STUDIES ON THE APPLICATION OF NATURAL MICROORGANISMS FOR OIL SPILL DEGRADATION AND POLLUTION CONTROL

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DEGREE OF **DOCTOR OF PHILOSOPHY** IN MARINE SCIENCE

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CERTIFICATE

This is to certify that the thesis entitled " **Studies on the application of natural microorganisms for oil spill degradation and pollution control** " submitted by **Jason Jeyachandran David** for the award of degree of Doctor of Philosophy in Marine Science is based on the results of investigations carried out by him under my supervision. The thesis or part thereof has not been submitted for any other degree or diploma of any University.

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1

Introduction

Oil spills in the marine environment causing ecological disasters is presently a cause for concern in the modern world. An increasingly staggering worldwide consumption of petroleum hydrocarbons estimated at 10^{12} US gallons/yr (United States Energy Information Administration, 1991) testifies to the modern day dependence on oil energy. Sea remains to be the major mode of transport for petroleum resources. Incidentally, the marine environment becomes susceptible to large and small releases of oil. Besides inputs from origins like offshore oil wells, urban and riverine run-offs also add to the process (Prince, 1993). The spatial and temporal distribution and eventually the ultimate fate of hydrocarbons in the marine environment is decided not only by physics and chemistry but also to a great deal by the biology of the oceans. Thanks to the indigenous microbes, especially the bacteria, yeasts and blue green algae, the original pristine nature is restored (Atlas, 1981). Marine environment is believed to be the ultimate sink for all anthropogenically introduced pollutants. As this last frontier is being explored and exploited for living and non-living resources, it would be prudent to bear in mind the damaging ecological impact caused mainly by the introduction of petroleum pollutants.

Till date, there is no single means of cleaning an oil spill effectively and completely in the marine environment. Though physical removal of oil and dissolution by chemical dispersants have been found to be the immediate response (Kelso and Kendziorek, 1991; McIntosh, 1989) to an oil spill today these are ridden with several logistic and management limitations or are known to be ecologically unsafe. Therefore,

much attention has been focused towards biodegradation, particularly after the historic *Amoco Cadiz* spill in 1978.

Although several bioremediation approaches were being looked into, success in field trials was limited and subjective. Due to the strong opinion that bioremediation could be the only inexpensive, nonintrusive and ecofriendly technology (Pritchard and Costa, 1991), there was promise in this area of science. The *Exxon Valdez* spill perhaps paved the way for success of bioremediation as an oil spill countermeasure at field level. This option was realised as a viable oil spill response requiring further refinement and standardisation to suit different ecological systems.

Natural microorganisms are known to possess a wide potential of degrading petroleum hydrocarbons (Floodgate, 1984). The understanding of metabolic pathways involved during its breakdown has shown that a number of enzymatic mechanisms are involved in the utilisation of the compounds found in petroleum mixture (Cerniglia, 1984). Hence microorganisms metabolising a wide range of substrates are required in a spill site to effectively increase biodegradation of these compounds. The most common metabolic constraint is the limited availability of nutrients. As the nitrogen and phosphorus replenishments fall short of carbon, the degradation rates are considerably reduced (Leahy and Colwell, 1990). To overcome the low ambient nutrient concentrations, application of fertilisers has met with some success. On the other hand, the use of such fertilisers in coastal environments have given rise to undesirable eutrophication effects (Atlas, 1991; Lee *et al*, 1995). Even in open sea floating oil slicks, it apparently had no success due to dissolution and dilution effects.

The extent of success using fertiliser application in tropical ecosystems have not been examined so far.

The potential of bioremediation has been well realised but the development of effective approaches are far from satisfactory for practical applications, especially in open and closed tropical ecosystems.

Since the marine environment is a repository for biodiversity, it is no wonder that various physiological traits could be harnessed for meeting nutrient demands. For example the presence of N_2 fixing bacteria capable of utilising hydrocarbon (Roy *et al*, 1988; Chen *et al*, 1993) has been documented but their role in degradation of crude oils have not been well examined. Similarly, though the abundance and activity of phosphate solubilising bacteria have been reported (Naik *et al*, 1982), their role in oil degradation has not been looked into. Hence, a need for a closer examination of these physiological groups and their simultaneous abilities to degrade petroleum hydrocarbons has been felt.

Initially, petroleum hydrocarbon degradation studies by microbes were conducted using individual cultures on pure hydrocarbons (Walker and Colwell, 1976a). The idea of using mixed cultures for studying their degradation potential was also looked into (Walker *et al*, 1975a; Rambeloarisoa *et al*, 1984). Since the majority of these laboratory studies lead to the conclusion that the oil degradation was limited by the availability of ambient nitrogen and phosphorus nutrients (Floodgate, 1979; Atlas, 1984), the practical realisation of the objectives was fluid. Present day technologies have aimed at inducing the growth of autochthonous flora by adding

fertilisers to achieve biodegradation. Not much attention has been paid to the use of selectively reconstituting natural marine microbial isolates which could degrade oil and also possess favourable physiological attributes (growth in nitrogen free medium, phosphate solubilisation). Moreover in the tropical environment of the Indian waters, apart from a few old, sporadic reports on oil degradation aspects of microorganisms, there seems to be very little information on the studies on developing microorganisms for application in bioremediation formulations for tackling hydrocarbon pollution. This new concept of such a selection could pave the way in finding a viable and novel technology in bioremediation.

Hence in the present study, a reconstitution of naturally active flora was thought to be a viable proposition to examine the biodegradation of petroleum hydrocarbons. To substantiate the potential of selected microorganisms, other important factors have also been examined in this study.

The following were the objectives of this study:

1. Isolation of bacteria and yeasts from varied marine environments for petroleum hydrocarbon degradation. Screening for ability of microbes to grow on nitrogen deficient medium and solubilise phosphates.
2. Biodegradation of crude oils, tar balls and other petroleum hydrocarbons by pure cultures.
3. Production of bioemulsifier and emulsifying activities of hydrocarbon degrading microorganisms.
4. Taxonomic characterisation of potential microbial strains.

5. Occurence and participation of extrachromosomal elements in hydrocarbon degradation.

6. Degradation of petroleum hydrocarbons by reconstituted and natural mixed cultures *in vitro* and simulated *in situ* conditions.

2

Review of Literature

2.1. INTRODUCTION

The role of microbes in the biological degradation of hydrocarbons has gained much attention. It is understood to be the major route for oil removal from the environment (Atlas, 1981). Microbial communities are known to be reponsible for the degradation of many of the introduced pollutants. Marine environment is increasingly becoming vulnerable to frequent petroleum releases by myriads of human activities. Fortunately this environment which acts as a sink to all types of anthropogenic pollutants including petroleum hydrocarbons is also provided with mechanisms of their removal over a period of time. Based on this understanding, microbial hydrocarbon degradation research has progressed considerably. But due to the complexity of petroleum hydrocarbons and their ultimate removal from the environment, this area of research has been still one of challenges to those who look for biological answers to pollution issues. This chapter would be detailing on the available literature on petroleum hydrocarbons and their biodegradation in the marine environment.

Hydrocarbon as the name denotes are compounds composed solely of carbon and hydrogen atoms. Petroleum hydrocarbons are major forms of fossil fuel and the raw material resource, is often referred to as the "crude oil". These have been formed as a result of complex physico-chemical and biological interactions (ZoBell, 1959). Crude oil is the primary source of petroleum hydrocarbons and encompasses a wide spectrum of compounds. Commercially crude oil is called 'light' or 'heavy' based on the low or high specific gravity.

2.2. CHEMICAL NATURE

The approximate elemental weight composition of crude is reported to be:

Carbon - 82%

Hydrogen - 12%

Oxygen and Sulphur - 0.1% each

Nitrogen - 0.05%

Cu, Ni, Va, Ca, Mg, Fe - trace (ZoBell, 1959).

Crude oil is a mixture of a host of components, starting from methane, to compounds with molecular weights in millions. Cycloalkanes are formed by joining to themselves to produce five- or six- membered ring compounds. Aromatic hydrocarbons are the most soluble among the components of oil; they are compounds having one or several benzene rings, with or without side chains. The two- to three-ring compounds are toxic while the four- to five- ringed compounds are known to be carcinogenic or teratogenic (Teal, 1993). Asphaltenes which are large molecules in crude oil, comprise of phenols, fattyacids, ketones, esters and porphyrins while resins contain pyridines, quinolines, carbozoles, sulphoxides, amides, etc. (Colwell and Walker, 1977). Some are toxic, but in general, they are compounds incapable of supporting biological activity.

Crude oils are classified into the following groups: i) aliphatics, ii) aromatics, iii) polars, and iv) asphaltenes based on the their molecular structure and solubility in organic solvents. The classification of various fractions obtained by adsorption column

chromatography are given in Chart 1. The composition as well as the percentage ratio of these groups vary considerably from one crude oil to another.

2.3. PETROLEUM HYDROCARBONS IN THE MARINE ENVIRONMENT

Petroleum concentration in water column ranges between 10,000 ppb to 5 ppm (Atwood and Ferguson, 1982). In 1991, the United States Energy Administration put the worldwide consumption of petroleum hydrocarbons in the order of 10^{12} US gallons/year for the year 1989. Annual global input of petroleum into the environment was estimated to be between 1.7 and 8.8 million MT in 1985. The National Academy of Science of the US have put the total annual input of petroleum into the sea at around 6.113 million tonnes in 1970 (NAS, 1975). Based on a world survey, Levy *et al* (1981) reported that distribution of petroleum in the oceans is, however not uniform. The United States National Research Council in 1985 estimated that about $9x10^8$ gallons of petroleum finds its way into the sea. They were quoted to be from the following sources:

(1) 10% - natural seeps,

(2) 10% - catastrophic releases,

(3) 40% - transportation losses (excludes accidents) and

(4) 40% - municipal wastes and run-offs.

An insight to the frequencies and intensities of some of the oil spills that have been locally significant in the recent past have been tabulated in the following page.

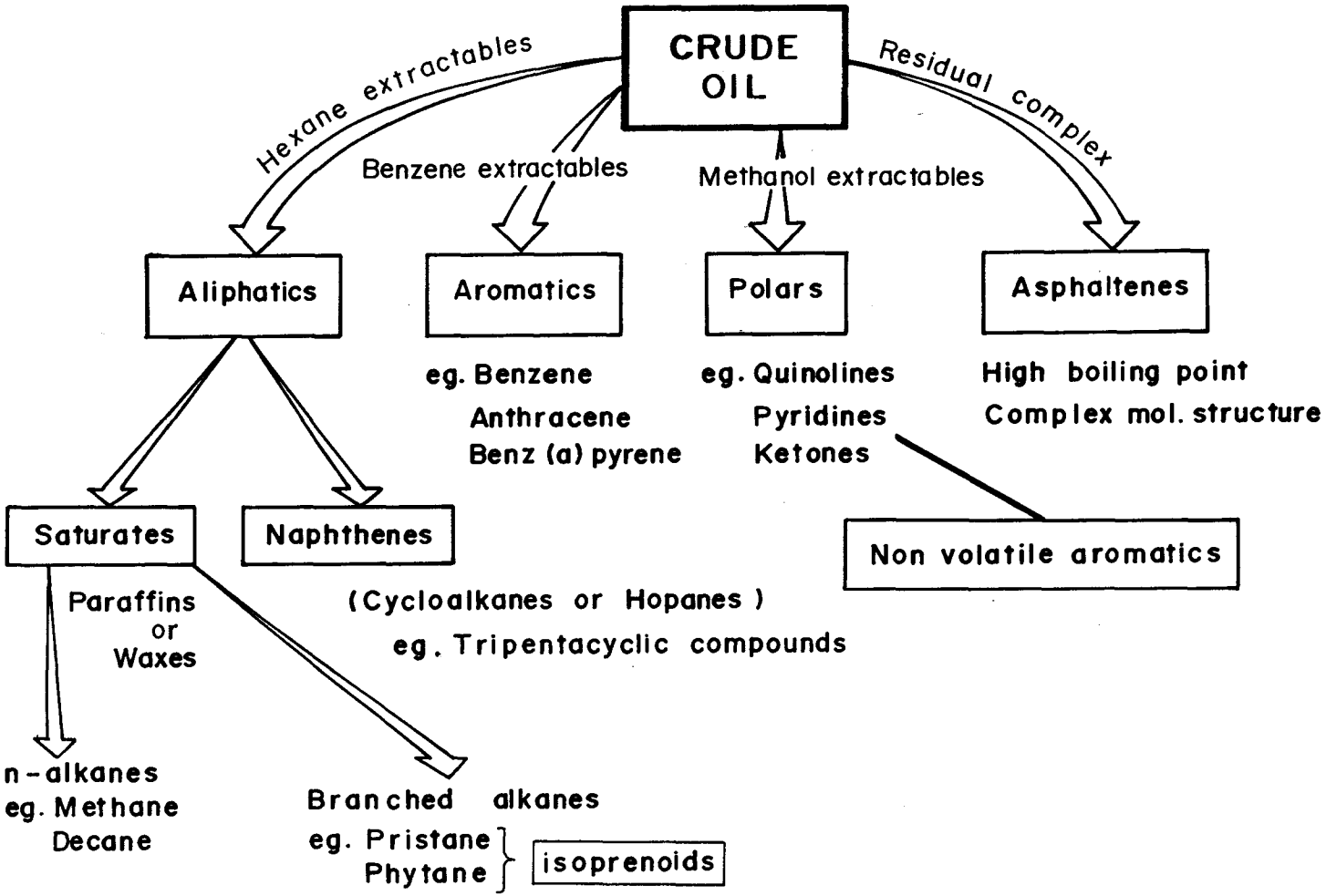


Chart. 1. Classification showing fractional composition of crude oils.

Major oil spills in the recent past.

Year	Cause	Site	Quantity	Type of cargo
1978	Grounding of oil tanker	Coast of Brittany	220,000 tons	Crude oil
1989	Grouping of the oil tanker	Coast of Alaska	35,000 tons	Crude oil
1991	Gulf war	Kuwait coast	1.36-2.04 million tons	Crude oil
1993	Collision	North east of Malacca strait	Large spill	Crude oil
1994	Grounding of freight carrier	Goa coast	2 tons	Furnace oil
1994	Sinking of ore carrier	Cape coast, South Africa	2,400 tons	Intermed-iate fuel diesel

In practice, it is not that crude oil alone gets transported in tankers but also the products of petroleum distillation. Some of these are: gasolines, heating fuels, diesels and heavy fuel oils.

Many of these oils are found to contain carcinogenic and mutagenic compounds. Normally it is the untreated crude that causes concern since it contains the entire range of volatiles and all the recalcitrant compounds. The sudden spill could affect localised water bodies. Hence, such sources have to be treated in a more exhaustive manner.

In India, an estimated 5 million tons of oil enters the Arabian sea which is much higher when compared to the Bay of Bengal whose estimated input is about 400,000 tons (Hinrichsen, 1990). Gupta *et al* (1989) have estimated tar deposits on the west coast of India to be between 750 and 1000 tons per year during a two year observation.

Unlike the terrestrial environment, horizontal spreading of oil is high in aquatic environments. Hence the affected area gets larger in time before containment measures are taken. Timely intervention is essential in incidents of oil spills in aquatic environments. But as they spread, they also tend to evaporate (Payne *et al*, 1991). The more soluble components like the polar compounds dissolve in seawater and on some occasions adsorption of oil onto particulates could cause sinking of the adsorbed oil into the sediment (Bartha and Atlas, 1977). Though the chemical composition of crude oil is not altered significantly through chemical reactions some photochemical reactions

oxidise hydrocarbons completely to carbon dioxide and water (Wise and Sancier, 1991).

Though the above mentioned fates of crude oil in the marine environment are observed initially, it is the biodegradation which is responsible for oil removal from the environment (Prince, 1993). In the marine environment, spilled oil normally is seen in any of the following states: 1) as films, 2) in solution, 3) as an emulsion, or 4) as tar balls (Atlas, 1981).

2.3.1. **Petroleum hydrocarbon as a pollutant**

Whatever be the state it enters the marine environment, the outcome is always deleterious to the life forms ie, it is more of a pollutant which is recalcitrant than an easily biodegradable matter. Group of Experts on the Scientific Aspects of Marine Pollution (GESAMP, 1993) defines pollution as an introduction by man, directly or indirectly of substances or energy into marine environment (including estuaries) resulting in such deleterious effects as harm to living resources, hazards to human health, hindrance to marine activities including fishing, impairment of quality for use of seawater and reduction of amenities. Crude oil and other petroleum products are considered as "pollutants" especially during catastrophies because of their toxic nature, immiscibility, lower specific gravity, slow dispersing property, ability to build anoxic environs, blocking of light which passes through water which disrupt photosynthesis and bioaccumulation at various trophic levels.

Though petroleum is removed by biological means it also causes serious effects on the biota. Solubilities of oil in seawater is lesser compared to freshwater. It is obvious that the accumulated oil on the surface would seriously affect marine life immediately below it (Morris, 1974). Hydrocarbons can affect photosynthesis or simply wipe away algal communities (Gordon and Prouse, 1973). Oil components were found to be present at dangerous levels in fish and shell fish harvested from foul waters after the Alaskan oil spill. Mere skin contact of oil to certain fauna can cause acute dermatitis. Necropsies on dead otters in Alaska revealed severe emphysema - presumably from breathing the fumes of the freshly spilled oil. Liver, kidney, intestinal, adrenal and bone marrow abnormalities, similar to damage previously found in studies of laboratory rats were also noted (Baringa, 1989).

Some of the sublethal effects of petroleum hydrocarbons are interference in chemoreception in microorganisms and bio-concentration. Bioaccumulation as reported by Finnerty *et al* (1973) could have more serious implication, if toxic aromatic compounds are sequestered and enter the food web.

2.3.2. **Microbial response to petroleum in nature**

Several types of responses by bacteria to hydrocarbons have been documented. They vary from community structure, biomass, biochemical characteristics and physiological changes. However, changes in an individual population, species composition of disturbed ecosystems have been hardly reported, due to the enormity and difficulty of identification and culture procedures. There is a report on the

changes in population densities of physiological groups of oil degraders (proteolytic, chitinolytic, cellulolytic) as result of exposure to oil (Walker *et al*, 1975c). Addition of different fractions of the same oil to seawater resulted in increases in the bacterial population of different bacterial species (Horowitz *et al*, 1975).

The ratio of hydrocarbon degraders are hundred times more in ecosystems exposed to petroleum than in pristine environments (Atlas, 1981).

2.4. DISTRIBUTION OF HYDROCARBONOCLASTIC MICROORGANISMS

Natural flora get enriched to combat the oil spills. Hence, different type of ecosystems that have been exposed to any hydrocarbon, harbour oil degrading microbes. As it is obvious that hydrocarbons occur both as natural products as well as pollutants the presence of hydrocarbon utilising microorganisms are also ubiquitous. As enormous quantities of crude and refined oils are transported over long distances and consumed in large amounts, hydrocarbons have now become a very important class of potential substrate for microbial oxidation. Hence it is not surprising that hydrocarbon oxidising bacteria are widely distributed in nature, although with large variations in their populations.

Moreover, biodegradation in the environment is primarily by bacteria and fungi although cyanobacteria have also been reported among the hydrocarbon utilising microorganisms (Leahy and Colwell, 1990; Sorkoh *et al*, 1992). The ratio of hydrocarbon oxidising bacteria to the total population of heterotrophic bacteria as well as the diversity of degrading organism found in a particular ecosystem may change

according to the time of sampling and/or extent of oil pollution. Atlas (1981) stated that 0.1% of a non-polluted marine microbial community are oil degraders whereas in a site which is chronically exposed to oil, 10% of the total heterotrophic population are hydrocarbon users. In another report (Prince, 1993), it is pointed out that in non-polluted sites they amount to 1% of the population.

Several investigators have demonstrated an increase in the number of hydrocarbon oxidising bacteria in oil polluted areas. In Helgoland North Sea coast the population was 0.9 to $4x10^2$ cells/ml (Gunkel, 1973) whereas in Colgate creek of the Atlantic waters it ranged from 10^3-10^5/ml (Walker and Colwell, 1976b; Horowitz and Atlas, 1977). There is only one study on the vertical distribution of hydrocarbon degrading bacteria in the Pacific ocean south of Japan (Venkateswaran *et al*, 1993) where the population of bacteria decreased from 10^3 cells/ml at the surface to 10^2 cells/ml at 1500 m but the ratio of this population to heterotrophic population increased from 21% (surface) to 80% at 1500 m. In beaches and sediments the populations are expectedly higher - 10^5-10^8/g (Jones and Edington, 1968; Gunkel, 1973; Atlas and Schofield, 1975; Crow *et al*, 1975; Walker and Colwell, 1976b). Leahy and Colwell (1990) and Rosenberg (1992) have provided a comprehensive account of the distribution of hydrocarbon oxidising bacteria in the marine environment.

The distribution of hydrocarbon utilising microbial population in Raritan Bay were directly correlated with presumed patterns of oil influx into the Bay (Atlas and Bartha, 1973). A number of studies along the shipping routes (Mironov, 1970),

sediment samples (ZoBell and Prokop, 1966), coastlines (Floodgate, 1976) have indicated a dominance of hydrocarbon oxidisers, due to the chronic nature of exposure to oil pollution.

The ability to degrade petroleum hydrocarbons is not restricted to a few microbial genera. ZoBell (1946) in his review has mentioned more than 100 species representing 30 microbial genera, capable of utilizing hydrocarbons. Bartha and Atlas (1977) have listed about 22 genera of hydrocarbon degrading bacteria and 14 genera of fungi and 1 algal genus capable of degrading hydrocarbons. Atlas (1981) listed 10 genera of bacteria, 3 genera of yeasts that are the most important ones in hydrocarbon utilisation based on frequency of isolation. Yeasts are ubiquitous in the oceans and are known to be present in considerable numbers in organic matter enriched waters (Uden and Fell, 1969). Yeasts are also known to be hydrocarbonoclastic (Ahearn *et al* 1971; Goma *et al*, 1973; Shennan and Levi, 1974; Kulkarni and Barnett, 1979; Cerniglia and Crow, 1981; MacGillivray and Shiaris, 1993). Twenty five genera of hydrocarbon degrading bacteria and 27 genera of fungi and yeasts that degrade hydrocarbons in marine or brackish water was listed by Floodgate (1984). Several higher fungi from the tropics to temperate regions were reported to utilise hydrocarbons (Kirk *et al*, 1991).

Several workers (Cundell and Traxler, 1974; Cook and Westlake, 1974; Walker *et al*, 1975a; 1976) have recorded the presence of *Pseudomonas* spp. among the oil degrading isolates. Austin *et al* (1977) have found that among the hydrocarbon degrading phenetic groups, two clusters of *Pseudomonas* were also recorded.

Interestingly, some of the known oil degraders like pseudomonads, flavobacteria and vibrios were the ones that responded adversely to some of the hydrocarbons in the studies of Cobet and Guard (1973).

2.5. MICROBIAL DEGRADATION

As the oceans cover 70% of the earth's surface, they are the ultimate recipients of the petroleum hydrocarbons. This makes the fate of oil and so also the degradation highly variable in space and time. It is needless to say that the fate of oil is also predominantly decided by biological mediation. Floodgate (1984) while using a novel assumption, concluded that about 1 to 10 tons of bacterial tissue could be accounted for in a volume of water occupying a space of 1 km^2 x 100 m depth. Thus that microorganisms should be the major agents in any organic degradation process is to be expected. Biodegradation of hydrocarbons by natural populations of microorganisms represents one of the primary mechanisms by which pollutants are eliminated from the environment (National Academy of Science (NAS), 1985). Their susceptibility to microbial attack varies from being the highest for saturates and to lowest for polar compounds.

Work on the biodegradation of petroleum hydrocarbons dates back to the 1940s. ZoBell as early as 1945 began working on the bacterial involvement in petroleum hydrocarbon chemistry. Thereafter several workers have carried out experiments to assess oil degradation rates, extent of degradation, mineralisation, conversion of various compounds, etc. using individual protocols. Most studies have

been laboratory based with recent validation on field trials. Estimations of oil degradation has been generally based on GC-MS, ^{14}C-uptake and respiration measurements. While some have used natural seawater, many have used other media of their choice. Thus a comprehensive glance on the microbial degradation is impossible. However, pertinent work find their place in this review.

Biodegradation studies have been carried out in two ways;

1) using individual isolates from petroleum contaminated sites and

2) using natural populations from seawater and sediment.

Prince (1993) in his review has mentioned three common mechanisms of bacterial degradation. They are,

* interaction with low levels of dissolved material

* direct contact with drops or surfaces of the insoluble phase

* interaction with hydrocarbon "solubilised" by means of surfactants.

Any or a combination of the above mechanisms could be involved during bacterial degradation.

2.5.1. Screening for individual hydrocarbonoclastic cultures

It is evident that any established genus or species *per se* need not be hydrocarbonoclastic. A routine screening procedure is therefore used to check for their potentiality before considering as potential candidates for oil degradation. To screen for microorganisms capable of petroleum hydrocarbon degradation, several techniques are employed. The use of selective plate counts with carbon sources of interest (Seki,

1973) have been the most commonly used method which are at times not reliable (Jain and Sayler, 1987). ^{14}C-labelled respirometry methods (Roubal and Atlas, 1978; Fedorak *et al*, 1982) are highly sensitive and are useful for monitoring the mineralisation of the specific compound. More recently methods using redox indicators (Hanson *et al*, 1993), improvised MPN techniques (Brown and Braddock, 1990) have also been reported for screening hydrocarbon degrading microorganisms. Perhaps another method by Rosenberg *et al* (1980) called the microbial adhesion to hydrocarbon (MATH) assay has found a better relevance in screening for microbes with hydrocarbonoclastic properties. As this assay principally measures cell surface hydrophobicity, and is considered as a crucial property for growth on hydrocarbons (Prince, 1993), MATH assay has been found to suit screening of hydrocarbon utilising organisms (Rosenberg, 1991).

2.5.2. **Degradation of crude oil and components by individual cultures**

Individual cultures have been isolated by plate method or enrichment methods from contaminated sites. Jobson *et al* (1972) have found that enrichments obtained with a high quality crude oil were not as effective in utilising a lower quality crude oil (North Cantal) as sole carbon source as a population enriched on the low quality crude oil (Lost Horse Hill) which contains high asphaltenic content.

Atlas and Bartha (1972) reported 57 and 40% utilisation of Swedish crude oil by *Flavobacteria* sp. and *Brevibacterium* sp whereas *Arthrobacter* sp. degraded 35% of Iranian crude oil (Reisfeld *et al* 1972).

Bailey *et al* (1973) have reported using mixed cultures of 4 bacterial strains namely *Pseudomonas*, *Flavobacteria*, *Achromobacter* and *Bacillus* and found that they degraded North Cantal crude oil.

Laboratory studies of Walker *et al* (1976) showed that the extent of degradation with two different crude oils using bacteria varied between 51 and 82%. Fuel oils with higher aromatic and sulphur content were degraded much less (11 and 55%) in their studies.

Walker *et al* (1975c) observed in laboratory studies that degradation could be as high as 82% in crude oils and upto 50% in fuel oils at an inoculum strength of 10^6-10^8 cells/ml. They also observed that low sulphur, high saturate South Louisiana crude was more susceptible to biodegradation than the dense, high sulphur, and high aromatic Bunker C fuel oil.

Bhosle (1981) compared the degradation of two crude oils using a bacterial and a yeast strain and concluded that yeast (*Candida tropicalis*) degraded 42.5% while bacteria (*Arthrobacter simplex*) degraded only 33%. These were among the 24 cultures that were isolated from seawater and sediments of Dona Paula Bay, Goa. He also found the predominance of vibrios and pseudomonads in enrichments.

Nocardia utilised 94% of n-alkane fraction of Arabian crude oil and 77% of the Venezuela crude oil (Mulkins-Phillips and Stewart, 1974).

Perry and Cerniglia (1973) reported 85-92% utilisation of a paraffin based crude oil by marine fungi (*Cunninghamella elegans* and *Penicillium* sp.). Four species of beach adapted fungi were successfully tried for ^{14}C-hexadecane mineralisation by

Kirk and Gordon (1988) and later several higher marine fungi were also found to mineralise alkanes (Kirk *et al* 1991). ***Fusarium*** sp. could degrade some of the petroleum hydrocarbons (Nair and Loka Bharathi, 1977). A group of fungi, the thraustochytrids have also found to degrade crude oil (67%) and tarballs (71%) (Raikar, 1992).

Among 7 genera of yeasts which were found to assimilate kerosene and hexadecane (Ahearn *et al*, 1971) 6 were isolated from oil slicks. A species of *Candida* was reported to be the fastest and heaviest in growth on Louisiana crude oil. *Candida* sp also produce surfactants to solubilise select n-alkanes (Goma *et al*, 1973; Kulkarni and Barnett, 1979). Ability of yeasts to oxidise aromatic compounds is also well established (Cerniglia and Crow, 1981; Wright and Ratledge, 1991; MacGillivray and Shiaris, 1993). Cernglia and Crow (1981) proved that yeasts could also oxidise aromatic hydrocarbons such as naphthalene, biphenyl and benzopyrene. Later, MacGillivray and Shiaris (1993) studied biotransformations of PAHs using yeasts isolated from coastal sediments. Four species of *Candida* and *Debaromyces* were found to oxidise PAHs. Transformation of individual aromatic hydrocarbons were as high as 8.15 micromol of PAH/g of sediment containing a population of 10^2-10^7 CFU of yeast (MacGillivray and Shiaris, 1993).

Though algae and protozoa are important members of the microbial community, limited evidences are available on their hydrocarbon degradative role. An alga *Prototheca zopfii* degraded 10-40% of motor oil and crude oil repectively (Walker

et al, 1975b). Cerniglia *et al* (1980) observed that a few cyanobacteria, green algae and two diatoms could oxidise naphthalene.

The degradation of hydrocarbons by microbial populations are confronted with limitations of nitrogen and phosphorus requirements. There are very few reports on the use of nitrogen fixing bacteria for hydrocarbon degradation. Besides there are no known reports of hydrocarbonoclastic bacteria capable of solubilising phosphate in the marine environment.

The use of hydrocarbon utilising bacteria capable of nitrogen fixing have been isolated on a few instances (Coty, 1967; Roy *et al*, 1988; Chen *et al*, 1993) and the feasibility of inclusion of such strains in mixed cultures have been suggested earlier (Rosenberg, 1992).

2.5.3. **Degradation by mixed population**

While individual cultures have been known for degradative abilities, mixed cultures attack the hydrocarbons synergistically. On synthetic medium containing 0.1% (v/v) of oils, the mixed population of yeast and bacteria were able to degrade; South Louisiana crude oil - 82%, Kuwait crude - 51%, No.2 fuel oil - 55%, No.6 fuel oil - 11% (Walker *et al*, 1975c).

In another work by Fedorak and Westlake (1981), they found that when Prudhoe Bay crude oil was used, with autochthonous sediment flora, in the absence of additional nitrogen and phosphorus (NP) nutrients, degradation of alkanes was reduced considerably but had little effect on aromatic fraction (after a 27-day period)

in ASW at 20°C on a rotary shaker. Hence under nutrient limiting conditions simple aromatics were readily degraded than n-alkanes.

From a chronically oil polluted zone of the French Mediterranean coast, Rambeloarisoa *et al* (1984) used native bacterial population and found that they degraded the Tunisian crude oil by 81% in 12 days using natural sea water supplemented with NP nutrients under shaking conditions, at 30°C. After 5 days 88% of the saturates had been utilised; about 83% of certain aromatics disappeared in 12 days. But 51.5% of asphaltenes were still reported in the culture medium.

Murukami *et al* (1985) found that Arabian crude oil could be degraded upto 36% in warm Japanese coastal waters and in sub-temperate north eastern waters. Venkateswaran *et al* (1991) used mixed population of bacteria to show that the flora of sedimental origin could degrade 58% of Arabian crude oil whereas the sea water flora could degrade 35-53% under laboratory conditions.

Amund and Akangbou (1993) used four Nigerian crude oils and found that degradation ranged from 55 to 85% depending on the fractional composition of the oil. The lighter oils containing about 2.5% of asphaltene were degraded upto 85% while that of heavy oils containing >10% of asphaltenes the degradation was just 55%.

Degradation of South Louisiana crude oil (0.1% v/v) by sediment flora of Chesapeake Bay revealed that saturates were removed at 0.18 mg per day while aromatics seemed to be partially attacked with a concomitant increase in the resins and asphaltenes after two weeks (Walker *et al* 1976). Commenting on the turnover periods of aliphatics and polynuclear aromatics, Lee and Ryan (1976) using radio-

labelled ^{14}C hydrocarbons have found that the latter took several years while the former was of the order of months. It is found that there is a general hierarchy in the degradation of the components and refined fractions of the crude oil (Prince, 1993). The outcome of similar work resulted in a general unananimity among workers (Oudot, 1984; Kennicutt, 1988; Chianelli *et al*, 1991) that degradation among petroleum compounds is firstly on alkanes and small aromatics followed by branched alkanes, multi-ringed and/or substituted aromatics and cyclic alkanes. There are reports of aliphatics being preferentially degraded as compared to aromatics by bacteria (Kimura *et al*, 1989). Only a couple of researchers attempted to estimate the extent of degradation *in situ*. ZoBell (1964) estimated that biodegradation rates of polluting oil in the open sea could be as high as 100-960 mg/m^3/day. Using ^{14}C radiolablelled compounds, Caparello and LaRock (1975) calculated the biodegradation rates of n-alkanes as 2500 to 25 gms/ m^3/day for polluted inshore and relatively unpolluted offshore water samples respectively. Studies by Dibble and Bartha, (1976) states that in moderately polluted coastal sea water the rate of degradation was 500/g/m^3/day, and in a relatively unpolluted coastal seawater degradation was only 200g/m^3/day and concluded that the efficiency is higher by two and a half times in the case of the former. In 1977, Bartha and Atlas however pointed out that any estimate on the *in situ* biodegradation potential of crude oil could be highly variable due to inherent difficulties.

2.5.4. Degradation of pure aromatic hydrocarbons

Aromatic hydrocarbons are reported to be utilised by many species of bacteria, cyanobacteria, filamentous fungi and yeasts either independently or in combination with other microbes (Gibson, 1972; Cerniglia, 1984). In the past decade much work has been carried out on the bacterial growth (Fredrickson *et al*, 1991), biodegradation (Bauer and Capone, 1985; Foght and Westlake, 1988; Garcia-Valdes *et al*, 1988; Konopka, 1993), metabolism (Bauer and Capone, 1985; Heitkkamp *et al*, 1987), mineralisation and oxidation (Barnsley, 1983; Guerin and Jones, 1988; Foght *et al*, 1989; Fredrickson *et al*, 1991) on aromatic hydrocarbons and heterocycles.

Foght *et al* (1989) found that all strains capable of degrading tricyclic aromatics could also degrade mono- and di-cyclic aromatics but not vice versa. They reported that phenanthrene degradation ranged between 61% and 65% after 14 days of incubation at 28°C. Pure single isolate experiments had a lower degradation potential compared to combined cultures.

Ability of naphthalene mineralisation seems to be more common than phenanthrene (Foght *et al*, 1989). Garcia-Valdes *et al* (1988) have reported several strains of bacteria that are known to degrade cmpounds such as naphthalene. Species belonging to *Pseudomonas* were always found to be the predominant degraders. Naphthalene degradation was found to be higher at 60-70% (Heitkamp *et al*, 1987). A similar high observation was observed by Barnsley (1983) also.

2.5.5. Role of biosurfactants in petroleum degradation

Microbes utilising hydrocarbons for metabolism are generally understood to produce a number of lipid surfactants or surface active agents (Cooper and Zajic, 1980). Zajic and Mahomedy (1984) have said that biosurfactants are considered important in the degradation of petroleum hydrocarbons as they allow hydrocarbon utilisers to efficiently use the substrates by emulsification and adhesion to oil/water interface. Cell wall hydrophobicity has been also documented among the oil degraders and are well correlated with the active growth of the cells and tended to decline with age (Fattom and Shilo, 1984; Rosenberg, 1991).

Surfactants are known to be produced in several species of microorganisms. Among the bacteria, *Acinetobacter* sp. (Reisfeld *et al*, 1972; Sar and Rosenberg, 1983) and *Pseudomonas* (Hisatsuka *et al*, 1977; Reddy *et al*, 1982; 1983) have been well studied for their synthesis, production, composition and activity of biosurfactants. Some emulsifying agents like Emulsan (surfactant produced from *Acinetobacter calcoaceticus* RAG-1) were found to emulsify mixture of petroleum hydrocarbons, but not individual aliphatics or aromatics (Rosenberg et el, 1979). Similarly, *Pseudomonas* sp. could produce emulsifiers in the presence of gaseous, liquid alkanes, aromatics such as alkyl benzenes (Reddy *et al*, 1983).

The ability of emulsification was found improved with oils as against individual hydrocarbons (Juwarkar and Khirsagar, 1991) with bacteria. High molecular weight surfactants are generally required to increase the emulsifying activity (Shoham and

Rosenberg, 1983). Thus the use of biosurfactants producing marine microorganisms find wide application (Bertrand *et al*, 1993).

2.6. FACTORS AFFECTING DEGRADATION

In general, degradation of differnt crude oils by various workers have ranged between 70-97% and is dependent on the environmental conditions (factors). Among the environmental parameters which might influence degradation, little emphasis is given on parameters such as salinity, pH and temperature (Shiaris, 1989) while oxygen availability is reported to be more significant in enzymatic degradation pathways (Bauer and Capone, 1985). Atlas (1981) has discussed many of the factors that limit oil degradation in nature i.e. **physical constraints** such as temperature, availability of oxygen, salinity, pH , habitat (whether open or closed system and **chemical factors** like nutritional requirement N and P, other elements, nature of the hydrocarbons and presence of other toxic substances.

2.6.1. Physical factors

2.6.1.1. Temperature

Hydrocarbon biodegradation can occur over a wide a range of temperature, from below 0°C (ZoBell 1973, Traxler, 1973; Delille and Vaillant, 1990) to 70°C (Mateles *et al*, 1967, Klug and Markoretz, 1967). The rate of degradation increses with increase in temperature (Gunkel, 1967, Mulkins-Phillips and Stewart, 1974). Atlas and Bartha (1973) believe that rate of degradation depends on the seasonal shift

in the composition of the microbial community. Further studies showed that effect of temperature depends on hydrocarbon composition and incubation temperature. It was the heavy oils which degrade faster than lighter crudes since the latter caused longer lag periods (Atlas and Bartha, 1972; Atlas, 1975). Horowitz and Atlas, (1977) concluded that at low temperature co-metabolism played a major role in degradation. Walker and Colwell, (1976a) in their study found that extensive degradation occured at low temperature and attributed it to low toxicity whereas Atlas and co-workers (1978) were of the opinion that at high temperatures the toxic substrates evaporates due to lower viscosity. Thus it appears that the influence of temperature on degradation is not a limiting factor except at low temperature for polyaromatic hydrocarbons in estuarine sediments (Shiaris, 1989).

