STUDIES ON THE BIODEGRADATION OF THE HETEROCYCLIC COMPONENT OF CRUDE OIL IN THE MARINE ENVIRONMENT.

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in

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by

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Certified that the student has incorporated all Corrections suggested by the referees.

Sangodkar 22/12/99

Sangodkar

CERTIFICATE

This is to certify that the thesis entitled "**Studies on the Biodegradation of the Heterocyclic Component of Crude Oil in the Marine Environment**" submitted by Ms. Joanita Coelho for the award of the degree of Doctor of Philosophy in Marine Biotechnology is based on the results of investigations carried out by the candidate under my supervision. The thesis or any part thereof has not been previously submitted for any other degree or diploma of any University or Institute. The material obtained from other sources has been duly acknowledged in the thesis.

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STATEMENT

I hereby state that this thesis for the Ph.D. degree on "**Studies on the Biodegradation of the Heterocyclic Component of Crude Oil in the Marine Environment** " is my original contribution and that the thesis and any part thereof has not been previously submitted for the award of any degree/diploma of any University or Institute. To the best of my knowledge, the present study is the first comprehensive study of its kind from this area. The literature pertaining to the problem investigated has been duly cited. Facilities availed from other sources are duly acknowledged.

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ABBREVIATIONS

NHA	nitrogen-heterocyclic aromatics
PANH	polyaromatic nitrogen heterocycles
NPAC	nitrogen polycyclic aromatic compound
PAH	polyaromatic hydrocarbon(s)
NSO	nitrogen-sulphur-oxygen
ASW	artificial seawater
TLC	thin layer chromatography
HQ	hydroxyquinoline
UV	ultra-violet
GC	gas chromatography
IR	infra-red
INT	2-(4-iodophenyl) –3-(4-nitrophenyl)-5 phenyl – 2H – tetrazolium chloride
PMS	phenazine methosulphate
NTG	N-methyl,N'-nitro, N-nitrosoguanidine
LTS	long termed starved (cells)
STS	short termed starved (cells)
BHCO	Bombay High crude oil
ε	absorption (extinction coefficient)
V_{max}	maximum velocity
λ_{max}	wavelength of maximum light absorbance
q	specific quinoline conversion rate
ppt	parts per thousand
ppm	parts per million
rpm	revolutions per minute
g	gram(s)
mg	milligram(s)
m	metre(s)
nm	nanometer
nmoles	nanomoles
A	absorbance
h	hour(s)
min	minute(s)
ml	millilitre(s)
l	litre(s)
M	molar
mM	millimolar
µM	micromolar
w/v	weight by volume
v/v	volume by volume
°C	degree Centigrade

CONTENTS

CHAPTER I

INTRODUCTION AND REVIEW OF LITERATURE

Waste is the natural by-product of man. For centuries, the sheer bounty of the environment allowed for an 'out of sight, out of mind' disposal policy. But the age of excess has passed and natural resources once abundant and forgiving, are now threatened. Until the advent of the chemical processing industry, man produced waste in manageable quantities. The post – world war II growth of the chemical industry has produced millions of tons of hazardous wastes. Large quantities of synthetic materials including dielectric fluids, flame retardants, refrigerants, lubricants, pesticides and petroleum products have been produced and they, their residues and off grade mixtures have entered the ecosystem.

Hazardous waste disposal today is a worldwide environmental problem. Many methods have been tried in the effort to treat and dispose of chemical wastes and their by-products to render them harmless to man and the environment. No single technology has proven to be economically and technically satisfactory.

Technologies for cleaning up hazardous wastes are often expensive, inappropriate for the site, or ineffective in handling complex mixtures of pollutants. Biotechnology has emerged as one of the most promising technologies to solve this enormous problem. Biological treatment systems use microorganisms such as bacteria or fungi, to transform harmful chemicals into less toxic or non toxic compounds. Biotransformation is an attractive option because it depends on natural processes, and process residues such

as carbon dioxide and water, can be cycled within the biosphere. In many cases, these technologies are also less expensive and less disruptive than options commonly used to remediate hazardous wastes, such as excavation and incineration. Bioremediation also holds a clear advantage over many technologies relying on physical or chemical processes because it involves the destruction of contaminants, not merely transference among media.

Nature itself has been providing the means of biological treatment due to the existence and evolution of organisms which can constantly degrade and mineralize all organic compounds in the environment. Alexander (1965) termed this phenomenon as the Principle of Microbial Infallibility. Gale (1952) supported this principle suggesting that organisms should exist which, under suitable conditions, oxidize any substance which is theoretically capable of being oxidized. It is true that microorganisms have evolved catalytic systems (enzymes) that degrade biogenic compounds or naturally occuring compounds that have been present in the biosphere for millions of years. Enzymes for catabolic pathways may have evolved by combining several gene clusters in the form of modules to which other peripheral genes may be added by processes like gene transfer, mutational drift and genetic recombination and transportation (Meer *et al.*, 1992).

To the microorganisms, pollutants can serve as energy sources that they can break down to obtain energy to live and reproduce. These organisms have a wide range of abilities to metabolize different chemicals;

scientists can tailor the technology to the pollutants at specific sites, by using an organism in the treatment system that breaks down a particular pollutant. Where possible, technologies are developed to utilize native microorganisms that have been demonstrated to metabolize the pollutants on the site. In these cases, the number and/or the rate of degradative activity of the microorganisms - and thus the speed at which a pollutant is broken down – may be increased by adding nutrients or other amendments to the site. In other cases, organisms known to metabolize the pollutants can be introduced and supplemented if necessary to accelerate biodegradation.

Biological treatment technology development is therefore based on an understanding of three principles:

- microbial physiology (biochemistry) – the metabolic processes leading to the detoxification of hazardous compounds and the genetics controlling these functions.
- microbial ecology – an appreciation of the structure and function of natural microbial communities and
- engineering – the capability to implement 'desired' microbial metabolic processes.

The potential of such biological treatments has only just been tapped, and more research is needed in various areas such as development of new biosystems for treatment of environmental pollutants. These would include naturally selected microorganisms, consortia, bioproducts and genetically

engineered microorganisms. It would also become imperative to evaluate and optimize the factors necessary for applying biological agents to detoxify or destroy pollutants and to determine the environmental fate and effects of, as well as the risks involved in the use of such agents.

Marine pollution and petroleum

The global pollution zone of the marine environment may be characterised as follows: highest pollution levels in the euphotic layer, high pollution levels in the neritic zone of inland seas, latitudinal distribution of toxic pollutants, a mosaic distribution pattern of toxicant concentrations in water, localisation of toxicants in the hyponekton and benthos biotope and a coincidence of the maximum pollution zones with zones of high biomass and productivity (Trivedi and Raj, 1992). Petroleum is one of the major pollutants associated with the marine environment besides other toxic organics such as herbicides, pesticides, radioactive wastes, toxic inorganics and solid wastes (Trivedi and Raj, 1992).

It is estimated that the annual global input of petroleum is between 1.7 and 8.8 million metric tons, the majority of which is derived from anthropogenic sources (National Academy of Sciences, 1985). Tanker accidents are major causes of oil pollution of marine environments on a large scale. Oceans and estuaries have generally been the sites of the largest and most catastrophic oil spills. The spill of more than 200,000 barrels of crude oil from the oil tanker Exxon Valdez in Prince William Sound, Alaska (Hagar,

1989), as well as smaller spills in Texas, Rhode Island and the Delaware Bay (Anonymous, 1989), has refocussed attention on the problem of hydrocarbon contamination in the environment. Other sources of petroleum contaminants include effluents from refineries, tank washings and operational discharge, and leakage through pipelines and tankers. The polluting oil that is released into the environment is a conserved, long lived contaminant of food webs and has long term dibilitating effects on organisms. The low molecular weight aromatics are responsible for the immediate toxic effects. However, the condensed aromatics tend to persist longer (Colwell *et al.*, 1978). There was a marked persistence of oil two years after the oil spill of the tanker VLCC Metula in the straits of Magellan (Colwell *et al.*, 1978).

Fate of oil in the marine environment

A contaminating crude oil is subject to many physical, chemical and biological processes which determine its fate in the marine environment. The typical fate of a contaminating oil is shown in Figure 1.1. Oil spilled in water tends to spread and form a slick (Berridge *et al.*, 1968). As a result of wind and wave action, oil in water or water in oil ("mousse") emulsions may form (Cooney, 1984). Dispersion of hydrocarbons in the water column in the form of oil in water emulsions increases the surface area of the oil and thus its availability for microbial attack. The most important physical process affecting oil is evaporation. Most of the lighter fractions of the oil volatilize off. As much of 50% of oil can be lost by evaporation (Cooney, 1984). The sunlight catlyzes oxidation of the hydrocarbons to polar compounds like ketones and

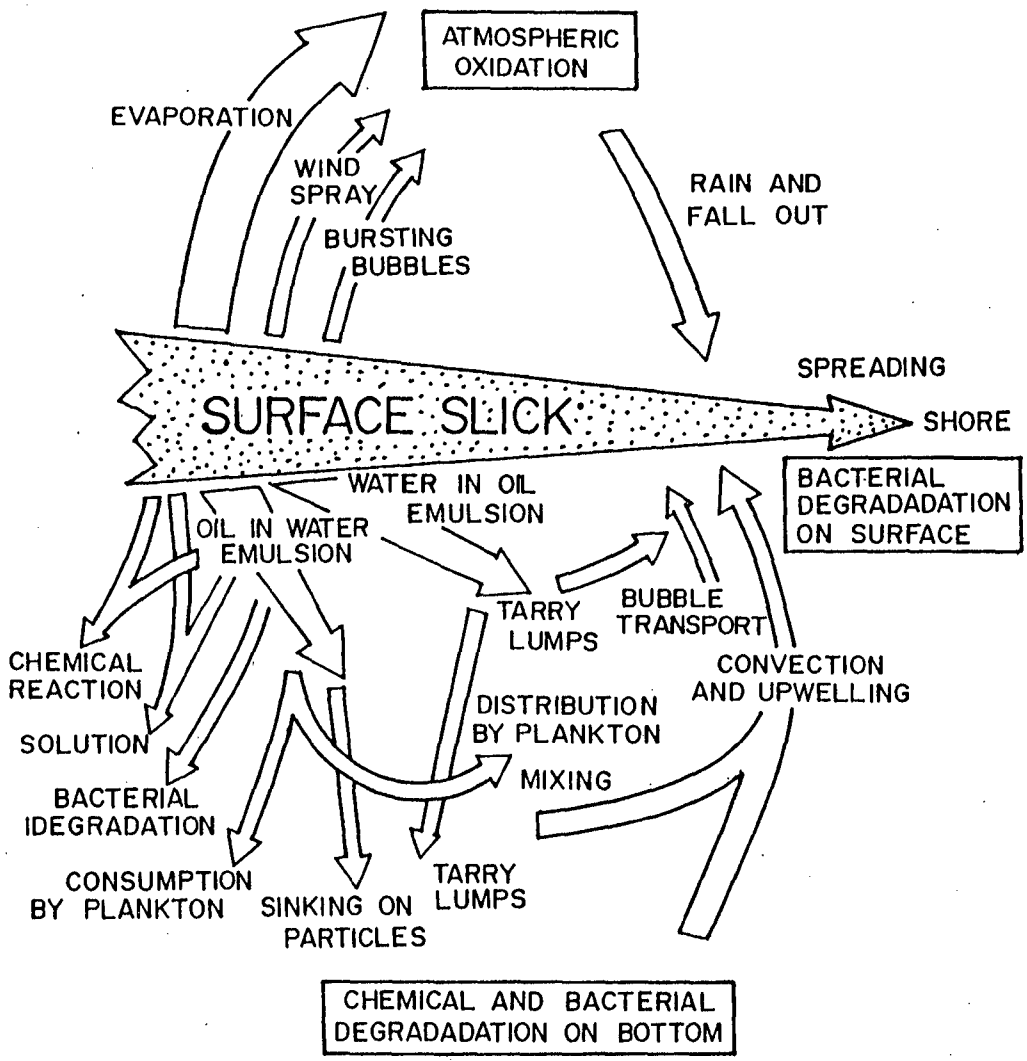


Fig. 1.1 : Fate of polluting oil in the marine environment (Carlberg, 1980).

esters. Other photocatalytic reactions result in polymerization leading to recalcitrance.

The processes of solubilization, volatilization, photochemical changes, emulsification and microbial attack are collectively called weathering of oil. As weathering proceeds, the hydrocarbons that are most readily metabolized are removed and the oil becomes more resistant to microbial attack (Cooney, 1984). After extensive weathering, petroleum hydrocarbons often occur in the environment as "tarballs". These are formed as tank washings dumped into cold water, but they also can be formed from slicks by a combination of wave action, weathering and degradation. Hydrocarbons in tar are quite resistant to microbial degradation and many have chemical structures which are not readily attacked by microbial enzymes (Atlas, 1981). They restrict access by microorganisms because of their limited surface area (Colwell *et al.*, 1978).

Composition of crude petroleum

Petroleum is an extremely complex mixture of tens of thousands of compounds that differ markedly in volatility, solubility and susceptibility to biodegradation. The petroleum mixture can be fractionated by silica gel chromatography into a saturate or aliphatic fraction, an aromatic fraction and an asphaltic or polar fraction (Brown *et al.*, 1969). The most common aliphatic components are the n-alkanes and in a crude oil, chain lengths of 5 to more than 35 carbon atoms may be found. Alkenes are relatively rare in crudes,

but may be present in significant quantities in some petroleum products. Branched alkanes are common and the isoprenoid derivatives pristane and phytane are commonly employed as aliphatic 'marker' compounds during crude oil analysis. Cyclic aliphatics are common. A wide range of aromatics are biologically available in petroleum and compounds containing both aromatic and cycloaliphatic rings are also present. The asphaltic fraction may be divided further to separate out a complex fraction which includes abundant compounds containing nitrogen, sulfur and oxygen and termed as NSO fraction (Westlake *et al.*, 1974). This fraction is also termed resins (Colwell and Walker, 1977) and the important components are reported to be typical heterocyclic compounds such as pyridines, quinolines, carbazoles, sulfoxides and amides. Representative compounds present in crude oil are illustrated in Figure 1.2. In addition to the petroleum hydrocarbons there are a number of heavy metals present in crude oils that have profound biological impact if they accumulate to high concentrations. These include Pb, Cu, Hg, Cd, Vn, Ni and Fe.

Hydrocarbon degrading microorganisms

A hydrocarbon utilizing microorganism was reported first in 1895 (Miyoshi, 1895). Sohngen (1906) described the use of crude oil, gasoline and kerosene by microorganisms. We know now that over 70 microbial genera contain organisms which can degrade one or more components of crude oil (Zobell, 1973; Berner *et al.*, 1975). Microorganisms capable of degrading hydrocarbon components of spilled crude or refined petroleum are present in

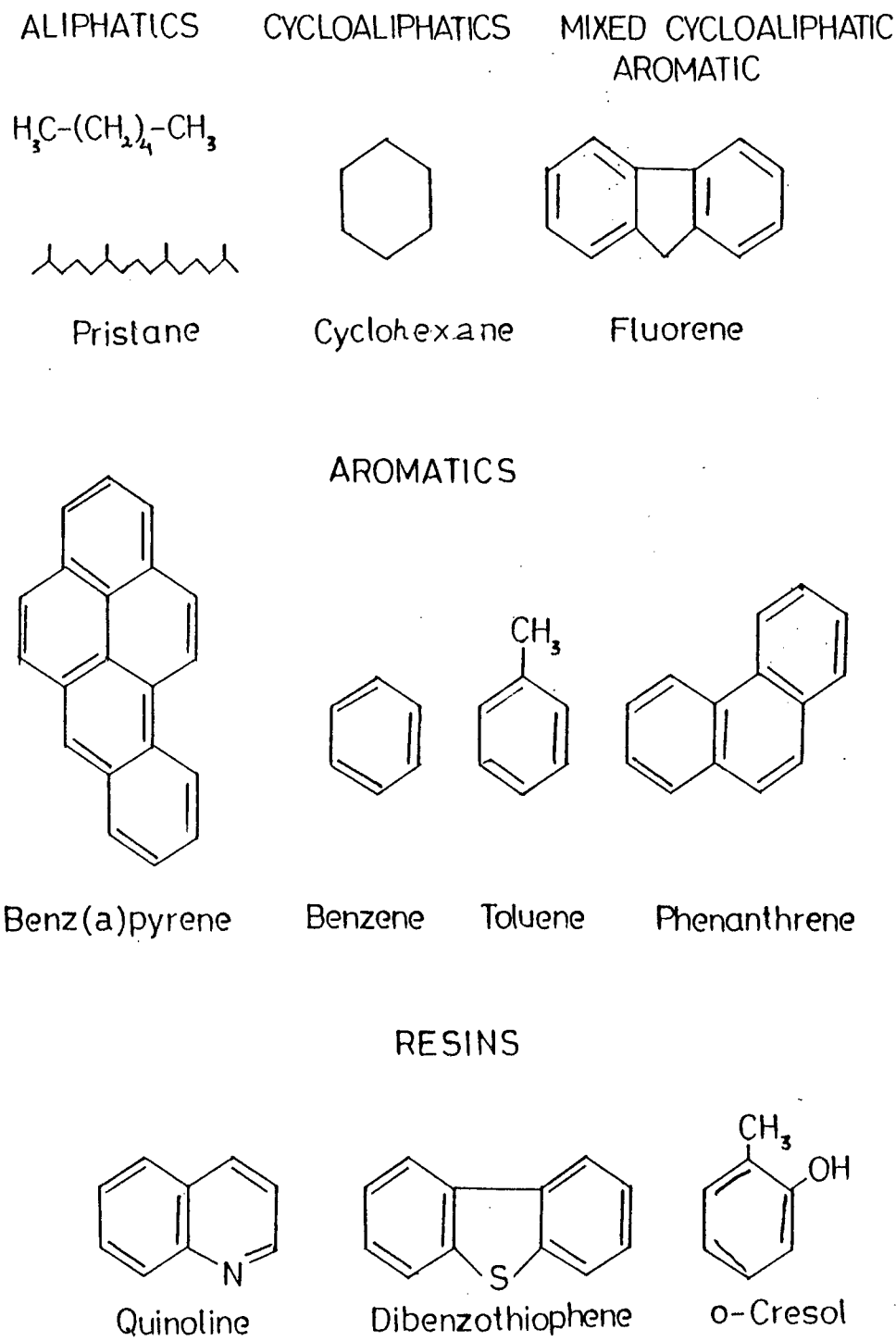


Fig. 1.2 : Representative compounds in crude oil.

the normal flora of fresh and marine waters. In waters which have not been polluted by hydrocarbons, hydrocarbon using bacteria make up 1% or less of the bacterial population whereas in most chronically polluted systems they make up 10% or more of the population as reviewed by Vestal *et al.*, 1984. This is because petroleum entering a body of water has two major effects on the indigenous microorganisms. It is toxic to some microorganisms while other microorganisms, including those which can use component(s) of the petroleum as a source of carbon and energy multiply (Atlas *et al.*, 1978). The result is a community of microorganisms with fewer species of non-hydrocarbon degraders and an increased number of hydrocarbon degrading organisms. This phenomenon, which results from increases in the hydrocarbon-oxidizing potential of the community, is known as adaptation (Spain *et al.*, 1980). The three inter related mechanisms by which adaptation can occur are,

(i) Induction and/or derepression of certain enzymes

(ii) Genetic changes which result in new metabolic capabilities, and

(iii) Selective enrichment of organisms able to transform the compound or compounds of interest (Spain et al, 1980; Spain and Van Veld, 1983).

Adaptation of microbial communities to hydrocarbons, i.e. increases in rates of transformation of hydrocarbons associated with oil contaminated environments has been reported in several studies. Kator and Herwig (1977) found that within few days after spillage of South Louisiana crude oil in a coastal estuary in Virginia, levels of petroleum-degrading bacteria rose by

several orders of magnitude. In experimental field studies in the Arctic, large increases in hydrocarbon utilizing microorganisms were found in marine ecosystems; concentrations of hydrocarbon utilizing microorganisms have been found to rise rapidly and dramatically in response to acute inputs of petroleum hydrocarbons (Atlas, 1981).

In marine environments contaminated with hydrocarbons, there is an increase in the proportion of bacterial populations with plasmids containing genes for hydrocarbon utilization. Plasmid DNA may play a particularly important role in genetic adaptation in that it represents a highly mobile form of DNA which can be transferred via conjugation or transformation and can impart novel phenotype, including hydrocarbon-oxidizing ability, to recipient organisms. Exposure of natural microbial populations to oil or other hydrocarbons may impose a selective advantage to strains possessing plasmids encoding enzymes for hydrocarbon catabolism, resulting in an overall increase in the plasmid frequency in the community (Leahy and Colwell, 1990).

The most important (based on frequency of isolation) genera of hydrocarbon utilizers in aquatic environments were *Pseudomonas, Achromobacter, Arthrobacter, Micrococcus, Nocardia, Vibrio, Acinetobacter, Brevibacterium, Corynebacterium, Flavobacterium, Candida, Rhodotorula* and *Sporobolomyces* (Bartha and Atlas, 1976). Bacteria and yeasts appear to be the prevalent hydrocarbon degraders in aquatic ecosystems.

A large number of *Pseudomonas* sps. have been isolated which are capable of utilizing petroleum hydrocarbons. The genetics and enzymology of hydrocarbon degradation by *Pseudomonas* sp. has been extensively studied (Chakrabarty, 1972; Chakrabarty *et al.*, 1973; Dunn and Gunsalus, 1973; Friello *et al.*, 1976). The genetic information for hydrocarbon degradation in these organisms generally has been found to occur on plasmids. *Pseudomonas* sps. have been used for genetic engineering and the first successful test case in the U.S. to determine whether genetically engineered microorganisms can be patented involved a hydrocarbon-utilizing *Pseudomonas* which was 'created' by Chakrabarty (Diamond V. Chakrabarty, 1980).

Features of typical marine bacteria

Most marine bacteria can utilize nutrients present in minute concentrations. This ability is the prerequisite for their growth in sea water, which throughout is very poor in nutrients.

Adaptation to the very small concentrations of nutrients in sea water might cause the marked pleomorphism of many marine bacteria in culture. Pure cultures, frequently show diverse cell shapes. Organisms which normally are rod shaped may occur as cocci, vibrios, filaments and spirals.

A great number of marine bacteria are capable of a wide variety of metabolic reactions and consequently can utilize a wide variety of nutrients (Rheinheimer, 1976).

Macleod (1965) believes that, although the ability to live in the sea is the only characteristic which clearly distinguishes them from other bacteria, this one characteristic is nevertheless sufficient to delimit them because under natural conditions – for example as a river enters the sea – adaptation of the freshwater bacteria which have been carried along does not occur. Macleod (1965, 1968) assumes that, on the other hand only a few mutational steps are needed to change a marine form into one which will survive in a non marine environment. Both marine and terrestrial may have developed from original marine ancestors.

Susceptibilities of different petroleum compounds to microbial attack

In an ideal situation, microorganisms would convert the hydrocarbons in a spill to carbon dioxide i.e. they would "mineralize" the hydrocarbons, or they would convert the hydrocarbons to non-toxic water soluble products. These and other biological activities which result in chemical transformations of hydrocarbons in a spill constitute biodegradation of the oil.

Since crude oils from different fields and from parts of the same field differ in their proportion of various classes of compounds it is not surprising that biodegradability varies from oil to oil (Atlas, 1975; Gilbert and Higgins,

1978). Atlas (1981), Cerniglia (1984) and Cooney (1984) reviewed the degradation of individual hydrocarbons in petroleum. Biodegradation rates have shown to be highest for the saturates, followed by the light aromatics, with high molecular weight aromatic and polar compounds exhibiting extremely low rates of degradation.

In the aliphatic fraction, n-alkanes are the most readily degraded. Short chain alkanes may evaporate rapidly. n-Alkanes as long as 44 carbons can be metabolized by microorganisms (Haines and Alexander, 1974). Branching makes an alkane more resistant to biodegradation (Schaeffer *et al.*, 1979). Similarly, the methyl-branched compounds such as pristane and pyltane though degraded by a number of organisms (Pirnik, 1977), they tend to be more persistent than other saturates in crude oils (Jobson *et al.*, 1974, Westlake *et al.*, 1974). Cycloalkanes are more refractory, some condensed cycloalkanes persist for very long periods after a spill (Atlas, 1981).

The biodegradation of n-alkanes has been reviewed by Atlas (1981) and Harayama *et al.* (1996) and normally proceeds by a monoterminal attack; usually a primary alcohol is formed followed by an aldehyde and a monocarboxylic acid. The enzymes carrying out this sequence of reactions are alkane monooxygenase, alkanol dehydrogenase, aldehyde dehydrogenase respectively. Further degradation of the carboxylic acid proceeds by *beta* oxidation with the subsequent formation of 2-carbon-unit shorter fatty acid and acetyl coenzyme A, with eventual liberation of CO_2.

Highly branched isoprenoid alkanes such as pristane, have been found to undergo *omega* oxidation, with formation of dicarboxylic acids as the major degradative pathway. Methyl branching at the *beta* position blocks *beta* oxidation, requiring an additional strategy such as *alpha* oxidation, *omega* oxidation, or *beta* alkyl group removal. The degradation of several unsubstituted cycloalkanes, including condensed cycloalkanes occurs via co-oxidation with the formation of a ketone or alcohol (Beam and Perry, 1974; Perry, 1979). Once oxygenated, degradation can proceed with ring cleavage. Degradation of substituted cycloalkanes appears to occur more readily than the degradation of the unsubstituted forms, particularly if there is an n-alkane substituent of adequate chain length (Perry, 1979). In such cases, microbial attack normally occurs first on the substituted portion, leading to an intermediate product of cyclohexane carboxylic acid or related compound.

Light aromatic hydrocarbons are subject to evaporation and to microbial degradation in a dissolved state. However, polycyclic aromatic hydrocarbons are relatively resistant to microbial attack. Walker and Colwell (1976) observed that monoaromatics were first attacked followed by di- and tri- aromatics. Substitutions on the rings can interfere with metabolism (Gibson, 1976; Cripps and Watkinson, 1978; Hopper, 1978). Herbes and Schwall (1978) found that polyaromatic hydrocarbons (PAH) have very high turnover times starting with 7.1h for naphthalene to more than 30,000 hours for benzo(a)pyrene.

The metabolism of aromatic hydrocarbons by living organisms is depicted in Figure 1.3 and has been reviewed by Atlas (1995). The aromatic hydrocarbon rings generally are hydroxylated to form diols; the rings are then cleaved with the formation of catechols which are subsequently degraded to intermediates of the tricarboxylic acid cycle. Interestingly, fungi and bacteria form intermediates with differing stereochemistries. Fungi, like mammalian enzyme systems, form *trans*-diols, whereas bacteria almost always form *cis*-diols. Many *trans*-diols are potent carcinogens whereas *cis*-diols are not biologically active. Since bacteria are the dominant hydrocarbon degraders in the marine environment, the biodegradation of aromatic hydrocarbons results in detoxification and does not produce potential carcinogens. Catechol that is produced is an intermediate in metabolism of aromatic hydrocarbons can be oxidised via the *ortho* pathway and *meta* pathway. The *meta* pathway results in the formation of 2-hydroxymuconic semialdehyde which is a yellow compound The complete biodegradation (mineralization) of hydrocarbons produces the non toxic end products CO_2 and H_2O as well as cell biomass (largely protein) which can be safely assimilated into the food web.

The metabolic pathways for the degradation of asphaltic components of petroleum are probably least well understood. These are complex structures which are difficult to analyze with current chemical methodology. No uniform pathway, comparable to the pathways established for aliphatic and aromatic hydrocarbons, has yet emerged for the asphaltic petroleum components.

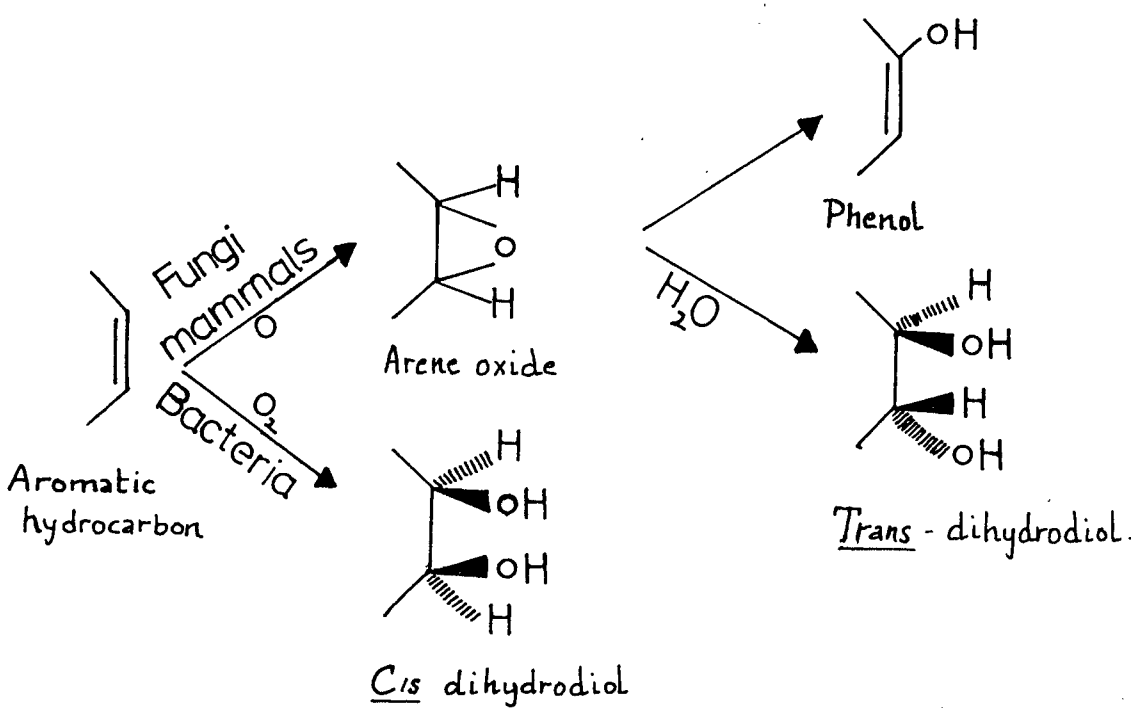


Fig 1.3 : Initial reactions used by mammals, fungi and bacteria to oxidize aromatic hydrocarbons (Cerniglia *et al.*, 1978).

It has been hypothesized that during the biodegradation of petroleum hydrocarbons, microorganisms are producing (synthesizing) hydrocarbons of different molecular weights or chemical structures. Walker and Colwell (1976) found that a wax was produced during microbial degradation of the oil. The possible production of such high molecular weight alkanes during petroleum biodegradation has also been reported by Pritchard *et al.*,(1976) and Seesman *et al.* (1976). Jobson *et al.* (1972) reported an increase in the polar nitrogen – sulfur - oxygen fraction during oil biodegradation in soil.

Mixed Culture Systems

Microorganisms in natural environments exist, with very few exceptions, as complex, multispecies communities. Laboratory experiments with pure cultures and single substrates have obvious advantages in terms of elucidation of genetic and biochemical bases of biodegradation. Yet these studies provide no information about the way in which microorganisms may interact under environmental conditions. In nature, organic compounds are not present singly but as components of a mixture of compounds. Degradation of these compounds is brought about by the concerted action of many microbes which form a community. No single species in this mixed culture contains the genetic and therefore enzymatic complement of the mixed culture. This means that any given compound can be sequentially mineralized or can be co-metabolized. Consequently microbial communities can use as sole sources of carbon and energy, compounds that cannot be degraded by any single organism alone (Grady, 1985).

Co-oxidation and sparing

Compounds which otherwise would not be degraded can be enzymatically attacked within the petroleum mixture due to the abilities of the individual microorganisms to grow on other hydrocarbons within the oil (Horvath, 1972). A petroleum hydrocarbon mixture, with its multitude of potential primary substrates provides an excellent chemical environment in which co-oxidation can occur. Co-oxidation was hypothesized to be responsible for the degradation of a number of compounds in oil (Jamison *et al.*, 1976; Horowitz and Atlas, 1977). Assesing the role of co-oxidation in natural environment is difficult since multiple microbial populations are present. In the above mentioned mixed population studies, synergism could be an alternative hypothesis to explain the observed results.

Another process which needs to be considered in the metabolism of petroleum hydrocarbons is 'sparing'. A diauxic phenomenon has been reported for the degradation of pristane in which pristane was not degraded in the presence of hexadecane (Pirnik *et al.*, 1974). Similar sparing effects undoubtedly occur for other hydrocarbons. Such diauxic phenomena do not alter the metabolic pathways of degradation, but rather determine whether the enzymes necessary for metabolic attack of a particular hydrocarbon are produced or active. These sparing effects have a marked influence on the persistence of particular hydrocarbons within a petroleum mixture and thus on the evolution of the weathered petroleum hydrocarbon mixture.

Nitrogen containing fraction of crude oil

During the last decade the skein of closely related processes involved with the interaction of oil with microorganisms in the sea has been unraveled to some extent. Compared with the wealth of information available on the biodegradation of aliphatic and aromatic compounds in oil, there is very little information on the ability of microorganisms to degrade the nitrogen compounds in oil.

Heterocyclic compounds have been estimated to represent more than two-thirds of organic compounds on earth (Kuhn and Suflita, 1989). Polynuclear azaheterocyclic compounds (azaarenes) are nitrogen containing analogs of polycyclic aromatic hydrocarbons (PAHs). The nitrogen atom in the ring system causes these compounds to be slightly polar and considerably more water soluble than related PAHs (Ondrus and Steinheimer, 1990). These classes of N-heterocyclic aromatic compounds (NHAs) along with many others are formed as combustion products in the generation of synthetic fuels from fossil fuels (Santodonato and Howard, 1981). NHAs also termed PANH (polycyclic aromatic nitrogen compounds) are ubiquitous environmental contaminants typically released in association with coal and oil energy conversion processes, aluminium smelting, asphalt production, wood treatment processes, automobile exhaust and hydrocarbon spills (Warshawsky, 1992). They are of particular concern because many are more biologically active than their homocyclic analogs (Warshawsky, 1992).

Petroleum contains between 0.1 to 2% nitrogen (Speight, 1980). Figure 1.4 lists some of the nitrogen compounds present in petroleum. The three major groups of nitrogen compounds in petroleum fractions boiling below 538°C (Snyder and Saunders, 1979) are,

- pyridine derivatives (including quinolines),
- indole derivatives (including pyrroles and carbazoles), and
- aromatic amides.

Although nitrogen containing compounds can be found throughout the boiling range, there is a tendency for such components to be found in the higher boiling fractions and residues. The presence of nitrogen compounds in oils can lead to the poisoning of catalysts used in the cracking of oil for commercial uses (Speight, 1980). As the availability of high quality, low boiling crude oils decreases, the trend is to the increased use of oils containing more nitrogen. An analysis of the basic N-compounds present in a virgin oil indicates that the mixture of bases is quite complex though the heterocyclic system is predominantly a pyridine (Jewell, 1980). 2-hydroxy quinolines have been identified in a California crude oil and constitutes 0.05% weight of the crude oil (Copelin, 1964). De Leon *et al.* (1982) have compared fresh unweathered oil from the Ixtoc-1 blowout with heavily weathered oil from that source. Qualitatively the nitrogen heterocycle profiles were almost identical, and they concluded that weathering does not adversely affect the distribution of higher molecular weight azaarenes such as C_3- and C_4-phenanthridines – benzoquinolines.

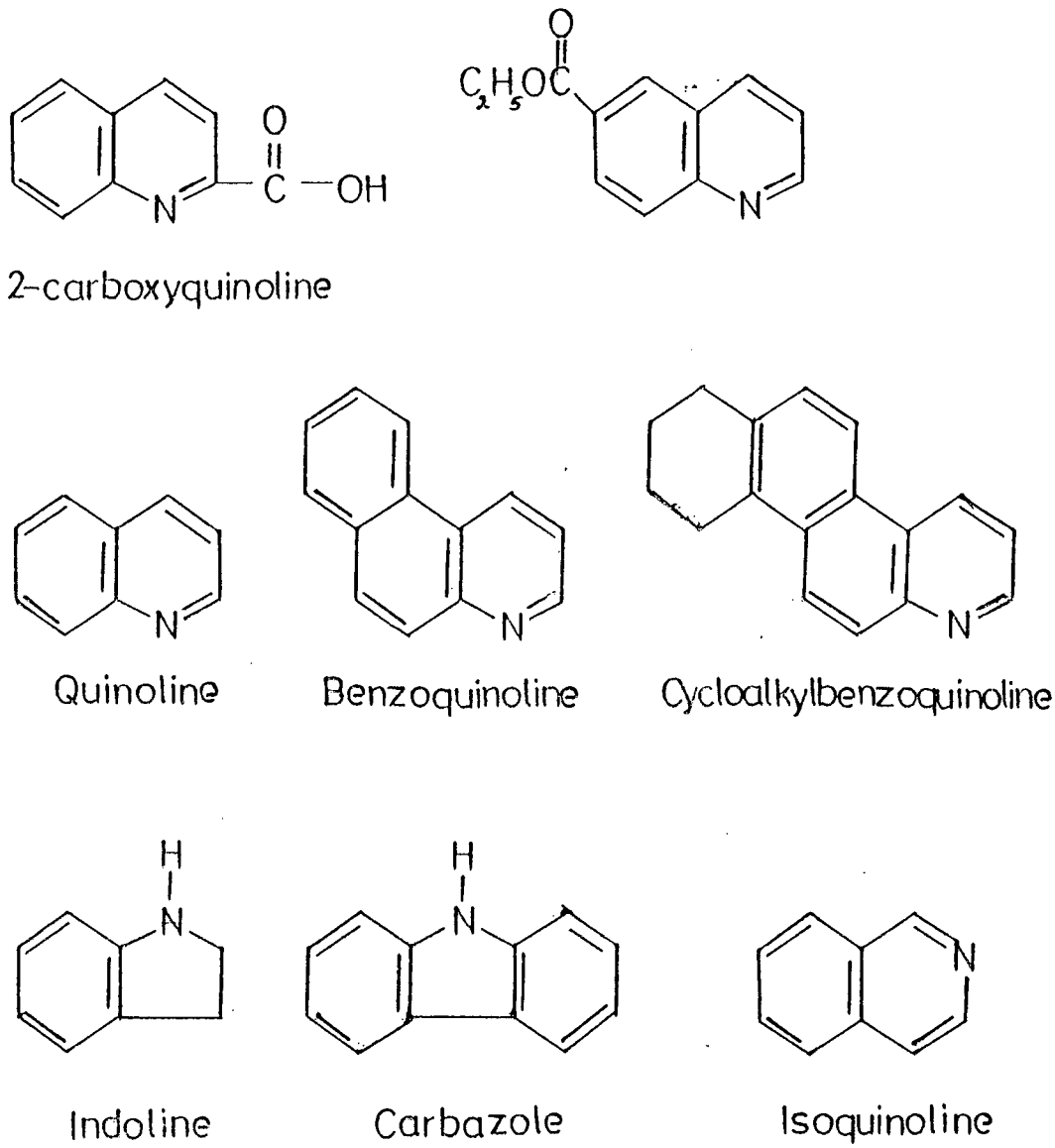


Fig 1.4 : Nitrogen compounds in crude oil.

Quinolines

Quinoline, Isoquinoline and Benzoquinoline co-exist in many types of polycyclic aromatics in coal tars and crude petroleum (Chou and Bohonos, 1978). Quinoline may enter the marine environment through spills of both natural and synthetic crudes (Ford *et al.*, 1981; Mckay *et al.*, 1976) and through industrial discharges since it is used to make dyes, medicinal drugs, catalysts, ion-exchange polymers, herbicides, insecticides, corrosion inhibitors, antioxidants and metallurgical chemicals; as a preservative for anatomical specimens, and as a solvent for resins and terpenes.

The environmental persistence of PAHs and NHAs is apparent from their detection in soils (Dupont *et al.*, 1990), groundwater (Ondrus and Steinheimer, 1990; Pereira *et al*, 1987), sediments (Barrick *et al.*, 1984) and tissues of fish living in polluted water (Vassilaros *et al.*, 1982). PANH have also been found in marine sediments (Furlong and Carpenter, 1982). Many components of the NSO fraction affect the biodegradation of other aromatic compounds (Dyreborg *et al.*, 1996a, 1996b). The aquatic toxicity of alkyl quinolines has also been reported using the Microtox assay with luminescent bacteria (Birkholz *et al.*, 1990).

Quinoline and all of its monomethyl isomers were found to be mutagens in the Ames Salmonella assay (Dong *et al.*, 1978). Quinoline, 4-methyl quinoline and 8-methyl quinoline have also been shown to initiate tumors in SENCAR mice (LaVoie *et al.*, 1984). Studies with the embryo larval

stages of *Salmo gairdneri* (rainbow trout) and large mouth bass (*Micropterus salmoides*) gave LC_{50} values of 11.0 mg/l and 7.5 mg/l respectively using quinoline (Black *et al.*, 1983). The LC_{50} of dimethyl quinoline to rainbow trout was found to be 6.2 mg/l, 7.6 mg/l and 2.6 mg/l for 2,6-dimethyl quinoline, 6,7-dimethyl quinoline and 6,8-dimethyl quinoline respectively (Birkholz *et al.*, 1990). Studies by LaVoie *et al* (1987) have shown that quinoline has greater carcinogenic potential – indicated by the development of hepatic tumors – than benzoquinolines in new born mice. The LD_{50} (oral – rat) is 331 mg/kg and LD_{50} (oral – rabbit) 5.40 mg/kg.

Quinoline is on the Hazardous substances list of many regulatory agencies worldwide. It has been designated 'hazardous' by US Clean Water Act (Omenn, 1988). The office of Environmental Health Hazard Assessment (OEHHA) of the California –Environment Protection Agency added quinoline to the list of chemicals known to the state to cause cancer, for purposes of the Safe Drinking Water and Toxic Enforcement Act of 1986 (Proposition 65). It is on the U.S. special Health Hazard Substance List (Aquire Database, 1988). Quinoline is fairly soluble in water (1g/l) and has a half life of 20-200 days in water; about 88.7% of quinoline will eventually end up in water; about 9.7% will end up in air and the rest will be divided about equally between terrestrial soils and aquatic sediments. The concentration of quinoline found in fish tissues is expected to be somewhat higher than the average concentration of quinoline in the water from which the fish was taken.

