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Research Guide.

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BIOCHEMISTRY AND CRYOPRESERVATION OF MARINE MICROORGANISMS INVOLVED IN THE BIODEGRADATION OF AROMATIC COMPONENTS OF CRUDE OIL WITH SPECIAL REFERENCE TO MINERALIZATION OF POLYCYCLIC AROMATIC HYDROCARBONS (PAH's).

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For the degree of

DOCTOR OF PHILOSOPHY

in

MARINE BIOTECHNOLOGY

by

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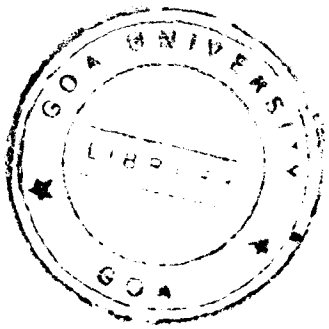
CERTIFICATE

This is to certify that the thesis entitled **"BIOCHEMISTRY AND CRYOPRESERVATION OF MARINE MICROORGANISMS INVOLVED IN THE BIODEGRADATION OF AROMATIC COMPONENTS OF CRUDE OIL WITH SPECIAL REFERENCE TO MINERALIZATION OF POLYCYCLIC AROMATIC HYDROCARBONS(PAH'S)"** submitted by Mr. Neil Fernandes 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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Certified that the Corrections are included to the satisfaction of the External examiner before the Vivavoce examination.

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Board for Vivavoce examination.

STATEMENT

I hereby state that this thesis for the Ph.D. degree on **"BIOCHEMISTRY AND CRYOPRESERVATION OF MARINE MICROORGANISMS INVOLVED IN THE BIODEGRADATION OF AROMATIC COMPONENTS OF CRUDE OIL WITH SPECIAL REFERENCE TO MINERALIZATION OF POLYCYCLIC AROMATIC HYDROCARBONS(PAH'S)"** 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.

11/5/2000

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Abbreviations

PAH's	Polycyclic aromatic hydrocarbons
NSO	Nitrogen-sulphur-oxygen
Tris-ASW	Artificial seawater containing Tris(hydroxymethyl) amino methane.
MSP	Marine salts phosphates
MNA	Marine nutrient agar
MPN	Most probable number
TVC	Total viable count
m-Tol	m-Toluic acid
TLC	Thin layer chromatography
GC	Gas chromatography
FID	Flame ionization detector
BHCO	Bombay High crude oil
AU	Absorbance units
λ_{max}	Wavelength at maximum light absorbance
rpm	revolutions per minute
g	Grams
mg	Milligram(s)
mts	Metre(s)
nm	Nanometer
ONGC	Oil and Natural Gas Commission
Flo	Fluorene
Xyl	Xylene
Pyr	Pyridine
Nah	Naphthalene
Quin	Quinoline
Phe	Phenanthrene
DBT	Dibenzothiophene
HMS	2-hydroxymuconic semialdehyde
NB	Nutient Broth
°C	Degrees Centrigade
v/v	Volume/volume
D/W	Distilled water
ml	Milliliter
mM	Millimolar
M	Molar
L	Litres
Min	Minutes
e.g.	For example

CONTENTS

Chapter I

INTRODUCTION AND LITERATURE SURVEY

PRESERVATION OF NATURAL MICROORGANISMS

Man has been using microorganisms since ages for industrial purposes such as the making of beer, wine, cheese, antibiotics, bread and milk products (Calcott,1985). More recently, the focus has shifted to the environment where microorganisms are used for the removal of waste, oil pollution and toxic pesticides.

In most of these processes, a microbial culture is inoculated into a nutrient medium and incubated for a specific time period. After this time period, the product of the process, whether it is in the cells or the culture fluid is harvested and purified to obtain a pure product. In the case of removal of pollutants, the biodegradative process is started with the inoculation of a small amount of starter culture.

For these processes to be economical, reproducible and continuous, the culture should grow very actively in a highly reproducible manner to yield a biomass with a high activity i.e. this culture has to be maintained in a state that allows very rapid growth once dormancy has been perturbed. Since serial subculturing is time consuming and can lead to genetic drift as smaller and smaller populations are selected, scientists and researchers are faced with the task of genetically stabilizing living cells.

Studies carried out have shown that subjecting them to cryogenic temperatures can stabilize populations of cells, which for all practical purposes stops time. Stabilizing cells at cryogenic temperatures is called "**cryopreservation**", an applied aspect of cryobiology, or the study of life at low temperatures. The importance of cryopreservation studies is extremely relevant today, as a number of industrial and biodegradative processes that are reaching the marketplace are based on genetically engineered microorganisms (GEMS). The increased potential for genetic instability

in these organisms dictates that the cultures used in these processes need to be held in a stable and steady state.

FUNDAMENTALS OF CRYOPRESERAVTION

Over the years, a number of methods have been used for the preservation of microorganisms such as direct transfers on agar media or agar slants (Heckly,1978), preservation under oil (Hesseltine *et al.*, 1960 ; Hesseltine and Haynes, 1974) as well as in distilled water (McGinnis *et al.*, 1974), however with all these methods, microorganisms are prone to very slow growth or turnover. This renders them potentially genetically unstable and prone to loss of vigor. On the other hand, cultures that have been preserved either by freezing or lyophilization are held in a suspended state of animation. If the population is able to survive the freezing process, then they can be stored for a very long time.

The freezing process per se is lethal to most living systems, yet it can also preserve cells and their constitutents, and it may some day permit long term storage of whole viable organs. It can slow or stop some biochemical reactions, but it can accelerate others. It is used both to preserve the fine structure of cells and to disrupt others. It is a challenge that is successfully met by some organisms in nature but not by others (Mazur, 1970).