Ninety percent of oceanic water mass has a temperature of 4°C or below. Higher hydrocarbon degradation was observed at 0°C than at higher temperatures among psychrophiles by ZoBell (1973) and Walker and Colwell (1976a). Rates of mineralisation doubled at every 5°C between 5°C and 25°C when crudes were examined by Atlas and Bartha (1972).

2.6.1.2. Salinity

Reports on the effect of salinity on degradation of hydrocarbons are limited. In 1978, Ward and Brock showed degradation decreased with increase in salinity and attributed to reduction in microbial metabolic rate. However Kerr and Capone (1988) attributed effect of salinity to the ambient regime of study area. Shiaris (1989) showed

positive correlation with salinity and phenanthrene mineralisers in an estuarine environment. Bertrand *et al* (1990) reported the ability of an halophilic archaebacterium which could grow on all the 5 alkanes and 4 aromatic hydrocarbons tested and recorded a high of 88% degradation on tetradecane and 24% on 9-methyl anthracene at a concentration of 225 g/L of sodium chloride. Gauthier *et al* (1992) isolated a halotolerant bacterium ***Marinobacter hydrocarbonoclasticus*** which could degrade hydrocarbon and grew on a wide range of salinity. It could be summarised that salinity does have a bearing in the biodegradation of crude.

2.6.1.3. **Pressure**

It is known that a very low percentage of spilled oil reaches the deep oceans and it is mostly the recalcitrant fraction. Very little work has been carried out on the effect of pressure on biodegradation. Jannasch and Wirsen (1973) and Schwarz *et al* (1974) have demonstrated degradation of hydrocarbons at high pressures (500 atm), albeit at extremely slower pace. Similar reports on the barophilic bacteria have been reported by Brooks *et al*, (1987). Thus it is undeerstood that hydrocarbons persist for decades due to slow microbial degradation (Schwarz *et al*, 1974; Colwell and Walker 1977).

2.6.2. **Chemical factors**

2.6.2.1. **Nature and substrate (oil) concentration factor**

The physical and chemical state of hydrocarbons and its effect on the ecosystem has been elaborated in the earlier section. In brief the susceptibility to microbial attack are related to the movement, distribution and the presence of particulate matter ie the chemical nature of the oil. The rate of uptake is proportional to the concentration of the compound except for low solubility, of high molecular weight aromatics (Wodzinski and Coyle, 1974; Thomas *et al*, 1986). High concentration of hydrocarbon can be associated with heavy, undispersed oil slicks causing inhibition of microbial degradation. Inverse relationship of oil degradation against concentration of oil have been reported (Rambelorisoa *et al*, 1984) and agree with the work of Atlas and Bartha (1973), Venkateswaran *et al* (1991). However the tested concentration range was less than 5 g/L.

2.6.2.2. **Oxygen**

Oxygen is required for aerobic microbial growth. Hyrocarbon as the name denotes are compounds composed solely of carbon and hydrogen atoms and hence much attention has been given to the effect of oxygen on oil degradation. Its importance is indicated by the fact that major degradation pathways of both saturates and aromatic hydrocarbons involve oxygenases. ZoBell (1969) calculated that 2×10^5 of dissolved oxygen in seawater is required for the complete oxidation of 1 litre of oil. For Kuwait crude oil the maximum rate of oxygen consumption was 0.45 $g/m^2/day$

corresponding to an oil degradation rate of 90 mg/m^2/day (Johnston, 1970). Biodegradation of oil in sediment has been found to be stimulated by bioturbation (Lee, 1977).

There have been general reports on degradation in anaerobic environments at negligible rates using nitrate and sulphate as electron acceptor (Bailey *et al* 1973; Ward *et al* 1980; Mihelcic and Luthy, 1988). Anaerobic degradation is considered to be minor. The fact is that oil which enters deep ocean will degrade very slowly and persist for long periods of time. Oxygen limitation normally do not exist in the upper levels of water column in marine environments. But the concentration of the element is rate limiting variable in sediments and anaerobic environments.

2.6.2.3. **Nutrients**

Besides oxygen nutrients are the other essential ingredients for the breakdown of hydrocarbons. Confusion and conflicts prevailed about nutrient requirements for oil degradation. It was widely observed and accepted that nitrogen and phosphorus are limiting in seawater for microbial degradation (Atlas and Bartha, 1972; Bartha and Atlas, 1973; Floodgate, 1979; LePetit and Barthelemy, 1968). Evidence has also been presented suggesting that the supplementation of certain ecosystems with N and P may increase the relative number of hydrocarbon degraders. (Atlas, 1981; Reisfeld *et al* 1972; Gutnick and Rosenberg, 1977). Some investigators (Kinney *et al*, 1969) were of the opposite view. Atlas and Bartha (1972) record 1 mg/L of N and 0.07 mg/L of P was needed, whereas Reisfeld *et al* (1972) have reported that 11 mg/L of N and 2

mg/L of P was required for the removal of crude oil. This could be summarised to a ratio of approx 10:1 of N/P. There have been reports (Gibbs, 1975) that certain environments supply the microbes with suficient N that could degrade 11-30 g of oil/m^3 per year.

Apart from N and P, oleophilic iron compounds effectively stimulated biodegradation (Dibble and Bartha, 1976).

Thus the importance of the key role nutrients play during oil degradation were reitereated. Keeping this as one of the triggering factors, bioremediation technologies gained a step forward as man fertiliser based bioremediation agents were formulated. Some of them are - Sun oil, CRNF (Atlas and Bartha 1973), PSF (Olivieri *et al*, 1976), Inipol EAP22 (Laddouse and Tramier, 1991), Customblen (Safferman, 1991) and F-1 (Rosenberg, 1992) containing N and P in bound oleophilic mixtures. The N and P elemental composition of these commercial preparations ranged from 4.5 to 26% and 0.2 to 16.6% respectively. Patents in USA hve been registered on product formulations with nutrient (N & P) containing compounds in capsulated forms (Townsley, 1975), slow release fertilisers (Marconi *et al*, 1978), microemulsions (Tellier *et al*, 1984) and in dispersant bases (LePain and Bronchart, 1984; 1985).

Interestingly, Fedorak and Westlake (1981) have noted that nutrient supplementation to crude oil had stimulated the attack on saturates and had little or no effect on aromatics. On the other hand, in the absence of nutrient supplementation aromatic degradation was not impaired but there was a retardation in the degradation of aliphatics.

2.7. METHODS OF OIL SPILL CLEAN UP

With the recent increase in the frequency and quantum of oil spills in the marine environment, several methods evolved in response. Moreover offshore oil platforms are a constant source of pollution in the marine environment (Lizarraga-Partida *et al*, 1982). The sudden catastrophic oil spills are of greater concern and thus several clean up methods are in use when oil is spilled in the marine environment.

2.7.1. Physical

The immediate response is to use physical methods of collection with the deployment of booms, skimmers, pumps and adsorbents (Kelso and Kendziorek, 1991). This type of recovery by containment is never known to be easy or complete. *In situ* burning of oil is another response to oil removal (Kerambrun, 1995), which also invariably leaves residues (Prince, 1993).

2.7.2. Chemical

The use of dispersants or other chemicals to promote dissolution and dispersion (U.S. National Reasearch Council, 1989) in several places have met with varying degrees of success. It has been shown that most chemical dispersants are toxic to marine life forms (McIntosh, 1989) and could cause undesirable effects in the environment (Harvey *et al*, 1990).

2.7.3. **Biological**

This realisation has paved way for other essentially biological means with least harmful side effects. Left to nature the process of biodegradation would proceed albeit slowly. It has been shown that of all the petroleum spilled in the world oceans during any one recent year (approx. 6 million MT) were left on the ocean surface, it could result in a covering two molecular monolayers thick over the entire ocean surface (Garrett, 1972). Therefore, microbial degradation is a principal process responsible for the elimination of petroleum pollutants from the aerobic marine environment. The extension of this process as an oil spill counter measure is referred to as bioremediation. The process is defined in several ways, each subtly different from the other. This is defined as the process of encouraging the natural process of biodegradation to clean up pollutants. Bioremediation has become a technology that is discussed, applied and considered in many different circumstances. Experience shows no single technique will be appropriate for all incidents requiring response after oil spills. Bioremediation can provide low impact clean up options for use in sensitive tropical environments (Hoff, 1993). Sometimes resident microbial community could oxidise the oil in water column in 10 days (Atwood and Ferguson, 1982). The rate of biodegradation is dependent on substrate type, temperature, microbial community. There is always a lag phase after oil is spilled before indigenous microbes begin to breakdown the oil molecules (Hoff, 1993; Francis *et al*, 1992). To make bioremediation a quick or first response technique it is suggested that this fact has to ably tackled. Biological clean up or bioremediation is not a new concept but has been

receiving additional attention and impetus recently. This is also because a majority of the molecules in crude oil and most refined products are biodegradable.

After the successful biological clean up of oil spilt following the *Exxon* grounding in Alaska, it is now considered that bioremediation has come of age (Bragg *et al*, 1994; Swannell and Head, 1994). This relatively inexpensive clean up technology has been found to be non-intrusive and increasingly effective (Pritchard and Costa, 1991).

Bioremediation of hydrocarbon mixture is a complex microbial process which gets greatly modified by the surrounding environmental factors. The general procedures consist of:

In situ

* Promoting the activity of the local microflora by adding nutrients - **biostimulation**

* Cultivating of microorganisms and introduction in the area to be decontaminated - **bioaugmentation**

Ex situ

* Fermentors and chemostat

There are different approaches involved in biostimulation procedures. They are: 1) adding nitrogen (N) and phosphorus (P) nutrients, 2) adding degradable carbon with N and P, 3) increasing the surface area by adding surfactants and 4) addition of exogenous organisms - natural isolates or by enrichment (Heitzer and Sayler, 1993).

Bioaugmentation involves seeding of allochthonous microorganisms into the environment for the purpose of increasing their rate and extent of biodegradation.

2.7.3.1. **Biostimulation by nutrient addition**

Other methods include addition of nutrients. This stimulates biodegradation by natural or autochthonous microflora and is in wider use in North America. Additon of nutrients aid in the extensive microbial degradation of large oil slicks as they reduce the wide C:N and C:P ratio resulting out of oil spills. Many types of nutrient formulations made of inorganic or organic compounds are now commercially available, some of which are listed in the review by Prince (1993).

Nevertheless bioremediation strategies based on nutrient enrichment are not without their disadvantages and adverse ecological effects. Excessive application of fish bone fertilisers have been shown to cause oxygen depletion and ammonia production within closed systems (Lee *et al*, 1995). In the case of open systems, soluble nutrients would get washed away or could cause undesired blooms of phytoplankton (Atlas, 1991).

In field conditions application of oleophilic fertilisers have not completely provided the nitrogen and phosphorus nutrients to speed up autochthonous microbial degradation of oil (Harvey *et al*, 1990).

Despite the availability of several nutrient formulations in encapsulated or as microemulsion forms there have apparently been no successful uses of nutrient addition to floating oil slicks (Sveum and Ladousse, 1989; Prince, 1993). Use of oleophilic

fertilisers like Inipol EAP22 have been found to be counter productive in few cases thus varying in stimulating biodegradation (Ladousse and Tramier, 1991; Lee and Levy, 1991) in temperate waters. But no effort on the use of such fertilisers in tropical waters have been reported so far.

Microbial degradation of oil is a surface phenomenon. Since nutrient addition is aimed at stimulating microbial biodegradation, it is unlikely to be dramatically effective when pooled oil is present in low energy shorelines and hence novel approaches to encourage biodegradation need to be realised.

2.7.3.2. **Advent of genetically engineered microorganisms**

With the advent of newer molcular tools and techniques genetically engineered microorganisms (GEMS) are developed through gene manipulation for use in bioremediation applications. Use of such GEMs for the degradation of various hydrocarbons have been documented (Friello *et al*, 1976; Lloydjones *et al*, 1994; Lee *et al*, 1994). Since certain hydrocarbon degradative genes are coded on plasmids (Chakrabarty, 1976) they are amenable for integration and regulation in desired organisms. Genetically improved microorganisms capable of rapid growth with crude oil have been constructed but have never been tested (Harvey *et al*, 1990). Despite the advancements of research in this area, there is still considerable controversy on the issue of introducing these GEMs into the environment (Leahy and Colwell, 1990). This is because the aspects of ecological safety, containment and damage by GEMs when introduced into the environment have not been resolved and the deliberate

release of GEMs is unlikely in the near future given the current international regulatory framework (Atlas, 1991).

2.7.3.3. **Bioaugmentation using allochthonous flora**

Consequently, the use of exogenous microorganisms with known degradative activities has been another principal way for bioremediation of oil spills. Naturally present microorganisms in an environment may not be capable of extensive degradation of the wide range of complex substrates in petroleum (Atlas, 1991). In such a scenario, introduction of exogenous seed cultures have been found to be effective and successful in removing the oil completely (Mangan, 1990).

The criteria to be met by effective seed organisms have been explained by Atlas (1977). Petroleum degrading bacteria with high degree of enzymatic activity and growth in the environment are preferred for selection of seed organisms. Inocula of mixed cultures having different and complementary hydrocarbon degrading capabilities have been looked into (Leahy and Colwell, 1990). Therefore the possibility of using allochthonous microflora which can synergistically break down different compounds of the crude oil mixture holds a good potential for current and future bioremediation formulations.

2.8. PLASMIDS AND PETROLEUM HYDROCARBON DEGRADATION

Some of the key catabolic functions are coded on extracellular chromosomal elements or plasmids in bacteria. Hydrocarbon degradative genes are also found among them (Singer and Finnerty, 1984b).

Hydrocarbon degradation by microbial community depends on its adaptive response to the presence of hydrocarbons. The mechanism involves both selective enrichment and genetic changes resulting in an increase in the genetic pool of hydrocarbon catabolising genes. Several workers have reported the involvement of plasmids in the degradation of aliphatics as well as in aromatics (Chakrabarty *et al*, 1973; Connors and Barnsley, 1982). Plasmids are extrachromosomal genetic elements that confer certain potential to a microorganism. They are dispensable as they are not required for viability or multiplication. Nevertheless, these carry a number of genes/determinants which permit their bacterial hosts to survive better under stressed conditions. They help them to compete more successfully with other microorganisms of the same or different species. Their molecular weight range from 2x 10^6 to 10^8 daltons and is approximately 1/20 of genomic DNA. Plasmids are also known to get horizontally transferred i.e. to bacteria belonging to different genera and species and they thus play an important role in the general adaptation and evolution (Boronin, 1992). The conferred potential helps in the evolution of integrated and regulated pathways (Singer and Finnerty, 1984a). Plasmids and their functions are thus transmissible both vertically and horizontally.

Diverse metabolic functions are coded on plasmids. The last decennia saw the description of new plasmid-borne resistances which were associated with metal-mediated selection pressures from clinical, agricultural or industrial origin (Collard *et al*, 1994). Some of these functions are also for degrading hydrocarbon in the environment mediated by diverse indigeous microbial community which contain naturally occuring D plasmids (Boronin, 1992). In fact, most of the degradative functions exhibited by bacteria are mediated by genes that are not located on the chromosome but on the extrachromosomal DNA elements - plasmids (Chakrabarty, 1972; Jain and Sayler, 1987). It should be stressed here that though the genetics of hydrocarbon utilising microorganisms has essentially occured since the mid seventies, the complexities involved therein are yet to be fully understood. However this is a rapidly developing field of microbial genetics.

The study of plasmid-specified aromatic hydrocarbon metabolism has advanced much more than aliphatics. This is perhaps due to the better understanding of the biochemistry and enzymology of aromatic hydrocarbon metabolism as compared to alkane metabolism (Singer and Finnerty, 1984a).

OCT plasmids which degrade n-alkanes and gene alk BAC for alkane hydroxylation and alkanol dehydrogenation located on plasmids have been studied by various workers (Baptist *et al*, 1963; Chakrabarty *et al*, 1973; Kok *et al*, 1989). Bacterial genes encoding degradation of low molecular weight aromatic hydrocarbons such as benzene, toluene, naphthalene and biphenyl have been localised to specific

plasmids which belong to TOL and NAH families (Burlage *et al*, 1989; Yen and Serdar, 1988; Furukawa *et al*, 1990).

The environment determines the extent of gene expression by influencing the activation and repression of the genes (Daubaras and Chakrabarty, 1992). Degradative genes thus evolves with the environmental conditions. High evidences of plasmid DNA as a consequence of adaptation to higher levels of hydrocarbons have been pointed out (Boehm and Fiest, 1980; Spain *et al*, 1980). Thus bacteria from oil polluted environments are more adept at degrading hydrocarbons than from unpolluted areas (Colwell *et al*, 1973). The frequency of occurence also increased in oil polluted environments (Hada and Sizemore, 1981; Burton *et al*, 1982; Day *et al*, 1988). However, Leahy *et al* (1990) reported otherwise. The reason attributed was that of the low degree of hydrocarbon contamination in the region of study. Besides, it has been observed that a certain amount of threshold concentration would be necessary to affect changes in plasmid incidence (Wickham and Atlas, 1988).

Incidence of plasmids are known to be common in a wide range of microorganisms, notably *Arthrobacter, Acinetobacter, Alcaligenes, Pseudomonas*, etc. The involvement of plasmid encoded genes in the catabolism of both alkane and aromatic hydrocarbons by strains of *Pseudomonas putida* and *Acinetobacter calcoaceticus* has been reported (Singer and Finnerty, 1984a and 1984b).

Alcaligenes eutrophus is known to harbour metal resistance conferring plasmids (Collard *et al*, 1994). *P. aeruginosa* possesses great diversity of plasmids which are incompatible and antibiotic resistant (Boronin, 1992). Incidentally they harbour TOL

and NAH7/NAH90 plasmids which are widespread but structurally stable (Foght and Westlake, 1991). Short chain alkane metabolism genetic studies have been conducted on *P. putida*. These studies have shown that the genes are localised on both OCT plasmids and chromosome (Lee *et al*, 1993).

3

Materials and Methods

3.1. ISOLATION OF PETROLEUM DEGRADING MICROBES

3.1.1. Sampling sites

Various marine ecological habitats were chosen for isolating microbes capable of degrading petroleum hydrocarbons, growing on nitrogen-free medium and solubilising insoluble phosphates. All the sites are part of the Indian peninsula, that receive petroleum hydrocarbons by some way or the other over a period of time.

3.1.1.1. Bacteria

The locations of sampling sites are shown in Fig. 1.

a) Kalpeni: This is one of the Lakshadweep islands in the Arabian Sea and is close to the main oil tanker route that connects the Mediterranean and the Indian Ocean. The island constantly receives inputs of highly weathered tarry lumps which are found washed ashore. Fishing and recreational activities are also partly responsible for the year round discharge of petroleum products into its lagoons.

b) Goa: The Dias beach situated at Dona Paula is a sheltered beach with rocky shores and is partly made of clayey sandy substratum. Beds of brown macroalgae (*Sargassum* sp.) are found along this beach which lies on the northern bank of the mouth of Zuari estuary confluencing with the Arabian Sea. The width of the estuarine mouth between the beach and the opposite Marmagao port is 5 kms. The beach experiences moderate oil pollution due to oil tanker operations at the harbour. Occurences of tar balls on the shores and oily sheens on water are common.

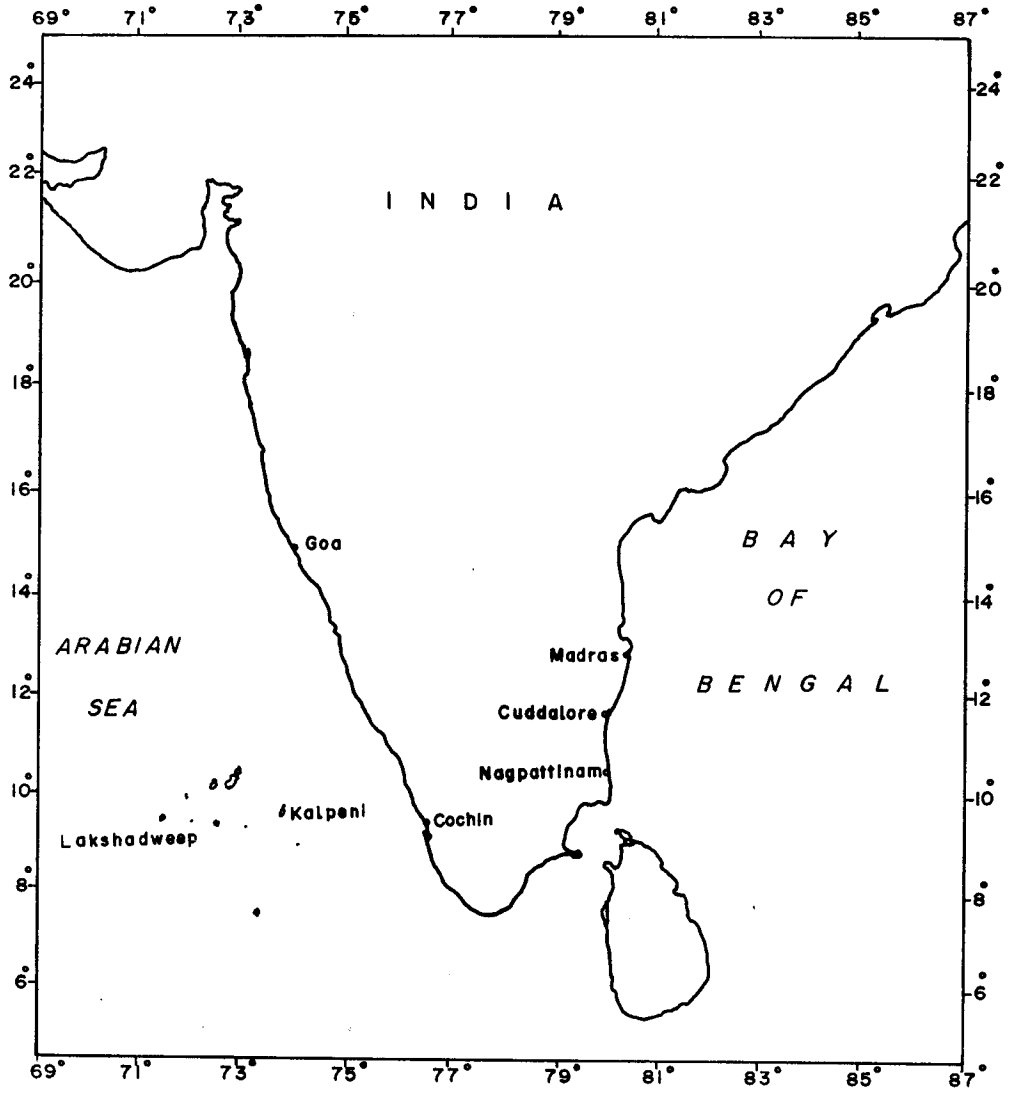


Fig.1. Location of the sampling site .

c) Madras: The site is very close to the area where Madras Refineries Ltd. discharges its refinery effluents into the adjoining Bay of Bengal. Due to shipping and other logistic activities of oil products close to the refineries, surrounding waters are subjected to chronic exposure of petroleum hydrocarbons.

d) Cuddalore: This is situated south of Madras and is an active fishing harbour. The adjoining waters receive considerable amount of oil due to port activities.

e) Nagapattinam: This is one of the major fishing ports in the south east coast which experiences chronic oil inputs due to the various port activities.

3.1.1.2. **Yeasts**

a) Cochin: This port city situated on the southwest coast of India, is active in oil tanker operations. Though the open sea is subjected to anthropogenic releases of petroleum hydrocarbons due to bilge washings, etc. the port and its back waters are also a perennial sink for petroleum hydrocarbons arising out of oil transport and storage activities. Besides, the port also serves as a Naval base and a major shipyard (Cochin shipyard) constructing bulk carriers is also situated near the collection site.

b) Madras: The location of the site for isolation of yeasts was the same as mentioned for bacteria in the earlier section.

3.1.2. **Sample collection and isolation**

3.1.2.1. **Isolation of bacteria**

3.1.2.1.1. **Sediment**

Sediment samples were collected from various locations as shown in table 1 using a sterile spatula. The collected samples were brought to the laboratory in ice boxes and subjected to serial dilutions (upto 10^{-6}) before plating on to nutrient agar in 50% seawater (NAS) plates. One ml of all the serial dilutions were inoculated into modified nitrogen-free mannitol (NFM) (appendix-1) broth (Coty, 1967). After 2-3 days 0.1 ml from the broth were spread plated onto NFM. Isolates which grew within 6 days were stored in NFM slants at 4 °C.

3.1.2.1.2. **Water**

Water samples were collected from different areas as mentioned in Table-1. Surface water samples were collected using sterile glass bottles and from shallow depths using J-Z samplers. After serial dilution upto 10^{-3}, aliquots of 0.1 ml were spread plated on NAS plates and subsequently morphological dissimilar colonies were randomly isolated and stored in slants at 4 °C. Bacterial cultures that were able to grow on nitrogen deficient medium were isolated from waters of Dias beach. One ml of all the serial dilutions were inoculated into modified nitrogen-free medium (NFM). After 2-3 days 0.1 ml from the broth were spread plated onto NFM. Isolates which grew within 6 days were stored in NFM slants at 4 °C.

3.1.2.1.3. **Seaweeds**

Specimens of the brown alga *Sargassum* sp. were collected from Dias beach. Holdfasts were cut using a sterile blade and were transferred to 100 ml of sterile sea water and serially diluted to 10^{-3}. A volume of 0.1 ml of all the above three dilutions were inoculated into modified NFM broth. After 2-3 days the broth was spread plated on a solid medium of the same composition. Those which were able to grow between 4 to 6 days and possessed the ability to grow on repeated sub-culturing were isolated, purified and stored on NFM slants at 4°C.

3.1.2.1.4. **Tar balls**

Tar balls were collected aseptically from the shores of Kalpeni Island using sterile plastic bags. The tar balls were washed with sterile seawater to remove attached sand and debris. Washed tarballs were stored in plastic bags in a refrigerator for further work. One or two tarballs (10-15 gms) were transferred to a flask containing sterile artificial sea water (ASW) (appendix-1), and kept on a rotary shaker for 2-3 hrs at room temperature (28 ± 2°C). At predetermined intervals water samples froem the flasks were streaked on NAS plates and were incubated for 2-3 days. The isolation procedure is illustrated in Fig. 2. Bacterial strains were isolated based on their phenotypic characteristics. After purification all the strains were subcultured onto NAS slants and stored in the refrigerator. These isolates were referred to as "tar ball associates".

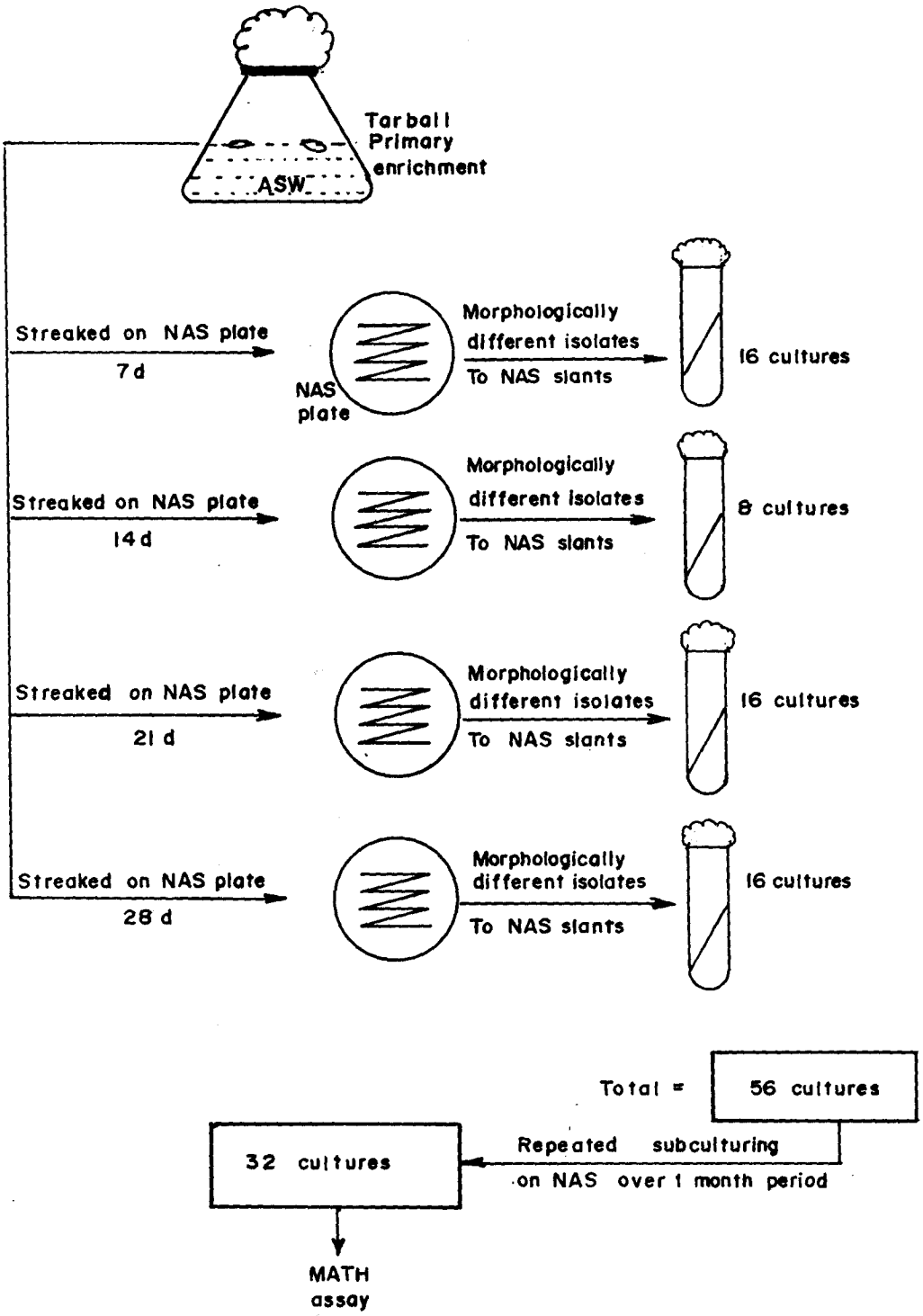


Fig.2. FLOW CHART FOR ISOLATION OF BACTERIA FROM TARBALL BY ENRICHMENT METHOD.

Isolates from all the above sources were tested for their ability to solubilise inorganic phosphate as hydroxy apatite medium (HAM) (Ayyakkannu and Chandramohan, 1970) (appendix-1). Cultures were streaked on plates containing HAM. After 3 days of incubation, halo formation in the media around the colony indicated the solubilisation of the insoluble hydroxy apatite precipitate. Those cultures that produced a halo around them were referred to as phosphate solubilising bacteria/yeasts or "phosphate solubilisers".

The bacterial isolates were tested for their growth in NFM before assesing their hydrocarbon degrading ability.

Thus bacteria were classified into 3 groups as:

a) "Phosphate solubilisers"

b) "Nitrogen fixers"

c) "Tar ball associates"

The term "nitrogen fixers" is used for those cultures that grew on the nitrogen deficient medium; however the nitrogen fixing ability was not tested.

3.1.2.2. **Isolation of yeasts**

3.1.2.2.1. **Sediment**

Samples were collected from Cochin backwaters and offshore, using a mud sampler (Petersen grab). The samples were serially diluted upto 10^{-4} and 10^{-3} for backwaters and offshore waters respectively. Isolation of yeasts from these two sampling stations was done using Malt Extract Agar (MEA) plates which were

prepared with 50% seawater (appendix-1). Morphologically different cultures were isolated, purified and were stored on MEA slants at 4°C.

3.1.2.2.2. **Water**

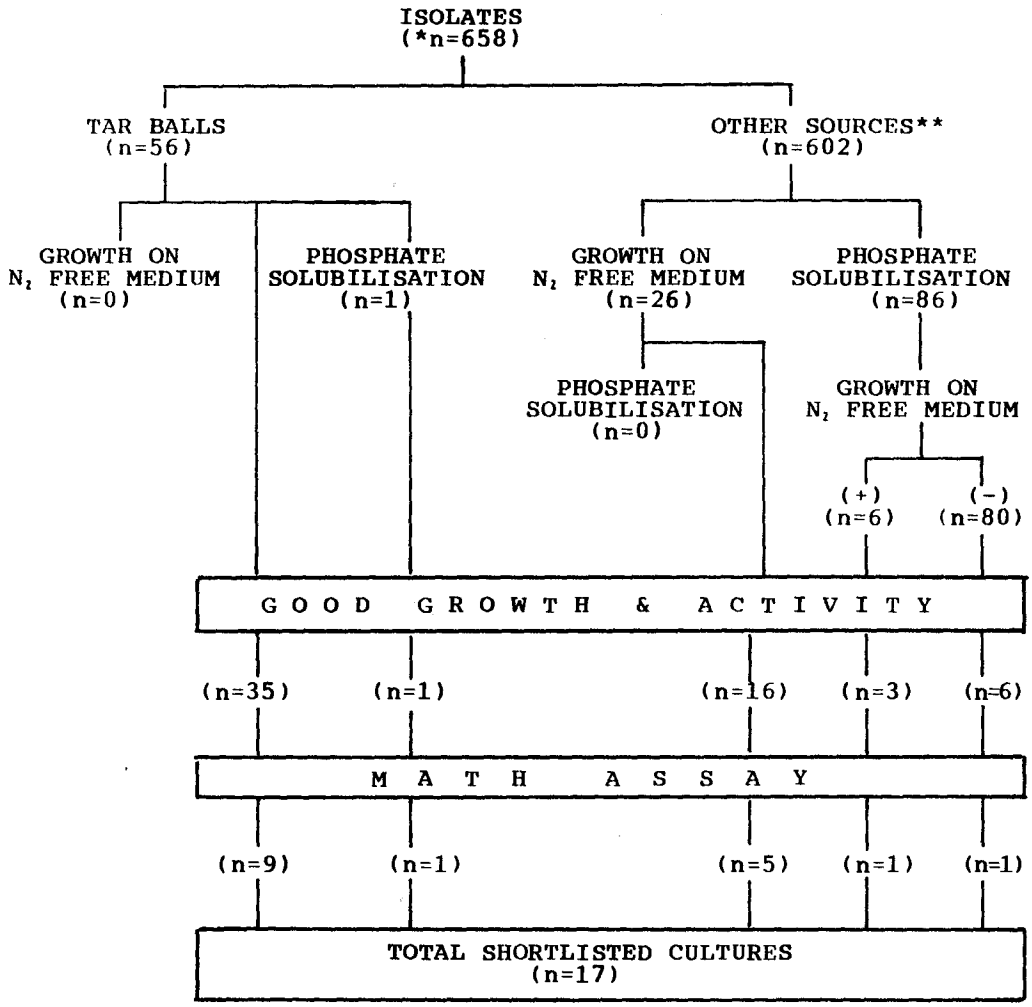
Samples from the Madras coast, close to Madras Refineries Ltd. were collected using ZoBell's water sampler. The samples were serially diluted to 10^{-3} and 0.1 ml of this dilution was spread plated on MEA medium (50% seawater). After 24-36 hours yeast colonies appeared on the plates which were isolated, purified and transferred to MEA slants and stored at 4°C. The cultures from sediment and water were tested for their ability to solubilise phosphate.

3.2. DEGRADATION STUDIES

3.2.1. Microbial adhesion to hydrocarbons (MATH) assay

All the selected bacterial strains (Fig. 3) from the 3 groups as mentioned earlier, were subjected to MATH assay for testing microbial cell wall hydrophobicity and surfactant production.

MATH assay was carried out following the procedure outlined by Rosenberg (1991). Bacteria were grown on nutrient agar (prepared in 50% seawater) plates for 24 hrs. A loopful of cells were harvested and suspended in 4 ml of membrane filtered (Millipore 0.2 μ) sea water in optically clean tubes (Corning) taking adequate care not to carry over any medium.



*n - number of cultures.
** - sediment, seawater, seaweed holdfast.

Fig.3 . Flow chart for selection of bacterial isolates for MATH assay and preliminary degradation experiments.

Initial turbidity of the cell suspension was measured at 550 nm (Beckman DU 6 Spectrophotometer). To this 0.5 ml of analar grade hexadecane was added and vortexed for 2 minutes. The suspension was allowed to stand for 20 minutes at room temperature for phase separation, the turbidity of the aqueous phase measured at 550 nm and the fraction of adherence was calculated as follows;

$$\text{Fraction of adherence} = \frac{A - C}{A}$$

where A = initial turbidity and

C = final turbidity.

Fraction of cells adhered was defined as the ratio of the difference between the initial and final turbidity over the initial value. The adhesion property is referred to as AP.

This method could also indicates the production of surface active agents referred to as SP exuded by the organisms which was observed by the persistence of fine globule-like formation in the organic phase.

3.2.2. **Degradation of crude oils by microbes**

Inoculum protocol

Bacteria

Bacterial strains were streaked on NAS plates and incubated for 24-30 hrs. Individual culture suspensions were prepared in 5 ml sterile ASW after harvesting the cultures such that it would yield a final $O.D._{600}$ of 1.0 in the experimental flasks.

Yeasts

Yeasts were streaked onto MEA plates and incubated for 24 hrs and then harvested. The cells were suspended into 5 ml of sterile ASW. The culture suspension was prepared to result a final $O.D._{600}$ of 1.0 in the experimental flasks.

Medium and substrates

Conical flasks containing 50 ml of sterile ASW (Fedorak and Westlake, 1981) was used as the medium. Details of the petroleum substrates used in this study are as follows: Iranian light crude oil (IC) was obtained from ONGC, Bombay which was over 2 years old. Bombay High crude oil (A) (BHC- A) was supplied from the Bombay High wells of the ONGC, Bombay and tar balls were collected from the shores of Kalpeni Island. Both IC and BHC-A were used at 1% v/v concentration while tar balls were used at 1% w/v.

Incubation

All experiments were carried out in duplicates for 7 days on a rotary shaker (200 rpm) at room temperature. Uninoculated controls were used in all the experiments.

Extraction and analysis

Experiments were terminated by extracting the residual petroleum hydrocarbons with CCl_4 as given in Fig. 4, and analyses were carried out by gravimetry and/or gas chromatography (GC).