Microbial transformation of quinolines

The microbial metabolism of quinoline has been reviewed by Kaiser *et al.* (1996). Quinoline can be transformed by microorganisms under both aerobic and anaerobic conditions (Aislabie *et al.*, 1990; Brockman *et al.*, 1989; Grant and AL-Najjar, 1976; Schwarz *et al.*, 1988). Although many bacteria are capable of transforming quinolines, most quinoline degrading organisms are *Pseudomonas* sp. Where aerobic bacteria introduce hydroxyl groups into the ring systems of their aromatic growth substrates by means of oxygenase enzymes, bacteria that utilize various heterocyclic compounds frequently initiate attack on these ring systems by using water as a source of oxygen for hydroxylation (Pereira *et al.*, 1988; Hirschberg and Ensign, 1971; Dagley and Johnson, 1963). Grant and Al Najjar (1976) isolated from garden soil a bacterium which could use quinoline as the sole carbon and nitrogen source. The authors suggested that the initial step in the transformation of quinoline by whole cells involved a hydroxylation at position 2 of the heterocyclic aromatic ring, leading to 2-hydroxyquinoline. Further transformation included additional hydroxylation steps leading to 2,6-dihydroxyquinoline and a trihydroxyquinoline, probably 2,5,6-trihydroxyquinoline.

Bohonos *et al.* (1977) used waters from lakes, streams and sewage plants as sources of inocula. When added to six water samples, quinoline was biodegraded in all six. The addition of quinoline along with benzo(f) quinoline yielded degradation of the latter in all six waters tested.

Shukla (1984) isolated from sewage an aerobic gram negative motile bacterium which was identified as a *Pseudomonas sp.* and was found to degrade quinoline by an alternate pathway. 2- hydroxyquinoline and 8-hydroxy coumarin were found in the culture medium and were further metabolized. Quinoline adapted cells were also able to transform these two compounds without a lag phase providing additional support for their intermediate role in the metabolism of quinoline. Further research demonstrated that 2,8-hydroxyquinoline and 2,3-dihydroxyphenylpropionic acid were additional intermediate products (Shukla, 1986). The proposed pathway for metabolism of quinoline by this *Pseudomonas sp.* Is presented in Figure 1.5.

Boyd *et al.* (1987) investigated the transformation of azaarenes such as quinoline, isoquinoline, quinazoline and quinoxalene by a mutant strain of *P.putida* which transformed naphthalene to form its *cis*-dihydrodiol metabolite. This bacterium transformed both the homocyclic and the heterocyclic moieties of quinolines. When the attack occurred on the homocyclic ring, the corresponding *cis*-hydrodiol derivatives were found in the culture medium, along with monohydroxylated derivatives such as 8-hydroxyquinoline or 5-hydroxyisoquinoline. *P. putida* also hydroxylated the heterocyclic ring to yield 3-hydroxyquinoline, 4-hydroxyquinazoline and 5-hydroxyquinoxaline. Hydroxylation of the heterocyclic ring was suggested by the following

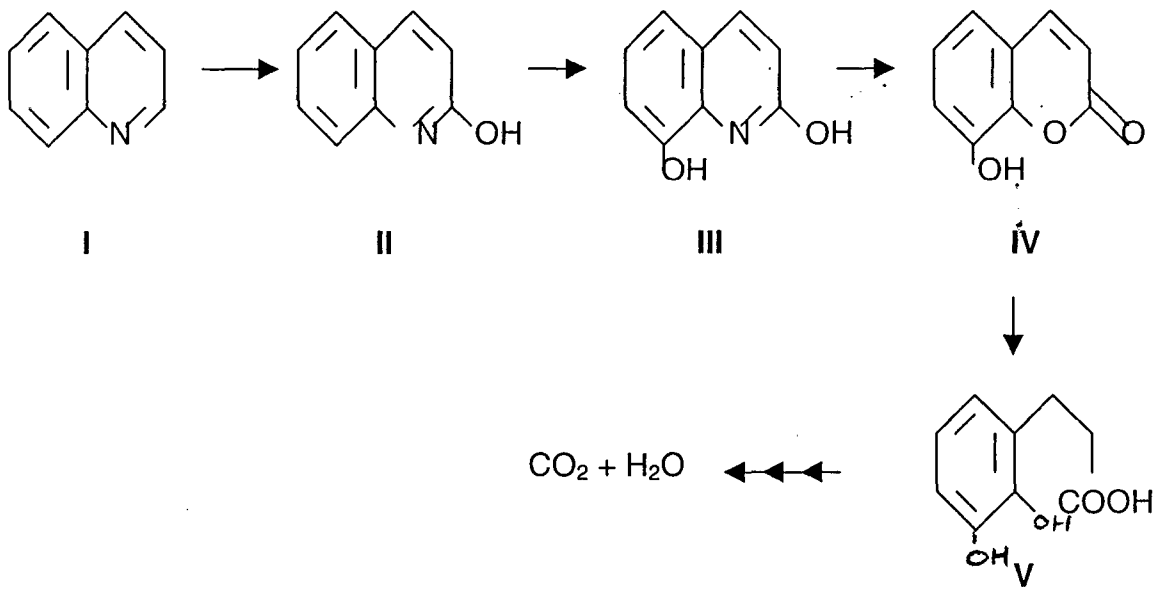


Fig. 1.5 : Pathway for the degradation of quinoline by a *Pseudomonas* sp. (Shukla, 1986).

I - Quinoline; II – 2-hydroxyquinoline;
III- 2,8- dihydroxyquinoline; IV- 8-hydroxycoumarin
V- 2,3- dihydroxyphenylpropionic acid

evidence:

(i) an unstable *cis*-dihyrodiol metabolite formed on the heterocyclic ring and

(ii) a spontaneous dehydration of this *cis*-dihydrodiol metabolite resulted in the formation of 3-hydroxyquinoline which led to anthranilic acid (2-aminobenzoic acid). Detection of anthranilic acid as a metabolite in the culture medium during the transformation of quinoline by *P. putida* suggested that cleavage of the heterocyclic ring had occurred.

Schwarz *et al.* (1989) investigated quinoline catabolism by several bacterial strains and two different degradation pathways for quinoline in different microorganisms were proposed. 2- oxo- 1,2- dihydroquinoline, 8-hydroxy-2-oxo-1,2-dihydroquinoline, 8-hydroxy-coumarin and 2,3-dihydroxyphenyl propionic acid were found as intermediates of quinoline transformation by *Pseudomonas fluorescens* 3 and *Pseudomonas putida* 86. This finding agrees with the results obtained by Shukla (1986). In the culture medium by a *Rhodococcus* strain (B1), 2-oxo-1,2-dihydroquinoline, 6-hydroxy-2-oxo-1, 2-dihydroquinoline and 5-hydroxy-6-(3-carboxy-3-oxopropenyl)-1H-2-pyridone were identified (Fig. 1.6).

Under both field and laboratory conditions Pereira *et al.* (1987a, 1987b) investigated the anaerobic transformation of quinoline in ground water contaminated by wood treatment chemicals. Quinoline, iso-quinoline, methylquinoline and acridines were found in the ground water samples. The

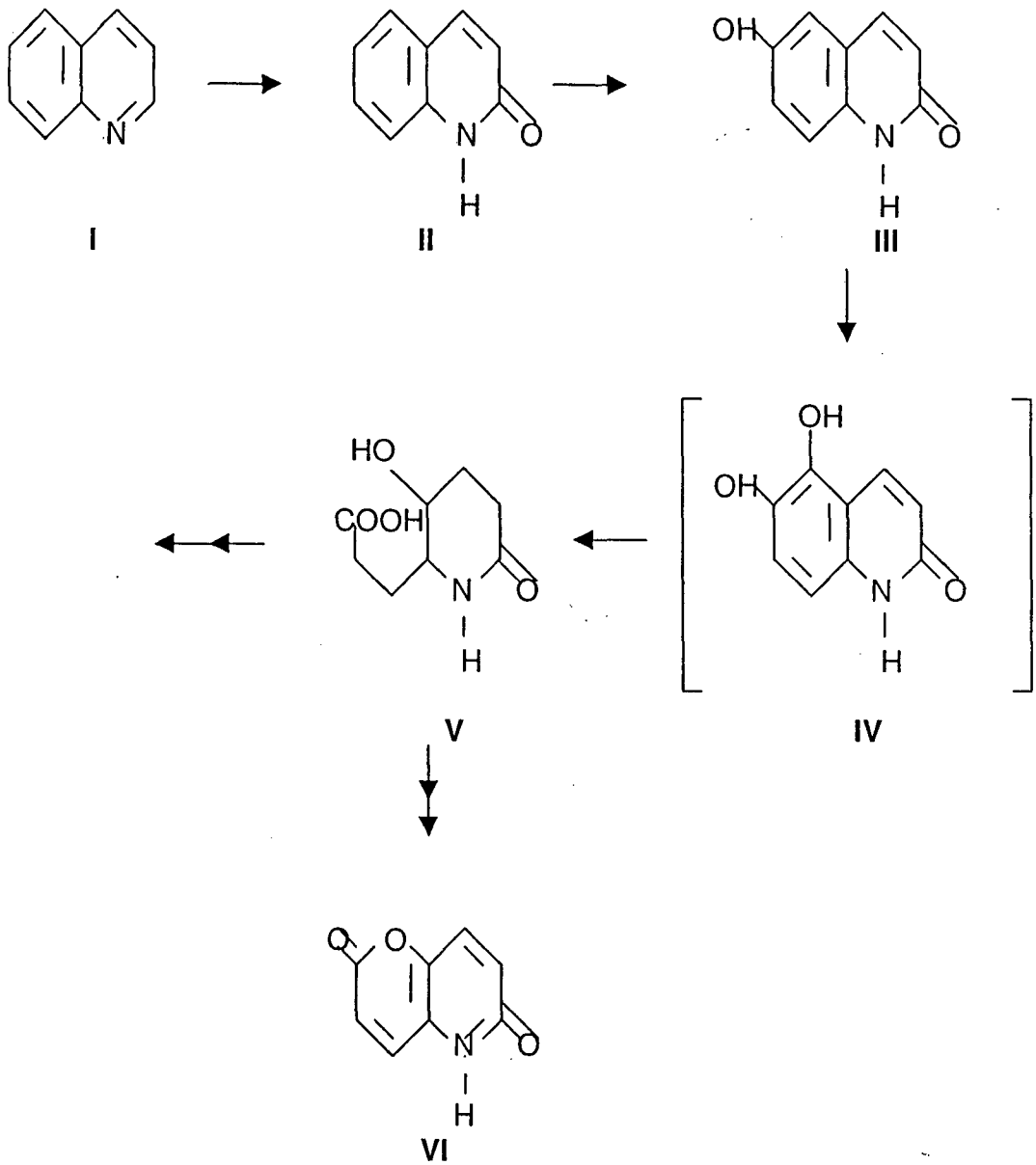


Fig. 1.6 : Proposed pathway for the transformation of quinoline in *Rhodococcus* strain B1 (Schwarz *et al.*, 1989).

I- Quinoline; II- 2-Oxo-1,2-dihydroquinoline;
III- 6-Hydroxy-2-oxo-1,2-dihydroquinoline;
IV- 5,6-Dihydroxy-2-oxo-1,2-dihydroquinoline;
V- 5-Hydroxy-6-(3-carboxyoxopropenyl)-1H-2pyridone
VI- 2-H-pyrano-2-one-(3,2B)-5H-6-pyridone

presence of oxygenated analogs of quinolines in the anaerobic zones demonstrated that the transformation of quinoline occurred under anaerobic conditions and involved hydroxylation at position 2 of the heterocyclic ring. This indicated that the anaerobic microorganisms use a pathway for the transformation of quinoline similar to that described above for aerobic bacteria.

Studies with H_2O^{18} showed that the oxygen for the hydroxylation reaction was derived from water (Pereira, 1988). The degradation of quinoline by *Pseudomonas aeruginosa* QP and *P. putida* QP also occurred via hydroxyquinolines (Aislabie *et al.*, 1990). Moreover, a limited number of methyl quinolines were hydroxylated by these two species but no further transformation of the hydroxylated products occurred. 2-methyl quinoline was not metabolized since the methyl group at position 2 apparently blocked the formation of 2-methyl quinoline. However the authors isolated a new strain of *Pseudomonas* (MQP) which was able to transform 2-methyl quinoline, leading to the formation of hydroxylated methyl quinolines and other unidentified metabolites.

As discussed in the preceding section, much work is needed to be done on the biodegradation of the NSO fraction of crude oil. The PANH components being polar are more water soluble and biologically active than their homocyclic analogs. Thus though they may occur at low concentrations (ppb range) they could cause problems considering the many toxic,

mutagenic, teratogenic and carcinogenic effects that have been demonstrated for these compounds, and the widespread distribution of these compounds in the environment is a cause for concern.

Hence, in this study we emphasize on the biodegradation of quinoline and quinoline derivatives by marine bacteria. The kinetics of quinoline biodegradation and substrate constants are evaluated as also the further use of these bacteria to degrade such compounds in whole crude oil itself. Finally the use of such bacteria as components of mixed microbial consortia for the effective degradation of crude oil is studied.

CHAPTER II

ISOLATION OF A MARINE BACTERIUM DEGRADING QUINOLINE AND OTHER NITROGEN-HETEROCYCLIC AROMATIC COMPOUNDS

A review of literature on petroleum biodegradation reveals the presence of diverse microorganisms involved in the breakdown of its many varied components. Bacteria also exist that have evolved so as to possess unique capabilities to degrade toxic heterocyclic compounds such as quinoline that is one of the components of the NSO fraction of crude oil. Although a low number of petroleum degrading bacteria exist in seawater that is constantly exposed to traces of oil components they are not able to amplify quickly in the event of an oil spill, owing to low rates of adaptation and also because the marine environment is basically a nutrient deficient system. Thus generation of actively degradative biomass in minimum period of time is one of the prime requisites to ensure quick clean up by bioremediation. Bacteria that degrade toxic and carcinogenic organic compounds such as NSO fraction could be assumed to be slow growing and rare as compared to the rapidly growing hydrocarbon degrading population and hence while screening for bacteria degrading this fraction we have employed the use of an enrichment culture system with quinoline as the enrichment substrate. The isolation and characterisation of a marine bacterium degrading quinoline is dealt with in the current chapter. Furthermore, the distribution of such bacteria in water samples collected in the Bay of Bengal is investigated.

MATERIALS AND METHODS

Media and Chemicals

Artificial sea water (ASW) of pH 6.8 was prepared with modifications, according to Gherna and Pienta (1989) and contained (per l) 17.52g NaCl, 5.0g $MgCl_2$, 3.0g $MgSO_4.7H_2O$, 0.5g $CaCl_2$, 1.0g KCl, 1.0mg $FeSO_4$, 1.0g $(NH_4)_2SO_4$, 0.5g KH_2PO_4 and 1.1g K_2HPO_4. The phosphate salts were prepared in double strength, autoclaved separately and added to a double strength, autoclaved solution of the remaining salts. For solid media agar was added at a concentration of 2%. Quinoline at a concentration of 0.02% (w/v) was used as sole source of carbon. The medium was devoid of $(NH_4)_2SO_4$ whenever quinoline was used as sole nitrogen source. Quinoline and its derivatives were obtained from Aldrich chemicals Co. Inc. and Sigma Chemical Co., USA. Bombay High crude oil was obtained from Oil and Natural Gas Commission (ONGC), Bombay, India. All other quinoline derivatives and aromatic compounds were at concentration of 0.02% (w/v). Crude oil, kerosene and petrol were used at concentrations of 0.1% w/v. Secondary carbon sources i.e. succinate or lactate were used at concentrations of 0.2% (w/v).

Media formulations used for growth of the culture included ASW supplemented with glucose (0.1g %) and quinoline (0.02%) ammonium-free ASW supplemented with quinoline and ammonium-free ASW supplemented with quinoline and nitrate (0.2g% $NaNO_3$).

To determine the molybdenum requirement for growth, $Na_2MoO_4.2H_2O$ or Na_2WO_4 (0.05 mg/l) were added separately to ASW medium.

Estimation of growth

Culture growth was monitored by measuring the absorbance of the cell suspension at 550nm in a spectrophotometer (Spectronic 1201), by viable counts on Zobell's marine agar or ASW-agar supplemented with quinoline.

Enrichment of quinoline degrading bacteria

Sea water samples were collected from the coastline of Goa and Maharashtra in sterile screw cap bottles and used for inoculation within 24h.

Quinoline degrading bacteria were isolated from marine water samples by enrichment culture in Artificial Sea Water (ASW) containing 0.02% quinoline. 5ml portion of sea water sample was inoculated in 100ml of medium and flasks were incubated on a shaker at 30°C and 200 rpm. After a week turbid flasks were inoculated with fresh medium and incubation was continued. Two more transfers at 48h intervals were performed. Marine water samples were also maintained in 5l bottles containing 2.5l of ASW with 0.02% quinoline and incubated at 30°C at static conditions and observed for growth. The strains were purified by streaking a loopful of turbid culture on Zobell's marine agar and preserved on ASW-agar slants containing quinoline either incorporated in the medium or supplied by addition of 1 drop to the lower portion of cotton plugs.

Strain Identification

All the tests for identification of the bacterial isolates was done according to the Bergey's Manual of Determinative Bacteriology (Krieg and Holt, 1984). Test for *ortho* and *meta* cleavage of catechol was done according to Stanier *et al.* (1966). Cells, cultivated in liquid medium with 0.01% (w/v) catechol, were suspended in 0.5ml of 0.02M tris.HCl buffer pH 8 and disrupted with 0.2ml toluene on a vortex, followed by the addition of 0.1ml of a 1% catechol solution. *Meta*-cleavage was revealed by the development of a bright yellow colour. After standing for 2h, the mixture was treated with 0.5g ammonium sulfate, 0.1ml of a 1% sodium nitroprusside solution and 0.4ml of ammonium hydroxide. *Ortho* cleavage was indicated by the formation of a deep purple colour.

Determination of quinoline in the medium

Quinoline concentration in ASW medium was determined spectrophotometrically by measuring the absorbance at 299nm ($\log\varepsilon = 3.51$). 1ml aliquot of the culture was centrifuged at 12,000rpm for 10min in a Beckman microfuge and the cell free supernatent was then analysed for the presence of quinoline.

Isolation of quinoline degrading bacteria from deep sea

Water samples were collected at designated sites and at depths from 0 to 2000m using a Niskin sampler mounted on a CTD (Conductivity, Temperature and Depth) device. 500ml of the water sample at each depth

was filtered through a millipore filter (0.22micron) and the filter was suspended in 5ml of ASW containing 0.1% sodium benzoate or 0.02% quinoline in sterile screwcapped tubes. Cultures enriched on benzoate were further tested to detect their growth on quinoline and crude oil by subculturing into ASW containing these substrates in appropriate concentrations.

RESULTS

Substituted quinolines and pyridines are principal components of nitrogen containing fractions of crude oil and tarballs. In order to isolate marine microorganisms capable of degrading these fractions, quinoline was used as the substrate.

Enrichment of quinoline degrading bacteria

The emphasis on enrichment cultures was to isolate quinoline degrading bacteria strictly from marine environment. Accordingly, marine water samples from the west coast were inoculated in ASW for enrichment cultures as described in materials and methods. The initial attempts at obtaining a quinoline degrading bacterium by enrichment cultures in shake flasks were not successful. To ascertain sufficient exposure of samples to enrichment conditions, a long term incubation of a mixture of all marine water samples in ASW with quinoline as sole carbon source was done in 5l autoclaved bottles. This was done to permit any slow growing bacteria to

adapt and multiply, and also enrich bacteria that may occur in the sample at a very low frequency. Quinoline degrading bacteria could not be immediately isolated by this method.

Figure 2.1 gives the sites on the west coast from where the marine waters were sampled. Only a single site (off Vengurla) on enrichment culture in ASW with quinoline as sole carbon source, resulted in a pure culture with distinct colony forming units on quinoline plates. The culture was able to grow on media containing quinoline as sole source of nitrogen as well. Interestingly, it was observed that the site from the coast had abundant tar ball deposits.

Characterization of the quinoline degrading culture

The quinoline degrading organism was a Gram negative rod (Fig.2.2a), motile and aerobic with presence of catalase, oxidase, citratase, urease and arginine dihyrolase activities. It also used nitrate as electron acceptor, hydrolyzed starch, and did not produce indole. Glucose, sorbitol, sucrose, xylose, mannitol and mannose were utilized as carbon sources, besides the organic acids such as acetate, lactate, fumarate, malonate, succinate and adipate. The strain metabolized catechol by *ortho* as well as *meta* pathway. On ASW containing quinoline, the cells form yellowish to cream colonies. On ASW media containing quinoline in addition to a secondary carbon source such as succinate, the bacteria produced green to pink to dark brown exopigment and the colonies attained a pink colour (Fig. 2.2b). The strain

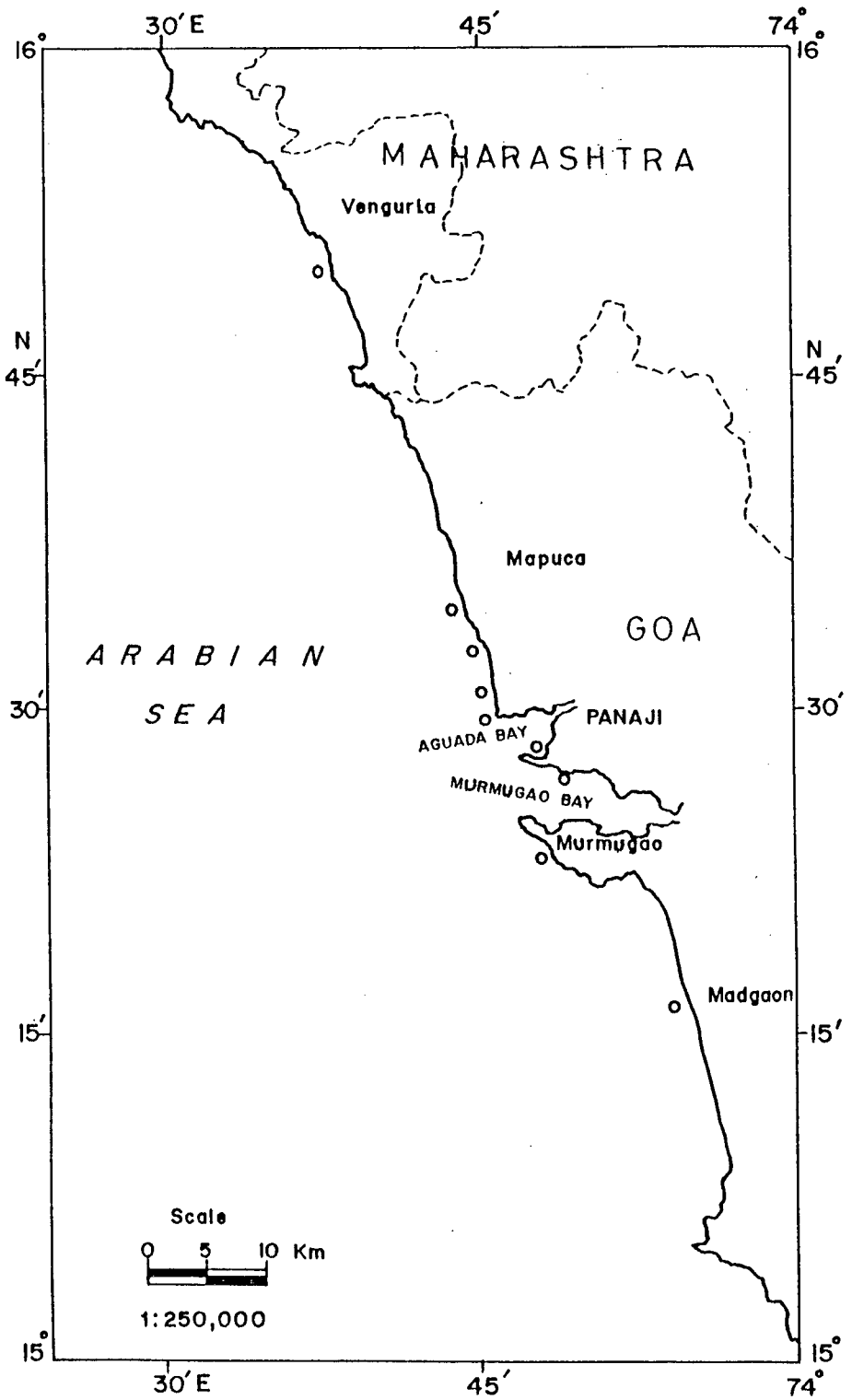


Fig. 2.1: Map showing the sites of sample collection along the west coast of India.

Fig.2.2a : Photomicrograph of the Gram stain of strain GU104 (magnification 100X).

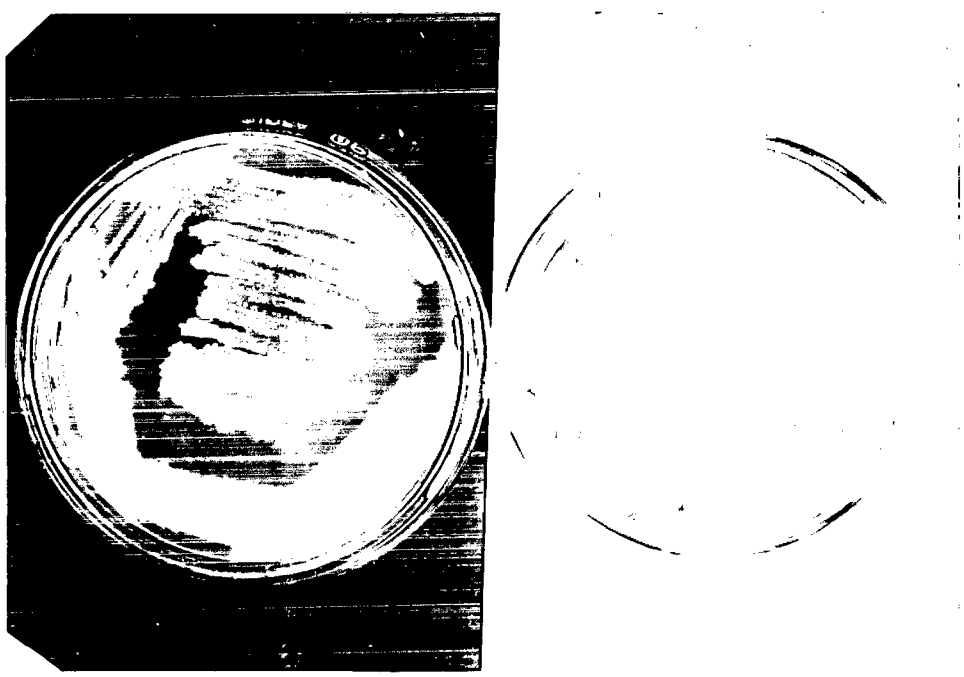


Fig 2.2b : Exopigment production by strain GU104 in ASW medium in the presence of secondary carbon sources. Greenish exopigment was observed after 1-2 days of incubation which attained a pink colour on further incubation.

exhibited pleomorphism typical of marine cultures. It dissociated rapidly into yellow or white, nucleated and non-nucleated colonies. It was resistant to kanamycin, streptomycin and ampicillin and did not have any growth factor requirements. Major characteristics of the strain were similar to those belonging to genus *Pseudomonas* as per Bergeys manual of determinative bacteriology. The isolate was designated as *Pseudomonas* sp. strain GU104.

Kinetics of growth of strain GU 104 and utilization of quinoline

Figure 2.3 shows the patterns of growth of strain GU104 in increasing concentrations of quinoline. 1ml of the culture pregrown in quinoline was inoculated in ASW media containing varying concentrations of quinoline from 0 to 0.04%. The general trend observed was that increasing quinoline concentrations resulted in increased cell mass of strain GU104 with simultaneous increase in lag period. At 0.01% (0.77mM) the lag was 3h which increased to 8h on increasing the concentration to 0.03% (2.3mM). Concentrations of 0.04% (3.1mM) and above proved to be toxic and completely abolished growth.

To understand the kinetics of mineralization of quinoline by strain GU104, it was grown on ASW at pH 6.8 in presence of 0.022g% quinoline as sole source of carbon (Fig. 2.4). Degradation of quinoline was followed spectrophotometrically at 299nm and was concomitant with increase in cell mass (A_{550nm}). The growth rate was $0.115h^{-1}$. Quinoline was reduced to 1%

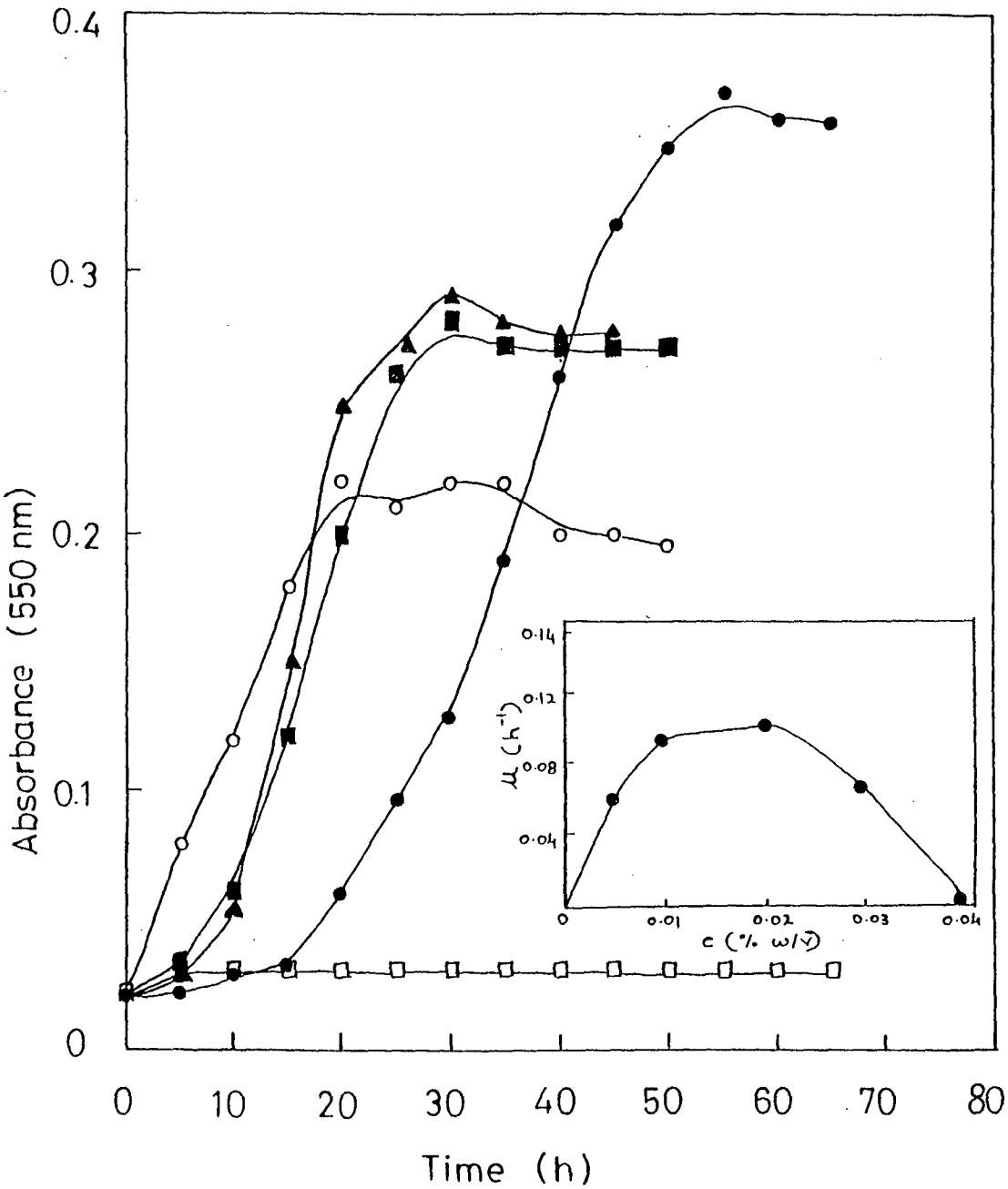


Fig. 2.3 :Growth profiles of strain GU104 in ASW medium with increasing concentrations of quinoline, (o) 0.005%, (■) 0.01%, (△) 0.02%, (●) 0.03%, (□) 0.04% quinoline. Inset is the profile of growth rates, μ (h^{-1}) with increasing quinoline concentrations, c (%).

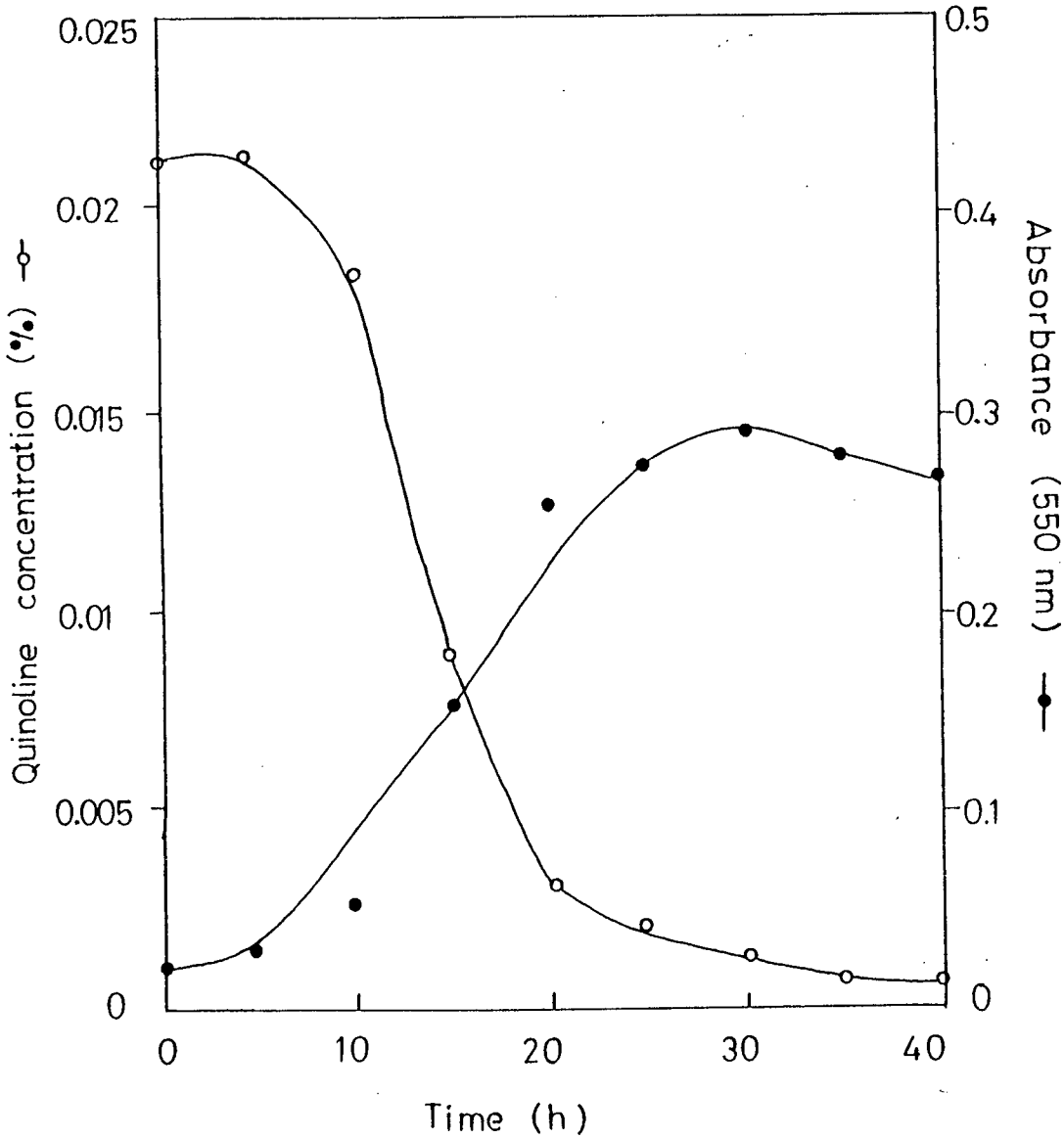


Fig. 2.4: Growth profile of strain GU104 in ASW containing quinoline (●), accompanied by depletion of quinoline (o) in the medium.

of the initial concentration at the end of 30h. Further incubation resulted in a decrease in cell mass and the cells entered death phase.

Strain GU104 grew much more rapidly with 0.1% glucose (Fig. 2.5) and reached a high cell density (A_{550} = 0.39) within 24h. When the growth medium contained quinoline in addition to glucose, it was seen that though initially growth was rapid, rate of quinoline depletion was very slow as compared to that grown on quinoline alone. After a considerable level of cell mass was reached, the log phase continued with a steady though slower increase in cell mass, along with the rapid depletion of quinoline.

Figure 2.6 demonstrates the growth of strain GU104 in ASW devoid of nitrogen salts and in which quinoline was incorporated as the sole source of carbon as well as nitrogen. From the graph it is apparent that the lag phase was prolonged with the cell mass not increasing appreciably for almost 24h. However, in relation to growth the level of substrate in the medium dropped quite rapidly and reached almost one third its initial concentration before the culture formally entered log phase. During the log phase the remainder of the quinoline was rapidly depleted and thus could only support a very low turbidity/growth.

The ability of strain GU104 to utilize the nitrogen from nitrates was tested in a medium containing quinoline as carbon source and nitrate as nitrogen source instead of ammonium salts. From Figure 2.6 it is apparent

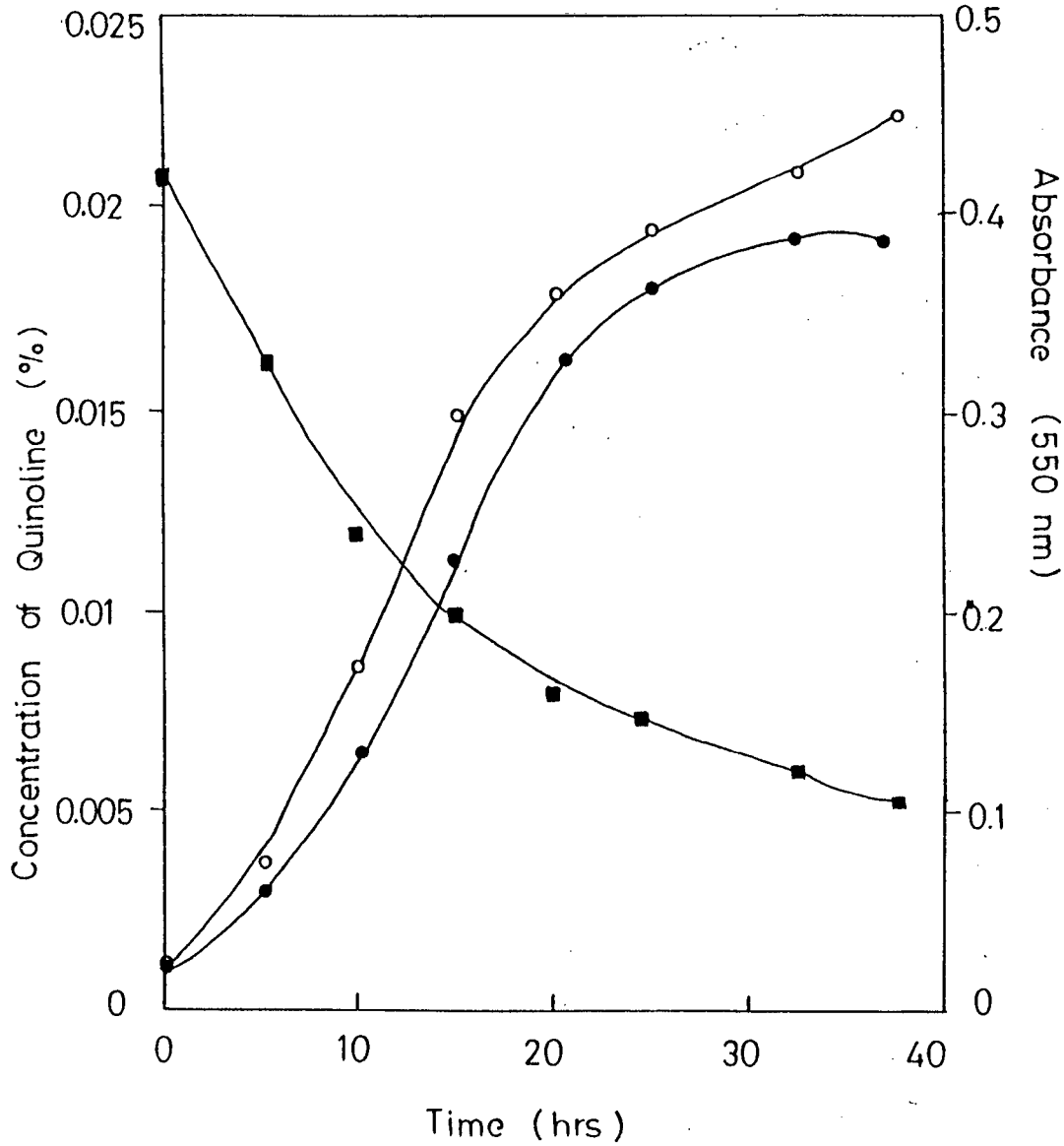


Fig. 2.5 : Growth of strain GU104 in ASW medium containing glucose (●) and in ASW medium containing glucose plus quinoline (o). The latter is accompanied by depletion of quinoline from the medium (■).

