh (1993) reviewed the role of n-alkane utilizing microorganisms which include **bacteria** *Vibrio, Flavobacter, Bacillus, Micrococcus, Pseudomonas*, **yeasts** *Candida*, *Torulopsis*, *Rhodotorula, Hansenula, Saccharomyces*, **fungi** *Penicillium, Aspergillus, Cladosporium, Fusarium, Mucor* and **algae** *Nostoc*, *Chlorella, Dunaliella, Anabaena, Oscillatoria*.

Actinomycetes are important oil degraders and have been isolated from oil wells, sedimentation ponds, storage tanks and soil and water polluted with hydrocarbons (Jones and Edington, 1968). *Rhodococcus, Mycobacteria, Nocardia, Corynebacterium, Streptomyces, Micromonospora* are known to degrade different fractions of crude oil (Lacey, 1988). Among the actinomycetes, the capacity of rhodococci to degrade various hydrocarbons suggests that they may play a significant role in the natural degradation of these compounds. Hydrocarbon degrading rhodococci have been isolated by Koronelli *et al*. (1988). *R. erythropolis* VKM AC-1339 D degraded hydrocarbons at 10°C (Yagafarova and Skvortsova, 1996). Warhurst and Fewson (1994) reviewed the biotransformation capabilities of rhodococci. The presence of aliphatic chains of mycolic

acids in the cell wall makes the *Rhodococcus* cells hydrophobic which may allow degradation of hydrophobic pollutants by allowing cells to adhere to oil/water interfaces (Neu, 1996).

Rhodococci can degrade hydrocarbons ranging from simple short chain alkanes to more complex herbicides. Acetylene (Rosner *et al.*, 1997) and propane degradation (Ashraf *et al.*, 1994) by rhodococci have been reported. *Rhodococcus rhodochrous* KUCC 8801 isolated from crude oil polluted soils and sea in Kuwait was a very effective degrader of C_{12} to C_{20} n-alkanes (Sorkhoh *et al.*, 1990). Degradation of variety of isoprenoid hydrocarbons such as pristane, 1-pristene, farnesane and hexadecanene by *Rhodococcus* sp. BPM 1613 has been discussed by Nakajima *et al.* (1985 a, b). Chlorinated phenols, which are recalcitrant to degradation and can be hazardous in soil and groundwater, have been reported to be degraded by rhodococci (Haggblom *et al.*, 1989). Degradation of various polychlorinated biphenyls by *R. erythropolis* and other unclassified strains of rhodococci is known (Maeda *et al.*, 1995; Seto *et al.*, 1995). Degradation of thiocarbamate herbicides and organophosphorous insecticides (Bekhi, 1994), sulphonated azo dyes (Heiss *et al.*, 1992), herbicides Atrazine, Propazine and Simazine (Bekhi and Khan, 1994) and triazine herbicide Metamitron (Parekh *et al.*, 1994) by strains of *Rhodococcus* is also reported. Rhodococci have also been used in the bioaccumulation of heavy metal ions. *R. erythropolis* CS98 and *Rhodococcus* sp. CS402 capable of accumulating high levels of cesium ions have been reported by Tomioka *et al.* (1994). Finnerty (1992) and Bell *et al.* (1998) reviewed the patents concerning *Rhodococcus* species useful in industrial syntheses.

Kilbane and Jackowski (1992) and Kayser *et al.* (1993) showed that *R. rhodochrous* IGTS8 utilized dibenzothiophene (considered to be a representative of a portion of an organic sulfur in coal) as a sole source of sulfur but not as a carbon source. Thus only C–S bonds are cleaved and C–C bonds remain intact, the calorific value of coal and petroleum is unaffected and sulfur emissions from combustion are reduced. Patents have also been filed for desulfurization of dibenzothiophene by *Rhodococcus* species (Grossman *et al.*, 1997; Gray *et al.*, 1998).

Hydrocarbon degrading bacteria have been reported from tropical soils (Lal and Khanna, 1996). Open ocean regions of Bay of Bengal (Gupta and Sankarnarayanan,

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1996) and the Arabian Sea (Zinjarde, 1996) are highly polluted with crude oil. Several isolates including yeasts have been obtained from these areas. Transport of petroleum products, refinery work and industrialization of the nearby area has resulted in crude oil pollution of this coastal region. In the interior regions, large freshwater lakes are used for cleaning of heavy vehicles and also as recreational spots contributing to the pollution of these sites. The aim of the present thesis is to study microbial hydrocarbon degradation from this region. Samples were collected from oil polluted coastal regions near Mumbai (18°55' N, 72°45'E) as well as from freshwater habitats in order to study the effect of contamination of crude oil on microbial population.

In the present thesis Chapter 2 deals with enrichment, isolation and identification of the best degrader on the basis of morphology, cell wall analysis, biochemical tests, fatty acid methyl ester pattern and G+C content of DNA. The organism has been classified to the genus *Rhodococcus*.

Chapter 3 deals with optimization of medium conditions for crude oil degradation by this organism. Degradation of n-alkanes, kerosene and pristane, identification of fermentation end products, cellular fatty acid analysis of *Rhodococcus* sp. grown on various hydrocarbons, comparison of growth of organism on different carbon sources was studied. Preparation and use of paraffinized fertilizers, use of encapsulated cells for crude oil degradation was carried out. Requirement of salts for crude oil degradation and growth of *Rhodococcus* sp. and its sulfur requirement was also studied.

Chapter 4 presents optimization of conditions for emulsifier production by *Rhodococcus* sp. Isolation, chemical and physical characterization of the emulsifier was carried out. Relationship between surface tension reduction and Critical Micelle Concentration was studied. Role of pesudosolubilization of hydrocarbons in this organism has also been discussed.

Chapter 5 is a general discussion of hydrocarbon degradation and emulsifier production with respect to actinomycetes. The recent trends in the classification of actinomycetes with reference particularly to *Rhodococcus* have also been discussed.

CHAPTER 2

Screening, Isolation and Identification of the Crude Oil Degrading Organism

SUMMARY

Enrichment of environmental samples on 1% (w/v) Assam crude oil resulted in six bacteria and yeasts degrading more than 10% of crude oil. The best degrader showing 30% degradation by gravimetry was selected for further studies. In terms of both Total Viable Count and oil determined as chloroform-soluble material showed that coastal areas were more contaminated than fresh water habitats.

Fatty acid methyl ester pattern of the whole cells of this orange coloured organism suggested that it was a nocardioform actinomycete. This was confirmed by the presence of *meso*-diaminopimelic acid, arabinose and galactose (cell wall chemotype IV) and mycolic acid in the cell wall of this organism. These results and biochemical tests compared with standard cultures, *Rhodococcus rhodochrous* ATCC 13808 and *Nocardia asteroides* ATCC 19247 helped to assign the isolate to the genus *Rhodococcus*. Further testing showed that our isolate was closest to *R. terrae*. The organism has been deposited in the National Collection of Industrial Microorganisms (NCIM) at National Chemical Laboratory (NCL) as *Rhodococcus* sp. NCIM 5126.

INTRODUCTION

Actinomycetes are Gram positive bacteria characterized by the formation of branching filaments which fragment into pleomorphic rodlike or coccoid elements (Waksman, 1959). They occupy a place between true bacteria and fungi. In 1875, Ferdinand Cohn published the first description of an actinomycete when he observed a filamentous organism in a concretion from a human lacrimal duct and named it *Streptothrix foersteri*. In 1877, C.O. Harz gave the name *Actinomyces bovis* to an organism isolated from the case of a bovine lumpy jaw. The word 'actinomyces', derived from Greek, means 'ray-fungus'. Actinomycetes may be separated into two large but unequal groups: the fermentative forms and the oxidative forms. The fermentative actinomycetes are simple in their morphology having neither aerial mycelia nor spores. They are usually found in soils or in the natural cavities of man and animals. The majority of actinomycetes are oxidative and soil inhabiting (Lechevalier and Lechevalier, 1981).

Actinomycete identification requires not only morphology but also cell wall analysis. Important morphological features include mycelia, which may be stable or fugacious, conidia, sporangia, sclerotia and synnemata (Lechevalier, 1989 a). Diaminopimelic acid (DAP) and whole cell sugar pattern (WCSP) are used to classify actinomycetes. Lechevalier and Lechevalier (1970) and Lechevalier *et al.* (1971) classified actinomycetes on the basis of chemical composition (Table 2.1).

Table 2.1 Major constituents of cell walls of actinomycetes (Lechevalier *et al.*, 1971)

Cell wall Type	Major amino acids	Sugar	Organism
I	L-DAP, glycine	none	*Streptomyces*
II	*meso*-DAP, glycine	xyl+ara (WCSP D)	*Actinoplanes*
III	*meso*-DAP	madurose (WCSP B)	*Microbispora*
III	*meso*-DAP	none (WCSP C)	*Nocardiopsis*
IV	*meso*-DAP	ara+gal (WCSP A)	*Nocardia*
–	lysine, aspartic acid	galactose	*Oerskovia*
–	DAB, glycine	none	*Agromyces*
–	*meso*-DAP etc.	none	*Mycoplana*

DAB–Diaminobutyric acid. All cellwall preparations contain major amounts of alanine, glutamic acid, glucosamine and muramic acid.

Actinomycetes have higher mol% G+C than other bacteria (60–75), with the only exception being that of DNA of thermophilic actinomycetes which has a mol% G+C of 44–54 (Lechevalier *et al.*, 1971).

Among the 8 groups of actinomycetes, 'Nocardioform' signifies actinomycetes that form a fugacious mycelium breaking up into rod shaped or coccoid elements (Lechevalier, 1989 b). Whole cell hydrolysates of this group show the presence of *meso*-DAP, arabinose and galactose as major sugars (cell wall chemotype IV, WCSP A). The peptidoglycan consists of *N*-acetylglucosamine, L-alanine, D-alanine, D-glutamic acid, *N*-glycolyl muramic acid (Goodfellow and Lechevalier, 1989; Goodfellow, 1989 b). The cell wall also shows the presence of major amounts of straight chain, unsaturated, 10-methyl tuberculostearic fatty acids with 12–18 carbon numbers (Lechevalier *et al.*, 1973; Collins *et al.*, 1982 b) and mycolic acids which are α-hydroxy acids having a long alkyl branch in β-position. They form three distinct groups depending upon the carbon number they possess, corynemycolic acids (22–38 carbons), nocardomycolic acids (34–60 carbons) and mycolic acids *sensu stricto* which have 60–90 carbons (Alshamaony *et al.*, 1976; Collins *et al.*, 1982 a). Nocardomycolic acids are present in nocardioform actinomycetes. Tetrahydroxymenaquinones with 8 or dihydroxymenaquinones with 9 isoprene units, (MK–8 (H_4), MK–9 (H_2), are also used as markers for identification (Collins *et al.*, 1977; Goodfellow and Minnikin, 1977; Collins and Jones, 1981) as well as phosphatidylglycerol, phosphatidylethanolamine, phosphatidylinositol and phosphatidylinositol mannosides (Minnikin *et al.*, 1977; Goodfellow and Minnikin, 1977).

Nocardioform actionomycetes are Gram positive to Gram variable, mesophilic, chemoorganotrophic, oxidase negative, catalase positive, oxidative organisms. *Nocardia*, *Corynebacterium*, *Mycobacterium*, *Dietzia* and *Rhodococcus* are the representative genera.

Rhodococci are aerobic, Gram positive, non-motile, non-sporing, mycolate-containing actinomycetes widely distributed in nature and have frequently been isolated from soil, fresh water and marine habitats as well from gut contents of blood sucking arthropods with which they may form mutualistic associations (Goodfellow and

Williams, 1983; Goodfellow, 1989 b). *Rhodococcus coprophilus* grows on herbivore dung (Rowbotham and Cross, 1977) whereas *R. bronchialis* is associated with sputa of patients with pulmonary tuberculosis and bronchiectasis (Tsukamura, 1971). *R. fascians* causes leaf gall in plants and fasciation in sweet peas and *R. marinonascens* has been isolated from marine sediments. *R. equi* is an important equine pathogen that can infect other domestic animals as well as human patients compromised by immunosuppressive drug therapy (Goodfellow, 1989 b). Recently, together with other nocardioforms, rhodococci have also been isolated from surface foams in activated sludge wastewater treatment plants (Schuppler *et al.*, 1995).

Rhodococci possess all the characteristics of nocardioform actinomycetes. The cocci may merely germinate into short rods or show elementary branching or extensively branched hyphae (Locci and Sharples, 1984). The colonies may be rough, smooth or mucoid and pigmented buff, cream, yellow, orange, pink or red although colourless variants do occur. They are unable to degrade casein, cellulose, chitin, or xylan but are able to use wide range of organic compounds as sole sources of carbon (Goodfellow and Minnikin, 1981).

Rhodococci have a long and confused taxonomic pedigree. Bisset and Moore (1950) isolated a new actinomycete and suggested a name *Jensenia* for it, whereas in 1971, Tsukamura proposed a new genus *Gordona*, for slightly acid fast organisms isolated from sputa of patients with pulmonary diseases. Later Gordon and Mihm (1957) reintroduced the epithet '*rhodochrous*' clubbing together various actinomycetes that shared properties with both *Mycobacterium* and *Nocardia*. On subsequent studies, after classifying their strain as *Mycobacterium rhodochrous*, they showed that the taxon was heterogeneous and could be distinguished from both *Mycobacterium* and *Nocardia*. On the basis of morphology and biochemical characters, Goodfellow and Alderson (1977) studied 3 clusters of organisms where cluster 1 comprised of organisms belonging to '*rhodochrous*' complex and gordonae, cluster 2 contained mycobacteria and cluster 3 belonged to nocardiae. They resurrected the genus *Rhodococcus* that reduced *Gordona* and *Jensenia* to the synonyms of *Rhodococcus*. However the use of molecular and genetic analyses has resulted in the reclassification of genera and species (Bell *et al.*, 1998). Ochi (1992) has separated *Gordona* and *Tsukamurella* from *Rhodococcus* on the

basis of electrophoretic heterogeneity of ribosomal protein AT–L30. Data from the partial sequencing of 16S RNA have placed *Rhodococcus* beside *Nocardia* and *Mycobacterium* among the nocardioform actinomycetes (Goodfellow, 1989 a). The work on reclassification, especially of nocardioform actinomycetes is not complete and recent developments in taxonomy make use of comparison of rRNA cistrons by RNA homology experiments and by 16S oligonucleotide similarities to create a unifying phylogenetic concept of these bacteria. However, we have used morphology, biochemical tests and cell wall analysis for the identification of an actinomycete isolated from coastal regions of the Arabian Sea near Mumbai.

The present chapter deals with isolation and identification of the isolate to the genus *Rhodococcus*.

MATERIALS AND METHODS

MATERIALS

Diaminopimelic acid, lysozyme, RNase, ethidium bromide and spraying reagents used in thin layer chromatography were purchased from Sigma Chemicals Co. (USA), Glutaraldehyde was from Aldrich Chemicals Co. (USA). Sugars were purchased from Hi Media (India). All reagents were analytical grade. Assam crude oil was obtained from Oil and Natural Gas Commission (ONGC), India.

METHODS

Sampling, enrichment and screening

For the isolation of crude oil degrading organisms scrapings, mud, decaying material and water were collected from the oil polluted coastal regions of Vashi and Panvel near Mumbai as well as from Pashan Lake and Khadakwasla Dam in Pune district. 1 ml of each sample was serially diluted and 100 μl of 10^{-4}, 10^{-5} and 10^{-6} dilutions were used to plate on nutrient agar for total viable count (TVC). For selective isolation of actinomycetes, 1 ml of all the samples was incubated in 1:150 dilution of phenol for 20 minutes. 100 μl aliquot of this material was used for TVC. Marine samples were plated on quarter strength seawater nutrient agar (0.125% peptone, 0.075% yeast extract, 2.5% agar and pH 8.0) and fresh water samples were plated on quarter strength nutrient agar prepared in distilled water (0.25% peptone, 0.075% yeast extract, 0.125% NaCl, 2.5% agar and pH 7.5). The Aquil® Artificial Sea Water had the following composition (g per litre deionized water): NaCl, 24.53; $CaCl_2$, $2H_2O$, 1.54; KBr, 0.1; NaF, 0.003; KCl, 0.7; H_3BO_3, 0.003; Na_2SO_4, 4.09; $NaHCO_3$, 0.2; $SrCl_2$, 0.017; $MgCl_2$, $6H_2O$, 11.1. All the plates were incubated at 30°C. Percentage of chloroform soluble material, which includes crude oil present in the environmental sample, was determined on 5 ml material, extracted with chloroform and dried to constant weight. Final values were expressed as mg % of chloroform soluble material present in the sample.

Artificial Sea Water supplemented with 0.5% $(NH_4)_2SO_4$ and 0.002% K_2HPO_4 at pH 8.0 with 1% (w/v) Assam crude oil was used for the enrichment. Marine samples

were inoculated into 50 ml medium in 250 ml conical flasks and incubated at 30°C at 150 rpm for 5 days. Resulting consortia were transferred twice into fresh media to adapt the cultures to crude oil. Organisms were streaked onto seawater nutrient agar plates and were incubated at 30°C. Colonies isolated from these plates were maintained on seawater nutrient agar slants and transferred at regular intervals. Preinocula of each isolate were developed in Artificial Sea Water nutrient broth and approximately 10^8 cells per ml were inoculated into the same medium containing 1% (w/v) Assam crude oil. Uninoculated flask with 1% (w/v) crude oil served as a control. All the flasks were incubated at 30°C at 150 rpm for 5 days. In these preliminary experiments, residual crude oil was extracted in chloroform and percent degradation of crude oil was determined gravimetrically with reference to an uninoculated control.

The best degrader showing the maximal degradation of crude oil was selected for further studies. For preliminary identification, Gram nature and shape of the cells of this organism were observed under oil immersion on a Leica model Leitz microscope at a magnification of 1000x. Biochemical tests were done in accordance with *Bergey's Manual of Systematic Bacteriology* (1989).

Fatty acid methyl ester pattern for the identification

The fatty acid methyl ester patterns of whole cells extracted in NaOH and Methanol were determined on a Microbial Identification System (MIS, Microbial Identification Inc., Newark, DE, USA) consisting of a Hewlett Packard Gas Chromatograph 5890, fitted with a 25 mm x 0.2 mm HP–Ultra 2 capillary column with a 0.33 μm film of cross linked 5% phenyl silicone using hydrogen as carrier gas and an FID detector programmed at 5°C per minute from 170–270°C and a 2 minute constant temperature at 270°C. The software in the MIS compared the patterns so extracted with standard ATCC cultures. The isolate was tentatively identified as an actinomycete.

Genus level identification of the isolate

Diaminopimelic acid was extracted by hydrolyzing dried cells in 6 N HCl and the material was chromatographed on Whatman No.1 paper using methanol : water : pyridine : 10 N HCl (80:17.5:10:2.5 v/v). Spots were visualized and compared with

diaminopimelic acid with 0.2% acetonic ninhydrin (Becker *et al.*, 1964). Sugars were extracted by hydrolysis of dried cells in 2 N H_2SO_4 and were chromatographed on Whatman No.1 paper using butanol : pyridine : water (60:40:10 v/v). The spots were detected by 0.3% phthalic acid and 0.4% *p*-anisidine in ethyl alcohol (Becker *et al.*, 1965; Yamaguchi, 1965). Mycolic acids were extracted from dried cells using methanol (5 ml), toluene (5 ml) and concentrated sulfuric acid (0.2 ml) at 50°C for 16 hr. The mixture was shaken with 2 ml hexane and chromatographed on pre-coated thin-layer Silica G plates using petroleum ether (b.p. 60–80°C) : diethyl ether (85 : 15 v/v). The spots were visualized by charring at 150–200°C after spraying with chromic acid solution (Minnikin *et al.*, 1975). Data obtained was compared to that from *Rhodococcus rhodochrous* ATCC 13808 and *Nocardia asteroides* ATCC 19247, purchased from the Institute of Microbial Technology (IMTECH), Chandigarh.

Biochemical tests were carried out in accordance with Goodfellow and Alderson (1977) and Goodfellow (1989 b). Colony morphology, oxidative and fermentative mode of carbohydrate utilization, growth on ethanol, starch, casein, xylan, cellulose, chitin, gelatin, glucose, fructose, mannose, xylose, sucrose, trehalose, cellobiose, rhamnose, arabinose, adonitol, dulcitol, glycerol and inulin were determined. Assimilation of sugars was checked using minimal agar medium, prepared in distilled water, supplemented with 0.002% K_2HPO_4 0.05% urea, 0.5% NaCl and 1% (w/v) test sugar as sole source of carbon.

Determination of mol% G+C (T_m)

To determine the T_m and mol % G+C content of the isolate, DNA was extracted by the method of Mordarski *et al.* (1976). Exponentially grown cells in nutrient broth were harvested and washed in saline EDTA (0.015 M NaCl, 0.1 M EDTA, pH 8.0). The washed cells were suspended in Buffer A (0.015 M Tris-HCl, pH 8.0; 0.45 M Sucrose and 0.008 M EDTA) containing 1 mg/ml lysozyme and incubated at 37°C for 18 hr. SDS at a final concentration of 2% was added and incubated at 60°C for 30 min. The cell debris was separated by centrifugation and protein was removed using Tris-saturated phenol and chloroform : isoamyl alcohol (24:1 v/v). DNA was precipitated with 0.15 M NaCl and 2 vol. of chilled ethanol, washed with 70% ethanol, dried and re-dissolved in Tris-EDTA ($T_{10}E_1$ – 10 mM Tris-HCl and 1mM EDTA, pH 8.0) buffer. Before treating

the samples with 50 μg/ml pancreatic RNase at 37°C for 1 hr to digest RNA, RNase was boiled for 15 min to inactivate contaminating DNase. DNA was re-precipitated and quality of the preparation was checked on 0.8% agarose gel stained with ethidium bromide.

DNA was dialyzed twice in 500 ml 0.1 x standard saline citrate (SSC, 1x SSC–0.15 M NaCl, 0.015 M tri sodium citrate, pH 8.0). DNA with a A_{260}/A_{280} ratio of 1.8–2.0 was used for T_m. Increase in absorbance at 260 nm was recorded in a Beckman DU–8B spectrophotometer with a temperature programming of 1 degree rise for ten minutes from 25°C to 102°C, to determine the T_m of the sample (Marmur and Doty, 1962). The ratio of A_t/A_{25} was plotted versus the temperature. The midpoint of the curve was used to calculate mol% G+C content, using the formula of Mandel and Marmur (1968),

$$G+C = 2.44\ (T_m - 53.9).$$

Species level identification of the isolate

Morphological features of the isolate were studied in comparison to *Rhodococcus rhodochrous* ATCC 13808 using Leica Stereoscan 440 model of scanning electron microscope, manufactured by M/s Leica Cambridge Ltd. UK. For this, slide cultures were developed and after 18 hr growth, the cells were fixed in glutaraldehyde (2% v/v) for further 18 hr and serially dehydrated by ethanol (10–95% v/v). The samples were mounted on specimen-mounting stubs by using conducting silver paste and then coated with a thin layer of gold in Polaron coating unit E5000 to prevent the charging of the specimens.

For species level identification, morphological features of the organism as well as biochemical characters were considered. Growth was determined on sodium citrate, sodium benzoate, sodium malate, sodium lactate, sodium pyruvate as a sole carbon source, at various temperatures, in presence of phenol, crystal violet, sodium azide and 7% NaCl (Goodfellow, 1989 b). Glucose yeast extract agar (Glucose, 0.5%, meat extract, 0.5%, peptone, 0.5%, yeast extract, 0.5%, agar, 2%) and Sauton's agar (L-asparagine, 0.4%, citric acid, 0.2%, K_2HPO_4, 0.05%, $MgSO_4$, 0.05%, Ferric ammonium citrate, 0.005%, glycerol, 4 ml, agar, 2%) were used to observe the colour of the organism.

RESULTS AND DISCUSSION

All the sample sites showed the presence of various bacteria, yeasts and actinomycetes. Samples treated with phenol showed overall low counts of organisms and powdery, sporulating colonies were considered as actinomycetes. Large numbers of red-orange colonies resembling *Rhodococcus* were also present. As seen from Table 2.2, among the sites, coastal Vashi had 56 mg % crude oil and higher TVC as compared to coastal Panvel, freshwater Khadakwasla and Pashan. It also showed greater number of actinomycete colonies and about 17% were *Rhodococcus*-like. There was a direct relationship between the amount of chloroform soluble organic material and the total number of cells present.