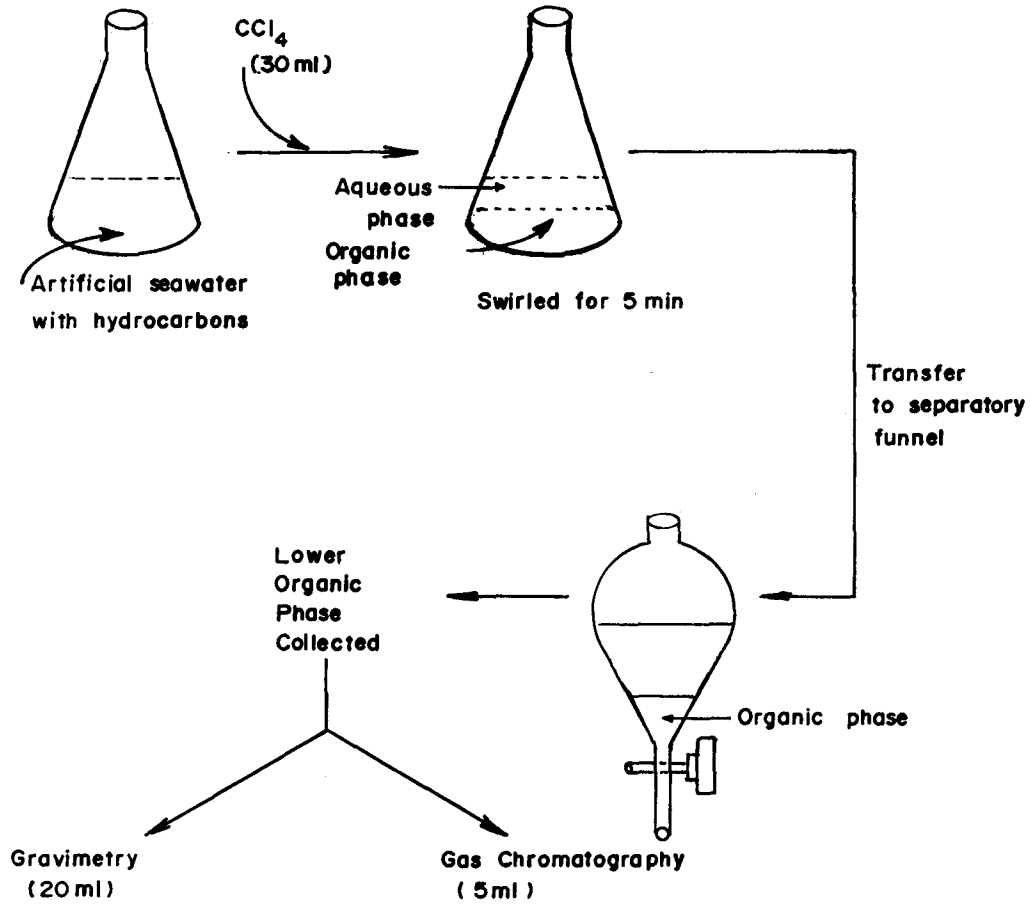


Fig. 4. Flow chart for extraction of petroleum hydrocarbons.

3.2.2.1. **Degradation of Iranian light crude oil**

Bacteria

Degradation of Iranian crude oil was studied for 17 individual bacterial cultures.

Yeasts

Yeast strains which showed good adhesion based on MATH assay were examined for their degradative ability on Iranian light crude oil. A few of the marine yeast isolates which did not show good adhesion were also examined for their degradation.

3.2.2.2. **Degradation of Bombay High crude (A) oil**

Bacteria

Bacteria which showed good degradation (60%) with Iranian crude oil were checked for their degradation of BHC-A.

Yeasts

All the yeast cultures were selected based on their degradation efficency of Iranian crude oil.

3.2.2.3. **Degradation of tar balls**

Bacteria

Two cultures which showed good utilisation of crude oils (>60%) were tested for their degradation of naturally weathered crude oil - tarballs.

Yeasts

All the three potent yeast cultures which were selected based on their degradation of BHC (A) were examined for their ability to degrade tar balls. Estimations of degradation were done by gravimetry.

3.3. FRACTIONATION OF PETROLEUM HYDROCARBONS BY COLUMN CHROMATOGRAPHY

Three crude oils (Bombay High (A), Bombay High (B) and Iranian light), a furnace oil and tar balls were fractionated following the procedure of ONGC (1993). While the source details of IC, BHC-A and tar balls are mentioned in section 3.2.2., the other petroleum sources were: Bombay High crude oil (B) (BHC-B) which was obtained from NCL, Pune; and furnace oil that was collected from a two-day old floating oil slick off Sinquerim, Goa.

Silica gel (60-120 mesh size) and alumina (neutral) were activated for 24 hours at 150°C. These were cooled to room temperature in a dessicator. A glass column (90 cm x 2 cm inner diameter), having glass wool at the bottom, with corresponding reservoir and standard joint, was filled with analytical grade petroleum ether and was packed with 50 gm of alumina followed by 50 gm of silica gel. The column was washed with petroleum ether once.

A known weight of crude oil/tar ball (approx. 1 gm) was taken in a 25 ml beaker and dissolved in about 4 to 5 ml of chloroform till complete dissolution. To this 2-3 gm of silica gel was added to make a slurry by mixing with a glass rod. The

mixture was allowed to stand at 60°C until the chloroform in the mixture completely evaporated and the crude oil adsorbed to silica gel.

The crude oil adsorbed to silica gel was charged at the top in the column. The saturate fraction was eluted with 200 ml of petroleum ether, the aromatic fraction with 200 ml of benzene and the polar fraction with 200 ml of methanol into separate round bottomed flasks. All solvents which were used were of HPLC grade. The fractions were concentrated using a Rotavapor at 60°C.

The concentrated fractions were transferred to tared vials and were allowed to dry and reach constant weight in a vacuum dessicator. Required concentrations of the two fractions of crude oil were prepared by dissolving in hexane for aliphatic fraction and benzene for aromatic fraction.

3.3.1. **Degradation of fractionated crude oils**

3.3.1.1. **Bombay High crude (A)**

Bacteria

Four bacterial cultures were grown according to the earlier inoculum protocol (section 3.2.2.). The cultures were inoculated into 250 ml conical flasks containing 50 ml of ASW and 1 % w/v of aliphatic fraction dissolved in 1 ml of hexane and another set of flasks containing 0.1% w/v of aromatic fraction dissolved in 1 ml of benzene. Other experimental details remained the same as mentioned in section 3.2.2. The hexane and benzene solvents which were added got evaporated totally within the first six hours of the experiment.

Yeasts

Among the yeasts, 3 strains were tested for degradation of the aliphatic and aromatic fractions of crude oil. The cells were raised in MEA medium. Inoculum preparation, experimental and analytical procedures were same as given in section 3.2.2..

3.3.1.2. **Bombay High crude (B)**

The degradation of aliphatic and aromatic fractions of Bombay High Crude (B) oil by four bacteria and 3 yeasts were tested.

All conditions of the experiment were same as mentioned in Bombay High crude (A) oil fractions for bacteria and yeasts except that in these experiments the concentration of the aromatic fraction was increased to 0.2% w/v.

3.3.1.3. **Tar balls**

Two bacteria and three yeast strains were examined for their ability to degrade aliphatic (1% w/v) and aromatic fractions (0.1% w/v) of tar balls under similar experimental conditions found in section 3.2.2.

3.4. DEGRADATION OF INDIVIDUAL PURE SOLID AROMATIC COMPOUNDS

Bacteria

The degradation of three pure, solid aromatic hydrocarbons namely naphthalene, anthracene and phenanthrene were examined at 0.1% w/v concentration in 50 ml of ASW for its degradation by two candidate bacterial strains. The inoculum was prepared according to the method mentioned previously (section 3.2.2.) and incubated for 7 days. After the incubation period, residual aromatics were extracted using 10 ml of analytical grade benzene and were estimated by both GC and UV spectrometry. The absorbance maxima for each of the three compounds were ascertained by scanning the entire UV spectrum. The absorbance maximum for naphthalene was 246 nm and for anthracene and phenanthrene it was 253 nm. The measurements were taken in a Beckman DU-6 UV-vis spectrophotometer.

Yeasts

Three candidate yeast cultures were also examined for degradation of the three pure solid aromatic hydrocarbons namely, naphthalene, anthracene and phenanthrene (0.1% w/v) individually. The experimental conditions of incubation and analyses were as mentioned for bacteria in the earlier section.

3.5. FACTORS AFFECTING PETROLEUM HYDROCARBON DEGRADATION

The effect of five variable parameters on the degradation of crude oil (Bombay High Crude oil (A)) was examined using five candidate cultures (2 bacterial strains and 3 yeast strains). All experiments were run in duplicates with suitable controls. Unless specified the incubation period was for 7 days on a rotary shaker (200 rpm). The extraction of residual crude oil after incubation was carried out using the extraction protocol.

3.5.1. Effect of temperature

All the 5 strains were tested for their individual ability to degrade Bombay High crude oil (1 % v/v) in ASW at $20\pm2^{\circ}C$ and $30\pm2^{\circ}C$ within a period of 7 days. One ml of the culture suspension (in ASW) having a $O.D._{600}$ of 1.0 was used as inoculum. Degradation was estimated by gravimetry.

3.5.2. Effect of period of incubation

The effect of 3 different periods of incubation (3,7,15 days) on the degradation of Bombay High crude oil (1% v/v) was studied by inoculating 1 ml of 1 $O.D._{600}$ culture suspension into 3 sets of flasks. Three sets of control flasks were also kept. The flasks were incubated on a rotary shaker at room temperature. At the end of 3, 7, and 15 days respective experimental and control flasks were removed and the residual oils were extracted and estimated using gravimetry.

3.5.3. Effect of substrate concentration

The effect of six concentrations of Bombay High crude oil for the above cultures were studied. Inoculum contained 1 ml of 1.0 $O.D._{600}$ culture suspension. The concentrations (v/v) of oil used were 0.5, 1.0, 1.5, 2.0, 2.5 and 4.0 %. Degradation was estimated gravimetrically.

3.5.4. Effect of inoculum size

Based on optical density at 600 nm, two different inoculum sizes (O.D. = 1.0 and 3.0) were used so as to get a cell density of 10^8 and 10^{12} cells/ml respectively for bacteria, while for yeasts the cell densities were 10^6 and 10^{11} cell/ml. They were inoculated into 50 ml of ASW containing 1% v/v crude oil. The experiment was terminated using the extraction protocol and the percentage of degradation was estimated gravimetrically.

3.6. CHARACTERISATION AND TAXONOMY OF MICROBIAL STRAINS

3.6.1. General characterisation of hydrocarbon degrading strains

Gram staining and motility tests

Standard methods were used for Gram staining and motility (hanging drop method).

Size and shape

The cultures were stained with crystal violet and the size and shape were observed and measured using an ocular micrometer.

Spore formation

Forty eight hours old cultures were stained with methylene blue for the presence of spores.

3.6.2. **Biochemical characterisation of bacteria using API strips**

Cultures were examined for their biochemical properties such as enzyme activity and sugar utilization using the API 20E #2010 strips (France). The cultures were streaked on NAS plates and after incubation for 24-36 hours, the cells were suspended in 5 ml of sterile sea water to arrive at an O.D. of 0.2 at 600 nm. About 20 tests were carried out by inoculating with the requisite volume of the cell suspension into each of the tubes and cupules. The strips were incubated at 35-37°C for 18-24 hours. Colour development was recorded and compared with the chart.

Other biochemical tests such as catalase, oxidase and phosphatase (Kobori and Taga, 1978) were also conducted using the standard procedures.

3.6.3. **Antibiogram**

Resistance to antibiotics was tested by checking the sensitivity of cultures to antibiotic discs (Himedia, Bombay). The following 14 antibiotics were tested: Ampicilin, Bacitracin, Chloramphenicol, Cloxacillin, Doxycycline, Erythromycin,

Gentamycin, Kanamycin, Nalidixic acid, Neomycin, Oxytetracycline, Penicillin, Rifampicin and Tetracycline. Cultures were grown in 5 ml of nutrient broth (NB) overnight in a shaker. Sterile cotton swabs were dipped into the broth and was spread uniformly on NAS plates for development of a "bacterial lawn". Antibiotic discs were placed on the medium using the antibiotic self dispenser and were evenly spaced out. Observations were made after 24 hrs. Cultures were considered sensitive to respective antibiotics when a halo was seen around the disc; and resistant, when growth was observed around the disc.

3.6.4. **Generic Identification of microbes using fatty acid profiles of cells**

All the strains selected after MATH assay were identified using a computer aided microbial identification system (MIS) (MIDI, USA). The MIS analyses and identifies microorganisms isolated in pure culture on a specific medium. It uses a sample preparation procedure and gas chromatography to yield qualitatively and quantitatively reproducible fatty acid composition profiles. The fatty acids extracted from unknown microorganisms are quantified and identified which are then compared to a library of reference organisms stored in the computer to determine the identity of the unknown. Samples could be identified to the species and subspecies level.

Sample processing

Bacteria and yeasts were grown on trypticase soyabean agar (TSBA) and Sabouraud dextrose agar (SDA) repectively by quadrant streaking of pure isolates on

plates. After incubation for 12-14 hours at room temperature the cells were harvested from the second and third quardrants for analysis.

Five major steps were involved in the preparation of cells for fatty acid composition analysis.

Harvesting

The cells were harvested from TSBA medium for bacteria and SDA medium for yeasts, to yield about 30 mg of the cells. This was transferred to the bottom of a glass tube with a teflon-lined screw cap.

Saponification

One ml of saponification reagent (appendix-2) was added and vortexed for 5-10 sec and the tube was placed in a water bath at 100°C for 5 min. This was vortexed for 5-10 sec and again kept in the water bath for a further period of 25 min after which it was cooled.

Methylation

Two ml of the methylation reagent (appendix-2) was added to the tube and vortexed for 5-10 sec placed in a 80 $\pm$ 1°C water bath for 10 $\pm$ 1 min. and cooled rapidly.

Extraction

After methylation the fatty acids in the aqueous phase were extracted using Methyl Tertiary Butyl Ether (MTBE) reagent (appendix-2). To the tube, 1.25 ml of the reagent was added and gently shaken end to end for 10 min for the extraction of

methylated fatty acids into the organic solvent phase. The bottom aqueous phase was removed and discarded. The top organic phase was retained.

Wash

After adding 3.0 ml of the base reagent (appendix-2) the tube was tilted end to end for 5 min to remove non methylated acids and residual reagents from the organic phase. Two-thirds of the top organic phase from the tube were transferred to a 2.0 ml GC vial. (When the upper organic phase was not clear a few drops of saturated NaCl was added to clarify the suspension).

The vials thus obtained were placed on the auto sampler of the MIS for analysis. The instrument was programmed for chromatographic runs, library comparisons and species identification. Chromatograms with report were obtained wherein the species was identified and its similarity index or Euclidian distance were given depending upon the relatedness to a genus and species found in the system library.

3.6.5. **Growth and generation time**

Selected bacterial (ND22, T17B, T44) and yeast cultures (CY6, CY8, MY11) were grown on nutrient broth and malt extract broth respectively prepared in 50% seawater and were incubated on a shaker at room temperature. At 3-hourly intervals the O.D. was measured at 600 nm using the spectrophotometer. This was plotted against time for growth and the generation time was calculated.

Experiments were also carried out to study the growth rate of these cultures on hexadecane (1% v/v) and crude oil (1% v/v). In the latter, the growth was measured by estimating the population by plate method initially every 6 hours upto 24 hrs and thereafter at 12 hourly intervals.

3.7. BIOSURFACTANT PRODUCTION AND OIL EMULSIFICATION

3.7.1. Production of biosurfactants

Production of oil emulsifier by culture T17B was estimated essentially following the procedure of Juwarkar and Khirsagar (1991).

The culture was inoculated into flasks containing 500 ml of mineral medium (Coty's medium - appendix-1) and the yield of emulsifier from substrates such as hexane, nonane, hexadecane and crude oil was examined. The flasks were incubated on a rotary shaker for 5 days. The cell-free culture medium was treated with 3 volumes of cold acetone and allowed to stand overnight at room temperature. The precipitate obtained was dissolved, after multiple serial washings in distilled water, acetone and dried in an incubator at 35°C.

3.7.2. Estimation of crude oil emulsifying activity

The total crude biosurfactant was dissolved in distilled water (10 ml), pH was adjusteed to 5.5 and was diluted to 10^{-5}. An aliquot of 10 ml of this surfactant solutiion was taken in a glass stoppered tube and 0.1 ml of Bombay High crude oil (A) was added. The contents were shaken vigourously and allowed to stand for 10 mins.

The $O.D._{610}$ of the stable suspension was measured using a spectrophotometer. The resulting O.D. signified emulsifying activity and is expressed as D_{610}.

3.8. SCREENING FOR PLASMIDS

3.8.1. Plasmid isolation

Bacterial strains and culture conditions

All hydrocarbonoclastic strains were grown on either nutrient or Luria Bertani (LB) broth (Himedia, Bombay, appendix-1) prepared in 50% sea water and incubated on a rotary shaker (200 rpm) at room temperature (RT). Cells were harvested at the exponential phase i.e. after 12-14 hrs of growth.

Chemicals and reagents

All reagents were prepared from chemicals procured from Himedia, Qualigens or Sisco Research Laboratories (Bombay). Phenol was equilibrated with TE buffer after adding hydroxyquinoline and stored frozen in 5 ml aliquots until use. The frozen phenol was liquefied and mixed with chloroform (50:50) before use. The composition of various reagents used are given in appendix-2.

Plasmid isolation

Different techniques were tried with all the isolates for the preliminary screening. Methods yielding consistently good results for particular strains were used.

1. Lysis by alkali

The method is essentially a modification of the methods of Birnboim and Doly (1979). About 5-10 ml of the culture was centrifuged (12,000 g) at RT for 5 min.

After carefully removing all traces of the medium, 100 μl of Solution I was added to suspend the cells. To this was added 200 μl of freshly prepared Solution II, the contents were mixed and kept for 5-10 min at room temperature; 150 μl of ice cold solution III was added before storing in ice for 10 min. After centrifugation for 5-10 min at 4°C, the supernatant was carefully removed and 2 volumes of ethanol was added and stored in the deep freezer overnight. The precipitated DNA was washed with 70% ethanol and then vaccuum dried and stored at -5°C until ready for loading.

2. Boiling lysis

This was essentially a method by Holmes and Quigley (1981) as outlined by Sambrook *et al* (1989) and was used for checking for smaller plasmids.

About 5-10 ml of an exponentially growing cell culture was centrifuged (12,000 g) and the pellet was suspended in 70 ml of STET. To this was added 5 μl of 10 mg/ml fresh lysozyme solution and vortexed. The contents were boiled for 40 sec, centrifuged for 10 min at RT and 5 μl of 3 M Sodium acetate (pH 6) was added. An equal volume of isopropanol was added and then incubated at -20°C for 15 min. The contents were centrifuged (12,000 g) for 5 min at 4°C . The supernatant was drained and the pellet was vacuum dried and stored at -5°C until use.

3. Sucrose method (lysis by sodium dodecyl sulphate)

In this method the cells are suspended in iso-osmotic solution of sucrose and lysozyme and this ensures the release of large plasmids with less damage. The cell pellet from 5-10 ml of the culture broth was resuspended and washed in TE buffer. After centrifuging the pellet is resuspended in 10 ml of ice cold solution of 10%

sucrose. To this 2 ml of freshly prepared solution of lysozyme was added followed by 5 ml of 0.25 M EDTA, mixed by inverting and kept on ice for 10 min. About 4 ml of 10% SDS was added and the contents mixed gently but thoroughly using a glass rod; then 6 ml of 5 M NaCl was added and the tubes kept in ice for 1 hr. The contents were then centrifuged at 30,000 g for 30 min at 4°C. The supernatant was then extracted twice with phenol:chloroform. To the aqueous phase 2 volumes of ethanol was added and allowed to stand for 1-2 hrs at room temperature. The DNA was then removed after centrifugation at 5,000 g for 20 min at 4°C. The pellet was washed with 70% ethanol before vacuum drying and storing to remove salts.

4. Rapid procedure for large plasmids

This procedure suggested by Kado and Liu (1981) was followed to screen for larger plasmids.

The cell pellet was suspended in E buffer, the cells lysed in lysing solution mixed by brief agitation. The contents were heated to 50-65°C for 20 min in a water bath, and 2 volumes of phenol chloroform solution (1:1 vol/vol) were added and shaken. The emulsion was later separated by centrifuging at 6,000 g for 15 min at 4°C. The upper aqueous phase was quickly used for electrophoresis.

3.8.2. Removal of RNA from plasmid DNA preparations

RNase was not used throughout the study to remove RNA; instead $LiCl_2$ was used to remove large molecular weight RNA which otherwise generally masked the low molecular weight bands of DNA.

The precipitated DNA was redissolved in 100 μl of TE (pH 7.8) buffer and 300 μl of ice cold 4M $LiCl_2$ was added and then stored in ice for 30 min. After centrifuging at 12,000 g for 10 min at 4°C. Supernatant was removed carefully and added into twice the volume of isopropanol. The contents were again stored in ice for 30 min. The plasmid DNA was recovered by centrifuging at 12000 g for 10 min at 4°C. The DNA pellet was then vacuum dried and stored. By this treatment, however, the DNA yield decreased by about 10-20%.

3.8.3. Curing of plasmids

Culture T17B was subjected to curing using acridine orange as an agent. A well isolated colony was suspended in sterile seawater upto 10^{-3} and exposed to different concentrations of acridine orange (50, 100, 150 μg/ml) and incubated at RT for 24 hrs. Growth was monitored by O.D. measurements. The CFU was estimated by spread plate method. The tubes that showed the maximum growth at the highest concentration of acridine orange was serially diluted and plated. At 10^{-5} dilution some 12 colonies were marked at random and then replica plated on LB containing acridine orange. Cured colonies were chosen and checked for the presence of plasmids. Simultaneously these were also examined for crude oil degradation.

3.8.4. Gel electrophoresis

Agarose gel electrophoresis was prepared on 0.5, 0.8 and 1.0% agarose in 1X TBE (pH 8) to check for large, medium and small plasmids. Stored DNA pellets were

suspended in 20-30 μl of stock TE buffer to which equal volume of tracking dye was added. Electrophoresis was carried out at 5 Volts/cm on a horizontal unit, and usually required about 5 hrs for bromophenol blue tracking dye in the sample to migrate 18 cms. The gel (5-6 mm thick) was stained with ethidium bromide (0.5 μg/ml) for 30 min, destained in buffer and viewed on a transilluminator (Mighty Bright UVTM 25, Hoefer). Photographs were taken using red filter and the distance of the bands were measured from the front edge of the well to the front edge of the band.

3.9. DEVELOPMENT OF CONSORTIA AND DEGRADATION STUDIES

The previous sections have described the experiments conducted on individual cultures, particularly in detail with the 5 candidate microbial cultures, on the degradation ability of petroleum hydrocarbons. The next step was to study the degradation behaviour of these cultures when mixed together in batch culture and laboratory microcosm studies.

Three types of mixed cultures were tried out in this study. The first type "Type I" was made essentially using the five potent candidate cultures (all the mixed cultures of this type are referred to as ***CONSORTIA*** as these were consistent in degradation and were selectively reconstituted), the second type "Type II" consisted of certain randomly selected bacterial cultures with medium and poor hydrocarbon degrading ability, and the third type "Type III" was made of uncharacterised tar ball enrichment bacterial cultures.

In Type I, the candidate cultures were reconstituted and named as follows:

a) Consortium #1: Mixture of the 2 candidate bacterial cultures (ND22 and T17B).

b) Consortium #1A: This consisted of consortium #1 with the addition of another potentially high degrading bacterial culture T44.

c) Consortium #1B: Besides the two candidate bacterial cultures ND22 and T17B, a candidate yeast culture MY11 was also added.

d) Consortium #2: Mixture of all the three candidate yeast cultures (CY6,CY8,MY11).

In Type II, four reconstituted mixed cultures were made and tried for their degradation of certain petroleum mixtures. They were;

a) Nitrogen fixers

i) Medium degraders (N1)

ii) Low degraders (N2)

b) Tar ball associates

i) Medium degraders (T1)

ii) Low degraders (T2)

In Type III, mixed bacterial cultures from tar ball enrichment were used. Two different enrichments were prepared and experiments on degradation of tar balls were conducted. The details of these experiments are given separately in flow charts (Figs. 7 and 8).

Unless specified all experiments were conducted for a period of 7 days on a rotary shaker (200 rpm) at room temperature. All the batch culture experiments were run with duplicates and uninoculated controls.

Inoculum preparation

All bacterial and yeast cultures were grown on NAS and MEA respectively for 24 hrs. Individual culture suspensions were prepared in ASW such that the final $O.D._{600}$ after inoculation was 1.0. This served as the source of inoculum for all the experiments except in microcosms where the inoculum $O.D._{600}$ was adjusted to 2.0, and 10 ml of this cell suspension was used. For continuous culture experiment, the inoculum of the 3 bacterial cultures were adjusted to yield a population of 10^6 cells/ml. Where mixed cultures were used the ratio of the volume of individual cell suspension was maintained at 1:1. The $O.D._{600}$ after inoculation was maintained at 1.0. For experiments with Type III, the inoculum preparation is mentioned therein.

Incubation details

All the batch culture and microcosm experiments were incubated for periods of 7 days. The continuous culture experiment was carried out for 27 days. While the incubation of batch culture experiments were on a rotary shaker (200 rpm) at room temperature ($28\pm2^{\circ}C$), the continuous culture experiment flasks were incubated with intermittent stirring using magnetic stirrer at room temperature.

Controls

Uninoculated flasks containing ASW and respective substrates served as controls for all the batch culture experiments. The microcosm and continuous experiments were also run along with uninoculated controls.

Extraction and analysis

The batch culture experiments were terminated by extracting the residual oil with HPLC grade carbon tetrachloride (CCl_4) as mentioned in Fig. 4. Analyses were done gravimetrically. The extraction of residual oils from the microcosm sand was by adding 250 ml of CCl_4 in small volumes to the decanted sand in a clean 1 L beaker. After each addition followed by swirling (5-6 times), the oil containing CCl_4 was collected in a beaker, to which all the extract washings were pooled. The extract was concentrated to 100 ml and aliquots were analysed for degradation by gravimetry. In the continuous culture experiment, the residual oil in the chemostat was extracted using 50 ml of CCl_4 and after separating the organic phase, an aliquot of it was analysed by gravimetry for degradation.

3.9.1. **Type I**

3.9.1.1. **Batch culture studies using consortium #1**

The consortium was used to examine the degradation of Bombay High crude oil (1% v/v), tar balls (1% w/v), aliphatic fraction of tar balls (1% w/v) and aromatic fraction of tar balls (0.1% w/v).

Strains were grown as per the inoculation preparation protocol given above. One ml of the mixed inoculum containing 0.5 ml of each culture was added as the inoculum.

3.9.1.2. **Microcosm studies using consortium #1**

To understand the way consortium #1 behave in a natural system, a laboratory microcosm was designed. The only variable parameter was the constant flow of filter sterilised ASW. The microcosm chamber was packed with 230 gm of acid washed beach sand. The sand was collected from a pristine beach area that was not exposed to any hydrocarbon contamination and was washed in conc. HCl for 24 hrs to remove organic matter. The microcosm was connected to a filter sterilised ASW reservoir in the upper part of the chamber (Fig. 5). The chamber was connected to an outlet from the bottom. The void volume of the packed sediment was determined by finding out the volume of water trapped between the sand grains. This volume was found to be 35 ml. The column was repeatedly flushed with sterile water for stabilisation. Then the sand column was filled with water till the surface; 3 ml of the Bombay High crude oil (A) was added and mixed with the surface layer of the sand.

The stabilised microcosm was inoculated with 10 ml of the culture suspension ($O.D._{600}$ = 2.0) containing the two bacterial strains (consortium #1) in a 1:1 proportion.

After inoculation the chamber was left undisturbed for 3 hours for the cells to get adsorbed to the sand. Later ASW was continuously passed through the chamber

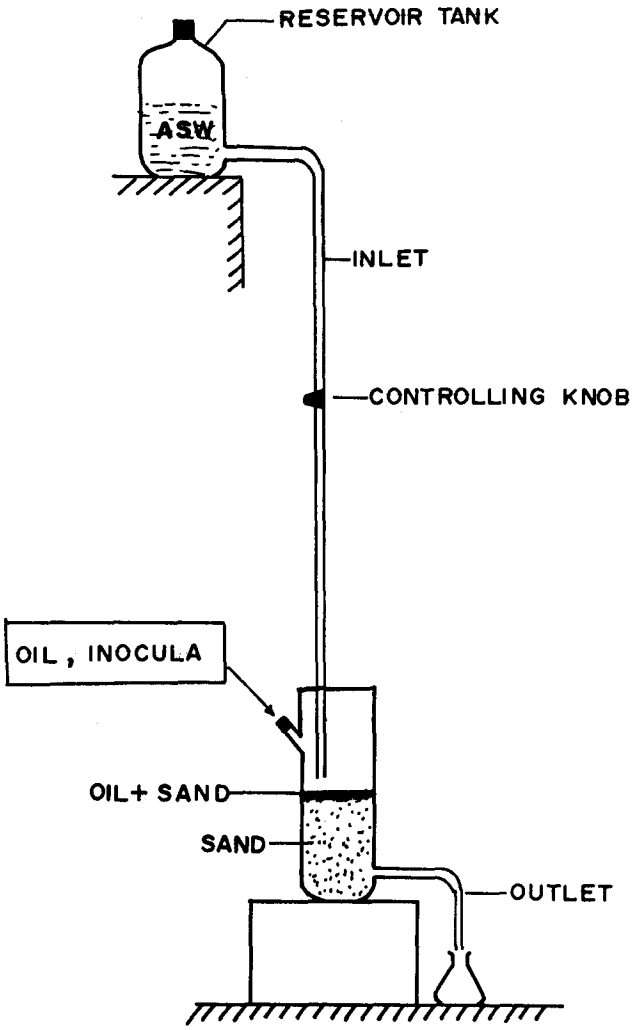


Fig. 5. Schematic diagram of the Laboratory microcosm

at a flow rate of 10 ml/hr for 7 days taking adequate care that the water level always stood 1 cm above the sand column.

3.9.1.3. **Batch culture studies using consortium #1A**

Degradation of Bombay High crude oil

In order to improve the degradability of crude oil, another active degrading culture T44 was added to consortium #1. The 3 bacterial cultures ND22, T17B, and T44, (consortium #1A) were grown on NAS plates and the cells were suspended in ASW. The substrate concentration was 1 % v/v of Bombay High crude oil.

3.9.1.4. **Continuous culture using consortium #1A**

A continuous culture system (chemostat) was designed and set up in the laboratory (Fig. 6). A reservoir containing ASW was connected to the chemostat with a adjustable flow cock. The volume of the chemostat was maintained at 150 ml and the flow rate was controlled at 2 ml/hr. There was an opening on one side of the chemostat for adding the cell inoculum, substrate (Bombay High crude oil A). The chemostat was sealed with a sterilisable rubber cork provided with a tubing for periodic sampling. The chemostat was aerated using a magnetic stirrer at hourly intervals for proper mixing and aeration.

Three bacterial cultures namely T17B, ND22 and T44 were grown on NAS plates and harvested. The cells were made into a suspension using ASW in such a way

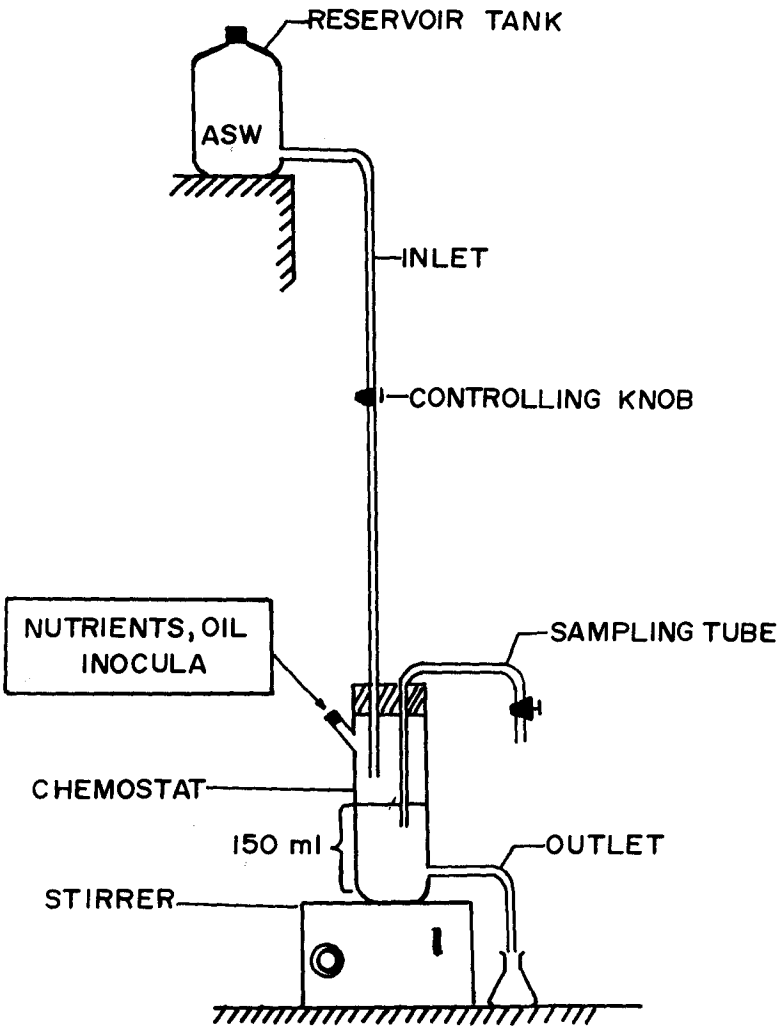


Fig. 6. Schematic diagram of the Continuous culture experiment set-up.

that the cell numbers were at a ratio of 4:1:2 in the chemostat, based on the observed lag phases of each culture in crude oil growth studies.

About 3 ml of the broth was drawn out using the sampling tube, on every third day. This was done immediately after 2 minutes of stirring with a magnetic rod.

The samples were monitored for the following parameters - pH, total viable counts by spread plating, plasmid transfer as well as oil degraation.

3.9.1.5. **Batch culture studies using consortium #1B**

Two of the candidate bacterial cultures (ND22, T17B) and a candidate yeast culture (MY11) were mixed (consortium #1B) to examine the degradation of Bombay High crude oil (1% v/v), tar balls (1% w/v), aliphatic fraction (1% w/v) and aromatic fraction (0.1%) of tar balls.

3.9.1.6. **Batch culture studies using consortium #2**

The degradation of Bombay High crude oil was studied using a consortium of three candidate yeast cultures CY6, CY8 and MY11 in batch culture.

3.9.1.7. **Microcosm studies using consortium #2**

The design of the microcosm remained the same as that mentioned in section 3.9.1.2.

This microcosm was used to study the degradation of crude oil by consortium #2 (3 candidate yeast strains-CY6, CY8, MY11). Three ml of Bombay High crude oil (A) was added and mixed on the surface layer of the sand in the microcosm.

3.9.2. **Type II**

Two groups of mixed cultures were prepared using a few cultures from the "Nitrogen Fixers". One was constituted by including 6 cultures having a medium degrading ability (N1) and the other composing of 4 cultures with low degrading ability (N2). Similarly two groups of cultures were prepared from the tar ball associates. T1 contained 7 cultures with medium hydrocarbon degrading ability and T2 contained 3 cultures with low degrading ability. These four mixed culture preparations were examined for their ability to utilise 3 petroleum mixtures (diesel, Iranian crude and Bombay High crude oils) at 1% v/v concentrations.

3.9.3. **Type III**

The degradation of tar balls by the natural flora was also tested. From a 1 week old primary enrichment flask (flow chart - Fig. 7), bacteria were plated. Different colonies of bacteria from the plate were scraped and transferred into 50 ml of ASW (resulting in a final $O.D._{600}$ of 2.0) containing 1% w/v of tar balls as the sole carbon and energy source. After 7 days of incubation, 1 ml of the broth was transferred to the next flask containing ASW and tarballs. The rate of degradation was monitored

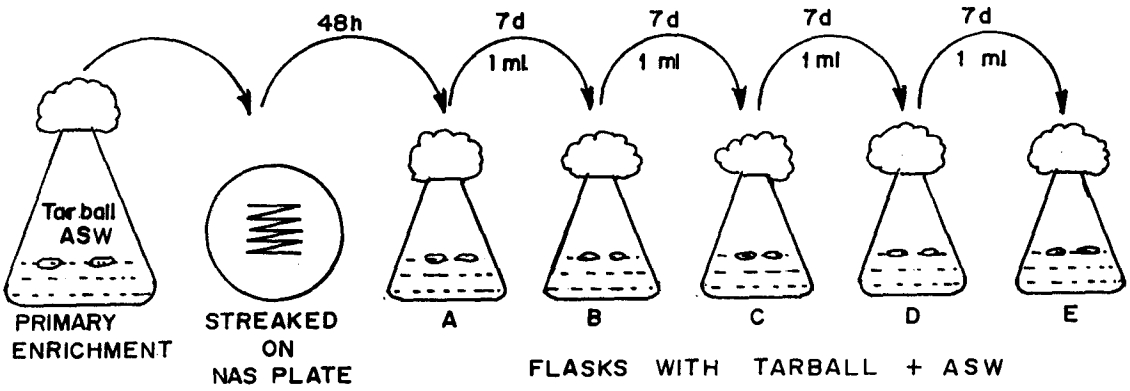


Fig. 7. Flow chart for tarball degradation studies using sequential enrichment by continuous sub-culture.

and the effect of repeated subculturing for 4 times (at weekly intervals) on the degradation were observed.

The efficiency of degradation of the repeatedly subcultured consortium of bacteria obtained at the end of the previous study, was tested for the ability to degrade tar balls (1% w/v) for a period of 35 days. Five sets of experimental flasks with controls were prepared with 50 ml of ASW (Fig. 8.). The degradation was monitored by gravimetry at weekly intervals.

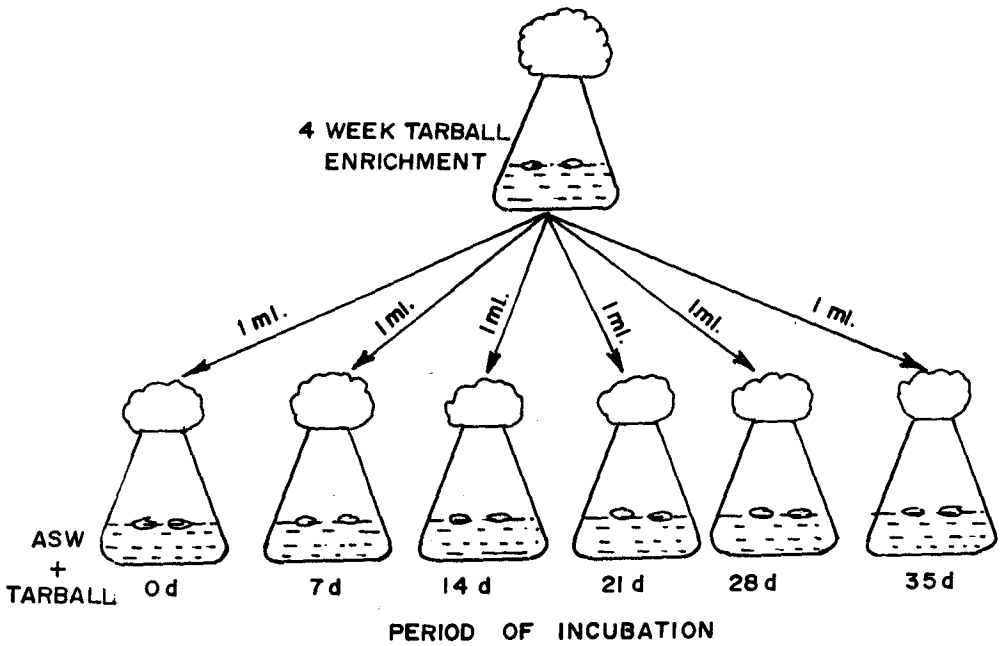


Fig. 8. Flow chart for tarball degradation studies by batch culture.