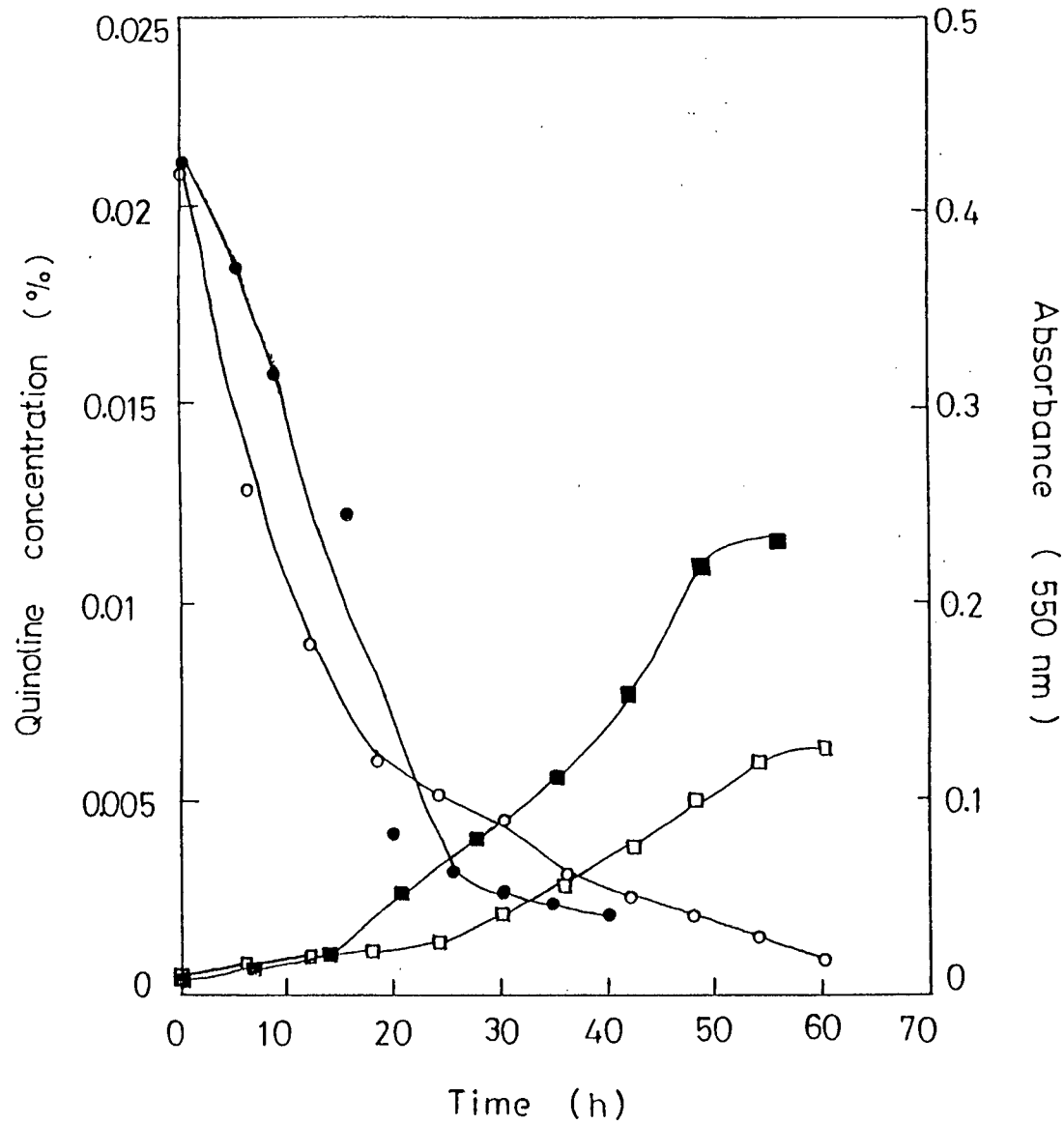


Fig. 2.6 : Growth of strain GU104 in ASW medium containing quinoline as sole source of nitrogen (□) accompanied by quinoline depletion (o), and growth of strain GU104 in ASW medium containing nitrate as nitrogen source (■) accompanied by quinoline depletion (●).

that the lag phase was reduced as compared to that in a medium containing quinoline as the sole source of nitrogen. The initial rate of quinoline depletion was also lower, but increased once the culture entered log phase. The final turbidity produced was relatively higher though not as high as in the case where ammonium salts are incorporated in the medium.

Growth of strain GU104 in ASW containing quinoline as sole carbon source was found to be dependent on molybdenum (Fig.2.7). Concentrations as low as 0.05mg/l of $Na_2MoO_4.2H_2O$ considerably decreased the lag period of growth. Growth was inhibited when molybdate was replaced by tungstate. When molybdate was not added to the medium, growth occurred with a lag and a lower biomass was obtained.

Utilization of other NHAs and aromatic compounds by strain GU104

Various compounds besides quinoline were tested to determine the catabolic versatility of strain GU104. Substituted quinolines form a part of the NPAC fraction of crude oil. The ability to utilize methyl substituted quinolines by strain GU104 besides quinoline was studied specifically to understand its versatility to mineralize oil components. All the carbon sources were provided at 0.01% (w/v) in ASW medium in shake flasks and incubated at 30°C. As can be seen from Table 2.1, among the methyl-substituted quinolines strain GU104 could grow on 4- and 7-methylquinoline. 2-methylquinoline was not degraded. Most chlorinated analogues of quinoline did not support growth except 7-chloro, 4-hydroxyquinolinic acid. Among other aromatic compounds

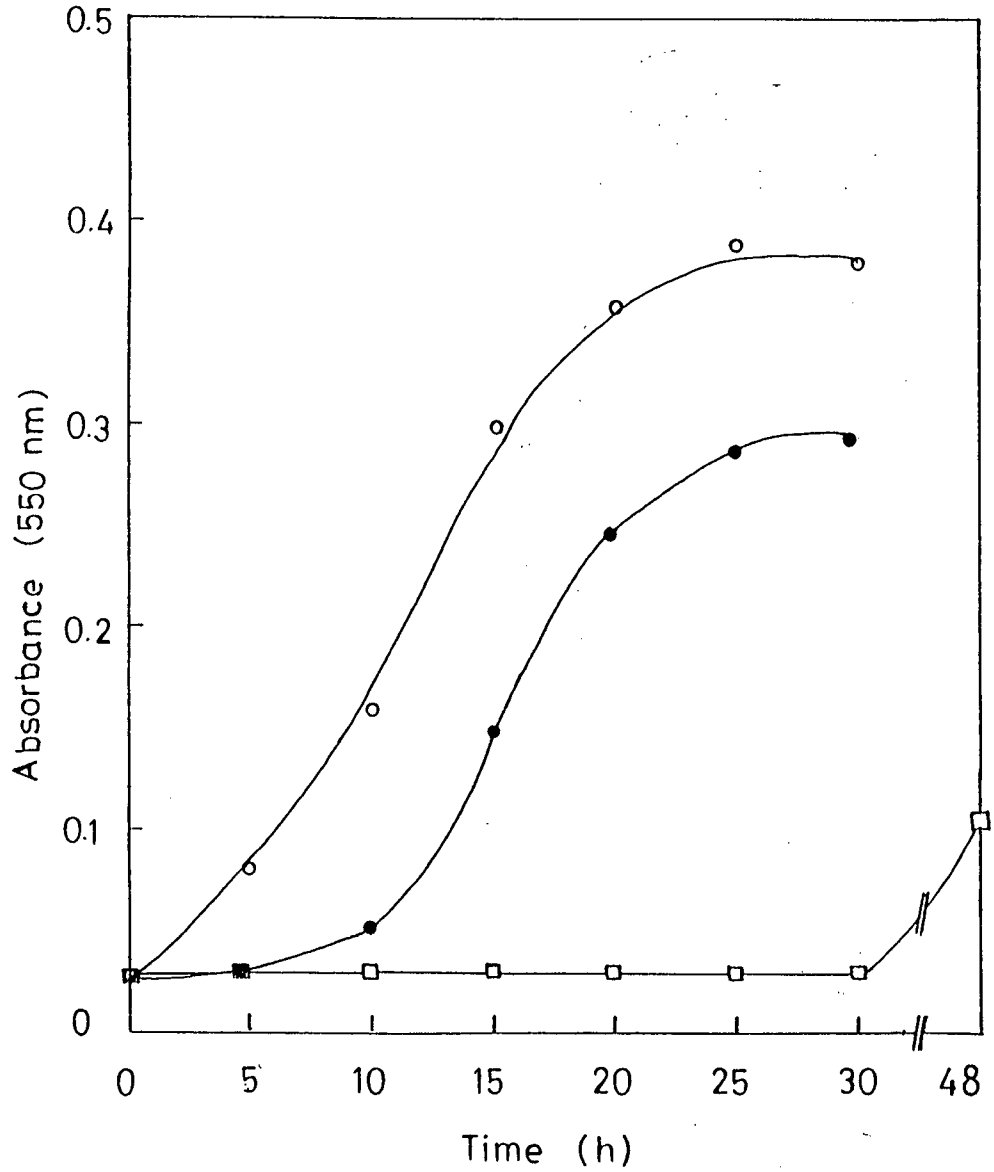


Fig. 2.7 : Growth of strain GU104 in ASW-quinoline containing 0.05 mg/l $Na_2MoO4.2H_2O$ (o), in the absence of Mo (●) and in ASW-quinoline containing 0.05 mg/l Na_2WO_4 (□).

Table 2.1 : NHAs and aromatic substrates utilized by strain GU104 as carbon source

Substrate	Growth*	A_{550nm} (48h)
Hydroxyquinolines:		
2-hydroxyquinoline	+	0.351
4-hydroxyquinoline	+	0.245
5-hydroxyquinoline	-	0.003
6-hydroxyquinoline	-	0.005
Substituted quinolines:		
2-methylquinoline	-	0.002
3-methylquinoline	-	0.043
4-methylquinoline	+	0.094
6-methylquinoline	-	0.073
7-methylquinoline	+	0.083
8-methylquinoline	-	0.003
2,4-dimethylquinoline	+	0.097
2,6-dimethylquinoline	-	0.080
2,7-dimethylquinoline	+	0.121
8-hydroxyquinoline	-	0.001
7-chloro, 4-hydroxy, 3-quinolinic acid	+	0.105
4,7-dichloroquinoline	-	0.003
4-hydroxy, 7-chlorquinoline	-	0.005
Other aromatic hydrocarbons:		
Benzoate	+	0.211
Naphthalene	+	0.201
Pthalate	+	0.110
7-hydroxycoumarin	+	0.225
catechol	+	0.141
nicotinate	+	0.221
hypoxanthine	+	0.210
pyridine	+	0.125
carbazole	+	0.105
isoquinoline	-	0.004
indole	+	0.112
Alkanes:		
Hexadecane (C16)	+	0·253
Heneicosane (C21)	+	0·171
Crude oil	+	0.231
Kerosene	+	0.126
Petrol	+	0.115

* - Absorbance values greater than 0.08 are indicative of positive growth.

utilized by strain GU104 are phthalate, naphthalene, benzoate, 7-hydroxycoumarin as well as nicotinate, hypoxanthine, chloroquinolinic acid, pyridine and carbazole demonstrating wide versatility of strain GU104 to mineralize NHA compounds.

Distribution of NHA degraders in the Bay of Bengal

Strain GU104 could mineralize catechol via *ortho-* as well as *meta-* pathway and could grow luxuriantly on benzoate. The strategy adopted to determine the distribution of quinoline degrading strains in deep sea waters involved the isolation of the benzoate utilizing strains followed by screening the same for quinoline/NHA degradation.

Cruise no. 106 aboard the research vessel Sagar Kanya was undertaken in Oct-Nov'95. Sea water samples were collected at 20 sites in the Bay of Bengal. Figure 2.8 shows the cruise track and the various sampling sites. The exact geographical location of the sites is listed in Table 2.2. Sea water samples were collected at the surface and at depths of 500m, 1000m, 1500m and 2000m. Sampling was done with a Niskin sampler mounted on a CTD device. The salinity and temperature of the water sample was monitored at each depth with the aid of a computer connected to the CTD device. The profiles of temperature and salinity at various depths is demonstrated in Figure 2.9. On an average, the temperature of sea water ranged from 2°C at depths of 2000m to 29°C at the surface. Salinity (34ppt) and pH (6.0 to 7.5) remained more or less constant in the water column,

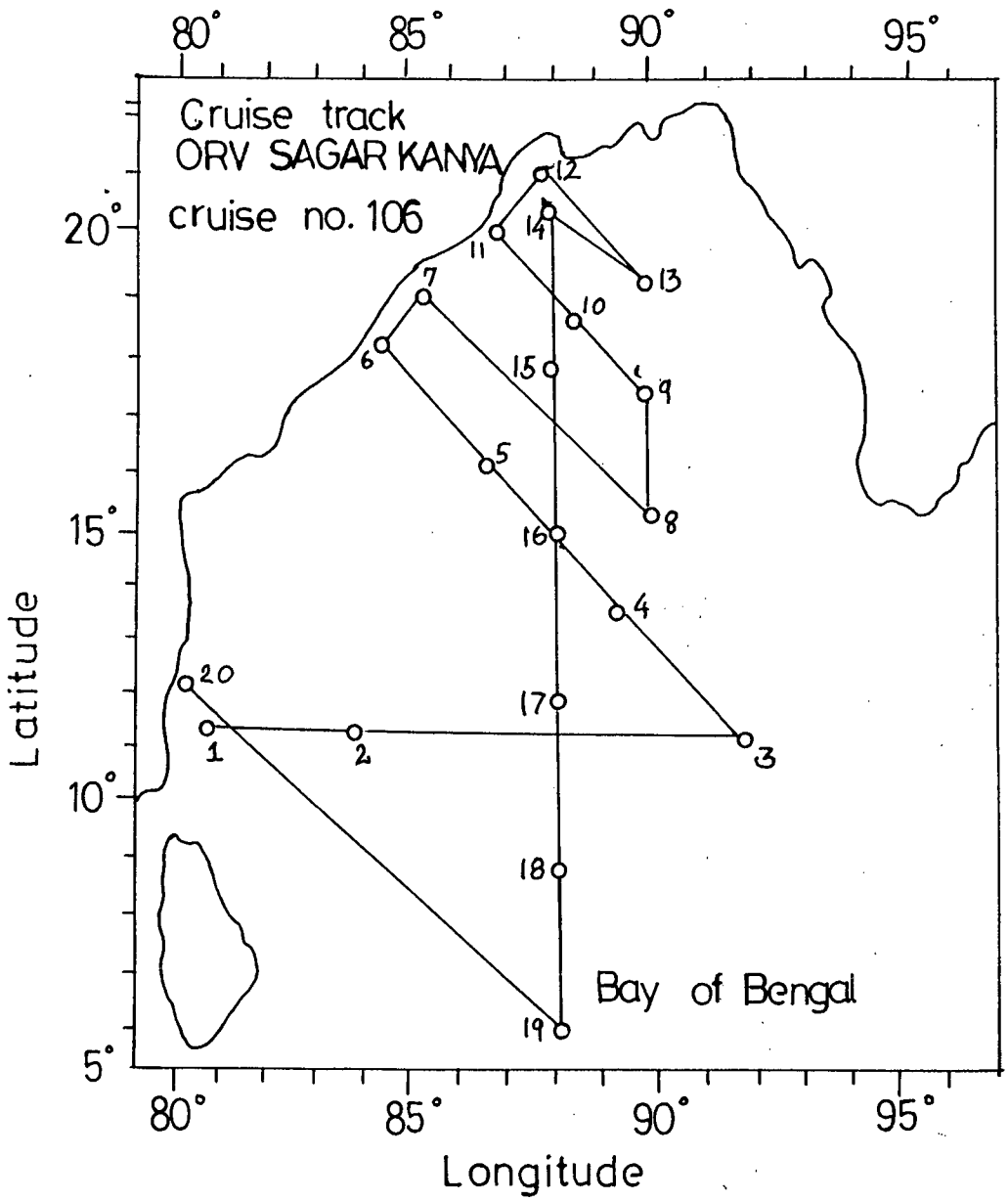


Fig. 2.8 : Route map showing sampling stations (o) in the Bay of Bengal aboard research vessel ORV Sagar Kanya Cruise No. 106 in October, 1995.

Table 2.2: General description of sampling stations.

Date yy.mm.dd	Stn. no.	Location		Depth (m)
		Latitude N	Longitude E	
95.10.4	1	11°29.98'	81°00.23'	0,500
95.10.5	2	11°30.33'	83°59.86'	0,500,1000,1500,2000
95.10.8	3	11°30.31'	92°00.15'	0,500
95.10.9	4	14°00.17'	89°00.068'	0,500,1000,1500,2000
95.10.11	5	16°48.99'	85°29.622'	0
95.10.12	6	17°58.72'	83°58.53'	0
95.10.12	7	18°37.609'1	84°47.60'	0,500
95.10.14	8	15°00.006'1	89°59.87'	0,500,1000,1500,2000
95.10.15	9	16°59.97'	89°59.54'	0,500,1000,1500,2000
95.10.15	10	18°35.789'	87°59.99'	0
95.10.16	11	19°47.90'	86°29.75'	0
95.10.17	12	20°43.71'	87°59.58'	0,500
95.10.18	13	19°00.045'	89°59.712'	0,500
95.10.18	14	20°00'	87°59.367'	0,500
95.10.19	15	17°00.39'	88°00.49'	0,500
95.10.21	16	13°59.94'	87°59.938'	0,500
95.10.22	17	11°02.2426'	87°59.1055'	0,500
95.10.22	18	7°59.6512'	87°58.558'	0,500
95.10.26	19	4°03.4490'	87°57.7371'	0,500
95.10.30	20	13°05.7317'	80°25.2835'	0

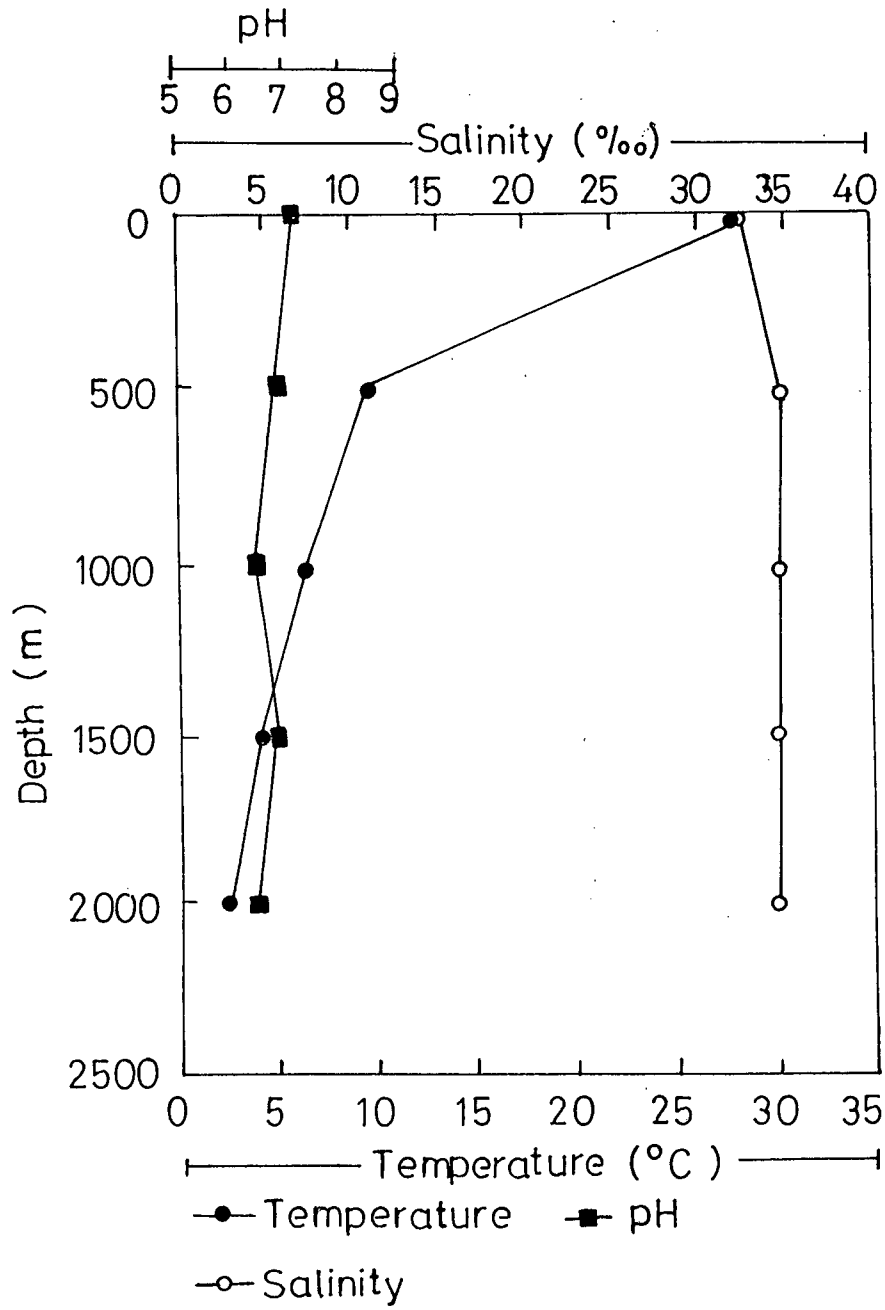


Fig. 2.9 : Profiles of temperature, salinity and pH at various depths in the Bay of Bengal.

although coastal waters exhibited a salinity as low as 13‰ due to the presence of numerous estuaries along the east coast of India. Total viable counts of the bacteria at the surface were obtained on Zobell's marine agar plates. Bacterial numbers ranged from 10 to 10^3 organisms per ml of sea water at surface and 10 to 10^2 organisms per ml at 500m depth.

Benzoate and quinoline degrading bacteria were isolated as per details in **Materials and Methods**. Water samples from all stations revealed the presence of benzoate degrading bacteria, while relatively fewer stations yielded quinoline degraders (Fig. 2.10). Benzoate degrading bacteria were prevalent at both surface as well as 500 m depths while quinoline degrading bacteria were found to be more prevalent in surface waters (Fig. 2.11).

In order to determine whether benzoate served as an enrichment substrate for quinoline degrading bacteria, enrichment cultures grown on ASW with benzoate as sole carbon source were inoculated in media containing quinoline and incubated at 30°C on a shaker. This was done by making 10 fold dilutions of an aliquot of the enrichment culture in such media. This not only served to detect quinoline degrading bacteria from the benzoate enrichment mixed culture, but also gave the MPN (most probable number) of the quinoline degraders in the mixture. Similarly the MPN of benzoate degraders was also determined. From this the ratio of quinoline to benzoate degraders was determined and was found to be low (Table 2.3). However, some of the sea water samples (sites 2 and 9) which did not yield quinoline

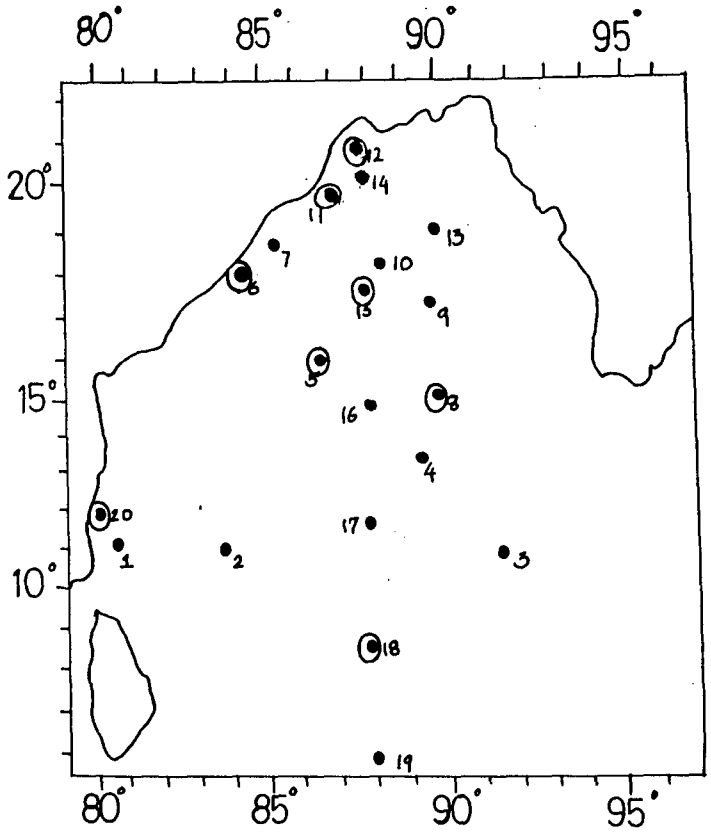


Fig. 2.10 : Horizontal distribution of benzoate (●) and quinoline (O) degrading bacteria in the Bay of Bengal.

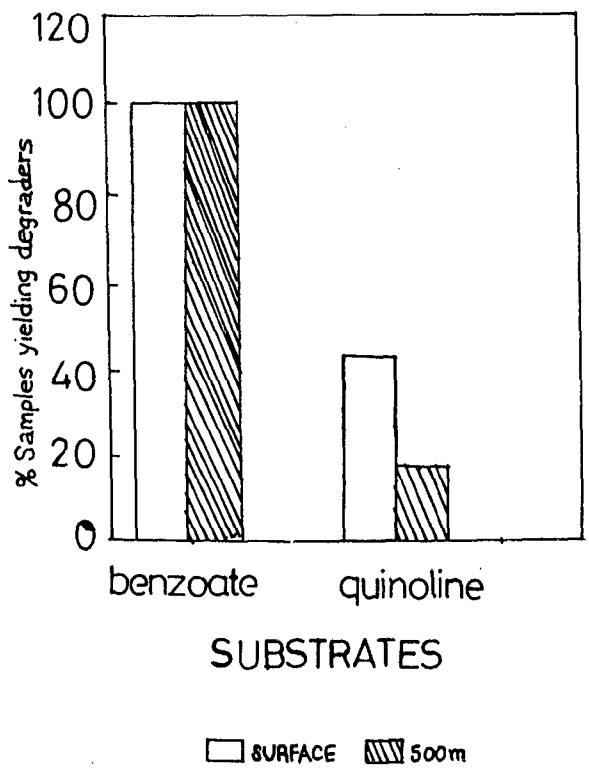


Fig. 2.11 : Percentage of positive marine water samples collected that yielded benzoate and quinoline degrading bacteria.

Table 2.3 : Frequency of quinoline degraders in benzoate enriched cultures

Stn. No.	Depth	No. of benzoate degraders / ml	No. of quinoline degraders / ml	Ratio Quinoline :benzoate degraders
2	0	10^6	0	--
	500	10^5	10^2	1:10,000
	1000	10^4	10	1:10,000
3	0	10^5	10^2	1:10,000
4	0	10^5	0	--
	500	10^5	0	--
	1000	10^5	0	--
5	0	10^5	10^2	1:1000
	500	10^4	10	1:1000
9	0	10^5	10^2	1:1000
	500	10^4	0	--
	1000	10^4	10^2	1:1000
12	0	10^5	10^2	1:1000

degrading bacteria on direct enrichment using quinoline as substrate, did yield such bacteria with prior enrichment on benzoate.

DISCUSSION

The rise in concentrations of hydrocarbon utilizing microorganisms after oil spills has been shown in several studies (Atlas, *et al.,* 1976; Oppenheimer *et al.*, 1977), but although some hydrocarbonoclastic bacteria have constitutive enzymatic systems, most of them use adaptive systems which appear only after a contact ranging from a few minutes to a few days with these hydrocarbons (Tagger *et al*, 1983). In order to provoke a rapid and high increase of the petroleum degrading bacteria in the early stage of an oil spill, it may become imperative to 'seed' the site of the oil spill with a heavy inoculum of a marine bacterial community previously adapted to crude oil biodegradation. With this aim in view, we attempted to isolate bacterial strains degrading various components of crude oil. Horowitz *et al.* (1975) observed that in most studies on pure cultures of oil degrading bacteria, the microorganism was obtained by enrichment culture procedure in which maximum specific growth rate or maximum final cell concentration was used as a selection criterion. Thus, paraffin oxidizers, which grow rapidly and reach high cell concentration of crude oil, would be selected and examined in detail. Bacteria which grow more slowly or oxidize minor components of crude oil, never come to fore in batch experiments, although the activity of

these microorganisms may be of special significance in natural environments. Therefore, since our studies were based on the heterocyclic component of crude oil which includes the N,S,O compounds and in particular on the 'NPAC' fraction, we used quinoline as the enrichment substrate. Also, isolation of quinoline utilizing strains may help in identifying strains that mineralize complex substituted quinolines.

As previously described in **Chapter I** hydrocarbon degrading microorganisms are present in marine environments. However, environments exposed to hydrocarbon contamination are more likely to harbour hydrocarbon degrading microorganisms and since these microorganisms have had a chance to adapt to the hydrocarbons, they would presumably exhibit better hydrocarbon utilization degradative capabilities (Leahy and Colwell, 1990). Therefore, the marine water samples collected in this study were from the intertidal zones of sites that were exposed to crude oil pollution due to transport of barges and tankers. Evidence for this, were the tarballs which were deposited along the shores of the beaches.

The method of enrichment of quinoline degrading bacteria by long term incubation of large sample volumes in stationary 5l bottles did not yield quinoline degrading bacteria presumably because of the lack of aeration and agitation which perhaps did not allow for the rapid growth of the aerobic bacteria. Only one of the samples collected from the west coast yielded a marine bacterium which was able to grow in ASW supplemented with

quinoline which served as the sole source of carbon, nitrogen and energy. The site (Fig. 2.1) was located off Vengurla and was densely polluted with tarballs. Quinoline degrading bacteria have been isolated previously from soil and sewage sludge (Grant and Al Najjar, 1976; Shukla, 1986; Schwarz *et al.*, 1988). To the best of our knowledge there have been no reports yet of a quinoline degrading marine isolate. The bacterium on biochemical characterization appeared to belong to the genus *Pseudomonas* and was designated as strain GU104. On membrane lipid analysis using computer aided taxonomy (CAT) the strain was shown to be phylogenetically closer to *Ochrobactrum anthropi* (*Achromobacter*) (D. Chandramohan, personal communication).

The isolate GU104 degraded quinoline under all cultural conditions tested. In ASW containing quinoline and supplemented with glucose, the growth rate and cell mass appeared to be higher than that with glucose alone. Such simultaneous metabolism of substrates is common in natural isolates (Sangodkar and Mavinkurve, 1991; Reber and Kaiser, 1981). Strain GU104 was able to use quinoline as source of nitrogen and this was seen as a rapid uptake of quinoline, since the culture scavanges nitrogen from the heterocyclic ring. However, the amount of biomass obtained was low owing to the fact that nitrogen was limiting. The isolate was able to use nitrate as the source of nitrogen.

Bauder *et al* (1990) have shown that growth and quinoline consumption are accelerated in the presence of molybdate. When molybdate was replaced by tungstate, an antagonist of molybdenum, growth did not occur and quinoline was consumed. Blaschke *et al.* (1991) also reported a molybdenum dependent degradation of quinoline by a *P. putida* strain. In strain GU104, the presence of low levels of molybdenum did result in a shorter lag phase and a higher turbidity, and inhibition of growth was seen in the presence of tungstate, indicating that quinoline degradation is dependent on molybdenum.

Strain GU104 showed a wide biochemical versatility and was found to degrade many quinoline derivatives as well as other N-heterocycles such as indole and carbazole proving our initial prediction that quinoline degrading marine microbes may infact carry traits to mineralize many substituted quinolines. Many quinoline degrading bacteria that have been previously isolated have been reported to possess only limited capabilities to degrade compounds closely related to quinoline and pyridine. Such a limited spectrum of degradative capabilities appears to be common among organisms that degrade nitrogen heterocycles, the class that includes quinoline and pyridine (O'Loughlin *et al*, 1995). There are also reports that there are bacteria that are capable of utilizing one or more quinoline derivatives in addition to quinoline. Schach *et al.*(1995) reported quinoline and 3-methyl quinoline degradation in *Coumomonas testosteronii* 63 and characterized the enzymes catalysing the first two steps in their metabolism.

Roger *et al.* (1993) also reported the degradation of 4-methyl quinoline and quinoline by *Pseudomonas putida* K1. Owing to the fact that the ocean is generally a nutrient limiting medium, marine bacteria may have a higher potential to scavenge scarce nutrients and therefore the wider biochemical versatility of strain GU104. Strain GU104 did have a limited potential of degrading aromatic compounds, with benzoate and naphthalene being degraded, while it could degrade alkanes such as hexadecane (C_{16}) and heneicosane (C_{21}) as well as whole crude oil, kerosene and petrol.

The distribution of hydrocarbon utilizing bacteria has been studied in a wide variety of niches, viz., marine, freshwater and soil habitats, although the majority of the studies dealt with horizontal distribution of such bacteria (Venkateswaran *et al.,* 1993). Furthermore, no reports are available on the prevalence of NHA degrading bacteria in the open ocean. Cruise no. 106 aboard the research vessel Sagar Kanya was undertaken in Oct-Nov'95 with the following objectives:

1. To study the prevalence of NHA degrading bacteria in the Bay of Bengal in surface waters as well as depths in the water column.
2. To study the prevalence of hydrocarbon degrading bacteria of which benzoate was used as the representative substrate.
3. To determine whether benzoate being a simple hydrocarbon substrate would serve in rapid enrichment of bacteria degrading various more recalcitrant hydrocarbons, e.g. crude oil as well as NHAs such as quinoline.

The results generated from our studies in the Bay of Bengal have shown that benzoate degrading marine bacteria were prevalent both horizontally and vertically to a depth of 2000 m in the Bay of Bengal. The incidence of NHA degraders however, was found to be low. Most of the samples collected from near the coast yielded quinoline degrading bacteria on direct enrichment using quinoline as substrate. This could be due to higher level of chemical pollution of coastal waters, in the Bay of Bengal, which would serve to select such bacteria. Moreover, surface waters yielded a higher degree of bacteria capable of degrading quinoline than deeper waters. Compounds such as benzoate used to enrich bacteria growing on more complex compounds such as quinoline as well as crude oil was found to be effective and such enrichment cultures did yield quinoline degrading bacteria on further subculture. NHAs, such as quinoline, are toxic and the isolation of bacteria degrading them from dilute water samples could prove to be difficult. The strategy devised in this study of preliminary enrichment on a simple and relatively nontoxic aromatic compound appear to help in increasing the initial cell numbers. It is understandable that the slow growing microorganisms could not get sufficient time to adapt and detoxify the compounds such as quinoline. Although the ratio of quinoline degraders to benzoate degraders in a population enriched on benzoate was found to be quite low, this does not rule out the effectiveness of benzoate as a substrate for preliminary enrichment, because as seen at sampling sites (2 and 9), the water samples which on direct enrichment on quinoline failed to yield

quinoline degrading bacteria, did infact yield such bacteria from benzoate enriched mixed cultures.

In conclusion, this study provides evidence that *Pseudomonas* sp GU104 is a marine bacterium capable of utilizing quinoline as sole source of carbon and nitrogen. It has a wide versatility in mineralizing the substituted quinolines, related NHAs and eventually major components in crude oil. It is also evident that such organisms are distributed, although at low frequencies, throughout the deep oceans and can be isolated by enrichment cultures on simple aromatic compounds as benzoate.

CHAPTER III

EFFICACY OF *PSEUDOMONAS* SP. STRAIN GU104 IN MINERALIZATION OF QUINOLINE IN CONTINUOUS CULTURE

Release of petroleum hydrocarbons in the environment is a widespread occurrence. One particular concern is the contamination of the aquatic environment by the toxic water soluble and mobile petroleum components. The environmental impact to an oil spill is determined in part by concentrations of toxic materials in the oil and by the rates at which the toxicants move from oil to water.

Several factors such as pollutant concentration, active biomass concentration, temperature, pH and microbial adaptation, influence the rate and extent of biodegradation of pollutants. The detoxification of environmentally relevant pollutants by microbial systems in industrial end of pipe abatement plants is an attractive proposition, but this approach cannnot be realized without kinetic data. Kinetic models are of value in investigating both the capacity and stability of biological processes which utilize inhibitory substrates. Haldane's expression has been widely used to study inhibition of biodegradation at high substrate concentrations. (Hill and Robinson, 1975; Yang and Humphrey, 1975).

Quinoline by itself is a toxic component and has been listed as one of the priority hazardous chemicals by the US Clean Water Act (Omenn, 1988). In this chapter we study the kinetics of mineralization of quinoline by the marine bacterium *Pseudomonas* sp. strain GU104. Most marine bacteria can utilize nutrients present at low concentrations and this study is aimed at determining the quinoline conversion rates during batch and continuous

cultivation with *Pseudomonas sp.* strain GU104 in order to ascertain the potentials of the strain for bioremediation of quinoline infested environments.

MATERIALS AND METHODS

Microorganism and growth conditions

Pseudomonas sp. strain GU104 was used to study the microbial degradation of quinoline in batch and continuous culture system. A loopful of the stock culture was transferred to 50 ml ASW containing 0.02% quinoline as sole source of carbon and energy. The culture was then incubated on a shaker at 200rpm for 24h at 30°C and fed 3-5 times with 0.02% quinoline. The above inoculum was used for the batch and continuous culture studies.

Continuous and batch culture experiments were carried out in a 1.6l laboratory bench top Bioflow fermentor (New Brunswick, USA) with a working volume of 1l. The reaction was equipped with a mechanism for regulating temperature, aeration and agitation. The cultivation temperature was 30°C and the pH was maintained at 6.8 - 7.0. The agitation speed was set at 200rpm but occasionally increased to 300rpm in order to prevent oxygen limitation. The aeration was carried out by compressed air at a rate of 0.4lpm.

Measurement of growth

The estimation of growth was done spectrophotometrically by measuring the absorbance of the culture broth at 550nm and by determining the dry weight on bacterial biomass. For this the absorbance and dry weight of *Pseudomonas* sp. strain GU104 growing in ASW-quinoline was determined at various cell densities and plotted. A linear relationship was obtained. The dry weight was obtained by centrifuging 5ml aliquot culture at 10,000 rpm for 15min. The pellet was washed twice in distilled water. Biomass was determined using a Mettler balance after drying the pellet to constant weight by heating at 80°C. An absorbance of 1 unit was found to correspond to a biomass of 0.4g/l.

Measurement of quinoline concentration and conversion rate

Quinoline concentration was determined spectrophotometrically at 299nm ($\varepsilon = 3.51$).

Quinoline conversion rate was determined according to Miethling *et al.*, (1993) and expressed as grams quinoline consumed per gram biomass per hour (g/gh) and given by

$$q = 1/X \; [\, DS_o - DS - ds/dt]$$

where X is biomass in grams per litre (g/l) ; ds/dt is grams per litre quinoline consumed per unit time (h); D is the dilution rate (h^{-1}); S_o is the quinoline concentration in feed (g/l); S is the quinoline concentration in culture broth (g/l).

Preparation of resting cells

Cells were grown on ASW medium containing either quinoline or glucose as carbon source and were harvested during the late exponential phase by centrifugation at 8000rpm for 15min at 4°C (Sorval RC5C centrifuge). The cells were washed with ASW and then were suspended in the same. Reactions with resting cells were carried out by incubating the reaction mixture at 30°C on a rotary shaker (150rpm). Absorbance at 550nm was adjusted to 1.0 unless otherwise stated.

Preparation of immobilized cells

Strain GU104 was grown in ASW supplemented with quinoline and fed 3-5 times with 0.02% quinoline. The cells were harvested by centrifugation, washed and resuspended in ASW to obtain OD of 2.0. The suspension was then mixed with equal volumes of 1% sodium alginate, and extruded dropwise through a syringe into ice-cold 0.1M $CaCl_2$ solution to form beads of calcium alginate containing immobilized cells of strain GU104 (Chibata and Tosa, 1977). The beads were then used as inoculum in flasks containing ASW supplemented with quinoline and the medium was analysed for disappearance of quinoline. For storage the beads were simply washed with ASW, resuspended in a small volume of ASW and stored in the refrigerator at 4°C.

RESULTS

As discussed in **Chapter I,** Quinoline concentrations above 11ppm (0.0011%) are reported to be lethal to fish and concentrations as low as 2ppm carcinogenic for mice. Tolerable levels although not specified, it is assumed that the levels below these would have to be achieved in any bioremediation process. Simulation of mineralization of quinoline in aquatic systems was done as depicted in the following results.

Mineralization of quinoline by strain GU104 in batch culture

Batch culture data for growth of strain GU104 at 30°C and pH 6.8 using a 1.6l bench top fermentor with quinoline as sole source of carbon is given in Figure 3.1. 50ml of primary culture pregrown in ASW was added to 950ml of ASW containing 0.02% quinoline. At defined intervals 1ml of sample was collected aseptically in a screw cap tube fitted to an outlet port. Cell concentration was determined spectrophotometrically at 550nm and quinoline concentration at 299nm. Increase in absorption at 550nm was accompanied by depletion of quinoline, however there was an initial lag of 5h after which linear exponential growth was observed. During the short stationary phase, quinoline was no longer detected in the medium, and there was then a decline at 550nm indicating advent of death phase. There was no appreciable change in pH throughout the growth of strain GU104. Quinoline concentration fell from 200ppm to non detectable levels in 24h.