Table 2.2 Population data of samples collected from different regions

Sample collection site	Total number of viable cells (per ml sample)	Actinomycetes (per ml sample)	*Rhodococcus*-like colonies (per ml sample)	Chloroform soluble material (mg %)
Coastal Vashi	14×10^6	35,000	6,000	56
Coastal Panvel	4×10^6	27,500	2,260	40
Freshwater Khadakwasla	13×10^5	26,200	800	32
Freshwater Pashan	12×10^5	5,000	600	28

Both marine and freshwater sites are contaminated. The Arabian Sea transports 54% of the total crude oil produced in the Gulf, and the interior freshwater lakes are oil contaminated because they are used for water sports. The Total Viable Count reflects the excess carbon available for growth. In the different ecosystems, oil-degrading flora, represented by the actinomycete populations decrease in direct ratio to chloroform soluble material as does the occurrence of red, orange colonies presumed to be representative of *Rhodococcus*-like organisms (Table 2.2). Actinomycetes were predominant in samples from crude oil polluted regions of Chesapeake Bay tested by Austin *et al.* (1977). In Indian waters, Gupta and Sankarnaraynan (1996) as well as Zinjarde (1996) had isolated mainly yeasts from oil polluted ecosystems. The present is a first report on the occurrence of actinomycetes.

10 isolates were obtained which degraded crude oil. Out of these, 6 showed degradation of 10% or more. Of these organisms, isolate 3 (Table 2.3) showed 30% degradation of Assam crude oil in 5 days as determined by gravimetry. This isolate was chosen for further study.

Table 2.3 Oil degrading bacteria and yeasts from the marine environment

Isolate Sr.No.	% degradation of crude oil (Gravimetric analysis)
Bacteria	
1	10
2	12
3	30
4	20
5	14
Yeast	
6	12

Medium: Artificial Sea Water supplemented with 0.5% $(NH_4)_2SO_4$, 0.001% K_2HPO_4, 1% (w/v) Assam crude oil, pH 8.0. Flasks were incubated at 30°C for 5 days at 150 rpm.

The organism produced orange coloured, convex colony with entire margin. It was Gram positive, non-motile, oxidase negative, catalase positive with a coccoid shape. Qualitative analysis of cellular fatty acids on a Microbial Identification System showed saturated and unsaturated fatty acids and tuberculostearic acid suggesting that the isolate was a nocardioform actinomycete (Goodfellow and Lechevalier, 1989; Goodfellow, 1989 b).

Genus level identification of the isolate

Paper chromatography of the whole cell hydrolysate of this organism showed only *meso*-DAP, placing it in the *Nocardia* group of organisms (Becker *et al.*, 1964). Both whole cell hydrolysate and cell wall preparations showed the presence of arabinose and galactose (Becker *et al.*, 1965; Yamaguchi, 1965). In accordance with the classification by Lechevalier and Lechevalier (1970), presence of *meso*-DAP and arabinose and galactose are characteristics of the nocardioform actinomycete group. Mycolic acid was also present in the whole cell hydrolysate of this isolate. The presence of *meso*-DAP (Fig.2.1), arabinose and galactose (Fig.2.2) and mycolic acid (Fig.2.3) in *Rhodococcus rhodochrous* ATCC 13808 and *Nocardia asteroides* ATCC 19247 as well as in the present isolate suggest that the unknown organism is a type IV actinomycete

Figure 2.1

Presence of *meso*-DAP in whole cell hydrolysates of

1. Isolate 3
2. *Rhodococcus rhodochrous* ATCC 13808
3. *Nocardia asteroides* ATCC 19247

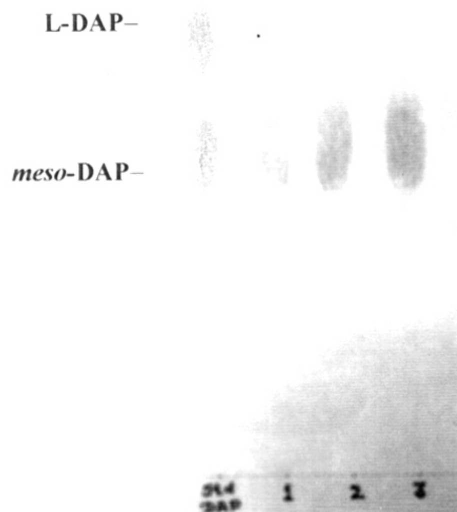


Figure 2.2

Presence of arabinose+galactose as characteristic sugars in whole cell hydrolysates of

1. Isolate 3
2. *Rhodococcus rhodochrous* ATCC 13808
3. *Nocardia asteroides* ATCC 19247

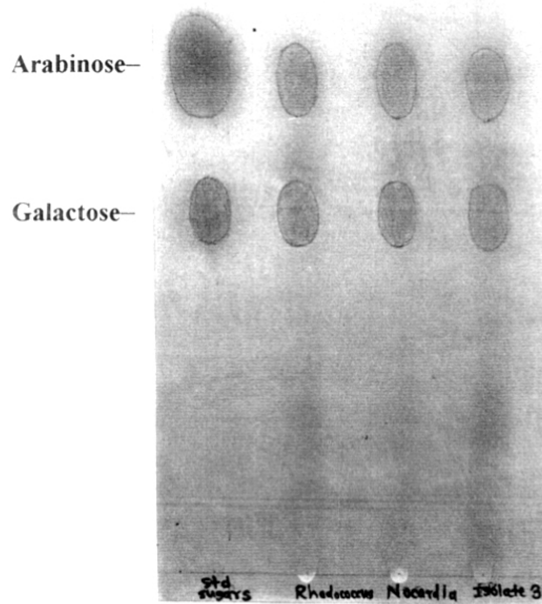
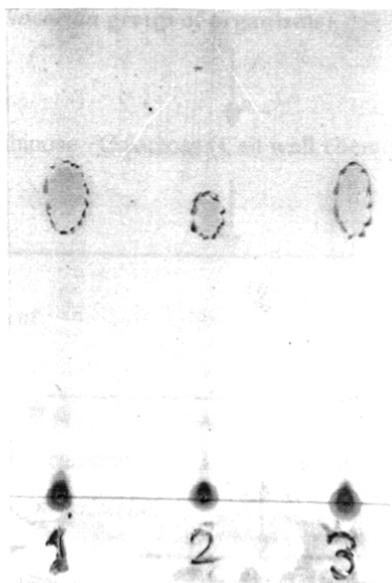


Figure 2.3

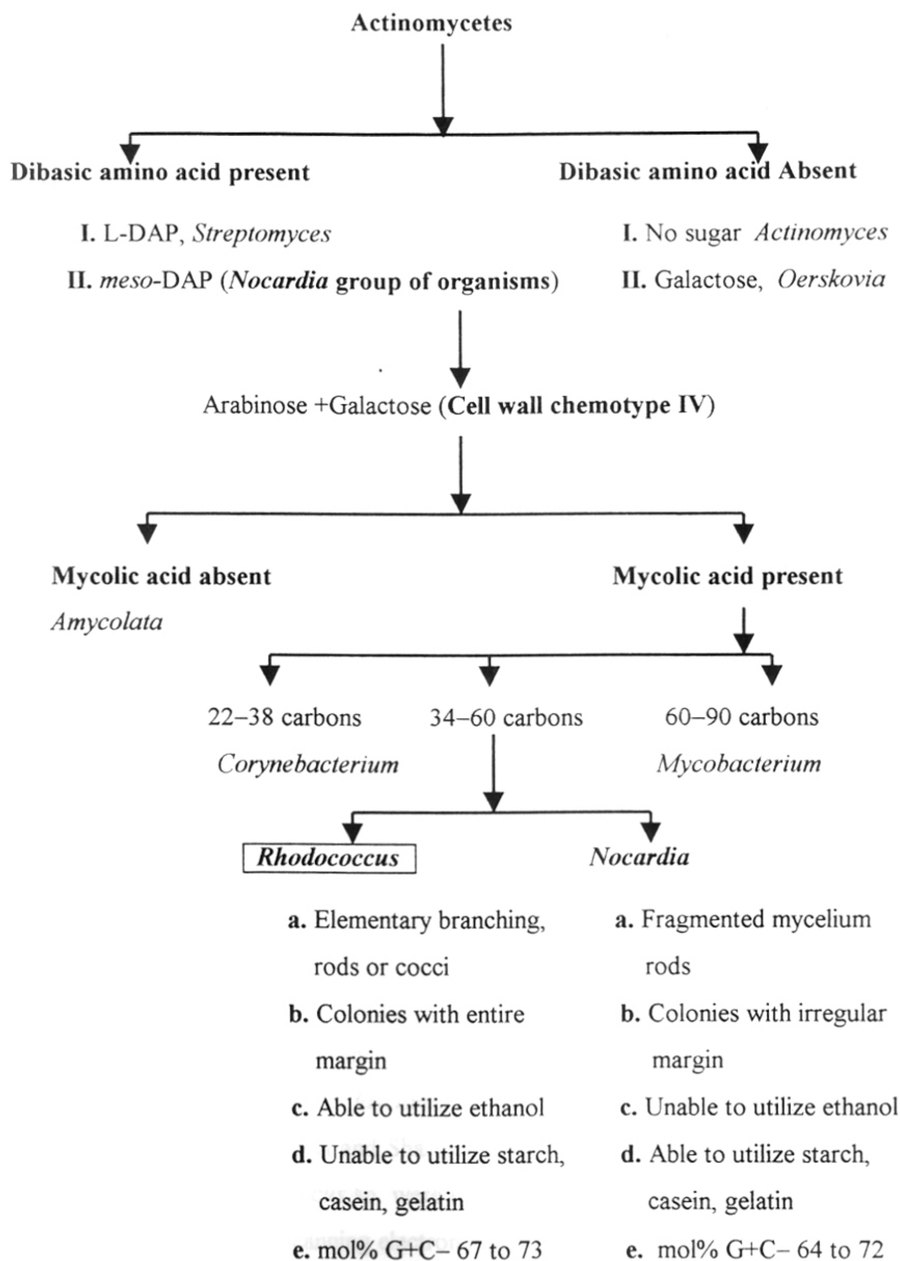
Presence of mycolic acid in the whole cell hydrolysate of

1. *Rhodococcus rhodochrous* ATCC 13808
2. Isolate 3
3. *Nocardia asteroides* ATCC 19247



belonging either to *Nocardia* or *Rhodococcus*. A key for the identification of nocardioform actinomycetes is presented.

A Key for the identification of nocardioform actinomycetes



Goodfellow and Alderson (1977) used numerical analysis on 92 characters for the identification of 177 organisms and defined three clusters at the 75 to 80% similarity levels. Organisms belonging to '*rhodochrous*' complex and gordonae were placed in cluster I whereas mycobacteria in cluster II and nocardiae in cluster III. The cluster I organisms were characterized by pink, red, orange colonies with entire margin and convex elevation. Further, about 94% of them utilized ethanol and 74% degraded *p*-hydroxybenzoic acid. As shown in Table 2.4, colony characteristics of the type species of *Rhodococcus rhodochrous* ATCC 13808 and isolate no.3 were similar. Neither utilized cellulose. The same sugars were assimilated by all three organisms with the single exception of xylose. Xylose, casein, starch, xylan and gelatin were assimilated only by *Nocardia asteroides* ATCC 19247. Ethanol was utilized as sole carbon source by *Rhodococcus rhodochrous* ATCC 13808 and isolate 3 but not by *Nocardia asteroides* ATCC 19247. On the basis of colony characteristics, cell wall and whole cell hydrolysates as well as biochemical tests, isolate 3 was identified as the genus *Rhodococcus* and is referred to as *Rhodococcus* sp. hereafter.

As shown in Fig. 2.4, the melting pattern of DNA from *Rhodococcus* sp. (T_m =82°C) was close to the value for *R. rhodochrous* ATCC 13808 (T_m=83°C). The calculated mol% G+C was 69 for *Rhodococcus* sp. as compared to 71 for *R. rhodochrous* ATCC 13808. These values are within the range for genus *Rhodococcus*, which according to Finnerty (1992) varies from 67 to 73.

Species level identification of the isolate

Morphologically, rhodococci are pleomorphic in nature. *R. bronchialis, R. chlorophenolicus, R. marris, R. rubropertinctus, R. sputi* and *R. terrae* are amycelial and exhibit rod-coccus growth cycle whereas *R. erythropolis, R. globerulus, R. rhodnii, R. rhodochrous* show elementary branching prior to fragmentation. The third group represented by *R. coprophilus, R. fascians, R. marinonascens* and *R. ruber* produce branched substrate mycelia (Locci and Sharples, 1984; Goodfellow, 1989 b). Only rod and coccoid forms of *Rhodococcus* sp. were observed in solid and liquid cultures, in all carbon sources as seen in the scanning electron microrgraphs (Fig. 2.5).

Table 2.4 Biochemical tests for genus level identification

Characters	*Rhodococcus* ATCC 13808	*Nocardia* ATCC 19247	Isolate 3
Morphology	Elementary branching-rods-cocci	Fragmented mycelium-rods	Rods-cocci
Cell wall Analysis	*meso*-DAP, ara+gal	*meso*-DAP, ara+gal	*meso*-DAP, ara+gal
Mode of utilization of carbon source	Oxidative	Oxidative	Oxidative
Colonies red, pink	+	–	+
Margin entire	+	–	+
Elevation convex	+	–	+
Catalase	+	+	+
Oxidase	+	+	+
Urease	+	–	+
Growth on sole carbon source (1%w/v)			
Ethanol	+	–	+
Casein	–	+	–
Cellulose	–	–	–
Starch	–	+	–
Xylan	–	+	–
Gelatin	–	+	–
Assimilation of sugars (1%w/v)			
Glucose	+	+	+
Fructose	+	+	+
Mannose	+	+	+
Xylose	–	+	–
Sucrose	+	+	+
Trehalose	+	+	+
Cellobiose	–	–	–
Rhamnose	–	–	–
Arabinose	–	–	–
Adonitol	–	–	–
Dulcitol	–	–	–
Glycerol	+	+	+
Inulin	+	+	+

+ Growth ; – No Growth

Figure 2.4

Melting patterns of DNA of *Rhodococcus rhodochrous* ATCC 13808 and *Rhodococcus* sp.

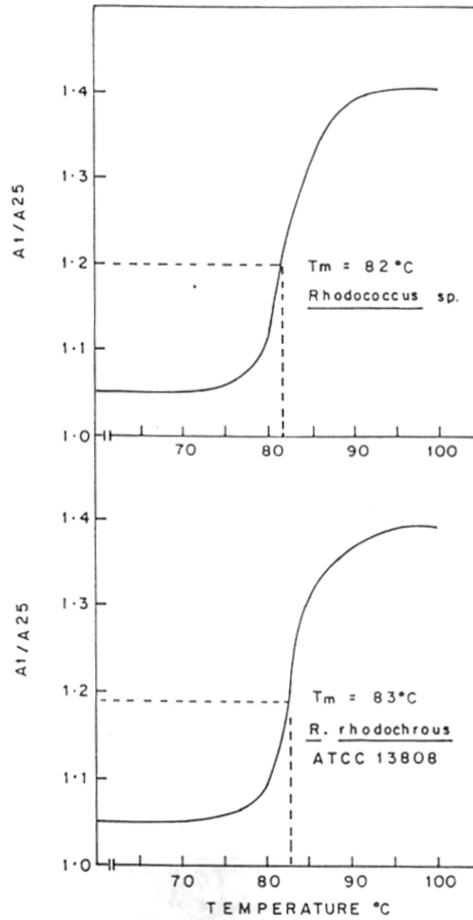
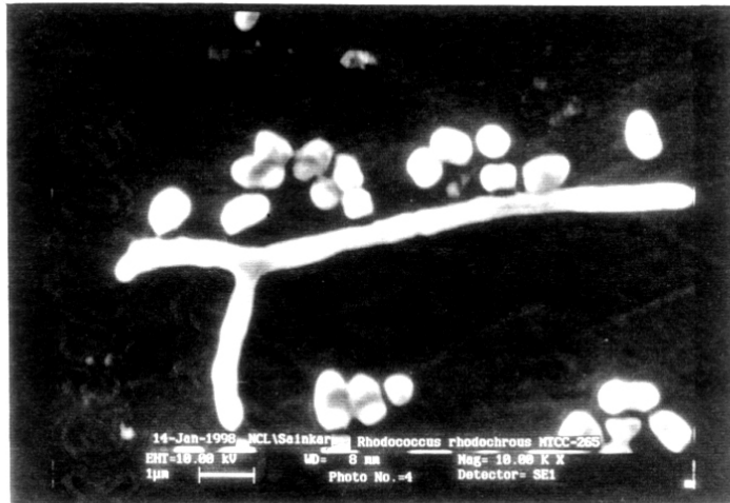


Figure 2.5

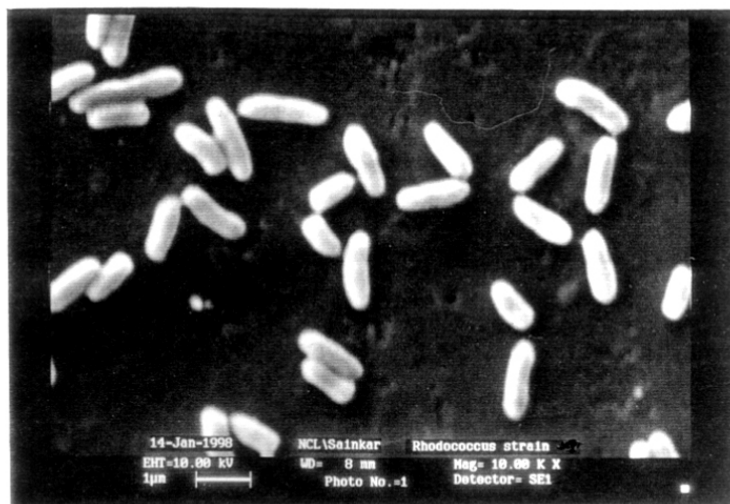
Scanning Electron Micrographs

The slide cultures of the organisms were incubated for 18 hr and treated as described in Materials and Methods.

1. *Rhodococcus rhodochrous* ATCC 13808 (10,000x)



2. *Rhodococcus* sp. (10,000x)



The biochemical test results for this organism were compared only with amycelial coccoid forms viz. *R. bronchialis, R. chlorophenolicus, R. marris, R. rubropertinctus, R. sputi* and *R. terrae*.

In terms of substrate decomposition and growth on carbon sources (Goodfellow, 1989 b) *Rhodococcus* sp. showed 17 matches out of 23 with both *R. terrae* and *R. bronchialis*, 14 matches with *R. rubropertinctus* (Table 2.5). *Rhodococcus* sp. differed from *R. terrae* in utilization of Na-benzoate, Na-citrate, *p*-hydroxybenzoic acid and inositol and ability to grow at temperatures 10 and 45°C (6 characters). It differed from *R. bronchialis* in utilization of tyrosine, Na-benzoate, Na-citrate and *p*-hydroxybenzoic acid and ability to grow at temperatures 10 and 45°C (6 characters). Our isolate differed from *R. rubropertinctus* in utilization of Na-benzoate, Na-citrate, *p*-hydroxybenzoic acid, tyrosine, inositol, Na-lactate, Na-pyruvate, and ability to grow at temperatures 10 and 45°C (9 characters). Thus, on the basis of the ability to utilize specific carbon compounds as sole carbon source, our isolate showed a closer resemblance to *R. terrae* and *R. bronchialis*. On glucose yeast extract agar and Sauton's medium, *Rhodococcus* sp. formed orange colonies. According to Goodfellow (1989b), *R. bronchialis* forms rough brownish colonies and synnamata of vertically arranged filaments on the surface of colonies after 12–18 hr incubation whereas *R. terrae* produces pink to orange colonies on both the media. Thus, *Rhodococcus* sp. showed closer resemblance to *R. terrae* on the basis of morphology and biochemical tests.

However, Stackebrandt *et al.* (1988) have transferred *R. bronchialis, R. rubropertinctus, R. sputi* and *R. terrae* back into the genus *Gordona* on the basis of dihydrogenated menaquinones with 9 isoprene units whereas genus *Rhodococcus* contains 8. Also, 16S rDNA sequencing has been used for identification of rhodococci (Stackebrandt and Goebel, 1994). DNA–DNA re-association is also a useful indication. In the absence of these data, we have preferred to retain the name *Rhodococcus* sp. to our isolate in spite of its close resemblance to *R. terrae* on the basis of morphological and biochemical tests. Isolate 3 has been deposited in National Collection of Industrial Microorganisms (NCIM) at National Chemical Laboratory (NCL) as *Rhodococcus* sp. NCIM 5126.

Table 2.5 Biochemical tests for species level identification

Characters	*R.* sp.	*R. bronc.*	*R. chlor.*	*R. marris*	*R. rubro.*	*R. sputi*	*R. terrae*
Decomposition of							
Tyrosine	+	–	ND	–	–	ND	+
Urea	+	+	ND	d	+	ND	+
Growth on sole carbon source (1% w/v)							
Inositol	+	+	+	–	–	–	–
Maltose	+	+	–	–	+	ND	+
Mannitol	+	d	+	–	+	+	+
Sorbitol	+	+	+	–	+	+	+
Growth on sole carbon source (0.1% w/v)							
p-Cresol	+	+	ND	ND	+	ND	+
m-Hydroxybenzoic acid	–	–	ND	ND	–	ND	–
p-Hydroxybenzoic acid	–	+	ND	ND	+	+	+
Na-adipate	+	+	ND	ND	+	ND	+
Na-benzoate	–	+	ND	–	+	–	+
Na-citrate	–	+	ND	d	+	+	+
Na-lactate	+	+	ND	–	–	ND	+
Na-malate	+	+	ND	+	+	+	+
Na-pyruvate	+	+	ND	+	–	+	+
Na-succinate	+	+	ND	+	+	+	+
Growth at							
10°C	+	–	–	ND	–	ND	–
40°C	+	+	ND	ND	+	ND	+
45°C	+	–	–	–	–	ND	–
Growth in presence of							
Crystal violet (0.001%)	+	+	ND	ND	+	ND	+
Phenol (0.1%)	+	+	ND	ND	+	ND	+
Na-azide (0.02%)	+	+	ND	ND	+	ND	+
NaCl (7.0%)	+	+	–	+	+	ND	+

+ Growth; – No Growth

d, 11–89% of strains positive; ND, not determined.

R. sp. –*Rhodococcus* sp; *R. bronc.* – *R. bronchialis*; *R. chlor.* – *R. chlorophenolicus*; *R. rubro.* – *R. rubropertinctus*.

The following presentation deals with crude oil and hydrocarbon degradation (chapter 3) and characterization of emulsifier (chapter 4) produced by *Rhodococcus* sp. NCIM 5126.

CHAPTER 3

Crude oil, Kerosene, n-Alkanes and Pristane Degradation By *Rhodococcus* sp. NCIM 5126

SUMMARY

Rhodococcus sp. NCIM 5126 degraded aliphatic and aromatic fractions but not the asphaltene fraction of three different crude oils. 72%, 60% and 35% of the aliphatic fraction of Bombay High crude oil, Assam crude oil and Gujarat crude oil respectively were degraded when the isolate was grown in optimized conditions viz. Artificial Sea Water supplemented with 35 mM N as urea, 0.114 mM P as K_2HPO_4, pH 8.0 at 30°C and 150 rpm for 72 hr.

The organism grew best in seawater nutrient broth with a protein specific growth rate of 0.09 hr^{-1} and a generation time of 7.7 hr. When supplied with crude oil or specific hydrocarbons, the μ decreased in consonance with the complexity of the supplied hydrocarbon and/or length of the carbon chains. The growth curves showed that as compared to growth in nutrient broth (0.09 hr^{-1}, 7.7 hr), fructose (0.031 hr^{-1}, 22 hr), n-tetradecane (0.018 hr^{-1}, 39 hr), crude oil (0.017 hr^{-1}, 41 hr), the organism was maximally stressed in the presence of pristane (0.016 hr^{-1}, 43 hr). Gas chromatographic analysis of n-alkanes showed that the organism degraded pure alkanes ranging from n-C_{14} to n-C_{18} (n-tetradecane being degraded maximum at 94%), mixed chain alkane (kerosene 92%) and the branched chain alkane pristane (30%). Cellular fatty acid methyl ester patterns of the organism grown on n-tridecane, n-tetradecane, n-hexadecane, n-heptadecane and pristane showed the predominance of the corresponding fatty acid. GC-MS analysis of the supernatant of the n-tridecane, n-tetradecane, n-hexadecane, n-heptadecane and pristane grown cells showed the presence of both odd numbered fatty acids and dicarboxylic acids indicating presence of α-oxidation and ω-oxidation pathways in addition to the usual β-oxidation pathway for alkane degradation in this organism.