4

Results

4.1. ISOLATION OF BACTERIA AND YEASTS

Bacteria and yeasts were isolated from six locations along the west and east coasts of India. A total of 658 bacterial cultures and 40 yeast cultures were isolated. Table 1 gives a detailed breakup of the number of cultures from the various sources. Sediment samples for bacterial isolation were collected from Goa, on the west coast and Madras, Cuddalore and Nagapattinam on the east coast. In all 522 bacterial isolates were from sediment samples. Seawater samples were collected from Goa and Lakshadweep on the west coast. A total of 70 cultures from water were isolated from these regions.

Additionally, sources like tar ball and holdfast of the brown alga *Sargassum* sp. were also used for the isolation of bacteria. Of the 66 bacterial cultures that were isolated from these sources, 10 were isolated from *Sargassum* sp. holdfast and 56 from tar balls.

Of the 40 yeast cultures isolated, 30 were from sediments of the Cochin region. Of these 15 were from offshore and the remaining from coastal regions. Ten cultures were isolated from seawater from Madras.

4.2. SCREENING FOR GROWTH ON NITROGEN FREE MEDIUM AND PHOSPHATE SOLUBILISATION

All the isolated bacteria and yeast cultures were checked for growth on nitrogen-free medium (NFM) and their ability to solubilise inorganic phosphate. The results are presented in Table 2.

Table 1. Isolation of bacteria and yeasts from different locations.

S.No.	Location	Lat/ Long	Number of isolates				
			Sediment		Water		Others
			Bacteria	Yeasts	Bacteria	Yeasts	Bacteria
I.	**WEST COAST**						
	1.GOA	15°25'N					
	Anjuna	73°48'E	11		3		
	Chorao island		7		2		
	Dias beach		46		12		10[a]
	Vasco harbour				15		
	2.COCHIN	9°58'N 76°16'E					
	Coastal			15			
	Offshore			15			
	3.LAKSHADWEEP ISLANDS						
	Kalpeni	10°04'N 73°38'E			38		56[b]

Table 1. (contd.)

S.No.	Location	Lat/ Long	Number of isolates				
			Sediment		Water		Others
			Bacteria	Yeasts	Bacteria	Yeasts	Bacteria
II.	**EAST COAST**						
	1.MADRAS	13°06'N 80°18'E	53			10	
	Coastal						
	2.CUDDALORE	11°45'N 79°45'E	215				
	Coastal						
	3. NAGAPATTINAM	10°46'N 79°51'E					
	Coastal		190				
	---	---	---	---	---	---	---
	Total		**522**	**30**	**70**	**10**	**66**

[a] = Seaweed holdfast
[b] = Tar balls
Total Bacterial isolates = 658
Total Yeast isolates = 40

Table 2. Source details of phosphate solubilisers and nitrogen fixers.

S.No.	Location/sample	Number of cultures			
		PS[1]		NF[2]	PS & NF
		Bacteria	Yeasts	Bacteria	Bacteria
1.	**WEST COAST**				
	1.1.GOA				
	Sediment	9		13	
	Seawater	1		3	
	Sargassum holdfast			10	
	1.2.COCHIN				
	Coastal Sediment		12		
	Offshore sediment		3		
	1.3. LAKSHADWEEP ISLANDS				
	Kalpeni				
	Tarball	1			
	Seawater	6		2	2
2.	**EAST COAST**				
	2.1.MADRAS				
	Water	10	3		
	2.2.CUDDALORE				
	Water	38		3	3
	2.3. NAGAPATTINAM				
	Water	22		1	1
TOTAL		87	18	32	6

1 - Phosphate solubilisers
2 - Nitrogen fixers

Among the 658 bacterial strains that were isolated, 32 strains were able to grow on NFM and are termed as "nitrogen fixers" (however, nitrogen fixing ability of these strains was not examined). The ability of phosphate solubilisation was observed in 87 bacterial strains. Six of these were able to grow on NFM thus having both the properties. The rest of the 81 cultures were exclusively phosphate solubilisers. The nitrogen fixers were predominantly (81%) from Goa; even though this group of organisms could be isolated from all the 3 different types of samples (sediment, seawater, seaweed holdfast) a greater number (13) were isolated from sediment.

It was interesting to note that none of the 56 cultures from tar balls (Table 2) could grow in NFM while only one could solubilise phosphate.

Out of the 40 yeast isolates, 18 strains were able to solubilise inorganic phosphate of which 12 were isolated from the coastal sediments of Cochin (Tables 1 and 2).

4.3. MATH (MICROBIAL ADHESION TO HYDROCARBON) ASSAY

A total of 61 bacterial cultures from the three groups and 27 yeast cultures were tested for their cell wall hydrophobicity and surfactant production using MATH assay. The results are tabulated in Table 3a (bacteria) and Table 3b (yeasts).

Among the bacterial cultures, which were grouped as (1) "phosphate solubilisers", (2) "nitrogen fixers" and (3) "tar ball associates", depending on the physiological characteristics, the fraction of adherence ranged upto 0.829. The fraction of adherence or cell hydrophobicity as observed by the ratio, showed that 4

Table 3a. Microbial Adhesion To Hydrocarbons (MATH) assay for bacterial cultures.

S.No.	Group/Source/ Number	Culture No.	Fraction of Adherence	Surfactant production
1.	PHOSPHATE SOLUBILISERS			
	Sediment (2)	P5	-	-
		P6	0.418	+++
	Water (7)	P20	0.207	-
		P25	0.038	-
		P43	0.326	+++
		P47	0.038	-
		P59	0.039	-
		P63	0.206	-
		P82	-	-
2.	NITROGEN FIXERS			
	Sediment (7)	ND2	0.687	-
		ND3	0.224	+++
		ND4	0.427	-
		ND5	0.221	-
		ND9	0.512	-
		ND11	0.255	-
		ND12	0.427	-
	Seawater (3)	ND13	-	-
		ND14	0.085	-
		ND15	0.156	-
	Sargassum holdfast (6)	ND16	0.047	-
		ND17	0.327	-
		ND18	0.156	-
		ND19	0.156	-
		ND20	0.178	-
		ND22	0.548	-

Table 3a. (contd.) MATH assay.

S.No.	Group	Culture No.	Fraction of Adherence	Surfactant production
3.	TARBALL ASSOCIATES (36)	T1	-	-
		T5	-	-
		T6	-	-
		T8	0.211	+++
		T11	-	+++
		T14	-	+
		T16	0.364	+++
		T17A	-	-
		T17B	0.829	+++
		T18	0.086	+++
		T19	0.08	++
		T21	0.004	+
		T24	0.394	+++
		T25	-	-
		T26	-	-
		T29	-	+++
		T30	0.565	+++
		T32	-	-
		T33	-	+++
		T34	-	+
		T36	0.486	+++
		T37	0.329	++
		T38	-	++
		T40	0.173	+++
		T41	0.458	+++
		T42	0.149	+++
		T43	-	+
		T44	0.579	++
		T45	0.807	+++
		T46	0.678	+++
		T47	0.183	+++
		T48	0.581	+++
		T49	0.243	+++
		T50	-	-
		T51	-	-
		T52	-	-

- no cell adhesion/surfactant production
+ average
++ moderate
+++ good

Table 3b. MATH assay for yeast cultures.

S.No.	Location/ Sample	Culture No.	Fraction of Adherence	Surfactant production
1.	COCHIN			
	Coastal sediment (15)	CY1	0.157	-
		CY2	0.409	-
		CY3	-	-
		CY4	0.206	-
		CY5	0.027	-
		CY6	0.54	-
		CY7	-	-
		CY8	0.681	-
		CY9	0.35	-
		CY10	-	-
		CY11	0.221	+
		CY12	-	-
		CY13	0.142	-
		CY14	0.198	+++
		CY15	0.092	-
	Offshore sediment (4)	MY1	0.315	-
		MY3	0.405	+++
		MY11	0.811	+++
		MY13	0.526	-
2.	MADRAS			
	Coastal water (8)	Y1	0.063	-
		Y3	-	-
		Y4	0.139	-
		Y6	0.161	-
		Y7	0.008	-
		Y8	0.009	-
		Y9	0.233	-
		Y10	0.252	+

of the 9 phosphate solubilisers had >0.1. Among the phosphate solubilisers the maximum adherence was found to be 0.418 by culture P6. Culture P43 was another phosphate solubilising strain that could produce surfactants as well as have a fraction of adherence of >0.1.

Similarly 13 of the 16 nitrogen fixers (81%) had >0.1 (fraction of adherence), whereas only 16 of the 36 cultures from tar balls (44%) had a fraction of adherence of >0.1. More than 50% of the cultures isolated from water in each of the first two groups had a low or no fraction of adherence. The highest values of fraction of adherence were observed among the "tar ball associates" (0.829) and those cultures in this group which showed cell hydrophobicity, in general, were ranging on the higher side (0.3 to 0.8). There were only 3 (8.3%) cultures from the first two groups that showed surfactant indication. In the case of tar balls nearly 70% of the bacterial isolates produced surfactants.

Yeasts on the whole, were negative for surfactant production, but those which showed the indication were found to be very good producers (+++) (CY14, MY3, MY11) (Table 3b). Most of the yeast cultures from Madras did not have a high fraction of adherence except Y9 and Y10 (0.233 and 0.252 respectively). It was seen that the fraction of adherence varied from 0 to 0.681 among the coastal yeasts, with a majority showing less than 0.2. All the offshore yeast isolates showed a high fraction of adherence (0.5 to 0.8) with MY11 giving 0.811 and also very good (+++) for surfactant production.

4.3.1. MATH grouping

Based on the MATH assay, the 61 bacterial isolates and 27 yeast isolates were grouped under 4 categories. These groups indicate the presence/absence of cell hydrophobicity and surfactant production ability. The results are compiled in Table 4. All those cultures which showed a fraction of adherence of $\geq$ 0.1 were categorised under AP+. Cultures which showed average surfactant indication (+) to very good (+++) are categorised under SP+.

Most of the bacterial cultures (31%) belonged to AP+SP+. Nearly 30% of the bacterial cultures were AP-SP-. There were 14 cultures (23%) which were AP+SP- and a smaller component (16%) was made of the AP-SP+ category.

Of the 27 yeast cultures which were also categorised alike, the major category was AP+SP- accounting for 44% of the cultures. While only 5 strains (18.5%) belonged to AP+SP+, there were as many as 10 cultures (37%) which were AP-SP-. Notably, there was not a single yeast which was AP-SP+.

4.4. SPECIFIC CHARACTERISTICS OF SELECTED CULTURES

Those cultures which showed a high fraction of adherence or surfactant production or a combination of both were used for further studies. MATH assay and key physiological characters of the selected strains are given in Table 5. A total of 17 cultures were chosen, of which 2 were phosphate solubilisers (PS), 5 were nitrogen fixers (NF) and 10 were tar ball associates (TBA).

Table 4. Grouping of cultures based on MATH assay.

S.No.	Organism	Total no. of strains	Assay characters			
			AP+SP+[a]	AP+SP-[b]	AP-SP+[c]	AP-SP-[d]
1.	Bacteria	61	19 (31%)	14 (23%)	10 (16%)	18 (29.5%)
2.	Yeasts	27	5 (18.5%)	12 (44%)	0	10 (37%)

[a] adhesion property positive (fraction of adherence $\geq$ 0.1) and surfactant property positive

[b] adhesion property positive (fraction of adherence $\geq$ 0.1) and surfactant property negative

[c] adhesion property negative (fraction of adherence < 0.1) and surfactant property positive

[d] adhesion property negative (fraction of adherence <0.1) and surfactant property negative

Table 5. Physiological characteristics of selected cultures.

S.No.	Group/nos.	Physiological Characters			
		Growth NFM[1]	Activity PS[2]	AP	SP
1.	PS[a] (2)				
	P6	-	+	0.418	+++
	P43	-	+	0.326	+++
2.	NF[b] (5)				
	ND2	+	-	0.387	-
	ND3	+	-	0.324	+++
	ND5	+	-	0.327	-
	ND9	+	-	0.412	-
	ND22	+	-	0.548	-
3.	TBA[c] (10)				
	T8	-	-	0.211	+++
	T17B	-	+	0.829	+++
	T29	-	-	0	+++
	T30	-	-	0.565	+++
	T36	-	-	0.486	+++
	T37	-	-	0.329	++
	T40	-	-	0.173	+++
	T44	-	-	0.579	++
	T45	-	-	0.807	+++
	T46	-	-	0.678	+++
4.	Yeasts (3)				
	CY6	NT	+	0.510	-
	CY8	NT	+	0.681	-
	MY11	NT	+	0.811	+++

[1] - Nitrogen Free Medium
[2] - Phosphate solubilisation
[a] - Phosphate Solubilisers; [b] - Nitrogen Fixers;
[c] - Tar Ball Associates
NT - not tested

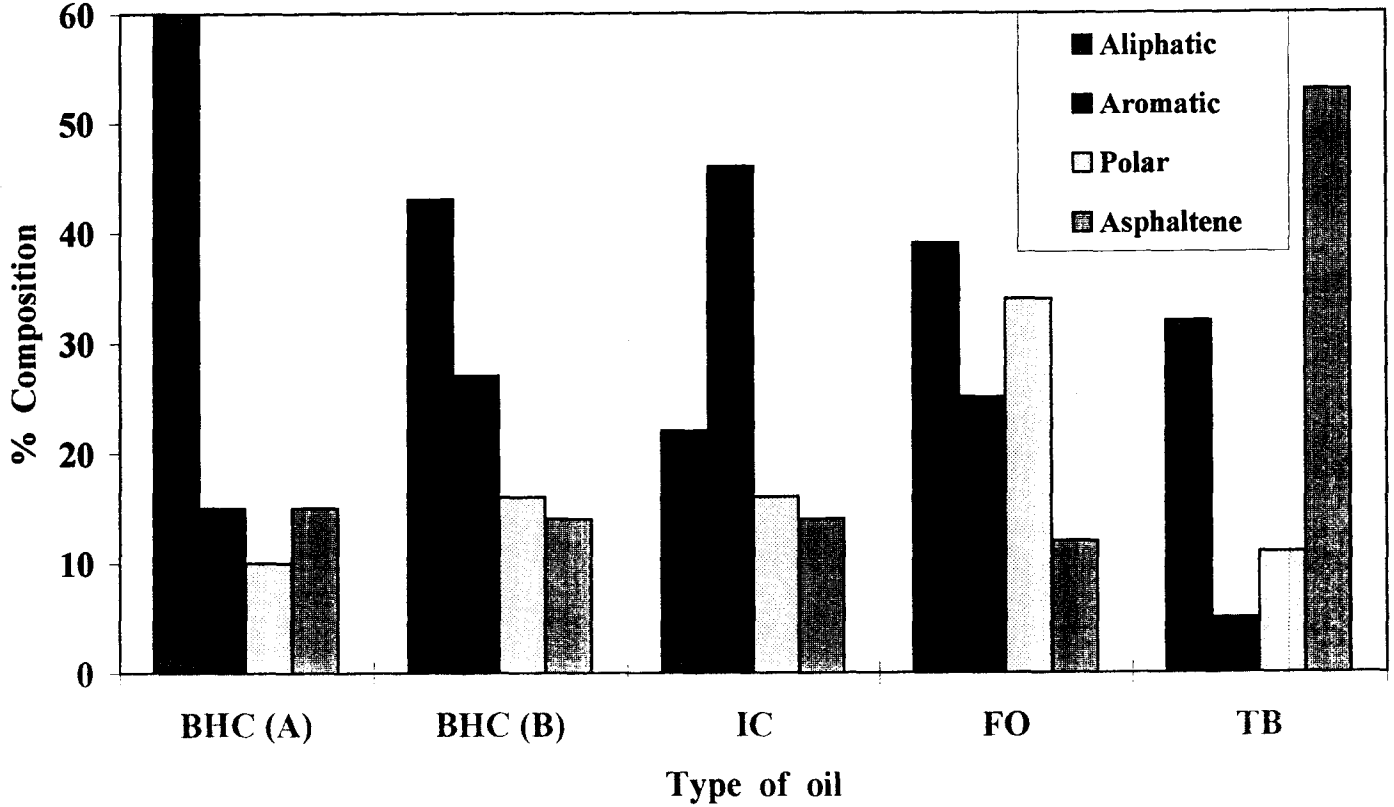
All the phosphate solubilisers and tar ball associates showed surfactant production, while only one nitrogen fixing culture (ND3) could produce surfactants. Except culture T29, all the cultures had a higher fraction of adherence (cell hydrophobic character). The 5 selected nitrogen fixers also showed a high cell adherence (0.324-0.548). Among the bacterial cultures besides P6 and P43 which were phosphate solubilisers, T17B also solubilised phosphate.

Three yeast cultures were selected on the basis of cell hydrophobicity and surfactant production. Two of these yeasts (CY6 and CY8) were isolated from coastal sediments and one (MY11) was from off-shore sediment (Table 3b). As seen from Table 5, culture MY11 showed the highest adherence as well as very good surfactant production, while both the selected coastal cultures did not indicate surfactant production, inspite of their high cell adherence. All the yeasts could solubilise inorganic insoluble phosphate.

4.5. COMPOSITION OF VARIOUS OILS

The method described by Oil and Natural Gas Corporation (ONGC, 1993) was used for the fractionation of crude oils. Three petroleum crude oils (Bombay High crude oil (A) (BHC-A), Bombay High crude oil (B) (BHC-B) and Iranian crude oil (IC)), a spilled furnace oil (FO) and tar balls (TB) were fractionated into aliphatics, aromatics, polars and asphaltenes. The composition of these petroleum products is given in Fig. 9.

Fig.9. Fractional composition of various petroleum oils.



The main constituent of all the oil products were aliphatics while in tar balls, asphaltene constituted the highest. Percentage composition of the aliphatics ranged between 22 and 60%, and aromatics between 5 and 46%. Polars contributed a lower percentage (10-16%) in all the oils except in a spilled furnace oil (34%). Similarly there was no wide difference in asphaltene percentage (10-14%) of the crude oils. In the case of tar balls which is a naturally weathered petroleum product upto 53% and furnace oil a refined petroleum product, upto 2% of asphaltenes were found.

Bombay High crude oil (A) contained the highest amount of aliphatics (60%). Iranian crude oil contained the highest amount of aromatics (46%) as compared to other fractionated oils.

4.6. DEGRADATION OF HYDROCARBONS BY SELECTED CULTURES

4.6.1. Iranian crude

The ability of 17 selected bacterial cultures to degrade Iranian crude oil was tested and the results of the experiment are given in Table 6. Three yeast cultures were also tested to examine their degradation capability.

It was found that the overall degradation ranged from 2-89% among bacteria and 66-94% in yeasts. The two strains of phosphate solubilising bacteria (P6 & P43) degraded 62 and 85% respectively, whereas only one nitrogen fixer (ND22) among the nitrogen fixers could degrade upto 71%. All the other cultures degraded about 50%. Culture T29 showing surfactant property but no adhesion property was able to degrade upto 89% of Iranian crude oil. Except 3 cultures from tar ball associates, the

Table 6. Degradation of Iranian crude oil by selected cultures.

S.No.	Group	Culture no.	Degradation of crude oil (%)
1.	BACTERIA		
	Phosphate Solubilisers (2)	P6	62
		P43	85
	Nitrogen Fixers (5)	ND2	52
		ND3	48
		ND5	49
		ND9	53
		ND22	71
	Tar Ball Associates (10)	T8	28
		T17B	75
		T29	89
		T30	74
		T36	81
		T37	2
		T40	7
		T44	71
		T45	82
		T46	71
2.	YEASTS (3)	CY6	89
		CY8	66
		MY11	94

remaining 7 cultures (T17B, T29, T30, T36, T44, T45 and T46) were able to degrade >70% of Iranian crude oil.

All the 3 selected yeast cultures were able to degrade Iranian crude oil. Culture MY11 showed the highest degradation among these 3 cultures, degrading 94% of the crude oil. This culture showed high adhesion and very good surfactant production. Hence fraction of adherence and surfactant production can be used as an useful index of crude oil degradation. Degradation of crude oil by cultures ND22, T17B and CY6, CY8 and MY11 are shown in Figs. 10 and 11. It could be seen from the figures that there was a total reduction of all the peaks in the crude oil after incubation.

4.6.2. **Bombay High crude oil (A)**

Ten bacterial cultures which showed good Iranian crude oil degradation belonging to all the 3 groups, were studied for the ability to degrade Bombay High crude oil (A). As seen from Table 7, the two phosphate solubilisers (P6 and P43) degraded 36 and 28% respectively, whereas the nitrogen fixer degraded 62%. All the cultures of the tarball associates that degraded Iranian crude well, were able to degrade Bombay crude oil also. Cultures T17B and T44 could degrade Bombay crude oil upto 73% and 71% respectively. From the chromatograms (Fig. 12), it is evident that even though all the fractions of crude oil were degraded, bacteria could utilise more of certain lower fractions, and yeast some of the higher fractions. Five microbial strains ND22, T17B, CY6, CY8 and MY11) were selected for further studies and these selected cultures were henceforth referred to as candidate cultures.

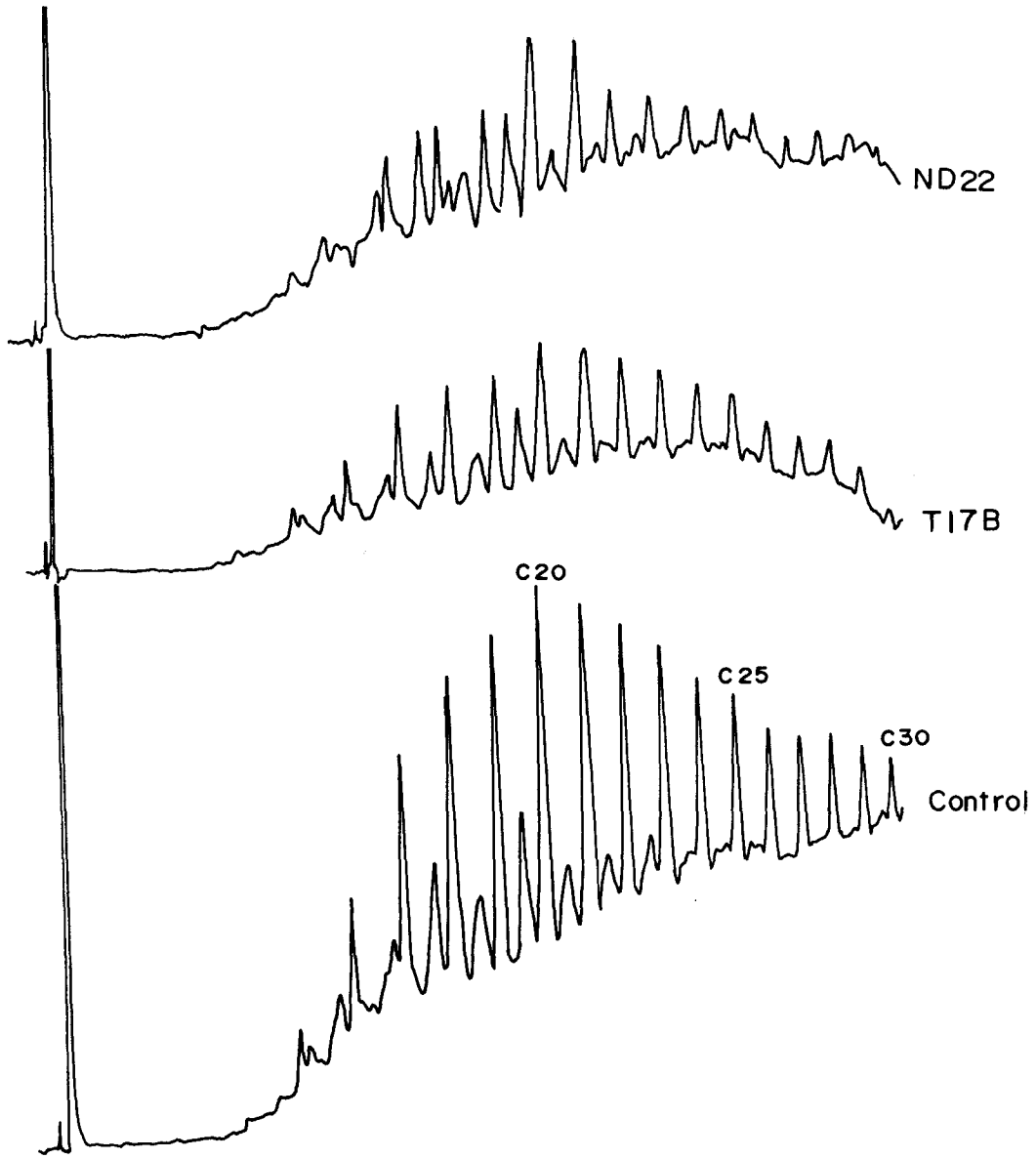


Fig.10. Gas chromatograms of Iranian Crude oil after degradation by bacterial strains in 7 days (packed column SE 30)

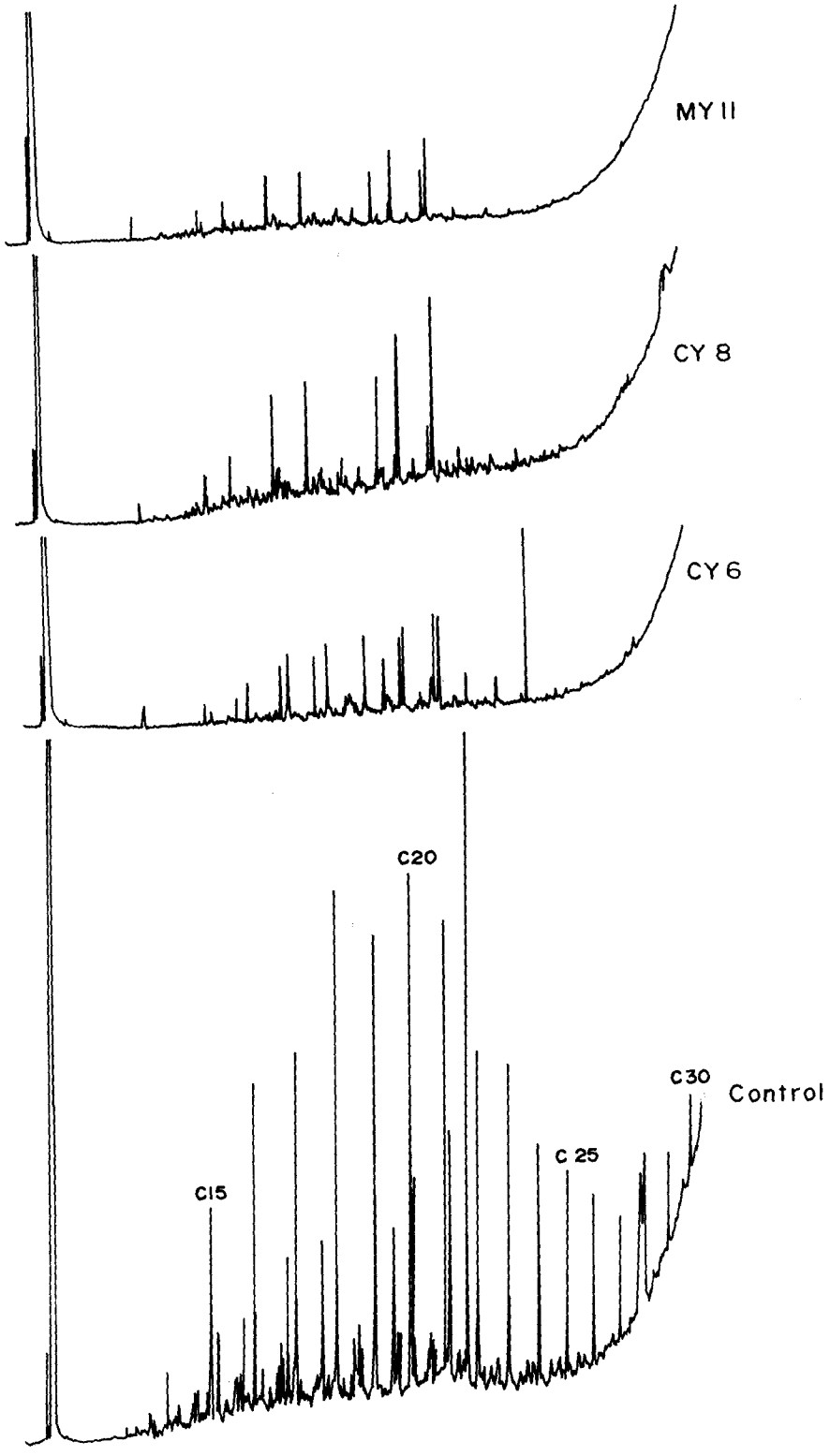


Fig.11. GC chromatograms showing Iranian Crude oil after degradation by Yeast cultures (Capillary column BP1)

Table 7. Degradation of Bombay High crude oil (A) by candidate organisms.

S.No.	Physiological group	Culture No.	Degradation (%)
1.	BACTERIA		
	Phosphate solubilisers	P6	36
		P43	28
	Nitrogen fixers	ND22	62
	Tar ball associates	T17B	73
		T29	53
		T30	39
		T36	48
		T44	71
		T45	43
		T46	50
2.	YEASTS	CY6	69
		CY8	65
		MY11	62

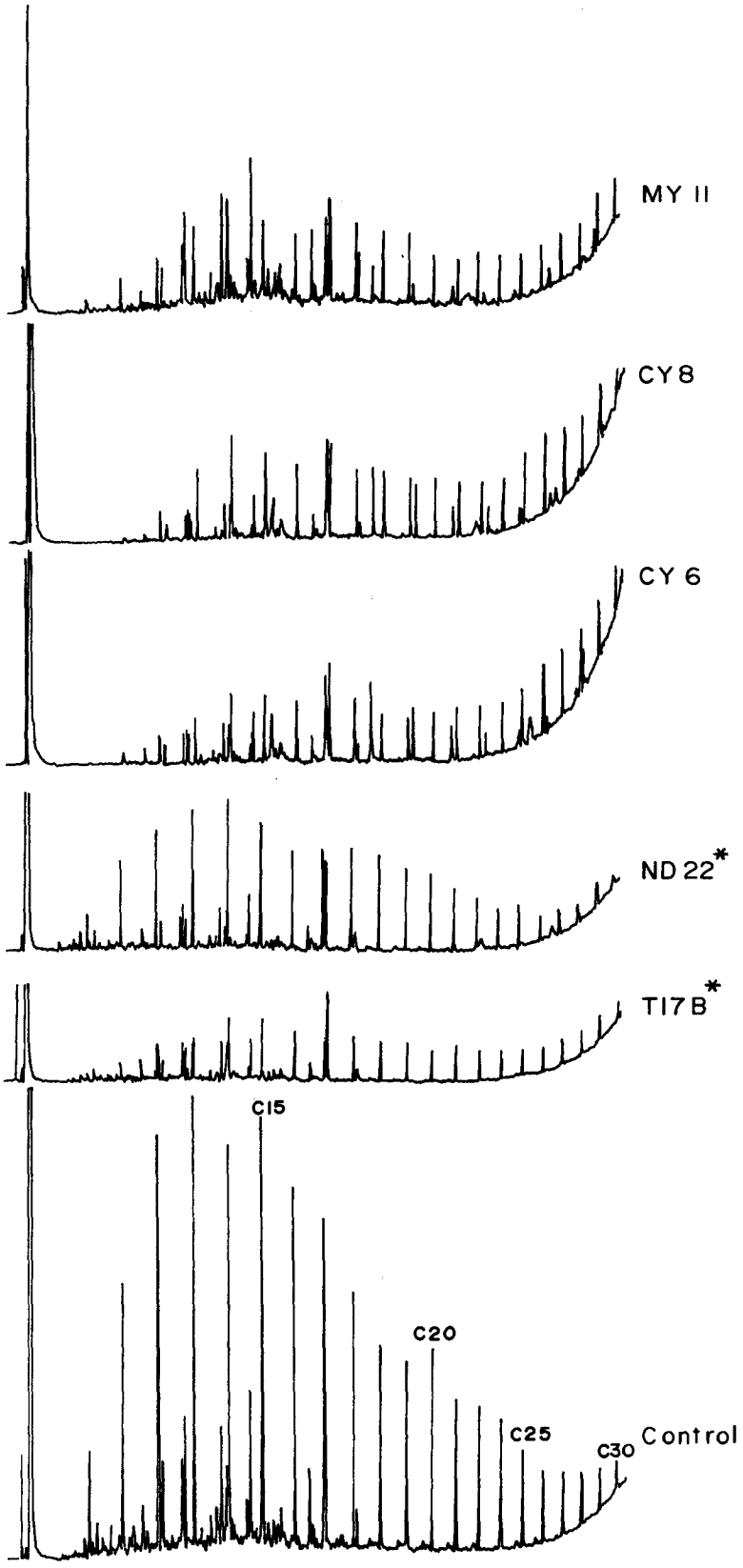


Fig. 12. Gas chromatographic pattern of BHC (A) after degradation by bacteria* and yeasts.

4.6.3. Tar balls

All the five candidate cultures were tested for their degradative ability on tar balls. Degradation of tar balls by bacteria and yeasts is shown in Fig. 13. Of the 5 cultures only T17B could degrade marginally more than the other cultures. However, the overall degradation of tar balls was between 24% and 31%.

From the degradation pattern of the above different petroleum sources, the preferential utilization of the hydrocarbons by certain cultures reflected the composition of the crude. As seen from the degradation studies on the 3 different petroleum substrates, it is clear that some cultures were specific in their preference to a particular type of hydrocarbon, while some cultures could degrade various types of hydrocarbon. For a candidate culture to be selected for consortia, the culture should exhibit a more extensive degradation of a broad range of crude oils.

4.6.4. Fractionated crude oils

Bombay High crude oil (A) and Bombay High crude oil (B) were fractionated and two fractions were tested for their degradation with pure bacterial cultures (4 strains) and yeasts (3 strains). The results are tabulated in Table 8. The degradation of aliphatic fraction of BHC (A) was between 65 and 95% by bacteria. Two of the cultures ND22 and T17B degraded 95% of the aliphatic fraction individually. Degradation of aromatic fraction ranged from 39-66% among bacteria; the highest degrader being ND22. Using BHC (B) aliphatic fraction as a substrate, the degradation was found to range between 92 and 96%, if T29 was not taken into

Fig.13. Tarball degradation by bacteria and yeasts.

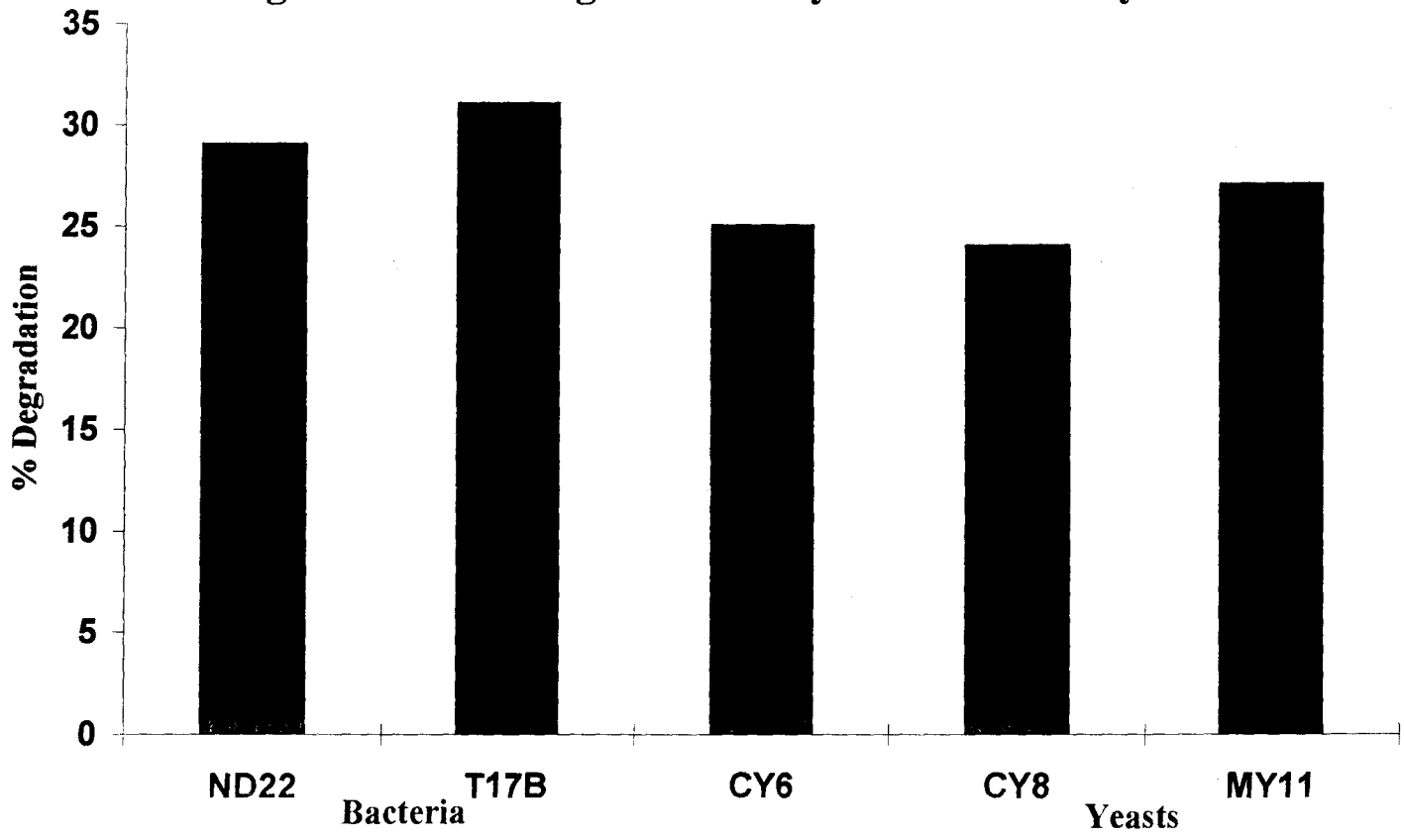


Table 8. Degradation of crude oil fractions by bacteria and yeasts.