EVENTS OCCURING DURING THE FREEZING PROCESS

Although the freezing point of cytoplasm is usually above -1°C, cells generally remain unfrozen, and therefore supercooled, to -10°C or -15°C, even when ice is present in the external medium (Mazur, 1965). This indicates that the cell membrane can prevent the growth of external ice into the supercooled interior. Two factors play an important role;

a) permeability of water into the cells and, b) the cooling velocity. Mazur (1970) in his study on yeast and human red cells has shown that when the water content of the cell was cooled slowly it would follow an equilibrum curve indicating that the cell would continuously maintain vapour pressure equilibrum with the external ice by dehydration. However, cells cooled at an infinite rate will contain more than the amount of water at a certain temperature and will therefore be supercooled, eg. yeast cells cooled at 100°C/min would contain 70% of the original water and that water would be supercooled. But such extreme supercooling cannot occur, for cell membranes apparently lose their ability to block the passage of ice crystals below -10°C to -15°C (Mazur, 1965). As a result, cells that are cooled fast enough to contain supercooled water below these temperatures will in fact complete their equilibration by intracellular freezing (Fig. 1.1).

Another important factor is the ratio of the volume of the cell to its surface area and its permeability to water (Mazur, 1963; Mazur, 1966). Regardless of whether cells equilibrate by the outflow of water or by intracellular freezing, they are subjected to a second class of events associated with the removal of water and its conversion to ice. As the temperature decreases, extra and intracellular solutes concentrate, solutes precipitate as the solubilities are exceeded (thus changing pH). These events, are referred to as solution effects. Fig. 1.1 (Lovelock, 1953).

In summary, when cells are subjected to subzero temperatures, they initially supercool. The manner in which they regain equilibrum depends chiefly on the rate at which they are cooled and on their permeability to water. If they are cooled slowly, and if their permeability to water is high, they will equilibrate by the transfer of intracellular water to the external ice, in other words, by dehydration. But if they are cooled rapidly or if

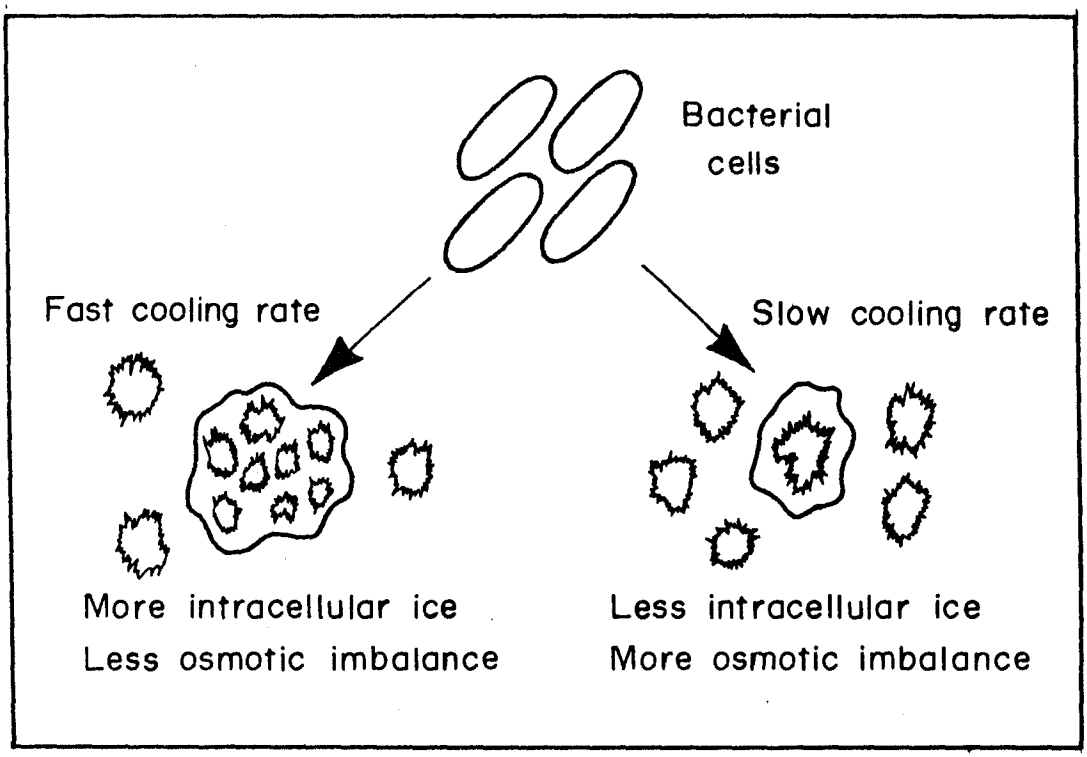


Fig. 1-1 Effect of slow and fast cooling on microorganisms. (Simione, 1998)

their permeability to water is low, they will equilibrate at least in part by intracellular freezing. Rapid cooling not only produces intracellular ice crystals, it also produces small crystals which are likely to enlarge during warming because of their high surface energies. Regardless of whether dehydration or intracellular ice crystal formation takes place, loss of liquid water is normally seen i.e. solution effects. Thus when freezing cells a fine balance must be established between slow freezing and rapid freezing so as to minimize the solution effects and intracellular ice formation. At the same time the type of organism, its permeability to water are other factors that must be considered.

FACTORS AFFECTING THE FREEZING PROCESS

(A) ***Age of the Culture*****:** The physiological condition of the microorganism has been considered by many investigators to be a factor in determining the ability to survive stress (Heckly, 1978). It is generally accepted that cells from the maximum stationary phase are more resistant to damage by freezing than cells from an early or midlog phase of growth. The percentage of cells surviving is also increased by an increase in cell density, possibly because lysed cells can yield cryoprotective substances (Bretz and Ambrosini, 1966).

Studies on *E.coli* have shown that aerobically grown cultures are more resistant to freezing than anaerobically grown cultures (Harrison and Ceroni, 1956). It has also been reported that *E.coli* is more resistant to death by rapid freezing and thawing as the concentration of cells is increased { Harrison *et al.*, (1952); Record and Taylor, (1953) ; McDougal (1954) ; Harrison (1955) }.

However, this generalization is probably not valid for all organisms, particularly viruses, in view of the observations made by Nyiendo *et al.*, (1974). They found that the percentage of survival of lactic *Streptococcus* bacteriophages is not correlated with the original titre before freezing. It thus appears that careful treatment of each microorganism is essential to maximize the recovery of a population. Although the fact remains that cells from young cultures do not survive freezing as well as the more mature cultures, it should be stressed that each type of cell appears to exhibit its own set of optimal conditions which render maximum survival.