The organism required 0.4 M NaCl concentration similar to that in Artificial Sea Water for growth and degradation although it tolerated upto 1.71 M NaCl, suggesting that the organism is a moderately halophilic organism. Sodium sulfate and sodium chloride were necessary both for growth and crude oil degradation. Sodium sulfate (0.07 M) and sodium chloride (0.085 M) in deionised water entirely replaced all other salts used in Artificial Sea Water. Sodium sulfate was essential for the growth of the organism in non-hydrocarbon medium also. Compared to *E. coli*, there was a higher requirement for sulfur by our organism in terms of sulfur per unit biomass.

Wax coated slow release fertilizers were prepared and their inclusion in the medium increased degradation of the aliphatic fraction of crude oil by about 8%. Cells immobilized on 2% κ-carrageenan degraded 10% more crude oil than free cells. The beads were reusable for 7 cycles of 4 days each over a period of one month.

INTRODUCTION

Arthrobacter, Streptomyces, Mycobacterium, Nocardia, Rhodococcus have been commonly isolated from oil wells and soils enriched with hydrocarbons (Jones and Edington, 1968). Jet fuel systems have yielded *Brevibacterium* and *Streptomyces* (Edmonds and Cooney, 1967) whereas *Corynebacterium, Micromonospora, Nocardia, Mycobacterium* and *Streptomyces* have been isolated from rolling mill emulsions (Genner and Hill, 1981). Lacey (1988) reported hydrocarbon degradation by actinomycetes. Of these, *Rhodococcus* is noted for its ability to degrade hydrocarbons in a variety of environmental conditions.

Rhodococci are frequently isolated from oil contaminated locations such as rivers in the Urals (Berdichevskaya, *et al.*, 1989) and soil and sea in Kuwait (Sorkhoh *et al.*, 1990). *Rhodococcus* strains were the most abundant and the most efficient in crude oil degradation among the oil degraders (Sorkhoh *et al.*, 1990; Bredholt *et al.*, 1998; Al-Hasan *et al.*, 1998). Sorkhoh *et al.* (1995) used naturally occurring bacterial consortium, removed from cyanobacterial mats found floating in oil-polluted waters to inoculate oil-contaminated sand. The consortium in which rhodococci were predominant increased the removal of the oil from the sand. Inoculation of hydrocarbon degrading strain of *R. erythropolis* into artificially contaminated soil plots resulted in increased rate of hydrocarbon degradation.

Rhodococcus rhodochrous KUCC 8801 was an effective degrader of straight chain alkanes containing 12 to 20 carbon atoms but could not degrade short-chain hydrocarbons (Sorkhoh *et al.*, 1990). However *Rhodococcus* sp. degrading n-C_{10} to n-C_{30} have also been reported (Milekhina *et al.*, 1998; Whyte *et al.*, 1998). McKenna and Kalio (1971), Cox *et al.* (1974) and Nakajima *et al.* (1974) demonstrated pristane metabolism in actinomycetes. Nakajima *et al.* (1985 a) observed that *Rhodococcus* sp. BPM 1613 metabolized pristane via both β and ω-oxidation pathways. This species of *Rhodococcus* was also able to grow on a variety of isoprenoid hydrocarbons such as 1-pristene, farnesane and hexadecanene (Nakajima *et al.*, 1985 b).

When growth of microorganisms on readily utilizable sugars is compared to growth on alkanes, a longer lag phase is observed on the latter. Usually enzymes

required for sugar assimilation are constitutive in nature whereas those for hydrocarbon assimilation are induced. In case of pure hydrocarbons, Bartha and Atlas (1977) stated that n-alkanes get readily utilized especially those between chain length C_{10} to C_{25}, on the other hand iso-alkanes are difficult to utilize as growth substrate if branching is extensive. Still, pristane is a biodegradable oil component because spacing of its 4 methyl groups at 2, 6, 10 and 14 positions is quite regular, which favours biodegradation (van der Linden, 1978). The comparison of growth of microorganism on sugars, n-alkanes or branched chain alkane easily demonstrates the stress experienced by the microorganism in terms of reduced biomass, lowered growth specific constant and increased generation time. In case of n-alkane grown yeasts, a longer lag phase was observed than glucose grown cells, however final biomass was similar in both the cases (Rodriguez and Dominiguez, 1984; Zinjarde, 1996).

When hydrocarbons are supplied as carbon source for the growth of the organisms, inorganic salts of nitrogen and phosphorus with favourable C/N and C/P ratios are necessary for maximal degradation. Apart from C, N and P microorganisms require K, Mg, Ca, and Na ions for their growth. Potassium is required by a variety of enzymes especially those involved in protein synthesis. Magnesium is necessary for the activity of many enzymes and it also stabilizes ribosomes, cell membranes and nucleic acids. Calcium helps in stabilizing bacterial cell wall. Sodium is required by some organisms and its need reflects the habitat of the organism. Seawater has all the major and minor ions required for growth and particularly high sodium content. Generally marine microorganisms require higher concentrations of sodium for growth, whereas fresh water species are usually able to grow on minimal sodium. Ward and Brock (1978) observed that rates of hydrocarbon metabolism decreased with increasing salinity in hypersaline salt evaporation ponds whereas Shiaris (1989) reported positive correlation between salinity and rate of metabolism of hydrocarbons in estuarine sediments.

Sulfur is another important nutrient required by the microbial cell. It is essential in some amino acids, polysulfated esters, some vitamins, coenzymes and is required for structural integrity of the cells. The incorporation of sulfate sulfur into thiol groups of amino acids and other compounds by microorganisms is termed as 'assimilatory reduction of sulfate'. The actual uptake of $SO_4^{=}$ takes place against a concentration

gradient and the process is temperature and energy dependent (Dreyfuss, 1964). The initial phase of sulfate uptake is described as 'sulfate binding'. Pardee *et al.* (1966) showed that cells of *Salmonella typhimurium* lose their ability to bind sulfate after they are converted to spheroplasts or osmotically shocked. They also suggested that surface location of binding might be a part of active transport system for sulfate. Sulfate-transporting system also appears to transport thiosulfate which is a potent inhibitor of sulfate transport. Siegel (1975) reviewed the regulation of sulfate assimilation in enterobacteria and yeasts. In enterobacteria *E. coli* and *S. typhimurium*, production of sulfur precursor of cysteine involves H_2S from sulfate and *O*-acetylserine from serine and acetyl-CoA. These two precursors joined to form cysteine with release of acetate. The sulfur atom of methionine is derived from cysteine, which represents a starting point for methionine biosynthesis. However, .cysteine also regulates sulfate assimilation by feedback inhibition and growth may be repressed in presence of cysteine as a sulfur source in these organisms. In yeasts, sulfate regulation is more complex than in enterobacteria. H_2S and *O*-acetylserine give cysteine which via cystathione and homocysteine produce methionine. Methionine can also get converted to cysteine. In yeasts also, methionine is a better sulfur source than either sulfate or cysteine.

Microorganisms oxidize hydrocarbons stepwise to fatty alcohols and fatty acids (Ratledge, 1978). Presence of these compounds in the culture filtrate of the hydrocarbon-grown organism is indicative of the pathway used. Organisms usually degrade n-alkane by α or β-oxidation pathway producing monocarboxylic acid or by ω-oxidation pathway producing dicarboxylic acid. Subterminal attack may also occur giving rise to shorter chain carboxylic acid (Boulton and Ratledge, 1987).

When grown in the presence of aliphatic hydrocarbon, the greatest physiological change occurs in the relative proportion of the various fatty acids found in the lipid of the microorganism. They contain more lipid than when grown on carbohydrate substrate. The increased lipid content reflects an increased cellular lipophilicity, which is required for hydrocarbon uptake and intracellular transport (Boulton and Ratledge, 1987). Cellular lipid composition of hydrocarbon utilizing bacteria (Yanagawa *et al.*, 1972 a, b), yeast (Mishina *et al.*, 1977) and fungi (Cerniglia and Perry, 1974) has been studied.

In this chapter, we deal with hydrocarbon degradation by *Rhodococcus* sp. NCIM 5126 isolated from oil polluted coastal regions of the Arabian Sea near Mumbai. The optimization of conditions for crude oil and hydrocarbon degradation as well as growth curves in presence of various carbon sources were carried out. Cellular lipid composition of *Rhodococcus* sp. grown on various hydrocarbons and degradation end products of hydrocarbons were determined. Requirement of salts, use of paraffin based fertilizers, use of immobilized cells of *Rhodococcus* sp. NCIM 5126 is discussed.

MATERIALS AND METHODS

MATERIALS

n-Alkanes (SD Fine, India), Eicosane (Aldrich Chemicals Co., USA), n-Tetradecane (Koch Light Laboratories Ltd., UK), Pristane (Sigma Chemicals Co., USA), Scintillation grade PPO and POPOP (Spectrochem, Mumbai), Scintillation grade Naphthalene and 1,4-Dioxan (SRL, Mumbai) were purchased. [$1-^{14}C$] n-hexadecane was purchased from Board of Radiation and Isotope Technology, Mumbai. All other reagents were analytical grade.

METHODS

Optimization for crude oil degradation

A preinoculum of *Rhodococcus* sp. NCIM 5126 was developed in seawater nutrient broth containing 0.5% peptone, 0.3% yeast extract, pH 8.0 at 30°C for 24 hours. Cells were centrifuged, washed and resuspended in seawater and approximately 10^8 cells per ml were inoculated in 50 ml Artificial Sea Water supplemented with nitrogen and phosphorus sources and 1% (w/v) Assam crude oil. Uninoculated flask served as a control. All the flasks were incubated at 30°C, at 150 rpm for 5 days.

Nitrogen sources, $(NH_4)_2SO_4$, NH_4NO_3, NH_4Cl, $NaNO_3$ and urea were supplied to give 70 mM nitrogen. Concentration of the best nitrogen source was varied as 17.5, 35, 70, 105 mM. K_2HPO_4 was supplied to give 0.06 mM to 0.3 mM P as a phosphorus source.

Using optimized medium, environmental conditions were varied to check their effect on crude oil degradation by the organism. Incubations were carried out at 15°C, 30°C, 37°C and 50°C. Initial pH of the medium was adjusted to 4, 6, 8, 10 and 12. Effect of aeration on degradation was determined by increasing shaker speed from 0 to 200 rpm. For all the above studies 1% Assam crude oil was used. Highest optimum concentration of crude oil was determined by varying crude oil concentration from 0.5 to 2.0% (w/v).

Time course of crude oil degradation by *Rhodococcus* sp. NCIM 5126

Growth of *Rhodococcus* sp. on crude oil in optimized medium was determined by removing aliquots for biomass estimation and crude oil degradation. Cells were washed with saline and treated with 1 N NaOH for ten minutes in boiling water bath. 0.1 ml aliquot was used to determine protein by the method of Lowry *et al.* (1951). All the experiments were done in duplicate. Final values of protein were calculated for total volume of the broth.

Total broth in the flasks was used for estimation of crude oil degradation by this organism. Residual crude oil was extracted in 200 ml chloroform and the solvent was evaporated. Residue was suspended in hexane and fractionated on Silica gel G (60–120 mesh) column, successively with 120 ml hexane, 120 ml benzene and 120 ml methanol to elute paraffin, aromatic and asphaltene fractions respectively (Atlas, 1975). Solvents were evaporated and weight of the fractions was compared with that of blank.

Hexane fraction was concentrated to 2 ml and 1μl of it was injected to Gas Chromatograph, Shimadzu GC–RIA model fitted with SE–30 column, coated with Chromosorb W. The carrier gas was nitrogen. Detection was done by flame ionization. Temperature was held at 60°C for 1 minute and programmed to 250°C at a rate of 6°C per minute where it was held for 15 minutes. Injector temperature was 250°C. Quantitation was done in accordance with Dibble and Bartha (1976) where total area under the peak, excluding the solvent peak, was considered.

Comparison of growth of *Rhodococcus* sp. NCIM 5126 on different carbon sources

Protein specific growth of *Rhodococcus* sp. NCIM 5126 was compared in individual media containing peptone, fructose, crude oil, n-tetradecane and pristane as sole carbon source. A series of preliminary experiments determined optimum concentration of these carbon sources. Using this information, preinocula of the organisms with about 10^7 cells per ml were inoculated in 100 ml media containing 0.5% peptone or 1.5% fructose or 1% crude oil or 1% n-tetradecane or 1% pristane. 1 ml samples were removed at 12 hour intervals from each flask and protein specific biomass was determined. Protein specific growth rate constant (μ) and mean generation time (g) were calculated using the formulae,

$$\mu = (\log_{10} Z - \log_{10} Z_0) / t - t_o \times 2.303$$

where Z and Z_0 represent the amount of protein at time t and t_0 and the generation time,

$$g = 0.693 / \mu$$

Degradation of various hydrocarbons by *Rhodococcus* sp. NCIM 5126

Using optimal conditions, degradation of n-alkanes, mixed chain alkane, kerosene and branched chain alkane, pristane were estimated by extracting residual hydrocarbon in test and control flasks in 200 ml chloroform and concentrating the extract to 50 ml. 1μl was injected to Gas chromatograph with isothermal programming at 150°C. Percent degradation was calculated as described earlier.

Cellular fatty acid extraction of *Rhodococcus* sp. grown on hydrocarbons

Cellular fatty acids of *Rhodococcccus* sp. grown on odd and even carbon number alkanes as well as fructose were obtained as described by Makula and Finnerty (1968). Total fatty acids were extracted by refluxing the washed cell biomass in 10 ml of 20% methanolic KOH for 1 hr. 200 ml of water was added, solution was acidified with concentrated H_2SO_4 and extracted with diethyl ether and dried over anhydrous Na_2SO_4. Fatty acid methyl esters were prepared in accordance with Zuckerberg *et al.* (1979). 3 ml methanol and 2–3 drops of concentrated H_2SO_4 were added to the extract and refluxed at 100°C for 4 hr. After cooling, 10 ml water was added and extracted with chloroform. Solvent was evaporated and samples were analyzed on a Shimadzu, GC–MS QP5000 on 30 m long SE–30 capillary column. Injector and detector were at 250°C. Initial temperature of the column was 140°C maintained for 2 minutes followed by a temperature increase of 20°C per minute and a final temperature of 250°C held for 10 minutes. The carrier gas was helium, injector mode split ratio was 70. The fragmentation pattern of each component was compared to the data stored in NIST62.lib library and was thus identified.

Degradation end products of *Rhodococcus* sp. NCIM 5126 grown on hydrocarbons

Degradation end products of *Rhodococcus* sp. grown on n-tridecane, n-tetradecane, n-hexadecane, n-heptadecane and pristane were determined on broth made

cell-free by centrifugation at 10,000 rpm and made emulsifier-free by passing through Amicon YM–10 membrane. The filtrate was made alkaline and extracted with hexane to remove residual alkane. Fatty acid methyl esters were prepared and identified as described above.

Rhodococcus sp. NCIM 5126 was also grown on radioactive n-hexadecane. Preinoculum of cells was prepared in Artificial Sea Water and 10^8 cells per ml were added in the medium containing optimal N and P sources and 73800 dpm [1–^{14}C] n-hexadecane (specific activity 0.428 μCi/ml; 0.951 x 10^6 dpm/ml). Flasks were incubated at 30°C at 150 rpm. At definite time intervals 1 ml cell suspensions were withdrawn from the flasks, filtered through 0.22 μ Millipore filters and washed with 20 ml of 50 mM phosphate buffer to remove radioactivity adhered on the surface of the cells. The filters containing cells as well as supernatant were placed separately in 20 ml scintillation vials and dried at 80°C. 10 ml cocktail mixture (10 g PPO, 0.25 g POPOP and 100 g Naphthalene per litre 1,4-Dioxan) was added to each vial and radioassayed in a LKB 1215 Rackbeta II liquid scintillation counter to quantify partitioning of radioactive n-hexadecane in cell biomass and supernatant.

Effect of salts on crude oil degradation in Artificial Sea Water medium

Specific salt requirement of the organism was tested by manipulating the salts in Aquil® Artificial Sea Water which was used as the base for medium preparation. First the salinity of Artificial Sea Water was varied from 7 ‰ (1/5th of the original) to 88 ‰ (2.5 times the original) and crude oil degradation was determined.

The NaCl concentration was varied from 0 M to 2.57 M to determine the effect on crude oil degradation. The Artificial Sea Water used was a mixture of 5 major salts in definite proportion and a further 5 as trace metals. The 5 major salts, NaCl, Na_2SO_4, $MgCl_2$, $CaCl_2$ and KCl were removed one at a time from Artificial Sea Water and effect on crude oil degradation was determined.

Effect of salts on crude oil degradation in deionised water medium

The major metal ions required for bacterial growth Na^+, K^+, Mg^{++} and Ca^{++} are also the dominant metal ions in seawater. 1% NaCl (0.17 M Na^+) was used as a

standard. Salts containing Na^+, K^+, Mg^{++} Ca^{++} were separately added to give final concentration of 0.17 M concentration of these ions in deionised water supplemented with N and P sources to determine effect on degradation of oil.

Effect of different SO_4^{--} containing salts on crude oil degradation was determined in deionised water supplemented with N and P sources. 1% Na_2SO_4 (0.07 M SO_4^{--}) was used as a standard. K_2SO_4, $MgSO_4$ and Na_2SO_4 were used separately to give final concentration of 0.07 M of SO_4^{--}.

The concentration of Na_2SO_4 was varied from 0.0175 M to 0.14 M in deionised water supplemented with N and P sources. Then keeping the optimum concentration of Na_2SO_4 constant, NaCl concentration was varied from 0.043 to 0.34 M and the effect of this variation was determined on the degradation of crude oil by *Rhodococcus* sp.

Effect of sulfur on the growth of the organisms

Effect of sulfur on growth in the form of sulfate in a fructose medium was determined and compared with that on *E. coli*. Cells were grown in seawater nutrient broth, harvested during exponential growth and washed with Artificial Sea Water lacking sulfate. The cells were resuspended in Artificial Sea Water lacking sulfate and A_{660} was adjusted to 1.0. These cells were incubated at 30°C for 2 hr to deplete the cells of intracellular sulfate. These preinocula were added separately in the media containing 1.5% fructose for *Rhodococcus* sp. and 1% glycerol for *E. coli* in Artificial Sea Water containing 0 to 0.06 M Na_2SO_4. Enough NaCl was added to maintain the final molarity of Na^+ of 0.12 M. Samples were removed periodically and biomass was determined as described earlier. Protein specific growth rate (μ) and generation time (g) were also calculated. Dry weight of *Rhodococcus* sp. and *E. coli* grown in Artificial Sea Water medium having 0 and 0.06 M sulfate was measured. These samples were analyzed for carbon and sulfur concentration on CHNS–O EA1108–Elemental analyzer, M/s Calro Erba Instruments, Italy at 1000°C using sulfanilamide as a standard.

Inorganic sulfur was replaced by 1 mM each of cysteine and methionine in order to determine the growth of *Rhodococcus* sp. NCIM 5126 on these amino acids. Cysteine is known to inhibit the uptake of sulfate. To check the effect of cysteine on sulfate uptake and consequently on growth of *Rhodococcus* sp., cells were grown in

presence of 0.015 M sulfate +1 mM cysteine, and growth was compared to with that in presence of 0.015 M sulfate and 1 mM cysteine added separately.

Effect of paraffinized fertilizers on crude oil degradation

Crude oil degradation by this organism with insoluble nitrogen was tested using paraffinized $(NH_4)_2SO_4$ and urea, both supplemented with K_2HPO_4. Paraffinized $MgNH_4PO_4$ was also used. Preparation of the paraffinized products was carried out in accordance with Olivieri *et al.* (1976) using paraffin wax (m.p. 60°C). Each preparation was supplied so as to give a final concentration of 35 mM N, the optimum concentration in the media used for shake-flask growth.

Oil degradation by entrapped *Rhodococcus* sp. NCIM 5126 cells

Immobilization of *Rhodococcus* sp. was carried out according to the method of Martinez-Madrid *et al.* (1989). Preinoculum of the cells was prepared as described earlier. 10 ml suspension containing 0.5 g wet weight cells, 0.5% NaCl and 2% κ-carrageenan was extruded dropwise in 100 ml of 4% KCl solution at 4°C with gentle stirring. After 2 hours, the spheres were washed with water and then used for crude oil degradation using 1% (w/v) Assam crude oil. After 4 days, beads were removed from the medium, washed and inoculated in fresh medium containing 1% (w/v) Assam crude oil. Residual crude oil was extracted and analyzed as described earlier in this chapter. Beads were used for crude oil degradation for one month.

RESULTS AND DISCUSSION

Rhodococcus sp. NCIM 5126 degraded crude oil in Aquil® Artificial Sea Water medium. Nitrogen and phosphorus supplementation resulted in dramatic increase in degradation and the nature of the nitrogen source also affected percent degradation. Table 3.1 shows that this isolate prefers urea as a nitrogen source.

Table 3.1 Effect of nitrogen sources on oil degradation by *Rhodococcus* sp. NCIM 5126

Nitrogen source (70 mM)	% degradation of aliphatic fraction (GC analysis)
Without nitrogen	10±2.2
$(NH_4)_2SO_4$	25±2.1
NH_4Cl	21±2.0
NH_4NO_3	28±0.5
$NaNO_3$	39±0.5
Urea	45±1.2

Medium: Artificial Sea Water containing 0.114 mM P as K_2HPO_4, respective nitrogen sources providing 70 mM nitrogen, pH 8.0, 1% (w/v) crude oil. Flasks were incubated at 30°C for 5 days at 150 rpm.

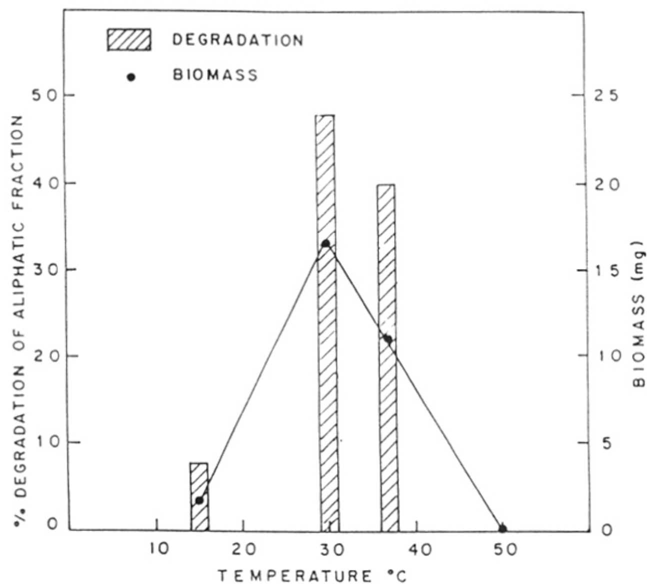
Apart from carbon source, microorganisms require nitrogen and phosphorus to incorporate into cellular material. *Rhodococcus* sp. used 35 mM nitrogen as urea to give best degradation of 50±3.0% of aliphatic fraction of crude oil. Absence of phosphorus source resulted in poor degradation. When K_2HPO_4 as a phosphorus source was added into the medium, degradation levels increased considerably although variation in concentration of this chemical had no significant effect on degradation. Therefore throughout these studies 0.114 mM P was used as K_2HPO_4. Thus, C/N ratio of 20:1 and C/P ratio of 2900:1 were favourable for crude oil degradation. Fedorak and Westlake (1981) also reported the extensive degradation of saturates and aromatics with the addition of nitrogen and phosphorus supplements.

The optimum temperature for crude oil degradation by this isolate was 30°C and percent degradation reduced on both the sides of the peak (Fig. 3.1). At lower temperatures, viscosity of oil increases and volatility of toxic compounds decreases, resulting in delayed biodegradation whereas at higher temperature cells could not grow.