S.No.	Culture number	Degradation (%)			
		Bombay High crude (A)		Bombay High crude (B)	
		Aliphatic (1%w/v)	Aromatic (0.1%w/v)	Aliphatic (1%w/v)	Aromatic (0.2%w/v)
1.	BACTERIA				
	ND22	95	66	92	28
	T17B	95	51	96	5
	T29	65	39	8	0
	T44	86	47	94	7
2.	YEASTS				
	CY6	65	41	71	11
	CY8	54	17	91	15
	MY11	97	22	94	48

account. The concentration of the aromatic fraction of BHC (B) was double that of BHC (A). It was perhaps due to their higher concentration that the degradation of the aromatic fraction of this crude oil was generally low, the highest being 28% by ND22.

Among the yeast cultures tested for degradation of aliphatic fraction of BHC(A) culture MY11 degraded 97%. The degradation range was wide (54-97%) among the 3 yeasts. Culture CY6 was able to degrade aromatics upto 41% as against CY8 (17%) and MY11 (22%).

Aliphatic fraction of BHC(B) was degraded between 71-94%, the range being smaller when compared with BHC(A). The aromatic fraction BHC(B) was degraded more by MY11 (48%) as against CY6 and CY8.

Fig. 14 shows that the yeast culture MY11 could degrade a range of higher aliphatic fraction, when compared to the other yeasts or bacteria. Two bacterial cultures (ND22 & T17B) were able to degrade the low carbon number compounds of the aliphatic fraction almost totally.

In comparison to the yeasts, the bacterial strains ND22 and T17B (both bacteria) showed a higher degradation of the aromatic fraction of BHC(A) as can be seen in Fig. 15 and Table 8. ND22 was the highest degrader of this fraction.

4.6.4.1. A Comparison of the aliphatic degradation pattern

Aliphatic fraction of BHC (A) has been arbitrairily grouped into lower and higher carbon compounds. Fig. 16 shows the degradation (in percentage) by the 5 candidate organisms. It can be seen that the aliphatic compounds of C_{13-21} were

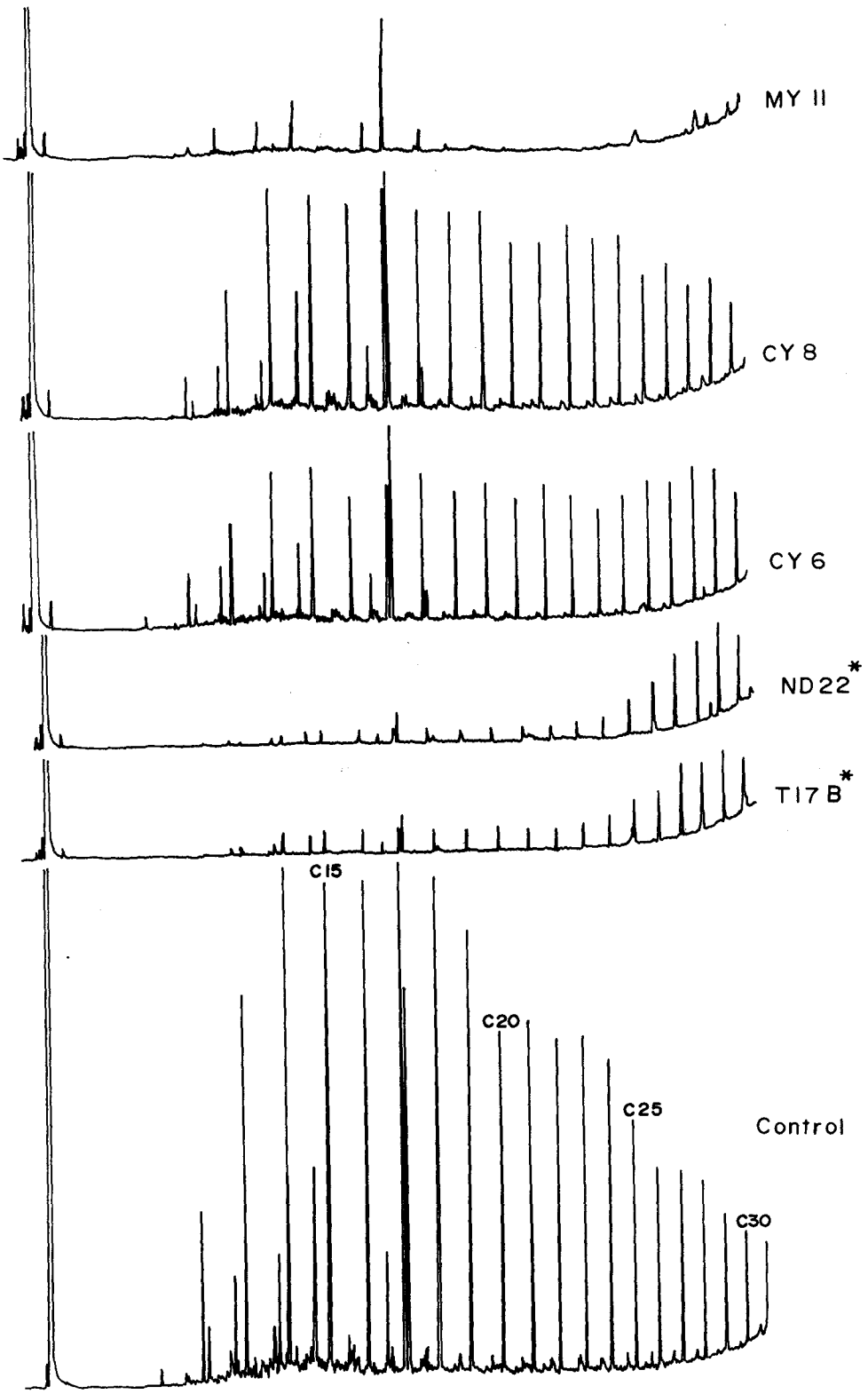


Fig.14. Gas chromatograms illustrating degradation of Aliphatic fraction of BHC (A) by bacteria* and yeasts.

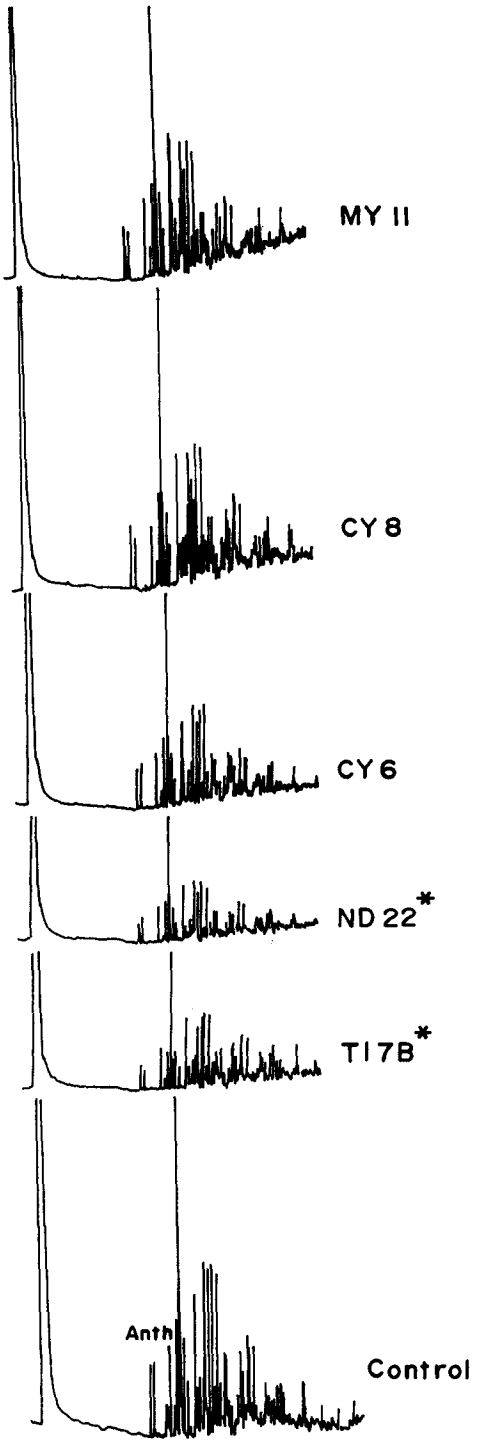
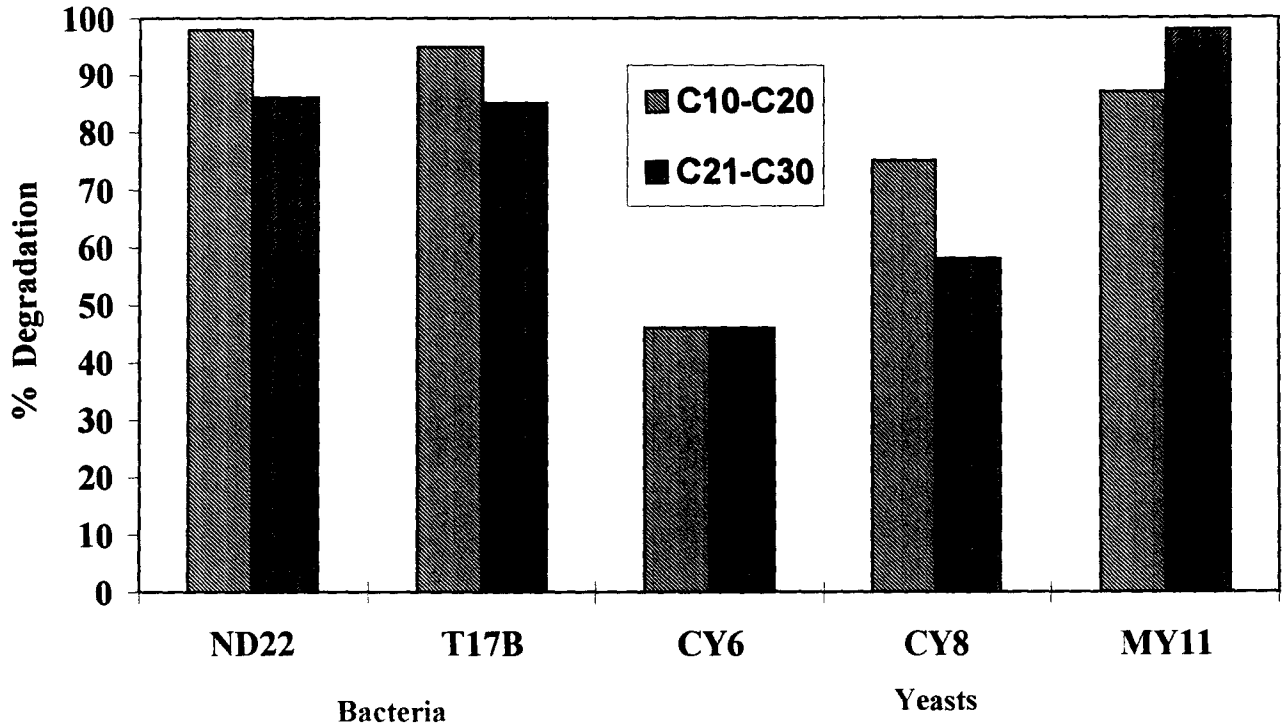


Fig.15. Degradation pattern of Aromatic fraction of BHC (A) by bacteria* and yeasts obtained by GC. (Anth - Anthracene)

Fig: 16. Degradation pattern of aliphatics (low and high carbon).



preferentially degraded by bacteria (95-98%) rather than by yeasts (46-87%). Though the percentage of degradation of higher compounds having carbon numbers C_{22-30} was reduced in both bacterial strains and CY8 (yeast), the yeast culture MY11 was found to degrade 98%. CY6 degraded the same amounts in both C_{13-21} and C_{22-30} groups (46%).

4.6.5. **Fractionated tar balls**

The 5 candidate cultures were tested for their degradation of aliphatic and aromatic fractions of tar balls also. The results are given in Table 9. The degradation of the fractions ranged from a minimum of 4 to a maximum of 38%. Aliphatic fractions of tar balls were degraded relatively higher by ND22 (38%). Both ND22 and T17B were able to degrade 33 and 28% of aromatic fraction respectively. Among yeasts, cultures CY6 and MY11 could degrade 29% and 32% respectively while CY8 was able to degrade only 4%. From the results it is obvious that bacteria were able to degrade aliphatic and aromatic fractions of tar ball with equal efficiency. The chromatograms in Figs. 17 and 18 show the degradation pattern of the candidate cultures.

4.6.6. **Polyaromatic compounds**

The degradation of 3 known individual polyaromatic hydrocarbon compounds were also examined using the five candidate cultures. The details of results are shown in Table 10.

Table 9. Degradation of aliphatic and aromatic fractions of tar balls by bacteria and yeasts.

S.No.	Culture No.	Degradation (%)	
		Aliphatic (1% w/v)	Aromatic (0.1% w/v)
1.	BACTERIA		
	ND22	38	33
	T17B	32	28
2.	YEASTS		
	CY6	30	29
	CY8	19	4
	MY11	35	32

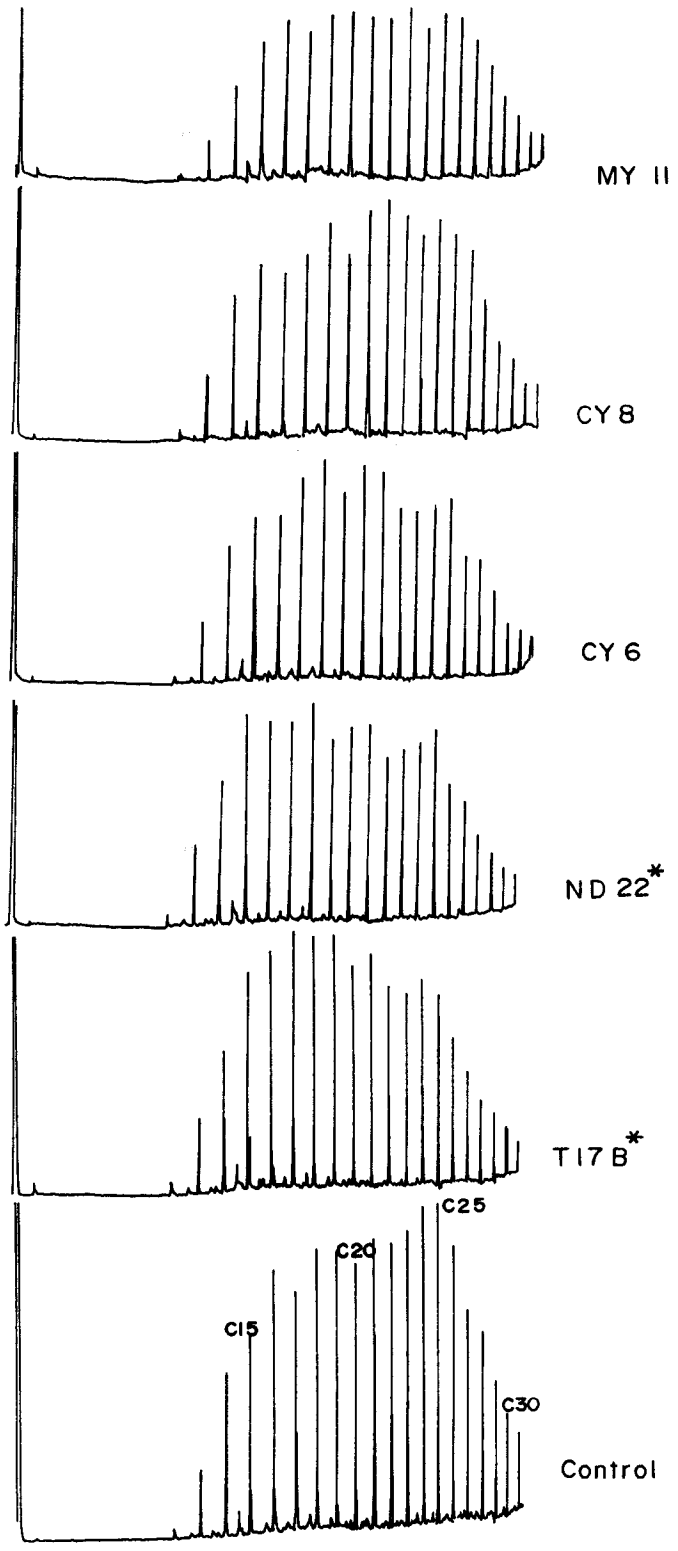


Fig.17. Degradation pattern of aliphatic fraction of tarball by bacteria* and yeasts.

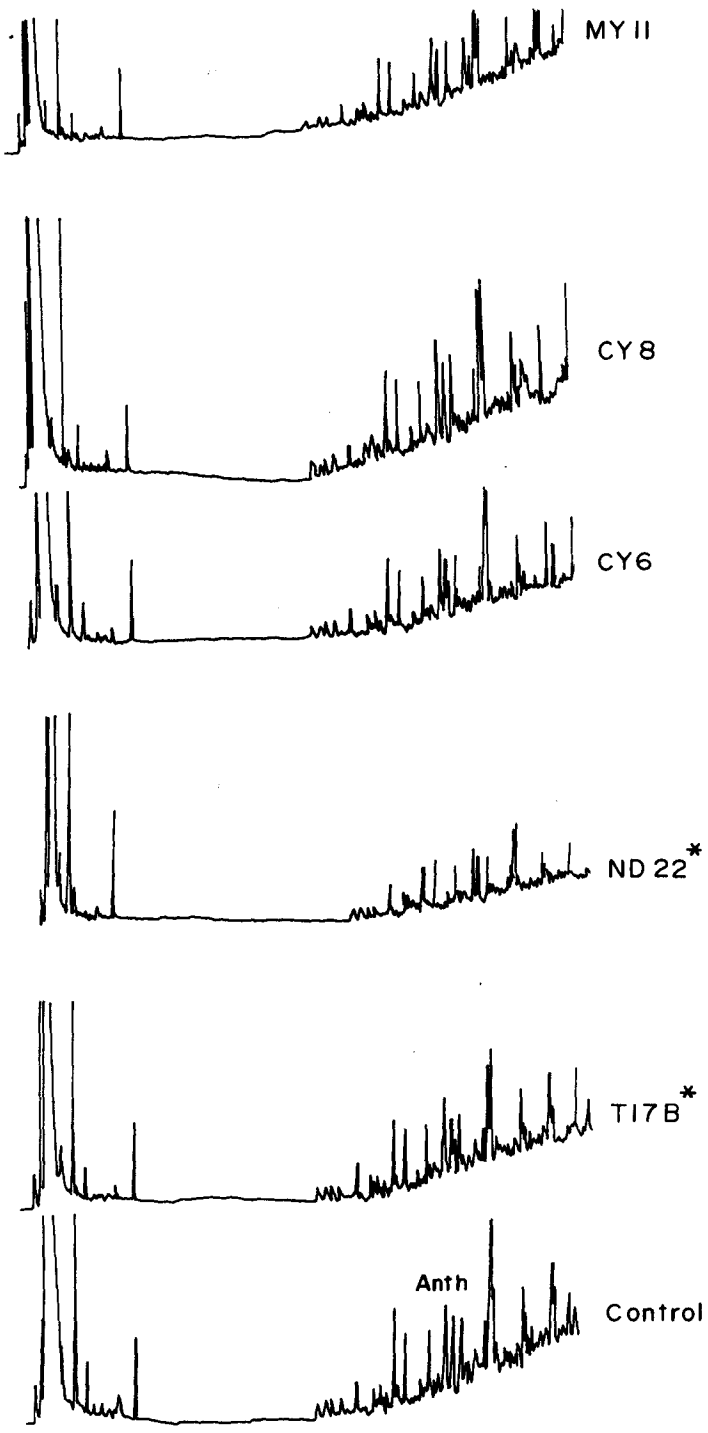


Fig. 18. Gas chromatograms showing degradation of Aromatic fraction of tarball by bacteria* and yeasts. (Anth - Anthracene).

Table 10. Degradation of known aromatic compounds by bacteria and yeasts.

S.No.	Culture No.	Degradation (%)					
		Naphthalene (0.1% w/v)		Anthracene (0.1% w/v)		Phenanthrene (0.1% w/v)	
		UV*	GC**	UV	GC	UV	GC
1.	BACTERIA						
	ND22	0	0	50	55	0	0
	T17B	83	80	0	0	16	16
2.	YEASTS						
	CY6	93	93	23	29	0	0
	CY8	0	0	9	10	0	0
	MY11	42	45	0	0	0	0

* = UV measurement at 246 nm
** = gas chromatography analysis

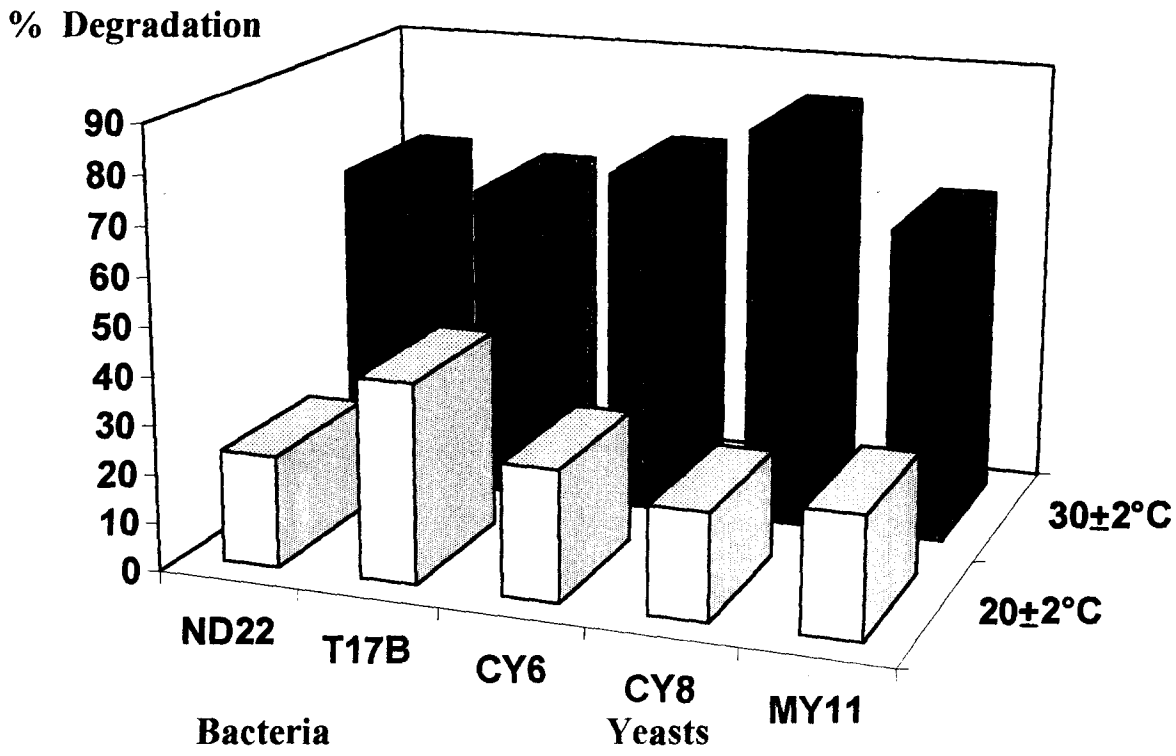
All the 3 compounds namely naphthalene, anthracene and phenanthrene were degraded by the organisms. Naphthalene was degraded by T17B, CY6 and MY11. Anthracene was degraded by ND22, CY6 and CY8. Phenanthrene was degraded by only one organism (T17B). Only the bacterial culture T17B and the yeast CY6 degraded at least two compounds. Culture CY6 was also able to degrade 93% of naphthalene, which is the highest value for aromatic compound degradation, among the 5 tested. The cultures which showed high degradation (T17B and CY6) of naphthalene were also able to degrade one of the other two aromatic compounds tested; for instance, culture T17B which degraded 83% of naphthalene was also able to degrade phenanthrene (16%); and culture CY6 which degraded 93% of naphthalene was able to degrade upto 29% of anthracene. Culture ND22 was able to degrade the triaromatic compound - anthracene (55%) but was not able to utilise naphthalene which was a diaromatic.

4.7. EFFECT OF VARIOUS FACTROS ON DEGRADATION

4.7.1. Temperature

The 5 candidate cultures were examined for the effect of different temperatures on their degradative capability of BHC(A). Two temperatures ($20\pm2^{\circ}C$ and $30\pm2^{\circ}C$) were examined and the results are given in Fig. 19. At $30^{\circ}C$ the degradation ranged between 64 and 82%. Bacteria ND22 (67%) and T17B (65%) did not show much variation in degradation. Among yeasts, culture MY11 degraded the least (64%) while

Fig.19. Effect of temperature on degradation.



the cultures CY6 (71%) and CY8 (82%) showed greater degradation. Yeast culture CY8 could degrade the maximum (82%) at this temperature.

There was an overall reduction in the degradation of the crude oil at 20°C. However, T17B was able to degrade 41% which was the highest among the 5 isolates for this temperature. Degradation ranged between 22 and 27% for other cultures. CY8 which showed the highest degradation potential (82%) at higher temperature, was notably the lowest degrading culture at lower temperature. These cultures which were isolated mainly from beaches and surface waters possess a greater ability of degradation at higher temperatures.

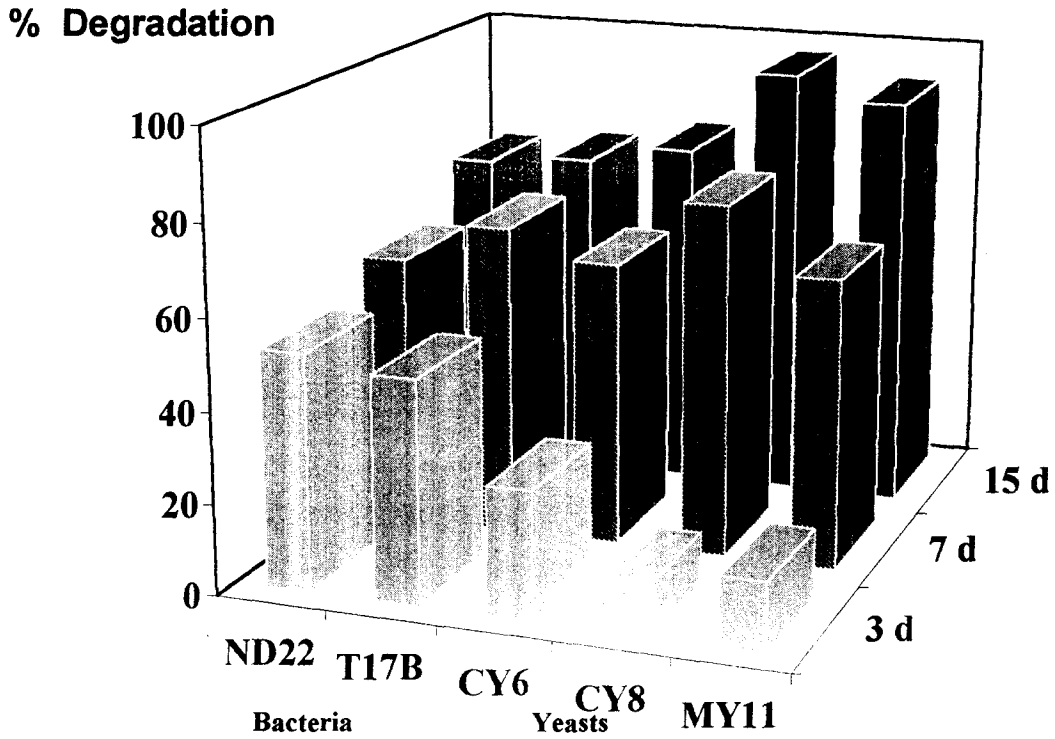
4.7.2. Period of incubation

The degradation of crude oil by the 5 candidate cultures after 3,7 and 15 days of incubation were tested and the results are given in Fig. 20.

Bacterial cultures ND22 and T17B did not differ much in their overall degradation pattern at any given incubation period however, ND22 degraded 52% within 3 days, T17B was lower marginally (49%). After 7 days, T17B could degrade more than ND22 (60%), but after the 15th day again the degradation was more or less uniform for ND22 (72%) and T17B (74%). Thus these two cultures appeared to be quick degraders.

The degradation of crude oil by yeasts showed a marked difference between the 3-day incubation (11-28%) and the 15-day incubation (78-97%). It took 7 days for the three cultures to degrade above 60%, and thus there was a sharp increase in

Fig.20. Effect of incubation period on degradation.



degradation from 3 days to 7 days (35-67%). The yeast cultures were clearly able to degrade higher amounts of crude oil compared to bacteria after 15 days of incubation.

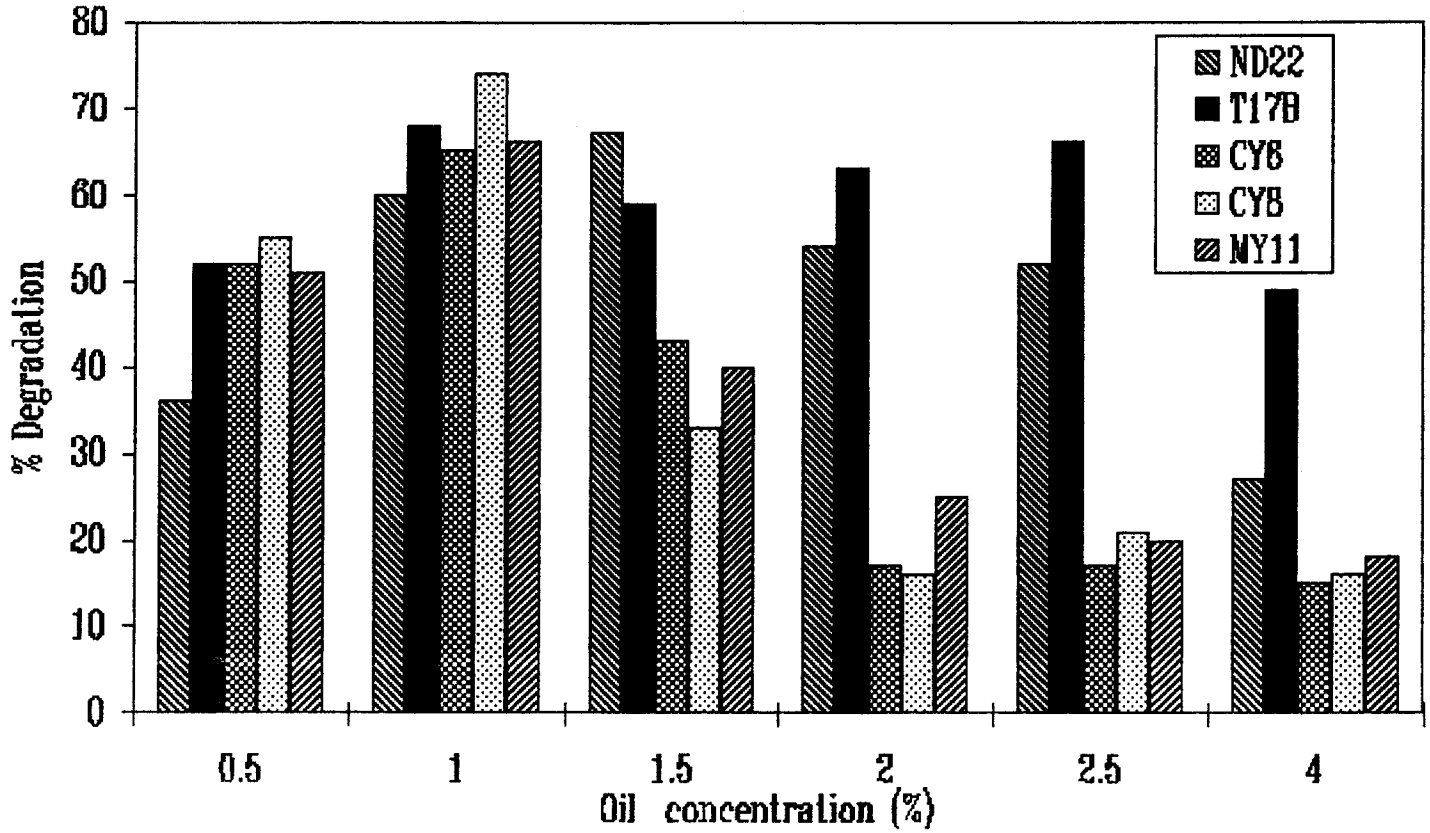
4.7.3. Substrate (crude oil) concentration

The effect of 6 concentrations of oil (0.5, 1.0, 1.5, 2.0, 2.5 and 4.0%) were tested and the results are presented in Fig. 21.

Bacteria and yeasts showed different optimal concentrations for degradation. Both the bacterial cultures were able to degrade >50% at 1.0%. The highest degradation was observed at this concentration for all the yeast cultures (65-74%). The degradation of crude oil at lower (0.5%) and higher (4.0%) concentrations, was retarded in culture ND22 (36% and 27% respectively). Culture T17B did not show such a drastic decline in degradation in any of the concentrations tested (the lowest (49%) being recorded at 4% concentration).

There was a steady decrease in degradation efficiency in all the yeast cultures when the concentration of crude was increased from 1.5 to 4.0%. Though culture CY8 showed the highest degradation of 74% at 1.0% oil concentration, this culture was not able to tolerate higher concentrations in comparison to the other two yeast cultures (CY6 and MY11) under similar conditions. This trend was observed when the oil concentration was increased to 1.5%. This was evident from the observed degradation by CY6 where the reduced from 43 to 15%; and for MY11 it reduced from 40 to 18%, whereas for CY8 the degradation came down from 33 to 16%.

Fig.21. Effect of oil concentration on degradation.



4.7.4. Inoculum size

Degradation of BHC(A) using low ($O.D._{600}$ 1.0) and high $O.D._{600}$ 3.0) inoculum sizes were examined for the five candidate cultures. Fig. 22 gives the percentage of degradation of BHC(A) under varying inoculum sizes.

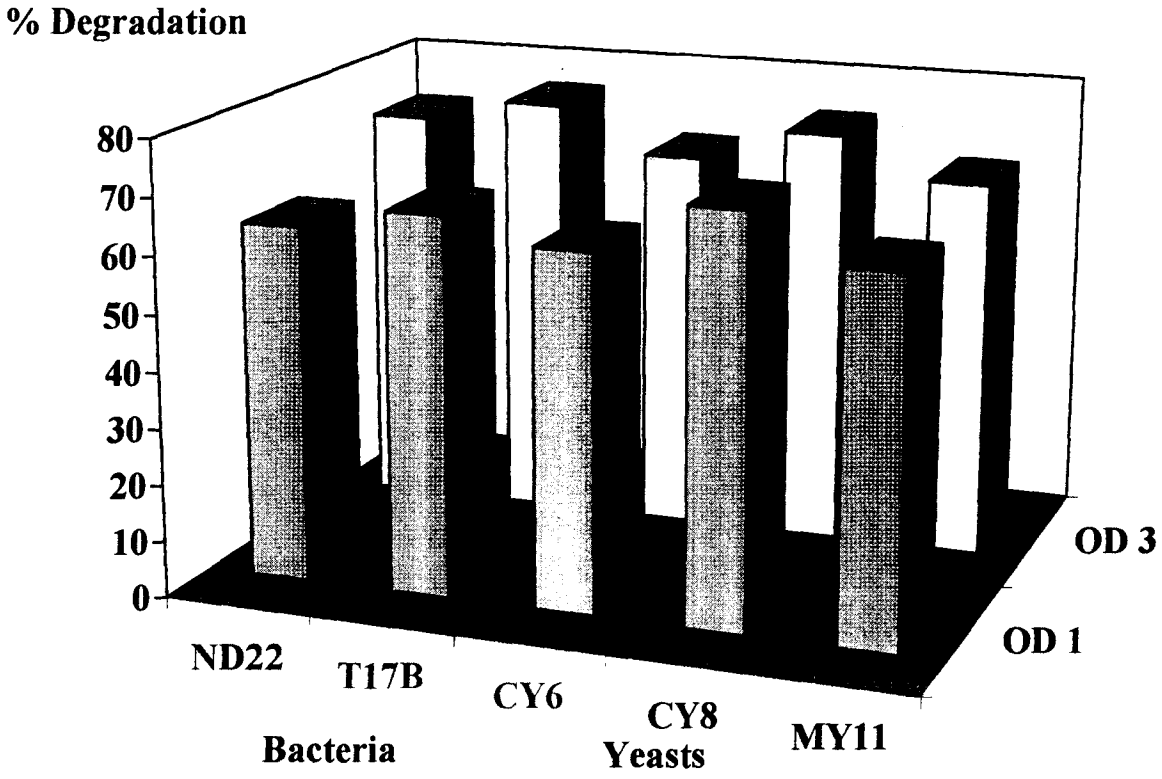
An increase in degradation of the crude oil was observed with all the strains, when a higher inoculum was used. The increase was higher in bacteria (9%) when compared with yeasts (2-5%).

With an inoculum size of $O.D._{600}$ at 1.0, CY8 degraded (78%) more than any other culture. When the inoculum size was higher, CY8 gave a high degradation of 74% but bacterial culture T17B was marginally more efficient (76%). When a lower inoculum size was used, the degradation among the candidate strains ranged between 63% and 72%, and at higher inoculum sizes it ranged between 67% and 76%. This shows that the overall increase in degradation was not significant among the chosen inoculum sizes.

4.8. TAXONOMY AND CHARACTERISATION

An attempt to study certain cultural details of the hydrocarbon degrading bacteria and yeasts was made. Seventeen bacteria and 3 yeast cultures were studied. Most of the bacterial cultures were Gram-negative (59%). The gram positive cultures were not restricted to any of the preliminary grouping of isolation. Most of the strains were motile except for cultures P6 and T44. T44 was a coccobacillus while the cell morphology of the rest of the cultures was small or large rods. The cell size ranged

Fig.22. Effect of inoculum size on degradation.



from 0.5-2.8 μm (Table 11). Six of the cultures were spore formers. Most of the cultures were off-white, opaque and glossy in appearance on NAS medium. Most of the pigmented cultures were orange in colour and were isolated from tar ball, except P43 which was a phosphate solubiliser from sea water.

All the bacterial cultures exhibited a wide range of enzymatic activity, hydrolysing polymers from gelatin to simple compounds like urea. The results of various enzymatic tests are given in Table 12. All the isolates were able to utilise amino acids as nitrogen sources by producing the respective enzymes, namely arginine dihydrolase (ADH), lysine decarboxylase (LDC), ornithine decarboxylase (ODC) and tryptophane deaminase (TDA). B-Galactosidase activity was observed in 10 strains. All the isolates were positive for catalase and oxidase except for T44 and P6 respectively. Among the nitrogen fixing group all the 5 cultures showed various enzymatic activities. In the case of tar ball associates, T30, T40, T45, and T46 were the cultures that could be put under a common bio-chemical group. Phosphatase activity was observed in 3 strains (P6, P43 and T17B).