(B) *Rate of freezing and thawing*: For a number of years it was considered essential to freeze organisms rapidly to obtain high survival. Thereafter a number of methods were developed to achieve ultrarapid freezing and thawing (Doebler and Rinfret, 1963). Thawing or warming of cells was mainly obtained by shifting the cells into saline at 37°C. However many investigators found out exactly the reverse i.e. slow freezing and rapid thawing yield the highest number of viable cells (Mazur,1966,1970; Johannsen, 1972; Rank,1973). Here the factors that come into play are mainly the " solution effects " which are caused by slow freezing and the formation of "intracellular ice" which is caused by rapid freezing which could enlarge on warming thereby damaging the cells (Mazur, 1966).

Thus a fine balance needs to be established between cooling and thawing rates for each particular type of cell. Despite the evidence cited, a cooling rate of 1°C/min seems to be widely used largely because it is impractical to determine the cooling rate for every organism.

(C) *Storage temperature*: Liquid nitrogen provides the lowest practical temperatures for storing microorganisms and, because viability is preserved so well, it is extensively

used for all sorts of organisms (Clark and Klein, 1966; Hwang, 1970; Norman *et al.*, 1970; Butterfield *et al.*, 1974). However, because liquid nitrogen is relatively expensive, it would be advantageous for viability to be preserved at higher temperatures.

There have been many studies on the effect of low temperature on the survival of organisms or types of organisms. Studies have shown that *Lactobacillus acidophilus* was viable after 6 months at either -10°C, -20°C or -60°C and some *Mycobacteria* survived quite well at -20°C(Kim and Kubica, 1972, 1973; Heckly, 1978). Thus the critical temperature is dependent on a number of factors and on the type of strain preserved, however, -70°C appears to be significantly low to preserve most organisms.

(D) *Cryprotective agents*: Many compounds have been tested as cryoprotective agents either alone or in combination. These include compounds such as glycerol, dimethylsulfoxide, sugars, serum and solvents. Although there are no absolute rules in cryopreservation, glycerol and dimethyl sulfoxide (DMSO) have been widely used as cryoprotective agents (Baumann and Reinbold, 1966; Wellman and Stewart, 1973; Heckly, 1978). Cryoprotective substances serve several functions during the freezing process. Freezing point depression is observed when dimethyl sulfoxide is used which serves to encourage greater dehydration of the cells prior to intracellular freezing. Cryoprotective agents also seem to be effective when they penetrate the cells and delay intracellular freezing and minimize solution effects (Farrant,1980). Glycerol and dimethylsulfoxide are generally used in concentrations between 5-10%(v/v), but are not used together. A large number of workers in the field of cryopreservation successfully used glycerol for preserving lactic acid starter cultures and brewing yeasts (Baumann and Reinbold, 1966; Wellman and Stewart, 1973).

However, not all studies found glycerol, an effective cryoprotectant. Barnhart and Terry (1971) found that as the glycerol concentration was increased, the percentage of *Neurospora crassa* surviving freezing decreased by 25-35% indicating the toxic effects of glycerol. Some workers used glycerol in combination with sugars such as 5% of either lactose, maltose or raffinose for *Saccharomyces cerivisiae*, *Pseudomonas aureofaciens*, *Streptomyces tenebrarius* and four species of algae (Daily and Higgens,1973).

Thus it can be concluded that the freezing of microorganisms requires a thorough study. Careful treatment of cells is essential to maximize the recovery of a population. It should be stressed that each type of cell appears to exhibit its own set of optimal conditions that renders maximum survival. The idea that 10% glycerol as a cryoprotectant and a cooling rate of 1°C/min are the optimal conditions for maximum survival is an example of over extrapolation of a formula for one cell line.

NATURE OF CRYOINJURY

It seems that the problem of identifying the nature of damage caused by freezing is similar to that of a blind man trying to characterize an elephant. The types of injury that can be demonstrated are varied. Some of the damages caused by freezing and thawing are loss of viability, membrane or cell wall damage, inhibition of respiration and active transport, retention of nutrients, inhibition of RNA, DNA and protein synthesis, inhibition of induction of operons or complex enzyme reactions.

It now appears that there at least two types of injuries that can result from the freezing of cells. Litvan (1972) believes that the injury produced by slow cooling rates is a result of dehydration and that rapid cooling produces intracellular ice formation, thereby

causing cell rupture. Calcott (1978) in his study on cryopreservation of microroganisms stated that there are four targets which are affected by freezing.

1) ***Cell Membrane***: The cell membrane is extremely vulnerable to freeze thaw. (Calcott, 1978; Beuchat, 1978). The type of damage can be detected in a number of ways. MacLeod and Calcott (1976) demonstrated the release of cellular constituents, UV absorbing material, potasium and β-galatosidase which were related to the loss of viability. While the mechanisms that cause the loss of membrane integrity are not understood, certain studies that have examined the effects of altered membrane composition on cryosensitivity have been useful.

   Cell membrane composition of microorganisms can be modified in a number of ways. Alteration of the cell environment (growth and temperature) can phenotypically alter the lipid composition of the cell. Calcott and Petty (1980) were able to phenotypically alter the lipid composition of the bacterial cell and observed that cryoresistance was altered. Organisms rich in cardiolipin and with a higher unsaturation of this fatty acid were more resistant to freezing in water and saline at both slow and rapid cooling rates. Studies have shown that *E.coli* mutants that are defective in unsaturated fatty acid synthesis show cryosensitivity that is dependent on the unsaturated fatty acid that is supplied in the medium.

   Release of an intracellular enzyme, β-galactosidase, a periplasmic enzyme, phosphodiesterase, and an outer membrane marker of LPS, ketodeoxy octoulosonic acid (KDA) were also observed in *E.coli* (Calcott and Petty, 1980).

   Calcott and Rose (1982) demonstrated that the tighter the membrane (stronger interactions) between sterols and phospholipids, the more resistant the cells were to freeze-thaw stress; longer the unsaturation chain, higher was the survival 20:1>

18:1 > 16:1). Substitution of the monosaturated fatty acids for diunsaturated fatty acids (eg. linoleyl versus oleyl) also increased the resistance to freeze-thaw stress, presumbaly by decreasing the melting temperatures of the membrane. Studies by Rose (1976) have shown that the membrane composition can have a profound impact on physiological functions such as retention of amino acids and ethanol tolerance. In cryopreservation studies, the trend as seen indicates that although the stresses are different, the involvement of the cell or cytoplasm membrane is common and an important structure for control of cell response to freezing.