Figure 3.1

Effect of temperature on crude oil degradation by *Rhodococcus* sp. NCIM 5126

The organism was grown in Artificial Sea Water containing 35 mM N as urea, 0.114 mM P as K_2HPO_4, 1% (w/v) Assam crude oil and pH 8.0. Flasks were incubated at temperatures 15, 30, 37 and 50°C for 5 days at 150 rpm.



Amund and Igiri (1990) showed that biodegradation of crude oil occurred at temperatures 25°C to 33°C, typical of mesophilic temperature range of tropical estuarine conditions. *Rhodococcus* is known to grow at temperatures 10 to 45°C. Psychrotrophic strains of *Rhodococcus* degrading aliphatics have also been described for bioremediation in cold climates (Yagafarova and Skvortsova, 1996; Whyte *et al.*, 1998).

In general, actinomycetes are known to prefer alkaline pH for their growth. This organism grew well on crude oil in the pH range of 6.0 to 10.0 although the optimum pH was 8.0, which is also the pH of natural seawater. Variation in initial pH of the medium (Table 3.2) clearly shows the effect on both biomass and degradation.

Table 3.2 Effect of initial pH on crude oil degradation by *Rhodococcus* sp. NCIM 5126

pH	% degradation of aliphatic fraction (GC analysis)	Biomass (mg)
4.0	18±1.0	5.0
6.0	35±0.3	14.0
8.0	48±0.3	16.5
10.0	31±0.4	12.5
12.0	3.8±0.1	1.0

Medium: Artificial Sea Water containing 0.114 mM P as K_2HPO_4 and 1% (w/v) crude oil. After autoclaving 35 mM N as urea was added, then initial pH of the medium was adjusted using sterile 1N HCl or 1N NaOH. Flasks were incubated at 30°C for 5 days at 150 rpm.

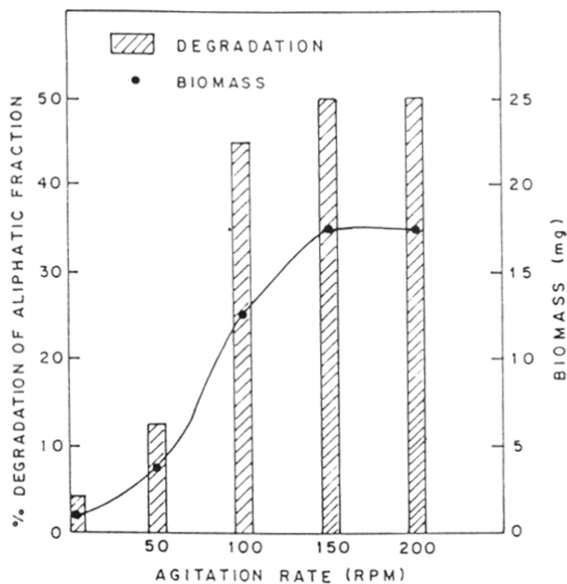
Hydrocarbon degradation is a highly oxidative process in which molecular oxygen is necessary. Therefore for maximal degradation both aeration and agitation are required. Agitation of medium maintains homogenous chemical and physical conditions, disperses the dissolved O_2 into smaller bubbles thereby increasing the interfacial area. As the agitation speed increases, oxygen transfer rate increases resulting in higher degradation. As shown in Fig.3.2, biomass and degradation increased gradually from 0 to 150 rpm and then remained steady. Almost 12% more degradation was observed at 150 and 200 rpm than at zero rpm.

This isolate showed maximum degradation of 47±0.8% at 1 % (w/v) crude oil. After that about 20 and 23% less degradation occurred at 1.5 and 2.0% (w/v) crude oil (Table 3.3). The higher concentrations of crude oil were inhibitory for degradation.

Figure 3.2

Effect of agitation rate on crude oil degradation by *Rhodococcus* sp. NCIM 5126

The organism was grown in Artificial Sea Water containing 35 mM N as urea, 0.114 mM P as K_2HPO_4, 1% (w/v) Assam crude oil and pH 8.0. Flasks were incubated at 30°C at 0, 50, 100, 150 and 200 rpm for 5 days.



For routine studies of crude oil degradation by microorganisms 1% (w/v) crude oil has often been used (Zinjarde, 1996; Lal and Khanna, 1996).

Table 3.3 Effect of concentration of crude oil on its degradation by the organism

Crude oil (% w/v)	% degradation of aliphatic fraction (GC analysis)
0.5	40±1.0
1.0	47±0.8
1.5	27±1.3
2.0	24±0.3

Medium: Artificial Sea Water containing 35 mM nitrogen as urea, 0.114 mM P as K_2HPO_4, pH 8.0 and various concentrations of crude oil. Flasks were incubated at 30°C for 5 days at 150 rpm.

The optimal conditions for degradation of Assam crude oil by *Rhodococcus* sp. NCIM 5126 were: **35 mM nitrogen as urea, 0.114 mM P as K_2HPO_4, 1% (w/v) crude oil in Artificial Sea Water, temperature 30°C, initial pH 8.0 and agitation rate 150 rpm.** Under these conditions there was about 50% degradation of the supplied crude oil. There was appreciable growth of *Rhodococcus* sp. on crude oil. Following a 12 hr lag phase the protein specific growth rate μ was 0.032 hr^{-1} till 72 hr. 50% degradation of the aliphatic fraction of supplied crude occurred by 72 hr after which there was no further increase in growth or degradation of crude oil. As shown in Fig.3.3, there was an exponential increase in biomass and degradation of crude oil. Figure 3.4 represents the gas chromatogram of the degraded crude oil by *Rhodococcus* sp.

As shown in Table 3.4, this isolate was capable of degrading aliphatic as well as aromatic fraction of Bombay High, Assam crude and Gujarat crude oil, but not the asphaltene fraction.

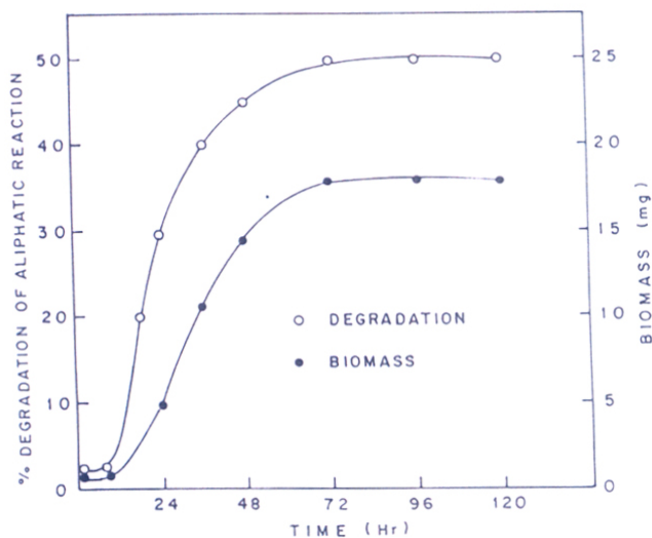
Table 3.4 Degradation of different fractions of crude oils by *Rhodococcus* sp.

Crude oil	Gravimetric analysis of degradation of		
	Aliphatic fraction (%)	Aromatic fraction(%)	Asphaltene fraction (%)
Bombay High	72	28	–
Assam Crude	60	20	–
Gujarat Crude	35	12	–

Figure 3.3

Time course of crude oil degradation by *Rhodococcus* sp. NCIM 5126

The organism was grown in Artificial Sea Water containing 35 mM N as urea, 0.114 mM P as K_2HPO_4, 1% (w/v) Assam crude oil, pH 8.0. Flasks were incubated at 30°C for 5 days at 150 rpm. Residual crude oil was extracted in chloroform and GC analysis was carried out as described in Materials and Methods.



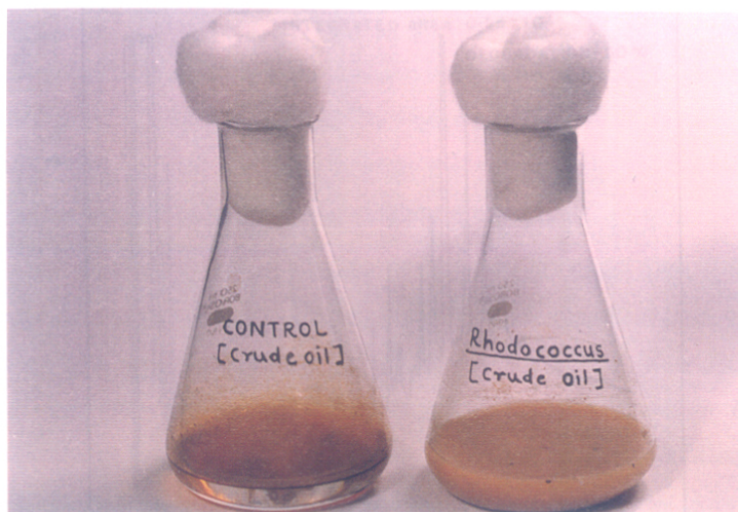
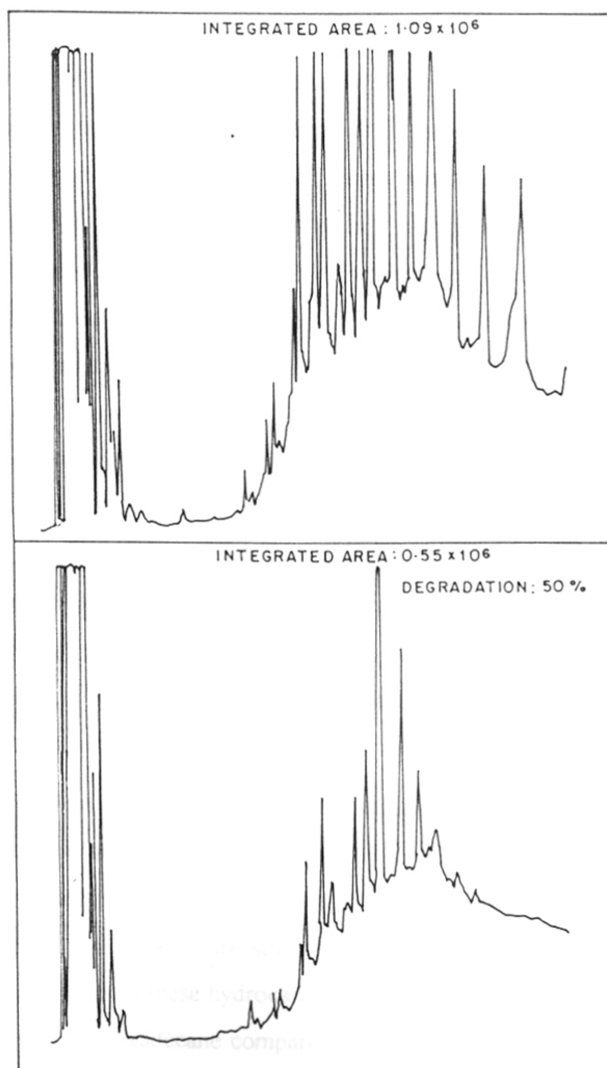


Figure 3.4

Gas chromatographic analysis of the aliphatic fraction of Assam crude oil by *Rhodococcus* sp. NCIM 5126

The organism was grown in Artificial Sea Water containing 1% (w/v) Assam crude oil, 35 mM N as urea, 0.114 mM P as K_2HPO_4, pH 8.0. Flasks were incubated at 30°C for 3 days at 150 rpm. Uninoculated flask containing 1% (w/v) crude oil was kept to monitor abiotic loss. GC analysis was carried out as described in Materials and Methods.



It is clear from Table 3.4 that composition of crude oil had a major effect on degradation of crude oil. Bombay High crude oil, which contains higher concentration of saturates and lower concentration of aromatics compared to Assam and Gujarat crude oil was better degraded. Gujarat crude oil was least degraded on account of its higher aromatic and asphaltene content.

Comparison of growth of *Rhodococcus* sp. NCIM 5126 on different carbon sources

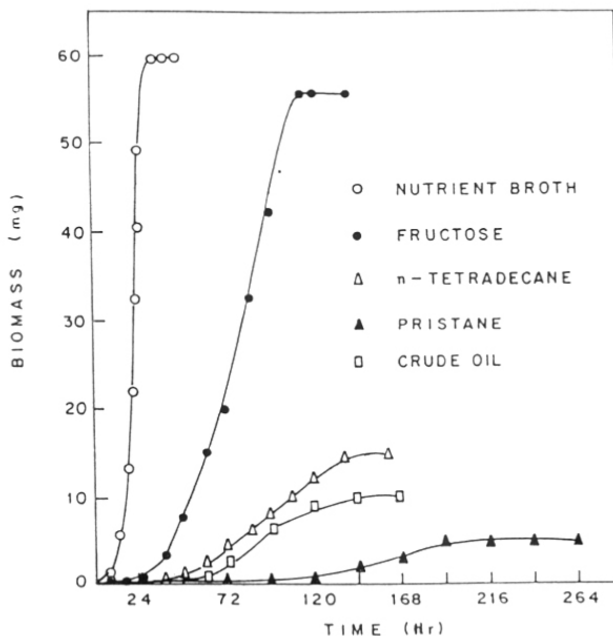
In order to determine growth of *Rhodococcus* sp. NCIM 5126 on nutrient broth, n-alkanes, crude oil and pristane, a starting inoculum of 10^7 cells per ml of the medium was used and biomass increase was monitored. As shown in Fig. 3.5, in nutrient broth, *Rhodococcus* sp. NCIM 5126 cells were in the lag phase till 6 hr and then grew at the protein specific growth rate of 0.09 hr^{-1} till 48 hr with a generation time of 7.7 hr. A lag phase of 24 hr was observed when fructose and urea were supplied in the medium and cells grew at the protein specific rate of 0.031 hr^{-1} with a generation time of 22 hr. *Rhodococcus* cells were apparently stressed in presence of crude oil and alkanes as observed by the increased lag phase and generation time and decreased protein specific growth rate. In n-tetradecane and urea medium, lag phase of 36 hr, μ of 0.018 hr^{-1} with a generation time of 39 hr was observed. The curve for crude oil was almost similar to that for n-tetradecane with a μ of 0.017 hr^{-1} and generation time of 41 hour. *Rhodococcus* cells were maximally stressed in presence of pristane where lag phase was extended to 120 hr, μ was 0.016 hr^{-1} with a generation time of 43 hr. All other nutritional, environmental conditions and initial biomass were identical in the treatment therefore increased lag period and generation time, decreased protein specific growth rate (μ) and decreased final maximum biomass can be attributed to the increasing complexity of the supplied carbon source and carbon number.

Enzymes required for the assimilation of peptone and yeast extract in nutrient broth are constitutive whereas systems for degradation of fructose, n-tetradecane, crude oil and pristane have to be induced. Furthermore fructose is soluble in water whereas n-tetradecane, crude oil and pristane are sparingly soluble in water. Therefore, fructose was utilized with less lag than these hydrocarbons. Extended lag phase was observed in case of yeast grown on n-hexadecane compared to glucose, however final biomass was similar in both the cases. Rodriguez and Dominiguez (1984) reasoned that yeast cells

Figure 3.5

Comparison of growth of *Rhodococcus* sp. NCIM 5126 on different carbon sources

The organism was grown in Artificial Sea Water containing 0.5% peptone and 0.3% yeast extract (nutrient broth) or 1.5% fructose or 1% n-tetradecane or 1% crude oil or 1% pristane as sole carbon source. Except nutrient broth, all other media contained 35 mM N as urea and 0.114 mM P as K_2HPO_4.



needed to adapt themselves to the hydrocarbon source resulting in extended lag period. In case of n-tetradecane and crude oil, growth curve was almost similar with slightly decreased μ and increased generation time for crude oil. Crude oil is a complex mixture of hydrocarbons and presence of other toxic, soluble hydrocarbons can be inhibitory to the cells as compared to pure n-tetradecane. n-Tetradecane is a straight chain alkane whereas pristane is a branched chain alkane consisting of methyl groups, which are known to inhibit degradation process. Final biomass was higher in n-tetradecane medium compared to pristane medium.

Degradation of various hydrocarbons by *Rhodococcus* sp. NCIM 5126

Rhodococcus sp. NCIM 5126 was able to degrade a variety of saturated aliphatic hydrocarbons. It degraded 94% of supplied n-tetradecane, though degradation decreased to 5% when n-C_{20} was used as a carbon source. Almost equal degradation was observed for n-C_{16} and n-C_{18} (Fig. 3.6). Kerosene, a mixed chain alkane, consisting primarily n-C_{10} to n-C_{13} was degraded to 92% (Fig.3.7). *Rhodococcus* sp. is known to degrade n-alkanes in the range of C_{12} to C_{20} (Sorkhoh *et al.*, 1990). However, rhodococci degrading n-C_{10} to n-C_{30} have also been reported (Whyte *et al.*, 1998; Milekhina *et al.*, 1998). Haines and Alexander (1974) have demonstrated metabolism of up to n-C_{44} by microorganisms. Pristane, which is a branched chain alkane, was also degraded by this species of *Rhodococcus*. In pristane, the straight chain is 15–carbon long with 4 methyl groups. It is degraded to only 30% compared to 94% of n-C_{14} having 14–carbon long chain without methyl branching. Increased branching of paraffins decreases their biodegradability. Nocardioform actinomycetes including *Rhodococcus* strains are known to metabolize pristane (Pirnik, *et al.*, 1974; Cox *et al.*, 1974; Nakajima *et al.*, 1985 a, b) but data regarding its percent degradation have not been reported.

When grown on hydrocarbons, major changes occur in the lipid composition of the microorganisms though variation in growth conditions and age of the culture also affect their cellular lipid composition (Ratledge, 1978). When all growth conditions were kept constant change in cellular fatty acid composition is probably due to the supplied carbon source. Table 3.5 represents the fatty acid composition of *Rhodococcus* sp. grown on fructose, odd and even carbon number alkanes and pristane.

Figure 3.6

Degradation of hydrocarbons by *Rhodococcus* sp. NCIM 5126

The organism was grown in Artificial Sea Water containing 1% (w/v) n-alkane, 35 mM N as urea, 0.114 mM P as K_2HPO_4, pH 8.0. Flasks were incubated at 30°C for 3 days at 150 rpm. Residual n-alkanes in test and control samples were extracted in chloroform and GC analysis was carried out as described in Materials and Methods.

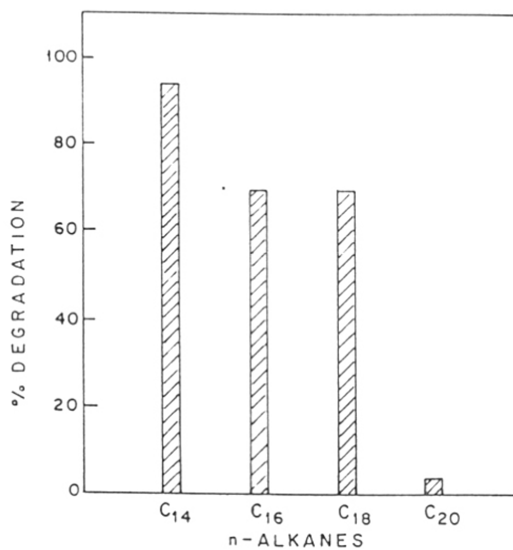


Figure 3.7

Gas chromatographic analysis of the kerosene degradation by *Rhodococcus* sp. NCIM 5126

The organism was grown in Artificial Sea Water containing 1% (w/v) kerosene, 35 mM N as urea, 0.114 mM P as K_2HPO_4, pH 8.0. Flasks were incubated at 30°C for 3 days at 150 rpm. Residual kerosene in test and control was extracted in chloroform and GC analysis was carried out as described in Materials and Methods.

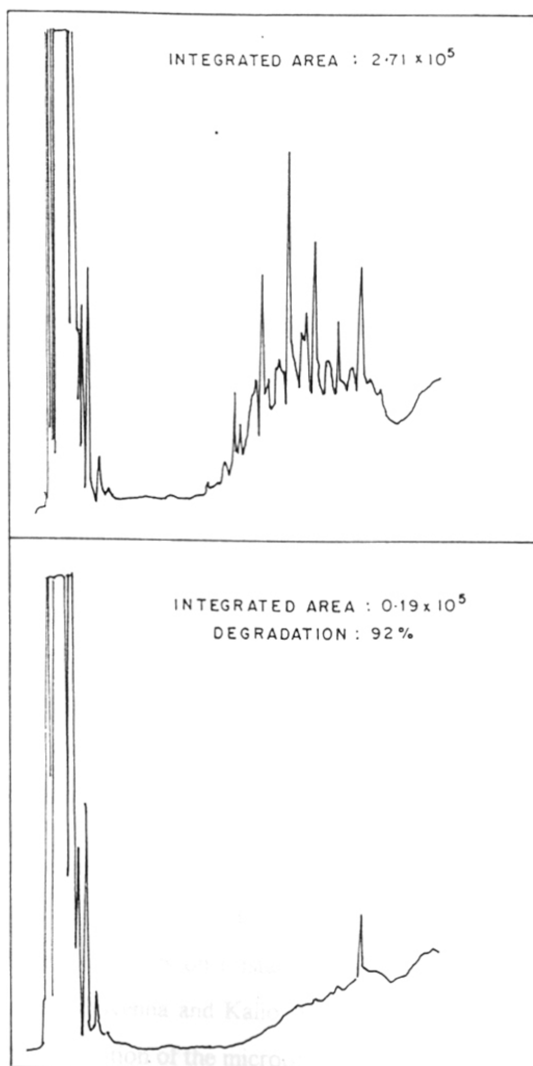


Table 3.5 Fatty acid composition of *Rhodococcus* sp. NCIM 5126

Fatty acid	Growth substrate					
	Fructose	n-C_{13}	n-C_{14}	n-C_{16}	n-C_{17}	Pristane
C_{13}	-	25.36	-	-	-	-
Br–C_{13}	-	-	-	-	-	6.02
C_{14}	-	-	30.26	-	-	-
C_{15}	-	-	-	-	-	10.02
Br–C_{15}	-	-	-	-	-	24.24
C_{16}	12.90	12.52	6.48	33.23	5.27	4.13
$C_{16:1}$	5.04	5.78	5.30	6.23	5.20	5.05
C_{17}	-	-	-	-	30.59	3.04
Br–C_{17}	-	-	-	-	-	3.03
C_{18}	6.69	6.53	5.03	5.78	5.14	0.20
Br–C_{18}	-	-	-	-	-	0.25
$C_{18:1}$	6.97	6.67	6.95	6.76	6.16	0.36
Total fatty acids	**31.60**	**56.86**	**54.02**	**52.00**	**52.36**	**56.34**

The organism was grown on various carbon sources under optimized conditions. Fatty acids are recorded as the percentage of the total fatty acids present. Br–branched chain fatty acid.

From Table 3.5, it is clear that total concentration of fatty acids was higher in hydrocarbon grown *Rhodococcus* cells than in fructose grown cells. In accordance with Boulton and Ratledge (1987), increased lipophilicity is required by the hydrocarbon grown cells to facilitate uptake and intracellular transport of hydrocarbons.

C_{16}, $C_{16:1}$ and C_{18}, $C_{18:1}$ fatty acids were predominant in fructose-grown cells. On even carbon number alkanes such as n-C_{14} and n-C_{16}, cell contained only even number carbon fatty acids and tetradecanoic acid and hexadecanoic acid were predominant.

When grown on n-C_{13} and n-C_{17}, odd carbon number fatty acids were predominant and the major fatty acids were tridecanoic acid and heptadecanoic acid. C_{16}, $C_{16:1}$, C_{18} and $C_{18:1}$ were uniformly present whether the cells were grown in presence of even or odd carbon number alkanes.

Similarly pristane grown cells produced 2,6,10,14-tetramethylpentadecanoic acid. Besides the presence of C_{15}, C_{16}, C_{17} and C_{18} acids, branched chain fatty acids were also present. Although there are reports on pristane metabolism by various microorganisms including actinomycetes (McKenna and Kalio, 1964, 1971), only few studies deal with the cellular fatty acid composition of the microorganisms grown on pristane. Hagihara *et*

al. (1977) reported pristanic acid as a major neutral lipid in *Candida lipolytica* NRRL Y-6795 grown on pristane as sole source of carbon and energy.