Most of the cultures were able to utilise more than one carbon source (sugar) (Table 13). Cultures ND5, T37 and T40 were able to utilise all the 9 sugars tested. All the cultures utilised the sugars by the oxidative pathway except for P6 (fermentative). Inositol was the only sugar alcohol tested and 50% of the isolates could utilise it. These cultures could degrade a range of simple compounds namely sugars and amino acids to complex polymers like gelatin. All the results of cell morphology and biochemical tests were subjected to stastical analysis based on the similarity index

Table 11. General characteristics of the bacterial strains.

S.No.	Culture No.	Gram reaction	Motility	Size (μ)	Shape	Spore formation	Colony character in NAS medium
1.	ND2	-	motile	1.1x0.7	curved rods	-	transluscent, round
2.	ND3	+	"	2.5x0.9	bacilli	+	opaque, round
3.	ND5	+	"	2.8x0.8	bacilli	+	opaque
4.	ND9	-	"	1.2x0.8	rods	-	transluscent
5.	ND22	-	"	0.7x0.5	small rods	-	transluscent, round, minute
6.	P6	-	non motile	1.3x0.5	rods	-	opaque, rhizoid
7.	P43	+	motile	2.6x0.9	bacilli	+	orange pigmented, round
8.	T8	-	"	1.4x0.8	rods	-	opaque, round
9.	T17B	-	"	1.3x0.9	rods	-	opaque, round, sticky
10.	T29	+	"	2.5x1.0	bacilli	+	orange pigmented, round
11.	T30	-	"	1.5x0.9	rods	-	transluscent, round
12.	T36	-	"	1.4x0.6	rods	-	transluscent
13.	T37	+	"	2.0x1.4	bacilli	+	yellow pigmented, round, glossy
14.	T40	+	"	2.5x1.0	bacilli	+	opaque, round
15.	T44	+	non motile	2.0x1.0	coccus rod	-	orange pigmented, round
16.	T45	-	motile	1.0x0.6	small rods	-	transluscent
17.	T46	-	"	0.9x0.5	small rods	-	pigmented, round, glossy, small

Table 12. Production of various enzymes by bacterial isolates.

S.No.	Culture No.	Enzyme production*									
		1	2	3	4	5	6	7	8	9	10
1.	ND2	-	+	+	+	+	+	+	+	+	-
2.	ND3	+	+	+	+	+	+	+	+	+	-
3.	ND5	+	+	+	+	+	+	+	+	+	-
4.	ND9	+	+	+	+	+	+	+	+	+	-
5.	ND22	+	+	+	+	+	+	+	+	+	-
6.	P6	+	+	+	+	+	+	-	+	-	+
7.	P43	-	+	+	+	+	+	+	+	+	+
8.	T8	-	+	+	+	+	+	+	+	+	-
9.	T17B	+	+	+	+	-	+	+	+	+	+
10.	T29	-	+	+	+	+	+	+	+	+	-
11.	T30	+	+	+	+	+	+	+	+	+	-
12.	T36	-	+	+	+	-	+	-	+	+	-
13.	T37	-	+	+	+	+	+	+	+	+	-
14.	T40	+	+	+	+	+	+	+	+	+	-
15.	T44	-	+	+	+	+	+	+	-	+	-
16.	T45	+	+	+	+	+	+	+	+	+	-
17.	T46	+	+	+	+	+	+	+	+	+	-

* 1 - ß-galactosidase
2 - Arginine dihydrolase
3 - Lysine decarboxylase
4 - Ornithine decarboxylase
5 - Urease
6 - Tryptophane deaminase
7 - Gelatinase
8 - Catalase
9 - Oxidase
10 - Phosphatase

Table 13. Utilization of various carbon sources by bacterial isolates.

S.No.	Culture No.	Carbon sources*								
		1	2	3	4	5	6	7	8	9
1.	ND2	O	+	+	-	+	-	+	-	+
2.	ND3	O	-	-	+	+	-	+	+	+
3.	ND5	O	+	+	+	+	+	+	+	+
4.	ND9	-	+	+	+	+	+	+	-	+
5.	ND22	-	-	-	-	-	-	+	-	+
6.	P6	F	+	+	+	+	+	+	+	+
7.	P43	O	-	-	-	-	-	+	-	+
8.	T8	-	-	-	-	+	-	+	-	+
9.	T17B	O	+	+	-	-	-	+	-	+
10.	T29	alk	-	-	-	-	-	+	-	+
11.	T30	alk	-	-	-	+	-	+	-	+
12.	T36	-	-	-	-	-	-	+	+	+
13.	T37	O	+	+	+	+	+	+	+	+
14.	T40	-	+	+	+	+	+	+	+	+
15.	T44	O	-	-	-	-	-	+	-	+
16.	T45	O	+	+	+	+	+	+	-	+
17.	T46	-	-	-	-	+	-	+	+	+

* 1-Glucose (GLU) 2-Mannose (MAN) 3-Inositol (INO)
4-Sorbose (SOR) 5-Rhamnose (RHA) 6-Sucrose (SAC)
7-Melibiose (MEL) 8-Amygdalin (AMY) 9-Arabinose (ARA)

and shown in a dendrogram (Fig. 23). The dendrogram showed the prominence of two major clusters A and B each having two sub-clusters. Most of the nitrogen fixing strains with the exception of ND22 belonged to cluster A while cluster B was dominated by tar ball associates.

Antibiogram pattern of the cultures showed a general multiple resistance (more than 3 antibiotics) (Table 14). Culture P6 was resistant to 10 out of the 15 antibiotics tested. Cultures T8 and T45 were sensitive to all the antibiotics whereas T46 was resistant to only one antibiotic - Nalidixic acid. All the cultures were resistant to chloramphenicol, gentamycin, and streptomycin. Nalidixic acid was insensitive to 11 of the cultures followed by ampicillin. In general, unlike the enzyme activity/carbon utilisation, none of the cultures were having the same antibiogram.

Based on the fatty acid profile, the 17 cultures were grouped into 4 genera with *Pseudomonas* being the dominant group. Table 15 gives the taxonomic identification of the cultures. The culture which showed maximum resistance to all tested antibiotics was P6 and was found to be *Klebsiella pneumoniae*. Among the pseudomonads, 3 belonged to *P. putida*. Table 16 brings out the distinguishing characters of the 2 candidate bacterial cultures (ND22 and T17B). As seen in Table 15, these belonged to *Pseudomonas* group but were biochemically different in their utilisation of sugars and enzyme activity. Using the fatty acid profiles of the 17 cultures a dendrogram was drawn (Fig. 24) with the MIDI software. This showed the presence of two clusters both of which contained representatives of all the three groups which were tested.

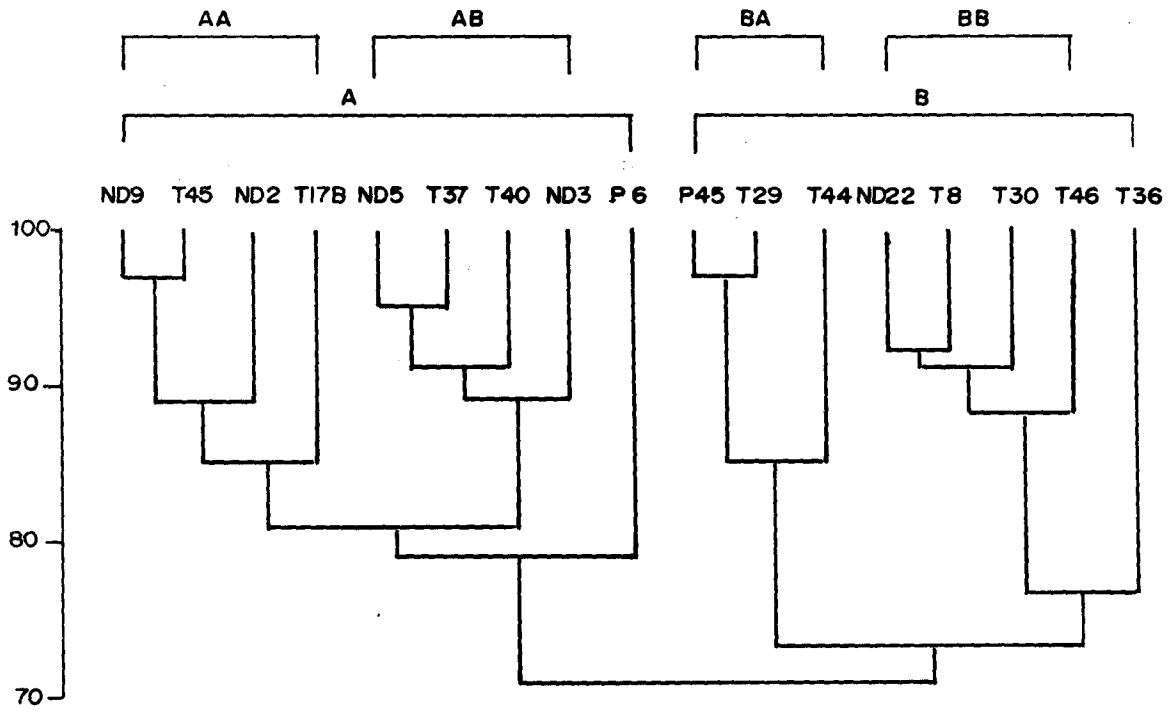


Fig. 23. Dendrogram of bacterial cultures based on morphological and biochemical characterisation.

Table 14. Antibiogram of bacteria.

S.No.	Cult.No.	Antibiotics*														
		A	B	C	Cx	Dx	E	G	K	Na	N	O	P	R	S	T
1.	ND2	R	S	S	S	S	S	S	S	R	S	R	S	S	S	S
2.	ND3	R	S	S	R	S	R	S	R	R	R	R	S	S	S	S
3.	ND5	R	R	S	S	S	S	S	S	R	S	S	S	S	S	S
4.	ND9	R	R	S	R	R	S	S	S	S	S	R	R	R	S	S
5.	ND22	R	R	S	S	S	R	S	S	R	S	S	S	S	S	S
6.	P6	R	R	S	R	R	R	S	S	S	R	R	R	R	S	R
7.	P43	R	S	S	S	S	S	S	S	R	S	R	S	S	S	S
8.	T8	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S
9.	T17B	S	S	S	S	S	R	S	S	S	S	R	R	R	S	S
10.	T29	R	S	S	S	S	S	S	R	R	S	S	S	S	S	S
11.	T30	S	R	S	R	S	S	S	S	S	S	S	S	S	S	S
12.	T36	S	S	S	S	S	S	S	R	R	S	S	S	S	S	S
13.	T37	R	S	S	R	S	S	S	S	R	S	R	S	S	S	S
14.	T40	R	S	S	R	R	S	S	S	R	S	R	S	S	S	S
15.	T44	R	S	S	S	S	S	S	S	R	S	S	S	S	S	S
16.	T45	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S
17.	T46	S	S	S	S	S	S	S	S	R	S	S	S	S	S	S

* A-Ampicilin (10) B-Bacitracin (10) C-Chloramphenicol (30)
Cx-Cloxacillin (1) D-Doxycycline (30) G-Gentamycin (10)
K-Kanamycin (30) Na-Nalidixic acid (30) N-Neomycin (30)
O-Oxytetracycline (30) P-Penicillin (10) R-Rifampicin (5)
S-Streptomycin (10) T-Tetracycline (30)

( ) all conc. in mcg; R - Resistant; S - Sensitive

Table 15. Taxonomic identification of bacterial and yeast isolates based on cellular fatty acid profiles.

S.No.	Culture No.	Species
	BACTERIA	
1.	ND2	*Pseudomonas diminuta*
2.	ND3	*Bacillus subtilis*
3.	ND5	*Bacilluspumilus*
4.	ND9	*Pseudomonas putida*
5.	ND22	*Pseudomonas diminuta*
6.	P6	*Klebsiella pneumoniae*
7.	P43	*Bacillus pumilus*
8.	T8	*Pseudomonas sp. (Chrysomonas luteola)*
9.	T17B	*Pseudomonas putida*
10.	T29	*Bacillus coagulans*
11.	T30	*Pseudomonas sp.*
12.	T36	*Pseudomonas putida*
13.	T37	*Bacillus brevis*
14.	T40	*Bacillus coagulans*
15.	T44	*Brevibacterium linens/epidermidis*
16.	T45	*Pseudomonas aeruginosa*
17.	T46	*Pseudomonas* sp.
	YEASTS	
1.	CY6	*Candida osorensis*
2.	CY8	*Candida albicans*
3.	MY11	*Candida canterelli*

Table 16. Distinguishing characters of bacterial cultures ND22 and T17B.

S.No.	Characters	Culture number	
		ND22	T17B
1.	Morphology Colony appearance	Transluscent	Opaque
2.	Enzyme production Urease Phosphatase	+ -	- +
3.	Carbon assimilation Glucose (O/F) Mannose Inositol	- - -	+ (O) + +
4.	Antibiotic resistance Nalidixic acid Oxytetracycline Penicillin Rifampicin	R S S S	S R R R
5.	Other characters Growth on NFM Bioemulsifier production	+ -	- +

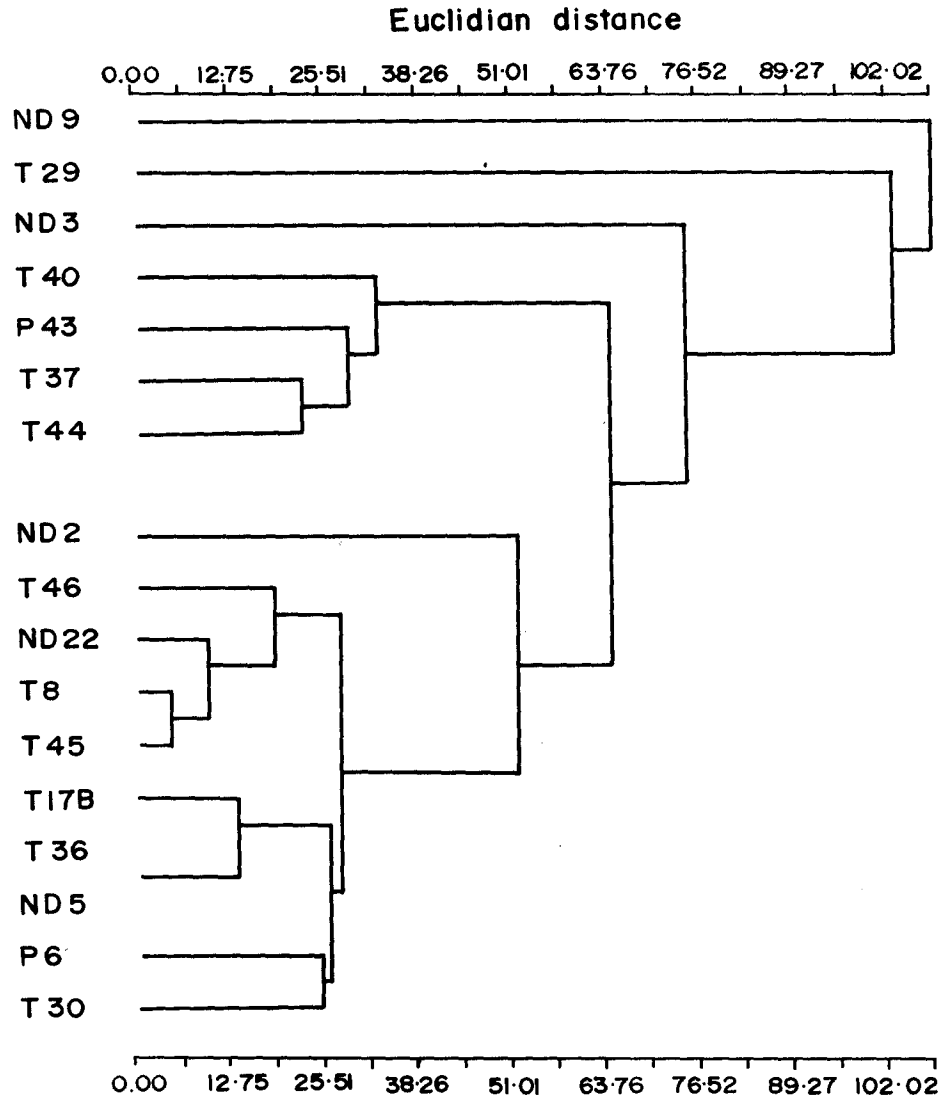


Fig. 24. Dendrogram obtained using fatty acid profiles of bacterial cultures.

The three selected yeast strains grew as white colonies on MEA medium. While the colonies of CY6 and CY8 were glossy and convex, culture MY11 showed a distinguishable colony surface pattern (dry type) with different from the other strains (Table 17). These cultures were identified by the fatty acid fingerprinting technique and were found to belong to *Candida* genus. Culture CY6 was *C.osorensis*, CY8 was *C.albicans* and MY11 was *C.canterelli*. These were tested for enzyme activities using 10 substrates and carbon utilisation (9 sugars) and the results are tabulated in Tables 18 and 19 respectively. All the 3 yeast cultures had the same enzyme activity showing positive for 6 enzymes (ADH, LDC, ODC, TDA, GEL, OX) except for CY8 which was negative for TDA. All the cultures in general showed a restricted enzyme activity compared to bacteria. The sugar utilisation was also less with culture CY6 which was able to utilise 6 of the 9 sugars; CY8 and MY11 were able to use 5 and 8 sugars respectively. All the 3 yeasts could utilise GLU, RHA, MEL, AMY and ARA. Inositol was not used by any of the culture and sorbitol was used only by culture MY11.

Table 20 gives the distinguishing characters of the 3 yeast cultures. Cultures CY6 and MY11 were positive for tryptophane deaminase whereas CY6 was negative for mannose, sorbose and sucrose utilisation. The distinguishing characters between CY8 and MY11 was their ability to utilise only sorbose or sucrose.

Table 17. Morphological characteristics of yeast strains.

S.No.	Characters	Cultures numbers		
		CY6	CY8	MY11
1.	Colony Size	5 mm	2 mm	4 mm
	Colour	White	White	Off white
	Appearance	Round Glossy	Round Glossy	Round Dry

Table 18. Production of various enzymes by yeast isolates.

S.No	Culture No.	Enzyme production									
		1	2	3	4	5	6	7	8	9	10
1.	CY6	-	+	+	+	-	+	+	-	+	-
2.	CY8	-	+	+	+	-	-	+	-	+	-
3.	MY11	-	+	+	+	-	+	+	-	+	-

* 1 - ß-galactosidase; 6-Tryptophane deaminase
2 - Arginine dihydrolase; 7-Gelatinase
3 - Lysine decarboxylase; 8-Catalase
4 - Ornithine decarboxylase; 9-Oxidase
5 - Urease; 10-Phosphatase

Table 19. Utilization of various carbon sources by yeast isolates.

S.No.	Culture No.	Carbon sources								
		1	2	3	4	5	6	7	8	9
1.	CY6	+	-	-	-	+	+	+	+	+
2.	CY8	+	-	-	-	+	-	+	+	+
3.	MY11	+	+	-	+	+	+	+	+	+

* 1-Glucose (GLU) 2-Mannose (MAN) 3-Inositol (INO)
4-Sorbose (SOR) 5-Rhamnose (RHA) 6-Sucrose (SAC)
7-Melibiose (MEL) 8-Amygdalin (AMY) 9-Arabinose (ARA)

Table 20. Distinguishing biochemical characters of the three yeast strains.

S.No.	Physiological characters	Culture number		
		CY6	CY8	MY11
1.	Enzyme production			
	Tryptophane deaminase	+	-	+
2.	Carbon utilization			
	Mannose	-	+	+
	Sorbose	-	-	+
	Sucrose	-	+	-

4.9. GROWTH AND GENERATION TIME OF BACTERIA AND YEASTS

The cultures (ND22, T17B and T44) when grown in nutrient medium exhibited, the typical growth curve of bacteria. As seen in Figs. 25 and 26, the generation times of the above three cultures namely ND22, T17B, T44 were 2 hours, 1 hr and 12 min and 1 hr and 36 min respectively. In the case of yeasts the periods of doubling were 1 hr, 1 hr 12 min and 1 hr for cultures CY6, CY8 and MY11.

The growth of the 5 candidate cultures and culture T44 in crude oil was measured and the results are shown in Fig. 27. The data clearly indicate that the growth of all the cultures on crude oil substrate did not show any common discernible trend. Culture T17B had a prolonged 24 hr lag phase when grown in crude oil, compared to T44 (12 hr) and ND22 (6 hr).

Growth pattern of the 5 candidate cultures on hexadecane was studied and the results are shown in Fig. 28. All the cultures showed a well defined growth curve in hexadecane containing medium. It was seen that hexadecane supported better growth of the two bacterial cultures ND22 and T17B (14 x 10^6 cells /ml) than any of the yeasts (3.1 - 5.7 x 10^6 cells/ml) as seen from the cell numbers.

4.10. BIOSURFACTANT PRODUCTION AND CRUDE OIL EMULSIFICATION

Culture T17B was the only culture which was examined for the production and activity of biosurfactant. Production of biosurfactant or bioemulsifier was examined using various hydrocarbon substrates. The results are seen in Table 21. Yields of

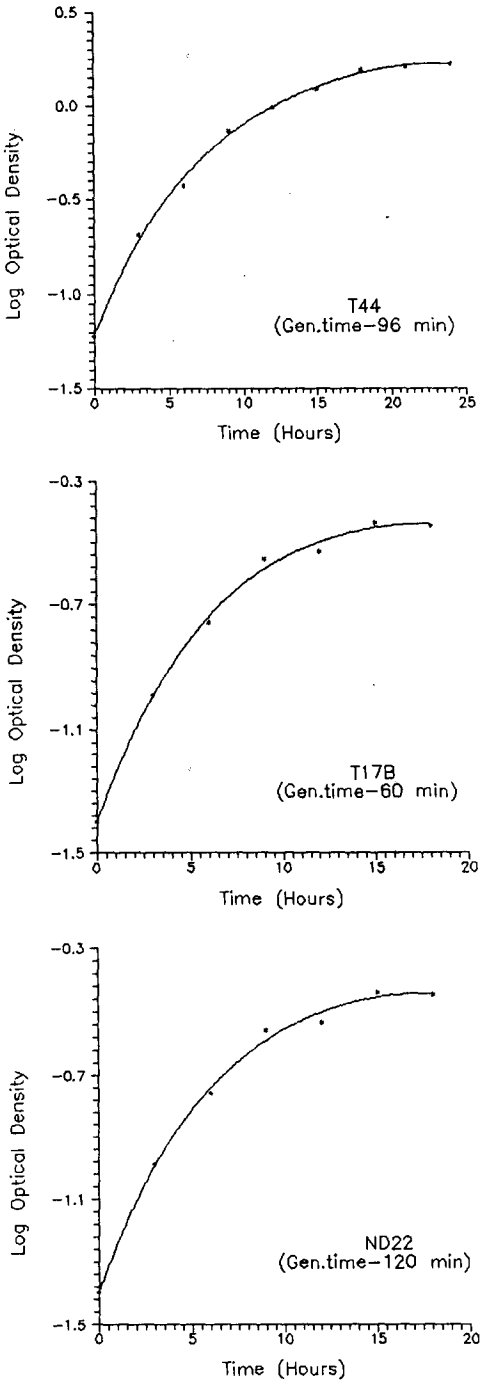


Fig.25. Growth curves of bacterial cultures in NAS medium.

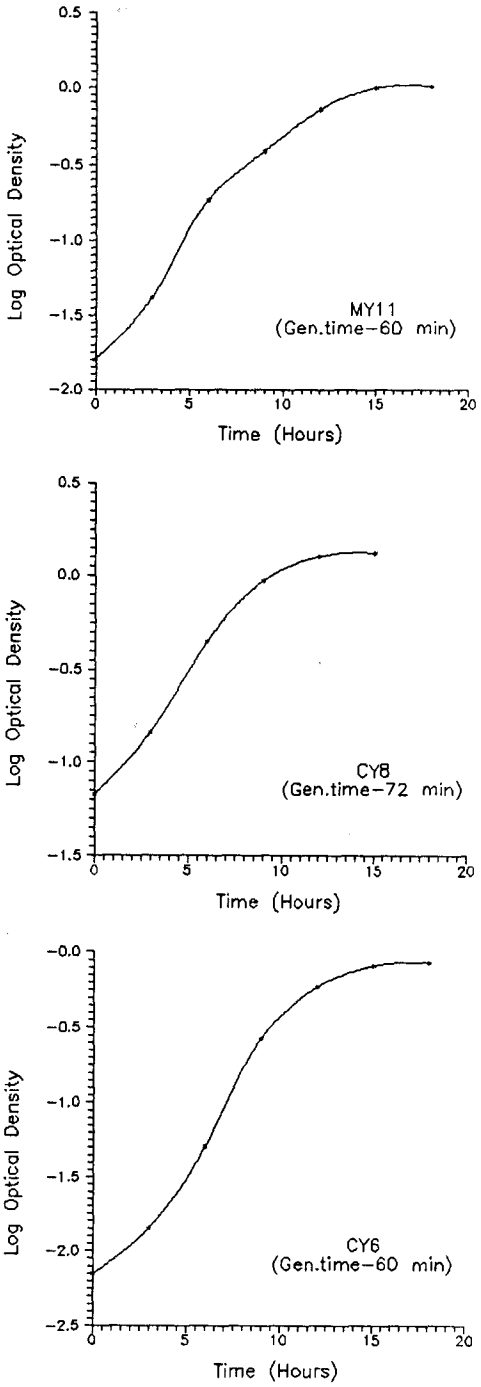


Fig. 26. Growth curves of yeast culturesin MEA medium.

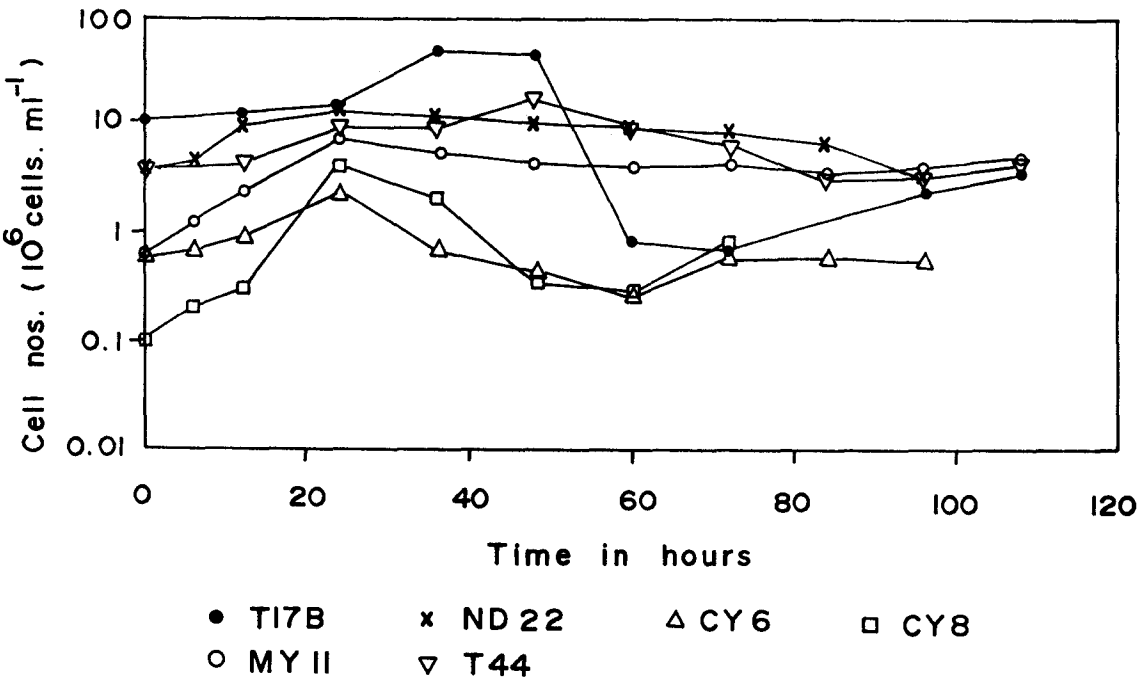


Fig. 27. Growth curves of bacteria and yeasts on BHC (A) oil.

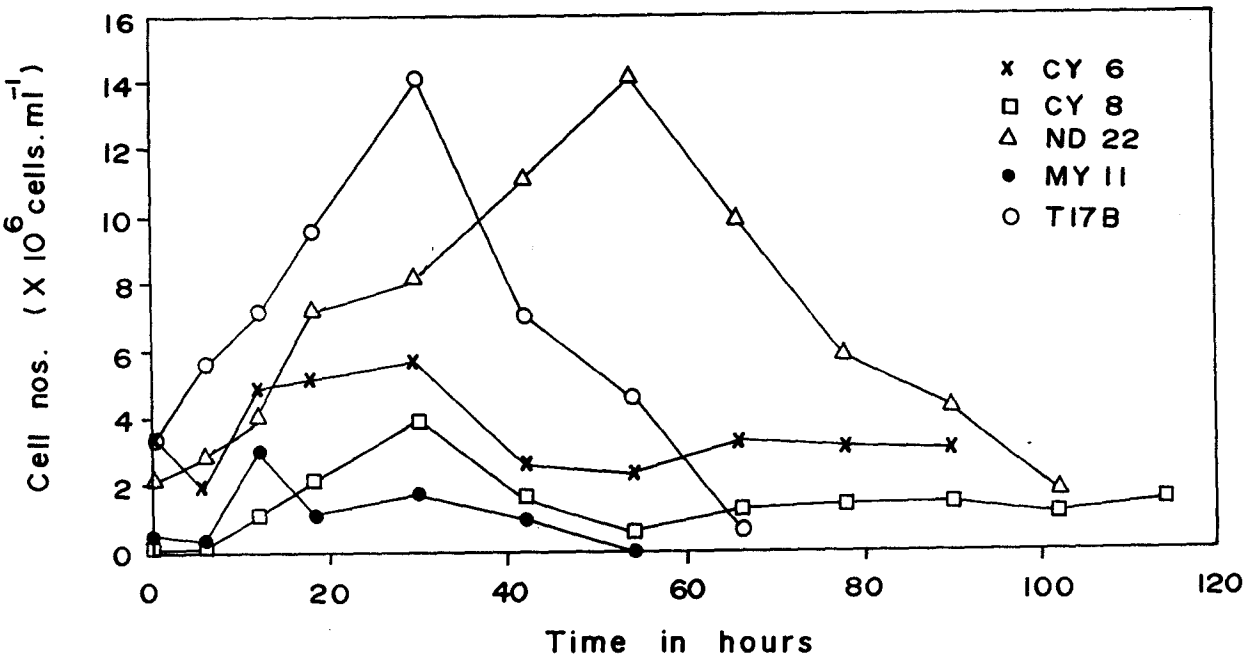


Fig. 28. Growth curves of bacteria and yeasts on hexadecane.

biosurfactant ranged between 0.9 g/L to 1.2 g/L with the tested alkanes and crude oil. There was no marked variation in yield with alkanes or crude oil.

Emulsifying activity of the various biosurfactants produced was tested using Bombay High crude oil and the results of which are shown in Table 22. It was found that the activity varied with respect to the hydrocarbon substrate used for production. Although the production of emulsifier was high (1.216 g/L) using hexane, oil emulsifying activity was low (0.174). The highest activity (D_{610}) of 0.552 was observed when the surfactant was produced using hexadecane. There was no relationship that could be perceived between emulsifier yield and emulsifying activity.

4.11. PLASMID SCREENING

Some 23 potential isolates from various sources especially those influenced by hydrocarbons were checked for extrachromosomal elements. These include strains capable of solubilising phosphates as well as those able to grow in nitrogen deficient medium. Preliminary screening showed that 12 of them harboured plasmids with 10 of them showing consistent hydrocarbonoclastic properties (Table 23).

The plasmid sizes have been derived using plasmid markers from 4.23 kb (pBR 322) to 23kb (pCP) and 51 kb (pRK). The migration of CCC of pBR 322 is taken as the lower end of the scale and that of pCP and pRK on the other end (Fig. 29). It has been assumed that single bands seen in these two are also CCC i.e. supercoiled forms. Likewise the migratory distance of the sample DNA is also assumed to be that of CCC. Plasmids are generally known to be isolated from the bacterial hosts as

Table 21. Production of bioemulsifier by T17B on different hydrocarbons.

S.No.	Substrate	Yield (g/L)
1.	Hexane	1.216
2.	Nonane	0.926
3.	Hexadecane	1.020
4.	Bombay High crude oil	1.006

Table 22. Emulsification of crude oil by bioemulsifiers.

S.No.	Hydrocarbon substrate for growth	Emulsifying activity (D_{610}) of BHC oil
1.	Hexane	0.174
2.	Nonane	0.237
3.	Hexadecane	0.552
4.	Bombay High crude oil (A)	0.321

Table 23. Plasmids of the bacterial cultures and their molecular weights.

S.No.	Culture No. (No. of plasmids)	Molecular weights (kb)
1.	ND2 (1)	18
2.	ND9 (1)	30
3.	ND22 (1)	80
4.	P6 (2)	< 4, 8
5.	T17B (3)	5, 10, 20
6.	T29 (4)	10, 15, 20, 23
7.	T30 (1)	< 20
8.	T36 (2)	2.4, < 4.3
9.	C7 (5)	4, 5.8, 7.6, 9.2, 12
10.	T46 (1)	5

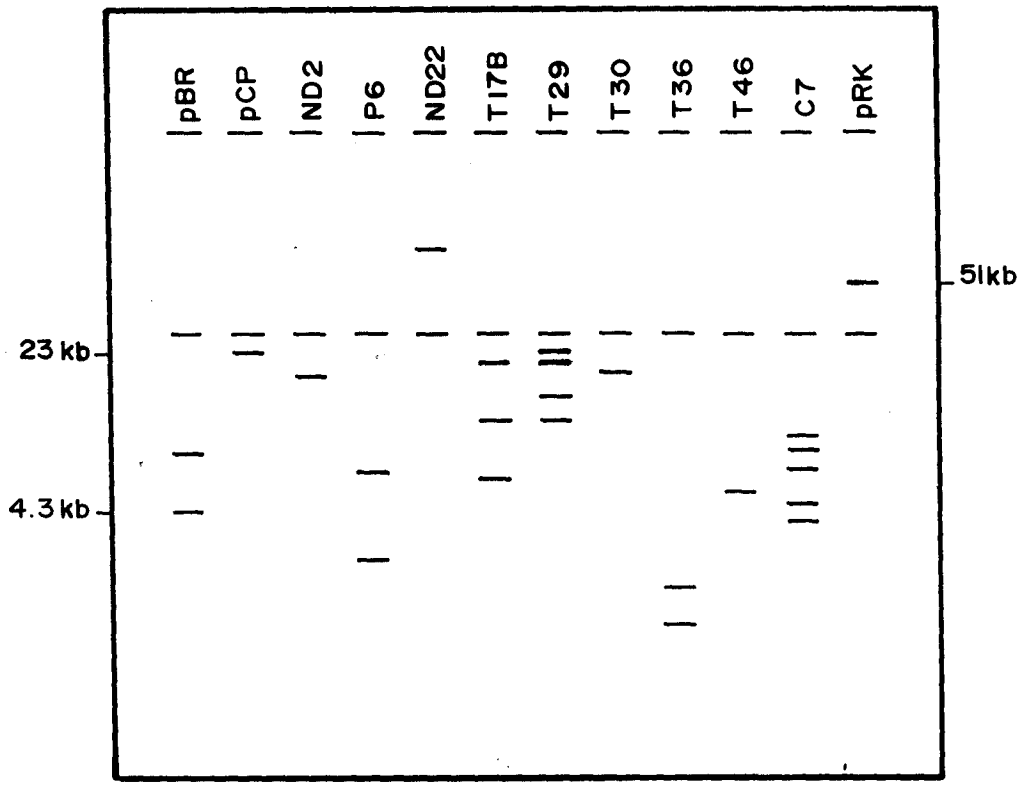


Fig.29. Schematic diagram showing plasmid mobility of Bacterial cultures against standard markers.

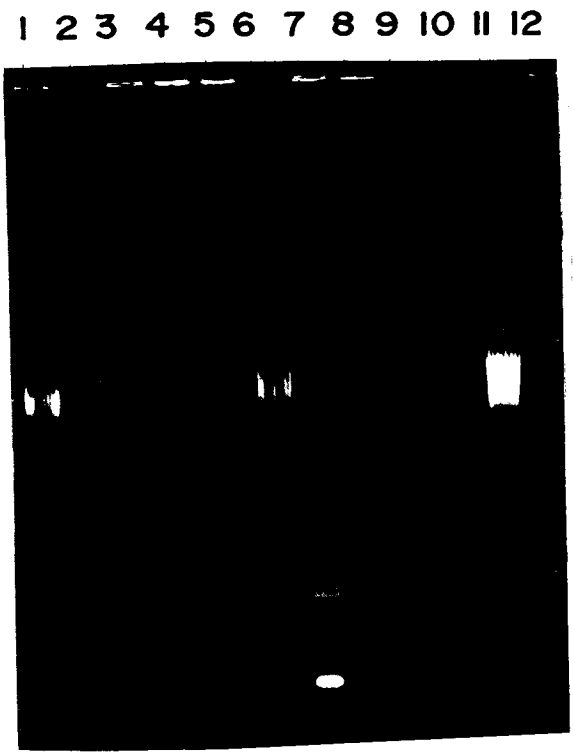
covalently closed circles CCC double stranded DNA molecules with a size range of 1.5 kb to 375 kb.

Several runs on agarose gels ranging from 0.5-1.0% clearly show a consistent migrating pattern of culture T17B (Fig. 30). The bands are equidistant, and for all purposes the band migrating the farthest is suggested to be a monomer of about 5 kb and others following are di and trimers of 10 and 15 kb. Twenty kb tetramers have also been noticed. Closely migrating bands could indicate that the open circular form of a monomer migrating just behind the SC of a dimer or it could be that the linear form migrating close behind the OC form.

The extrachromosomal DNA in cultures T29 and T30 show bands ranging from 20-25 kb. Culture T36 showed a varied molecular weight of the plasmid band. Perhaps it could be a plasmid of 20 kb getting sheared. Nitrogen fixers harboured plasmids ranging in weight from 18-80 kb. Culture P6 showed a ladder from 2-20 kb. Perhaps this too is a sheared large plasmid.

While hydrocarbonoclastic bacteria are known to harbour mega plasmids (Sanseverino *et al*, 1993) the present analogues showed very few large ones. Only the cultures capable of growing in nitrogen deficient medium (culture ND22) showed some large plasmids of 80 kb. Some long runs showed extra bands migrating late (culture nos. T29, T36) but is yet to be confirmed.

Preliminary curing experiments with T17B showed that when the strain was stripped of its extrachromosomal DNA, the ability to degrade hydrocarbon was greatly reduced to a value of $<5\%$.