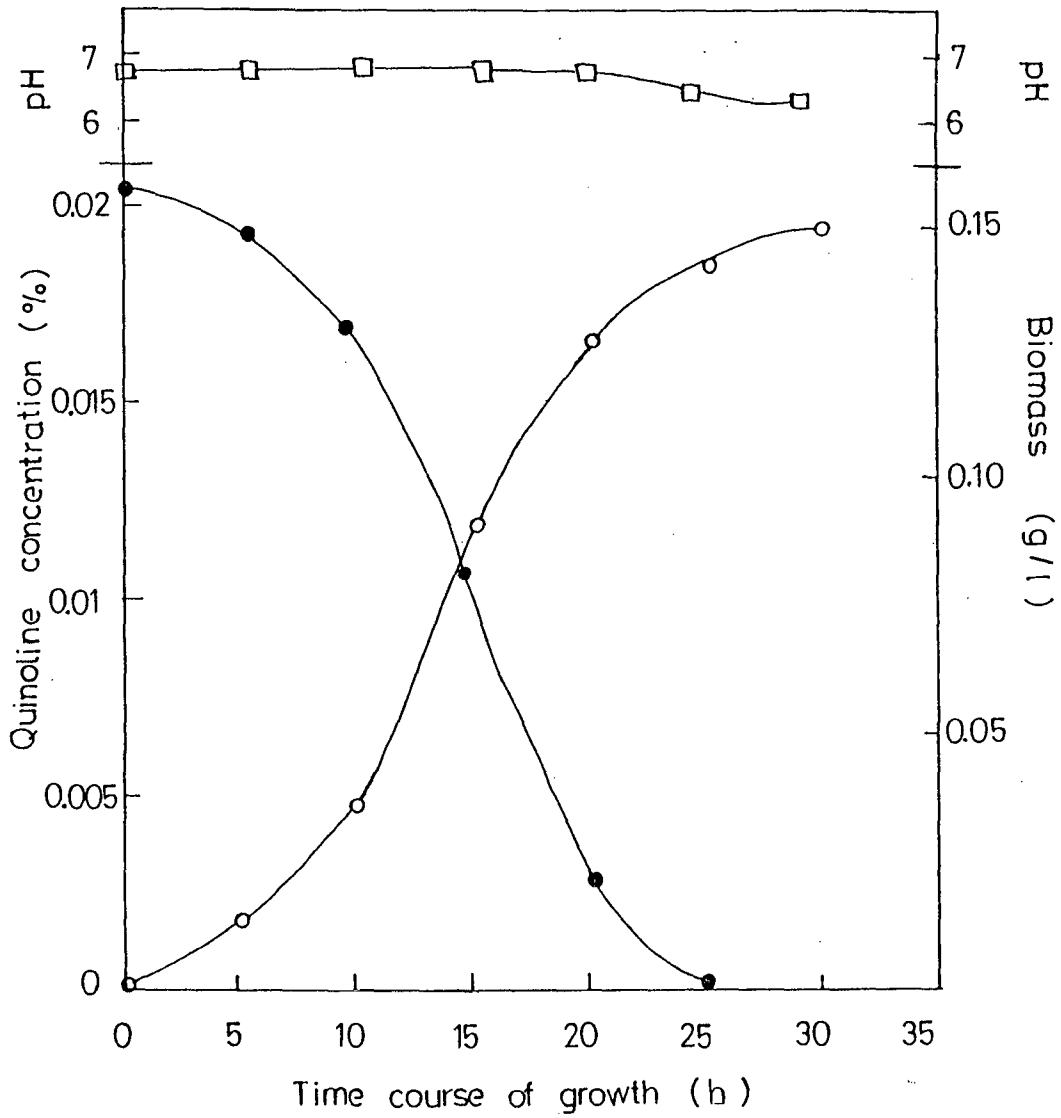


Fig. 3.1 : Batch culture of strain GU104; growth (-o-) indicated by absorbance at 550 nm was accompanied by depletion of quinoline (-●-). No drastic change in pH (□) was observed during growth.

Resting cell preparations showed a rapid depletion of quinoline with 100% removal of quinoline within 1.5h. Quinoline depletion was followed at 299nm, and TLC analysis of the extracts as per **Materials and Methods** in **Chapter IV**, indicated the appearance of intermediates of quinoline biodegradation. Observation of intermediates was characteristic of biodegradation of quinoline. The quinoline conversion rate calculated for resting cells was 0.25g/gh.

Continuous cultivation of *Pseudomonas* strain GU104

In continuous culture experiments, cultivation conditions were similar to those of batch experiments as mentioned above. Batch culture was initiated by inoculating 50ml of the primary inoculum and continued with batchwise addition of quinoline (0.02%) until a final biomass of approximately 0.3g/l was obtained. The fermentor was then operated in the continuous mode by means of a peristaltic pump at various flow rates to give dilution rates of 0.02 to $0.3h^{-1}$. The quinoline concentration in the inlet medium was 0.5g/l.

Pseudomonas strain GU104 was cultivated continuously for several weeks. The dilution rate was varied over a range of 0.02 to $0.3h^{-1}$. In all cases the aeration rate of 0.6lpm and agitation speed of 200rpm were kept constant. Since no appreciable pH change was observed during growth there was no requirement of artificial regulation of pH. The growth in continuous culture showed the advent of steady state condition by adjustment of the cell mass as per the dilution rate after initial drop in density of cells. The same

behaviour was observed for all the dilution rates. Eight different steady states were studied upto a dilution rate of $0.2h^{-1}$. At steady states, cell mass reached a stable value as observed from constant absorbance at 550nm as well as constant dry weight of cells per litre (Fig. 3.2). Constant rate of quinoline utilization was also seen with more than 98% of the quinoline being degraded upto dilution rate $0.25h^{-1}$. As seen in Figure 3.3, the quinoline, concentration remaining in the medium ranged from 0 to 7ppm at different dilution rates. At very low dilution rates quinoline was not detected in the medium. The maximum degradation rate of quinoline that could be achieved under steady state conditions at a dilution rate of $D= 0.25h^{-1}$ was 0.37g/gh. The operational data, including calculated yield coefficients and specific quinoline conversion rates are summarized in Table 3.1.

The yield coefficient (Y) can be split into a "true yield" and a maintenance coefficient (m), expressed as grams substrate per gram biomass per hour (g/gh). The true yield is the value which would be obtained in the absence of any maintenance requirement. To determine the maintenance requirement, the specific quinoline conversion rates (q) were plotted against the dilution rate (D), which equals the specific growth rate (μ) under steady state conditions. From Figure 3.4, the maintenance requirement for quinoline (m) was calculated from the following equation:

$$q = (1/Y)\mu + m$$

From this the maintenance coefficient was found to be 0.006 g/gh.

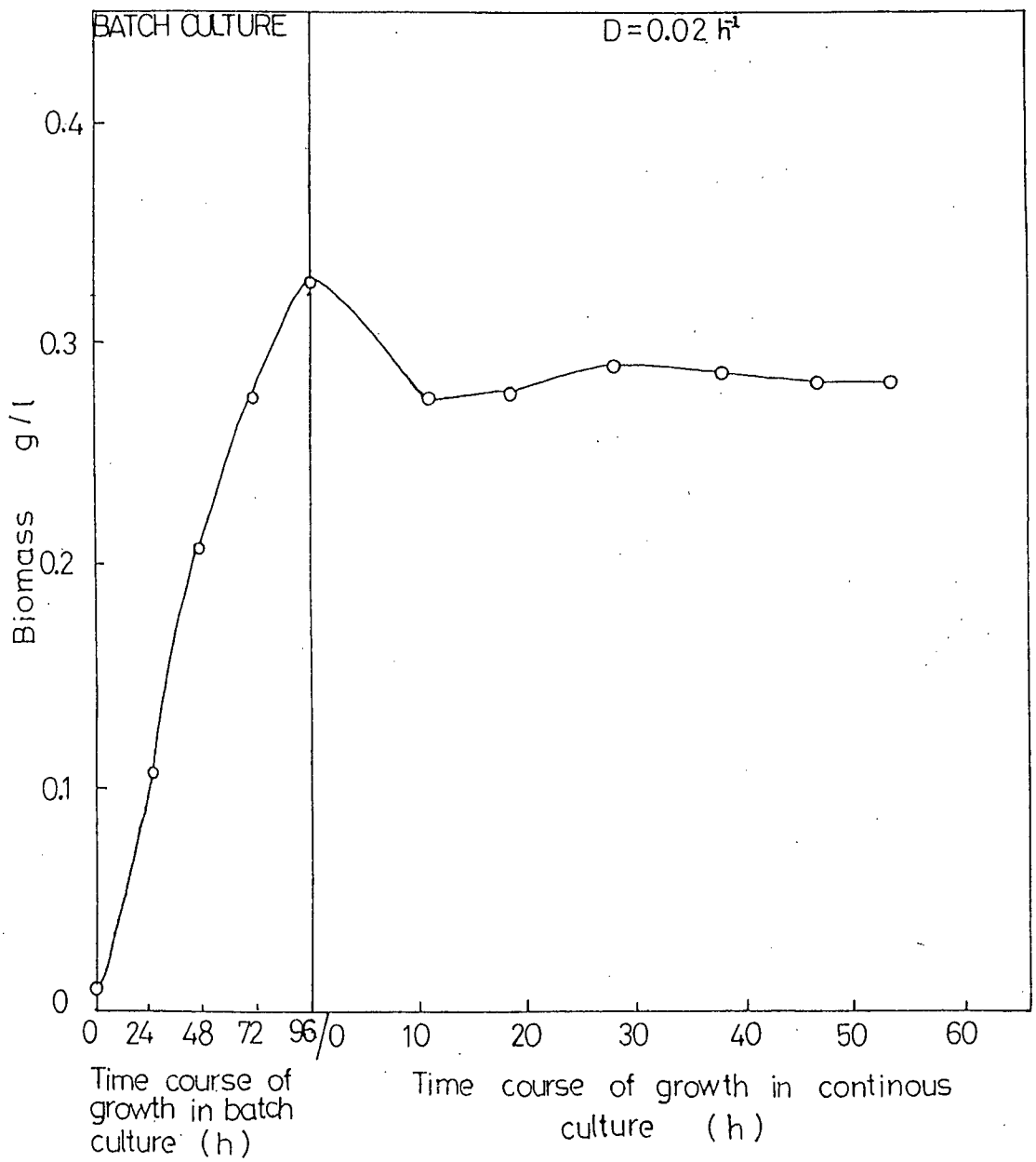


Fig. 3.2 : Steady state of growth of strain GU104 at D = 0.02h^{-1}in a chemostat with quinoline as limiting substrate. The culture was grown in batch with periodic addition of quinoline (0.02%) to achieve a biomass of 0.32g/l before continuous culture was initiated.

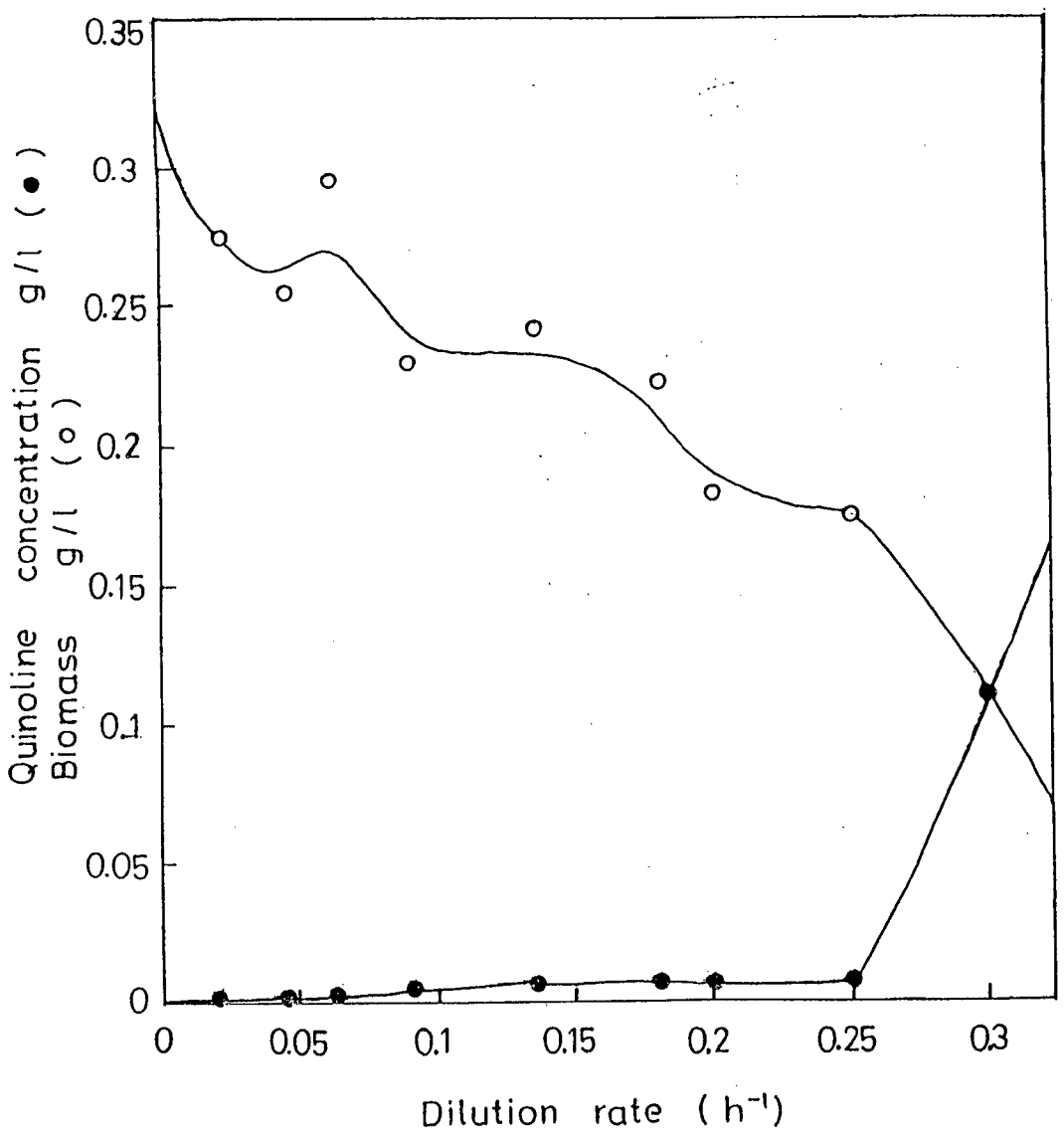


Fig. 3.3 : Biomass, Xg/l (o), obtained at steady states at various dilution rates, D (h^{-1}) and residual quinoline, Sg/l (●) in the fermentation broth.

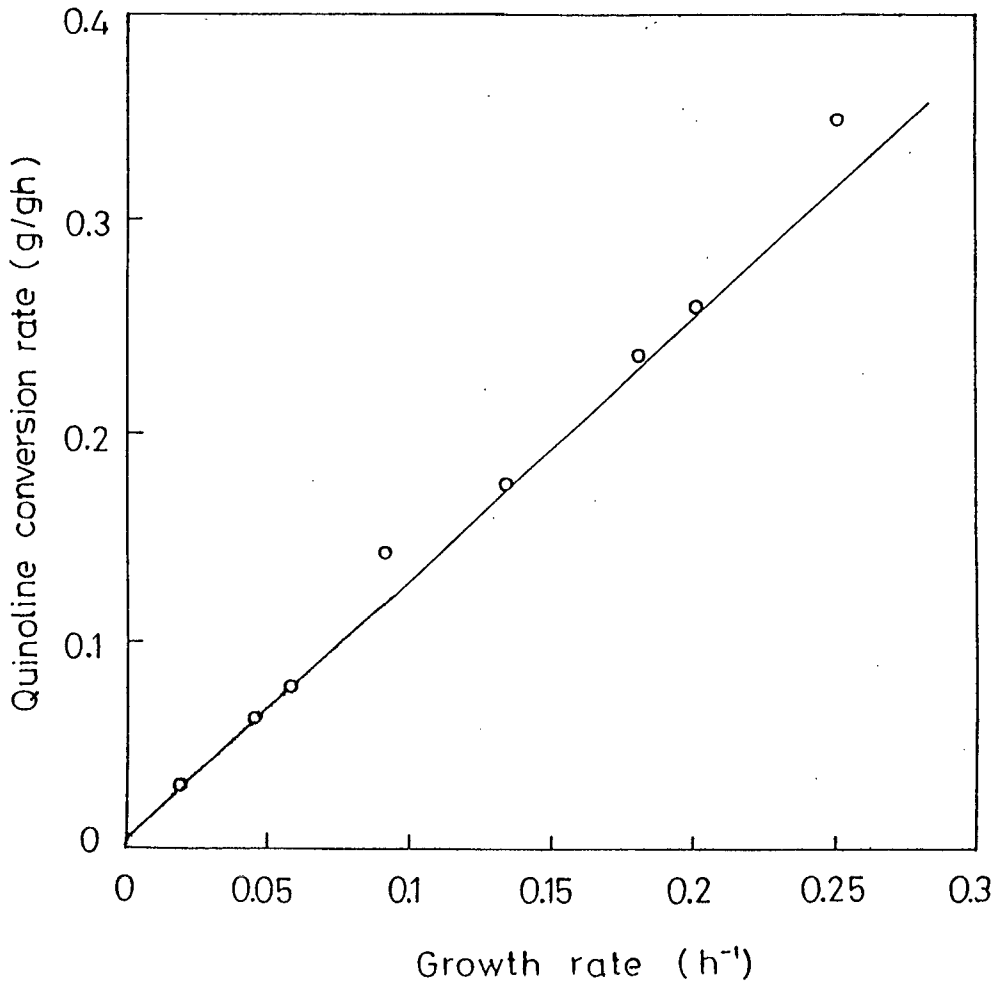


Fig. 3.4 : Determination of the maintainence requirement "m" for quinoline, given by the y intercept of the plot of quinoline conversion rate (q) v/s growth rate (μ). "m" is calculated from the equation, $q = (1/Y)\,\mu + m$ to be 0.006g/gh.

A further increase in dilution rate from $0.25h^{-1}$ to $0.3h^{-1}$ caused an exponential decrease in bacterial cell mass indicating washout. Correspondingly, this led to an accumulation of quinoline in the medium. From the measured values for biomass and quinoline, the specific growth rate, μ, was calculated by,

$$\mu = (1/X).\ dx/dt + D$$

Figure 3.5 gives an analysis of turnover of quinoline during critical washout period at dilution rates of $0.3h^{-1}$. At critical washout, the accumulation of quinoline was accompanied by a decrease in the specific growth rate and biomass. A very low quinoline conversion rate however was evident. Inhibition of quinoline degradation at high quinoline concentration was modelled by a Haldane's expression which incorporates a second order inhibitory term K_i into the Michaelis-menton expression. The substrate inhibition model proposed by Haldane is as follows,

$$\mu = \mu_{max} \frac{S}{K_s + S + (S^2/K_i)}$$

Assuming $S >> K_i$,

$$1/\mu = \{1/(K_i.\mu_{max})\}\ S + 1/\mu_{max}$$

Figure 3.6 shows the application of the linearized Haldane function to the data of Figure 3.5 from which the values of K_i and μ_{max} were calculated to be 95mg/l and $0.29h^{-1}$, respectively.

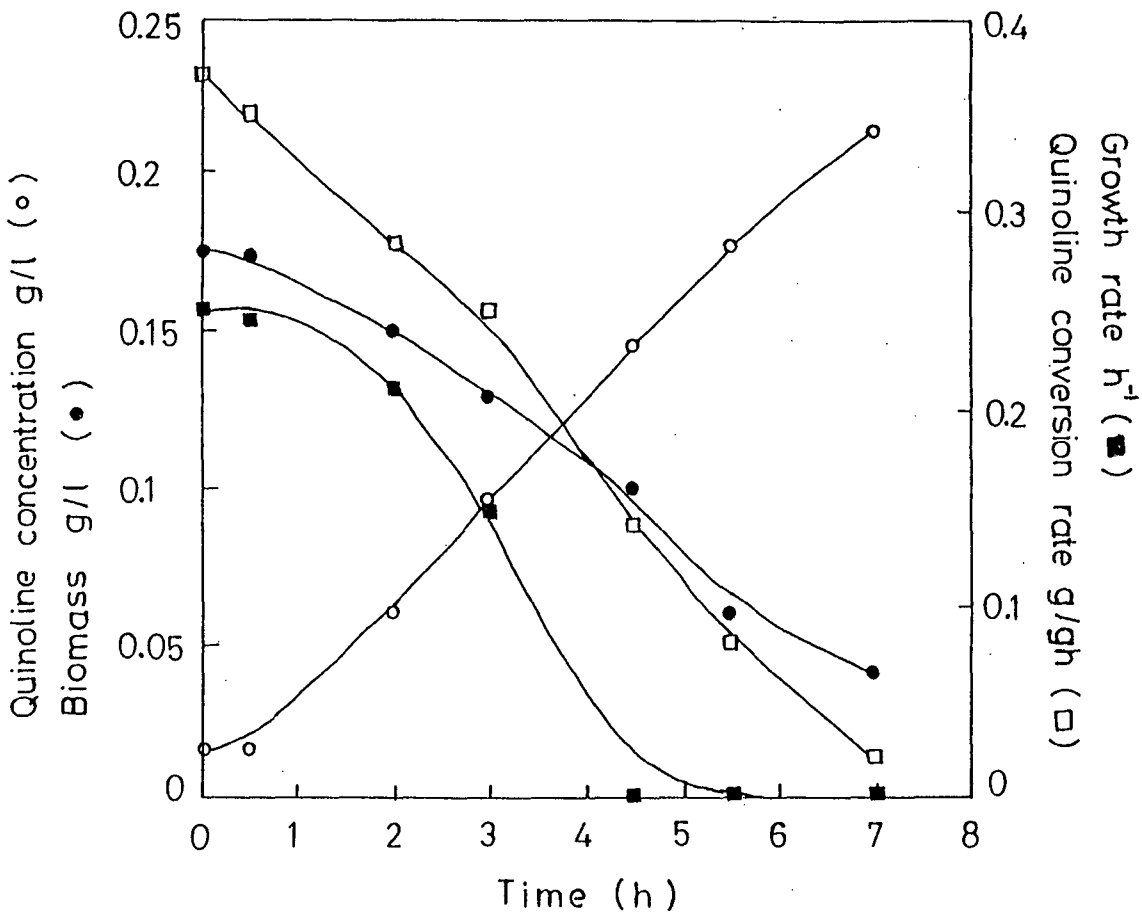


Fig. 3.5 : Analysis of growth of strain GU104 during critical washout in the chemostat at dilution rate (D) of 0.3h-1 with quinoline as limiting substrate. Salient features are the decrease in quinoline conversion rate, q (□), accumulation of quinoline (o), decrease in specific growth rate of GU104 (■) and washout of biomass (●).

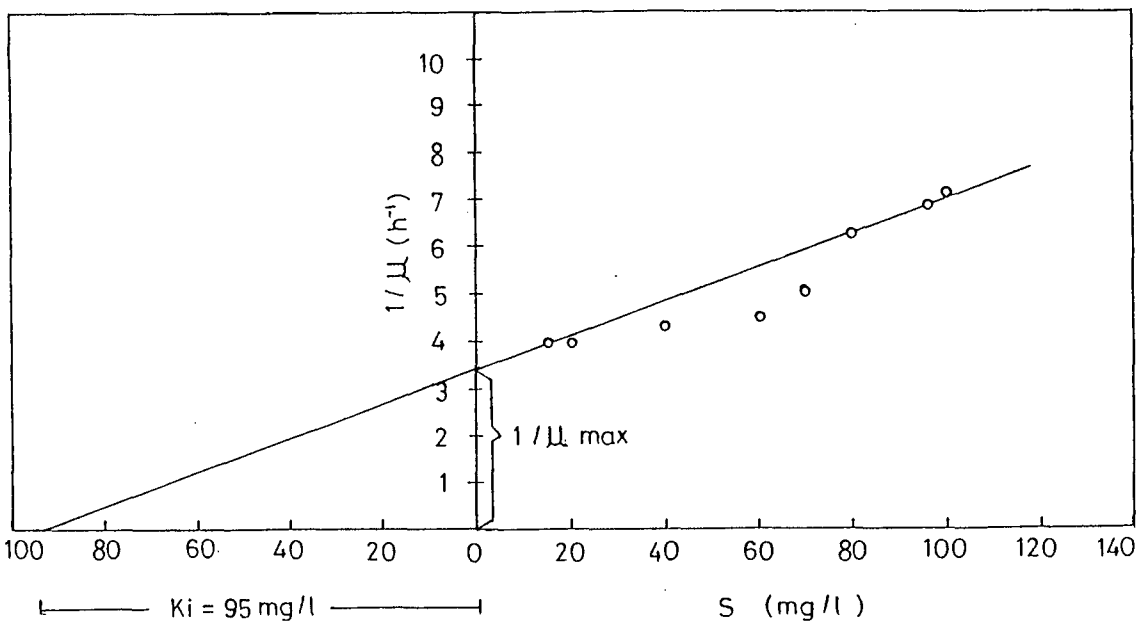


Fig. 3.6 : Determination of the inhibitory constant for quinoline (K_i) and maximum growth rate (μ_{max}) for strain GU104 growing on quinoline.

From Figure 3.5 it can be seen that a concentration of quinoline above 0.015g/l inhibits the specific growth rate. By setting the first derivative of the Haldane's expression to 0, the following relation is obtained,

$$S^* = (K_i.K_s)^{1/2}$$

where, K_s is saturation constant for quinoline and expressed as mg/l. This gives the quinoline concentration at highest attainable growth rate (S*). Assuming that S* would be equal to lower than 15 mg/l, K_s can be calculated as < 2.3 mg/l. Setting K_s= 2.3 mg/l, μ_{max}^* which is the maximum attainable growth rate is found to be 0.296h^{-1}.

Immobilization of cells of strain GU104

Cells of strain GU104 were immobilized by the gel entrapment method using calcium alginate beads. The beads were stable for upto 45 days and the cells gave high quinoline conversion rates of 0.78g/gh. Substrate was added to the flasks daily and the entrapped cells could tolerate upto 0.05% of quinoline without any toxic or inhibitory effects. The beads in the test flasks turned dark yellow as compared to the control in which the substrate was not added. The medium however remained clear. The beads could be stored by draining out the media and washing with buffer and refrigerating. In this manner the cells remained stable upto 45 days and gave the same quinoline conversion rates.

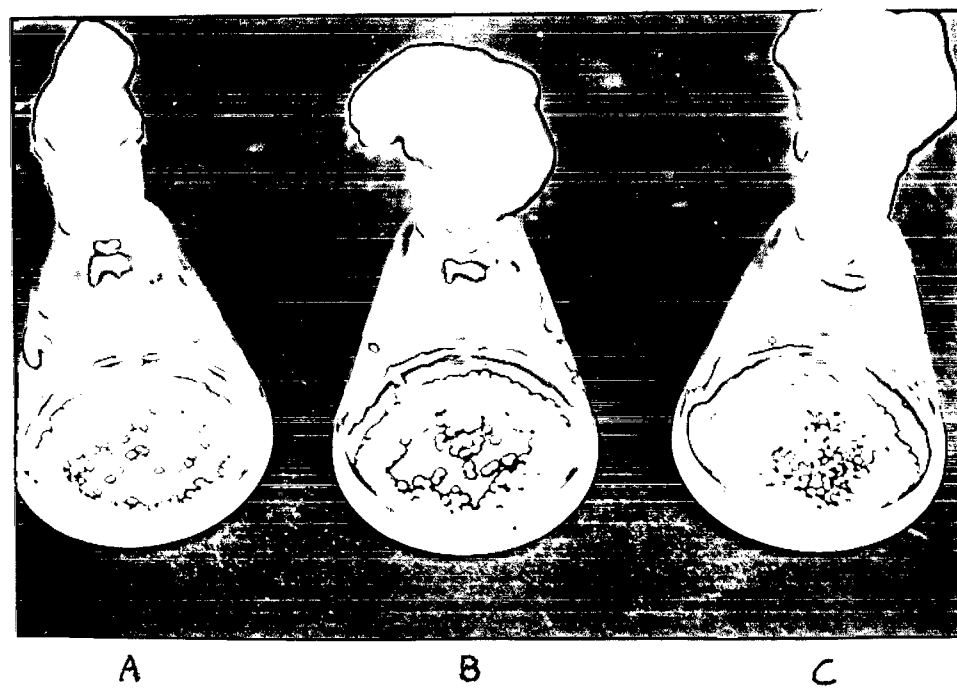


Fig.3.7 Cells of strain GU104 immobilized using calcium alginate beads; Flask B (control) : immobilized cells in ASW medium without quinoline; Flasks A and C (Tests): immobilized cells in ASW medium containing quinoline.

DISCUSSION

The widespread use of quinoline in industry, as well as its presence in both natural and synthetic crudes and its relatively high solubility in water makes it a cause for environmental concern. In the last decade, there have been various studies on the detection of azaarenes such as, quinoline, isoquinoline and acridine in ground water and aquatic habitats (Ondrus and Steinheimer, 1990; Pereira *et al.*, 1987) and their effects on aquatic life has been investigated.

Pseudomonas sp. strain GU104 isolated from marine water samples had the ability to utilize quinoline as sole carbon and nitrogen source. In addition it could also utilize various methyl-quinolines, as well as components in crude petroleum, diesel and kerosene. A great number of marine bacteria are capable of a wide variety of metabolic reactions and consequently, can utilize a wide variety of nutrients. Our studies in batch cultures indicated that strain GU104 could grow at concentration upto 0.03% quinoline. In order to check the practical feasibility of using strain GU104 in bioremediation of aquatic wastes containing quinoline, simulated conditions in batch and continuous cultures were established with quinoline as limiting substrate.

The removal of quinoline from the medium in batch culture was apparent from the UV spectra of cell free fermentation broths which showed the depletion of the quinoline peak at 299nm. There was no drastic

change in pH with growth and thin layer chromatograms showing the formation of intermediates. Resting cells pregrown on quinoline, showed a more rapid removal of quinoline at a rate of 0.25g/gh.

One means of assessing the bioremediation potential of GU104 is to subject the culture for continuous removal of toxic quinoline. Stimulation of such a situation was done by continuous cultivation of strain GU104 in a bench top chemostat which showed fairly high conversion rates of upto $0.25h^{-1}$, with the highest conversion rate being $0.37g/h^{-1}$ at a dilution rate of $0.25h^{-1}$. The maximum yield coefficient obtained was 0.76 and the maintainance coefficient was very low (0.006g/gh). The maintainance energy requirement of various bacteria degrading aromatic compounds has been found to be quite low Hill and Robinson (1975), for example, estimated no significant maintainence energy requirement for *P. putida* ATCC 17484 grown on phenol. The marine environment is a nutrient deficient system, and indigenous bacteria are believed to exist in a state of physiological stress (Truex *et al.*, 1992). This state is characterised by low energy reserves, a small size and a very low maintenance energy requirement or rate of respiration (Morita, 1988). The low maintenance energy requirements would explain the fact that even at low growth rates, there is no significant decrease in yield (Table 3.1).

On further increasing the dilution rate, the quinoline began accumulating rapidly in the chemostat. This led to an inhibition of growth and

decrease in the specific growth rate (μ). Consequently, there was a washout of bacterial biomass. The maximum specific growth rate obtained for the marine strain GU104 was $0.29h^{-1}$. After the growth rate fell to zero a very low rate of quinoline conversion was still seen. This is attributed to the fact that the enzymes that transform quinoline probably have a lower inhibition concentration for quinoline than the cells. The saturation constant for quinoline K_s would be expected to be low considering the low concentration of quinoline inhibiting growth. K_s was found to be 2.3mg/l and K_i or the inhibition constant was 95mg/l. The biodegradation of quinoline in continuous culture using a chemostat has been reported thus far by Miethling *et al.* (1993) using *Comomonas acidovorans* DSM 6426 isolated from garden soil. They reported a K_s of 1.45mg/l, a K_i of 69mg/l and a μ_{max} of $0.48h^{-1}$. A conversion rate of $0.53g/gh^{-1}$ was reported for *C. acidovorans* DSM 6426.

Yet another means of application of the strain GU104 is by subjecting its immobilized cells to achieve continuous removal of quinoline. Alginates have been used to immobilize whole cells of *Candida tropicalis* for phenol degradation (Hackel *et al.*, 1975). In our studies, strain GU104 was entrapped in Ca-alginate beads and was employed to transform quinoline in shake flasks (< 100rpm). The fact that the suspension media did not get extensively turbid until 2-3 weeks of incubation suggest that the beads were mechanically stable. The activity of the immobilized cells with respect to quinoline conversion as compared to that of free cells was found to be very high. This has been reported with methods where natural high polymers are

used to prepare the entrapment network and applies to methods of ionotropic gelation (carrageenan, alginate) and has been demonstrated for microbial cells (Klein and Vorlop, 1985). The cells of strain GU104 are able to withstand and metabolize higher concentrations of quinoline than that tolerated by free cells. Storage stability of the cells was also enhanced due to immobilisation. Even after 45 days of storage, the cells remained viable and were able to transform quinoline. On prolonged storage however the suspension turned turbid owing to the disintegration of the beads. Immobilization of cells degrading quinoline by colonization using porous glass beads as support has been studied by Ulonska *et al.*, (1995). The immobilized cells remained viable and active and could survive quinoline overloads and could bring about a complete conversion of quinoline as long as the specific quinoline feed rate did not exceed maximum degradation rate. Similar results were also observed by Rothenburger and Atlas (1993). The cells could achieve faster conversion rates of quinoline and higher substrate concentration were tolerated.

In conclusion strain GU104 appears to hold promise in the biological treatment of wastes and bioremediation of contaminated aquatic systems containing quinoline, owing to the high conversion rates of quinoline in continuous culture. For effective removal, the reactors should be operated at quinoline concentrations below 15mg/l to avoid inhibition. Furthermore, the use of immobilized cells holds promise in bringing about an even faster quinoline mineralization.

CHAPTER IV

MODE OF BIODEGRADATION OF QUINOLINE BY STRAIN GU104

Chapters II and **III** have dealt with the isolation of quinoline degrading bacteria from marine environments, characterization of strain GU104 and the kinetics of quinoline biodegradation in batch and continuous cultures. There have been reports of bacteria that transform and degrade quinoline, and it has been found that no unique pathway exists for the metabolism of quinoline. It is thus essential to understand the sequence of metabolic steps of quinoline degradation in different environmental backgrounds. *Pseudomonas* sp. strain GU104 being a typically marine microorganism provides scope for investigation of the evolution of the metabolic pathway of quinoline in marine bacteria. Attempts have been made in this chapter therefore, to identify the intermediates of quinoline metabolism, the enzymes involved in quinoline degradation in this strain, as well as to study the genetic implications of quinoline biodegradation.

MATERIALS AND METHODS

Chemicals

Acrylamide, bis acrylamide, INT, Sephadex G-25(M) were obtained from Sigma Chemical Co., USA. Cesium chloride was of analytical grade and purchased from SRL-SISCO Chemical Lab. Quinoline and its derivatives were obtained from Sigma Chemical Co. and Aldrich Co. Inc., USA. N-methyl, N'-nitro, N-nitrosoguanidine was a gift from Dr. K. Dharmalingham, MKU, ~~Madras~~ Madurai. Acridine orange and agarose were obtained from Hi-Media

Ltd. All other chemicals used for preparation of buffers and media were either of AR or GR grade and purchased from local suppliers.

Large scale cultivation of strain GU104

Large scale cultivation of strain GU104 in ASW containing 0.02% quinoline was achieved in a 1.6l bench top Bioflow fermentor (New Brunswick, USA) with a working volume of 1l. The cultivation temperature was 28-30°C and the pH was maintained at 6.8 to 7.0. The agitation speed was set at 200 rpm and aeration was carried out at a rate of 0.4 lpm.

Extraction and analysis of intermediates of quinoline biodegradation

An aliquot of the culture supernatant obtained by centrifugation at 8000 rpm for 20 mins at 4°C (Sorval Centrifuge RC5C), was extracted several times with one tenth volume ethyl acetate. The aqueous part was acidified with HCl to pH 2.0 and reextracted with ethyl acetate. The ethyl acetate extracts were pooled, dried over anhydrous sodium sulphate and concentrated by evaporation at 50°C. The concentrated extract was applied and analysed by paper chromatography using a solvent system of 5% sodium formate : formic acid (200 : 1) and by thin layer chromatography (TLC) using silica gel G plates (0.5 mm). After development, using solvent systems chloroform : methanol (50:2.5) or toluene : dioxane : methanol : acetic acid (72:16:20:15), the compounds were detected under UV-illumination. Paper chromatograms were sprayed with Gibbs reagent to detect phenolic compounds.

Preparative chromatography

Preparative TLCs were performed on 2mm thick silica gel plates and the ethyl acetate extract was spotted in the form of a fine band. For isolation of the metabolites from TLC, the metabolite band (after visualizing under UV) was scraped off, extracted twice with warm ethyl acetate, filtered through whatman paper, concentrated as described above and analysed spectroscopically. Column chromatography was used to obtain significant quantities of intermediates for IR analysis. A column (30 x 1.5cm) was packed with silica gel (grade AR) and 1-2ml of concentrated ethyl acetate extract from 200ml of culture supernatant was adsorbed onto silica gel and layered over the column to form a band 3mm thick. The column was then eluted with chloroform followed by chloroform : methanol (9:1) and gradually increasing the polarity to 1:1 chloroform : methanol. The solvent was evaporated and tubes were checked for the presence of compounds by rapid TLC's performed on microscopic slides. Samples that showed a single spot with identical Rf values were pooled, concentrated by evaporation and crystallised.

Gas Chromatography

Gas chromatographic analysis of the ethyl acetate extracts was done on Shimadzu GC-14B gas chromatograph using a DB1 coated fused silica capillary column (30m x 0.25mm) attached to a flame ionization detector, programmed for initial temperature 60°C to 300°C increasing with 6°C per minute. Nitrogen was used as carrier gas. The chromatograms were

developed after correcting the volumes of extracts with appropriate dilution factor.

Spectroscopic analysis

UV analysis

Quantitative determination of quinoline and 2-hydroxyquinoline in the culture broth was done based on their extinction coefficient at 299nm ($\log\varepsilon$ = 3.51) and 328nm ($\log\varepsilon$ = 3.84), respectively.

IR analysis

IR spectra of the isolated metabolites was recorded as KBr pellet using a FT-IR spectrophotometer (JASCO FT/IR 300E).

Oxygen uptake analysis

Exponentially growing cells (1l) of *Pseudomonas* sp. strain GU104 were harvested by centrifuging at 8000 rpm for 10min at 4°C (Sorval RC5C Centrifuge). The pellets were washed with 50mM phosphate buffer (pH 7.0) and resuspended in the same buffer to give a final absorbance of 5.0 at 550nm. The cells were maintained at 4°C at all times by immersing in an ice bath. Rates of oxygen uptake by such resting cells of strain GU104 in presence of various aromatic heterocyclic and other substrates were measured on a Gilson 5/6 oxygraph at 30°C with a Clarke electrode. 100µl of cell suspension was added to 1.6ml of oxygen saturated phosphate buffer and respective substrate added. The hydroxy- and methyl- derivatives of quinoline, including other aromatics such as naphthalene, benzoate, pyridine,

indole and carbazole were used at a concentration of 0.01% (w/v). Glucose and lactate were used at a concentration of 0.1% (w/v). Washed cells were treated with Chloramphenicol at a concentration of 100µg/ml for 2h for induction experiments. Rates of oxygen uptake were corrected for the endogenous respiration of cells and expressed as nmoles of oxygen/min/mg cells.

Washed cells prepared from strain GU104 pregrown in ASW containing 0.02% quinoline were used to calculate oxygen uptake rates using different concentrations of quinoline. The K_s and V_{max} values were estimated from the Linweaver-Burke plot. Resting cells were also used to determine the moles of oxygen consumed per mole of substrate using quinoline and 2-hydroxyquinoline. All values reported are means of duplicate measurements.

Oxygen uptake studies with starving cells of strain GU104, cured cells of strain GU104 as well as metabolically blocked mutants were also determined by the above method.

Preparation of starving cells

Short term and long term starving cells of strain GU104 were produced according to a modified protocol of Truex *et al.* (1992). Short Term Starving (STS) cells were produced by growing the culture in ASW and supplied with 0.1% sodium lactate to stationary phase, harvesting the cells by centrifugation, washing and resuspending the cells in sterile ASW media. To

reduce endogenous nutrient reserves and sorbed nutrients, the cells were rested in ASW (30°C / 150 rpm) for 2 days after the washing procedure. Cells were then concentrated by centrifugation and resuspended in sterile ASW (A_{550} = 5.0) and used for STS experiments.

For Long Term Starving (LTS) rested cells (50ml) were washed as described above, transferred to 250ml Erlenmeyer flasks and stored at 10-16°C for 2 months, with intermittent aeration using an aerator fitted with an air filter. Aliquots from starvation flasks were concentrated by centrifugation to the appropriate density (A_{550} = 5.0) and used as inoculum in LTS experiments.

Isolation of mutants of strain GU104

Washed cells were suspended in a citrate-phosphate buffer (37mM citrate, 127mM Na_2HPO_4, pH 6.0) and adjusted to an absorbance of 1.0 at 550nm. The suspension was diluted with an NTG solution (final concentration 100µg/ml). After 45 to 60 minutes cells were plated in appropriate dilutions on ASW agar containing lactate (0.2%). After incubation at 30°C, colonies were replica plated on ASW agar containing lactate and quinoline respectively. Those colonies which did not grow on quinoline were picked from lactate replica plates subcultured on lactate-ASW media and processed as metabolically blocked mutants.

Curing of plasmid DNA

A stock solution of acridine orange was prepared in sterile distilled water and added to exponentially growing cultures in ASW medium containing lactate at a concentration of 20µg/ml. The initial bacterial count was 10^4 cell/ml. Incubation was at 30°C on rotary shaker (200 rpm) for 24h. Appropriately diluted broth was plated out on ASW-lactate agar, incubated overnight and this served as the master plate. The colonies were replica plated on ASW agar plates containing quinoline and screened for the inability to metabolize quinoline. Such colonies were then subcultured and subjected to plasmid isolation and agarose gel electrophoresis.

Plasmid Isolation

Plasmid DNA was isolated according to the method of Kado and Liu (1981). The plasmids were further purified by cesium chloride-ethidium bromide density gradient ultracentrifugation as mentioned by Sambrook *et al.* (1989). The plasmid band was visualised under UV, extracted with a syringe and the DNA was precipitated after removal of ethdium bromide.