Predominance of corresponding fatty acid of the parent alkane in the cellular lipids has been reported (King and Perry, 1975; Hug and Fiechter, 1973). In case of n-alkane grown *Rhodococcus opacus* strain PD630, the composition of fatty acid of intracellular inclusions was related to the substrate and intermediates of β-oxidation pathway such as hexadecanoic and pentadecanoic acid. Hexadecanoic acid was also the major fatty acid when these cells were grown on gluconate, indicating β-oxidation and *de novo* fatty acid biosynthesis (Alvarez *et al.*, 1996).

Thus, in case of n-alkanes as well as pristane, monoterminal oxidation of the parent hydrocarbon gives rise to monocarboxylic acid of equivalent chain length which is incorporated into the cellular lipids by the phenomenon known as 'intact/direct incorporation'. The occurrence of C_{16}, $C_{16:1}$, C_{18} and $C_{18:1}$ fatty acids in *Rhodococcus* sp. is constitutive and can be attributed to *de novo* biosynthesis independent of the supplied carbon source.

Degradation end products of *Rhodococcus* sp. NCIM 5126 grown on hydrocarbons

Fatty acids isolated from the broth of n-tridecane grown cells of *Rhodococcus* sp. showed the presence of undecenoic acid and hexadecenoic acid whereas tridecanoic acid was the major cellular fatty acid. Thus, monoterminal oxidation and further decarboxylation, desaturation and chain elongation are involved in the formation of these acids.

In case of n-tetradecane grown cells, undecanedioic acid was recovered. Presence of dicarboxylic acid and its odd carbon number suggest the operation of a diterminal and α-oxidation pathway after the initial attack on C_1 carbon.

Tridecanedioic acid and undecanedioic acid were identified from the supernatant of n-hexadecane grown cells. Recovery of odd carbon number fatty acids from the even carbon number n-alkane is itself indicative of α-oxidation and presence of dioic acid is indicative of the diterminal pathway.

When n-heptadecane was used as sole carbon and energy source, hexadecenoic acid was recovered from the culture filtrate. Production of even carbon number fatty acid from odd carbon number n-alkane indicates the operation of α-oxidation and its unsaturated nature suggests desaturation of fatty acid.

Thus, in this species of *Rhodococcus*, monoterminal oxidation is probably a major route of oxidation of alkanes. Recovery of dioic acids and odd carbon number fatty acids from even carbon number alkanes and vice versa also indicate the operation of ω and α-oxidation pathways for the breakdown of supplied carbon source. Whyte *et al.* (1998) showed that *Rhodococcus* sp. Q15 oxidized n-alkanes by both the terminal oxidation pathway as well as subterminal oxidation pathway and both primary and secondary alcohols of n-hexadecane and n-dodecane were recovered from the supernatants. In the present case however only fatty acids were detected.

The culture filtrate of pristane grown cells showed the presence of 2,6,10,14-tetramethylpentadecanoic acid, suggesting attack at a single terminus. This result is similar to that reported by Nakajima *et al.* (1985 a) who showed that pristane was metabolized to pristanic acid (2,6,10,14-tetramethylpentadecanoic acid) by *Rhodococcus* sp. BPM 1613, followed by β-oxidation. They also proposed another pathway in which pristanic acid gave rise to pristanedioic acid (ω-oxidation) followed by β-oxidation. McKenna and Kalio (1964, 1971) suggested two pathways of pristane utilization by soil *Corynebacterium* sp. giving rise to 4,8,12-trimethyltridecanoic acid and a-methylglutaric acid. *Nocardia* accumulated pristanol and pristanic acid as major products and pristyl pristanate and pristyl aldehyde as minor products in the culture broth (Nakajima *et al.*, 1974). Cox *et al.* (1974) isolated 2,6,10,14-tetramethylpantadecane-1-ol, 2,6,10,14-tetramethylpentadecanoic acid and 4,8,12-trimethyltridecanoic acid as oxidation products of pristane from *Mycobacterium fortuitum* suggesting attack at only one terminus of this substrate. *Brevibacterium erythrogens* accumulated a series of dicarboxylic acids suggesting attack at both the termini of pristane (Pirnik *et al.*, 1974). Pristane oxidation has been reported mainly for the *Nocardia* group of organisms of which *Rhodococcus* is also a member. In the present species however, no dioic acid seems to be produced.

In the radioactivity experiments, after 72 hr, about 4520 dpm were observed in particulate matter (about 6% radioactivity) and about 980 dpm remained in the supernatant (1% radioactivity). Probably about 93% radioactivity is released as CO_2.

Effect of salinity on crude oil degradation by *Rhodococcus* sp. NCIM 5126

The salinity of the Artificial Sea water is 35‰. As shown in Table 3.6, as the salinity of the medium increased from 7‰ to 35‰, crude oil degradation also increased, maximum with a 35‰. Thereafter as the salinity increased, crude oil degradation decreased. Ward and Brock (1978) also reported decrease in rates of hydrocarbon

Table 3.6 Effect of salinity of the Artificial Sea Water on crude oil degradation

Salinity of the Artificial Sea Water (‰)	% degradation of aliphatic fraction (GC analysis)
7	30±1.0
9	42±0.5
17.5	46±1.0
35	50±2.0
52	44±2.0
70	38±1.0
88	30±1.5

Medium: Salinity of the Artificial Sea Water was varied as mentioned above. Flasks containing 35 mM N as urea, 0.114 mM P as K_2HPO_4, 1% (w/v) crude oil, pH 8.0 were incubated at 30°C for 3 days at 150 rpm.

metabolism when salinity of the medium was increased for the samples collected from salt evaporation ponds and Great Salt Lake, Utah. They have attributed these results to a general reduction in microbial metabolic rates at high salt concentration.

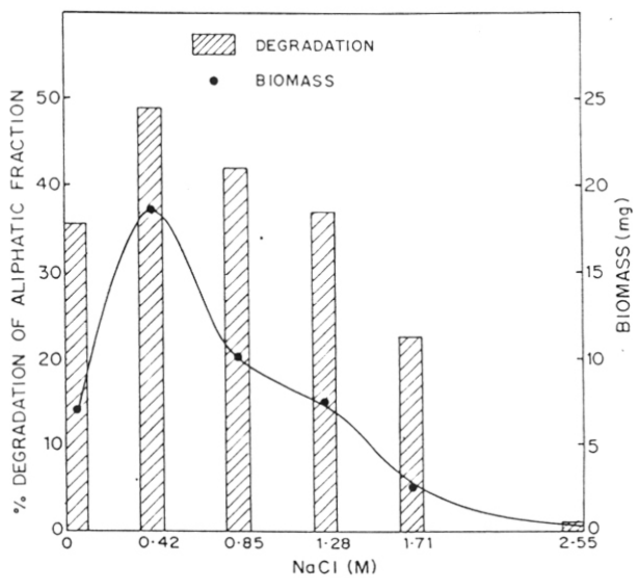
Effect of NaCl on crude oil degradation by *Rhodococcus* sp. NCIM 5126

NaCl is the main constituent of the Artificial Sea Water (0.42 M). As shown in Fig. 3.8, maximum degradation of 48.7±0.1% occurred at 0.42 M NaCl, which is the original concentration of NaCl in Artificial Sea Water. For *Rhodococcus* sp., as molarity of NaCl in Artificial Sea Water medium was increased, crude oil degradation decreased. Bertrand *et al.* (1990) showed that growth of an archaebacterium did not occur below 1.8 M NaCl concentration whereas Mille *et al.* (1991) observed that amount of crude oil degraded by a mixed bacterial community decreased with increasing NaCl concentration.

Figure 3.8

Effect of NaCl on crude oil degradation by *Rhodococcus* sp. NCIM 5126

Concentration of NaCl varied from 0 to 2.57 M in Artificial Sea Water medium containing 35 mM N as urea, 0.114 mM P as K_2HPO_4, 1% (w/v) Assam crude oil, pH 8.0. Flasks were incubated at 30°C for 3 days at 150 rpm.



Zajic *et al.* (1974) proposed that at high concentration of NaCl emulsification of oil as well as enzymatic reactions get hampered resulting in decreased biodegradation. We also observed decreased emulsification and degradation of crude oil in the form of adherence of oil to the sides of the flasks at high salt concentration.

Depending upon the tolerance to NaCl, Stanier *et al.* (1981) divided bacteria into four broad categories, non-halophiles (NaCl tolerance 0–0.2 M), marine forms (0.03–0.85 M), moderate halophiles (0.4–3.5 M) and extreme halophiles (upto 6 M). As *Rhodococcus* sp. NCIM 5126 tolerated about 1.7 M NaCl, it can be categorized as a moderate halophile. In these organisms, NaCl is required for the maintenance of cellular functions and transport mechanisms.

Artificial Sea Water is a mixture of 10 different salts. The 5 major salts are NaCl, Na_2SO_4, $MgCl_2$, $CaCl_2$ and KCl. Table 3.7 shows that removal of Na_2SO_4 from Artificial Sea Water medium resulted in a sharp decrease to 28% degradation of crude oil. Removal of NaCl on the other hand also decreased degradation from 50% to 40%. Removal of $MgCl_2$, $CaCl_2$ and KCl did not affect crude oil degradation by this organism.

Table 3.7 Effect of salts in Artificial Sea Water medium on crude oil degradation

Type of medium	% degradation of aliphatic fraction (GC analysis)
Artificial sea water	49±0.2
Without NaCl	40±0.4
Without Na_2SO_4	28±0.4
Without $MgCl_2$	49±1.2
Without $CaCl_2$	48±0.4
Without KCl	46±0.5

Medium: Artificial Sea Water with omission of a specific salt at a time, 35 mM nitrogen as urea, 0.114 mM P as K_2HPO_4 and 1% (w/v) crude oil, pH 8.0. Flasks were incubated at 30°C for 3 days at 150 rpm.

To determine effect of these salts on crude oil degradation, equimolar concentrations of Na^+, K^+, Mg^{++} and Ca^{++} (0.17 M) were supplied individually in a medium using deionised water. In addition to deionised water and crude oil, the medium contained optimum concentrations of N and P sources. As presented in Table 3.8, in the presence of NaCl and Na_2SO_4 the organism showed 12 to 25% degradation of crude oil. Mg^{++}, Ca^{++} and K^+ containing salts did not increase the degradation also the various concentrations of

Cl^- and HCO_3^- were ineffective in increasing crude oil degradation. These results emphasize the need of SO_4^- as a sulfur source and Na^+ for crude oil degradation by this organism.

Table 3.8 Effect of salts on crude oil degradation in deionised water medium

Added Salt	% degradation of aliphatic fraction (GC analysis)
NaCl	12±0.4
$NaHCO_3$	8±0.9
Na_2SO_4	25±0.1
$MgCl_2$	6±0.4
$CaCl_2$	6.5±0.1
KCl	7.5±0.5
Deionised water	6.4±0.4

Medium: 35 mM N as urea, 0.114 mM P as K_2HPO_4, pH 8.0 and 1% (w/v) crude oil with respective salt in deionised water. Flasks were incubated at 30°C for 3 days at 150 rpm.

Na_2SO_4 served as the best source of SO_4^- (0.07 M) with 38±0.5% degradation compared to K_2SO_4 and $MgSO_4$, which gave 30±0.3% and 23±0.2% degradation respectively. Na_2SO_4 concentration greater than 0.07 M did not increase crude oil degradation.

Maintaining a molarity of 0.07 M Na_2SO_4 the concentrations of NaCl was varied in the medium. A concentration of 0.086 M NaCl was sufficient to give 50±2.5% degradation of the crude oil which was equal to the degradation in usual Artificial Sea Water medium. Increasing the concentration of NaCl did not affect degradation. Thus, 0.07 M Na_2SO_4 and 0.086 M NaCl in deionised water medium were sufficient to replace all other salts in Artificial Sea Water for degradation of 50% of aliphatic fraction of crude oil.

Effect of sulfur on the growth of the organism

The obvious need of sulfate ions as the sulfur source, for crude oil degradation by *Rhodococcus* sp. NCIM 5126 led us to determine its effect on media which did not contain hydrocarbon for example, fructose and to compare its requirement by *Rhodococcus* sp. to the requirement by *E. coli*. Sulfur plays a key biological role in

providing –SS– linkages between amino acids within protein molecules thus giving 3-dimensional structure to proteins and mechanical structure to the cells.

In sulfate-less medium, protein specific growth rate of *Rhodococcus* sp. was 0.014 hr^{-1}, a generation time of 50 hr after a lag phase of 30 hr with a maximum final biomass of 6 mg. As shown in Fig. 3.9, in media containing sulfate, μ for all tested concentrations of sulfate was about 0.032 hr^{-1}, a generation time of 21 hr in the exponential phase which followed a lag phase of 18 hr. The maximum final biomass increased from 43, 46, 49 to 51 mg with increased concentrations of sulfate.

Growth of *E. coli* showed almost similar protein specific growth rate 0.09 hr^{-1}, generation time of 7.7 hr and a lag phase of 6 hr in sulfate-less medium as well as in media containing sulfate. There appears to be a lower apparent requirement of sulfur and almost no difference in final biomass in *E. coli* growth.

Results of C and S analysis of both the organisms in different media are presented in Table 3.9.

Table 3.9 Carbon and Sulfur analysis of the organisms

Organism	Medium	Dry weight (mg)	C (mg)	S (mg)
***Rhodococcus* sp.**	No sulfate	26	9.82	0.114
	With 0.06 M sulfate	90	39.381	1.778
E. coli	No sulfate	2.95	1.23	0.051
	With 0.06 M sulfate	3.4	1.42	0.059

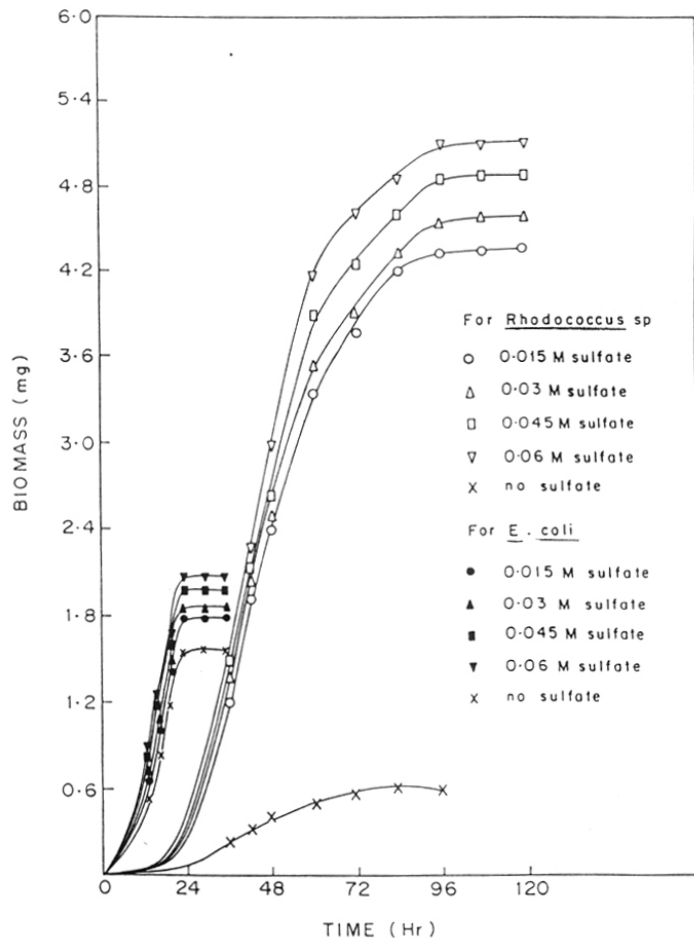
It appears that sulfate in the medium is assimilated by *Rhodococcus* cells and used to build up biomass. Depending on the medium, not only the total biomass gets affected but also the total concentration of the sulfur in *Rhodococcus* sp. as compared to *E. coli*. Cuhel *et al.* (1981 a, b) have also obtained increase in total cellular sulfur as well as protein content and have attributed the increased cellular sulfur to the change in protein sulfur. Assimilatory sulfur metabolism has been known to occur in *E. coli* K-12

Figure 3.9

Effect of sulfate on the growth of the organism

Rhodococcus sp. NCIM 5126 (1.5% fructose) and *E. coli* (1% glycerol) were grown separately in Artificial Sea Water in the absence of sulfate, 0.015, 0.03, 0.045 and 0.06 M sulfate medium. Values for Na^+ were corrected by adding appropriate amount of NaCl to give a final concentration of 0.012 M Na^+.

1/10th of original mg values of protein biomass are plotted in case of *Rhodococcus* sp. NCIM 5126 in order to compare the results with those of *E. coli* on the same scale.



(Springer and Huber, 1972), *Saccharomyces cerevisiae* (McCready and Din, 1973) and in marine microorganisms *Pseudomonas halodurans* and *Alteromonas luteo-violaceus* (Cuhel *et al.*, 1981 a, b). Poole and Haddock (1975) observed sulfate limited growth in *E. coli* K-12 and growth yield was six fold and two fold lower with respect to the amount of glycerol and oxygen consumed. The authors suggested that sulfate limited cells lack the proton translocating oxidoreduction segment of the electron transport chain between NADH and the cytochromes and the loss of site–I phosphorylation. Similarly for the sulfate-limited growth of yeast, Light (1972) showed loss of one site of oxidative phosphorylation in the respiratory chain.

Sulfur sources are assimilated mostly to provide sulfur for the amino acids cysteine and methionine. Smaller amounts of sulfur are also required in some coenzymes such as biotin, coenzyme A, ferredoxin, lipoic acid and thiamine. Sulfur limitation probably decreases the synthesis of these key compounds and affects their function in the cell. As seen in Fig. 3.10, sulfate limitation in *Rhodococcus* sp. affected steady state dry weight, carbon and sulfur concentration. Methionine replaced sulfur in *Rhodococcus* sp. with a protein specific growth rate of 0.028 hr^{-1} and a generation time of 25 hr. The maximum final biomass was 34.5 mg. On the other hand, cysteine inhibited growth by extending the lag phase upto 60 hr and a negligible growth of about 4.5 mg was observed at the end of 120 hr. Methionine is known to be a better sulfur source than either sulfate or cysteine for enterobacteria as well as yeasts (Siegel, 1975). It appears that in the presence of cysteine as sole source of sulfur, growth of *Rhodococcus* sp. is getting inhibited.

As cysteine is known to inhibit the uptake of sulfate in microorganisms, *Rhodococcus* sp. was grown in 3 different media containing 0.015 M sulfate (minimum amount of sulfate giving considerable growth of *Rhodococcus* sp., Fig. 3.9) or 1 mM cysteine or 0.015 M sulfate + 1 mM cysteine in Artificial Sea Water medium lacking sulfate. As shown in Fig. 3.11, in the presence of 0.015 M sulfate and 1 mM cysteine, protein specific growth rate of about 0.024 hr^{-1} was obtained with a generation time of 29 hr whereas μ for that of 0.015 M sulfate was 0.032 hr^{-1}, generation time was 21 hr. The maximum final biomass was 27 mg which was considerably less than 44 mg in

Figure 3.10

Effect of sulfur containing amino acids as sole source of sulfur on the growth of *Rhodococcus* sp. NCIM 5126

1.5% (w/v) Fructose

Rhodococcus sp. NCIM 5126 was grown in Artificial Sea Water containing 35 mM N as urea, 0.114 mM P as K_2HPO_4 and 1 mM methionine or cysteine as a sole source of sulfur.

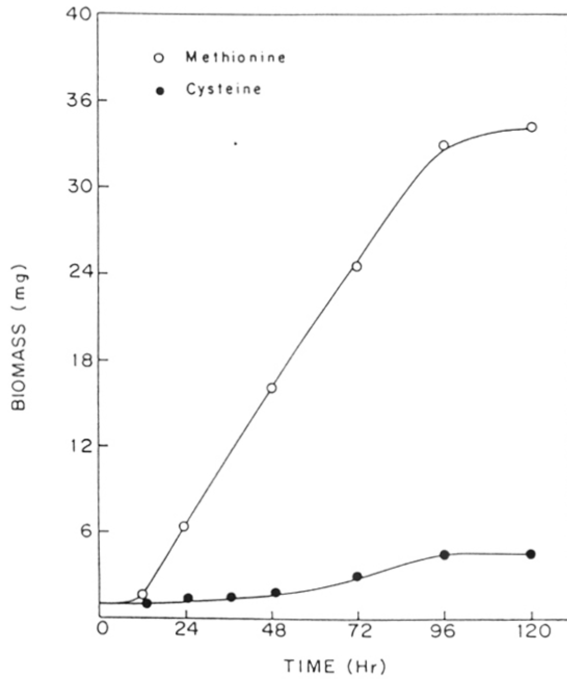
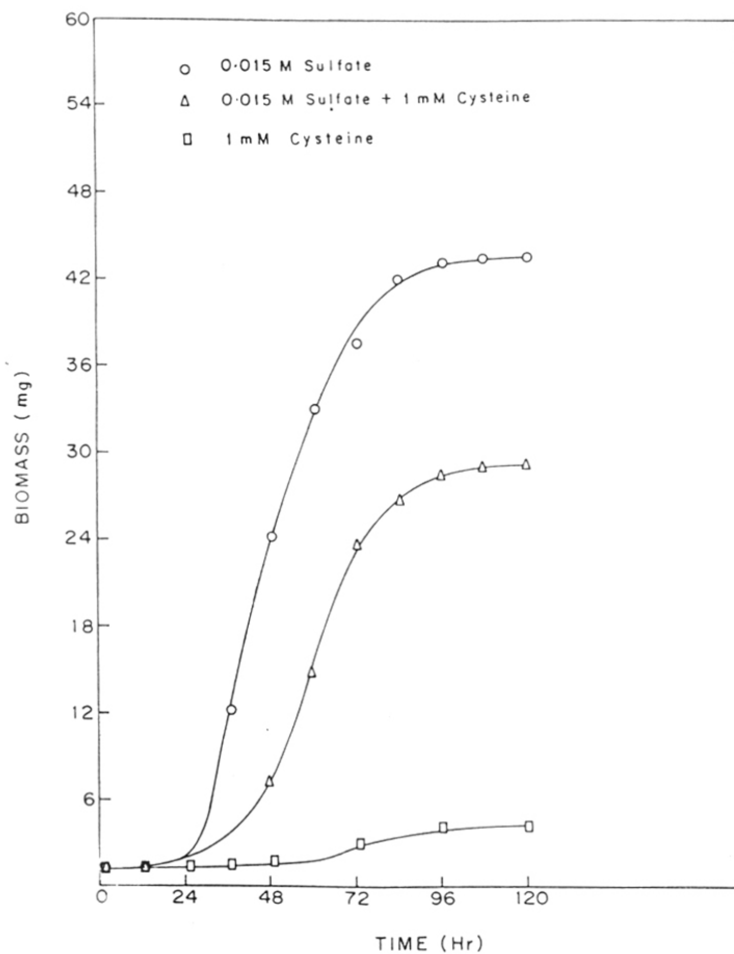


Figure 3.11

Sulfate uptake inhibition by the presence of cysteine in the medium for *Rhodococcus* sp. NCIM 5126

Rhodococcus sp. NCIM 5126 was grown in the presence of 0.015 M sulfate, 1 mM cysteine and 0.015 M sulfate + 1 mM cysteine added separately in Artificial Sea Water lacking sulfate with 1.5% fructose, 35 mM N as urea and 0.114 mM P as K_2HPO_4, pH 8.0. Flasks were incubated at 30°C at 150 rpm.



presence of 0.015 M sulfate. It is clear that in presence of cysteine, sulfate uptake is getting hampered in case of *Rhodococcus* sp. NCIM 5126. Pasternak (1961, 1962) showed that cysteine inhibited sulfate uptake in *E. coli*. Cysteine is known to repress the key components of sulfate uptake and it regulates sulfate assimilation by feedback inhibition. He concluded that cells preferred cysteine to sulfate as a source of cellular sulfur.

Thus presence of sulfur was essential for good growth of *Rhodococcus* sp. NCIM 5126 in non-hydrocarbon and hydrocarbon media. Insufficient growth of the organism in the absence of sulfate resulted in hampered crude oil degradation, emphasizing the interdependence of biomass and degradation.

For bioremediation of crude oil, specific conditions are applied which includes addition of oxygen under pressure, aqueous solution of hydrogen peroxide or nitrogen and phosphorous sources. Slow release fertilizers are also useful for this purpose. Oil degrading organisms and slow release fertilizers (oleophilic fertilizers) associate themselves at oil/water interface and are therefore available to the degrader population. As shown in Table 3.10, in the experiment when crude oil degradation was compared using soluble nitrogen and phosphorus sources to paraffin supported nitrogen and phosphorus sources, about 8% increased degradation was observed in case of paraffin supported urea.