2) ***Cell wall structures and its role in Cryosurvival*** : Gram-negative bacteria are characterized by possesing a complex cell wall. Outside the cell membrane, the cell is bound by a second unit membrane, the outer membrane. The space between the outer membrane and the cell membrane is called the periplasm, a region that contains the peptidoglycan and a number of enzymes. The outer membrane is composed of a bilayer with specific proteins and lipolysaccharides embedded in it (Fig. 1.2).

Marine gram-negative bacteria on the other hand are notably pleomorphic and their pleomorphism together with a low content of amino sugar in the cell has led to the suggestion that the cell walls of these organisms are relatively weak. Studies on a number of marine bacteria have shown a number of common characteristics, the only one which distinguishes them from bacteria in other habitats is the capacity to grow and survive in the sea (MacLeod, 1965). When gram-negative bacteria are subjected to the stress of freeze-thaw, damage can be detected which can be directly related to the cell wall e.g. leakage of periplasmic enzymes, entrance of large molecules such as lysozyme and trypsin (MacLeod and Calcott,1976; Calcott,1978; Beuchat,1978). Both the studies showed the importance of the outer membrane in determining the

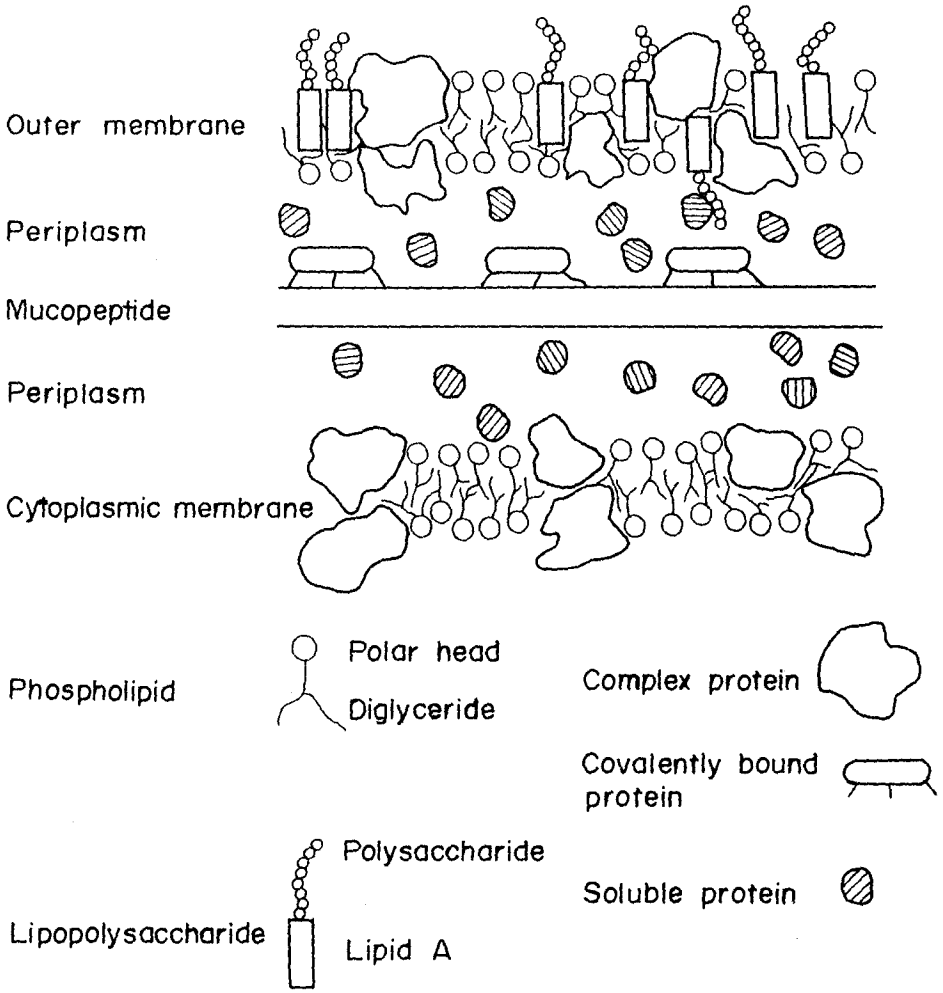


Fig. 1·2 Model Structure of a gram-negative bacterial cell wall (Clarke and Ornstein, 1975)

resistance of microorganisms to freezing and thawing. Further, the importance of the component structure is emphasized in determining cryoresistance. This may explain why different strains isolated form different environments, which probably differ in the chemical makeup and composition of individual components show differences in the susceptibility to stresses such as freeze-thaw.

3) ***DNA damage and the mutagenicity of freezing and thawing***: As early as the 1960's Postgate and Hunter (1961) questioned whether the process of freezing and thawing was mutagenic. At that time, they were able to detect three auxotrophic mutants in a population after the stress. However, the techniques used did not allow a quantitative evaluation. From the studies it is still not clear how the stress of freeze-thaw can cause damage. It is conceivable that a lesion similar to that introduced by X-rays or ionization radiation may be involved.

4) ***Stability of plasmids during freeze-thaw***: Since DNA appears to be a primary target of freezing and thawing stress, it was thought that plasmids present in bacteria might be damaged, and if damage did occur, plasmids could also be cured on freeze-thaw. The answers to these questions can be very relevant particularly to biotechnology where a large number of gene products have been cloned into artificially constructed or engineered plasmids, many of which are unstable under routine conditions.