Preparation of crude extract for enzyme studies

Wet packed cells grown on appropriate substrates were suspended in an equal volume of 50mM Tris. HCl buffer pH 8.0 and disrupted by ultrasonication for 10 pulses of 20 seconds each at 4°C by a Vibra Cell Sonicator (Sonic and Material Inc. Connecticut, USA). Cell debris was removed by centrifugation at 12,000rpm for 20min at 4°C on Sorval

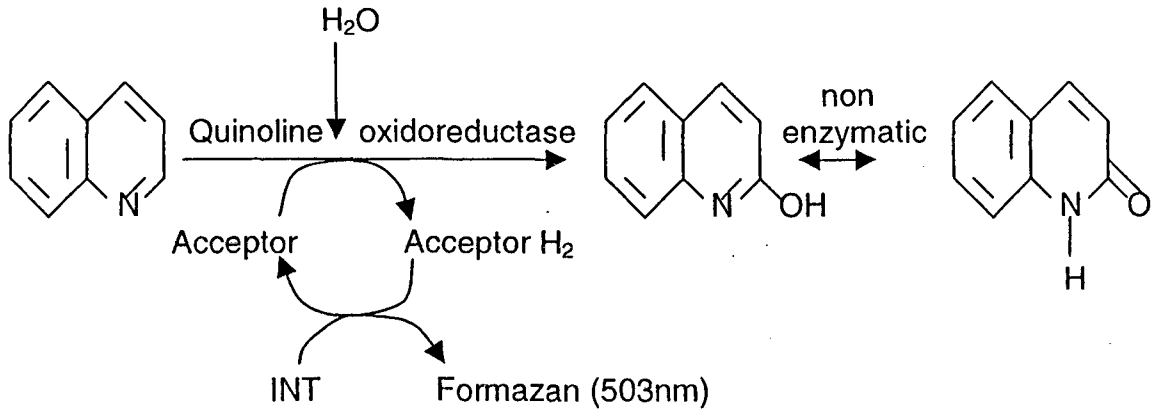
refrigerated Centrifuge RC5C. The supernatant was used as the crude extract for further investigations.

Purification of quinoline oxidoreductase

The crude extract of the enzyme was precipitated with ammonium sulphate and the precipitate formed at 35-55% saturation was taken. It was dissolved in Tris.HCl buffer (pH 8.0) and passed through Sephadex G-25 M column for rapid desalting and group separation. The column had a diameter of 1.5cm and a bed height of 6cm. It was equilibrated with 25ml of 100mM of Tris.HCl pH 8.0 and eluted with the same at a flow rate of 0.5 ml/min. The volume of the fractions collected was 1ml. The protein was eluted in the void volume (Wilson, 1994). Active fractions were pooled and subjected to heat precipitation at 55°C for 5min in a circulatory water bath (LKB Bomma 2219 Multitemp II).

Quinoline oxidoreductase assay

Quinoline oxidoreductase activity was assayed by measuring the change in absorbance at 503nm due to the reduction of INT to formazan (Peschke and Lingens, 1991).



The standard assay mixture contained; 730µl test buffer (100 mM Tris/HCl, pH 8, with 0.3% Triton X100), 200µl INT (2.5mM in double distilled water), 20µl phenazine methosulphate (PMS) (2.3mM in test buffer), 50µl quinoline (100mM in ethanol) and 50µl of crude extract or enzyme solution. The reaction was started by addition of enzyme and performed at 25°C. One unit of enzyme activity was defined as the amount of enzyme that produced 1µM formazan per minute using an absorption coefficient of $19.3x10^3$ $M^{-1}cm^{-1}$. Specific activity of quinoline oxidoreductase was expressed as unit per mg protein. Protein estimation was done by the Lowry method using bovine serum albumin as standard.

pH optimum for the enzyme was determined using the following buffers, 0.1M citric acid – 0.2M Na_2HPO_4 (pH 2.6 to 7.6), 0.1M each KCl – H_3BO_3 – NaOH (pH 8.0 to 10.2) and 0.1M each Na_2CO_3 – $NaHCO_3$ (pH 9.2 to 10.8). Temperature optimum was determined by incubating the enzyme assay mixture in a circulatory water bath (LKB Bomma 2219 Multitemp II).

In situ detection of quinoline oxidoreductase protein

Native polyacrylamide gel electrophoresis was carried out using 10% acrylamide and a mini gel running chamber (Bangalore Genei Pvt. Ltd.). Protein staining of the gels was performed with Coomassie Brilliant Blue R-250. For activity staining, gels were immersed in standard enzyme assay mixture.

Agarose gel electrophoresis for plasmid DNA

DNA samples were examined on horizontal gels containing 0.7 w/v electrophoresis grade agarose. The gels were prepared and run in TBE buffer. Electrophoresis was carried out in a submarine electrophoresis unit at 80V. The gels were stained by ethidium bromide solution (1µg/ml) and viewed on a UV transilluminator. The gels were intermittently checked under UV and electrophoresis was carried out until the plasmid bands were visualized.

RESULTS

Identification of intermediates of quinoline biodegradation

Ethyl acetate extracts of the fermentation broth of the culture grown on ASW with 0.02% quinoline revealed four spots on paper chromatography when sprayed with Gibbs reagent. However, the same extracts revealed the presence of 3 metabolites having R_f values 0.47, 0.62 and 0.78 on thin layer

chromatograms developed with chloroform : methanol (50:2.5) and visualized under UV. The spots were scraped off, eluted and scanned spectroscopically (Fig.4.1). Of these, spots having R_f values 0.62 and 0.47 were the most prominent. The R_f value of the former was equal to authentic 2-hydroxyquinoline (2HQ) and had an absorbance spectrum identical to the same. The spot with R_f = 0.47 could also be extracted from TLC plates and had an absorbance spectrum identical to that reported for 8-hydroxycoumarin (8HC) (Shukla, 1986). The third spot was extremely faint under UV, however its concentration increased slightly when arsenite was added to the medium at a concentration of 5mM. It was characterized as 2,8-dihydroxyquinoline (2,8 DHQ) based on its λ_{max} at 258nm. (Shukla, 1986).

In order to obtain substantial quantities of the intermediates, preparative TLCs and silica gel column chromatography were performed. Silica gel column eluted with chloroform : methanol (9:1) yielded substantial quantities of colourless needle shaped crystals with a melting point of 199-200°C. Figures 4.2 and 4.3 show UV and IR spectra of the crystals. The spectra were identical with authentic 2-hydroxyquinoline. The IR spectrum of the compound having UV absorbance spectrum identical to 8-hydroxycoumarin was also obtained (Fig.4.4) and the pattern of peaks obtained were found to be similar to those reported for 8-hydroxycoumarin (Shukla, 1986). However, an IR spectrum of the authentic sample could not be obtained for comparison due to lack of the same. 2,8-dihydroxyquinoline although identified based on its UV spectrum, could not be purified in

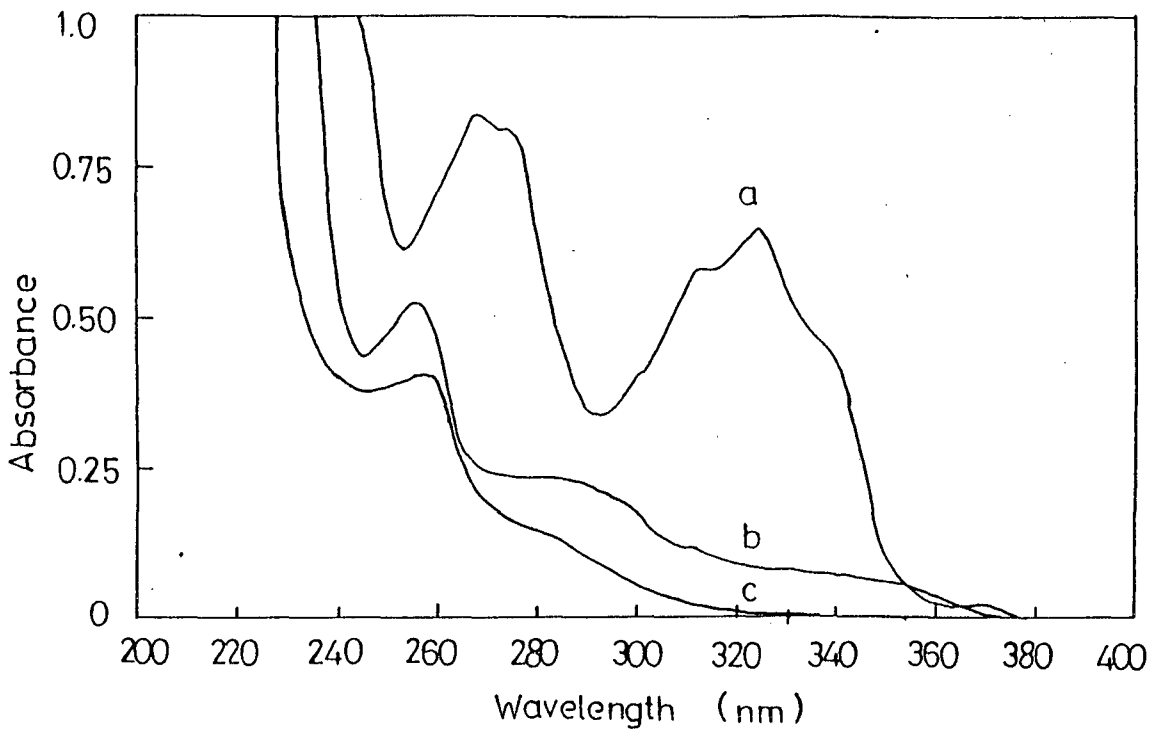


Fig. 4.1 : UV spectra of compounds extracted from preparative TLC; a: 2-hydroxyquinoline (λ_{max}= 328nm); b: 8-hydroxycoumarin (λ_{max}= 255nm); c: 2,8-dihydroxyquinoline (λ_{max}= 258nm).

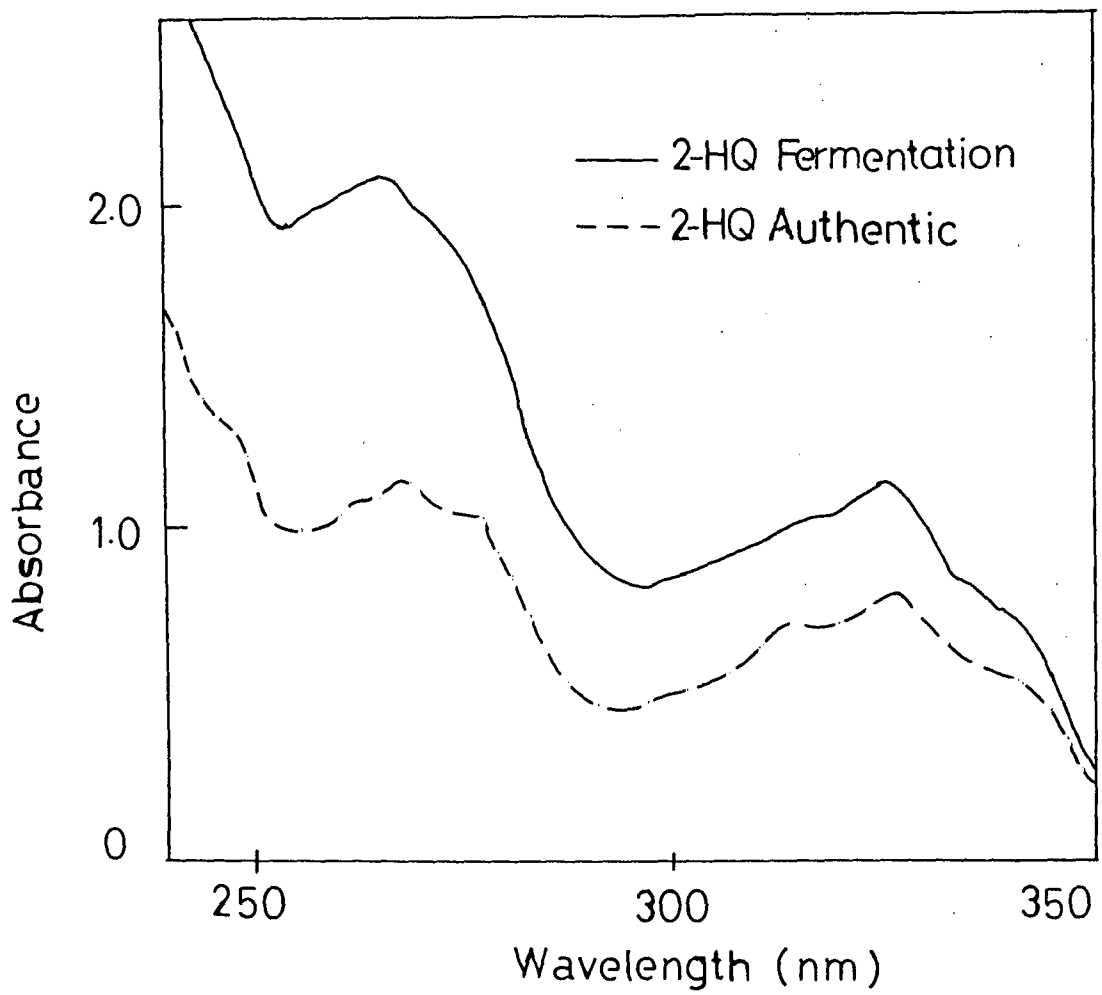


Fig.4.2: UV spectra of purified 2-hydroxyquinoline extracted from fermentation broth (—) and that of authentic 2-hydroxyquinoline (– –).

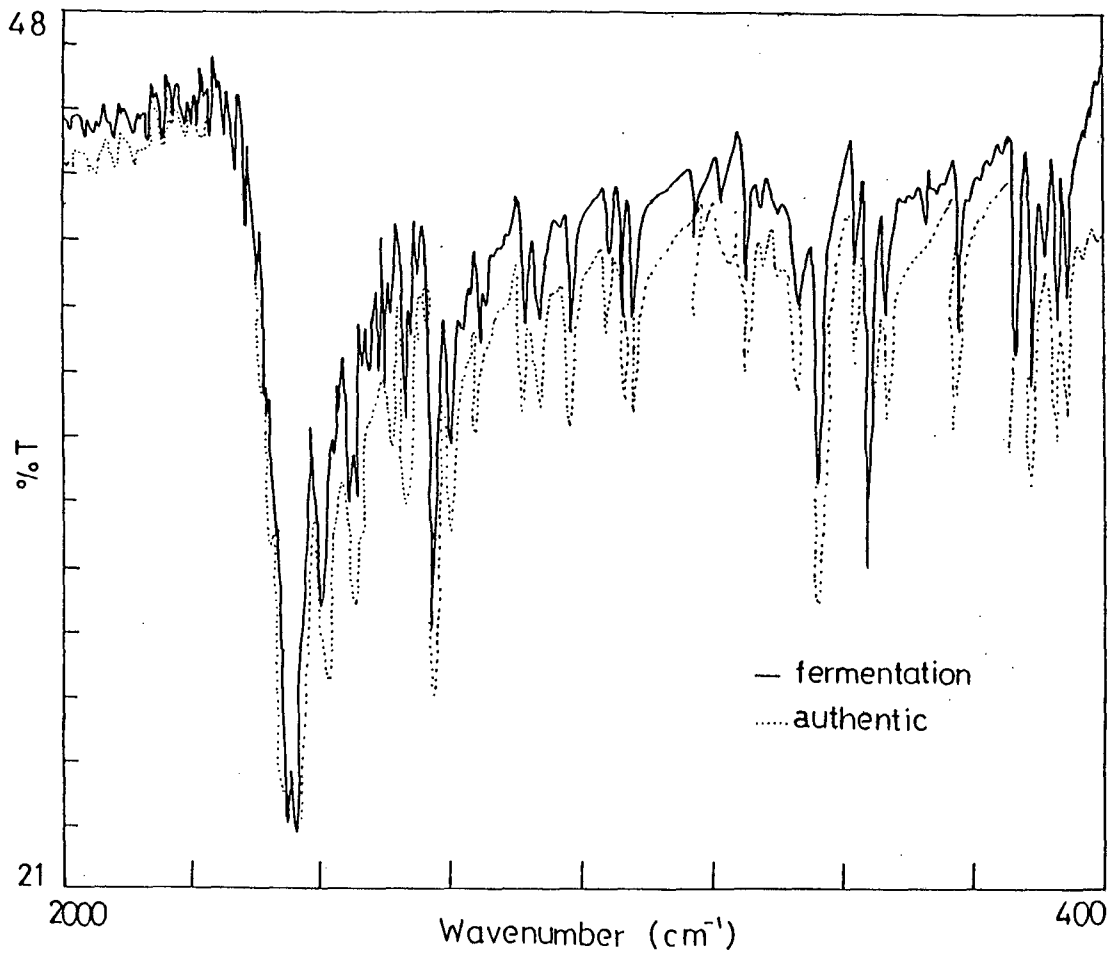


Fig.4.3 : IR spectra of purified 2-hydroxyquinoline extracted from fermentation broth (—) and that of authentic 2-hydroxyquinoline (·····).

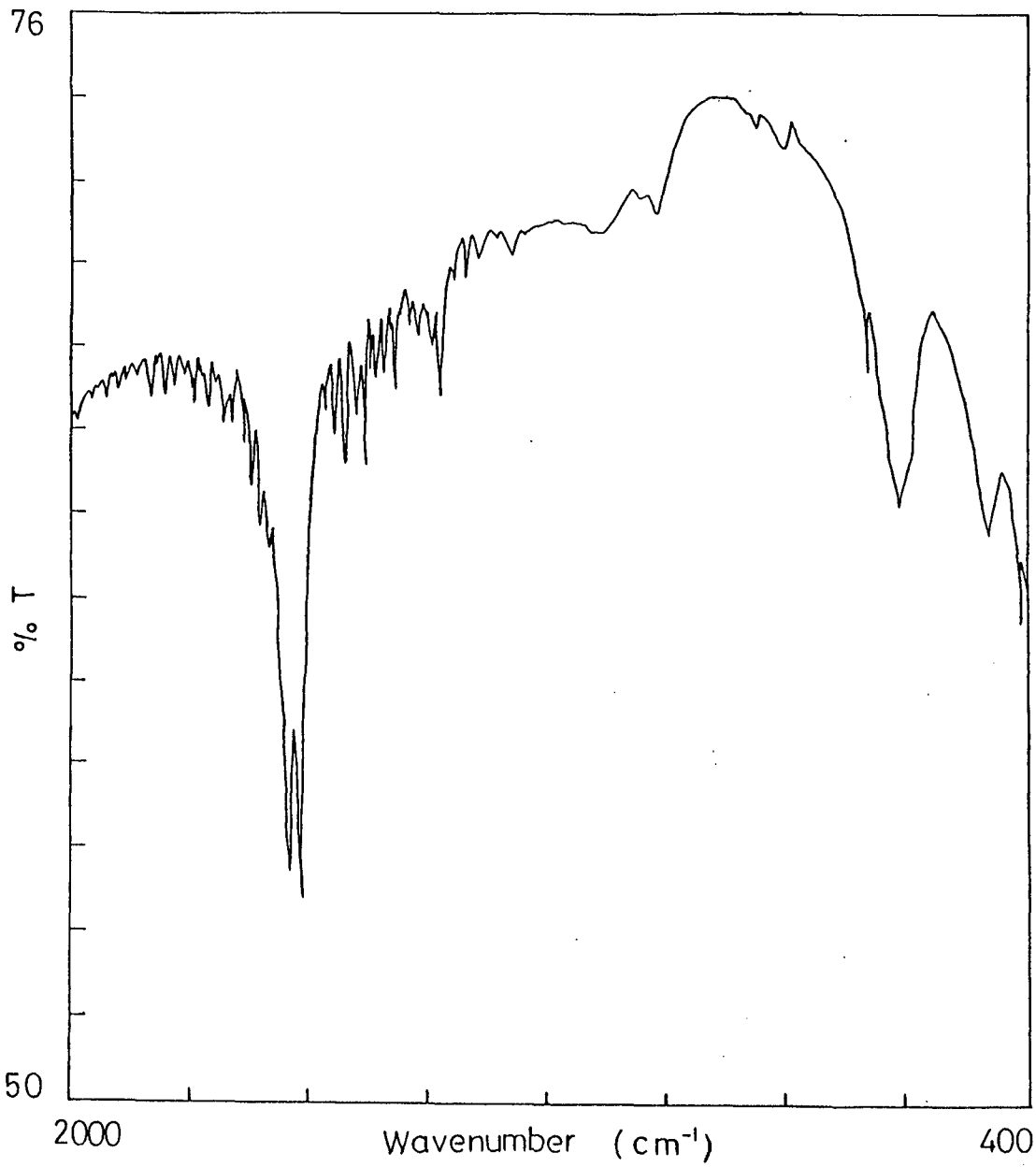


Fig. 4.4 : IR spectrum of purified 8-hydroxycoumarin.

sufficient quantities by column chromatography. Ethyl acetate extracts of cell free fermentation broths on GC analysis showed the presence of compounds having retention times identical to 2-hydroxyquinoline and 8-hydroxycoumarin (Fig.4.5).

Quantitative determination of 2-hydroxyquinoline in the culture broth was done based on its extinction coefficient at 328nm ($\log\varepsilon = 3.84$). Figure 4.6 shows that strain GU104 forms 2-hydroxyquinoline as a transient intermediate with maximum accumulation at 20h. 2-hydroxyquinoline was metabolized without accumulation of any of the pathway intermediates as determined by TLC and gas chromatography of ethyl acetate extracts of fermentation broths of cultures grown on 2-hydroxyquinoline.

Oxidation of substituted quinolines and related compounds by resting cells of strain GU104

To confirm the wide versatility of strain GU104, the resting cells were checked for their oxidation of several methyl- and hydroxymethyl- substituted quinolines and related heterocyclic and aromatic compounds. The results of oxygen uptake by strain GU104 pregrown in quinoline, with quinoline and other related compounds is shown in Table 4.1. Many hydroxy derivatives of quinoline including 2, 4, and 6-hydroxyquinoline were oxidized. Mono methyl quinolines particularly 3-, 4-, 7-, and 8- methylquinolines were oxidized. Naphthalene, pyridine and isoquinoline were oxidized at variable rates. Other heterocyclic compounds oxidized included indole and carbazole though the

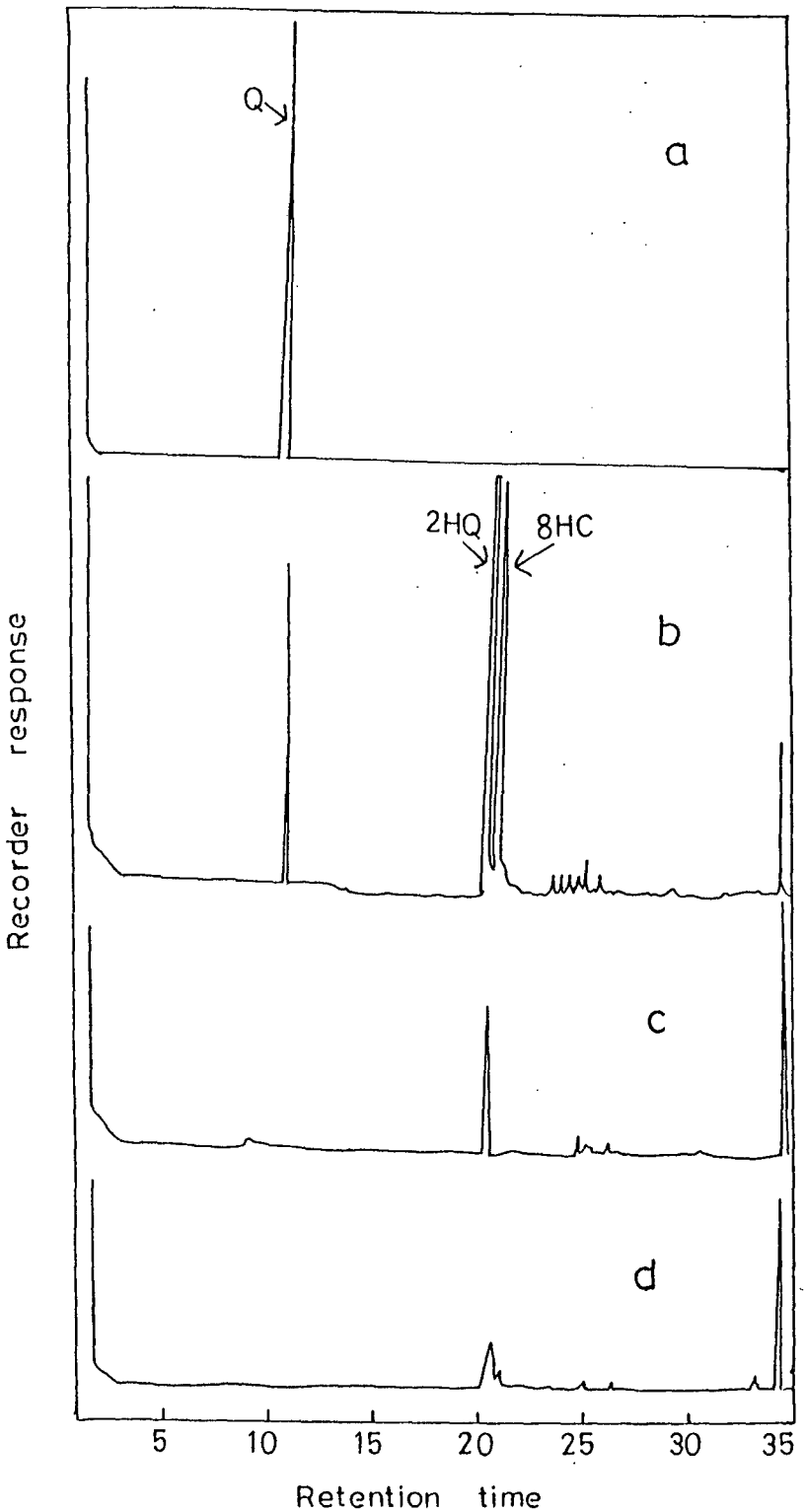


Fig.4.5: Gas chromatograms of ethyl acetate extracts of fermentation broths of strain GU104 growing on quinoline at different times: a) 0h, b) 18h, c) 24h and d) 30h.
Q-quinoline; 2HQ- 2hydroxyquinoline and 8HC- 8-hydroxycoumarin.

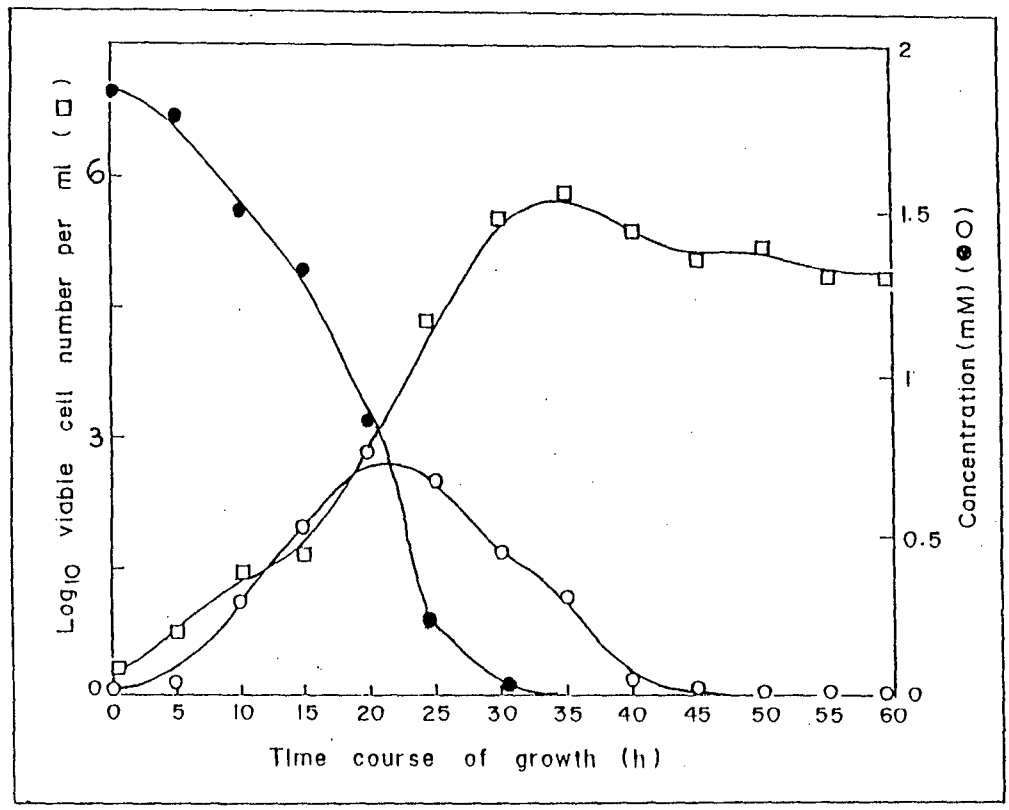


Fig.4.6: Transient accumulation of 2-hydroxyquinoline measured at 328nm (O) along with growth of strain GU104 (□) and depletion of quinoline (●).

Table 4.1 : Oxygen uptake by quinoline grown cells of strain GU104 due to oxidation of various organic compounds.

Substrate	Oxygen uptake (nmoles/min/mg dry wt.)
glucose	20.03
quinoline	27.5
2-hydroxyquinoline	34.3
4-hydroxyquinoline	13.56
5-hydroxyquinoline	5.6
6-hydroxyquinoline	9.2
3-methylquinoline	9.69
4-methylquinoline	3.25
7-methylquinoline	9.5
8-methylquinoline	12.5
2,4,dimethylquinoline	13.7
isoquinoline	9.09
pyridine	4.20
indole	6.45
carbazole	5.0
benzoate	7.04
naphthalene	16.6

rates of oxidation were quite low with quinoline grown cells. However, the rates increased when strain GU104 was pregrown in indole as carbon source (Table 4.2).

Inducibility of quinoline degrading enzymes in strain GU104

To understand whether the quinoline metabolic trait of GU104 is inducible, the washed cells were prepared after repeated growth on sodium lactate or glucose. It was observed that lactate and glucose grown cells gave non detectable rates of quinoline oxidation without induction with quinoline. An exposure period of 2h with 25µM quinoline was sufficient to induce the cells to oxidize quinoline to reach the oxygen uptake levels to approximately 27.5nmoles/min/mg dry wt. To further confirm the inducibility of the enzymes for quinoline metabolism, resting cells of strain GU104 grown on lactate were maintained in four batches. Cells were incubated for 2h with quinoline as inducer with or without chloramphenicol (100µg/ml). Other two batches were controls. The results of this experiment are depicted in Table 4.3 and clearly show that the rate of quinoline oxidation is considerably lowered in quinoline induced cells in the presence of chloramphenicol.

Kinetics of oxidation of quinoline by strain GU104

In order to determine substrate constant (K_s) and maximum rate (V_{max}) of oxygen uptake by strain GU104 in response to quinoline, resting cells pregrown in quinoline were used to determine quinoline oxidation rates. Since intact whole cells were used instead of enzyme, the term 'K_s' is

Table 4.2 :Oxygen uptake by strain GU104 cells grown on indole and lactate.

Growth substrate	Substrate oxidized	nmoles of O_2 consumed/ min per mg dry wt .
indole	Indole	13.6
	Carbazole	10.08
	Quinoline	20.14
lactate	Glucose	27.4
	Benzoate	22.8
	Quinoline	ND

ND – not detectable

Table 4.3 : Inducibility of quinoline oxidation in strain GU104.

	nmoles of O_2 consumed/min/mg dry wt.	
	Quinoline	Lactate
Lactate grown cells	ND	32.98
Quinoline grown cells	28.25	32.08
Lactate grown washed cells Induced with quinoline	23.09	33.43
Lactate grown washed cells Induced with quinoline in presence of 100 µg/ml chloramphenicol	16.13	31.68

ND – No detectable oxygen uptake.

employed instead of 'K_m'. Figure 4.7 indicates the Lineweaver Burke plot for the same. The K_s and V_{max} values so obtained from the plot were 0.04mM and 45.45nmoles O_2/min/mg dry wt, respectively.

Table 4.4 depicts the stoichiometry of oxygen uptake by strain GU104 in response to quinoline as substrate. Resting cells pregrown in quinoline showed consumption of 1.44 moles of O_2 per mole of quinoline and 1.86 moles of O_2 per mole of 2-hydroxyquinoline.

Experiments with starving cells (STS and LTS) gave the following results. During the first 20 days of the starvation period, the cell numbers as obtained on Zobell's marine agar plates decreased, indicating cell death and lysis. The cells were therefore washed intermittently with ASW to remove any nutrients that may be released in the form of cellular contents of dead cells. However, after this period the cell numbers remained approximately constant at 4×10^5 cfu/ml, till the end of the experiment i.e. 60 days.

STS and LTS cells were checked for their ability to oxidize quinoline. As the length of the starvation period increased so did the induction time with STS showing a much lesser time of induction than LTS. However, the rates of quinoline oxidation were much higher for LTS cells (Table 4.5).

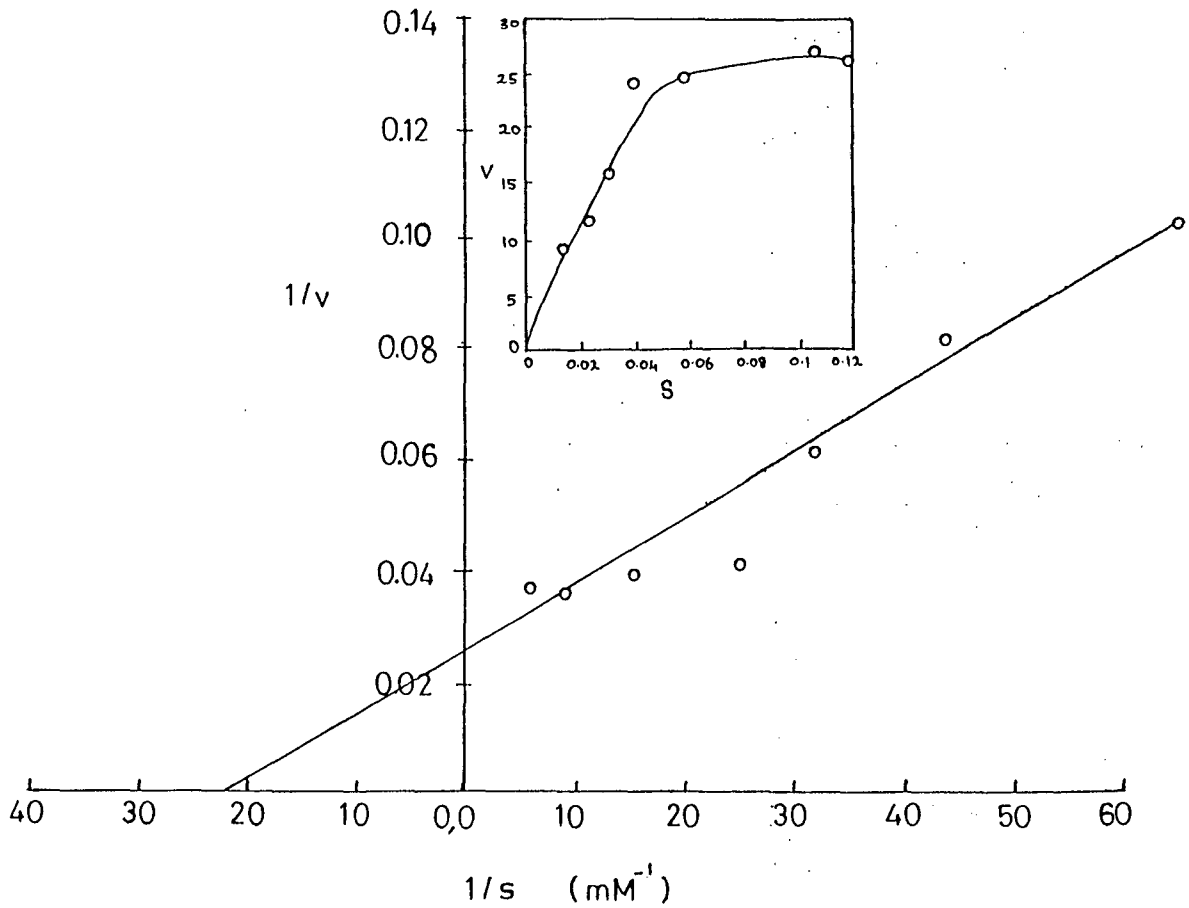


Fig.4.7: Lineweaver Burke plot to determine substrate constant, K_s (mM) for quinoline and maximum rate of oxygen uptake, V_{max} (nmoles of O_2 consumed/min/mg dry wt.) by cells of strain GU104. Inset shows the increase in rate of quinoline oxidation as the concentration increases.

Table 4.4: Stoichiometry of oxygen uptake in GU104

Substrate	moles of O_2 consumed/ mole of substrate
quinoline	1.44
2-hydroxyquinoline	1.86

Table 4.5 : Oxidation of quinoline by starved cells of GU104.

Control	Time of Induction	nmoles of O_2 consumed/min/mg dry wt.
Unstarved	2 h	27.68
STS	30 h	33.49
LTS	80 h	41.06

Study of enzyme catalyzing oxidation of quinoline

Quinoline oxidoreductase catalyses the oxidation of quinoline to 2-oxo-1,2-dihydroquinoline. The enzyme showed no activity toward NAD^+ or $NADP^+$. For the reaction *in vitro*, the presence of electron acceptors such as INT and PMS was obligatory. No oxygen consumption measured with a Clarke electrode cell was observed when crude extract containing the enzyme was incubated with quinoline in the absence of electron acceptors. The K_m value with quinoline as substrate and INT as electron acceptor determined by Lineweaver Burke plot was 2.54×10^{-6} mM for quinoline (Fig.4.8a) and 2.2×10^{-4} mM for INT (Fig. 4.8b).

a) Optimal conditions for quinoline oxidoreductase

The optimum temperature of assay was found to be 55°C (Fig. 4.9) and the optimum storage temperature 0°C. The enzyme had a pH optimum of 9.0 (Fig.4.10). It was more stable when stored at alkaline pH (9.5 to 10.0).

b) Substrate specificity of quinoline oxidoreductase

Table 4.6 shows the relative activities of the enzyme with different substrates. The enzyme showed activity with various methyl derivatives such as 3-,4- and 8-methylquinoline and with 6-hydroxyquinoline. However, 2-,4- and 5-hydroxyquinoline were not accepted as substrates. Isoquinoline, pyridine, indole and naphthalene were not accepted as substrates, although with xanthine the crude enzyme showed a relative activity of 28%. The inhibitors of the enzyme include methanol where the inhibition is dependent on its concentration. A concentration of 0.98M reduced the activity of the enzyme by 50%.

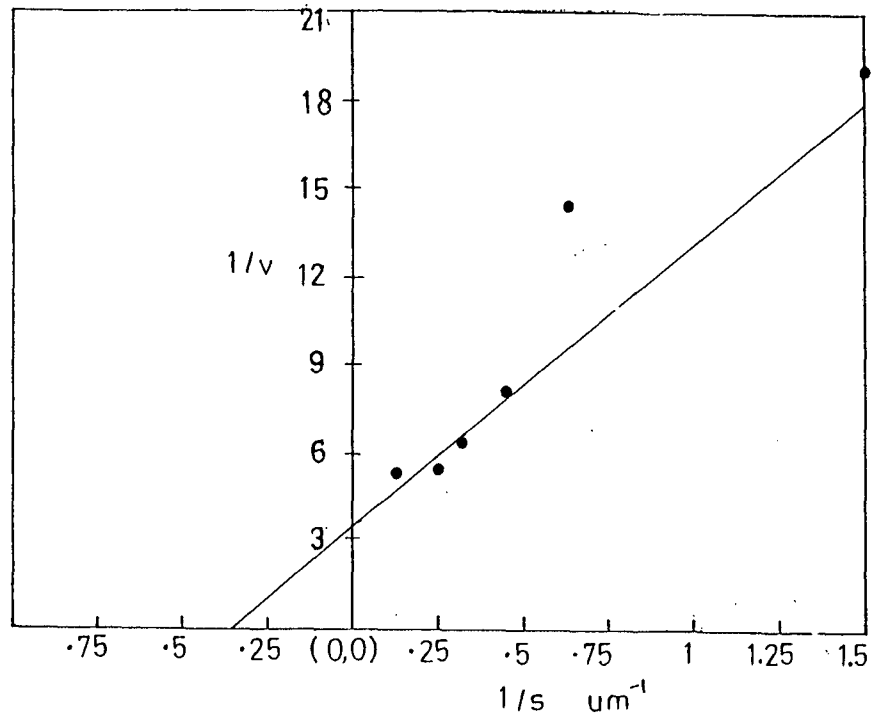


Fig.4.8a: Lineweaver Burke plot for the determination of the K_m value for quinoline of the enzyme quinoline oxidoreductase extracted from strain GU104.

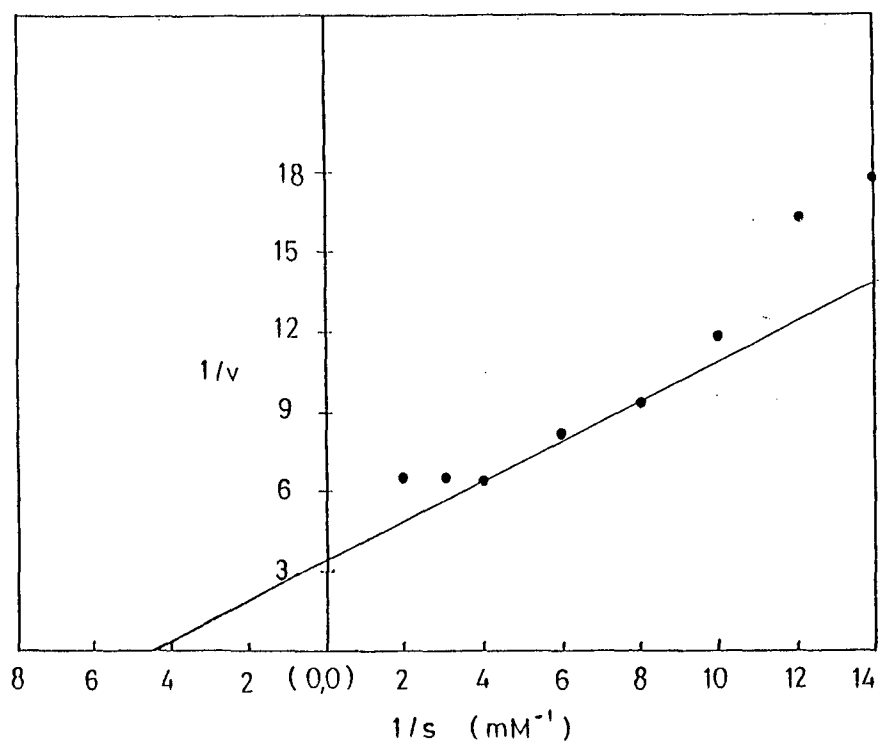


Fig. 4.8b: Lineweaver Burke plot for the determination of the K_m value for INT of the enzyme quinoline oxidoreductase extracted from strain GU104.