Table 3.10 Effect of slow release fertilizer on crude oil degradation

Type of fertilizer	% degradation of aliphatic fraction (GC analysis)
Soluble urea	50±2.5
Soluble $(NH_4)_2SO_4$	25±0.3
Paraffinized urea	58±0.5
Parafinized $(NH_4)_2SO_4$	29±0.8
Paraffinized $MgNH_4PO_4$	04±0.9

Medium: Artificial Sea Water containing soluble nitrogen and phosphorus sources, paraffinized nitrogen and phosphorus sources, pH 8.0. Flasks were incubated at 30°C for 3 days at 150 rpm

Atlas (1991) described the use of an oleophilic fertilizer Inipol EAP22® constituting oleic acid, urea and lauryl phosphate, in laboratory conditions as well as for the cleanup of the shoreline of Prince William Sound polluted with crude oil. Olivieri *et al.* (1976) described a slow release fertilizer containing paraffin supported magnesium ammonium

phosphate ($MgNH_4PO_4$) for stimulating biodegradation of Sarir crude oil in shake flask and field conditions. They observed that after 21 days 63% of the oil had disappeared when fertilizer was added as compared to only 40% in control area. Zinjarde (1996) reported better utilization of Bombay High crude oil by *Yarrowia lipolytica* in the presence of magnesium ammonium phosphate in shake flask cultures. However, *Rhodococcus* sp. NCIM 5126 did not utilize magnesium ammonium phosphate as N and P source and only 4% degradation was observed.

Oil degradation by entrapped *Rhodococcus* sp. NCIM 5126 cells

Oil degradation by *Rhodococcus* cells entrapped in κ-carragennan was observed for 7 cycles each of 4 days. For the first four cycles, 60% degradation of aliphatic fraction of Assam crude oil, which was 10% more than free cells, was obtained. After that degradation reduced gradually to 45% till the 7th cycle. Zinjarde (1996) also reported increase in degradation of aliphatic fraction of Bombay High crude oil from 78±2.5% to 92±1.5% by immobilized *Yarrowia lipolytica* cells and then degradation decreased to 64% by the fifth cycle. Although, there are no reports on use of immobilized cells of *Rhodococcus* for crude oil degradation, Valo *et al.* (1990) immobilized *Rhodococcus* cells on polyurethane for chlorophenol degradation whereas Iborra *et al.* (1994) immobilized *R. fascians* cells in κ-carrageenan for continuous limonin degradation. Pai *et al.* (1995) used *Rhodococcus* sp. immobilized on granular activated carbon and in calcium alginate for continuous phenol degradation. Ehrhardt and Rehm (1985) stated that immobilization of cells leads to increased tolerance for the substrates as reported for phenolic compounds. The entrapped cells are not constantly exposed to the toxic components and are therefore better able to degrade the oil than the free cells.

In conclusion, *Rhodococcus* sp. NCIM 5126 is a moderately halophilic actinomycete able to degrade aliphatic and aromatic fractions of crude oils, n-alkanes, mixed chain alkanes and branched chain alkane. The major route of n-alkane degradation is by β-oxidation although end products also suggest the possibility of operation of α and ω-oxidation. Fatty acid methyl ester patterns showed the predominance of corresponding fatty acid when grown on specific n-alkane. Sulfate was necessary for growth as shown by using non-hydrocarbon source as well as for crude oil degradation.

Methionine supported growth of the organism whereas cysteine repressed the growth of the organism individually as well as in the presence of sulfate suggesting feedback inhibition. Paraffinized fertilizers increased the degradation of crude oil by 8%. Entrapped cells degraded 10% more crude oil than free cells for 7 cycles of 4 days each.

CHAPTER 4

Production of Emulsifiers by *Rhodococcus* sp. NCIM 5126

SUMMARY

Rhodococcus sp. NCIM 5126 produced extracellular emulsifier in the presence of hydrocarbons, during exponential phase of growth. Under optimal conditions, 225 mg/50 ml emulsifier was produced in Artificial Sea Water supplemented with 35 mM N as urea, 0.114 mM P as K_2HPO_4, 1% (w/v) n-tetradecane, pH 8.0 at 30°C and 150 rpm. Only 35 mg/50 ml emulsifier was produced when the organism was grown in deionised water medium. The surface tension of the broth was reduced from 66 dynes/cm to 32 dynes/cm.

Pseudosolubilization of hydrocarbon appears to be the mode of uptake of alkane by this organism. In the presence of the supernatant of n-tetradecane grown *Rhodococcus* sp., the solubility of n-tetradecane increased from normal aqueous solubility of $7x10^{-3}$ mg/l to 50 mg/l.

The emulsifier was concentrated by ultrafiltration and isolated by Sepharose-4B gel filtration. It was a glycolipid with about 92% lipid and 5% carbohydrate. Depending on the supplied n-alkane, corresponding fatty acid was incorporated in the lipid fraction of the emulsifier. In all cases, trehalose was the carbohydrate moiety.

Emulsifiers produced on n-tetradecane and pristane were stable from pH 6 to 11 and at temperatures from 10 to 80°C. 100 μg of the emulsifier in 5 ml of total volume of assay, gave 1 unit activity. 1 unit of emulsifier activity is defined as the amount of emulsifier producing an absorbance of 1.0 at 410 nm under the assay conditions. Critical Micelle Concentration (CMC) was 12 mg/l.

INTRODUCTION

Water is the most abundant and important substance in living systems. The dipole has a tendency to ionize to H^+ and OH^-. The nearly tetrahedral arrangement of orbitals about the oxygen atom allows each water molecule to form hydrogen bonds with neighbouring water molecules. These hydrogen bonds are constantly and rapidly being broken and formed. Polar molecules such as NaCl, dissolve readily in water because they can replace energetically favourable water–water interactions with even more favourable water–solute interactions. In contrast when hydrocarbon is added into water, these hydrophobic molecules interfere with the hydrogen bonding among water molecules. Water tends to repel the non-polar solute molecules, which then cluster together as a separate layer. Thus the clustering of hydrocarbon molecules in water arises from the unusually strong mutual attraction of water molecules and not from mutual attraction of non-polar solute molecules (Lehninger *et al.*, 1993).

Since hydrocarbons are utilized by microbes at the oil/water interface, the oil/water interface itself becomes the limiting factor for the rate of biodegradation (van der Linden, 1978). Emulsifiers enhance the rate of biodegradation because they are amphipathic molecules containing a hydrophilic 'head' group and a hydrophobic 'tail'. In the presence of immiscible liquids, the emulsifiers orients itself appropriately and lowers the surface tension of water promoting dispersion and emulsification as droplets of the hydrocarbon (Swisher, 1987). This increases the surface area of the dispersed material, which is now accessible to the microorganism for degradation. Owing to their amphipathic nature, emulsifiers tend to associate at surfaces between different polarity phases such as air/water, water/oil or water/solid where they reduce surface tension, interfacial tension or bring about wetting. The terms 'surfactant' and 'emulsifier' are often used as synonyms in biological systems: the difference being that surfactants have a defined molecular structure whereas the term 'emulsifiers' describes all surface active compounds secreted by cells to facilitate uptake of insoluble substrates (Fiechter, 1992). We have preferred to use the term 'emulsifier' in our studies.

In dilute aqueous solution, emulsifier molecules orient themselves at interfaces as monomers. At a certain concentration these molecules in solution associate to form larger units called micelles. The concentration at which this association phenomenon

occurs is known as critical micelle concentration (CMC) (McBain and Salmon, 1920). Addition of emulsifier to the solution beyond the CMC gives rise to further micelles, keeping surface tension properties constant. Emulsifier at these concentrations can solubilize large quantities of non-polar molecules such as hydrocarbons. This is known as pseudosolubilization. In micelles the hydrophilic group is extended into the water and the hydrophobic end is hidden within the structure. Hydrocarbons and other non-polar compounds get incorporated in the micelle interiors whereas polar compounds may occupy positions at the micelle surfaces. When one liquid phase is dispersed as microscopic droplets in another liquid continuous phase, an emulsion is formed. In emulsions the size of micelles are in the range of 0.03–10 μm (Kaccholz and Schlingmann, 1987). The emulsification activity is assayed by measuring the turbidity generated due to micelle formation by the addition of emulsifier to oil/water systems (Rosenberg *et al.*, 1979 a; Reddy *et al.*, 1983).

Chemical surfactants are useful in oil recovery, cleaning of oil spills, paper and pulp industry, leather industry. Gogarty and Olson (1962) patented the combination of alkylnaphthenic acid as surfactant and isopropyl alcohol as cosurfactant for secondary oil recovery from oil well. In chemical surfactants, the hydrophobic group consists of fatty acids, paraffins, alkanes, alkylbenzenes or alkylphenols and the hydrophilic group may be a sulfonate, sulfate, carbohydrate, polypeptide or carboxylate. Depending upon the nature of the hydrophilic group they are classified as anionic, nonionic or cationic. Natural surfactants of plant and animal origin are also known. The plants produce saponines and sulpholipids. In animals lecithin (phosphatidylcholine) is present in membranes. Lung surfactant found at the air/liquid interface of lung alveoli is a complex mixture of phospholipids, proteins, carbohydrates and lipids and is necessary for the normal inflation and dilation mechanism of lungs, its deficiency can lead to alveolar collapse in premature babies. Sodium salts of taurocholate and deoxycholate are bile salts, the surface-active secretions of the liver in higher animals, essential for the emulsification of digested food (Swisher, 1987). Microorganisms are also known to produce emulsifiers. Bacteria, yeasts and actinomycetes producing bioemulsifiers are listed in Table 4.1.

Table 4.1 Microorganisms producing emulsifiers

Organism	Type of emulsifiers	Reference
Bacteria		
Bacillus licheniformis	peptide lipid	Lin *et al.* (1994)
B. subtilis	surfactin	Arima *et al.* (1968)
B. subtilis	subtilisin	Bernheimer and Avigad (1970)
B. brevis	gramicidins	Maraheil *et al.* (1977)
B. polymyxa	polymixins	Suzuki *et al.* (1965)
Thiobacillus thioxidans	phospholipids	Beeba and Umbreit (1971)
Acinetobacter calcoaceticus	emulsan	Rosenberg *et al.* (1979a, b)
Pseudomonas aeruginosa	Rhamnolipid	Guerra-Santos *et al.* (1986)
P. aeruginosa	protein P A	Hisatsuka *et al.* (1972, 1977)
P. fluorescens	carbohydrate-protein-lipid	Desai *et al.* (1988)
Serratia marcescens	serrawettin	Matsuyama *et al.* (1991)
Yeasts		
Candida antarctica	mannosylerythritol lipid	Kitamoto *et al.* (1993)
C. bogoriensis	sophoroside	Cutler and Light (1979)
C. tropicalis	mannan lipid	Kappeli *et al.* (1984)
C. lipolytica	liposan	Cirigliano and Carman (1985)
Saccharomyces cerevisiae	mannoprotein	Cameron *et al.* (1988)
Torulopsis apicola	sophorose lipid	Hommel *et al.* (1987)
T. bombicola	sophorose lipid	Gobbert *et al.* (1984)
Ustilago sp.	cellobiolipid	Boothroyd *et al.* (1956)
Actinomycetes		
Rhodococcus erythropolis	trehalose lipid	Rapp *et al.* (1979)
Nocardia erythropolis	trehalose lipid	Margaritis *et al.* (1979)
Corynebacterium lepus	fatty acids	Cooper *et al.* (1978)
C. hydrocarboclastus	fatty acids	Zajic *et al.* (1977)
Mycobacterium sp.	trehalose lipid	Cooper *et al.* (1989)

Bioemulsifiers can be produced in the exponential phase of growth, in the stationary phase or by resting cells. The production of rhamnolipid by *Pseudomonas aeruginosa* 44T (Robert *et al.*, 1989), glycoprotein AP-6 by *P. fluorescens* 378 (Persson *et al.*, 1988) and surface active agents by *Bacillus cereus* and *Bacillus* sp. IAF-343 (Cooper and Goldenberg, 1987) is growth-associated. Espuny *et al.* (1996) reported growth-associated emulsifier production by *Rhodococcus* sp. whereas Singer and Finnerty (1990) reported stationary phase emulsifier production by a different strain of *Rhodococcus*.

Velankar *et al.* (1975) presented a model, in which presence of micelles of surface-active agents was essential for transport of hydrocarbons to growing cells of *Pseudomonas aeruginosa*. Reddy *et al.* (1982, 1983) reported predominant role of hydrocarbon solubilization in yeasts and *Pseudomonas* PG-1. They isolated crude ES factor comprising of emulsifier and solubilizing factor from *Pseudomonas* sp. growing on pristane. They proposed that solubilizing factor was essential for substrate solubilization and transport to cells and the role of emulsifying factor could be the facilitation of the action of the solubilizing factor on the substrate. Goswami and Singh (1990) studied mode of n-hexadecane uptake by *Pseudomonas* M1 and *Pseudomonas* N1. They showed that cells of *Pseudomonas* M1 were attached to the hydrocarbon droplets and substrate uptake was facilitated without extracellular emulsifier. Growth of *Pseudomonas* N1 was associated with the production of extracellular emulsifier which was a lipoprotein and a pseudosolubilizing factor which was a glycoprotein. The synergistic action of these factors was responsible for good growth of the cells.

Major classes of bioemulsifiers include glycolipids, lipopeptides and lipoproteins, phospholipids and fatty acids, polymeric emulsifiers and particulate emulsifiers. Among these, the best studied are glycolipids, which include rhamnolipids from *Pseudomonas*, sophorose lipids from *Torulopsis bombicola* and trehalose lipids from actinomycetes *Rhodococcus, Nocardia, Mycobacteria* (Desai and Banat, 1997). The biosynthesis of glycolipids is possible in 4 different ways (Syldatk and Wagner, 1987): i) constitutive synthesis of both the carbohydrate and the lipid portion as shown in rhamnolipid production by *Pseudomonas* (Burger *et al.*, 1963). ii) constitutive synthesis of the sugar moiety and hydrocarbon dependent chain length of the lipid moiety as in trehalose lipid production by *Rhodococcus* sp. (Espuny *et al.*, 1996). iii) constitutive production of the lipid moiety and substrate influence on the sugar moiety as in *Arthrobacter paraffineus* (Suzuki *et al.*, 1969, 1974) and iv) substrate dependent synthesis of both sugar and lipid portions.

Rhodococcus is known to produce glycolipid emulsifier with trehalose as a sugar moiety and long chain aliphatic fatty acid or hydroxy aliphatic fatty acid as a lipid, when grown on n-alkanes. The trehalose lipid is also known as 'cord factor', which contains disaccharide trehalose linked to mycolic acids. It differs from organism to organism in

size, number of carbon atoms and degree of unsaturation (Desai, 1987). Although there are several reports of *Rhodococcus* sp. producing trehalose mycolic acid emulsifiers (Rapp *et al.*, 1979; Kretschmer *et al.*, 1982; Singer *et al.*, 1990), some authors have reported that rhodococcal emulsifiers do not have mycolic acid in their structure. Of these, Uchida *et al.* (1989 a) reported mono and disuccinoyl trehalose lipids from *Rhodococcus erythropolis* SD-74 on the basis of ^{1}H-NMR spectra. Espuny *et al.* (1996) reported trehalose tetraester from *Rhodococcus* sp. 51T7 on the basis of ^{1}H-NMR and ^{13}C-NMR spectra. Most of the work has been carried out using strains of *Rhodococcus erythropolis*, isolated from soils or oil containing soils.

In this chapter, we describe the growth conditions for emulsifier production, mode of uptake of alkane, isolation and characterization of emulsifiers produced by *Rhodococcus* sp. NCIM 5126.

MATERIALS AND METHODS

MATERIALS

Eicosane, 1-Methylnaphthalene (Aldrich Chemicals Co., USA), Pristane, Sepharose-4B (Sigma Chemicals Co., USA), n-Tetradecane (Koch-Light Laboratories Ltd., UK), n-alkanes (SD Fine, India) were used. All chemicals and solvents were analytical grade.

METHODS

Organism, growth media and cultivation

All the studies regarding emulsifier production by *Rhodococcus* sp. NCIM 5126 were carried out in 250 ml flasks containing 50 ml medium. Artificial Sea Water containing nitrogen and phosphorus sources and 1% (w/v) alkane as sole source of carbon was inoculated with "preinoculum" of *Rhodococcus* sp. NCIM 5126 developed in a manner similar to that described in chapter 3. The flasks were incubated at 30°C for 5 days at 150 rpm.

Determination of emulsifier activity

After incubation, the culture broth was centrifuged at 10,000 rpm for 10 minutes and the supernatant was passed through a Whatman No.1 filter paper. The filtrate was used to determine emulsifier activity. 20 µl aliquot of crude broth was diluted to 5 ml with distilled water. After the addition of 0.1 ml of benzene, toluene, cyclohexane, n-tetradecane, n-hexadecane, pristane or 1-methyl naphthalene, the mixture was vortexed for 1 minute, allowed to stand for 1 hr at room temperature (ambient temperature 28–30°C) and absorbance at 410 nm was recorded. 1 unit of emulsifier activity is defined as the amount of crude broth producing an absorbance of 1.0 at 410 nm under these conditions. Toluene gave maximum emulsifier activity of 20 units/ml and 0.1 ml of toluene was used as the standard hydrocarbon for measuring emulsifier activity.

The emulsifier was isolated by centrifuging the cells grown on hydrocarbons at 10,000 rpm and concentrated using a YM–10 membrane. The concentrate was dialyzed thrice against 1000 ml double distilled water and 1 ml sample was loaded on a Sepharose-4B gel filtration column (25 cm x 1 cm) equilibrated with phosphate buffer

(20 mM, pH 7.0) containing 100 mM NaCl. The same buffer was used for elution and 1 ml fractions collected. Emulsifier activity was determined using 0.1 ml toluene as standard. Absorbance at 660 nm was also measured by diluting the samples to 10 times (Fig. 4.2). Active fractions were pooled, concentrated and dialyzed thrice against 500 ml double distilled water. Emulsifier was lyophilized to dry powder.

20 μg to 140 μg and 20 to 200 μg of the lyophilized emulsifier was used to determine emulsification activity and surface tension reduction measurement respectively. Weight of emulsifier plotted against activity (Fig. 4.6) showed that 100 μg of emulsifier corresponded to an absorbance of 1.0 at 410 nm. Activity units were converted to mg of emulsifier for total volume of the broth after filtration through Whatman No.1 paper (50 ml).

Time course for emulsifier production

Using optimal conditions for crude oil degradation, protein specific biomass, emulsifier production and hydrocarbon degradation were studied. Amount of protein was measured by the method of Lowry *et al.* (1951). Emulsification activity was determined as described earlier. Residual alkane in the medium was extracted in chloroform and quantified on a Shimadzu model GC–RIA Gas Chromatograph on a SE–30 stainless steel column, using FID with an isothermal program at 150°C. Uninoculated control was treated in a similar way. The surface tension of the broth was measured using Nima Trough Model 611 tensiometer. The hydrocarbon-grown cells were checked for attachment to hydrocarbon droplet with a Leitz microscope (400x).

Determination of pseudosolubilization activity

0.38, 0.76, 1.52 and 1.9 μg of n-tetradecane were injected onto SE–30 column in Shimadzu Gas Chromatograph, program and conditions as described earlier. A standard graph of area under the peak versus μg of n-tetradecane was plotted. This graph was used to determine unknown concentration of n-tetradecane. Psudosolubilization activity was determined in accordance with Reddy *et al.* (1983). Cell free culture broth of n-tetradecane grown cells was filtered through 0.22 μ Millipore filter. This supernatant was used to determine pseudosolubilizing activity. 1% (v/v) n-tetradecane was added to 20 ml supernatant and flask was kept on shaker at 150 rpm for 5 minutes. This

hydrocarbon-saturated mixture was taken in a separating funnel, kept for 10 minutes to separate the phases and lower aqueous phase (upto 18 ml) containing solubilized hydrocarbon was filtered through 0.22 μ Millipore filter. The filtrate containing solubilized hydrocarbon was extracted in 3 volumes of chloroform, concentrated to 0.5 ml and 5 μl was used to determine area under the peak. The amount of n-tetradecane solubilized is given as mg/l of the culture broth.

Composition of the emulsifier

The emulsifier was refluxed in ethyl methyl ketone at 80°C for 1 hr and the lipid fraction was quantified gravimetrically (Reddy *et al.*, 1983). Fatty acid methyl esters prepared in accordance with Zuckerberg *et al.* (1979) were chromatographed on Shimadzu, GC–MS QP5000 on 30 m SE–30 capillary column. Injector and detector were at 230°C. Initial temperature of the column was 140°C maintained for 2 minutes followed by a temperature increase of 20°C per minute and a final temperature 250°C held for 10 minutes. The carrier gas was helium, injector mode split ratio was 70. Fatty acids were identified on the basis of fragmentation pattern obtained with the data stored in NIST62.lib.

For the isolation of sugar moiety, the emulsifier was kept in 0.1 M methanolic KOH at 50°C for half an hour, made acidic and extracted in diethyl ether (Singer *et al.*, 1990). The aqueous phase was concentrated and sugar was qualitated by the method of Collmer *et al.* (1988). Identification of the sugar was carried out by Infra-red spectroscopy using FT–IR, ATI–Mattson (UK), Model Research Series 1. Total carbohydrate content was estimated by the phenol sulphuric acid method of Dubois *et al.* (1956) using trehalose as a standard.

^{13}C-high resolution CPMAS (Cross Polarization Magic Angle Spinning) NMR spectra of lyophilized emulsifier was scanned on Brucker MSL–300 spectrometer operating at 75.476 MHz for ^{13}C.

Properties of emulsifier

The pH stability of the emulsifiers produed on n-tetradecane and pristane was determined by incubating samples of known activity in 100 mM buffers of pH ranging

from pH 2.0 to 7.0 in Citrate phosphate buffer; pH 7.0 to 9.0 in Tris HCl buffer, pH 9.0 to 11.0 in Glycine–NaOH buffer for 24 hr.

The emulsifier samples were incubated at temperatures 10 to 100°C for 1 hr to determine temperature stability.

Effect of culture conditions on emulsifier production

$(NH_4)_2SO_4$, NH_4Cl, NH_4NO_3, $NaNO_3$ and urea were individually supplied in the Artificial Sea Water to give 70 mM nitrogen. Concentration of the best nitrogen source (urea) was varied from 7, 21, 35, 49, 70 and 89 mM.

1% (w/v) glucose or fructose or C_{11} to C_{20} n-alkanes were used as sole source of carbon in the Artificial Sea Water containing 70 mM N as urea, 0.114 mM P as K_2HPO_4 at pH 8.0 which were also the optimum conditions for degradation of supplied hydrocarbons. The concentration of the best carbon source was varied from 0.2 to 1.2% (w/v).

The organism was incubated at 17°C, 30°C, 37°C and 50°C with optimized carbon and nitrogen sources. Initial pH of the medium was adjusted to 4.0, 6.0, 8.0, 10.0 and 12.0. Effect of aeration was determined by varying agitation rate from 0 to 200 rpm.

Using optimal conditions emulsifier production by *Rhodococcus* sp. was compared in deionised water medium and Artificial Sea Water supplemented with C, N and P sources.

RESULTS AND DISCUSSION

Crude oil and hydrocarbon degradation by *Rhodococcus* sp. NCIM 5126 was associated with extracellular emulsifier production. The ability of the crude emulsifier to emulsify various hydrocarbons is given in Table 4.2.

Table 4.2 Selection of the best hydrocarbon for emulsifier activity

Test Hydrocarbon	Emulsifier activity (U/ml)
Benzene	8±0.5
Toluene	20±1.0
Cyclohexane	5±0.4
n-Tetradecane	3±1.0
n-Hexadecane	3±1.0
1-Methyl naphthalene	9±1.5
Pristane	2±0.5

Assay: Aliquot of crude emulsifier diluted to 5 ml, 0.1 ml test hydrocarbon added, vortexed for 1 minute and tubes were kept for 1 hour at room temperature. A_{410} recorded.