Calcott and Calcott (1984) using two natural (non-genetically engineered) plasmids as models, examined the incidence of loss of plasmids from the survivors after freeze-thaw. As predicted, freezing and thawing did cure the plasmid, but at a low rate. As the number of freeze-thaw cycles (slow, rapid, ultrarapid) increased, the percentage of populations containing the plasmid decreased. Studies have not been

conducted so far with a chimeric plasmid that contains a foreign structural gene. With the advent of the construction of plasmids that contain multiple copies of foreign structural genes flanked by multiple promoters, there will be a situation where the number of regions of homology in the plasmid is going to become very large, this can only result in the instability of the plasmid under growth conditions. This instability could wreck havoc on the economics of the process of not being able to control the genetics of the organism under question.

CRITERIA FOR PRESERVATION

In the previous sections, the process of freezing and the types of damage that occurs have been discussed ,but how is one to establish the quality of the preservation method. This is done on the basis of the following criteria:

A) ***Ability to Reproduce***: Quantitative measurements such as colony-forming units or plaque forming units are taken as an indication of the quality of the preservation technique; these assays are made before and after preservation. However, in 1970's; several workers observed an unusual phenomena termed as metabolic injury (Ray and Speck,1973; MacLeod and Calcott, 1976 ; Beuchat,1978). The essential features of this phenomenon were that of the survivors of the stress of freezing and thawing, a percentage of those able to form colonies on a nutrient-rich medium were unable to grow on a minimal salt medium. Incubation of the cells in a nutrient medium without growth allowed the cells to regain the ability to grow on a poor medium indicating that these cells were going through a reversible metabolic injury. Thus the selection of a proper medium for evaluating the microorganism's ability to reproduce should receive serious consideration. A large number of workers (Kuo

and Macleod,1969; Gomez *et al.*,1973) have shown that microorganisms that have been injured during freeze-thaw could be repaired by a particular medium or a specific compound supplemented in the growth medium.

B) ***Maintenance of Functional Properties***: Viability, even it is based on the number of organisms surviving, is not an entirely satisfactory criterion for evaluation of the effectiveness of culture preservation. Studies on preservation described in Heckly (1978) demonstrated that the efficiency of two different tuberculosis vaccines made for the antivirulent *bacillus* of Calmette and Guerin (BCG) were not co-related to viability. Other workers also demonstrated that the numbers of viable organisms provide a poor index of preservation. It is particularly important that criteria other than the number of viable cells be used in the development of preservation methods. Effectiveness of the preservation methods used in the cheese industry has been evaluated by simulating proceedures used in making cottage and cheddar cheese or buttermilk (Lamprech and Foster,1963; Keogh,1970).

These few examples show that biochemical or biological activity after storage may be an appropriate criterion for evaluating preservation efficiency. However, viability assays should not be abandoned because they provide sensitive and quantitative measures of quality control which can be used to predict when a culture needs to be reprocessed.

C) ***Maintenance of Genetic Properties***: Another important factor in this concept of culture preservation is the fact that the genetic composition of the progeny must be the same as that of the original culture. Normally, such conditions are not studied in detail. However, gross changes such as pigmentation (Servin-Massieu and Cruz-Camarillo, 1969), changes in morphology, temperature requirements, fermentation

medium should be noted. Kubica *et al.* (1977) concluded that although mycobacteria stored at -70°C for 2-5 years appeared to be sluggish in diagnostic tests, culturing restored their vigor and key functions were retained. It was thus observed, that the damage to the genetic composition is not much as most of the microorganisms have the ability to repair the damage.

In summary, one can conclude that the freezing process is one of the best solutions for genetically stabilizing cells without loss of its genetic and functional properties. However, careful treatment of cells is essential for maximum recovery of a population. The old idea that 10% glycerol as a cryoprotectant and a 1°C /min cooling rate are the optimal conditions for maximum survival cannot be used for all types of organisms. What might hold good for gram-negative bacteria may not hold good for gram-positive organisms. In the case of algae, fungi and marine bacteria careful understanding of their growth conditions/nutritional requirements etc. need to be studied, which have not been done. Table 1.1 shows a quick reference chart for cryopreservation of various types of organisms. Futhermore, most of the studies have dealt with the preservation of a single culture, there are few reports which deal with the preservation of mixed microbial consortiums which are now being used in many industrial processes as well as in the removal of toxic pollutants from the environment. Preservation is relevant in the early stages of any industrial or biodegradative process and can affect the entire process itself. Although cryopreservation is very important, little research on this topic has been carrried out.

Table. 1.1 : Quick reference chart for the preservation of living cells(Simione,1998)

Cell Type	Number of Cells	Cryoprotective Agents	Minimum Storage Temperature
Bacteria	10^7/ml	Glycerol(10%)	-60°C*
Bactriopahge	10^8pfu/ml	Glycerol(10%)	-60°C
Fungi			
a) Hyphae	#	Glycerol(10%)	-150°C
b) Spores	10^6/ml	Glycerol(10%)	-60°C
Yeast	10^7/ml	Glycerol(10%)	-150°C
Protozoa	10^5-10^7/ml	DMSO(5-10%) or Glycerol(10-20%)	-150°C
Algae	10^5-10^7/ml	Methanol(5-10%)or DMSO(5-10%)	-150°C
Plant Cells	**	DMSO(5-10%) + Glycerol(5-10%)	-150°C
Animal Cells	10^6-10^7/ml	DMSO(5-10%)	-150°C
Hybridomas	10^7/ml	DMSO(5-10%) + Serum (20%)	-150°C
Plant Viruses	++	None	-60°C
Animal Viruses			
Cell Free	++		-60°C
Infected cells	10^6/ml	DMSO(7%) + Fetal Bovine Serum (10%)	-150°C
Plasmids	10^7/ml	Glycerol(10%)	-150°C
Phage libraries	++	Glycerol(10%)	-150°C

* While -60°C is adequate for most organisms in the groups noted some sensitive cells may not survive long periods of storage at this temperature.

Mycelial masses are prepared for freezing of the hyphae of fungi without regard to the number of cells.

**Plant cells are generally packed to 3-20% cell volume for freezing.

++The number of infectious particles has little effect on the recovery of viruses and bacteriophages.

MARINE POLLUTION WITH SPECIFIC REFERENCE TO PETROLEUM POLLUTION

For centuries, the sea has been used as a huge reservoir for the disposal of all kinds of wastes. However, during the last decade it has become obvious that the balance between the input of autochthonous and allochthoneous substances and decomposition processes in the sea i.e. the "self-purification" process has been disturbed. Of all species, humans seem to have made the greatest impact on the disturbance of this self-purification process through activities such as over fishing, disposal of waste, toxic pollutants and oil pollution (Hoppe, 1986).