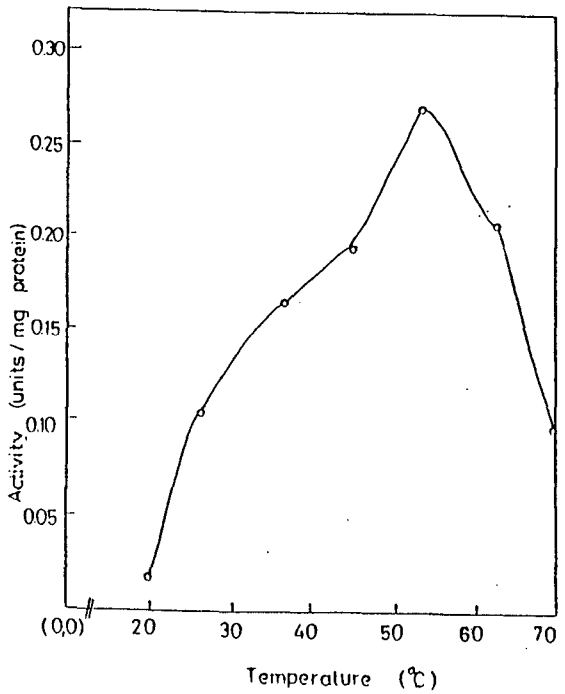


Fig.4.9: Graph indicating the optimum temperature of assay for the enzyme quinoline oxidoreductase from strain GU104.

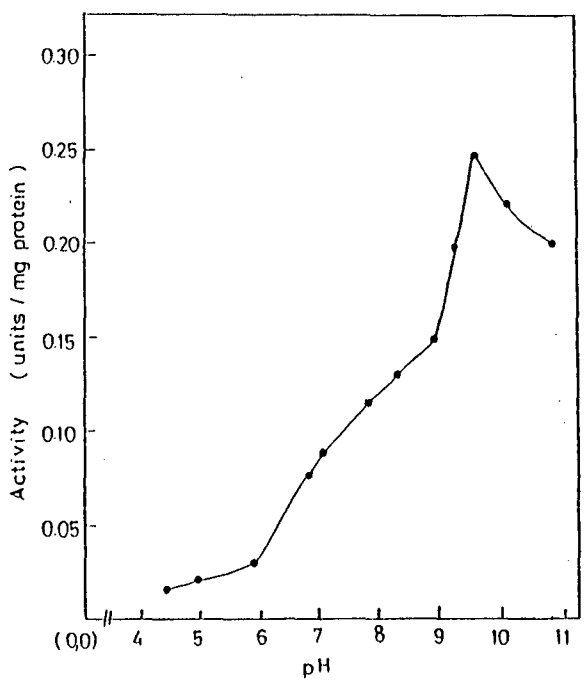


Fig.4.10: Graph indicating the optimum pH of assay for the enzyme quinoline oxidoreductase from strain GU104.

Table 4.6 : Specificity of quinoline oxidoreductase.

Substrate	Relative activity of quinoline oxidoreductase (%)
quinoline	100
2-hydroxyquinoline	ND
4-hydroxyquinoline	ND
5-hydroxyquinoline	ND
6-hydroxyquinoline	43.84
2,4-dimethylquinoline	ND
4-methylquinoline	56.92
3-methylquinoline	37.69
8-methylquinoline	50.77
xanthine	31.79
isoquinoline	ND
pyridine	ND
naphthalene	ND

ND – Not detectable

c) Inducibility of quinoline oxidoreductase

Table 4.7 lists the substrates used to check for their ability to induce quinoline oxidoreductase. It was seen that the enzyme was induced by quinoline and 2-hydroxyquinoline while 4-hydroxyquinoline, pyridine, benzoate, naphthalene and indole failed to induce the enzyme. The inducible nature of the enzyme was confirmed by the lowered activity of the enzyme extracted from chloramphenicol treated cells (Table 4.8).

d) Purification profiles of quinoline oxidoreductase

A preliminary purification procedure which included ammonium sulfate precipitation, desalting and group separation using Sephadex G-25(M) column, followed by heat treatment at 55°C/5min resulted in obtaining a partially pure preparation with an increase in specific activity of the enzyme to 0.92 (Table 4.9). Figure 4.11 shows the 10% native PAGE profiles of the enzyme detected by activity staining indicating high molecular weight of enzyme complex.

Detection of plasmid DNA in strain GU104

The plasmid profiles by agarose gel electrophoresis indicated the presence of at least two plasmid bands (Fig. 4.12). The plasmids appeared to be of a high molecular weight, and the gel had to be electrophoresed even after the tracking dye had reached the end of the gel in order to draw out the plasmid from the wells.

Table 4.7 : Inducibility of quinoline oxidoreductase.

Growth substrate	Specific activity of quinoline oxidoreductase (units/mg protein)
Quinoline	0.136
2-hydroxyquinoline	0.145
4-hydroxyquinoline	ND
lactate	ND
pyridine	ND
benzoate	ND
naphthalene	ND

ND – Not detectable

Table 4.8 : Confirmation of induction of *de novo* synthesis of quinoline oxidoreductase

Source of crude enzyme	Specific activity (units/mg)
Lactate grown cells	ND
Quinoline grown cells	0.15
Lactate grown cells induced with quinoline (2 h)	0.085
Lactate grown cells induced with quinoline in presence of chloramphenicol (100 µg/ml)	0.03

ND – No detectable activity

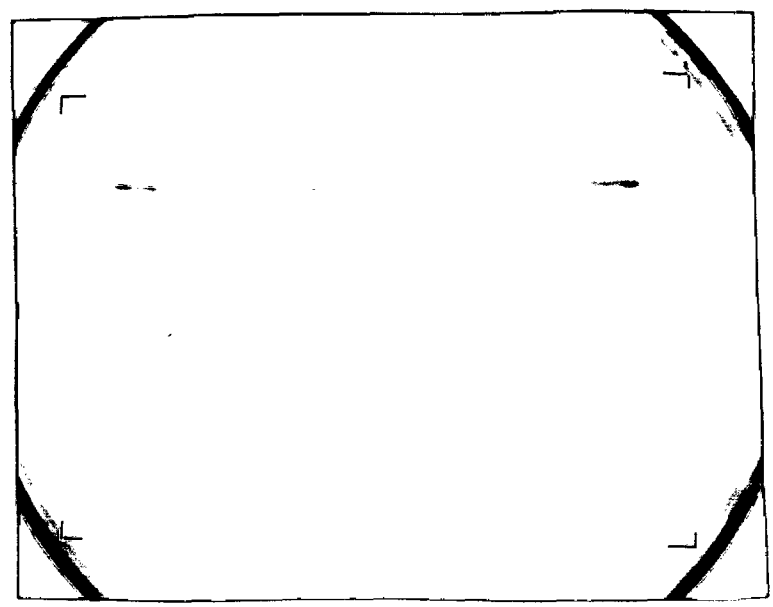


Fig.4.11: Activity staining of 10% native polyacrylamide gel to detect quinoline oxidoreductase. Red coloration of the band is due to the formation of 'formazan' the reduction product of INT.

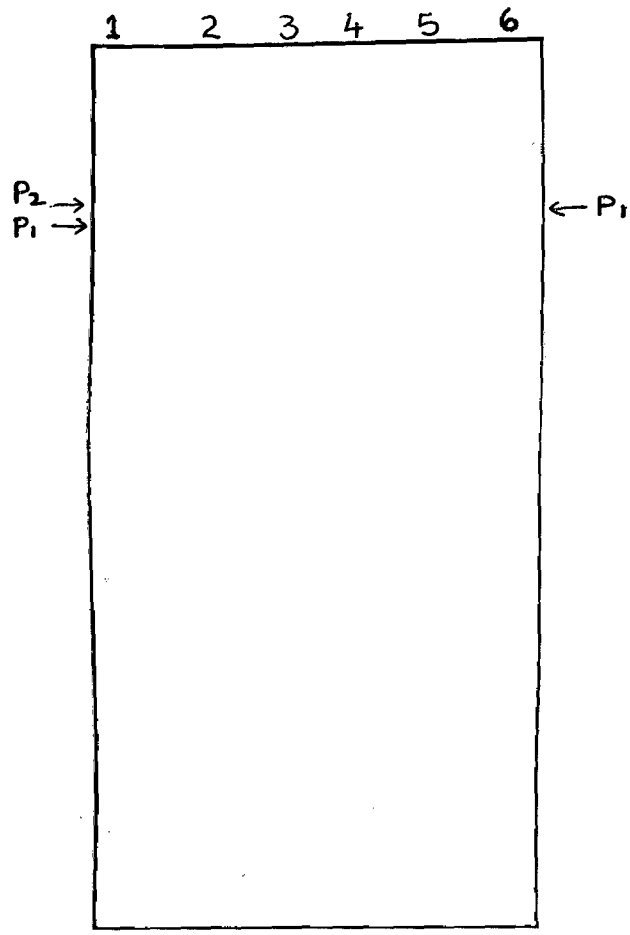


Fig.4.12: Agarose gel electrophoresis of the plasmid preparation from strain GU104. Lanes 1 to 4 - plasmid preparation from wt GU104, Lanes 5 and 6 - cured strains A01 and A02 respectively.

Isolation of metabolically blocked strains of GU104

To determine whether enzymes for quinoline degradation are plasmid encoded, curing of the plasmid was attempted using acridine orange. Of 150 colonies replica plated, 2 colonies were obtained that had lost the ability to metabolize quinoline. Examination of the plasmid profile did indeed reveal the absence of the larger molecular weight plasmid in both strains (Fig. 4.12). The cured strains were examined for their ability to utilize other compounds i.e. indole, hexadecane as well as crude oil. Both strains designated as AO1 and AO2 retained their ability to utilize all the substrates. Oxygen uptake studies revealed that the two strains were able to oxidize quinoline but not 2-hydroxyquinoline, indicating lack of enzyme required for metabolism of 2-hydroxyquinoline. Furthermore, both the strains exhibited quinoline oxidoreductase activity (Table 4.10).

In another experiment NTG mutagenesis of strain GU104 was attempted in order to obtain metabolically blocked mutants accumulating certain intermediates of quinoline metabolism. Of 300 colonies replica plated, 4 colonies were obtained that were unable to utilize quinoline as growth substrate. The oxygen uptake profiles of the washed cells of mutants with quinoline and 2-hydroxyquinoline, as well as the quinoline oxidoreductase activity is given in Table 4.10. Mutants NTG3 and NTG 4 were able to oxidize 2-hydroxyquinoline but could not oxidize quinoline. However they showed some quinoline oxidoreductase activity. By contrast mutant NTG1 was able to oxidize quinoline and not 2-hydroxyquinoline. Mutant NTG2

Table 4.9 : Purification of quinoline oxidoreductase.

	Protein (mg)	Activity (units)	(units/mg)	Yield (%)	Purification (fold)
Crude	110	12.27	0.112	100	1
$(NH_4)_2SO_4$ precipitation 35 – 55%	21.66	10.55	0.49	85.98	4.45
Sephadex G-25	8.1	6.5	0.8	52.97	7.27
Heat precipitation (55°C/5min)	7.04	6.5	0.92	52.97	8.3

Table 4.10 : Oxidation of substrates, and quinoline oxidoreductase activity by mutants cells of GU104 induced with quinoline.

Type Strains	nmoles of O_2 consumed/min/mg dry wt.		Specific activity of quinoline oxidoreductase (units/mg protein)
	Quinoline	2 hydroxyquinoline	
Wild type	39.6	44.1	0.12
Cured strains			
AO1	42.1	ND	0.072
AO2	39.9	ND	0.085
NTG mutants			
NTG1	36.2	ND	0.081
NTG2	ND	ND	ND
NTG3	ND	58.1	0.031
NTG4	ND	52.4	0.020

ND – no detectable oxygen uptake.

neither oxidized quinoline nor 2-hydroxyquinoline. The results indicate that the genes for specifying oxidoreductase and those specifying the enzymes for oxidation of 2-hydroxyquinoline are not linked.

DISCUSSION

The pathway for quinoline biodegradation in soil *Pseudomonas* is reported (Shukla, 1986). However, *Pseudomonas* sp. strain GU104 is a typical marine culture that utilizes quinoline as sole source of nitrogen as well as carbon. 2- hydroxyquinoline was identified as the metabolite of quinoline biodegradation in strain GU104, on the basis of TLC, UV spectrophotometry, GC and IR analysis. Its transient accumulation permitted its extraction and purification. 2-hydroxyquinoline supports growth of strain GU104 as sole source of carbon and nitrogen indicating that the first step in the degradative pathway in strain GU104 is similar to that in terrestrial bacteria. Many previous researchers too have stated the formation of 2-hydroxyquinoline as the first step in the biodegradation of quinoline (Grant and Al Najjar, 1976; Schwarz *et al.*, 1988; Shukla, 1986). The other intermediates were identified as 8-hydroxycoumarin and 2,8-dihydroxyquinoline and thus the quinoline metabolism in *Pseudomonas* strain GU104 proceeds according to the pathway identified by Shukla (1986), i.e. via 2-oxo-1,2-dihydroquinoline, 8-hydroxy-2-oxo-1,2-dihydroquinoline, 8-hydroxycoumarin and 2,3-dihydroxy-phenyl propionic acid (DHPP). No absorbance at 356nm was seen indicating absence of 2,6 -dihydroxyquinoline as an intermediate.

Regarding the overall degradation of quinoline it can be assumed that DHPP as well as the nearly insoluble 2,8-dihydroxyquinoline will remain intracellular during the degradation (for e.g. enzymatically bound) and are converted more rapidly than 2-hydroxyquinoline. The transient accumulation of 2-hydroxyquinoline and lack of accumulation of other metabolites as seen when 2-hydroxyquinoline was used as carbon source suggests that the rate limiting step of quinoline degradation by *Pseudomonas* strain GU104 is the conversion of 2-hydroxyquinoline. An identical observation has been made by Miethling *et al.* (1993) regarding the degradation of quinoline by *Comamonas acidovorans* DSM 6426.

It is apparent that quinoline metabolism is inducible from comparing the oxidation uptake rates for quinoline oxidation using cells pregrown on glucose, lactate and quinoline respectively. Glucose and lactate grown cells show no detectable uptake rates, while quinoline adapted cells showed highest oxygen uptake rates for quinoline oxidation (Table 4.3). Adaptation of cells with quinoline in presence of chloramphenicol lowered the oxygen uptake rates with quinoline confirming that quinoline induced de novo synthesis of enzymes for its degradation. Similarly quinoline oxidoreductase activity was not seen in glucose and lactate grown cells and quinoline induced cells treated with chloramphenicol showed lowered enzyme activity.

The fact that strain GU104 oxidizes various hydroxy and methyl derivative of quinoline as well as pyridine, naphthalene, indole and carbazole indicates the possibility of presence of broad substrate-specific enzymes.

Induction of a biodegradative pathway is of importance because it involves a large commitment of energy and other cellular reserves. During starvation the endogenous nutrient reserves of cells are decreased. In addition the initial reactions in the degradation of recalcitrant compounds rarely yield energy for metabolism and in some cases may even require energy in the form of reduced NAD^+ as a cofactor. Cells that have undergone long term starvation in the laboratory closely approximate the morphology and physiology of cells existing in the environment. The starvation process is believed to induce physiological stresses that approximate those experienced by indigenous bacteria.

In this study therefore, the *in situ* physiology of strain GU104 was simulated by subjecting the bacteria to starvation and checking for the time of induction of quinoline uptake as well as the rate of quinoline oxidation by starved cells.

Our results indicate that strain GU104 is well adapted to survival under nutrient limiting conditions as in the natural environment. Although the induction response is longer for starved cells they were able to scavenge low concentrations of quinoline and oxidize it at a faster rate than unstarved cells

or short term starved cells. This could be attributed to the fact that LTS cells reach a stable starvation survival state characterized by very low energy reserves, low maintainence energy requirement or rate of respiration and small size and these factors prompt a more efficient utilization of low concentrations of substrates than STS.

Cells of strain GU104 are able to scavenge fairly low concentrations of quinoline as is apparent from the low K_s value of 0.4mM and oxidize it efficiently as seen from the stoichiometry ratio of 1.44 moles of O_2 per mole of quinoline.

The enzyme quinoline oxidoreductase showed activity only in the presence of artificial electron acceptors. It showed no activity toward NAD^+ or $NADP^+$. The physiological electron acceptor is unknown. Studies by Peschke and Lingens (1991) and Bauder *et al* (1990) have shown that the enzyme is associated with FAD. During the enzyme reaction, no oxygen consumption could be detected suggesting that the oxygen of the hydroxyl group may be derived from water as reported by Pereira *et al.*(1988). Methanol acts as an inhibitor of the enzyme quinoline oxidoreductase. Methanol is a known inhibitor of molybdo enzymes and traps molybdenum in the pentavalent state (Bauder *et al.* 1990). It has already been established earlier (**Chapter II**) that molybdenum enhances growth of strain GU104 in quinoline. These results therefore indicate that quinoline oxidoreductase from strain GU104 is a molybdoenzyme as has been reported by Peschke and

Lingens (1991) and Bauder *et al.* (1990) with *Rhodococcus* and *Pseudomonas* strains respectively. Quinoline oxidoreductase from strain GU104 showed activity with 6-hydroxyquinoline but not with 4- and 5-hydroxyquinoline (Table 4.6). Bauder *et al.* (1990) suggests that 5-hydroxyquinoline can exist in the beta form as 1-4-oxoquinoline and in this form hydroxylation in 2-position is difficult. 6-Hydroxyquinoline cannot exist in this tautomeric form and hence oxidation is facilitated. The enzyme also gave some percentage of activity with 3-,4- and 8-methylquinolines and a low activity with xanthine. Activity with xanthine was not reported with the quinoline oxidoreductase from bacterial isolates (Bauder *et al.*, 1990 and Peschke and Lingens, 1991). It was quite possible that quinoline oxidoreductase may function as a xanthine oxidase in this strain. There have been reports where quinoline oxidoreductase occurs together with xanthine dehydrogenase in the same organism (Hettrich and Lingens, 1991).

Bauder *et al* (1990) reported a K_m value of 18.2 x 10^{-5} M for quinoline with the enzyme from a *Pseudomonas putida* strain, while Peschke and Lingens (1991) reported a K_m value of 2.5 x 10^{-6} M for quinoline with the enzyme from a *Rhodococcus* strain. The enzyme from the marine isolate of this study had a K_m which was in the range of the latter.

Enzyme assay studies using methyl derivatives such as 3-, 4- and 8-methyl quinoline indicated that the enzyme quinoline oxidoreductase did show some amount of activity with these substrates. However, aromatic

compounds such as pyridine, naphthalene, indole and carbazole were not accepted as substrates. This would mean that strain GU104 has different oxidizing enzymes for these substrates. Furthermore, enzyme assay studies with quinoline oxidoreductase showed that the enzyme was induced only by quinoline and 2-hydroxyquinoline.

Agarose gel electrophoresis of DNA preparation revealed the presence of at least two mega plasmids in strain GU104. Plasmid curing by acridine orange resulted in the loss of the higher molecular weight plasmid and this was accompanied by the loss of the ability of strain GU104 to utilize quinoline as growth substrate. Oxygen uptake studies revealed that the cured strain retained the ability to oxidise quinoline. Quinoline oxidoreductase activity was also exhibited by the cured strain. However, 2-hydroxyquinoline was not oxidized . These results suggest that the genes for quinoline metabolism are fragmented and perhaps distributed at least on two plasmid and perhaps partly on chromosomal DNA. Hence the loss of a plasmid though preventing utilization of the quinoline as growth substrate, did not result in the loss of the genes of quinoline oxidoreductase responsible for conversion of quinoline to 2-hydroxyquinoline. The predicted model mechanism is depicted in Figure 4.13 which is based on the reported pathways.

NTG mutagenesis resulted in 3 types of mutants, one which did not oxidize quinoline nor 2-hydroxyquinoline, the other oxidized 2-hydroxyquinoline and not quinoline, while the third oxidized quinoline and not

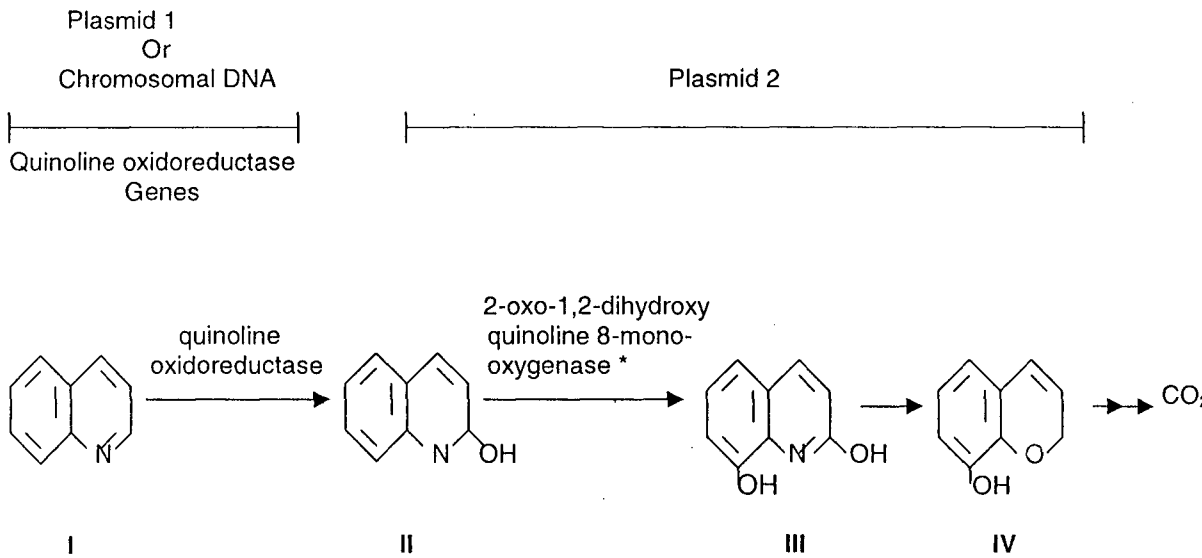


Fig.4.13: Predicted model of distribution of genes specifying for the degradation of quinoline in *Pseudomonas* sp. strain GU104.

I - Quinoline;
II - 2-hydroxyquinoline;
III- 2,8- dihydroxyquinoline;
IV- 8-hydroxycoumarin

* Rosche *et al.*, 1995.

2-hydroxyquinoline. Two out of the four mutants obtained were of the second type. Our studies therefore reveal that majority of the blocks had occurred at the first step i.e. the conversion of quinoline to 2-hydroxyquinoline thus affecting the genes coding for quinoline oxidoreductase. The ability to oxidize 2-hydroxyquinoline indicates that the genes for 2-hydroxyquinoline are clustered on a specific region, which is not linked with the region carrying quinoline oxidoreductase genes. On induction with quinoline however, the mutant strains did show minimal quinoline oxidoreductase activity. A mutation in the regulatory gene could explain this low level of activity. Recent studies by Bldse *et al.* (1996) have revealed that there are 3 genes coding for the 3 subunits of quinoline oxidoreductase from *P. putida* 86. If the same is true for the marine isolate of this study, a mutagen affecting any of the 3 genes would result in decrease/ loss of its activity.

In conclusion, the marine isolate of this study, strain GU104, metabolized quinoline via 2-hydroxyquinoline and 8-hydroxcoumarin. It was capable of oxidizing various NHAs and aromatic compounds. At least some of the genes for quinoline metabolism are located on a plasmid in this strain and the genes are not linked i.e. situated at different loci.

CHAPTER V

CONTRIBUTION OF *PSEUDOMONAS* SP. STRAIN GU104 IN MINERALIZATION OF CRUDE OIL

Microorganisms are considered to be the best agents for the destruction of organic material originating from petroleum spills (Colwell and Walker, 1977; Van der Lynden, 1978; Atlas, 1981). Microorganisms with specialized degradative capabilities may be obtained by selective enrichment techniques. *Pseudomonas* species strain GU104 was isolated by such techniques from the marine environment and had the ability to degrade quinoline, quinoline derivatives, various other NHAs and PAHs, alkanes as well as crude oil, kerosene and diesel. Emulsification is an important phenomenon among these parameters which influence the fate of petroleum. In this chapter therefore, the ability of strain GU104 to cause a dispersion of crude oil by production of emulsifying agents and bacterial adhesion to crude oil as well as the ability to utilize various components of crude oil with emphasis on the NPAC fraction of crude oil is investigated.

Many compounds of the NSO fraction of oil have been shown to be inhibitors of biodegradation of other aromatic compounds (Dyreborg, *et al.*, 1996). Our studies investigate the effects of quinoline on biodegradation of certain specific aromatic compounds as well as the role of strain GU104 in binary and mixed culture systems in lifting the repressive effects of quinoline.

The success of inoculation of bacterial cultures isolated on pure components for bioremediation of a complex mixture of components such as oil depends on several criteria: the organisms must retain their specialized metabolic capability, the organisms must come into contact with the

contaminant and nutrient and environmental conditions must be conducive to contaminant degradation. These criteria were studied in specially constructed microcosms, in which a 7 membered mixed culture system including strain GU104 were used and their ability to utilize tarballs was investigated.

MATERIALS AND METHODS

Media and chemicals

Bombay High Crude Oil (BHCO) was obtained from ONGC, India. Tarballs were collected along the Bombay coast and were a gift from NIO, India. All other chemicals used in media or buffers and organic solvents were of AR and LR grade and obtained from local suppliers. HPLC grade solvents were used for GC analysis.

Growth and culture conditions

Strain GU104 was routinely cultured in ASW supplemented with 0.1% (w/v) crude oil. Tarballs when used in the medium as a source of carbon were at a concentration of 0.1% (w/v), hexadecane was used at a concentration of 0.1% v/v. Viable counts of the culture grown on crude oil and tarballs, were determined on Zobell's marine agar plates. Other substrates such as quinoline, benzoate, xylene, naphthalene, m-toluic acid, pyridine and dibenzothiophene whenever used for growing the mixed

bacterial consortium were used at concentrations between 0.02% to 0.1% w/v.

Extraction and fractionation of crude oil

Fractionation of BHCO into aliphatic, aromatic and Nitrogen Polycyclic Aromatic Compound (NPAC) fractions, was done according to Fedorak and Westlake (1984). Before extraction, 25µl of dichloromethane solution containing 150mg of 7,8-benzoquinoline per ml (Aldrich Chemical Co. Inc) as a GC standard was added to each culture flask. Growth medium (200ml) containing crude oil was extracted at neutral pH 2 to 3 times with dichloromethane, the extracts were pooled to 40ml, dried and concentrated as outlined by Fedorak and Westlake (1981). A 10ml aliquot was concentrated at 80 – 85°C using a reflux column. 2ml hexane was added and dichloromethane was displaced by concentrating the sample further to 0.7 to 1ml. Concentrated extracts from duplicate flasks were adsorbed on 3g of neutral alumina and this was added to 6g of neutral alumina in a column (30 x 1cm). The column was eluted sequentially with 30ml hexane, 50ml benzene and 70ml chloroform to give respectively, the saturate, aromatic and NPAC fractions as described by Later *et al.* (1981). Fractions were concentrated and were analyzed by GC as described in **Materials and Methods** of **Chapter II.**

Fractionation of whole crude oil into aliphatic fraction, aromatic fraction and polar fraction was done according to the protocol from ONGC Dehradun

using silica gel - alumina column chromatography. 1g of crude oil was adsorbed onto 2g silica gel and overlayered over 50g silica gel and 50g alumina in a glass column (90 x 1.8cm). The column was developed sequentially by addition of 200ml petroleum ether, 200ml benzene and 200ml methanol to elute the saturates, aromatics and polar NSO compounds respectively. The residues were weighed on a Mettler balance in pre-weighed crucibles after drying to constant weight by evaporation of the solvents at 50°C.

Gravimetric determination of crude oil:

Extraction of oil for gravimetric determination was done as per Fedorak and Westlake, 1981. Growth medium (200ml) was extracted repeatedly with dichloromethane in a 500ml separating funnel. The flask was rinsed several times with the same and the extracts were pooled together and filtered through anhydrous Na_2SO_4. A control was also extracted in the similar way each time. The solvent was evaporated by heating in an oven at 50°C and the residue was weighed using a Mettler balance till a constant weight was obtained, and expressed as percentage residual crude oil.

Determination of oil emulsifying activity

Oil emulsifying activity was determined according to the method of Reddy *et al.* (1983). Strain GU104 was grown in ASW supplemented with crude oil and culture broth filtered first through Whatman paper and then through millipore (0.22 micron) to get a cell free supernatant which was then

assayed for its ability to form a stable emulsion with a hydrocarbon such as hexadecane (1ml supernatant + 10µl hexadecane) at 610nm. The stability of the emulsion over time was measured at 610nm (Roy *et al.*, 1979), using a 1201 Spectronic spectrophotometer. A similar reaction system prepared from artificial sea water served as control. Emulsifying activity is expressed as D_{610} under the condition of measurement.

Measurement of surface tension

The surface tension was measured at 25°C with a Traube's Stalagnometer
Stagalometer (surface tension (γ) = γ_o x m/m_o) where γ_o is the surface tension of double distilled water; m_o is the mass of the double distilled water per drop and m is the mass of sample per drop (Morikawa *et al.*, 1993).

Estimation of hydrocarbon solubilizing activity

For the measurement of hydrocarbon solubilizing activity (Reddy *et al.*, 1983), 50ml of the cell and oil free supernatant was taken along with 0.5ml of the hydrocarbon in a glass stoppered separating funnel and shaken by hand for the desired period of time (10min). The emulsion was allowed to stand for half an hour and the lower layer was filtered through 0.45 micron millipore membrane filter. A suitable aliquot (30-40ml) of the filtrate was extracted thrice with 15ml portions of n-hexane and the extracts were pooled, dried under anhydrous Na_2SO_4, concentrated to a fixed volume and analysed by GC.

MATH assay or Cell surface hydrophobicity test

Cells of strain GU104 pregrown on crude oil were washed with hexane to remove any adhering hydrocarbons. Cell surface hydrophobicity, or microbial adhesion to hydrocarbon (MATH) assay was performed according to Deziel, 1996. Hexadecane (0.5ml) and washed cells of strain GU104 pregrown on crude oil (2ml) were vortexed in a test tube. After equilibration the loss in absorbance of the aqueous phase relative to that of the initial cell suspension was measured and hydrophobicity was estimated by calculating the percentage of cells adhering to hexadecane. Reported results are means of duplicate measurements.

Formulation of GU104 based mixed marine bacterial consortium

The cultures used to formulate the mixed marine bacterial consortium are listed in Table 5.1. 0.5ml of each pure culture pregrown on its principal substrate was inoculated into 100ml of artificial sea water medium containing a mixture of m-toluic acid (0.07%), naphthalene (0.04%), xylene (0.04%), quinoline (0.02%), pyridine (0.04%), phenanthrene (0.08%) and dibenzothiophene (0.05%) and incubated at 30°C on a rotary shaker (200rpm). The mixed culture so grown was preserved at –70°C as per the cryopreservation procedure established in the department (N. Fernandes, personal communication). 100 ml of the mixed culture was then amplified to 1 litre in Erlenmeyer flasks and used as inoculum in the microcosms designed to study tarball biodegradation.

Table 5.1: List of bacterial cultures used in this study to formulate a mixed culture system.

Location	Marine Bacterial Strain No.	Principal substrate degraded	Reference
Goa	GU101	m-toluic acid	S. Chakravarty (unpublished)
Vengurla	GU102	Naphthalene	Coelho *et al.*, 1995
	GU103	Xylene	Coelho *et al.*,1995
	GU104	Quinoline	This study
	GU105	Pyridine	Coelho *et al.*,1995
Gulf of Kutch	GU109	Phenanthrene	N. Fernandes (unpublished)
Goa	GU110	Dibenzothiophene	J. Rodrigues (Unpublished)

Design of marine microcosms

Glass tanks of over 300l capacity each, were filled with filtered natural sea water. The tanks were equipped with mechanical stirrers and independent aeration line and were maintained in temperature controlled rooms (25-30°C). Tarballs (0.15% w/v) from Bombay High was used as substrate. A parallel microcosm supplemented with nitrogen and phosphorus (8mM $(NH_4)_2SO_4$ and 5mM each K_2HPO_4 and KH_2PO_4) was also maintained. The mixed culture was inoculated (0.5% v/v) and the tarball was monitored over time to detect changes due to bacterial activity. Water samples were taken at regular intervals for measurement of growth. Figure 5.1. shows a line drawing of the marine microcosm.

Determination of growth and degradation of oil in microcosms

50ml aliquots of water sample were filtered using a millipore filter, which was then washed in 5ml ASW medium. Viable counts of each of the components of the mixed culture were established by the MPN (most probable number) method. The individual components of the mixed culture were enumerated by carrying out seven series of ten fold dilutions of the sample (after filtration and resuspension in ASW). Each series of tubes contained the respective principal substrate. The highest dilution showing growth was taken as a measure of the viable count. Viable counts were also determined using benzoate (0.08%) as substrate since it supports growth of all the 7 cultures used in the microcosms. Growth was also measured turbidometrically at 550nm on a spectronic 1201 spectrophotometer.

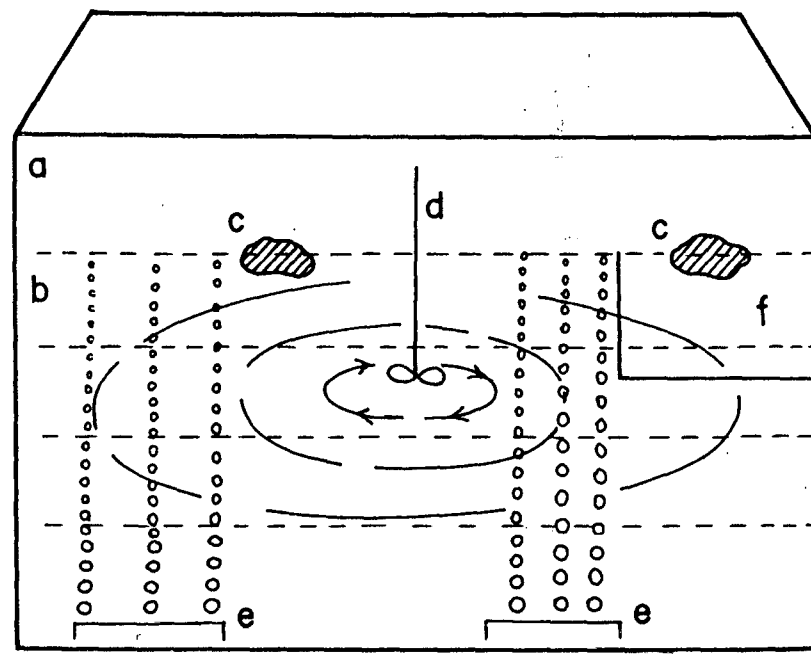


Fig.5.1 : Line diagram of the typical microcosm used in this study (Coelho *et al.*, 1995); a: 324l glass aquarium; b: filtered sea water; c: tarballs; d: stirrer; e: aerators and f: sampling site.

To study degradation of the tarball, 100mg of the tarball from each tank was dissolved in 5ml of carbon tetrachloride and chromatograms were obtained on a Shimadzu Gas Chromatograph 14B as described in **Materials and Methods** of **Chapter II**.

Catechol 2,3-dioxygenase assay

Catechol 2,3-dioxygenase was assayed according to Nozaki (1970) by following the change in absorbance at 375nm due to formation of α-hydroxymuconic semialdehyde. The assay system in a final volume of 3ml contains 2.7ml 50mM Phosphate acetone buffer (pH 7.5), 100µl of 10mM catechol, and 100µl enzyme. The reaction was started by addition of enzyme and performed at 25°C. One unit of enzyme corresponds to an absorbance increase of 14.7/min. The specific activity was expressed as units per mg protein. Protein was determined by the Lowry method.

Determination of oil degrading ability in benzoate enriched culture from the Bay of Bengal

The enrichment of benzoate degrading cultures at various depths in the Bay of Bengal, aboard the research vessel ORV Sagar Kanya was dealt with in **Chapter II**. In order to determine the presence of oil degrading bacteria in these cultures, subcultures were done in ASW supplemented with 0.1% crude oil. The cultures were observed for their ability to cause a dispersion of oil and growth was determined by an increase in viable counts obtained on Zobell's marine agar plates.

RESULTS

Chemical composition of oil

The chemical composition of BHCO is listed in Table 5.2. The oil was fractionated by alumina – silica gel column chromatography as described in **Materials and Methods**. It was found to contain 61.16% aliphatics, 22.41% aromatics and 8.43% polar compounds.

Potential of strain GU104 for biodegradation of crude oil and tar balls

The marine culture *Pseudomonas* species strain GU104 was initially isolated on quinoline as a sole source of carbon. To test whether the culture effects mineralization of crude oi, it was inoculated in 200ml ASW media containing 0.1% of BHCO. At 24h intervals contents of duplicate flasks were extracted with dicholoromethane. The organic layer was evaporated and the residue measured gravimetrically as described in **Materials and Methods**. Figure 5.2 shows the percent of residual oil in the medium as a function of time during growth of strain GU104 on crude oil as sole source of carbon. Upto 40% of the oil was degraded by the fourth day and the figure reached 48% after 7 days. Maximum degradation of oil occurred during the log phase of growth. Tar balls (0.1%) used as substrate also supported the growth of strain GU104 (Fig.5.2) indicating unequivocally that the strain could utilize certain components from BHCO as well as tar balls.

Table 5.2: The chemical composition of BHCO

Fraction No.	Component	Weight of fractions per gram of crude oil (g)
I	Aliphatic	0.6116
II	Aromatic	0.2241
III	Polar	0.0843

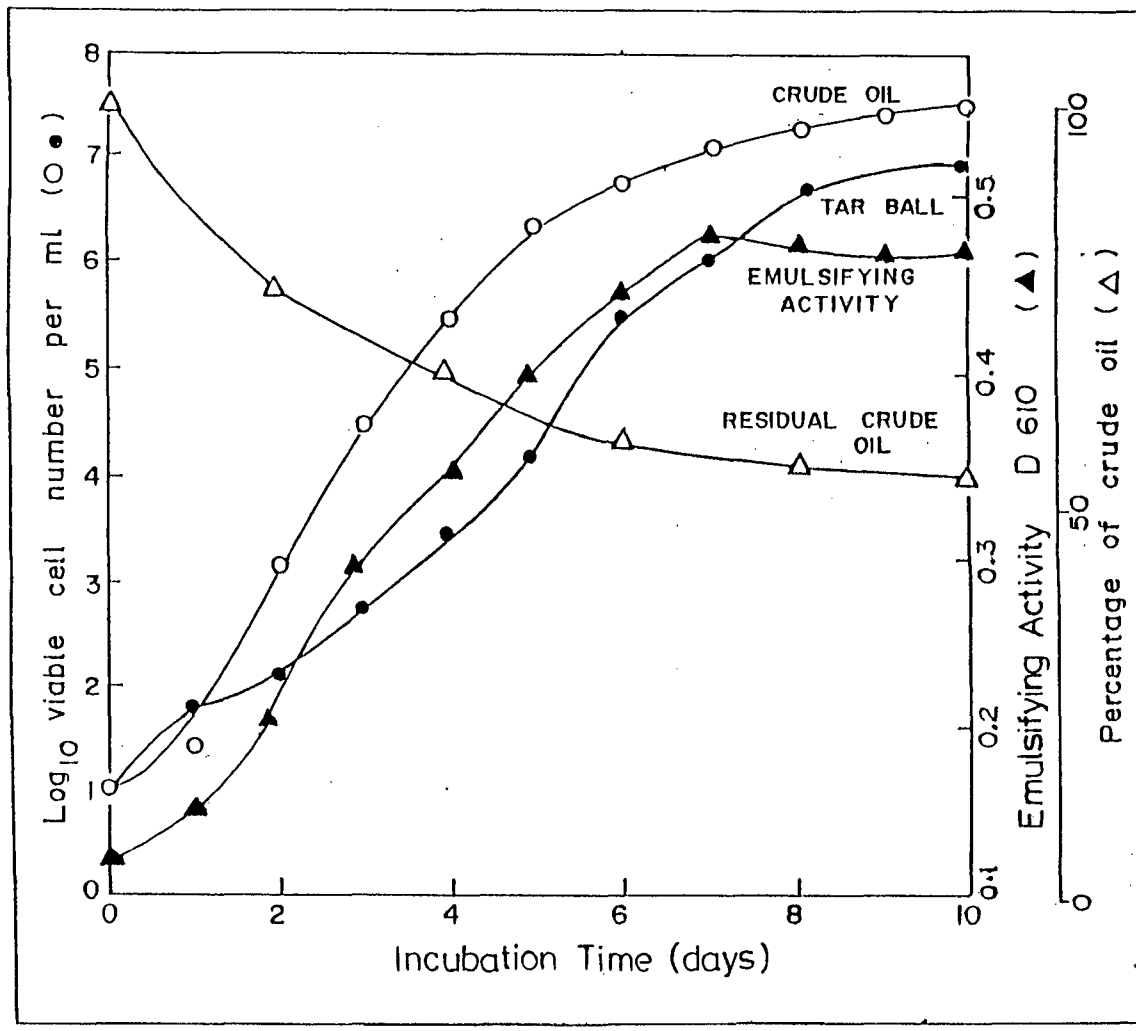


Fig.5.2: Time course of growth of strain GU104 on crude oil (O) and tarballs (●). The increase in emulsifying activity is shown as absorbance units at 610nm (▲). (△) indicates percentage of residual crude oil in the medium during growth.

Changes in the profiles of oil components as a result of growth of strain GU104

Emulsification of crude oil by strain GU104

To determine the physical as well as chemical changes brought about in crude oil as a result of attack by strain GU104, the culture was grown in ASW medium with crude oil as sole source of carbon. The first evidence of bacterial activity on BHCO was a quick and extensive emulsification of oil (Fig. 5.3a). Similarly disruption of the tarball into smaller particles was seen when tarballs were used as sole source of carbon in ASW medium (Fig. 5.3b). The surface tension of the ASW medium used for growth, decreased from 67 mN/m to 38 mN/m within 5 days of growth. the emulsifying activity was monitored as described in **Materials and Methods**. The cell free supernatant was able to form a stable emulsion at 610nm with hexadecane, which was stable for atleast 6h (Fig 5.4). The maximum emulsifying activity was demonstrated after 6 days of growth (Fig.5. 2).