Panel A
Lanes

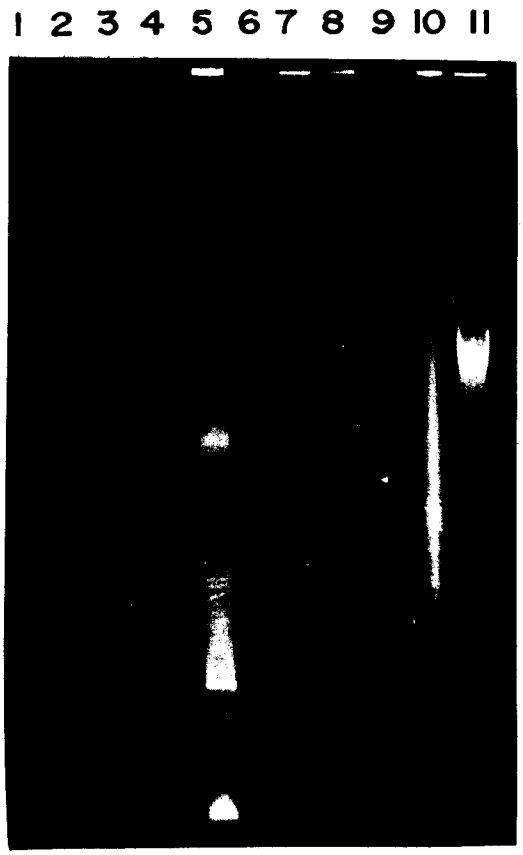
1 T17 B

2 T17 B

7 pBR

8 pCP

12 P6



Panel B
Lanes

3 C7

4 C7

5 pBR

7 pRK

Fig.30. Isolation of plasmids from bacteria

Culture T44 did not show the presence of plasmids during initial screening. Subsequent to growing it in a consortia along with T17B and ND22 in continuous culture system, it (renamed as culture no. C7) seemed to have acquired segments varying in size from 4.7-12 kb (Fig. 29). Experiments on the ability to improve hydrocarbon degradation has been tested on a preliminary level and has been found to be positive.

4.12. DEGRADATION OF BOMBAY HIGH CRUDE OIL (A) USING MIXED CULTURES

4.12.1. Type I

4.12.1.1. Batch culture and microcosm studies of Consortium #1

The results of studies on the degradation of Bombay High crude oil(A), tar ball and tar ball fractions conducted with consortium #1 are given in Table 24.

The consortium could degrade 70% of BHC(A) which was 3 % less than that when T17B degraded individually and 8% higher than that of ND22 (Table 7). In the case of tar balls, the degradation was slightly higher (33%) when compared to individual cultures (29% and 31% for ND22 and T17B respectively). Among the two fractions (aliphatic and aromatic) of tar ball, there was a distinct preference of degradation to the aliphatic fraction (51%) than the aromatic fraction (35%).

When degradation of BHC (A) was studied in the laboratory microcosm, it was observed that the consortium degraded only 30% of the crude oil (Table 24). As seen

Table 24. Degradation of petroleum hydrocarbons using Consortium #1.

S.No.	Substrate	Degradation (%)
1.	Bombay High crude oil (A)	70
2.	Tar Ball	33
	Aliphatic fraction	51
	Aromatic fraction	35
3.	Bombay High crude oil (A) in microcosm	30

from the table, the degradation was half compared to batch culture experiments (70%).

4.12.1.2. Batch culture studies using Consortium #1A

Consortium #1 was supplemented with another active degrading culture (T44) in order to assess the improvement in its degradability, if any. The newly constituted consortium (consortium #1A), degraded 50% of the BHC(A) in batch culture. It appears that modification of the consortium with a culture that showed 70% degradation did not improve the degradation substantially but only had a marginal effect.

In order to understand the reason for the observed decrease in degradation, a study on the changes in population was carried out in a continuous culture system. A continuous culture was used so that there was no accumulation of any toxic metabolic products and a steady state of culture could be maintained to observe the degradation.

The behaviour of the 3 cultures in the chemostat is shown in Fig. 31. Unlike the batch culture wherein the population was 1:1:1, the ratio in the chemostat was made up to 4:2:1 (T17B:T44:ND22) based on their growth pattern in crude oil. Despite initial population fluctuations observed in ND22 and T17B all the three cultures maintained their population at a steady state upto 9 days. Culture T44 was the only culture which maintained its population steadily from day 0 to day 9. The population of ND22 increased gradually from the 9th day to 18th day and thereafter a decline was seen until 24 days after which it reached a plateau. Culture T17B increased in population till 12 days reaching a maximum of 14.8×10^7 cells/ml and

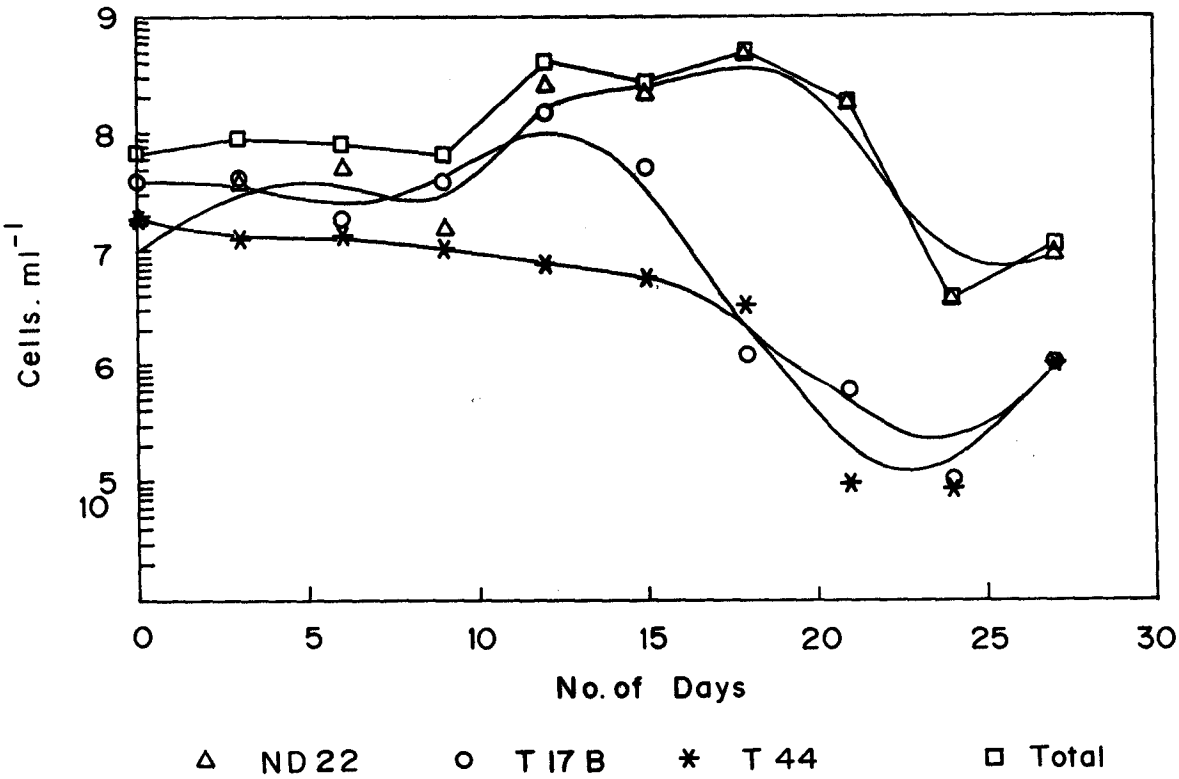


Fig. 31. Changes of bacterial populations in a continuous culture system.

declined till the 24th day. The population of T44 declined similarly after 15 days but increased again after 24 days. Thus, the total population, with the exception of the period from 12 days to 24 days was maintained at 6-9x10^7 cells/ml. It was intersting to note that at the end of 27 days culture ND22 which was less in number initially as compared to the other two cultures became the dominant group and the population of T17B which was the highest in number initially became low.

It is also noteworthy that none of the populations was totally eliminated and they appeared to co-exist, except with some changes in individual population ratio. Emulsifiying activity (D) of the chemostat broth also increased from 0.246 to 0.495 (Fig. 32).

4.12.1.3. **Batch culture studies by Consortium #1B**

An attempt was made to develop a modified consortium of #1 by adding the yeast culture, MY11, which not only showed high percentage of degradation but also degraded the higher fractions of crude oil in pure culture. This consortium was called consortium #1B and as seen from the results (Table 25) it degraded 74% of Bombay High crude oil (A) and 35% of tar ball. The degradation of aliphatic fraction of the tar ball was 25% which was lower compared to the individual degradation observed in these cultures (32-38%) (Table 9). However the degradation of aromatic fraction of tar ball was shown to be 35% which was higher than that observed for any of these individual cultures (4-33%).

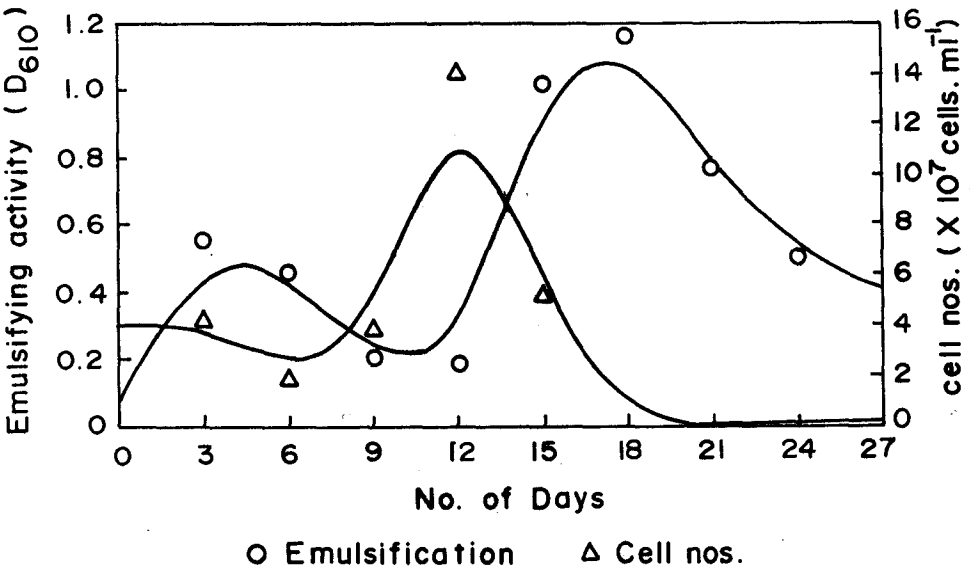


Fig. 32. Emulsification activity of culture no.T17B in a continuous culture system.

4.12.1.4. **Batch culture and microcosm studies by Consortium #2**

When all the 3 candidate yeast cultures were mixed and the degradation by this consortium was studied in batch culture, the degradation of Bombay High crude oil(A) increased to 85%; the highest degradation recorded for any consortium. When degradation was estimated using a laboratory microcosm, BHC(A) was degraded upto 81%. which was slightly lower than that observed in batch culture. Thus the 3 yeasts appear to degrade Bombay High crude oil (A) efficiently as a consortium.

4.12.2. **Type II**

Degradation by uncharacterised mixed cultures

The degradation of three petroleum hydrocarbons namely Diesel, Iranian crude and Bombay High crude (A)) was tested using 4 mixed cultures. The results are given in Table 26. Mixed cultures N2 and T2 were relatively less competent in degradation of all the three substrates when compared to the mixed cultures N1 and T1 respectively. Mixed culture T1 was able to degrade more of diesel (78%), Iranian crude (71%), BHC(A) (34%) when compared with the mixed culture T1 which degraded 45% of diesel, 26% of Iranian crude and 12% of BHC(A). Among these 4 mixed cultures, increased degradation by mixed culture T1 was observed. However these mixed cultures were not able to degrade the crude oil to the extent as observed in the earlier section using consortia.

Table 25. Degradation of petroleum hydrocarbons using Consortium #1B in batch culture.

S.No.	Substrate	Degradation (%)
1.	Bombay High crude oil (A)	74
2.	Tar Ball (TB)	35
	Aliphatic fraction	25
	Aromatic fraction	35

Table 26. Degradation of various oils by mixed cultures.

S.No.	Group	Degradation (%)		
		Diesel	Iranian light crude oil	Bombay High crude oil
1.	Nitrogen fixers			
	N1	24	16	8
	N2	68	50	26
2.	Tar Ball Associates			
	T1	45	26	12
	T2	78	71	34

4.12.3. **Type III**

Sequential enrichment of naturally occuring mixed cultures

Degradation of tar balls by naturally occuring mixed cultures were tested by sequential inoculation (continuous subculture) of the enriched inoculum at weekly intervals. The results of degradation are tabulated in Table 27a.

The total degradation of tar balls after 7 days was 10%; but at the end of 14 days, which was after 7 days of incubation with the 1 week old enriched inoculum, the degradation went up more than 3 times to 36%. Likewise, there was a 12% increase in the total degradation after 21 days. At the end of 28 days, which was after incubation using 3-week oil enriched inoculum, the degradation of tar balls was 63%. This was a substantial increase compared to the degradation using the initial inoculum. Hence repeated subculturing onto fresh tar balls improved the efficiency of the mixed culture by 53%. The chromatographic pattern of degradation is showed in Fig. 33. It can be noted that the lower carbon number compounds had almost been completely degraded in 28 days. There was also a distinct reduction of some of the higher carbon compounds after 21 days, whereas in 7 and 14 days these compounds were degraded only to a limited extent.

A mixed culture inoculum of bacteria which was enriched for 28 days (weekly subculturing) was tested for its ability to degrade tar balls for an extended period (35 days) in batch culture. The degradation of tar balls after every 7 days was estimated and the results are shown in Table 27b. Maximum degradation (80%) was seen in 4 weeks (28 days). Thereafter, the total degradation did not increase. There was a

Table 27a. Effect of incubation period on tar ball degradation by mixed cultures (continuous subculture).

S.No.	Incubation period (Days)	Tarball degradation (%)
1.	7	10
2.	14	36
3.	21	48
4.	28	63

Table 27b. Effect of incubation period on tarball degradation by mixed cultures (batch culture).

S.No.	Incubation period (Days)	Tarball degradation (%)
1.	7	67
2.	14	62
3.	21	74
4.	28	80
5.	35	78

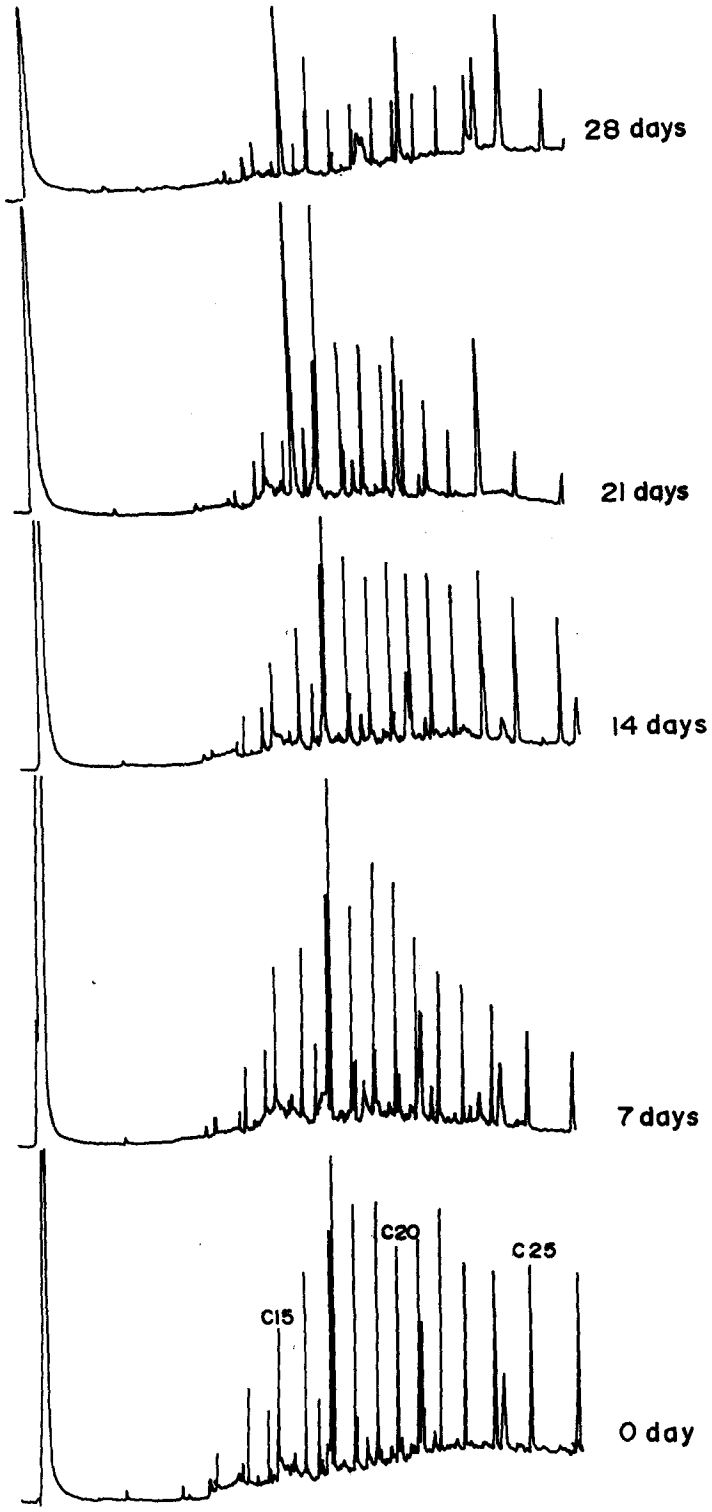


Fig. 33. Gas chromatograms showing the degradation of tarballs by a natural mixed bacterial cultures after sequential enrichment.

distinct increase in the degradation of tarballs after 14 days and a 12% increment was observed with 21-day old flasks.

5

Discussion

It is well established that the main natural mechanism by which the residual petroleum hydrocarbon pollutants are removed from the environment is by microbial degradation. Though hydrocarbonoclastic bacteria are ubiquitous in nature, their function is limited by the availability of two important nutrients, namely, nitrogen and phosphorus. Hence sometimes fertilisers containing these nutrients have been used to enhance and hasten the natural process of biodegradation. Using fertilisers to enhance the oil biodegradation is a relatively simple technology, but optimal conditions are rarely uniform in the environment to obtain maximum efficiency. Nonjudicious use of such tertilizers could lead to eutrophication and release of toxic metabolites back into the environment. Need for alternate technologies has been realised to mitigate oil pollution with the least intrusive and most environmentally sound means. Indigenous flora possess capabilities like surfactant production, cell adhesion and plasmid encoded enzymatic mechanisms which favour the utilisation of petroleum hydrocarbons. This study highlights the need for use of selected indigenous microorganisms from tropical marine environment in developing oil degrading consortia which possess physiological abilities to overcome key ecological limitations. Yeasts were also studied in the present work as they are known to degrade hydrocarbons, although their potential has not been examined for bioremediative measures as yet.

5.1. ISOLATION AND SCREENING

In the light of the above context, and with a view to develop self sustaining microbial consortia, attempts have been made to isolate both bacteria and yeasts from various sources and screen them for growth in nitrogen-free medium (only bacteria), phosphate solubilisation and hydrocarbon degradation. It is well known that oil biodegradation is limited due to nitrogen and phosphorus deficiencies in the marine environment. While the common bioremediation strategy is to advocate the use of inorganic and organic fertilisers, in this study bacterial cultures which were able to grow in nitrogen deficient medium, and those which were able to solubilise inorganic/organic phosphates were primarily screened and their oil degrading efficiencies subsequently elucidated.

In order to isolate bacteria with apparently nitrogen fixing potential, strains growing on medium devoid of any added nitrogen were isolated. The ability of hydrocarbon oxidising bacteria to grow on nitrogen deficient medium has been documented earlier (Coty, 1967). It was found that in the present study, 32 bacterial strains were able to grow on such a medium containing mannitol as the C source. These strains were termed as "nitrogen fixers" (please refer Materials and Methods) and such organisms could perhaps convert atmospheric nitrogen into cellular material. The strains were predominantly from seaweed holdfast and organically rich sediments collected from the Goa coast. It is well known that nitrogen is found in nature in both organic and inorganic forms. It is available to microorganisms in the form of organic

nitrogen (aminoacids, nitrogenous bases) from dead organic matter. The bulk of the availbale N in nature is in the inorganic form either as NO_3 or NH_4. Most bacteria are capable of using NH_4 as the sole N source as also with NO_3. Average concentration of NO_3 ranges from 5-8 μg at NO_3-N/L in coastal waters and about 0.8 μg at NO_3-N/L in oceanic waters. The amount of N required to degrade 1 g of crude oil as shown by previous workers, varies from 1 mg/L (Atlas and Bartha, 1972) to 3.2 mg/L (Bridie and Bos, 1971) and 11 mg/L (Reisfeld *et al*, 1972). When both inorganic and organic sources of nitrogen is depleted, an ecosystem depends on indigenous nitrogen fixers for nitrogen replenishment.

The nitrogen fixing marine organisms can be of 3 types; 1) the photosynthetic aerobic blue-green cyanobacteria anaerobic photosynthetic bacteria and 3) heterotrophic nitrogen fixers. Besides these, there are other heterotrophs which contribute to the nitrogen economy of an ecosystem. The nitrogen fixing ability in estuarine waters is calculated to range between 146 and 547.5 mg $N/m^2/yr$ while that of open oceanic waters ranges from 2-90 mg $N/m^2/yr$. The heterotrophic component was shown to be about 0.277 mg $N/m^2/yr$. The ambient concentration of nitrogen sources being low compared to the actual requirement for oil degradation, nitrogen fixing ability of oleoclastic heterotrophs gains significance.

The phosphate solubilising property of bacteria was examined using hydroxy apatite medium. These bacteria were found to be more uniformly distributed in all the sampling sites. Free phosphates are also generally low and are in limiting concentrations in the marine environment. It is estimated that 0.07-2.0 mg/L of

phosphorus is required to degrade about 1 g of crude oil (Atlas, 1981). Report of Naik *et al* (1982) demonstrated that certain phosphate solubilising microorganisms were able to leach upto 296 mg of phosphorus in 5 days under laboratory conditions, which suggests that phosphate solubilisers may be useful in making available soluble phosphates required for oil degradation.

As for the specific group of hydrocarbonoclastic bacteria, they were isolated from tar balls which were washed ashore in Kalpeni Island. Very few workers have looked into this niche (Naganuma and Horikoshi, 1993). They found that due to the sticky nature of tar balls it could serve as an useful source for hydrophobic bacterial cultures. They have also pointed out that tar ball harbours diverse microflora and therefore could serve as a source of natural enrichments.

In contrast to bacteria which are ubiquitous, population of yeasts is restricted to selected niches like organically rich polluted waters or nutrient-rich haloclines in estuaries (Hagler *et al*, 1979; MacGillivray *et al*, 1993). Yeasts were isolated in MEA medium from selected ecosystems like backwaters (Table 1). The randomly isolated colonies were screened for their PO_4 solubilising ability and oleophilic property. It was found that a majority of the yeast cultures which solubilised phosphates occured in coastal sediments. Twelve out of fifteen strains were found to solubilise phosphates. Effective solubilisation of PO_4 in the form of fish bones and hydroxy apatite by yeasts have been recorded by Naik *et al* (1982).

5.2. SCREENING OF MICROBIAL CULTURES FOR HYDROPHOBIC CHARACTER

Cell wall hydrophobicity

Several methods are employed to screen organisms capable of degrading specific hydrocarbons. The common methods are MPN technique by sheen screen method (Brown and Braddock, 1990), microtiter plates and redox indicators (Hanson *et al*, 1993), specialised oil containing isolation media (Seki, 1973; Venkateswaran *et al*, 1991), spray plate method (Kiyohara *et al*, 1982), ^{14}C labelled hydrocarbons (Roubal and Atlas, 1978; Brown *et al*, 1991) and MATH assay (Rosenberg, 1991). MATH assay which mainly measures the cell surface hydrophobicity has become a recent technique to screen for petroleum hydrocarbon degrading organisms also. Adhesion and emulsification/surfactant property which are examined in MATH assay are considered as key characteristics of hydrocarbon utilising microorganisms. Microorganisms which degrade hydrocarbons mainly act at the oil-water interface. Two major mechanisms are involved in microbial degradation of hydrocarbons (Prince, 1993). 1) By direct contact with the insoluble organic phase and 2) by interaction with surfactants. It is imperative that microbes exercise one mechanism or the other to utilise oil. Kennedy *et al* (1975) described that the ability of microorganisms to partition at the oil:water interface was a property unique to petroleum degrading bacteria.

In the present study, the most potential isolates which were positive for phosphate solubilisation and growth in the absence of nitrogen were checked for

hydrophobicity by MATH assay. The results showed that the cultures which were isolated from sources other than water had a fairly good cell wall hydrophobicity (Table 3a). The maximum extent of this character was found to be lower in nitrogen fixers (0.4) isolated from sediment, water or seaweed holdfast and phosphate solubilisers (0.6) isolated from sediment or water. Tar ball isolates which showed cell wall hydrophobicity had generally higher fraction of adherence compared to these. Hydrophobicity is a hypothesised mechanism for hydrocarbon degradation (Rosenberg, 1992; Prince, 1993). Such a high fraction of adherence in these isolates is apparently related to the fact that they were isolated from tar balls. It might have been the mechanism of hydrocarbon uptake and utilisation as these organisms showed considerable degradation of various crude oils (Tables 6 and 7). Rosenberg and Rosenberg (1981) have also found that adhesion is crucial for growth on hydrocarbons when mixing is poor. As tar balls are found at the intertidal area where there is minimum water dynamics, adhesion could be perceived to be the principal mechanism by which these hydrocarbon utilisers obtained their carbon. Foght and Westlake (1988) have also shown that there was a positive correlation between adherence and aromatic degrading phenotypes.

Although isolation was done on non petroleum hydrocarbon based medium (NAS), MATH assay obviously serves as an appropriate selection procedure for hydrocarbon degraders. Therefore this assay has been considered to be an useful index in the classification of organisms based on the type of mechanism employed by them in the degradation of petroleum.

Phosphate solubilisers like P43 were found to possess not only adhesive property but also could produce surfactants. Most of the nitrogen fixers showed cell wall hydrophobicity albeit at low values. The presence of hydrophobic ability in both these strains suggest the history of petrogenic inputs in the niche of their origin.

Surfactant property

Yet another mechanism for hydrocarbon utilisation is by surfactant production. It has been recorded that many hydrocarbon degrading microorganisms produce extracellular emulsifying agents and it has been opined that the substance is induced by growth on hydrocarbons (Hisatsuka *et al*, 1977). The production of biosurfactant substances either at the cell surface or into the medium to enhance hydrocarbon emulsification has been reported by earlier workers (Leahy and Colwell, 1990). Bacteria belonging to the nitrogen fixing and phosphate solubilising group from water or sediment or seaweed holdfast did not show much surfactant property probably due to the fact that these organisms were isolated from water or sediments where other hydrophilic substrates might have been available.

Since most of the bacterial cultures from tar balls were also able to produce surfactants it is probable that this mechanism is also in use by most of the organisms for the efficient utilisation of substrates from tar balls. The indication of surfactant production by the formation of fine oil globules in the organic phase (hexadecane phase) was observed to be much higher among the tar ball associates.

In contrast to bacteria, many isolates of yeasts did not show any surfactant property. This has not been a common observation among other researchers (Reddy

et al, 1982; Cameotra *et al*, 1984). More strains of yeasts expressed only adhesive property (Table 3b). Such adhesive properties among yeasts have been observed by some workers (Rosenberg, 1986). Poor growth among the strains which lacked surfactant property has also been noted in our strains as elsewhere (Itoh and Suzuki, 1972). Rosenberg (1992) has recorded a by-product of cell/hydrocarbon detachment process which was an extracellular emulsifier.

Another classification of the bacterial cultures based on both the hydrophobic and surfactant characters is presented in Table 4. This table shows that cell wall hydrophobicity and surfactant production are not necessarily related. Organisms showing just one or the other were still excellent hydrocarbon degraders (Table 6). Therefore the mechanism of hydrocarbon degradation might be carried out by cell adhesion to hydrocarbons or by production of surfactants. So it seems appropriate that for screening bacterial and yeast isolates for hydrocarbon degradation, either cell wall hydrophobicity or surfactant production may be used. However, it is preferable to use both the methods together. It is also found that on the whole, cell surface hydrophobicity of the cultures and an additional ability to produce surfactants is present irrespective of the ecological origin of the isolates. This would probably enable the cultures to compete for difficult hydrophobic carbon sources.

5.3. PETROLEUM HYDROCARBON DEGRADATION BY INDIVIDUAL CULTURES

5.3.1. Bacteria

The expression of adhesive and/or surfactant properties is a prerequisite for hydrocarbon degradation. In the tropical marine environment, studies on the biodegradation of crude oil are limited (Higashihara *et al*, 1978; Venkateswaran *et al*, 1993; Amund and Akangbou, 1993). Previous studies have shown that in aquatic ecosystems bacteria and yeasts are the prevalent hydrocarbon degraders (Atlas, 1981).

Ecotype and degradative abilities

From the results obtained for the degradation of Iranian crude oil (Table 6), it was evident that in general, the bacterial isolates from tar ball were able to degrade about 70% of the oil. In the case of the nitrogen fixers, *Ps. diminuta* (ND22) from seaweed holdfast showed a degradation of 71%. Among the phosphate solubilisers, culture P43 which is *Bacillus pumilus* from coastal sediment was another strain which could degrade Iranian crude oil considerably up to 85%. Thus hydrocarbonoclastic property was seen in isolates irrespective of the source of isolation.

It has been observed that regions affected by recalcitrant tar balls give rise to high bacterial populations including hydrocarbonoclastic ones (Naganuma and Horikoshi, 1993). Though total hydrocarbon utilising bacteria were not quantitatively estimated in any of the sampling sites in this study, MATH assay indicated that most of the microbes from tar balls exhibited both the hypothesized mechanisms of hydrocarbon utilisation, namely adhesion property (AP+) or surfactant production

(SP+). In the case of isolates from other sources, only one or none of these two properties was observed. In such cases the bacteria were probably not chronically affected by petroleum inputs at the time of isolation. Such environments where only sporadic discharges of petroleum occur, could have a fluctuating oil degrading population depending on the extent of pollution in that environment (Stewart and Marks, 1978; Atlas and Bronner, 1981).

If one goes by the index of the hydrophobic character of the cultures, it could be concluded that by virtue of their isolation from tar ball most of the cultures could degrade oil. Atlas (1981) has stated that the distributory pattern of hydrocarbonoclastic bacteria reflected the hydrocarbon exposure history of the site. The sediments from the beaches of Goa showed isolates having a higher incidence of hydrophobicity which could indicate a higher degree of exposure to hydrocarbon contaminants in these ecosystems during the period of collection.

Yeasts

The role of yeasts in the degradation of crude oil and its components has not received much attention. Degradation or biotransformation experiments have been undertaken by workers like Komagata *et al* (1964), Ahearn *et al* (1971), Walker *et al* (1975a), Cerniglia and Crow (1981) on individual alkanes and aromatic compounds, Blasig *et al* (1989) and Sorkoh *et al* (1990) on straight chain alkanes and MacGillivray and Shiaris (1993) on polycyclic aromatic hydrocarbons. *Candida* has been reported to be the prominent yeast genus in the marine environment capable of hydrocarbon utilization (Komagata *et al*, 1964; Ahearn *et al*, 1971; Sorkoh *et al*, 1990).

In the present study, 17 of the 27 yeast cultures from both coastal and offshore sources showed a value of > 0.1 in MATH assay indicating the general prevalence of hydrophobicity among yeasts. Degradation studies using yeasts was restricted to 3 cultures and these cultures belong to the genus *Candida*.

Relative degradation of various hydrocarbons

Bacteria

The degradability of crude oil depends on its type and origin. Of all the three petroleum mixtures tested, namely Iranian light crude oil, Bombay High crude oil and tar balls, bacteria degraded the former best (Fig. 10). The 7 isolates tested were able to degrade more than 70% of this oil in 7 days. This is probably related to the composition of the oil particularly of the saturates. The chromatograms showed that saturates in Iranian light crude were degraded to a much higher extent than Bombay High crude oil. Jobson *et al* (1972) have found that the enrichment cultures obtained from using low quality petroleum (containing more asphaltenes) could readily metabolise high quality crude oil. The 7 cultures tested were isolated from recalcitrant high asphaltenic tar balls. This might be the reason why these isolates were able to degrade Iranian light crude oil appreciably. The other 3 cultures from tar balls which were not able to degrade Iranian crude oil were possibly bacteria that were dependent on breakdown products/extra-cellular products of crude oil degradation. These 3 bacterial cultures were probably co-inhabitants in tar ball either deriving energy from metabolised products of hydrocarbon degraders (Venkateswaran *et al*, 1993) or were active degraders with unstable genetic elements (Lee *et al*, 1993).

Tar balls are known to be washed ashore and remain on the beaches for a long time (Nair *et al*, 1972). There has been a rise in tar ball deposits along Indian coasts in the recent past (Gupta *et al*, 1989). Reddy and Singbal (1973) while characterising these materials found the persistence of asphaltene content reflecting the age of the material. Elsewhere Morris (1974) among others also observed floating tar in the Atlantic waters, but it was considered as a recalcitrant residue after physical and microbial degradation of crude oils. The degradability of this material, not surprisingly, has not been examined except for an *in vitro* study conducted by Wyndham and Costerton (1981) who found that the aliphatic and aromatic fraction of bitumen supported growth of microorganisms while the asphaltene fraction did not. Their results yielded low total degradation (23%) of the aliphatic, aromatic and polar fractions in 120 days. However ready colonisation by sediment microorganisms on the bitumen have been reported by these workers. Thus an environmental adapatability has been observed among the organisms in the presence of such refractile compounds. This is perhaps the reason why the bacterial culture *Ps.putida* (T17B) in addition to being isolated from tarball also showed a higher degradation of this substrate on the whole as well as individual aliphatic and aromatic fractions. A high oil degrading culture (ND22) capable of growing and isolated from seaweed hold fast was also able to degrade tar ball considerably. These two hydrocarbonoclastic bacterial cultures were chosen for further detailed studies after determining their degradative abilities on light crude oil (Iranian), heavy crude oil (Bombay High) and fractionated mixtures. Alhough these isolates were able to degrade tar balls, it was relatively lower compared

to other crudes. It is well known that the hydrocarbons in tar are quite resistant to microbial attack. In addition, the surface area to volume ratio is not favourable for ready degradation. Tar balls are considered inert substances resulting after the physical and biological degradation (Morris, 1974). However, using reconstituted tar ball enriched isolates, a degradation of upto 60% of tar ball substrate was achieved in 7 days. This is perhaps a remarkable degradation with this substrate. On the other hand, earlier workers have neglected or sidelined the microbial degradation of tar ball as they were thought to be less accessible or conducive to microbes (Colwell *et al*, 1978). As such, except for a few studies by Wyndham and Costerton (1981) and Naganuma and Horikoshi (1993), there are hardly any reports examining microbial degradation of tar balls.

Yeasts

The overall petroleum hydrocarbon degradative abilities of the 3 *Candida* strains are significant, when we consider the percentages of degradation observed with Iranian light crude oil (ave. 83%) and Bombay High crude oil (ave. 65%) within a period of 7 days. However, yeasts were not able to utilise tar balls to the same extent as bacteria. There was a decrease in the percentage degradation (ave. degradation - 25%) of tar balls. Tar balls have a low aliphatic fraction (32%) and a very high asphaltenic fraction (52%). Among the yeast strains, the only instance where the degradation of tar ball or its constituents by yeasts was nearly in par with bacteria was when culture MY11 was used to test the degradation of aliphatic fraction of tar ball. The percentage degradation with the isolates in this study were higher than those

reported by Wyndham and Costerton (1981) who conducted their experiments with a lower concentration of bitumen and over a longer period of incubation of 14-15 days. In spite of the ability of culture MY11 to degrade higher aliphatic compounds in BHC oil, it was unable to attack this fraction in tar balls. This could be because of the absence of low molecular weight compounds (C_{10-15}) and a higher proportion of complex unresolvable mixture of recalcitrant asphaltenes. The conclusion by Walker *et al* (1975a; 1975c), that there was decrease in the degradation with an increase in carbon chain length among yeasts may well be the only explanation.

Pritchard *et al* (1976) reported that certain compounds which appeared during the microbial degradation of Altamont crude were similar to tarry components which are high molecular, recalcitrant and persistent in the environment. However, from the chromatographic analyses (Figs. 17 and 18) of the present studies, it was found that during degradation of the tested petroleum products there were no accumulation or synthesis of such high molecular weight compounds.

Yeasts were found to degrade the tested crude oils with relative ease. The degradation pattern obtained using GC with Iranian crude oil (Fig. 11) and fractionated Bombay High crude oil (Figs. 14 and 15) suggests that culture MY11 was able to utilise the higher molecular compounds (mainly the aliphatics) completely and in general the yeasts showed abilities of degradation of higher chain compounds. This result differed from the observation of Walker *et al* (1975a) who found a decrease in degradation with pure alkanes of increasing chain length. They examined the degradation of mixed hydrocarbon substrates by yeasts and found that degradation

showed a decrease with increase in chain lengths and patterns for hydrocarbon utilization were similar for yeasts and bacteria. However in the present study the pattern of utilisation between the two groups was different. Hence, it could be concluded that this variation in the degradative abilities could be strain dependent as suggested by Ahearn *et al* (1971).

Floodgate (1984) while considering the relative importance of yeasts and bacteria on degradation, concluded that though hydrocarbonoclastic yeasts may not be globally important, they may locally be responsible for a greater flux of petroleum derived carbon and energy during blooms. As yeasts are also known to convert certain paraffinic hydrocarbons into cell protein (Ahearn *et al*, 1971) these potentials could form the basis for using yeasts in formulating bioremediative measures.

Studies on the aromatic hydrocarbon degradation by yeasts have been carried out earlier (Cerniglia and Crow, 1981; Walker *et al*, 1975a; MacGillivray and Shiaris, 1993). While Walker *et al* (1975a) described the utilisation of compounds like phenanthrene, pyrene and perylene, MacGillivray and Shiaris (1993) have detailed transformation of phenanthrene and benz (a) anthracene. It was noted that the aromatic fraction of crude oil does not appear to be toxic to the yeast strains in the present study. Upto 41% of the fraction was degraded in 7 days by culture CY6. This demonstrates the relatively comparable degradation potential of aromatic hydrocarbons by yeasts and bacteria. It was also observed in the recent past that the importance of the biotransformation of polyaromatic hydrocarbons (PAHs) by yeasts on a per cell basis, is comparable to bacterial capability (MacGillivray and Shiaris, 1993).

PAHs such as naphthalene, anthracene, phenanthrene, and 1,2-benzanthracene are some of the classsical examples of petroleum components. Their utilisation in the marine environment by yeasts have been studied to a certain extent (Cerniglia, 1984). The selected yeast cultures were tested with 3 PAHs besides the aromatic fraction of crude oils. It was found that CY6 could utilise naphthalene (95%) and anthracene (29%) while cultures CY8 and MY11 were able to degrade anthracene and naphthalene respectively. The reason for the inability to utilise phenanthrene, a pure hydrocarbon could be due to the toxicity of the compound to the strain as these polyaromatics are considered to be toxic when dissolved (Cerniglia, 1984).