However, the marine microbial population has a great flexibility in its response towards materials introduced into the sea due to pollution. It is not surprising therefore, that microorganisms have evolved mechanisms for the conversion of these toxic pollutants i.e. nature serves us by working by itself. It is thus the duty of conservationists and scientists to observe and understand these processes in order to understand the limits of this self-purification process.

One such area moving in that direction is the field of biotechnology which draws on a range of sciences, together with engineering, to exploit the properties of microbes, plants and animals. It harnesses the biological processes that occur in cells to provide products such as medicines, food etc. and services such as pollution control and sewage treatment. It is particularly relevant in the field of pollution control where the use of organism's natural mineralization properties are made use in cleaning up toxic pollutants, thereby providing us pure water and a clean environment which we hope will be more esteemed in the near future.

Pollution occurs when something harmful is added into the environment be it land, air or sea. In the case of marine pollution, the causes are many. Waste products from industry, agriculture and oil pollution which may contain highly toxic substances are often to blame. These are either discharged into the marine environment or seep down through the ground into the underground sources. In the United States alone, scientists estimate that one three-quarter of the waste-disposal sites in the country may be producing hazardous chemicals that find their way into water sources. Many of these chemicals are toxic or suspected carcinogens. However, many of these chemicals that have been exploited by modern industry are closely related to biogenic material and are therefore biodegradable, on the other hand, many others are strangers to the biosphere (xenobiotics) having been present for only an instant on an evolutionary scale (Grady, 1985). These chemicals could pose a problem as many of them persist in the environment thereby having deleterious effects on living systems due to their toxicity.

Of importance, is the subject of oil /hydrocarbon pollution which is probably one of the most emotive subjects in any discussion on environmental pollution. Hydrocarbons may arise from oil fields following geological disturbances or they may be formed biosynthetically. The amount of oil released into the environment is given by several authors (Hooper, 1978; Dart and Stretton, 1978).

Despite the input of hydrocarbons, the marine ecosystem has always been able to cope with naturally occurring amounts of hydrocarbons mainly because it is spread over a wide area, diluted in extremely large volumes of water and usually released gradually over a period of time. The major problem, however is dealing with the stress of massive local pollution over a short period of time following serious accidents. Table 1.2 lists

Table.1.2. List of tanker accidents, in the marine environment (International Tankers Owners Pollution Federation)

Ship	Year	Location	Oil Lost(Tonnes)
1) Torrey Canyon	1967	Scilly Isles	119,000
2) Wafra	1971	Off Cape Agulhas, South Africa	65,000
3) Metula	1974	Magellan Straits, Chile	53,000
4) Jakob Maersk	1975	Oporto, Portugal	80,000
5) Uriquiola	1976	La Coruna, Spain	108,000
6) Hawaiian Patriot	1977	300 nautical miles off Honolulu	99,000
7) Amoco Cadiz	1978	Off Brittany	27,000
8) Atlantic Empress	1979	Off Tobago, West Indies	280,000
9) Independenta	1979	Bosphorous, Turkey	93,000
10) Castillo de Belliver	1983	Off Saldhana Bay, South Africa	257,000
11) Assimi	1983	55 nautical miles off Muscat,Oman	65,000
12) Nova	1985	Gulf,20 nautical miles off Iran	70,000
13) Odyseey	1988	700 nautical miles off Nova Scotia	132,000
14) Khark 5	1989	120 nautical miles off Atlantic coast of Morocco.	80,000
15) Exxon Valdez	1989	Prince William Sound, Alaska	37,000
16) Have	1991	Genoa, Italy	140,000
17) ABT Summer	1991	700 nautical miles off Angola	260,000
18) Aegean Sea	1992	La Coruna, Spain	72,000
19) Katina P	1992	Off Maputa, Mozambique	72,000
20) Braer	1993	Shetland Islands	85,000
21)Maersk Navigator	1993	Indonesia/Malaysia	2million barrels
22) Sea Prince	1995	South Korea	700
23) Erika	1999	France	15,000
24) Al Jazya 1	2000	United Arab Emirates	980

some of the major tanker accidents at sea (International Tanker Owner's Pollution Federation).

Accidents of such type have tended to happen in areas close to the coast, and the major problem has been to stop the oil before it reaches the coastline, which is one of intense biological activity (Bartha and Atlas,1977, Baker,1978). The Gulf war during January and Feburary 1991 also resulted in the largest and most intense oil pollution event of all time. A minimum of 1.0-1.3 billion L (6-8 million barrels) of crude oil was spilt into the sea off the shore of Kuwait and at the head of the Gulf between January and May 1991 and a minimum of nearly 16 billion L(100 million barrels) were burned or spilled at the 702 sabotaged oil well heads in Kuwait's terrestrial oil field between February and November 1991 (Evans *et al* .,1993).

Crude oil spilt into the sea comes from a variety of sources (Fig 1.3) (United Nations Environment Programme. Report No. 15, 1993).

CHEMICAL COMPOSITION OF OIL

The chemical composition of crude oil is extremely complex and variable consisting paraffins, naphthenes, aromatics and the heteroatoms (nitrogen, sulphur and oxygen-NSO's). However, the elemental composition varies over a small range (82% C, 12-15% H) the remainder being oxygen, nitrogen and sulphur (2-4% weight basis). Fig.1.4A & B shows the classification of oil (Schobert,1990).