The cell surface hydrophobicity was monitored by the MATH assay for the culture grown in crude oil supplemented media. Cell surface hydrophobicity increased with the time course of growth with a maximum adherence to hydrocarbons observed in 6 days (Fig. 5.5). Microscopic examination of the culture grown on crude oil revealed that initially the cells attach to droplets and the cells start dividing resulting in groups of cells surrounding the oil droplets (Fig. 5.6). The density of cells per oil droplet was usually found to be highest at 3-5 days (log phase) and as the culture

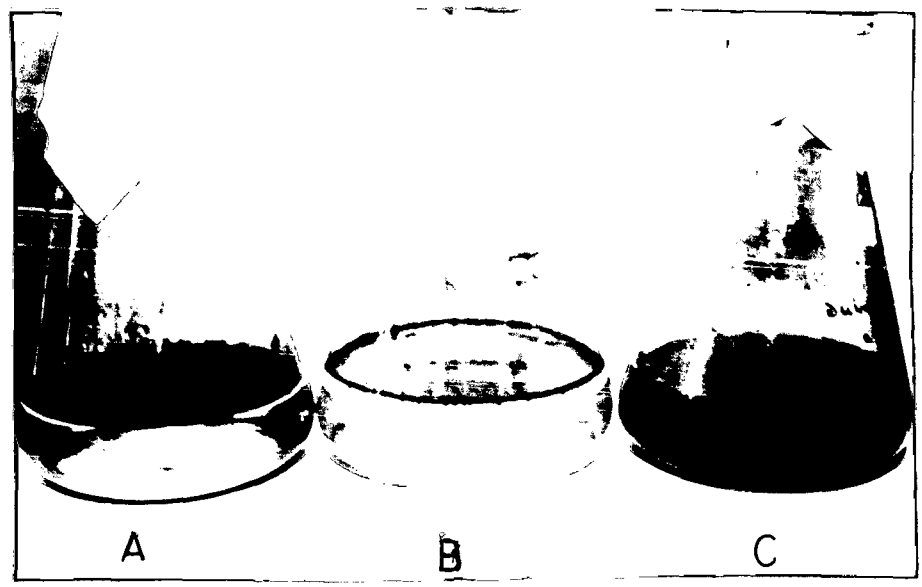


Fig.5.3a: Dispersion of crude oil during growth of strain GU104 in ASW medium containing 0.1% crude oil; A: control flask; B: 1 day oil culture; C: 4 days old culture.

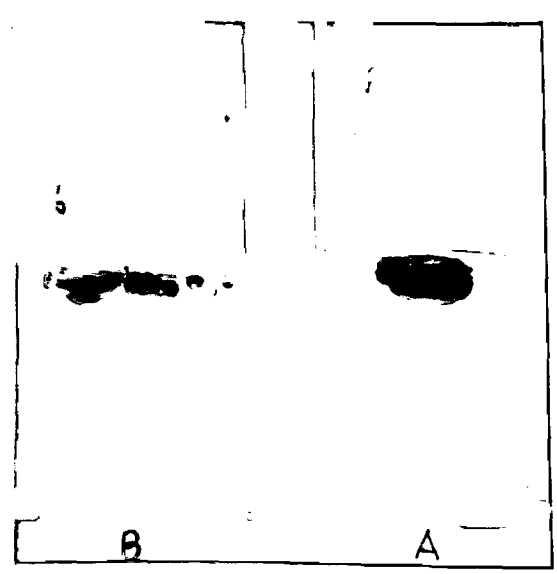


Fig.5.3b: Dispersion of tarballs during growth of strain GU104 in ASW medium containing tarballs as sole source of carbon; A: control; B: 3 day old culture.

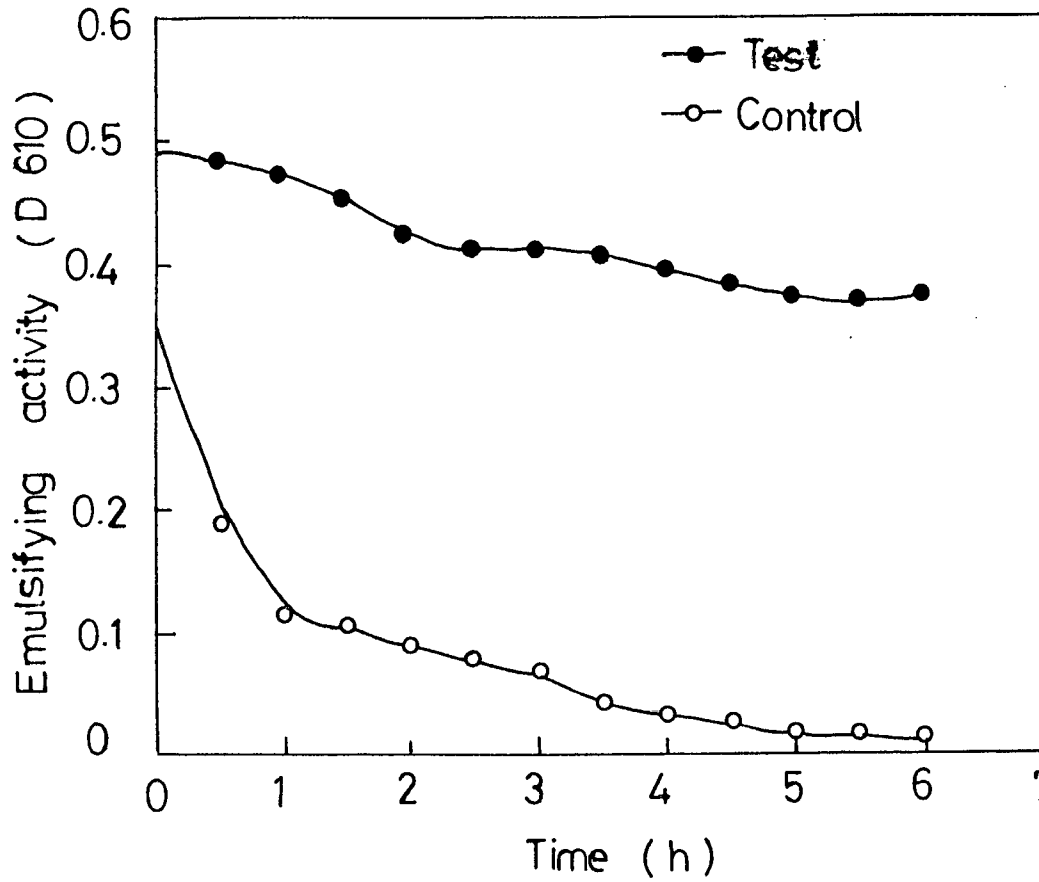


Fig.5.4: Stability of the emulsion formed by agitation of the cell free supernatant of strain GU104 grown on crude oil with hexadecane. The control (o) which was ASW agitated with hexadecane showed a decline in absorbance at 610nm while the emulsion formed by the cell free supernatent with hexadecane was stable upto 6h (●).

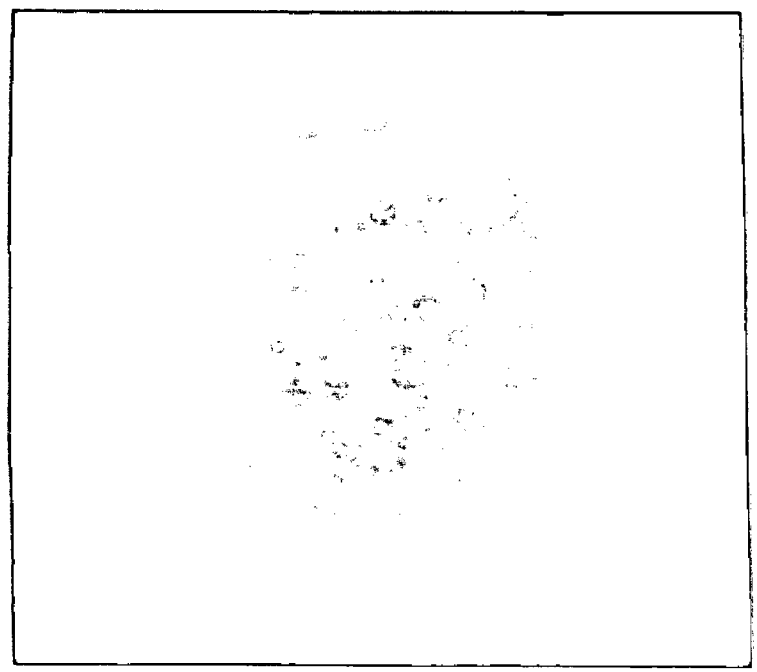


Fig.5.6: Photomicrograph showing the adherence of cells of strain GU104 to crude oil droplets under Phase Contrast Microscope (magnification 40X).

usually found to be highest at 3-5 days (log phase) and as the culture approached stationary phase, fewer cells were seen per oil droplet. The oil droplet decreased in size along with growth. The presence of a soluble emulsifying factor was apparent when 100µl of the culture grown in ASW containing crude oil, was placed in a well at the centre of the petriplate containing ASW agar incorporated with 1% crude oil. Although bacterial growth was not immediately apparent, a clearance zone devoid of 'oil was formed around the well within 8-10h. The zone increased in size on further incubation (Fig. 5.7). The confirmation of hydrocarbon solubilizing activity of the biosurfactant produced by strain GU104 was demonstrated by the appearance of a peak corresponding to hexadecane in gas chromatograms of the extracts of cell free supernatant agitated with hexadecane (Fig 5.8). The controls did not show the presence of solubilized hexadecane.

Degradation of crude oil components by strain GU104

Sequential chemical analysis of the oil as microbial growth proceeds was done by fractionating the extracts by column chromatography followed by GC analysis. The results indicate that components in all the fractions of crude oil were used at different rates and to different extents. The n- alkane components of the saturated fraction and the aromatic fraction were utilized with 74% of the saturates and 60% of the aromatics being degraded within 4 days of growth (Fig. 5.9 and 5.10). The NPAC fraction was also simultaneously utilized and was reduced by 43% (Fig. 5.11). Thus the peak areas calculated from the chromatograms show that after 12 days of growth,

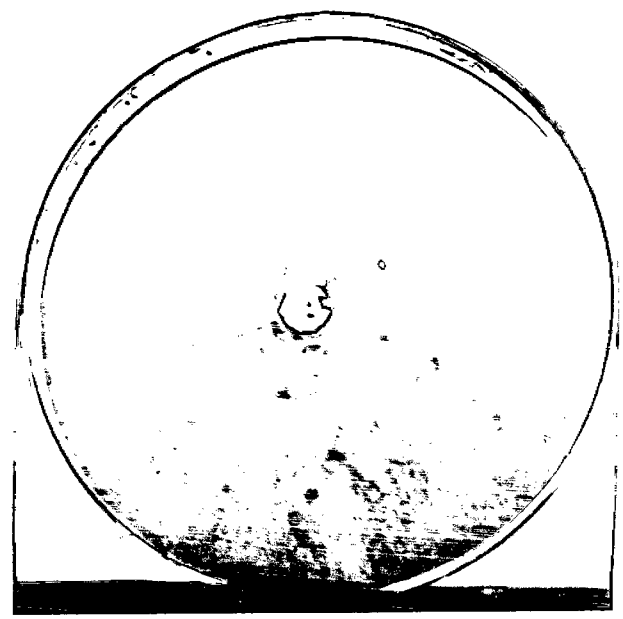


Fig.5.7: Formation of a clearance zone by strain GU104 on ASW agar plates containing 1% crude oil.

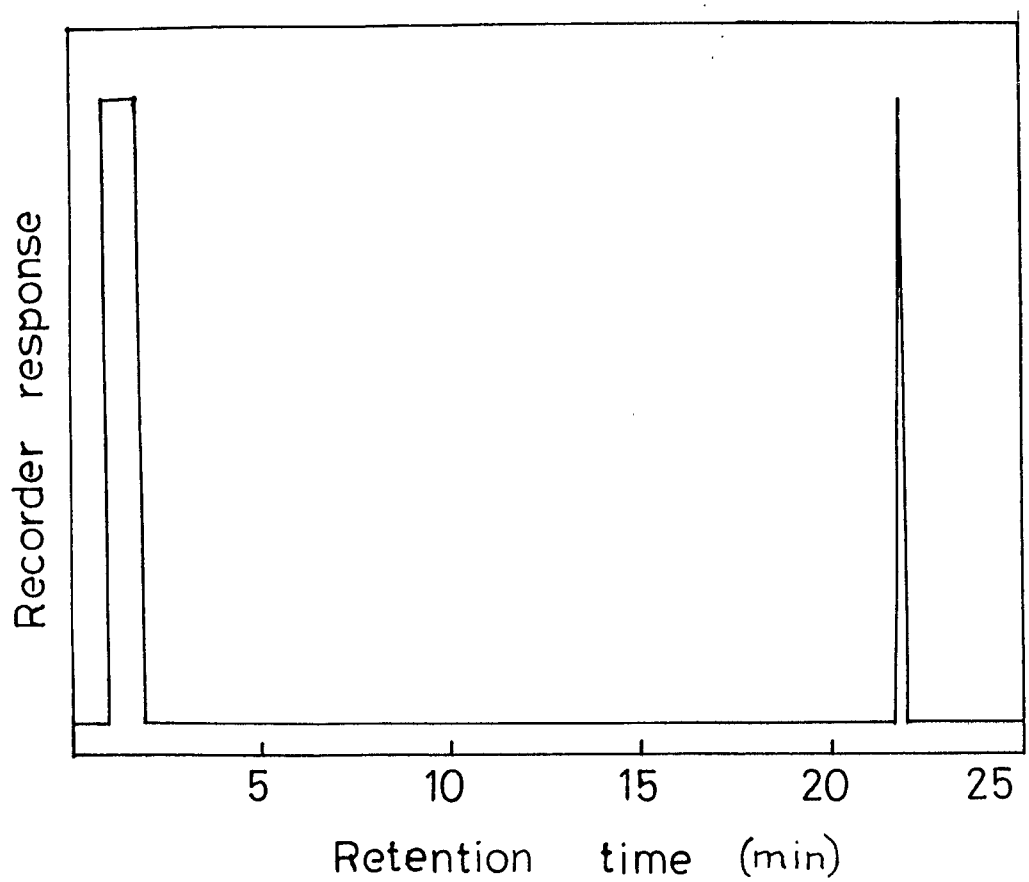


Fig.5.8: Gas chromatograph of the hexane extract of the cell and oil free supernatant of strain GU104 grown on crude oil when agitated with hexadecane. The peak signifies solubilization of hexadecane by the cell free supernatent. The peak was absent in control.

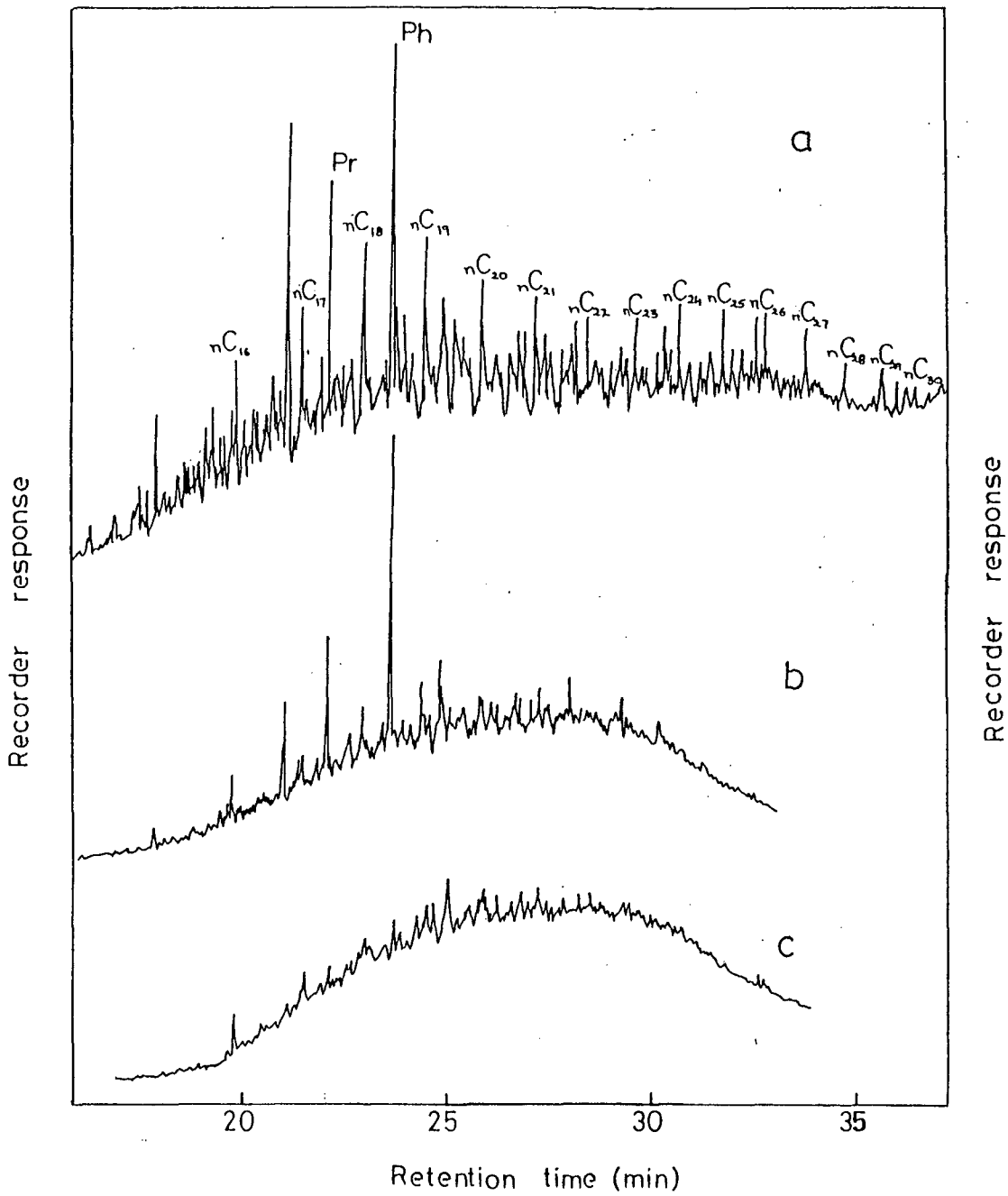


Fig.5.9: Gas chromatographs of the aliphatic component of BHCO; a: control; b: after 4 days of growth; c: after 10 days of growth.
nC – number of carbon atoms, Pr – Pristane , Ph- Phytane.

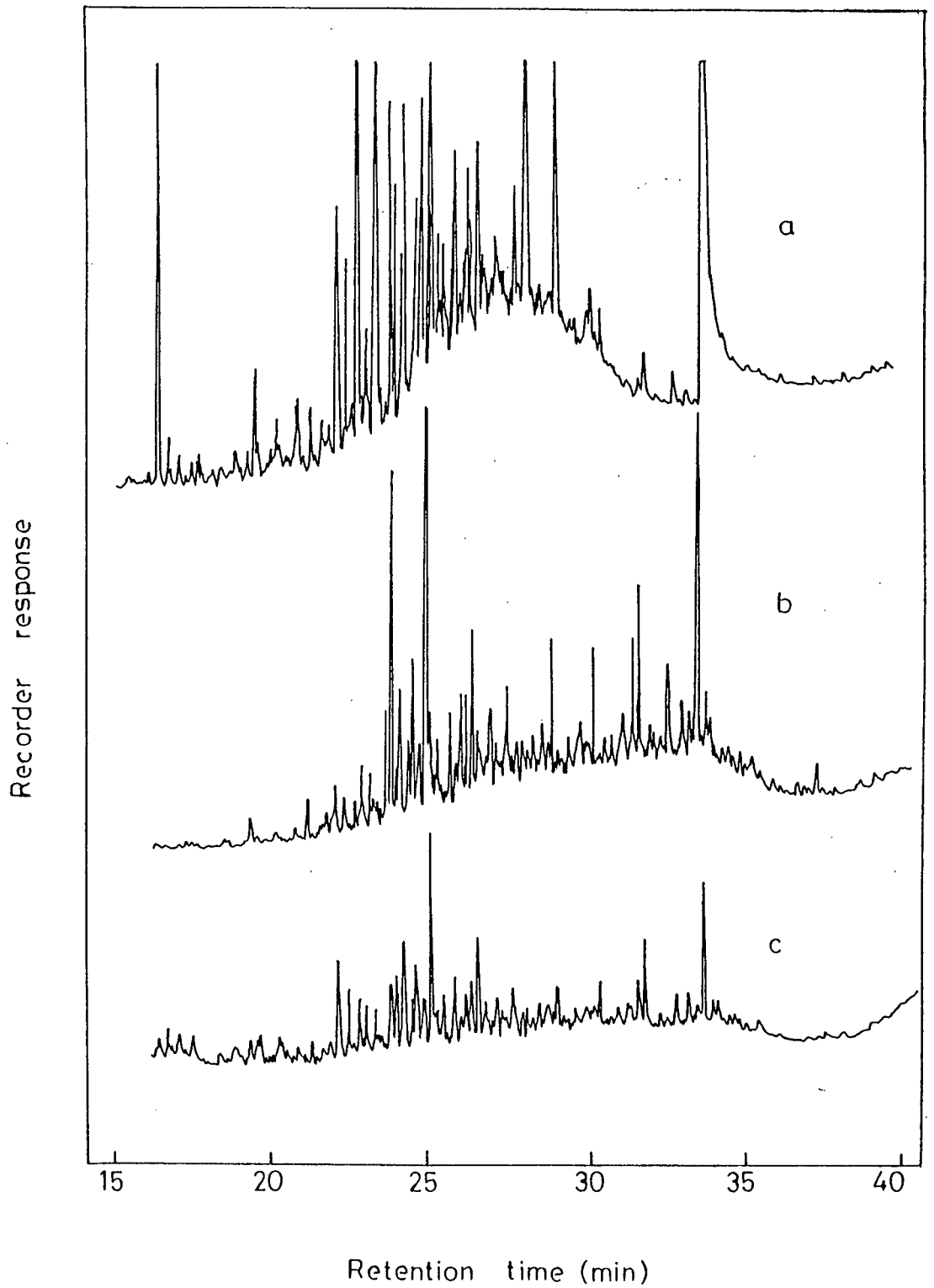


Fig 5.10: Gas chromatographs of the aromatic components of BHCO; a: control; b: after 4 days of growth; c: after 10 days of growth.

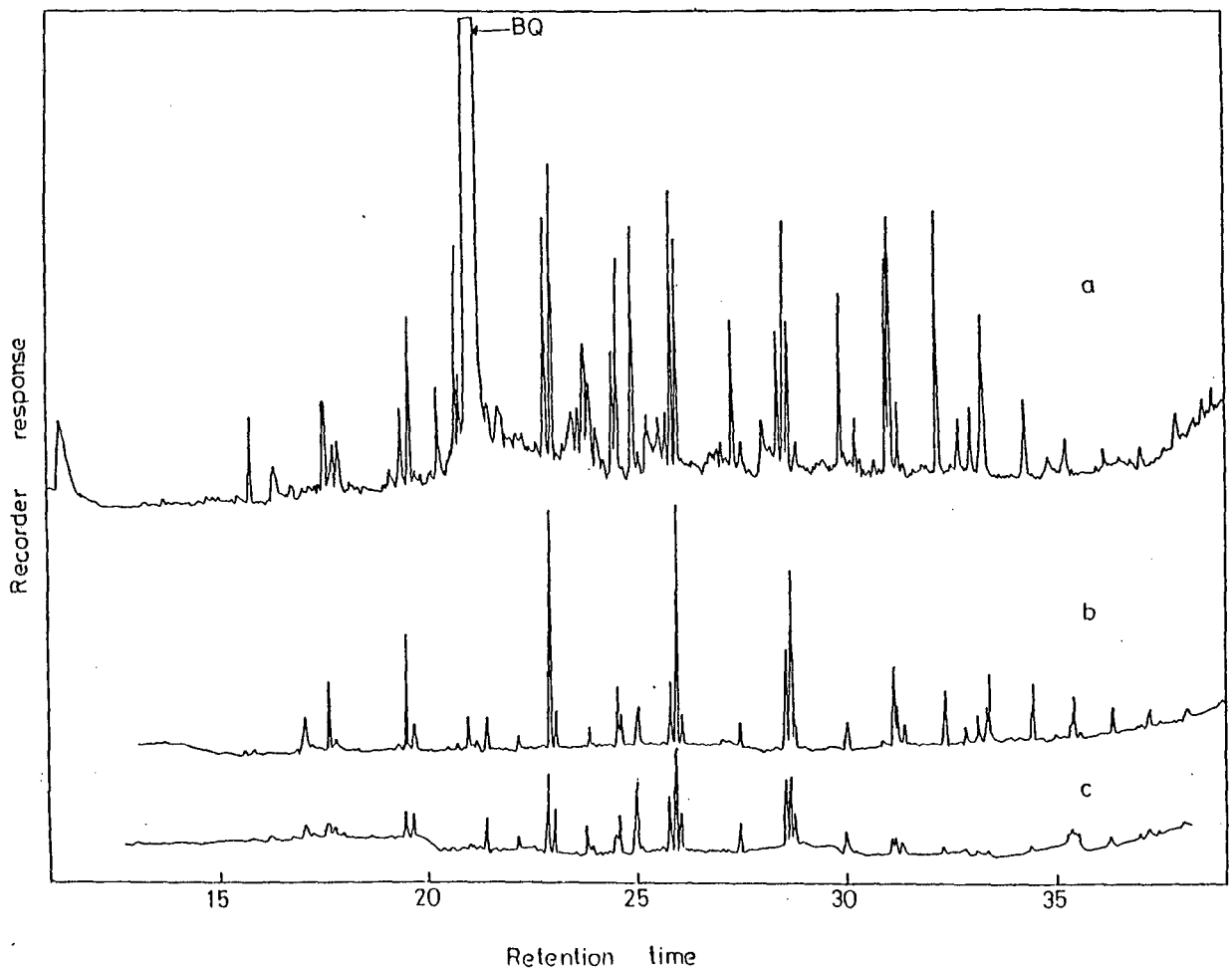


Fig.5.11: Gas chromatographs of the NPAC fraction of BHCO; a: control; BQ: benzoquinoline (internal marker), b: after 4 days of growth; c: after 10 days of growth.

91% of the saturates, 70% of the aromatics and 71% of the NPAC fraction was utilized.

Figure 5.12 shows the final biomass (mg/l) as a function of crude oil concentration. The amount of biomass obtained at the end of 7 days of growth, was directly proportional to the concentration of crude oil in the growth medium upto 1% (w/v). Strain GU104 was able to grow at higher concentrations tested (upto 3%). The maximum cell yield obtained was 0.7g/l.

Impact of quinoline utilizing ability of GU104 on other marine bacteria involved in mineralization of aromatic hydrocarbons

Strain GU104 besides being able to utilize quinoline also utilizes other substrates particularly methyl and hydroxyl substituted quinolines and NHAs. To determine whether GU104 displays any positive effect on the growth of other marine bacteria involved in crude oil degradation, by clearing quinoline contents, strain GU104 was grown in presence of strain GU104, GU102 and GU103 on binary substrates containing quinoline. m-Toluic acid (0.07%), naphthalene (0.04%) and xylene (0.04%) were used as substrates respectively for strains GU101, GU102 and GU103 in batch cultures. Parallel batch cultures were grown by inoculating strain GU104 in addition to the respective strains. In either case, stress was applied in the form of increasing concentrations of quinoline in the medium, while maintaining a constant concentration of the principal hydrocarbon substrate. The growth of the

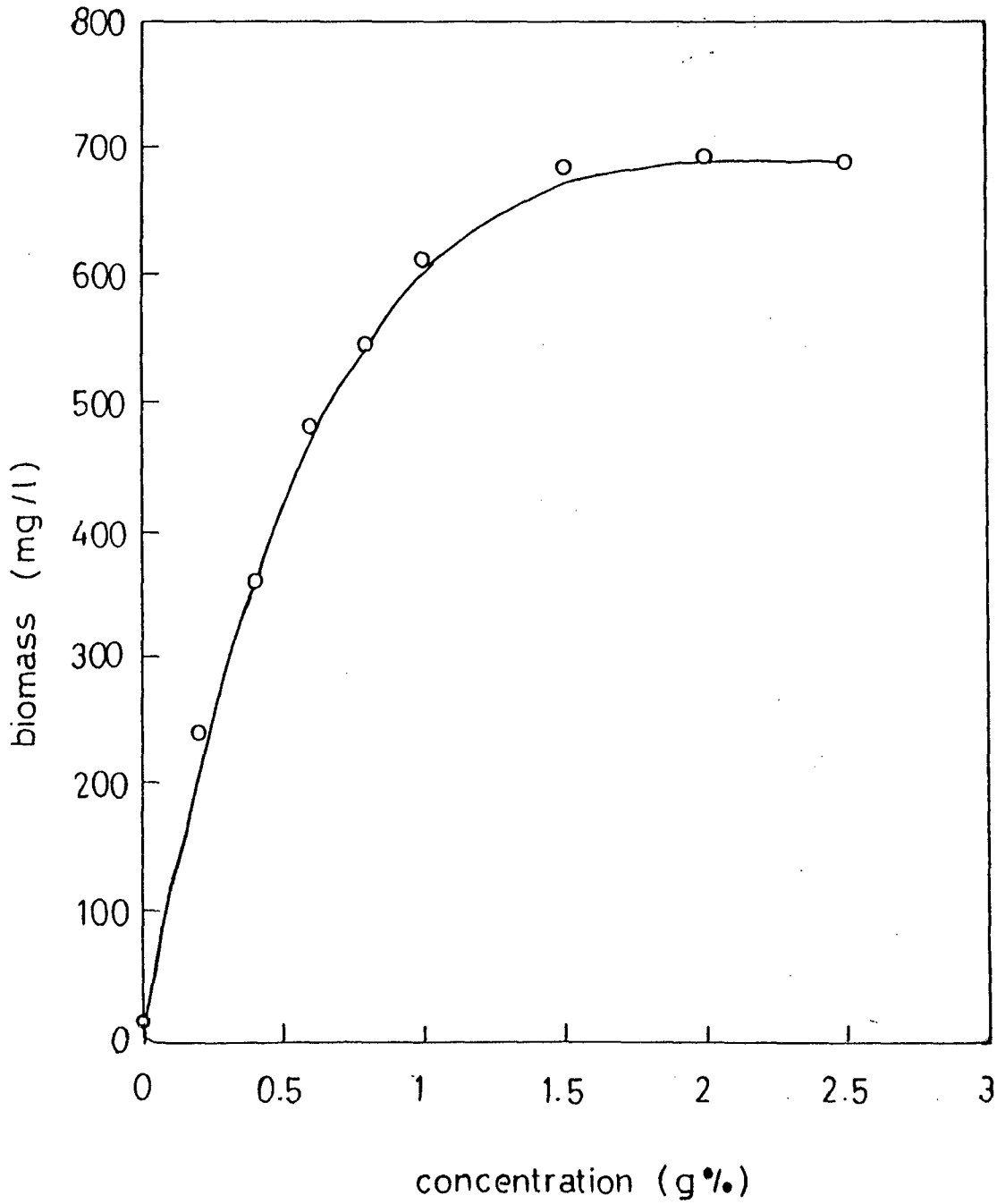


Fig.5.12: Biomass of strain GU104 as a function of concentration of BHCO in the medium.

individual strains was determined from their viable counts on media containing the respective substrates as sole carbon sources. Figures 5.13a, 5.14a and 5.15a indicate that none of the strains showed growth on levels of quinoline higher than 0.005%. The inhibition of growth was obvious from a lack of increase of turbidity in the media and viable counts on respective media. In the case where each of the cultures was grown in combination with strain GU104 in the form of a binary culture system, it was observed (Fig 5.13b, 5.14b and 5.15b), that the strains of GU101, GU102 and GU103 could grow well along with strain GU104 at concentrations as high as 0.02% of quinoline.

To check the effect of quinoline on the mixed culture system, a medium containing the three respective substrates in the appropriate concentrations and in addition 0.01% of quinoline was inoculated with the three member mixed culture system. Figure 5.16 shows that after 20h growth was still not evident as indicated by no increase in turbidity and viable counts. At this juncture, strain GU104 was introduced into the culture vessel at a concentration of 2 mg/l. After a short lag of 4-5h there was an increase in the viable counts of strain GU104 accompanied by rapid depletion of quinoline in the medium. After short lags of differing duration from the time of addition of strain GU104, the viable counts of the rest of the cultures showed a steady increase.

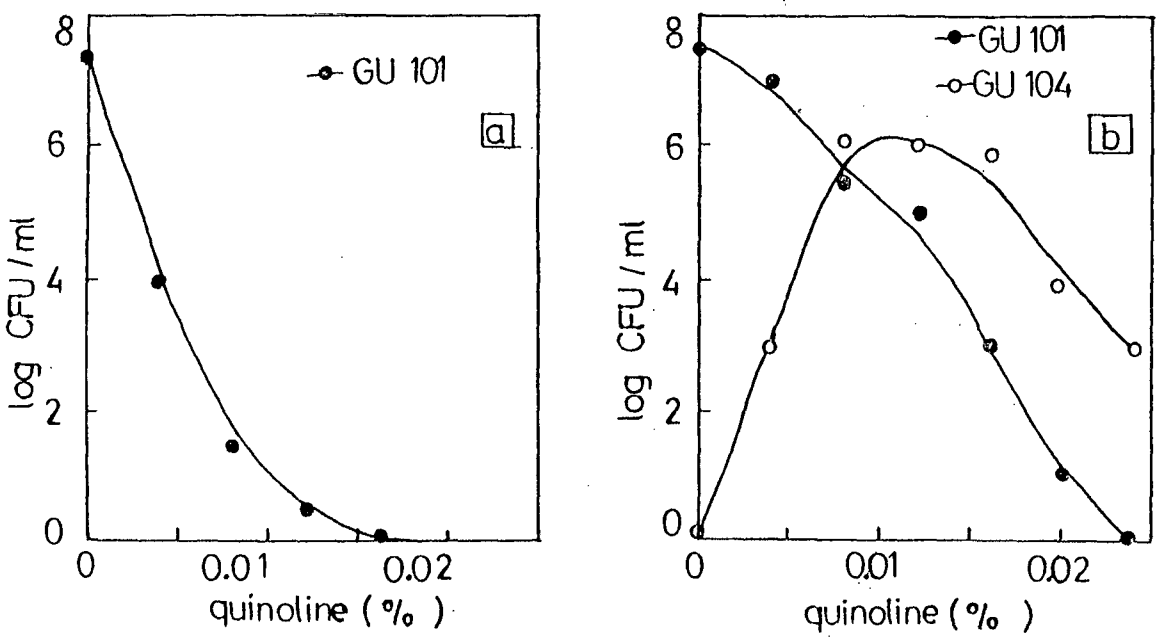


Fig.5.13a: Growth of strain GU101 (●) (m-toluic acid degrader) in the presence of increasing concentrations of quinoline. Concentration of m-toluic acid was 0.07%

b: Growth of strain GU101 (●) and GU104 (o) in the presence of increasing quinoline concentrations. Concentration of m-toluic acid was 0.07%

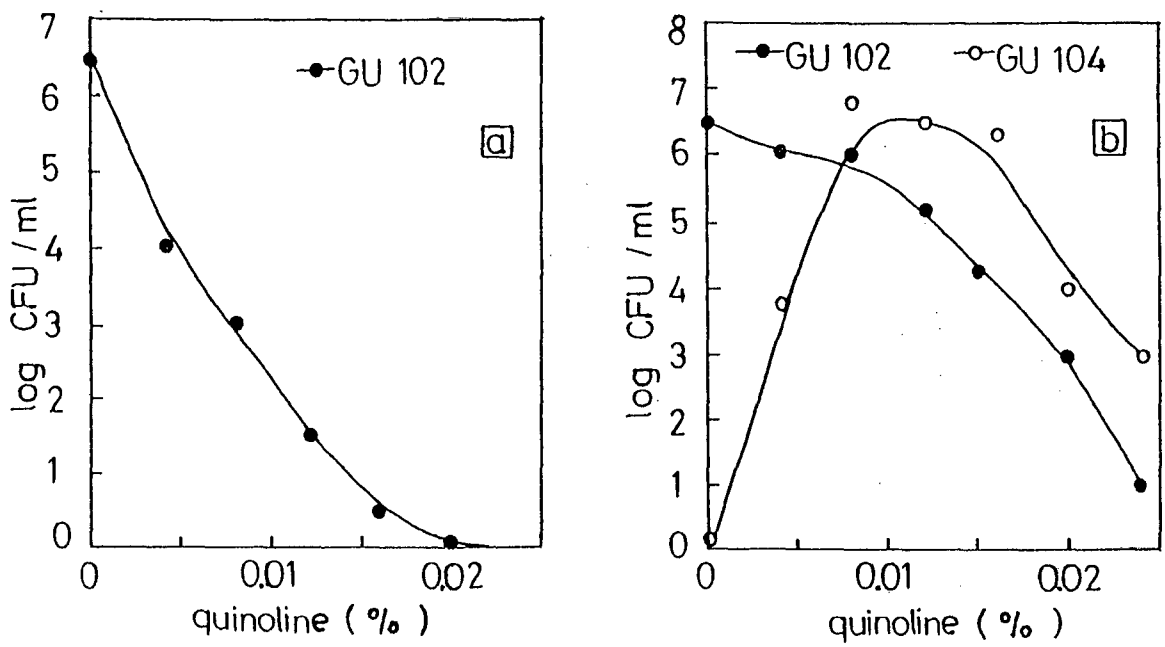


Fig.5.14a: Growth of strain GU102 (●) (naphthalene degrader) in the presence of increasing concentrations of quinoline. Naphthalene concentration was 0.04%

b: Growth of strain GU102 (●) and strain GU104 (o) in the presence of increasing concentration of quinoline. Naphthalene concentration was 0.04%.

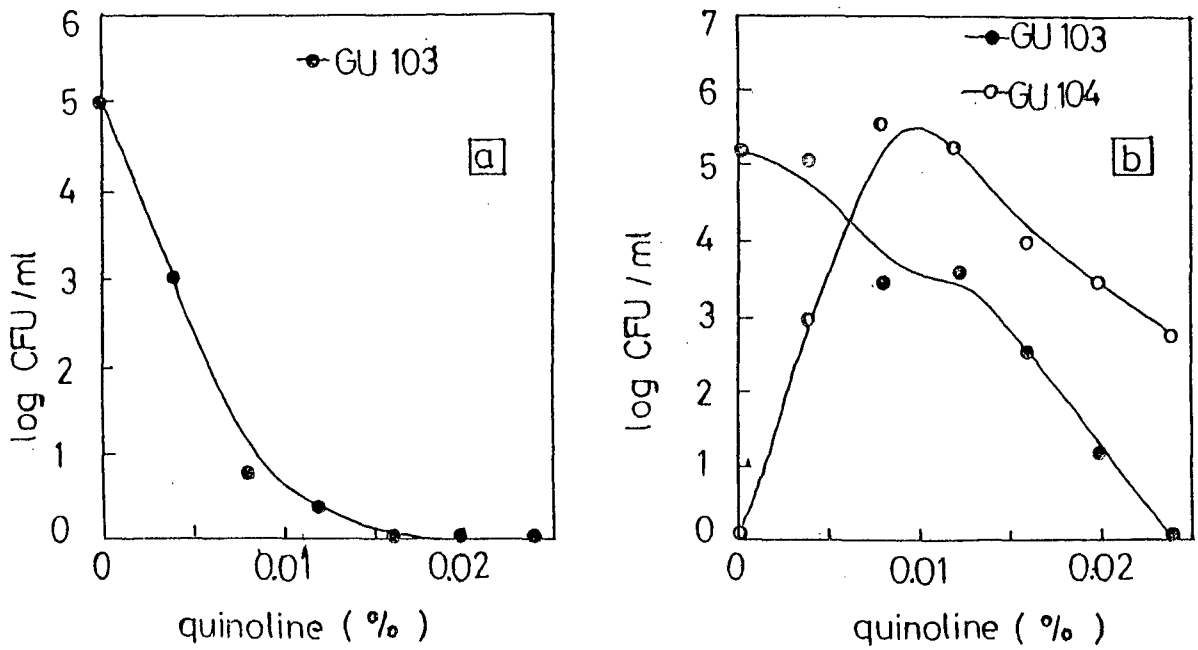


Fig.5.15a: Growth of strain GU103 (xylene degrader) in the presence of increasing concentrations of quinoline. Xylene concentration was 0.04%

b: Growth of strain GU103 (●) and GU104 (o) in the presence of increasing concentrations of quinoline. Xylene concentration was 0.04%

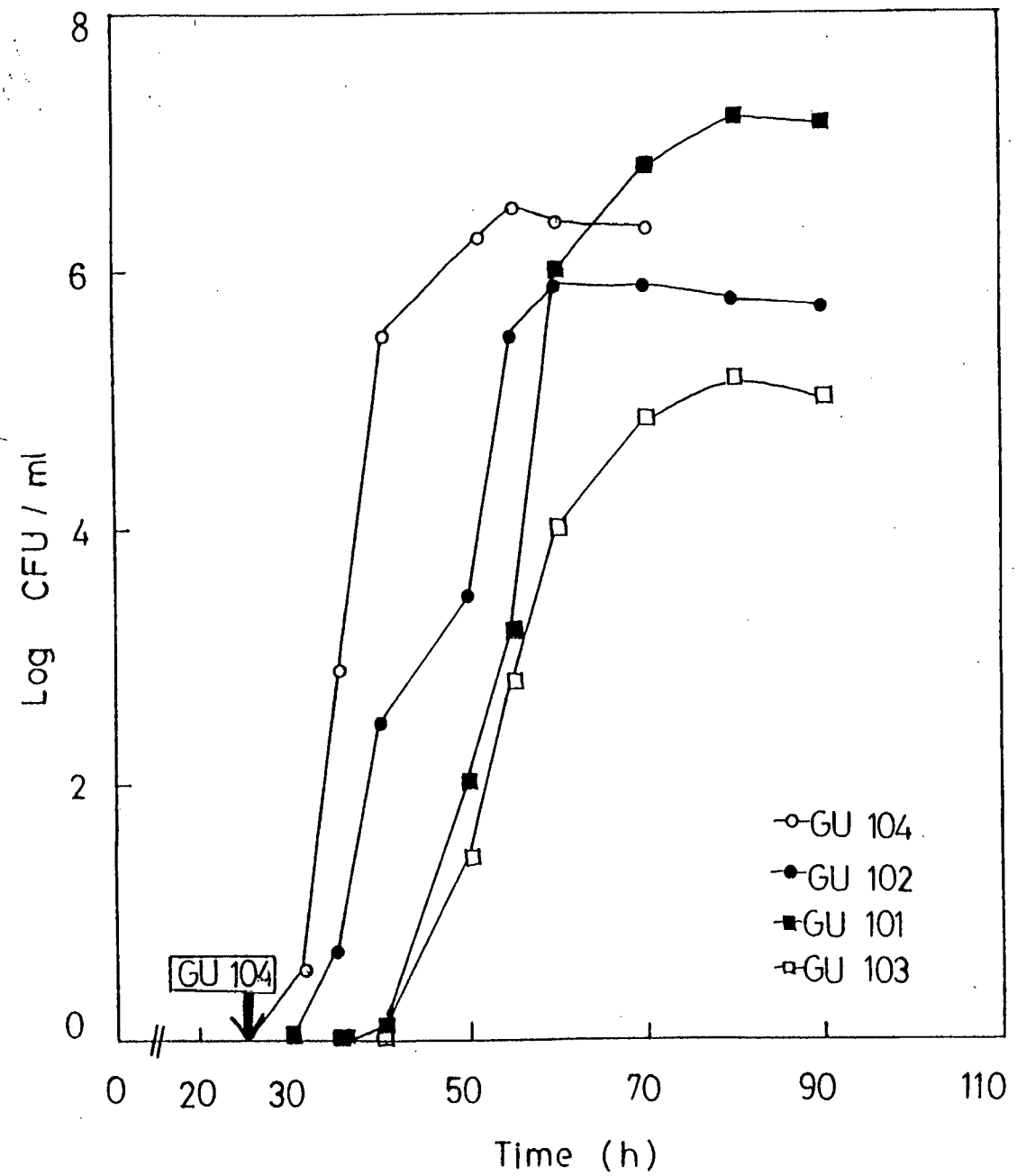


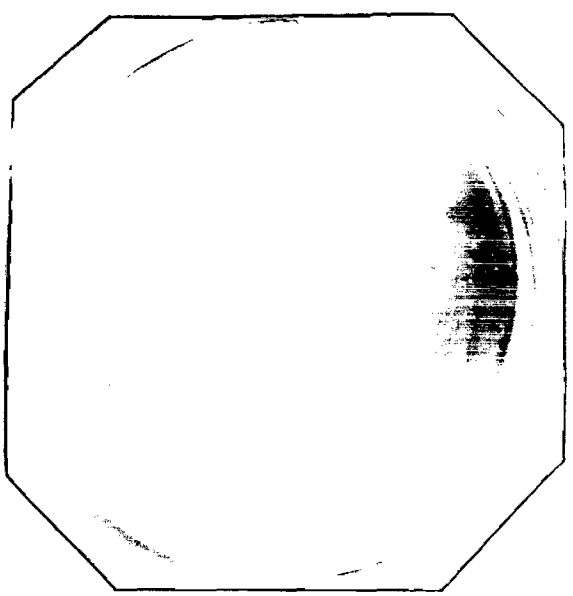
Fig.5.16: Growth of strains GU101, GU102 and GU103 in the presence of quinoline was characterized by a prolonged lag/absence of growth. Addition of GU104 at 24h followed by its growth and depletion of quinoline resulted in an increase in counts of the 3 cultures.