Yeast culture MY11, among the tested individual aromatic hydrocarbons could degrade only the diaromatic naphthalene. The degradation of aromatic fraction of crude oil was found to be lower than that compared of tar ball. These findings could probably indicate that MY11 had aromatic degradative enzymatic mechanisms which operated at conditions when there are either less toxic compounds or when there is a lower proprtion of soluble aromatics ehich are considered to be more toxic (Cerniglia, 1984) as observed here in the case of tar balls.

The poor aromatic fraction degrading ability by the yeast culture CY8 was also substantiated when individual polyaromatic hydrocarbons (PAHs) were used. This culture could not degrade naphthalene or phenanthrene and was poorly effective on anthracene also. Such poor degradative performances could be attributed to the absence of required aromatic enzymatic mechanisms which are different for the utilisation of

aliphatics. Hence as the mechanisms for aliphatic and aromatic fraction degradation are discrete and their functioning in combination could be strain specific (Atlas, 1984).

On the whole it is clear that marine bacteria and yeasts are efficient in degrading different types of petroleum hydrocarbons including the more resistant fractions. While bacteria were efficient with unaltered crude oils yeasts were good at attacking the aliphatic fractions (C20-C30) of the crude oils.

5.4. EFFECT OF CERTAIN PARAMETERS ON THE DEGRADTION OF BOMBAY HIGH CRUDE (A) OIL

As soon as oil is spilled in the environment its degradation removal is under the control of several parameters such as temperature, oil composition and ambient nutrient concentration. Atlas (1991) has emphasised that petroleum biodegradation rates are determined by the population of indigenous microorganisms, the physiological capabilities of the populations and other abiotic factors that influence the growth of these organisms. The effect of various factors like varying temperatures in temperate waters (Gibbs *et al*, 1975; Atlas *et al*, 1978; Shiaris, 1989), nitrogen and phosphorus concentrations (Boehm and Fiest, 1980), salinity (Shiaris, 1989; Bertrand *et al*, 1993), pressure (Colwell and Walker, 1977), pH (Dibble and Bartha, 1979) and oxygen (Atlas, 1984) have been extensively studied in space and time. However, some of the important variables that can play a key role in the degradation of crude oil such as temperature variations in tropical environment, quantitative microbial populations, period of incubation and substrate concentration have found very little attention.

Temperature

Temperature based studies showed that culture T17B was the only culture that could degrade upto 41% of the crude oil at 20°C while other candidate cultures degraded only about 25%. This is in contrast to the observations seen at 30°C, the degradation being much more at this temperature. The yeast CY8 was the most sensitive culture to low temperature. Atlas (1975) found that microbial degradation accounted for 26-50% loss of crude oils during 42 days incubation at 20°C. However when the crude oils were incubated at 10°C, degradation ranged between 16-28%. Thus, nearly a decrease by half the degradation was observed at the lower temperature. This is in agreement with our finding. There are two possible reasons why bacteria in the present study showed this trend; 1) All the bacterial and yeast strains which were isolaed from tropical waters with an average temperature of 28°C might be better adapted to ambient temperature conditions. This points out to the need for using indigenous bacteria in oil degrading consortia, for use in microbial seeding in tropical marine environment. 2) As Atlas (1991) has pointed out, the reduction could be due to high viscosity and reduced volatalisation of toxic compounds at low temperatures. These alterations of the physical and chemical nature of the oil leads to a lower degradation at lower temperatures (Atlas, 1991).

Substrate Concentration

Substrate concentration was an important factor that decided the percentage of degradation. Atlas (1981) observed that different metabolic pathways are expected under different hydrocarbon concentrations. On the contrary, Leahy and Colwell

(1990) opined that biodegradation rates for many hydrocarbons do not display the dependence on concentration but substrate solubilities. Venkateswaran *et al* (1993) based on their own findings as well as that of Rambeloarisoa *et al* (1984) have concluded that the increase in concentration to 1,2,4 or 6 g/L of crude oil had a decreasing trend in the microbial degradation capacity. The above observation was found to be partly true in the present study. The percentage degradation increased when substrate concentration was increased from 0.5-1.0%. Thereafter however the degradation decreased. The decline in percentage degradation with increase in substrate was steeper with yeasts than with bacteria. Thus the tolerance range of concentration was wider in bacteria than yeasts. It should be noted that among the cultures tested, CY8 was sensitive to higher oil concentrations (>1.5%) as well as lower temperature (20°C) thus showing a narrow range of adaptability. However it showed higher degradation than other cultures under ambient temperatures and at ideal oil concentrations (1%).

Period of incubation

The period of incubation crucially decides the percentage of degradation. The importance of length of incubation period on the degradation of crude oil has also been stressed by Venkateswaran *et al* (1993). As seen from the results of this study (Fig. 20), bacterial degradation was optimal between 3-7 days while yeasts showed an optimum between 7-15 days. Earlier reports show that an incubation period of 7 to 10 days was found to be optimal for oil degradation studies Venkateswaran *et al* (1991).

Thus the candidate microbial cultures seemed to fall within the range prescribed by previous workers.

5.5. BIOEMULSIFIER PRODUCTION AND EMULSIFICATION

The importance of emulsification in influencing the fate of petroleum has gained scientific and commercial interest (Harvey *et al*, 1990; Bertrand *et al*, 1993; Basseres, 1995). The production of bioemulsifiers by hydrocarbonoclastic bacteria has been reported by several workers (Chakrabarty, 1985; Goutx *et al*, 1987; Bertrand *et al*, 1993). These emulsifiers are either fatty acids or their derivatives or other complex polymers (Rosenberg, 1992). Surfactants are known to increase the surface to volume ratio and reduce the surface tension of the hydrophobic substances thus increasing the dissolution into the aqueous phase. The production of such emulsifying agents by *Pseudomonas* sp. has been studied by Reddy *et al* (1982; 1983) and Harvey *et al* (1990).

It has been shown by Reddy *et al* (1982) that certain microorganisms developing on hydrocarbons produce surface active agents which emulsify the substrate enabling its transfer into cells. Culture T17B was the only strain among the bacterial candidate cultures which showed indication of surfactant production in the MATH assay. This culture was identified as *Pseudomonas putida*. The production was examined with various hydrocarbon sources. The organism was able to produce emulsifying substances on all the alkanes and Bombay High crude oil tested. Chakrabarty (1985) found that the presence of surfactants in a medium by one strain

can restore the growth and utilisation of hydrocarbons by other oil degrading organisms incapable of surfactant production. Rambeloarisoa *et al* (1984) have observed a 120% increase in the oxygen utilisation of a mixed culture in the presence of added biosurfactant. This shows the positive role of biosurfactants on the hydrocarbon catabolic activity. Thus inclusion of culture T17B into any mixed cultures might provide biosurfactants helpful in enabling other nonsurfactant producing organisms to utilise hydrocarbons as well. The yield of biosurfactants under varying conditions are known to fluctuate (Shabtai and Wang, 1990; Goswami and Singh, 1991). An average yield varying from 0.9 to 1.2 g/L was obtained in the present studies. This was lower than that observed by Juwarkar and Khirsagar (1991), who observed a 3 g/L yield of the biosurfactant. The emulsifying activity of these water soluble biosurfactants was also examined in the present study. The emulsifier produced from any one of the substrates was able to emulsify Bombay High crude oil. However, the emulsifying activity varied with the substrate used to produce the emulsifier. Emulsifying activity was maximum with the bioemulsifier produced with hexadecane as the substrate. This study indicates that these emulsifiers are capable of interspecific substrate emulsification. Such properties exhibited on mixtures of aliphatic and aromatic petroleum hydrocarbons have been reported earlier (Rosenberg *et al*, 1979; Schulz *et al*, 1991) and are recognised to be an advantage for oil spill clean up (Harvey *et al*, 1990).

Though the yield of bioemulsifier per liter basis in this study was relatively lower than that obtained by Juwarkar and Khirsagar (1991), it should be emphasised

that the emulsifying activity with crude oil was close to their values (0.552 at 1 mg % concentration of the emulsifier). Therefore less than 1/2 the concentration of the bioemulsifier produced by culture T17B, in comparison with that of Juwarkar and Khirsagar (1991) was sufficient to emulsify the crude oil with comparable values. The most remarkable features of the biosurfactant as a crude oil emulsifying agent in this study are: 1) it was found to be effective at low concentrations (0.001%) and 2) it showed a broad range of substrate specificity from simple alkanes to more complex crude oil.

The inclusion of culture T17B as a candidate culture was based on this additional property of biosurfactant production. In the natural environment, use of chemical dispersants to clean oil spills have caused major pollution problems (McIntosh, 1989). Hence the use of surfactant producing microorganisms are preferred. Biogenic surfactants have shown good environmental compatibility and also could emulsify various types of petroleum hydrocarbons. (Schulz *et al*, 1991).

Another candidate organism (MY11) also showed an indication of surfactant production by MATH assay. This strain identified as *Candida albicans* was not further examined. The production of biosurfactant in *Candida* has been recorded by Kappeli and Fiechter (1977). Reports of yeasts belonging to *Candida* sp. showing surfactant production have been reviewed by Rosenberg (1991). Certain organisms are known to contain polysaccharide-lipid complex on the cell surface which show strong affinity for hydrophobic substrates. This is possibly the reason why cultures CY6 and CY8 showed a strong cell wall adhesion in MATH assay. For MY11, extracelllular

emulsifier production could be anticipated due to the stable hexadecane emulsion seen during MATH assay. Though the yield/activity were not studied for MY11, the bioemulsifier would possibly aid in stabilising hydrophobic emulsions during degradation in hydrocarbon polluted environments.

5.6. TAXONOMIC CHARACTERISATION OF POTENTIAL CULTURES

Most of the bacterial strains were mostly Gram negative and motile. A similar observation has been made by other workers studying oil degrading marine bacteria (Janiyani, 1993). *Pseudomonas* and *Bacillus* were the most prevalent genera among the key hydrocarbon degrading bacteria in this study (Table 25). Other genera such as *Klebsiella* and *Brevibacterium* were also found to be significant hydrocarbon degraders in this study. These organisms have also figured in the table of hydrocarbon degrading groups enlisted by Rosenberg (1992). There are innumerable reports on the hydrocarbon degrading *Pseudomonas* and *Bacillus* species. Rosenberg (1992) has stated that one of the most frequently isolated genera in hydrocarbon enrichments is *Pseudomonas*, and hence their predominance in the present study is not surprising.

It was observed that most of the good crude oil degrading bacterial cultures (like ND22, P43, T17B, T44) were able to utilise less number of sugars compared to those which degraded hydrocarbons less efficiently. Whether this preferential nutritional aspect of such organisms has any relevance to hydrocarbon metabolism is not known. However, the enzymatic properties of the hydrocarbonoclastic strains seem to be highly diverse (Table 12). The findings of this study were similar to those

observations of Janiyani (1993) on the catalase and oxidase activities of the crude oil degrading bacterial strains. Such observations have also been noted from oil degrading populations isolated from sea foams (Rambeloarisoa *et al*, 1984). The evolution of such diverse mechanisms could be due to the complex localised environmental niches demanding varied metabolic requirements. Such environmental factors lead to cross acclimation enabling the organisms to metabolise different compounds of similar structure (Bauer and Capone, 1985).

The cluster pattern based on the dendrogram (Fig. 23) showed that most tar ball isolates (60%) belong to one group (cluster B) while the nitrogen fixing strains (80%) to another (cluster A) with the exception of ND22. Light crude oil degradation was found to be higher among cluster B strains because they were originally isolated from tar balls.

All the 5 candidate cultures had a rapid generation time of 1 hr to 1h 36 min (Figs. 25 and 26). Such high rates are desirable characters for use as oil spill degrading organisms. Colonies had morphologically different traits aiding in visually recognising one from the other. There were clear changes in the growth pattern of all the five cultures in hexadecane as compared to crude oil. Though crude oil was able to support the growth of the test microorganisms, hexadecane could serve as a better carbon source for growth and energy compared to crude oil.

Multiple resistance to antibiotics was generally observed in most of the bacterial cultures in this study and it is well known that antibiotic resistance is coded on plasmids.

5.7. PLASMIDS IN HYDROCARBONOCLASTIC BACTERIA

Bacteria by their sheer number and turnover rates decide the fate of a pollutant in an environment. They respond to different types of pollutants by adapting themselves. Consequently new phenotypes emerge. It has been shown after the Alaskan oil spill by Sotsky et al (1994) that different genes find expression during the different phases of oil degradation. As the period of degradation advanced, equal expression of the observed genes (Alk and Xyl) was replaced by XylE lacking AlkB after enrichment with naphthalene. In this adaptive process the bacteria are greatly assisted by the extrachromosomal DNA (ECD), namely the plasmids. Plasmids are supposed to be ubiquitous and their frequency of distribution depends upon the species and environment (Boronin, 1992). They are known to be involved in the biodegradation of hydrocarbons. The involvement of plasmid encoded genes in the degradation of both aliphatic and aromatic hydrocarbons by strains of *Pseudomonas putida* has been reported earlier by Singer and Finnerty (1984b). The genes in the plasmids could code for 1) the catabolism of the hydrocarbons, 2) physical interaction with the cells (adhesion), 3) protection of the cell from toxic elements in the oil, or 4) encode the process of internalisation of the hydrocarbon as a growth substrate. Many of the degradative pathways seen in *Pseudomonas* sp have been shown not to be due to the functions coded by the genes on the chromosome, but those on the extrachromosomal elements - plasmids (Chakrabarty, 1972). Our results show that the strains are able to degrade the oil components and grow on it as substrate; however the function for which the plasmids were encoding could not be discerned. It has been

established that under most stressed conditions, the frequency of plasmid carrying bacterial population increase. In the light of this context, it is little surprising that most of the potential strains which were isolated from tar ball in the present study were found to harbour plasmids. Some of these strains constantly showed the presence while in a few others the frequency of detecting the ECD from the derivatives of the same isolate were less.

Keeping in view that hydrocarbonoclastic plasmids came in various size range, different techniques were employed. The potential strains showed plasmids in the range of <2 to about 80 kbs. Besides, only small plasmids and multimers of these were detected in bacterial culture T17B. It has been argued that conjugative plasmids are supposed to be small but have genes coding transfer, replication and non essential genes for antibiotic resistance.

Strain T17B identified as *Pseudomonas putida* has a small CCC with multimers. It has been recorded that such small plasmids could be cryptic with no known functions (Chakrabarty, 1976). On the other hand experiments in this study showed different results with this culture. Visual observation of experiments conducted with acridine orange cured cells of T17B showed that emulsification was very poor. Consequently the crude oil degradation was negligible. Hence it could be suggested that the small plasmids seen in culture T17B could be reponsible for degradation of the oil. Boronin (1992) has found the frequency of occurence of such degradative plasmids (D plasmids) to be significant in *Ps. putida* and *Ps. fluorescens* spp. The D plasmids are known to control the degradation of various compounds including

xenobiotics. In nature, the D plasmid-host combination have been recorded to give a greater diversity of bacterial strains capable of degrading xenobiotics than previously believed (Frantz and Chakrabarty, 1986). Frantz and Chakrabarty (1986) found that sixteen out of 30 strains of Pseudomonas sp. growing on naphthalene were found to harbour conjugative plasmids (NAH) controlling the degradation/oxidation of this substrate. The NAH plasmids are also known to be resistant to heavy metal ions and in general, hydrocarbonoclastic properties have been linked to metal resistance but these properties have not been tested in this study.

Thus, though, the D plasmids have sometimes been linked to metal tolerance they have rarely been connected with antibiotic resistance. In fact, there have been very few references that link the hydrocarbon degradative genes with antibiotic resistance. The present studies have shown that most of the hydrocarbonoclastic strains were resistant to one or more antibiotics (chapter on Taxonomic characteristics). In particular, culture ND22 was resistant to 3 out of 16 antibiotics and T17B to 4. (Table 14). Perhaps some plasmids could be termed as both "D" and "R" because they have both the phenotypes. Degradative plasmids have frequently been isolated as CCC and in this study, plasmids have been recorded as CCC forms. However, other forms i.e. OC or L configurations have also been detected consistently with T17B.

Besides plasmid mediation, certain strains are able to degrade using genes in the chromosomal DNA (Singer and Finnerty, 1984). It is perhaps due to this reason that some of the strains like T44 could degrade hydrocarbons without plasmid involvement. Some of the bacteria are known to carry two separate sets of genes; one

on the chromosome and one on the plasmid (Chakrabarty, 1972). Probably this duplication in the chromosome and its maintenance would enable the host for adapting better in degrading the hydrocarbon substrate.

5.8. DEGRADATION OF BOMBAY HIGH CRUDE (A) OIL BY MICROBIAL CONSORTIA AND MIXED CULTURES

Degradative studies using microbial flora have been approached in different ways using either mono or mixed cultures (with varying number of strains) which are either allochthonous or autochthonous. Mixed microbial cultures are known to degrade complex hydrocarbons much better compared to individual pure cultures (Miyachi *et al*, 1993). An approach has been to use engineered microorganisms for the same purpose (Chakrabarty, 1985; Lee *et al*, 1994). However, the present trend is to reduce or totally eliminate the use of genetically manipulated microorganisms and to use plurispecific microflora without altering their genetic makeup.

When referring to mixed cultures, very often naturally occuring indigenous flora from a particular site is meant (Walker *et al*, 1975a; Tagger *et al*, 1983; Venkateswaran *et al*, 1993; Amund and Akangbou, 1993). Very seldom does it refer to selectively reconstituted microbial cultures based on degradative or physiological advantages. The aliphatic and aromatic compounds of crude oil are understood to be either degraded by different organisms or different pathways in the same organism which may not be active at the same time (Prince, 1993). Hence in the present study,

emphasis has been laid on isolating, testing and combining different types of strains for the degradation of petroleum hydrocarbons.

Three types of approaches have been used to look at the degradative performance of different petroleum hydrocarbons in batch and microcosm studies. These were: 1) Selectively reconstituted microbial cultures or Consortia (Type I), 2) Randomly reconstituted mixed cultures (Type II) and 3) Serially enriched natural cultures (Type III).

Type I: Selectively reconstituted Consortia of microbial cultures

Consortium #1B consisting of 2 selected bacteria (cultures T17B, ND22) and a yeast (MY11) was found to degrade BHC oil slightly more (74%) than mixed cultures containing only the bacteria (Consortium #1) which degraded 70% in batch studies. Similarly degradation of tar ball was also slightly higher by 2% when the yeast was included. The inclusion of the yeast slightly improved the degrading ability of the mixed culture. However, the degradation of the aliphatic fraction of tar ball was reduced by 26% in the presence of yeast.

Degradation of the heavy fraction is generally aided by the presence of lighter fraction. The cells are thus able to grow and multiply in the low molecular weight compounds and then attack the larger molecular weight forms (Walker *et al*, 1975a).

In the case of aromatic fraction of tar ball the percentage degradation was slightly better than the degradation by individual bacterial cultures. The inclusion of yeast culture MY11 in this Consortium did not improve the tar ball aromatic fraction degradation further probably because yeasts in general have a low transforming ability

for aromatics (MacGillivray and Shiaris, 1993). In this study degradation was observed using the following experimental setup; 1) continuous flow through microcosm with sediment inocultated with the Consortium and 2) batch culture The microcosm was a harsher condition compared to the batch culture experiments in terms of aeration. In spite of this, Consortium #2 containing 3 candidate yeast cultures was able to degrade a substantial quantity (81%) of the crude which was only marginally less than the 84% degradation observed in batch culture experiments. The bacterial Consortium #1 fared differently. It was able to degrade 30% of BHC oil in 7 days in the microcosm which was less than half the degradation achieved in batch culture experiments (70%). The significant quantitative removal of crude oil by the yeast consortia was found to be highly remarkable compared to other studies which report a lower range (Mulkins-Philips and Stewart 1974; Amund and Akangbou, 1993; Venkateswaran *et al*, 1991). There have been reports where the amounts of degradation were about the same as the present study. The values reported by Bertrand *et al* (1993) was about 80-95% for saturates and 17-34% for aromatics. Still others have reported higher degradation of 92% and 83% of saturates and aromatics respectively (Rambeloarisoa *et al*, 1984), but these experiments have been carried out at 1/10th of the concentration used in the present study.

The consortium reconstituted with the 3 yeast cultures could be a viable bioremediative bioadditive compared to bacteria in cases where ciliate predatory fears are anticipated. In addition, yeast are considered more resistant to stress conditions of

UV rays and osmotic pressure changes (Ahearn *et al*, 1971) due to their more developed cell wall.

Another Consortium of 3 selected bacterial cultures (Consortium #1A) was examined for degradation in a continuous culture system at higher (2%) BHC oil concentration compared to batch cultures (1%) using individual strains. At the end of 27 days a degradation of 50% was observed. This was lower than the percentage degradation of individual bacterial cultures at 2% v/v concentration at the end of 7 days (54% and 63% for ND22 and T17B respectively). Reduction in degradation values could be due to the reduced aeration and the possible decrease in the number of active cells. A reduced flow rate could perhaps have given a higher value.

Significant variation in the individual population was observed over the period. The ratio of T17B:T44:ND22 changed from 4:2:1 to 2:1:10, the cultures T17B and ND22, reversing their population trends. The bacterial culture T44 was found to be more stable in population in spite of an overall decrease between 18 and 24 days (Fig. 31) among the three populations. This decrease could be due to a brief accumulation of toxic metabolic products in the aqueous phase, after 18 days and took about 6 days to get flushed out of the system. It should be emphasised here that the percentage degradation (50% of 2% v/v) was higher than 60% degradation of 0.6% w/v of crude oil reported by Venkateswaran *et al* (1993).

Type II: Randomly reconstituted mixed cultures

In another experiment, some of the uncharacterised bacterial cultures were examined in a random combination. Among the 4 groups of mixed cultures, T2

degraded Diesel > Iranian light crude oil > BHC oil. The other groups T1, N1 and N2 also reflected the same trend but degraded less than T2. It is presumed that T2 degraded better because the cultures were originally isolated from tar balls and it was composed of more number of cultures which could have increased the cooxidation effect of substrates (Venkateswaran *et al*, 1991). The possibility of different strains contributing to degrading hydrocarbons of different nature (saturated, unsaturated, branched, linear compounds) was higher in a 7 membered mixed culture than a culture with less members. Some cultures may not degrade directly but are indispensable in combination (Rambeloarisoa *et al*, 1984). Mixed culture N2 did not lag very much behind in the extent of degradation. This could be due to certain physiological adaptability of the constituent strains acquired from the ecotype of origin.

Type III: Serially enriched naturally occuring mixed cultures

A progressive increase in the ability to degrade tar balls was seen when an uncharacterised mixed bacterial culture was sequentially subcultured into fresh tar ball-containing flasks. The weekly subculturing of the inoculum increased degradative ability by 6 times at the end of 4 weeks. This 4-week old sequentially enriched mixed culture was found to degrade nearly 80% of tar balls at the end of 28 days. This observation was quite interesting in terms of the total loss of solvent extractables at the end of the experiment. Nevertheless much of the tar ball was found emulsified in the medium and stayed at the interphase while extracting the residual tar, possibly as a result of extracellular emulsifier production.

Amund and Akangbou (1993) studied hydrocarbon biodegradation using four different crude oils in estuarine microcosms. Natural populations of bacteria and fungi of the tropical ecosystem were able to degrade between 55-85% in 10 weeks. This is certainly a long period for degradation. It is noteworthy that in the present study, the serially enriched cultures were able to degrade about 60-80% of crude oils within 1 week. This is the period reported by Tagger *et al* (1983) for the autochthonous flora to respond in the event of a spill. Therefore, it may be suggested that a serially enriched mixed culture could be used as a starter seed inocula to overcome the initial delay in the growth of native microflora.

Some of the following justifications for the use of consortia could be drawn from the present work which are summarised as below:

1. Addition of single or pure cultures has, in the past, failed to elicit significant degradation in natural ecosystems. Kimura *et al* (1989) while using naturally enriched monospecific cultures of bacteria found that they were able to utilise barely 40% of the aliphatics of Class C fuel oil in 14 days and a maximum of 5.5% of the aromatic fraction. Though these organisms were isolated from an oil contaminated region they could not serve as candidates for oil clean up in the environment. *Moraxella* sp. and certain coryneforms isolated from the oil spill region were also not found to be suitable to utilise the aromatic compounds of petroleum hydrocarbons (Kimura *et al*, 1990).

2. As regards the use of added nutrients, Lee and Levy (1991) have observed that a semi-continuous addition of fertilisers in an oil contaminated sand beach

environment did not enhance oil degradation when the use of oil content was low (0.3% v/v), although at higher oil concentrations (3% v/v) degradation was rapid upon use of the fertilisers.It may be argued that application of chemical fertilisers at times is a successful mitigation measure. However, toxicity issues have also to be borne in mind (Lee, 1995). The components of fertilisers used during clean-up operations could be toxic (US congress, 1991; Hoff, 1993). Hence natural organic fertilisers like fish meal, fish bone meal etc. were tried to supplement the nutritional requirements. Under excessive application, it was learnt to suppress oil degradation rates and cause production of ammonia and depletion of oxygen (Lee *et al*, 1995). In addition, Swannel *et al* (1995) have found that application of organic nutrients was comparably less effective than inorganic nutrients for oil biodegradation. This could be perhaps of the absence of organic solubilisers like the phosphate solubilising microbial flora. In such cases, use of phosphate solubilising bacteria could be beneficial. One of the isolates in the present study, T17B could very well be used under such circumstances because it can solubilise both inorganic and organic phophates. Even the candidate yeast strains in the present study could solubilise inorganic phosphates. Thus these organisms have a versatility which could be made use of as bioadditives in suitable nutrient formulations.

3. When seeding the environment for bioremediative purposes, the size of the population is also important; likewise the potential of the autochthonous flora should be duly considered. Normally, there is a delay in the growth of oil degrading bacteria by nearly a week. Moreover, several workers (Mahadi and Watkinson, 1988; Amund

and Igiri, 1990) have also noticed that hydrocarbon utilising population in pristine ecosystem increased in two weeks but stayed constant, thus indicating localised thresholds of hydrocarbon degrading populations. Sometimes, as Tagger *et al* (1983) point out, the autochthonous population utilise certain fractions of crude oil, and soon after its depletion record a decrease in degradation rates. Therefore, there is a need for seeding with high populations of selected hydrocarbon degrading organisms which can be achieved by growing the candidate organisms in a consortium in the laboratory. Once seeded, the oil degrading population stays at 25% of the total heterotrophic population (Tagger *et al*, 1983) and thus it could aid in abating oil pollution biologically.

The experiments of the present study have shown that some of the candidate bacteria and yeasts qualify for use in bioremediation measures of oil spills in tropical regions. They could be tried *per se* or as formulations with fertilisers as the case may be.

6

Summary and Conclusions

Objectives

The following were the objectives of this study:

1. Isolation of bacteria and yeasts from varied marine environments with a potential for petroleum hydrocarbon degradation in addition to the ability to grow on nitrogen deficient medium and solubilise phosphates.

2. Biodegradation of crude oils, tar balls and other petroleum hydrocarbons by individual cultures.

3. Biosurfactant production of hydrocarbonoclastic organisms.

4. Occurence and involvement of extrachromosomal elements in oil degrading bacteria.

5. Taxonomic characterisation of active isolates.

6. Reconstitution of petroleum degrading strains having additional physiological attributes into consortia and evaluation of crude oil degradation in the laboratory and simulated field conditions.

Isolation and Screening

A total of 602 bacterial and 40 yeast cultures were isolated from 6 locations of the Indian coast and screened for phosphate solubilisation and growth in nitrogen deficient medium (only for bacteria). All the microbial cultures were isolated from water, sediment, or seaweed holdfast.

Cultures scoring positive for these abilities along with another 36 bacterial isolates from tar balls were assayed for hydrocarbon degrading potential using

Microbial Adhesion to Hydrocarbon (MATH) assay. Specialised habitats like tar balls yielded cultures which showed a significantly high cell hydrophobicity and/or surfactant indication.

Degradation studies using pure cultures

A total of 17 bacterial isolates and 3 yeasts showing hydrophobicity or biosurfactant producing properties or both were then used for light oil (Iranian crude) degradation studies.

Screening for degradation of a heavy oil (Bombay High crude) resulted in 10 active bacterial cultures and 3 yeast cultures.

The actively degrading microbial cultures were examined for the utilisation of the following petroleum mixtures:

i) highly weathered crude oil residues like tar balls

ii)diesel oil

iii) aliphatic and aromatic fractions of crude oils and

iv) solid polyaromatic hydrocarbons.

Analyses were mostly carried out by gas chromatography.

It was found that the degradation by bacteria was highest on Iranian light crude oil (89%) and lowest on tar balls (31%).

There were a few bacterial cultures which were able to degrade Bombay High crude oil considerably (71-73%) whereas yeasts were less competent in degradation.

Both bacteria and yeasts could degrade >90% of the aliphatic fractions. The pattern of degradation of aliphatic fractions of crude oil indicated the preferential removal of the low molecular aliphatic compounds (upto C18) by bacteria and high molecular aliphatics by yeasts (C19-C30). Bacteria could apparently use aromatic fraction to a greater extent (upto 66%) when compared to yeasts (41%).

The degradation of tar ball fractions remained low and there was no preference for either low or high aliphatic compounds of tar balls by both group of microorganisms. The degradation range of aromatic fraction of tar ball was narrow (28-33%) for bacteria while for yeasts it was wide (2-32%). Naphthalene was attacked upto 83 and 93% by bacteria and yeasts while anthracene degradation was lower (55 and 29% respectively).

Degradation of crude oils by bacteria and yeasts was observed to vary from one petroleum mixture to the other depending on the fractional composition of the oils.

Effect of some pertinent variables

Effects of 1) inoculum size, 2) incubation period, 3) substrate concentration and 4) temperature on the degradation of Bombay High crude oil by bacteria and yeasts were studied.

There was only a marginal increase in the oil utilisation by both bacteria and yeasts when the inoculum was increased from 10^8 cells/ml and 10^7 cells/ml to 10^{12} cells/ml and 10^{11} cells/ml respectively.

Bacteria could degrade a higher quantity of oil in a shorter period (3 days), but after 15 days, yeasts were found to degrade most of the crude oil.

Concentrations of oil upto 2.5% did not have any drastic bearing on the bacterial degradation, while for yeasts the optimal oil concentration was found to be lower (1% v/v) and an increase in concentration therafter resulted in a decrease in degradation.

All the cultures showed a higher degradation at 30°C than at 20°C. A yeast strain showed the highest degradation of 82% at 30°C but was notably the lowest at the lower temperature.

Biosurfactants

Biosurfactant production and activity of the emulsifier from a tar ball isolate (T17B) was studied and found to vary when grown on pure alkanes and crude oils. Emulsifier yield was similar with different substrates but emulsifying activity was high in crude oil.

Plasmids

All potential bacterial strains were checked for plasmids using alkali and boiling lysis. Out of 17 hydrocabonoclastic bacteria, 8 harboured plasmids. The molecular weights of these extrachromosomal DNA determined with reference to standard plasmid markers showed that they ranged from 2 to 80 kilobases.

Physiology, Biochemical grouping and Taxonomy

Doubling time of selected bacterial cultures were from 72 minutes to 120 minutes and for yeasts, it ranged between 60 minutes and 72 minutes. Distinct clusters comprising 3-4 isolates were observed when the biochemical characteristics and fatty acid profiles of the cultures were statistically analysed. Fatty acid profiles showed that bacteria belonged predominantly to *Pseudomonas* and *Bacillus* species and yeasts to *Candida* sp.

Mixed cultures and oil degradation

Six uncharacterised mixed cultures and four selectively reconstituted mixed cultures (or consortia) were examined for the degradation of Bombay High crude oil using selected bacteria and yeast cultures.

Mixed cultures from tar ball and mixed cultures capable of growth in nitrogen deficient medium (selection of cultures was at random) were found to degrade only upto 32%.

Tar ball associated bacterial mixed cultures could degrade upto 80% of tar balls in 28 days.

Degradation in laboratory microcosms showed that selecively mixed yeast cultures could degrade as high as 81% and a consortium of selectively mixed bacterial cultures could degrade about 30%. Continuous culture experiments with bacteria showed that mixed bacterial populations co-existed although degradation extent was reduced. However the culture T44 appeared to have acquired plasmids as a result of

mixing plasmid bearing and non-bearing cultures in a chemostat. A mixed consortium of bacteria and a yeast could degrade 70% of crude oil in laboratory batch cultures.

Conclusions

From the present study it could be concluded that the candidate marine microorganisms with physiologically favourable traits were efficient at degrading different groups of hydrocarbons in seawater and sediment. The bacteria and yeast cultures were found to co-exist and be amenable when reconstituted, in terms of their degradation potential. This qualifies the selected microorganisms to be effective seed organisms in oil spill bioremediation. This new concept of such a selection could pave the way in finding a viable and novel technology in oil bioremediation.

7

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Appendix

APPENDIX - 1

Media composition

A. Nutrient Agar (NAS) (Himedia, Bombay)

Peptone	5.0 g
Sodium chloride	5.0 g
Beef extract	1.5 g
Yeast extract	1.5 g
Agar	15.0 g
50% sea water	1000.0 ml
pH	7.4

B. Nitrogen-free medium (NFM) (Coty's medium, 1967)

Na_2HPO_4	300.0 mg
KH_2PO_4	200.0 mg
$MgSO_4$	100.0 mg
$FeSO_4.7H_2O$	trace
$Na_2MoO_4.2H_2O$	trace
NaCl	10.0 g
Mannitol	10.0 g
Distilled water	1000 ml
pH	7.5

C. Hydroxy apatite (HAM) (Ayyakkannu and Chandramohan, 1970)

Yeast extract	0.2 g
Ammonium sulphate	0.5 g
Magnesium sulphate	0.1 g
Potassium chloride	0.2 g
Dextrose	10.0 g
50% sea water	1000.0 ml
Calcium chloride 10%	60.0 ml
Pot. dihydogen phosphate 10%	40.0 ml
1N NaOH	45.0 ml
Agar	18.0 g
pH	7.6 to 8.0

$CaCl_2$, KH_2PO_4 and NaOH were sterilised separately, cooled and added.

D. Malt Extract (MEA) (Himedia, Bombay)

Malt extract	30.0 g
Mycological peptone	5.0 g
Agar	15.0 g

50% sea water.....1000.0 ml
pH.....7.0

E. Luria Bertani (LB) (Himedia, Bombay)

Bacto tryptone.....10.0 g
Bacto yeast extract..... 5.0 g
Sodium chloride.....10.0 g
Agar.....15.0 g
50% seawater.....1000 ml
pH.....7.5

F. Soyabean Casein Digest Agar (TSBA) (Himedia, Bombay)
(Tryptone Soya Agar)

Tryptone.....15.0 g
Soya peptone..... 5.0 g
Sodium chloride..... 5.0 g
Agar.....15.0 g
50% seawater.....1000.0 ml
pH..... .....7.3

G. Sabouraud Dextrose Agar (SDA) (Himedia, Bombay)

Mycological peptone.....10.0 g
Dextrose.....40.0 g
Agar.....15.0 g
50% seawater.....1000.0 ml
pH.....7.0

H. Artificial Sea Water (ASW)

NaCl.....23.4 g
KCl.....0.75 g
$MgSO_4.7H_2O$.....7.0 g
Distilled water.....1000.0 ml
pH .....adjusted to 7.3

It was sterilised, cooled and 1% v/v of N+P source was added;

Nitrogen and Phosphorus (N+P)

K_2HPO_4.....70.0 g
KH_2PO_4.....30.0 g
NH_4NO_3..... 100.0 g
Distilled water..... 1000.0 ml
pH.....7.3

It was either filter sterilised or autoclaved.

APPENDIX - 2

Reagents

A. Microbial Identification System

1. Saponification reagent

NaOH	15.0 g
Methanol (AR)	50.0 ml
Glass distilled water	50.0 ml

Water and methanol were added to sodium hydroxide pellets in a bottle and stirred until the pellets have dissolved.

2. Methylation reagent

6.0 N HCl	108.0 ml
Methanol (AR)	92.0 ml

The acid was added to methanol and stirred alongwith.

3. Extraction solvent reagent

Hexane (HPLC)	75.0 ml
Methyl Tertiary Butyl Ether (MTBE) (HPLC)	75.0 ml

MTBE was added to hexane and stirred.

4. Base Wash reagent

NaOH (AR)	3.6 g
Glass distilled water	300.0 ml

Water was added to NaOH pellets in the bottle and was stirred until the pellets dissolved.

100 ml of saturated NaCl was also prepared.

B. Plasmid Extraction Reagents

1. Solution I

50 mM glucose
25 mM Tris-Cl (pH 8)
10 mM EDTA

It was autoclaved for 15 minutes at 10 psi.

2. Solution II

0.2 N NaOH (freshly diluted from a 10 N flask).
1% of SDS

3. Solution III

5 M potassium acetate	60.0 ml
Glacial aceetic acid	11.5 ml
Distilled water	28.5 ml

C. Buffers

1. Tris-Boric acid-EDTA (TBE) buffer 5X

Tris base	54.0 g
Boric acid	27.5 g
0.5 M EDTA (pH 8)	20.0 ml

2. Tris-EDTA (TE) buffer

33 mM Tris (pH 8)	200 ml
1 mM EDTA (pH 8)	50 ml

3. Gel loading buffer (6X buffer)

0.25% Bromophenol blue (BPB)
0.25% Xylene-cyanol FF
40% Sucrose in distilled water

APPENDIX - 3

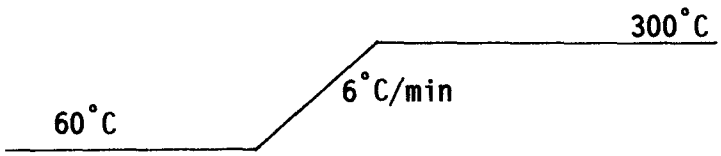
GAS CHROMATOGRAPH DETAILS

	Capillary column	Packed column
Column:	BP1 (5% dimethyl siloxane) SGE (Australia)	5% SE 30 Chemito (India)
Length:	25 m	8 ft (SS)
Internal dia.:	0.32 mm	1/8 in
Carrier gas:	Hydrogen	Nitrogen
Inlet pressure:	6 psi	46 psi
Sample size:	0.4 μl, split 60:1	1 μl
Sensitivity:	8x1	16x10
Detector:	FID	FID

Operating temperatures

Injector ..... 300°C
Detector ..... 310°C

Oven temperature programme



Degradation quantification

$$\text{Degradation (\%)} = \frac{(C - E)}{C} \times 100$$

C - total peak area of residual oil in control flask
E - total peak area of residual oil in experimental flask