Young shallow crude oils are often sour , have a high aromatic content, a high viscosity and a high sulphur content. Old shallow crudes are less viscous in comparison, have a lower boiling point range and have short paraffin chains. However, the most desirable crudes are the old deep crudes which have a low viscosity, low sulphur content and a

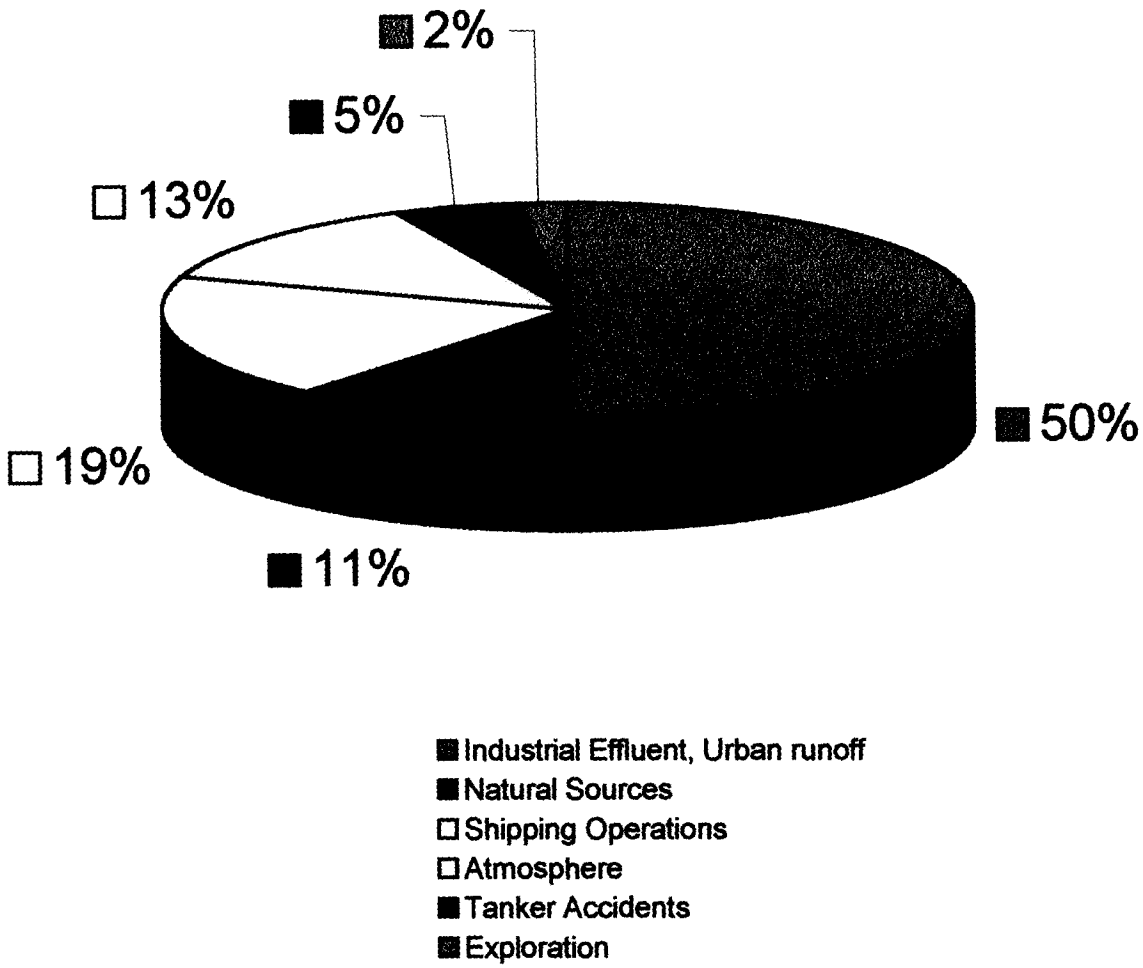
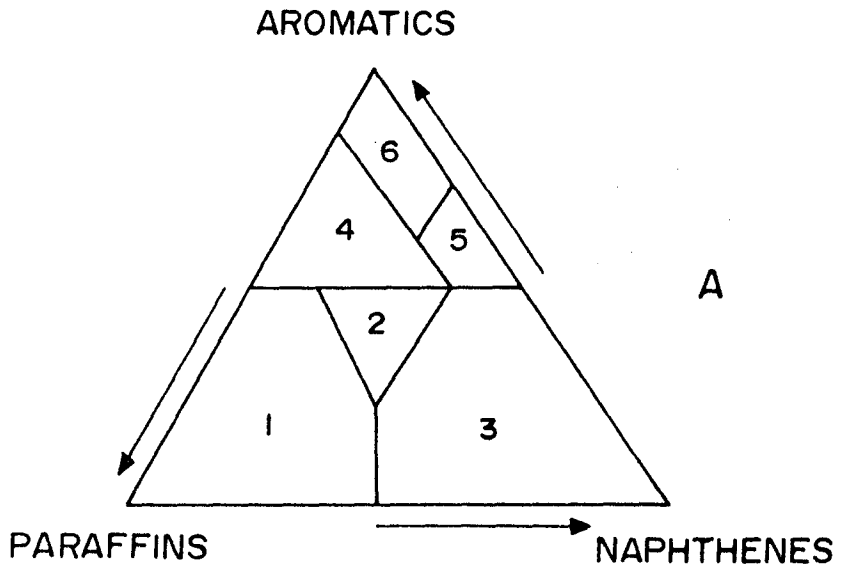
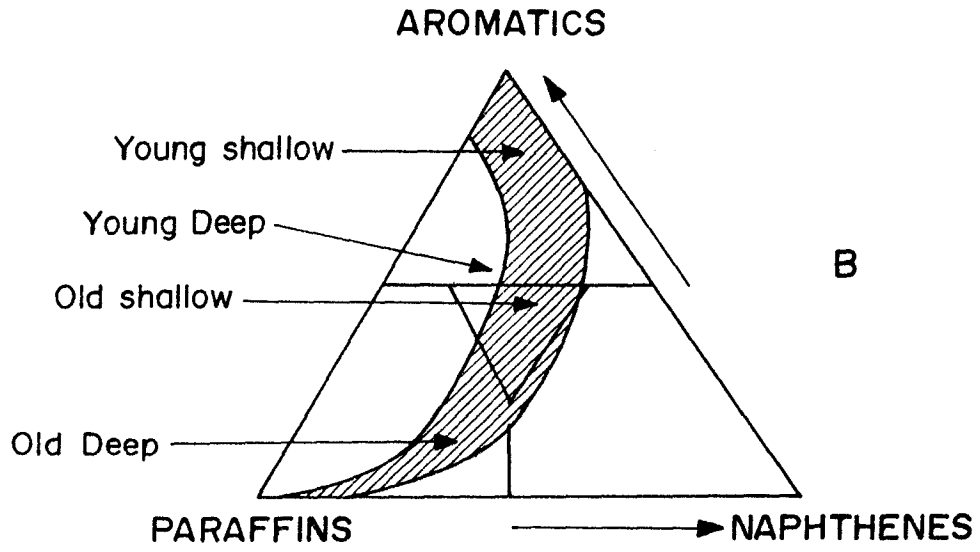


Fig. 1.3. Sources of oil in the Marine environment (United Nations Environment Programme, Report No15, 1993).