Genotoxic effect of quinoline on hydrocarbon degrading microorganisms:

Strain GU101, capable of using m-toluic acid as substrate, when grown in presence of low sublethal levels of quinoline in the medium (0.005%) resulted in the emergence of colonies that accumulated a brown intermediate in ASW medium with m-toluic acid. This was more pronounced in plates containing m-toluic acid as sole carbon source and quinoline (0.005%). The colonies turned darker in colour on prolonged incubation. On minute observation it was found that pinpoint colonies of mutants emerged that accumulated a brown coloured compound extracellularly (Fig. 5.17a). The colonies would not grow further after accumulation of the brown compound. The colonies however grew to a substantial size, (like wild type) when grown in the presence of a secondary carbon source such as succinate, in addition to m-toluic acid.

To demonstrate the mutagenic effect of quinoline, strain GU101 was seeded on ASW plates containing m-toluic acid and quinoline (1µl) was spotted in the centre of the seeded plate. Interestingly a zone of inhibition of growth was observed after 24-28h and on subsequent incubation brown coloured mutants emerged (Fig. 5.17b).

a



b

c

Fig.5.17: Emergence of mutants of strain GU101; a: when cultured in the presence of sublethal levels of quinoline; b: on m-toluic acid-agar plates spotted with quinoline and c: in presence of tarball components.

Genotoxic effects of tarballs and induction of metabolically blocked mutants

On placing a few mg of tarballs from Bombay High in the centre of a petri plate seeded with strain GU101, a clear zone of growth inhibition was seen in the immediate vicinity of the tar ball spot. An aqueous extract of the tarball when spotted in the similar manner led to an emergence of metabolically blocked mutants around the periphery indicating the genotoxicity of tarball components (Fig.5.17c) (Ballal, 1995).

On assaying the early enzymes of the m-toluic acid degradative pathway of the colonies of mutants it was observed that there was no detectable catechol 2,3-dioxygenase activity, unlike the wild type which showed a specific activity of 0.092 units/mg protein, confirming that the brown coloured colonies were metabolically blocked mutants possibly accumulating catechols.

Role of strain GU104 in mineralization of tarballs in marine microcosms

The cultures used in the formulation of the mixed culture system are listed in Table 5.1 together with the principal substrate on which they were isolated. All cultures could grow confluently on benzoate. Studies with this seven membered mixed culture in batch cultures of 100ml containing all the aromatic substrates showed that all the cultures grew simultaneously at different rates reaching an equilibrium in 5-7 days. The mixed culture so developed when cryopreserved at -70°C could remain viable even after 2

years without losing its degradative ability (Neil Fernandes, personal communication).

To study the efficacy of the seven membered mixed culture to mineralize tarballs in the marine environment, microcosms containing 324l of sea water were maintained in the laboratory and used to create a simulation of an oil spill scenario (Fig.5.1). Tarballs, which are the eventual fall out of such a scenario, were used as a substrate. The seven membered mixed culture was amplified to 1.6l in ASW medium containing the principal substrates. Tank A contained filtered natural sea water supplemented with nitrogen and phosphorus and Tank B contained only filtered natural sea water. Tarball (0.15% w/v) was introduced in both these tanks. Microcosms were seeded with 0.5% inoculum of the amplified mixed culture. The bacterial counts in Tank A increased and remains stationary upto 50 days whereas in Tank B the decline in the total benzoate utilizing cultures began after 20 days but reached an equilibrium at a lower level by 50 days (Fig. 5.18). Figures 5.19a,b and c show the dynamics of growth of the cultures utilizing aromatic compounds i.e. m-toluic acid, xylene and naphthalene. Counts of strains GU103 and GU102 degrading xylene and naphthalene decline after 20 days. Figures 5.19d and e show the growth analysis of components of the mixed culture utilizing quinoline and pyridine as growth substrates. Strain GU104 showed reasonably high counts throughout the test period. Srains GU109 and GU110 were also stably maintained, in the microcosms throughout the experiment. The gas chromatographic profiles of

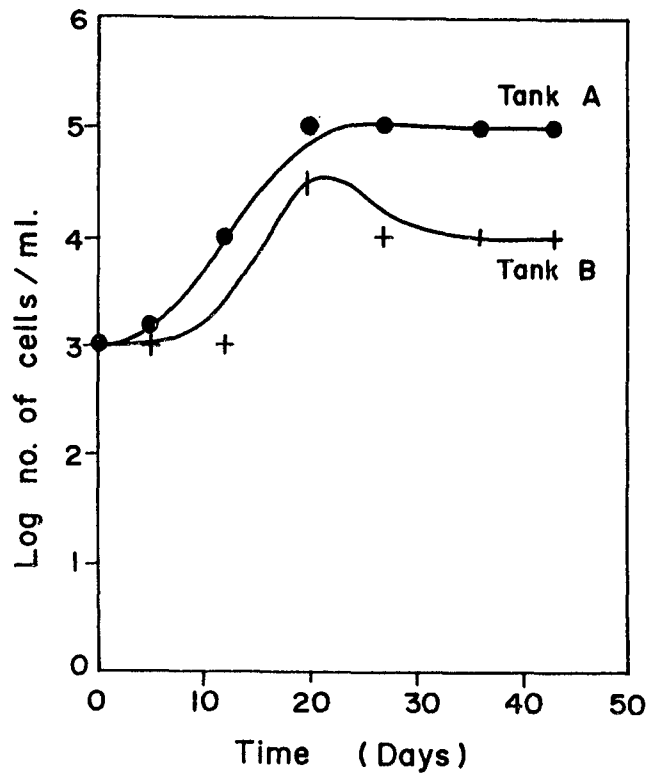


Fig.5.18: Bacterial counts of benzoate utilizing population in microcosms supplemented with N and P, Tank A (●) and without N and P, Tank B (+).

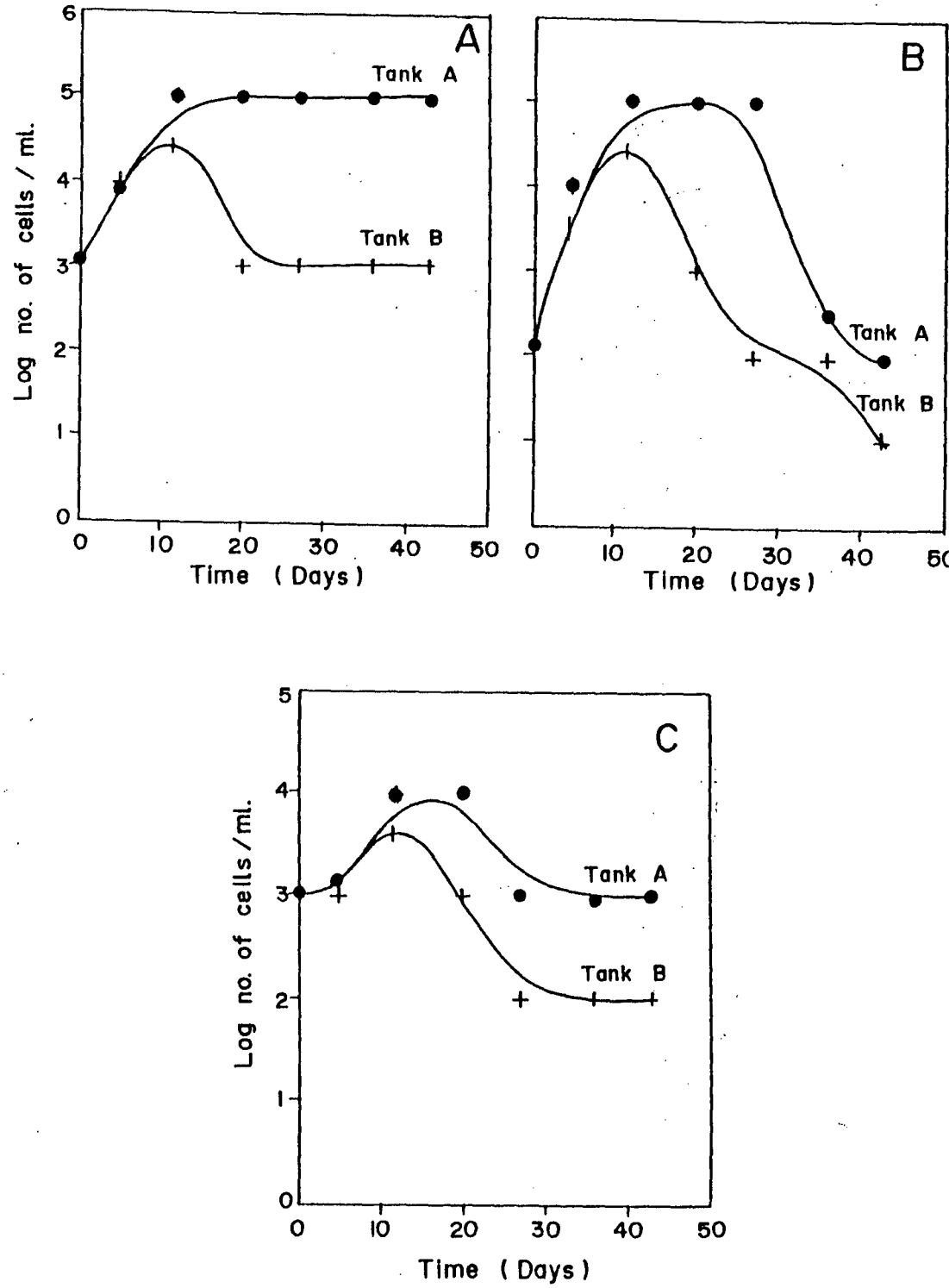
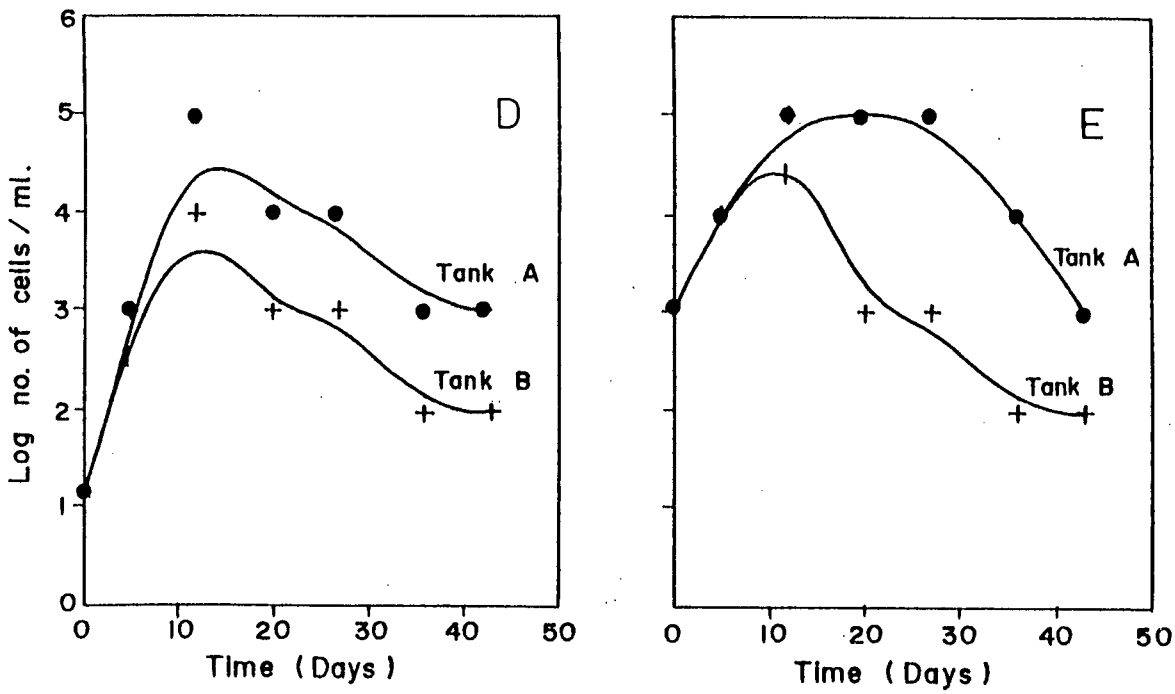


Fig.5.19: Counts of bacteria in microcosms supplemented with N and P, Tank A (●) and without N and P, Tank B (+); A: m-toluic acid degrading population; B: naphthalene degrading population; C: xylene degrading population; D: quinoline utilizing population and E: pyridine utilizing population.


D
E
Tank A
Tank B
Log no. of cells / ml.
Time (Days)
0
1
2
3
4
5
6
0
10
20
30
40
50

the tarball samples analyzed from each microcosm after exposure to the mixed culture for 50 days suggested that certain peaks are considerably reduced in both Tank A and B as compared to the control with Tank B showing a greater decline. The results indicated clear microbial attack on tarball components. At the end of 50 days growth the consistency of the tarball underwent a visible change. It lost its sticky nature and became more crumby. Characteristic evidence of destruction of tarballs and growth of the cultures over the surface (Fig. 5.20) as well as penetrating into the core of the tarball was noticed after microscopic analysis of the cross section.

Water samples were collected from various sites in the Bay of Bengal aboard Research Vessel ORV Sagar Kanya and enrichment cultures were obtained using benzoate as enrichment substrate (**Chapter II**). These mixed bacterial cultures enriched on benzoate were used to determine their oil degrading ability. Figure 5.21 shows that almost 60% of samples that yielded benzoate degraders also yielded oil degrading bacteria; the first evidence for this being a rapid emulsification of the oil. Thus benzoate served to enrich bacteria capable of degrading a more complex substrate i.e. petroleum.

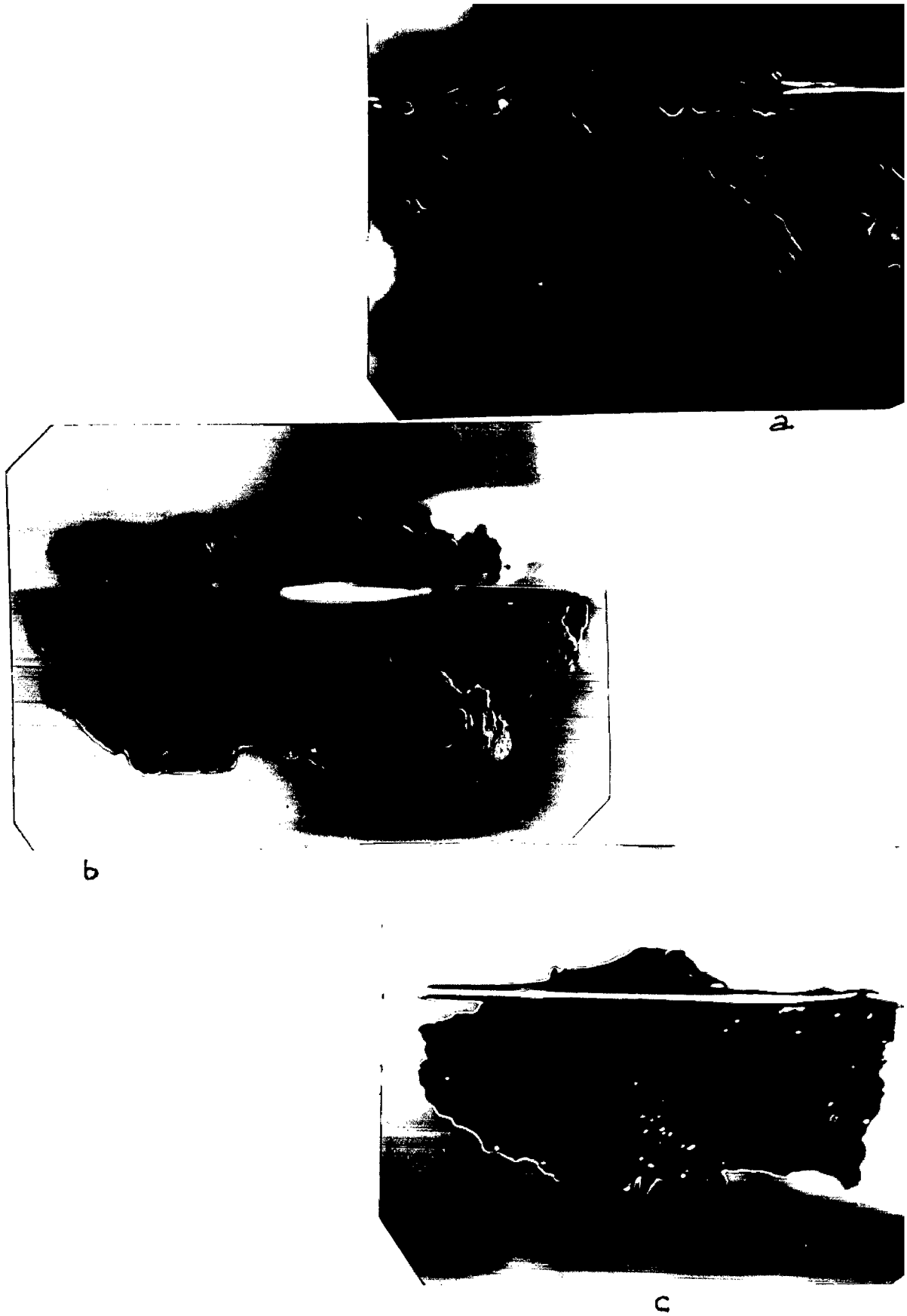


Fig.5.20: Bacterial growth on the surface and core of the tarball from microcosms (a) supplemented with N and P, Tank A ; (b) without N and P, Tank B and (c) Control Tank.

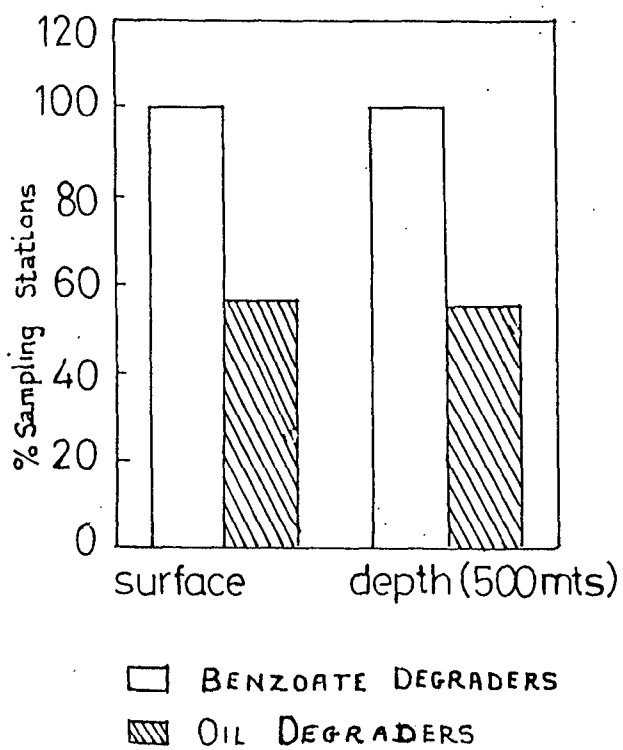


Fig.5.21: Percentage of marine water samples that yielded crude oil degrading bacteria from benzoate enriched cultures.

DISCUSSION

The biodegradation of crude oil is largely dependent on the susceptibility of its constituent hydrocarbons to microbial attack, which in turn dictated by the chemical nature and structure of the hydrocarbons (Atlas, and Bartha, 1992). The degradation of crude oil by pure and mixed cultures has been studied extensively. Horowitz *et al.* (1975) reported 40 to 66% degradation of crude oil using pure cultures. The degradation of crude oil by mixed cultures was reported to be in the range of 21-68% (Leahy and Colwell, 1990), while Atlas and Bartha (1972) and Venkateswaran *et al.* (1991) reported 58-70% degradation. In the present study with strain GU104 48% degradation of crude oils was obtained, using an oil concentration of 1g/l.

Microbial degradation of crude oil has been shown to occur by attack on alkanes while the high molecular weight aromatics resins and asphaltenes are considered to be recalcitrant or exhibit very low rates of biodegradation (Jobson *et al.*, 1972; Herbes and Schwall, 1978; Lal and Khanna, 1996). Fractionation of the residual BHCO into discreet chemical classes following degradation by strain GU104 for various time periods revealed that the saturate fraction was most rapidly and extensively biodegraded. However, the aromatic as well as NPAC fraction was also decreased considerably although to a lesser extent. On further incubation there was a further decrease in all fractions with 91% of the alkanes, 70% of the aromatics and 71% of the NPAC fraction being degraded. Walker *et al.* (1975) reported

decrease by 83.4% and 70.5% of saturates and aromatics respectively with normal and branched chain alkanes decreasing by 96.4%. They reported an increase in the asphaltene and resin fraction by 28%. In order to explain low degradability of the saturate fraction of Prudhoe Bay oil, Westlake *et al.* (1974) proposed that the bacterial metabolism of an oil and the utilization of the n-saturate fraction is not only dependent upon the genetic capability of the bacteria present but also upon the chemical composition of the remaining fraction of crude oil. Thus the presence of a higher polar NSO content in Prudhoe Bay oil could be responsible for the low biodegradability of its saturate fraction. The high biodegradability of BHCO crude oil by strain GU104 could therefore be attributed to the fact that strain GU104 is not affected by the polar compounds present due to its ability to utilize them for growth. This fact also has implications in the event of using strain GU104 in a mixed culture system, since it would contribute indirectly to the biodegradation of oil by the other cultures by reducing the polar N-content of the oil.

The isolate used in this study was enriched using quinoline as the model compound of the N-heterocyclic fraction of crude oil. This, and the fact that the marine strain GU104 also proved to be biochemically versatile attacking many aliphatic compounds and some aromatics, enhanced its ability to degrade all three fractions of BHCO. The apparent increased utilization of the aromatics in the presence of whole crude oil which contains the readily utilized saturates, as opposed to the lack of growth when the aromatics were

used in pure form as sole carbon source suggest that co-oxidation is involved in their metabolism. The works of many other investigators (Jamison et al., 1971 and Jobson *et al.*, 1972) also confirms the requirement for the presence of an assimilable substrate in order to obtain utilization of certain model aromatic compounds.

As already discussed, one of the major factors that limit crude oil degradation is the toxicity of its hydrocarbon components. Thus the composition of crude oil determines the concentration of crude oil that can be tolerated and degraded by microorganisms. Studies with varying BHCO concentrations showed that the growth of strain GU104 was proportional to crude oil concentration upto 1.5% after which it remained constant (Fig.5.12). There was no growth inhibition even upto concentrations of 3% crude oil.

Biodegradation of hydrocarbons depends on their dispersion state, the formation of emulsions, surface tension and viscosity (Bertrand *et al.*, 1993). The process of biodegradation is enhanced using various detergents by lowering the surface tension. Surfactants are the most important components of such synthetic dispersants and detergents (Koskova and Kozlovskaya, 1979). The first microbial attack that occurs on oil components is generally its emulsification. Strain GU104 brought about an effective emulsification of the oil. Our results indicate that the cells show a marked adherence to crude oil as well as alkanes i.e. hexadecane, in addition to the production of a soluble dispersing factor which lowered the surface tension of the medium. It has

been accepted that the microorganisms developing on hydrocarbons produce certain surface active agents or biosurfactants which are responsible for emulsification of the substrate, enabling its transfer into cells (Roy *et al.*, 1979; Reddy *et al.*, 1983). Broderick and Cooney (1982) showed that bacteria, which effectively degrade crude oil also, exhibit strong emulsifying activity. Strain GU104 showed maximum emulsifying activity (Fig.5.2) and adherence to crude oil (Fig.5.5) after 6 days of growth. Although the MATH assay showed a maximum adherence during the log phase, microscopic examination showed a maximum adherence to the oil droplets during the early to midlog phase. This could be explained by the fact that the oil droplets also decrease considerably in size along with growth. Therefore during late log phase although division of cells is slower the surface area for adhesion is increased accounting for the lower density of cells per oil droplet on visual examination. Thus the production of biosurfactant was found to be growth associated in strain GU104, where a parallel relationship exists between growth substrate utilization and biosurfactant production. Other reported examples of growth associated biosurfactant production include the production of Rhamnolipid by some *Pseudomonas* species, glycoprotein AP-6 by *P. fluorescens* 378, surface active agent by *B. cereus* 1 AF - 346 and Biodispersan by *Bacillus* sp. Strain 1AF – 343 (Desai and Banat, 1997). The peak of emulsifying activity at the late log phase could be attributed to the fact that the biosurfactant production is growth associated. The biosurfactant appears to remain attached to the cell membrane and is dislodged mainly towards the late log or stationary phase showing why maximum emulsifying

activity in the cell free supernatant. The release of the surface active agent would therefore lead to deadhesion of the bacterium from the interface (Neu, 1996) which is why many cells are seen in the medium during stationary phase. Another explanation, which takes into account that emulsifying activity is seen in the cell free supernatant even during log phase, could be the production of a separate dispersing factor by the bacteria that have adhered to the oil droplets. This breaks up the oil droplets into smaller units thereby producing new surface area, necessary for the increasing population. Such a phenomenon has been reported by Horowitz *et al.* (1975). The fact that emulsifying activity and adherence to hydrocarbons in strain GU104 show a positive correlation supports this view.

Our studies in the Bay of Bengal have proved that oil degrading microbes can be efficiently obtained by enrichment of bacteria on a simple aromatic compound such as benzoate. This finding is based on the assumption that most bacteria from natural environments having evolved enzymes to metabolize an aromatic compound such as benzoate may also possess more versatile metabolic traits. We have already seen in **Chapter II** that quinoline degrading bacteria were isolated from benzoate enrichment cultures. Thus, it would follow, that bacteria would exist in these enrichment cultures, that would attack other hydrocarbons, both simple and perhaps more complex, such as those existing in crude oil.

Many organisms have a wide range of hydrocarbons that they utilize while others are more restricted, however no one organism could be expected to utilize a mixture as complex as crude oil as effectively as mixed populations (Hughes and Mc Kenzie, 1975). Button (1984) observed that individual organisms could metabolize only a limited range of hydrocarbon substrates. This has also been shown in Brevibacterium and Flavobacterium which degrade 40 and 51% of crude oil in 12 days respectively as analyzed by GC and both the isolates were capable of degrading the aliphatic fraction only (Atlas and Bartha, 1972). Mixed microbial communities have distinct advantages over pure cultures. Firstly, this is because biodegradative capacity of a community is much greater, both quantitatively and qualitatively. Furthermore the resistance of a community to toxic substances may be much greater because there is a greater likely hood that an organisms that can detoxify them will be present (Grady, 1985). Our results have shown that quinoline inhibits the growth and hence the biodegradative potential of various bacterial strains (Figs. 5.13, 5.14 and 5.15). However, when any of these strains were co-incubated with strain GU104 in the presence of quinoline, the repressive effects of quinoline were lifted due to depletion of quinoline from the medium by strain GU104 and thereby clearing the way for the stable growth of the other species. Dyreborg *et al.* (1996a) reported the inhibiting effect of NSO compounds on the aerobic degradation of toluene. They also reported the inhibitory effect of NSO compounds on the aerobic degradation of benzene (Dyreborg *et al.*, 1996b), where in the lag phase increased, the degradation rate decreased, and a residual concentration of benzene was

observed in microcosms when NSO compounds were present. This shows that NSO compounds can have a potential inhibitory effect on the degradation of many compounds, and that inhibitory effects in mixtures can be important for the degradation of different compounds.

A mixed culture can be employed to achieve a greater degree of utilization of all the components of oil, however the disadvantage of such mixtures is because of the problems of interaction among different microorganisms leading to survival of only a few strains (Friello *et al.*, 1976). In the absence of external stress all the individual cultures used in this study (strains GU101, GU102 and GU103) maintained significant numbers during their growth in a mixed culture system reaching an equilibrium between 5-7 days. However, in the presence of quinoline, the additional presence of strain GU104 is required in order to eliminate the toxic effects of quinoline. The use of a mixed culture is particularly advantageous in the case where the cometabolic substrate is toxic and cometabolism leads to detoxification. Our experiments clearly proved that cometabolism occurred in the chemostat allowing for the survival of the degradative bacterial strains and broadening the biodegradative potential of the mixed culture system (Fig. 5.16).

In addition to decreasing the survivability of strains in a mixed culture, quinoline was also demonstrated to cause certain mutations in genes displaying its genotoxic effect on bacteria. This could result in the growth of a mutant population which though able to survive, may not be effective in

degrading the substrates for which it had originally been employed. Such a situation would dramatically disturb the equilibrium of the mixed culture, resulting in loss of a particular population, or worse, the loss of an important degradative function thereby belittling the use of bioremediation for environmental clean up. Our results have shown that the presence of quinoline caused accumulation of an intermediate of m-toluic acid metabolism by strain GU101. Furthermore no detectable activity of the enzyme catechol 2,3-dioxygenase was seen. This pattern is characteristic of mutants that have either lost the complete plasmid or those which suffer deletions in catabolic genes. m-Toluic acid, which contains a methyl group at the C-3 position of the benzene ring, cannot be utilized by the *ortho*-pathway, indicating that the mutants were blocked at some point of the *meta* pathway. From the assay of enzyme catechol 2,3-dioxygenase, it was confirmed that the mutants did not show any activity even on induction with benzoate (Ballal, 1995) which is an inducer of the *meta*-pathway (Cerniglia, 1984). Mutants with deleted catabolic genes are known to give similar results (Williams *et al.*, 1988). Similar mutants of GU101 accumulating brown coloured intermediates were also obtained when the water soluble leacheate of tarballs were used as the source of the mutagen. This indicates the presence of quinoline or quinoline type mutagenic compounds in tarballs which would thus affect its biodegradation by bacterial strains.

Our studies have demonstrated the role of strain GU104 in the removal of the toxic and mutagenic effects of quinoline. The use of such strains in

mixed culture systems employed for the bioremediation of hazardous chemical mixtures thus has great implications since they would allow for the survival of other bacterial strains, thus resulting in a much more efficient biodegradation of the mixed substrate.

To overcome both, the inconvenience of small volume *in vitro* experiments and the difficulty of studying the same oil slick for a long period in the open sea, we used 300l controlled microcosms to check the efficacy of the seven membered mixed culture consortium formulated in this study. As Marty *et al.* (1979) have stated: "The conditions existing in these ecosystems are different from those existing in a 'natural oil spill, but they make up an acceptable compromise between laboratory experiments and the simple observation of uncontrolled phenomena in a natural environment."

A seven-membered mixed culture was formulated with marine bacterial strains degrading m-toluic acid, naphthalene, xylene, pyridine, phenanthrene, dibenzothiophene as well as strain GU104 utilizing quinoline (Table 5.1). This culture system was used to study the biodegradation of tarballs, as it is the persistent component of crude oil, in specially constructed microcosms, as described in **Materials and Methods**. As already discussed earlier because of the toxic and mutagenic effects exerted by quinoline at very low concentration, strain GU104 is introduced to selectively clear quinoline and other NPACs from the tarballs and crude oil making them more susceptible to biodegradation by other bacterial strains. Our results showed that in batch

cultures of 100ml all the 7 members of the mixed culture system grew at different rates and reached an equilibrium in 5-7 days. The rationale behind this approach of seeding the microcosms with the mixed culture system is that the autochthonous microbial populations may not be capable of degrading the wide range of potential substrates present in complex mixtures such as petroleum. The higher total bacterial counts were observed in microcosm supplemented with N and P. Nutrient availability has been widely demonstrated to overcome a critical rate limiting factor in aerobic oil contaminated marine environments as reviewed by Leahy and Colwell (1990), Atlas (1981) and Atlas (1995). Interestingly, strain GU104 was present in the microcosms throughout the 50-day test period.

It is clear from the results obtained that the mixed culture system works effectively in scavenging and utilizing the carbon sources derived from tarballs. This is also evident from GC studies of the tarball and fractionated crude oil samples as well as the emulsification of crude oil and disintegration of tarballs. Although the mixed culture used in this study had limited members, the cumulative action was very significant. The criteria to be met by effective seed organisms (Atlas, 1977) which include the ability to degrade most petroleum components, genetic stability, viability during storage, rapid growth following storage, a high degree of growth in the environment, the ability to compete with indigenous microorganisms, and the inability to produce toxic metabolites are fulfilled by the cultures used in this study.

Thus, the data suggests that the mixed culture system is effective and offers a promise to be employed in large scale applications.

CHAPTER VI

SUMMARY

For years the oceans have been considered as the ultimate sink for all kinds of pollutants. The rationale was that dilution would serve as an effective means to reduce the risks rising from exposure to these chemicals. The spillage of tons of crude oil in the oceans in the last few decades has been of common occurrence and the effects of these spills on marine life and environment cannot be ignored.

Our studies have focussed on the biodegradation of petroleum in the environment with emphasis on essentially the Nitrogen-Heterocyclic Aromatic (NHA) components of oils. Most of the previous studies by numerous workers have mainly concentrated on the aliphatic and aromatic compounds, while very little information is available on the biodegradation of the heterocyclic compounds which are major components of asphaltenes and NSO fractions. While it is correct to emphasize the major components in petroleum it is misleading to limit analysis to them. It has very often been assumed that the nitrogen fraction in oil is unavailable to bacteria. However, many of these polar compounds are more water soluble than their homocyclic analogs and have toxic and carcinogenic properties. They have been shown to be toxic to marine fish and may exert their toxic effects to bacteria as well. This could result in the disruption of the community structure of the microbial population. Thus they may selectively inhibit or kill organisms capable of carrying out ecologically important activities, which may affect the total ecosystem.

Quinoline and its derivatives are the predominant constituents of the neutral and basic fractions of oils. We initiated our studies with the development of quinoline degrading marine bacteria by enrichment culture. Obtaining bacteria selectively enriched on quinoline could result in a population which would attack the N-heterocyclic fraction in crude oil. This is rarely the case with bacteria isolated on whole crude oil because this strategy would serve to select the rapidly growing bacteria attacking the aliphatic fraction and thus the N-heterocyclic fraction would remain unattacked.

Having established a pure culture of a marine bacterium degrading quinoline we found it to have major similarities with genus *Pseudomonas* and designated it as *Pseudomonas* sp. strain GU104. On membrane lipid analysis using computer aided taxonomy (CAT) the strain was shown to be phylogenetically closer to *Ochrobactrum anthropi* (*Achromobacter*) (D. Chandramohan, personal communication). Its growth under different cultural conditions was studied. Strain GU104 was able to utilize many other methyl and hydroxy derivatives of quinoline for growth including some aromatic compounds, crude oil, kerosene, petroleum and diesel. Many quinoline degrading bacteria previously reported are selective with respect to the range of substrates they can utilize for growth. However, the marine strain GU104 was biochemically very versatile. Results generated from different regions of Bay of Bengal showed that quinoline degrading cultures are quite rare in the ocean and the same could be isolated if benzoate is used as substrate for enrichment.

The kinetics of quinoline metabolism was studied by continuous culture in order to determine conditions of maximum rates of quinoline conversion. The K_i value (inhibitory constant for quinoline) and K_s (saturation constant) was also determined by these studies. These studies are especially significant in the case of natural isolates such as marine bacteria that survive in nutrient limiting conditions of the oceans. The immobilization of cells of strain GU104 gave high conversion rates of quinoline and the high concentration used were not inhibitory to the entrapped cells.

It was observed that quinoline was metabolized via 2-hydroxyquinoline. Oxidation of quinoline and its derivatives as also other NHAs and aromatic compounds were studied with cells of strain GU104 grown on quinoline and lactate in ASW medium. Cells grown on quinoline are able to oxidise many monomethyl and hydroxy derivatives of quinoline. However, glucose and lactate grown cells did not show any uptake with quinoline, unless subjected to induction with quinoline as inducer. The inducible nature of quinoline metabolism was also confirmed using chloramphenicol.

The first enzyme quinoline oxidoreductase carrying out the conversion of quinoline to 2-hydroxyquinoline was assayed. The K_m values of the enzyme as well as its pH and temperature optima were investigated. The enzyme was able to give a lowered activity with some methyl- and hydroxy-derivatives of quinoline. Partial purification of the enzyme was attempted and

it was found to be of a high molecular weight as apparent from native polyacrylamide gels.

Strain GU104 was found to contain two high molecular weight plasmids. Curing the larger molecular weight plasmid resulted in the loss of the ability of the strain to utilize quinoline as carbon source. Oxygen uptake and enzyme assay studies with mutants of strain GU104 indicate that the genes for quinoline metabolism are not linked.

Finally the ability of strain GU104 to mineralize various components of crude oil and tarballs was investigated. Growth on crude oil and tarballs was accompanied by a rapid emulsification of the oil and disruption of the tarballs.

Cell free supernatants of strain GU104 grown on crude oil were able to emulsify and solubilize hydrocarbons. All fractions of crude oil were attacked by strain GU104. A prominent feature was the simultaneous depletion of the NPAC fraction of oil which is normally attacked to a very low extent after primary attack on other hydrocarbons and sometimes not at all. Strain GU104 isolated by enrichment culture using quinoline as substrate was able to attack the NPAC fraction as well resulting in a 70% decrease of the same.

Many NSO compounds present in crude oils are known to affect the biodegradation of other aromatic compounds being toxic to many bacteria. Our studies have shown that quinoline affects the biodegradation of

compounds such as xylene, toluene and naphthalene since it prevents the growth of other bacteria degrading these compounds in batch cultures. Presence of strain GU104 lifts these repressive effects by effecting the removal of quinoline from the medium. The above result was demonstrated in binary as well as mixed culture systems.

One of the effects of quinoline was the formation of metabolically blocked mutants of m-toluic acid degradation which resulted in the accumulation of a brown coloured intermediate. Such a phenomenon was also seen when aqueous extracts of tarballs known to contain the remnants of crude oil after weathering and biodegradion were placed in the centre of petriplate plated with the m-toluic acid degrading bacterium. This indicates that such compounds may be present in severely degraded crudes.

The efficacy of a seven membered mixed culture consortium of which strain GU104 was a component was tested in 300l microcosms. The mixed culture worked effectively, all the members maintained stable numbers during the experiment and were able to scavenge and utilize carbon sources derived from tarballs.

In conclusion the biochemical versatility of strain GU104 as well as its ability to degrade the heterocyclic components of crude oil warrants its use in bioremediation of marine environments either singly or as part of a mixed bacterial culture.

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APPENDIX

Tris-Borate-EDTA Buffer (TBE) 5X, pH 8.0

Tris-base	54.0g
Boric acid	27.5g
EDTA(0.5N)	20.0ml

The volume was made upto 1000ml with distilled water.

Before use, the working solution was made 1X by adding distilled water.