Although the organism used n-alkane for growth and emulsifier production, the emulsifier was better able to form stable emulsions with aromatic hydrocarbons rather than with straight chain compounds. Similar results have been reported by Singh and Desai (1988) for emulsifier produced by *Acinetobacter calcoaceticus* and *Arthrobacter paraffinieus* and Zinjarde (1996) for emulsifier produced by *Yarrowia lipolytica*. Emulsifier produced by *Pseudomonas* sp. could emulsify aliphatic as well as branched chain hydrocarbons (Reddy *et al.*, 1983). *Acinetobacter* RAG-1 could not emulsify either pure aliphatic or pure aromatic hydrocarbons but only the mixtures of these (Rosenberg *et al.*, 1979 b).

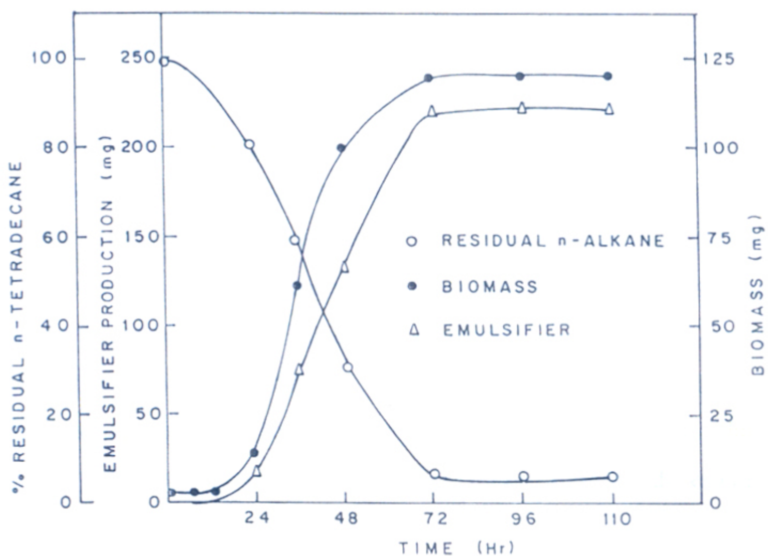
Time course for emulsifier production in the optimized medium

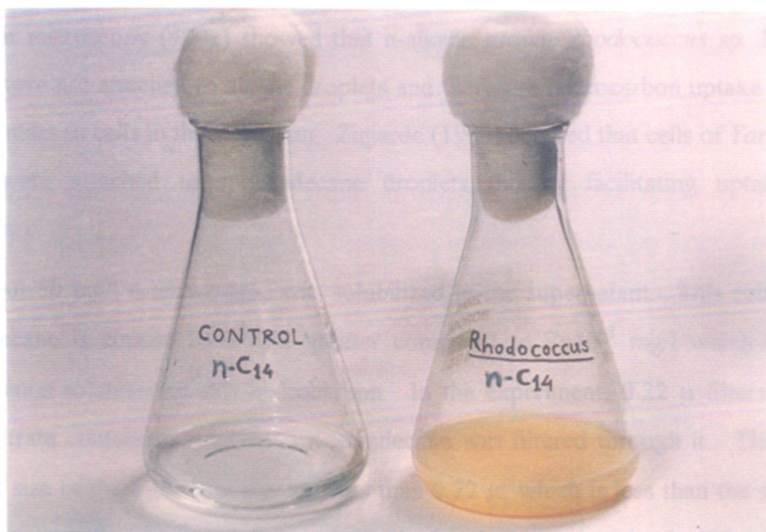
Emulsifier production by *Rhodococcus* sp. was growth-associated (Fig. 4.1). Cells entered the exponential phase after 12 hr with a protein specific growth rate of 0.087 hr^{-1}. Simultaneously emulsifier production entered the exponential phase after 12 hr with a production rate of 0.095 hr^{-1}. Under the growth conditions used, maximum emulsifier production of 225 mg was obtained after 72 hr. At this time only 6% residual

Figure 4.1

Time course of emulsifier production by *Rhodococcus* sp. NCIM 5126 grown on n-tetradecane

The organism was grown in Artificial Sea Water containing 1% (w/v) n-tetradecane, 35 mM N as urea, 0.114 mm P as K_2HPO_4, pH 8.0. Flasks were incubated at 30°C for 5 days at 150 rpm.





n-tetradecane was present in the medium. Simultaneous growth and emulsifier production in *Rhodococcus* sp. 51T7 and *Corynebacterium* sp. has been reported (Zajic *et al.*, 1977; Espuny *et al.*, 1996). However, our *Rhodococcus* sp. differs from *R. erythropolis* DSM 43215 in which cell growth is not associated with increase in trehalose-lipid (Rapp *et al.*, 1979). It also behaves differently compared to *R. erythropolis* and *Rhodococcus* sp. H13-A, both producing emulsifier in the stationary phase under the conditions of nitrogen limitation (Ristau and Wagner, 1983; Singer and Finnerty, 1990).

Surface tension of *Rhodococcus* sp. grown n-tetradecane broth was reduced from 66 dynes/cm to 32 dynes/cm. Kretschmer *et al.* (1982) reported surface tension reduction to 35 dynes/cm by *R. erythropolis*. *Nocardia* (30 dynes/cm, Margaritis *et al.*, 1979), *Corynebacterium* (40 dynes/cm, Margaritis *et al.*, 1979) and *Mycobacterium* (38 dynes/cm, Cooper *et al.*, 1981) are known to reduce surface tension of the broth in the range of 30–40 dynes/cm. The fall in the surface tension of the broth by our organism is comparable with the reported values of other nocardioform actinomycete. The surface tension reduction by a chemical surfactant Tween 80 (0.25 g/50 ml medium) was 41 dynes/cm which is less compared to reduction obtained by our organism. However, purified emulsifier reduced the surface tension of water from 72 dynes/cm to 56 dynes/cm only.

Mode of uptake of hydrocarbon by *Rhodococcus* sp. NCIM 5126

Light microscopy (400x) showed that n-alkane grown *Rhodococcus* sp. NCIM 5126 cells were not attached to alkane droplets and therefore hydrocarbon uptake is not by direct contact to cells in this organism. Zinjarde (1996) showed that cells of *Yarrowia lipolytica* were attached to n-hexadecane droplets thereby facilitating uptake of hydrocarbon.

About 50 mg/l n-tetradecane was solubilized in the supernatant. This solubility of n-tetradecane is almost 1000 fold greater compared to $7x10^{-3}$ mg/l which is the normal aqueous solubility of this hydrocarbon. In the experiment, 0.22 μ filters were used and filtrate containing solubilized n-tetradecane was filtered through it. This fact implies that size of the n-tetradecane was less than 0.22 μ, which is less than the size of the *Rhodococcus* cells (about 1–1.5 μ as shown in the electron micrographs, Chapter 2).

Roy *et al.* (1979) also have reported particle size between the range of 0.22 and 0.45 μ for n-dodecane solubilized in the cell-free culture medium of *Endomycopsis lipolytica*. The high growth rate of 0.087 hr^{-1} and 94% degradation of n-tetradecane or 50% degradation of aliphatic fraction of crude oil in case of *Rhodococcus* sp. NCIM 5126 can be attributed to pseudosolubilization.

The supernatant of n-tetradecane grown cells, which acts as a pseudosolubilizing factor consists of dioic acid (chapter 3). The emulsifier produced by this organism is a glycolipid. Apparently two chemically different factors are produced by *Rhodococcus* sp. one of which is the emulsifier and the second which is a solubilizing agent. Goswami and Singh (1991) reported pseudosolubilization during the growth of *Pseudomonas* N1. They isolated an emulsifying factor (lipoprotein) and a pseudosolubilizing factor (glycoprotein). They proposed that initial dispersion of hydrocarbon brought about by emulsifying factor and agitation would be necessary for pseudosolubilizing factor to act efficiently and the synergistic action of emulsifying and pseudosolubilizing factors may provide enhanced transport of hydrocarbon to cells. In case of *Rhodococcus* sp., the surface tension reduction to 32dynes/cm by emulsifier was necessary to break the barrier between water/hydrocarbon interface resulting in micelle formation and the dispersion of hydrocarbon, thereby increasing the surface area of hydrocarbon for the microbial attack. Further solubilization of the hydrocarbon may be brought about by the pseudosolubilizing factor. Furthermore, in the presence of hydrocarbons, *Rhodococcus* cells were more lipophilic and according to Boulton and Ratledge (1987) increased lipophilicity is a prerequisite for hydrocarbon uptake and intracellular transport. Thus a combination of pseudosolubilizing factor, emulsifier and increased lipophilicity is necessary for the uptake of hydrocarbons by this species of *Rhodococcus*. Both the emulsifier and pseudosolubilizing factor did not support growth of *Rhodococcus* sp. when supplied separately as sole source of carbons. Although there are several reports on emulsifier production by *Rhodococcus* strains (Uchida *et al.*, 1989 a, b; Singer and Finnerty, 1990; Singer *et al.*, 1990; Espuny *et al.*, 1996), these studies primarily deal with structure elucidation of the emulsifier.

Composition of emulsifier

The emulsifier eluted from a Sepharose-4B column (Fig. 4.2) is a glycolipid. About 92% of the compound is lipid in nature. Identification of methyl esters of fatty acids on GC–MS showed that tridecanoic acid, tetradecanoic acid, hexadecanoic acid, heptadecanoic and 2,6,10,14-tetramethylpentadecanoic acid were each dominant when the corresponding straight or branched alkane was provided in the growth medium. The Infra-red spectra (Fig.4.3) of standard trehalose matched with the non-reducing sugar in the water-soluble fraction of the refluxed material. The total carbohydrate content was 5% as determined by phenol sulfuric acid method of Dubois *et al.* (1956). Free trehalose was not present in the supernatant of either fructose or n-alkane grown *Rhodococcus* sp.

The absence of mycolic acid makes this emulsifier different from other reported rhodococcal emulsifiers. Rapp *et al.* (1979) reported α,α-trehalose-6,6′-dicorynomycolates when grown on n-hexadecane whereas α,α-trehalose-6-monocorynomycolates have been reported by Kretschmer *et al.* (1982). Singer *et al.* (1990) reported trehalose and C_{10} to C_{22} saturated and unsaturated fatty acids, C_{35} to C_{40} mycolic acids, hexanedioic acid, dodecanedioic acid, 10-methyl hexadecanoic and 10-methyl octadecanoic acids in the emulsifier by n-hexadecane grown *Rhodococcus* sp. H13-A. Although, Ristau and Wagner (1983) and Uchida *et al.* (1989 a, b) reported mycolic-acid-free-emulsifiers from *Rhodococcus*, these contained succinoyl moieties, which are not present in the emulsifier reported here. Bailey and Bailey (1997) reported *R. erythropolis* ST-2 producing glycoprotein emulsifier in the presence and absence of crude oil.

Syldatk and Wagner (1987) have suggested 4 different possibilities for the synthesis of biosurfactants. Emulsifier produced by this species of *Rhodococcus* can be categorized in the second group where the trehalose moiety is constitutively produced and the chain length of the fatty acid of glycolipid depends upon the supplied alkanes. Change in composition of the lipid fraction depending upon the carbon source has also been reported in *Rhodococcus* sp. 51T7 by Espuny *et al.* (1996).

Boulton and Ratledge (1987) discussed the intermediary metabolism related with biosurfactant precursor synthesis from hydrocarbon substrates. The oxidation of alkanes

Figure 4.2

Sepharose-4B gel filtration profile of the emulsifier

Sepharose-4B gel filtration column was equilibrated with phosphate buffer (20 mM, pH 7.0) containing 100 mM NaCl. 1 ml crude emulsifier was loaded and eluted using the same buffer. 1 ml fractions were collected, diluted to 1:10 and A_{660} was measured. Emulsification assay was carried out as described in Materials and Methods.

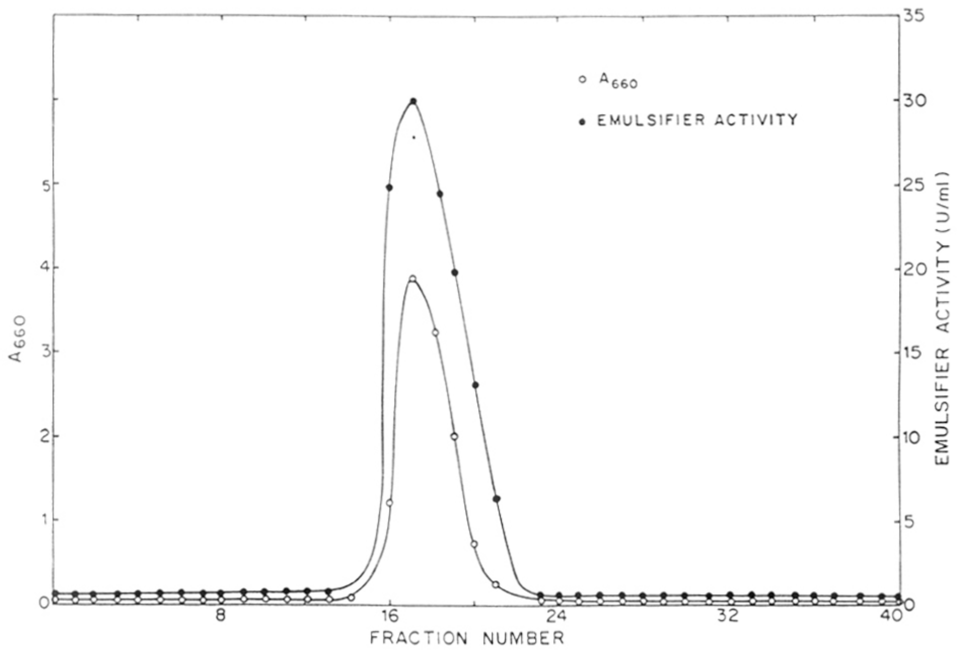
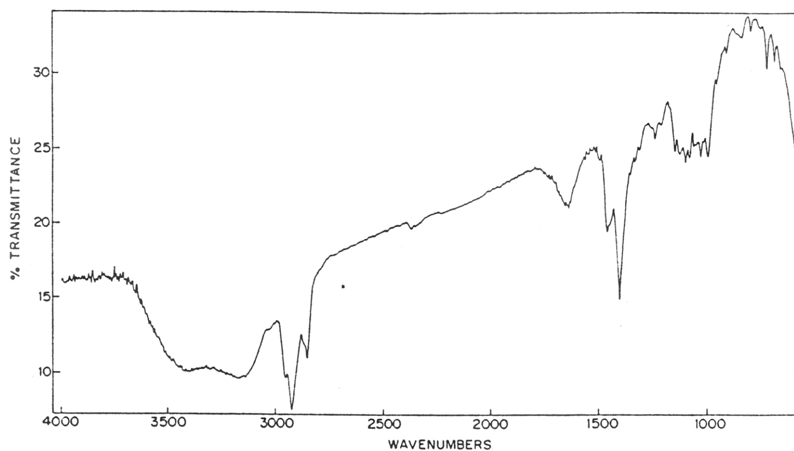


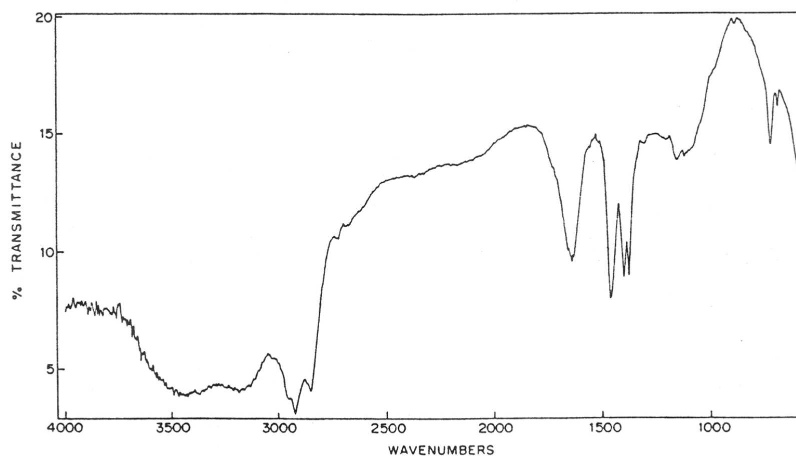
Figure 4.3

Infra-red spectra of the standard trehalose and test sample

A) Standard Trehalose



B) Aqueous portion of n-Tetradecane grown emulsifier



gives rise to corresponding fatty acids. These may be modified and incorporated into surfactants. Also, operation of another pathway must occur for the synthesis of carbohydrate moiety. It includes the oxidation of fatty acid via β-oxidation to form acetyl-CoA (or propionyl-CoA in the case of odd-numbered fatty acids). From acetyl-CoA, glucose 6-phosphate, a precursor for polysaccharide synthesis will form through a series of gluconeogenesis reactions. The linkage of lipid and carbohydrate precursors will result in the formation of glycolipid.

Lang and Wagner (1987) proposed that when saponification gives rise to water-soluble carbohydrate and water insoluble lipid moieties, it confirms the ester bond between the two. In this case also after mild treatment with methanolic KOH at 50°C for half an hour, to the emulsifier, we could isolate carbohydrate moiety. ^{13}C-NMR spectra conformed the presence of ester bond between lipid and carbohydrate moieties.

Properties of emulsifier

As shown in Fig. 4.4, emulsifiers obtained by growing *Rhodococcus* sp. on n-tetradecane and pristane were almost 100% stable in the pH range of 6.0 to 11.0. At pH 2.0, 35% activity and 52% activity was retained in case of n-tetradecane and pristane respectively. Drop in the activity of the emulsifier below pH 5.0 can be attributed to the protonation of a carboxyl group as described by Rosenberg *et al.* (1979 a). Singer *et al.* (1990) also have reported surface activity at pH values between 4.0 to 12.0 whereas an unusual emulsifier produced by *Rhodococcus erythropolis* was stable from pH 1.0 to 14.0 (Kretschmer *et al.*, 1982).

n-Tetradecane and pristane emulsifiers were stable at temperatures 10 to 80°C (Fig.4.5) retaining full activity. At 100°C, 60% and 75% activity was respectively retained. Emulsifier reported by Kretschmer *et al.* (1982) is also stable upto 90°C whereas emulsifier discussed by Singer *et al.* (1990) is stable at 120°C.

As shown in Fig. 4.6, increasing concentrations of emulsifier resulted in increased emulsifier activity upto 100 µg, which gave 1 unit activity. Surface tension was reduced to 56 dynes/cm at a concentration of 60 µg of emulsifier per 5 ml, then it did not decrease further giving a CMC of 12 mg/l. CMC values of 1 to 165 mg/l has been reported for rhodococcal emulsifiers (Kretschmer *et al.*, 1982; Singer *et al.*, 1990).

Figure 4.4

pH stability of the emulsifiers

Samples of the emulsifiers were incubated in 100 mM buffers of pH ranging from 2.0 to 11.0 (pH 2.0 to 7.0–Citrate phosphate buffer, pH 7.0 to 9.0–Tris HCl buffer, pH 9.0 to 11.0–Glycine NaOH buffer) for 24 hr and emulsification assay was carried out as described in materials and Methods.

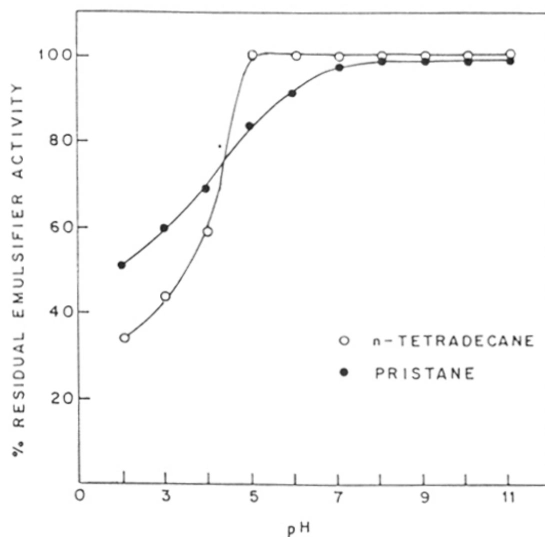


Figure 4.5

Temperature stability of the emulsifiers

Samples of the emulsifiers were incubated at temperatures 10 to 100°C for 1 hr and emulsification assay was carried out as described in Materials and Methods.

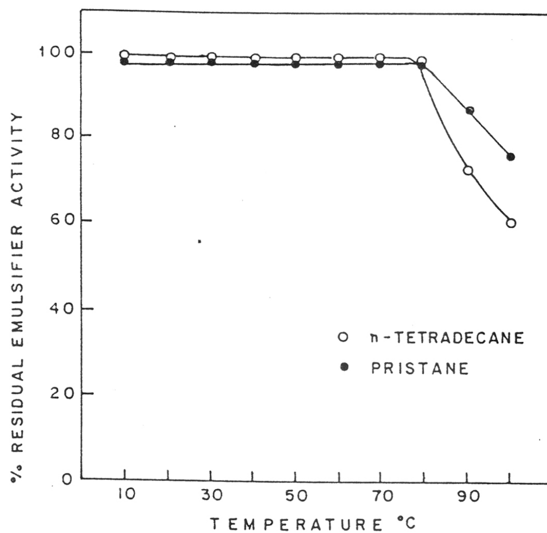
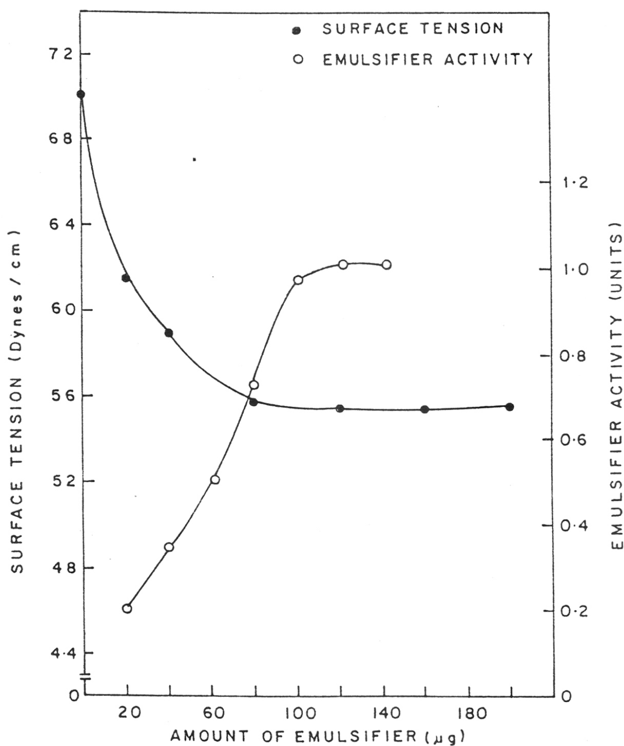


Figure 4.6

Relationship between amount of emulsifier, emulsifier activity and surface tension reduction

20 μg to 140 μg of emulsifier were used in a final volume of 5 ml of emulsification assay and activity units were determined. 20 to 200 μg of emulsifier were used for surface tension reduction measurement.



Effect of culture conditions on emulsifier production

To determine the effect of culture conditions on emulsifier production, nutritional and environmental conditions were varied. In every case, simultaneous production of biomass and emulsifier was observed.

This strain of *Rhodococcus* did not use ammonium salts as nitrogen source for growth and emulsifier production (Table 4.3). Incorporation of NH_3 and efflux of H^+ during ammonium ion metabolism resulted in the lowering of pH of the medium and subsequently it affected emulsifier production. Similar results have also been reported for *Corynebacterium* species in which surfactant production was hampered at higher concentrations of $(NH_4)_2SO_4$ following the drop in pH of the medium (Gerson and Zajic, 1979). Only urea and sodium nitrate supported emulsifier production presumably because this organism is urease and nitrate reductase positive.

Table 4.3 Effect of nitrogen source on emulsifier production

Nitrogen source (70 mM N)	Emulsifier production (mg)	Biomass (mg)
$(NH_4)_2SO_4$	-	-
NH_4NO_3	-	-
NH_4Cl	-	-
Urea	190±1.5	90
$NaNO_3$	150±1.0	75

Medium: 1% (w/v) n-tetradecane, 0.114 mM P as K_2HPO_4, nitrogen source at 70 mM concentration in Artificial Sea Water, pH 8.0. Flasks were incubated at 30°C for 5 days at 150 rpm.

R. erythropolis producing emulsifier using ammonium sulfate under growth limiting conditions in the stationary phase has been reported (Ristau and Wagner, 1983; Singer and Finnerty, 1990). Our strain differs in the preference of nature of nitrogen source as well as time of emulsifier release in the medium. Uchida *et al.* (1989 b) and Abu-Ruwaida *et al.* (1991) described *Rhodococcus* sp. which used nitrate as a nitrogen source. Urea was used as a nitrogen source by *Acinetobacter* RAG-1 (Rosenberg *et al.*, 1979 a).

Urea providing 35 mM of nitrogen was optimum for emulsifier production of 225 mg per 50 ml growth medium. 35 mM N as urea was also an optimum concentration for

crude oil degradation by this *Rhodococcus* sp., again emphasizing the interdependence of emulsifier production and hydrocarbon degradation.

Table 4.4 Variation in concentration of nitrogen source

Nitrogen (urea) (mM)	Emulsifier production (mg)	Biomass (mg)
7	-	-
21	40±1.0	15
35	225±1.0	115
49	200±0.5	100
70	190±1.5	90
89	150±1.0	75

Medium: 1% (w/v) n-tetradecane, 0.114 mM P as K_2HPO_4 and various concentrations of urea in Artificial Sea Water, pH 8.0. Flasks were incubated at 30°C for 5 days at 150 rpm.

In general, 70 mM nitrogen (0.1g% N) is provided in the medium. This strain of *Rhodococcus* requires just 35 mM nitrogen as urea for maximum emulsifier production, in contrast to *Acinetobacter* RAG-1 which utilizes almost 88 mM nitrogen as urea (Rosenberg *et al.*, 1979 a).

This *Rhodococcus* sp. did not produce emulsifier using water soluble carbon sources such as glucose or fructose. As shown in Table 4.5, it used only intermediate chain length n-alkanes C_{13} to C_{18} for growth and emulsifier production.

Table 4.5 Effect of carbon source on growth and emulsifier production

Carbon Source (1.0% w/v)	Emulsifier production (mg)	Biomass (mg)
Glucose	-	40
Fructose	-	100
n-C_{11}	-	-
n-C_{12}	-	-
n-C_{13}	60±1.0	30
n-C_{14}	190±1.2	90
n-C_{15}	110±0.3	65
n-C_{16}	95±0.5	60
n-C_{17}	60±1.0	40
n-C_{18}	50±0.5	30
n-C_{19}	-	-
n-C_{20}	-	-

Medium: 70 mM N as urea, 0.114 mM P as K_2HPO_4 with carbon source in Artificial Sea Water, pH 8.0. Flasks were incubated at 30°C for 5 days at 150 rpm.

As *Rhodococcus* sp. did not produce emulsifier when water-soluble substrates were used, emulsifier production is inducible for this organism. Inducible emulsifier production has also been reported for *Corynebacterium* (Cooper *et al.*, 1979). However, Bailey and Bailey (1997) reported constitutive production of emulsifier by *R. erythropolis* ST-2 in the absence of crude oil.

n-Alkanes <10 carbons are more soluble in water than long chain alkanes. Their higher solubility exerts toxic effect on the cells resulting in the poor growth, whereas n-alkanes >18 are inhibitory for the microorganisms. Therefore, microorganisms prefer n-alkanes containing 12–18 carbons. Nocardioform actinomycetes like *Rhodococcus erythropolis* and *Corynebacterium hydrocarboclastus*, produce surface-active agents on intermediate chain length n-alkanes (Rapp *et al.*, 1977). *Rhodococcus erythropolis* strains producing emulsifier using mixtures of n-alkanes ranging from C_{12} to C_{18} or on n-hexadecane are also known (Uchida *et al.*, 1989 b, Singer and Finnerty, 1990).

Emulsifier production was observed only for 0.8, 1.0 and 1.2% (w/v) concentration of n-tetradecane (Table 4.6). 190 mg emulsifier was obtained at 1.0% (w/v).

Table 4.6 Variation in concentration of n-tetradecane

n-tetradecane (% w/v)	Emulsifier production (mg)	Biomass (mg)
0.2	-	-
0.4	-	-
0.6	-	-
0.8	150±1.5	60
1.0	190±1.0	90
1.2	140±2.0	55

Medium: 70 mM N as urea, 0.114 mM P as K_2HPO_4 in Artificial Sea Water with various concentrations of n-tetradecane, pH 8.0. Flasks were incubated at 30°C for 5 days at 150 rpm.

According to Velankar *et al.* (1975) lower substrate concentrations are unable to produce hydrocarbon-saturated micelles sufficient for growth and emulsifier production whereas higher substrate concentrations are inhibitory. Usually optimum concentration of 1–2% of n-alkane has been used for growth of yeasts (Zalashko *et al.*, 1983; Zinjarde, 1996).

Phosphate as K_2HPO_4 was necessary for growth and emulsifier production, although increasing concentration above 0.114 mM P as K_2HPO_4 did not have considerable effect on emulsifier production. Phosphate concentration of 0.114 mM P as K_2HPO_4 was used throughout the studies.

Maximum emulsifier production of 225 mg was observed at 30°C, which was also the optimum temperature for growth (Fig. 4.7). This organism did not grow at 50°C and consequently there was no emulsifier production. Most of the reports for emulsifier production by *R. erythropolis* are at 28–30°C and the present strain is no exception in this respect. However, Abu-Ruwaida *et al.* (1991) reported temperature of 37°C for maximal production of emulsifier by a strain of *Rhodococcus*. Syldatk and Wagner (1987) reported optimal production of trehalose tetraesters by *R. erythropolis* at 22°C while the best growth was at 30°C.

As shown in Table 4.7, maximum emulsifier production (225 mg) as well as growth was observed at pH 8.0, which is also the pH of the seawater.

Table 4.7 Effect of pH on growth and emulsifier production

Initial pH	Final pH	Emulsifier production (mg)	Biomass (mg)
4.0	7.3	115±0.5	60
6.0	7.4	120±0.6	65
8.0	7.5	225±1.0	115
10.0	8.9	125±0.2	60
12.0	10.3	-	-

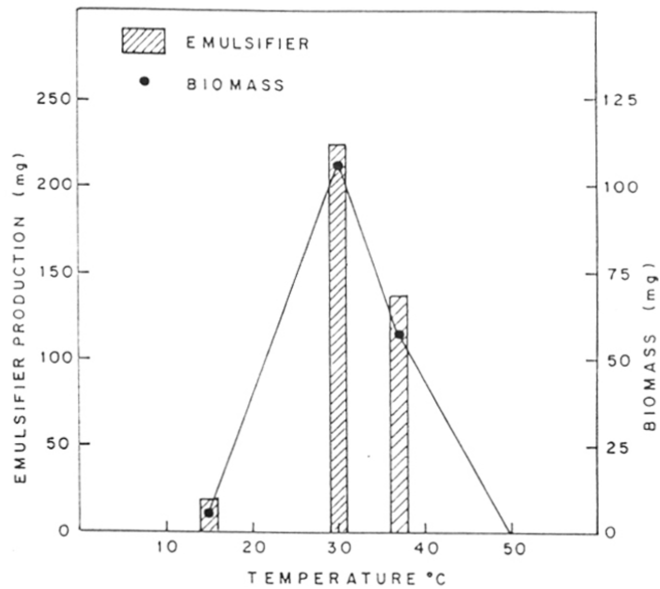
Medium: 1% (w/v) n-tetradecane, 0.114 mM P as K_2HPO_4 in Artificial Sea Water. After autoclaving 35 mM N as urea was added and then initial pH of the medium was adjusted using sterile 1N HCl or 1N NaOH. Flasks were incubated at 30°C for 5 days at 150 rpm.

Emulsifier production was also observed at pH 4.0, 6.0 and 10.0 although in less quantity. Uchida *et al.* (1989 b) reported *R. erythropolis* strain which grew best at pH 7.0 to 7.5 and produced maximum emulsifier at pH 6.8 to 7.0. Most reports state the pH value of 6.8 to 7.0 for emulsifier production by *R. erythropolis*. The present strain prefers alkaline pH for emulsifier production compared to reported strains.

Figure 4.7

Effect of temperature on growth and emulsifier production by *Rhodococcus* sp. NCIM 5126

The organism was grown in Artificial Sea Water containing 1% (w/v) n-tetradecane, 35 mM N as urea, 0.114 mM P as K_2HPO_4, pH 8.0. Flasks were incubated at temperatures 15, 30, 37 and 50°C for 5 days at 150 rpm.



Increase in agitation rate from 0 to 150 rpm, increased biomass as well as emulsifier production (Fig. 4.8). As hydrocarbon utilization is a highly oxidative process, aeration and agitation are necessary, therefore at zero rpm, growth and emulsifier production were not observed. Velankar *et al.* (1975) suggested formation of more micelles at higher agitation rates thereby increasing the number of growth sites. It results in the effective concentration of the substrate in the vicinity of the cells leading to higher growth.

Addition or limitation of Fe^{++}, Mg^{++} or Ca^{++} containing salts is known to influence bioemulsifier production. Emulsifier production of only 35 mg was observed in deionised water medium compared to 225 mg in Artificial Sea Water medium. Growth as well as degradation of n-tetradecane hampered in deionised water medium (Table 4.8).

Table 4.8 Emulsifier production in deionised water and Artificial Sea Water medium

Medium	Emulsifier production (mg)	Biomass (mg)	% degradation of n-tetradecane
Deionised water	35±0.5	25	25
Artificial sea water	225±1.0	115	94

Medium: 1% (w/v) n-tetradecane, 35 mM nitrogen as urea, 0.114 mM P as K_2HPO_4 added in deionised water as well as in Artificial Sea Water to prepare respective media. In both the cases, pH was 8.0. Flasks were incubated at 30°C for 3 days at 150 rpm.

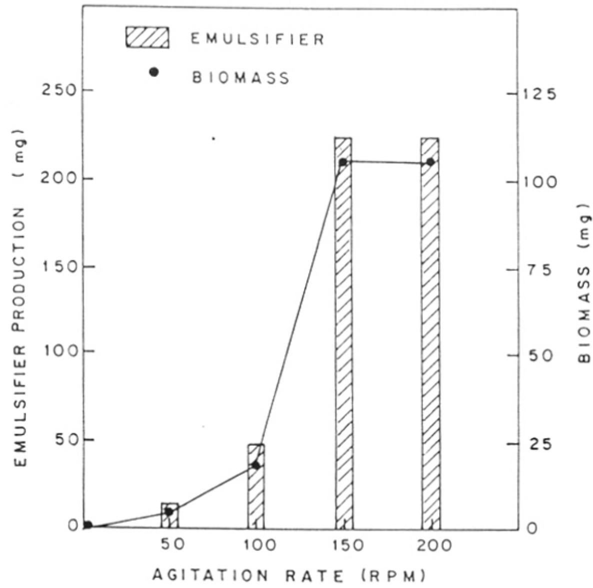
As discussed in chapter 3, this strain of *Rhodococcus* requires sulfate and sodium ions for its growth in hydrocarbon and non-hydrocarbon media. Absence of these salts in deionised water medium could not support either growth or emulsifier production. Uchida *et al.* (1989 b) showed that K^+ was superior to Na^+ for emulsifier production by *Rhodococcus* sp. SD-74.

In conclusion, *Rhodococcus* sp. NCIM 5126 produced extracellular emulsifier in the presence of crude oil and hydrocarbons. Increase in biomass, emulsifier production and hydrocarbon degradation were interdependent. Optimal conditions for hydrocarbon degradation and emulsifier production were similar. The emulsifiers were glycolipids. Nature of lipid moiety was dependent upon the supplied hydrocarbon and sugar moiety was trehalose in all cases. Emulsifiers were stable at wide range of pH and temperature.

Figure 4.8

Effect of agitation rate on growth and emulsifier production by *Rhodococcus* sp. NCIM 5126

The organism was grown in Artificial Sea Water containing 1% (w/v) n-tetradecane, 35 mM N as urea, 0.114 mM P as K_2HPO_4, pH 8.0. Flasks were incubated at 30°C for 5 days at agitation rates of 0, 50, 100, 150 and 200 revolutions per minute.



Pseudosolubilization appears to be the mode of uptake for crude oil and hydrocarbons by this organism. About 50 mg/l n-tetradecane was solubilized by the supernatant of n-tetradecane grown *Rhodococcus* sp. NCIM 5126.

CHAPTER 5

General Discussion

Rhodococcus has been shown to be grouped in a coherent clade whose members are exclusively mycolate containing genera. Ruimy *et al.* (1994) suggested that the term 'CMN group'which refers to the genera *Corynebacterium, Mycobacterium* and *Nocardia* can be expanded to include the genera *Rhodococcus, Gordona, Tsukamurella* and *Dietzia.* One more genus, *Turicella*, a novel clinical isolate, has been proposed, however its validity has been disputed and Ruimy *et al.* (1995) state that it belongs to *Corynebacterium.* On the basis of the complete 16S rRNA sequences, Chun *et al.* (1996) suggested that the genera *Gordona, Mycobacterium, Nocardia, Rhodococcus* and *Tsukamurella* should be grouped with *Mycobacterium* in the family Mycobacteriaceae while *Corynebacterium, Dietzia* and *Turicella* make up the family Corynebacteriaceae. Using the partial sequences of the 16S rRNA genes of the *Nocardia* species and comparing them with the sequences of representatives of genera *Corynebacterium, Gordona, Mycobacterium, Rhodococcus* and *Tsukamurella*, Chun and Goodfellow (1995) reported that *Nocardia* formed a distinct clade which was most closely associated with the genus *Rhodococcus*, the only difference being that nocardiae contain hexahydrogenated menaquinones with 8 isoprene units in which the two end units are cyclized. Rainey *et al.* (1995 b) carried out 16S DNA analysis of species of the genera *Corynebacterium, Rhodococcus, Nocardia, Mycobacterium, Dietzia, Gordona* and *Tsukamurella* and observed that the genus *Nocardia* does not appear to be a sister taxon of *Rhodococcus* but branches off from within the radiation of *Rhodococcus*.

In *Bergey's Manual of Systematic Bacteriology*, Goodfellow (1989 b) has described twenty genera of *Rhodococcus* along with four *Species Incertae Sedis* which includes *R. aichiensis, R. auranticus, R. chubuensis and R. obuensis.* The sequence analysis of the 16S rRNA gene is responsible for the considerable changes in the classification of this genus recently. Now *R. obuensis* has been determined to be synonymous with *R. sputi*, making the former name obsolete (Zakrzewska-Czerwinska *et al.*, 1988). *R. aichiensis* and *R. chubuensis* have been transferred to *Gordona* (Klatte *et al.*, 1994 a) whereas *R. auranticus* and *Corynebacterium paurometabolum* are proposed to be a single species and is reclassified into a new genus, *Tsukamurella* (Collins *et al.*, 1988). *R. chlorophenolicus* is transferred to the genus *Mycobacterium* on the basis of composition of mycolic acid (Haggblom *et al.*, 1994). *R. luteus* is determined to be a synonym of *R. fascians* (Klatte *et*

al., 1994 b) and *R. maris* has been transferred to the new genus *Dietzia* as *D. maris* (Rainey *et al.*, 1995 a).

In our study, the selected crude oil degrading organism was identified as 'nocardioform actinomycete' on the basis of morphology, fatty acid methyl ester pattern, cell wall analysis for the presence of *meso*-DAP as diamino acid, arabinose+galactose as characteristic sugars (cell wall chemotype IV) and mycolic acid (Lechevalier and Lechevalier, 1970). When compared with *Rhodococcus rhodochrous* ATCC 13808 and *Nocardia asteroides* ATCC 19247, on the basis of entire margin, convex elevation of colonies, inability to use starch, gelatin, xylan, chitin as sole carbon source and ability to use alcohol as sole carbon source, we separated isolate 3 from *Nocardia*. Although, similarity in mol % G+C does not necessarily indicate genomic similarity, mol% G+C value of 69 (T_m =82°C) was well within the published range for the genus *Rhodococcus* (mol% G+C 67–73). It also showed close resemblance to *Rhodococcus terrae*, on the basis of morphology and biochemical tests. However Stackebrandt *et al.* (1988) transferred *R. terrae*, *R. bronchialis* and *R. rubropertinctus* to the genus *Gordona* on the basis of menaquinone pattern and 16S rDNA sequencing. In the absence of these data isolate 3 has been retained in the genus *Rhodococcus* and deposited in National Collection of Industrial Microorganisms (NCIM) as *Rhodococcus* sp. NCIM 5126.

This species of *Rhodococcus* was able to use various n-alkanes, kerosene, pristane and aliphatic and aromatic fractions of crude oils as sole source of carbon and energy in Artificial Sea Water supplemented with optimum N and P sources. In the presence of peptone, fructose, n-tetradecane, crude oil and pristane separately as sole source of carbon and energy, protein specific growth rate (μ) and generation time (g) of 0.09 hr^{-1}, 7.7 hr; 0.031 hr^{-1}, 22 hr; 0.018 hr^{-1}, 39 hr; 0.017 hr^{-1}, 41 hr and 0.016 hr^{-1}, 43 hr were observed respectively indicating that as the complexity of carbon source in terms of number of carbon atoms and methyl branching increased, duration of lag phase and generation time increased with decrease in protein specific growth rate.

Cellular fatty acids of *Rhodococcus* sp. NCIM 5126 grown on fructose, n-tridecane, n-tetradecane, n-hexadecane, n-heptadecane and pristane showed the presence of C_{16}, $C_{16:1}$, C_{18} and $C_{18:1}$ fatty acids indicating *de novo* biosynthesis of these fatty acids. For every hydrocarbon supplied, predominance of corresponding monocarboxylic acid was observed suggesting monoterminal attack on n-alkane and subsequently 'intact/direct incorporation' mechanism for cellular fatty acids. There are few reports on cellular fatty acids of organisms grown on pristane (Hagihara *et al.*, 1977) and in this species of *Rhodococcus*, apart from branched chain fatty acids it also showed the predominance of corresponding monocarboxylic acid 2,6,10,14-tetramethylpentadecanoic acid, emphasizing the operation of monoterminal oxidation of alkanes in this organism. The fatty acid composition of intracellular inclusions in *Rhodococcus òpacus* strain also varied according to the supplied carbon source and gluconate and n-hexadecane grown cells showed the presence of intermediates of β-oxidation fatty acids (Alvarez *et al.*, 1996). The alkane-grown *Rhodococcus* cells were more lipophilic than fructose grown cells and according to Boulton and Ratledge (1987), increased lipophilicity of alkane grown cells helps in uptake and intracellular transport of the hydrocarbons.

Presence of dioic acids and unsaturated fatty acids in the supernatant of n-alkane grown cells indicate the operation of ω-oxidation pathway besides usual β-oxidation pathway and desaturation of fatty acids in this organism. When grown on long chain n-alkanes *Rhodococcus* sp. NCIM 5126 produced undecanedioic acid and tridecanedioic acid with about 25% conversion of supplied carbon source. Broadway *et al.* (1993) have reported production of dioic acids by *Corynebacterium* sp. strain 7E1C. Dioic acids are used in perfume industry (Fukui and Tanaka, 1980). Supernatant of pristane-grown medium of *Rhodococcus* sp. showed the presence of monocarboxylic acid, 2,6,10,14-tetramethylpentadecanoic acid, which is used as cardiovascular agent.

The organism utilized n-hexadecane to about 70%. Partitioning of [1–^{14}C] n-hexadecane activity among different components showed that particulate uptake represented only about 6% of supplied reactivity at the end of 72 hr. About 1% radioactivity remained

in the cell supernatant in the experimental flasks. These results suggest that 93% of the radioactivity supplied was released as CO_2.

This *Rhodococcus* sp. is isolated from crude oil polluted regions of the Arabian Sea near Mumbai. It required salinity for growth and crude oil degradation and tolerated about 1.71 M NaCl in Artificial Sea Water medium. In accordance with Stanier *et al.* (1981), on the basis of NaCl tolerance, the present organism can be described as a moderately halophilic organism. Gauthier *et al.* (1992) described *Marinobacter hydrocarbonoclasticus* tolerated NaCl from 0.08 to 3.5 M and they have described their organism as 'extremely halotolerant'.

Combination of sodium sulfate (0.07 M) and sodium chloride (0.085 M) in deionized water medium replaced all other salts used in the preparation of Artificial Sea Water to give 50% degradation of paraffin fraction of crude oil by this species of *Rhodococcus*. These results indicated that along with sodium, sulfur is essential for the growth of this organism. These results led us to check the effect of sulfate on the growth of non-hydrocarbon source, fructose. Final dry weight of biomass as well as sulfur content showed that both increased in case of sulfur rich medium to that when sulfur was absent. This indicates 'assimilatory sulfate reduction' and uptake of sulfur for building up of biomass and other biomolecules. Sulfur is never limiting (about 25 mM) in natural seawater and high requirement of sulfur by this organism emphasizes the adaptation of this organism to its habitat.

Rhodococcal strains have been used for bioremediation purpose (Yagafarova and Skvortsova, 1996). Along with addition of oxygen, hydrogen peroxide or easily utilizable carbon sources, paraffinized fertilizers are also useful for this purpose. Paraffinized fertilizers do not get washed out in the ocean and act at oil/water interface to be selectively available to the degrader population. As this species of *Rhodococcus* can utilize nitrogen and phosphorus source of this kind, in case of application for oil spill management, this organism will be particularly useful helping in degradation of variety of hydrocarbons including aromatics. As *Rhodococcus* sp. uses urea which is a cheaper source of nitrogen compared

to ammonium sulfate and ammonium chloride, the process will be cost effective which is the basis of any bioremediation programme.

For the assimilation of hydrocarbons by microorganisms, cells either attach themselves to the hydrocarbon droplets or emulsify them. *Rhodococcus* sp. NCIM 5126 produced emulsifier in the presence of alkanes but in assay conditions it emulsified aromatics better than alkanes. The surface tension reduction is necessary to break the barrier between oil and water, subsequently dispersion of oil increases the surface area favouring microbial attack (Swisher, 1987). In this case, surface tension reduction of the broth was from 66 dynes/cm to 32 dynes/cm which is considerable and within the range reported for other nocardioform actinomycetes (Margaritis *et al.*, 1979; Cooper *et al.*, 1981). Apart from reducing the surface tension, emulsifier is not directly involved in the solubilization of n-tetradecane. Koch *et al.* (1991) showed that mutants of *Pseudomonas aeruginosa* lacked extracellular rhamnolipid making them unable to take alkanes. Addition of small amount of rhamnolipid restored their ability to grow on alkanes. This showed that rhamnolipids play a major role in alkane uptake, however they also suggested the necessity of production of a factor similar to 'protein like activator' proposed by Hisatsuka *et al.* (1977). Goswami and Singh (1991) isolated an emulsifying factor (lipoprotein) and a pseudosolubilizing factor (glycoprotein) from n-hexadecane grown *Pseudomonas* N1 and synergistic action of both the factors was essential for utilization of alkane by this organism. We observed that supernatant of n-tetradecane grown *Rhodococcus* sp. solubilized n-tetradecane from 7×10^{-3} mg/l to 50 mg/l broth. 94% degradation of n-tetradecane and 50% degradation of aliphatic fraction of crude oil by *Rhodococcus* sp. can be attributed to the pseudosolubilization of hydrocarbons. The combination of emulsifier, pseudosolubilizing factor and increased lipophilicity are required for effective utilization of alkanes by *Rhodococcus* sp. NCIM 5126.

Rhodococcus sp. produced glycolipid emulsifiers. On various alkanes as a carbon source, corresponding fatty acid was a lipid moiety and trehalose was a sugar component in the emulsifier. In accordance with Syldatk and Wagner (1987) the emulsifier produced by *Rhodococcus* sp. can be categorized in the second group where sugar moiety of emulsifier is produced constitutively and fatty acid production is dependent upon the supplied alkane.

The oxidation of alkanes produces fatty acid which is incorporated in the emulsifier but for the production of sugar moiety, operation of other pathway is necessary. A series of gluconeogenesis reactions from fatty acid forms the precursor for carbohydrate synthesis. The linkage of lipid and carbohydrate moieties will result in the formation of glycolipid. The emulsifier produced by *Rhodococcus* sp. NCIM 5126 was different from other reported rhodococcal emulsifiers in that it did not contain mycolic acid or succinic acid as reported by Singer *et al*. (1990) and Uchida *et al*. (1989 a, b). Growth conditions for hydrocarbon degradation and emulsifier production were similar in *Rhodococcus* sp. NCIM 5126. A simultaneous emulsifier production, hydrocarbon degradation and biomass increase suggests interdependence of these processes in this organism.

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