1. - paraffinic, 2 - paraffinic-naphthenic, 3 - naphthenic,
4 - aromatic intermediates, 5 - aromatic-naphthenic,
6 - aromatic - asphaltic

Fig. 1·4 A & B. General classification of crude oil (A) composition of most crude oils (B) (Schobert, 1990)

high content of alkanes. The standard crude by which others are rated is the Pennsylvania crude which is an old deep crude. This means that no two spillages are identical in terms of microbial substrate even before weathering, physical and chemical changes occur.

CLEAN UP OF OIL SPILLS: PHYSICAL AND CHEMICAL METHODS

The corrective measures taken may be of several types. One such measure is the physical treatment such as containment of the oil by floating booms, followed by the addition of an absorbent such as hay or straw which has been used in enclosed waters for small amounts of oil (Beastall,1977; Dart and Stretton,1978). Chemical treatment is another such measure whereby chemicals are added to disperse oil. These chemicals may be of two types, those causing sinking e.g. chalk or sand coated with silicones) and those causing dispersal (e.g. surfactants). However, chemical treatment suffers from the drawback that the chemicals used are generally recalcitrant and toxic to the marine environment.

Degradation of oil may also take place aided by physical factors such as UV light, which cause the formation of peroxides which can be degraded or dimerized to complex molecules (Dean,1968; Freegarde *et al.*,1971).

Autooxidation also takes place, the rate of which is dependent on the presence of various chemicals, cations are generally thought to accelerate the process, whereas phenolics and sulphur containing compounds act as inhibitors (Atlas and Bartha, 1972a). . The solubility of hydrocarbons in aqueous is low and various workers have reported that the rate limiting factor in the oxidation of hydrocarbons may be solubility. In aqueous solutions, the solubility of hydrocarbons decreases as the chain length and

molecular weight increases (Johnson, 1964). Hydrocarbons are also less soluble in seawater than distilled water so that soluble hydrocarbon substrate available for microbial attack is limited. However, as a consequence of solubililty, the short chain n-alkanes (< C9) are toxic to many organisms and this toxicity is usually attributed to their greater solubility and higher concentrations in the aqueous phase (Ratledge,1978). At the same time, in an event of an oil spill, these toxic hydrocarbons will be the main component that will evaporate most readily. Thus it is probable that toxicity is not a serious consideration in the degradation of oil (Linden, 1978).

BIOLOGICAL TREATMENT OF OIL

The biogenic nature of petroleum however allows for another corrective measure i.e. bioremediation whereby the degradative ability of the indigenous microbial population is made use of in detoxifying components of oil. Although, bioremediation appears to be a relatively cleaner technology as compared to chemicals and surfactants, its limitations are mainly due to its slow speed in oil removal. Moreover, the biological and chemical reactions that attack oil could give rise to additional structures that are more toxic than the parent compound. Therefore as a prerequisite to such an approach, the mechanisms of microbial degradation and the limitations with respect to the structure of the chemicals to be degraded need to be understood. Furthermore, these hydrocarbonoclastic microorganisms (i.e. microorganisms capable of degrading hydrocarbons) are ubiquitously distributed in marine and freshwater ecosystems with the relative abundance varying according to environmental conditions and the pollution history. Prior exposure or adaptation of a microbial community is clearly important in determining how rapidly hydrocarbon inputs are degraded (Bartha and Bosset, 1984).

According to Leahy and Colwell (1990), the induction and/or derepression of specific enzymes, genetic changes resulting in new genetic capabilities (Ghadi and Sangodkar, 1994a) and selective enrichment are important mechanisms by which adaptation can occur.

The genetic manipulation of hydrocarbonoclastic microorganisms through recombinant DNA technology, molecular breeding and forceful evolution of catabolic pathways are clearly newer approaches towards enhanced bioremediation of oil. Timmis *et al.* (1985) suggested three basic experimental approaches to accomplish laboratory evolution of metabolic pathways.

a) Long term chemostat selection, which often involves progressive replacement of a mineralizable substrate by a recalcitrant molecule (Dorn *et al.*, 1974).
b) In vivo genetic transfers in which genes of critical enzymes of one organism are recruited into a pathway of another organism through natural genetic processes such as transduction, transformation and conjugation (Reineke and Knackmuss, 1979).
c) In vitro evolution, in which cloned and well characterised genes are transferred into different organisms in order to evolve a new pathway (Harayama *et al.*, 1986).

The second approach is sometimes facilitated by the fact that the genetic information for recently evolved pathways is frequently located on transmissible plasmids, transposons or distinct long segments of DNA flanked on both sides by unique insertion sequences, characteristic in transferring long segments of genome or activity to silent genes by derepression (Haugland *et al.*, 1990; Ghadi and Sangodkar , 1994b).

These approaches generally lead to an expansion of the substrate profile of the existing pathway where the extension could be either horizontal in which more analogs of a single class of compounds are metabolized due to recruitment of isofunctional enzymes

or due to alterations of substrate specificities of key enzymes by mutation (Campbell *et al.*,1973; Clarke, 1978) or the expansion could be vertical whereby the existing pathway is used as a base onto which are grafted additional enzymes that extend the pathway upwards (Lehrback *et al.*, 1984; Timmis *et al.*, 1985). These approaches clearly enhance the prospects of using natural and recombinant microorganisms for mineralization of crude oil.

BIODEGRADATION OF OIL IN THE MARINE ENVIRONMENT

Crude oil spilt into sea undergoes a variety of changes due to various factors. Evaporation is one such variable factor which results in the loss of the volatile components of oil. However, evaporation will depend on the type of oil as well as the weather conditions (rates at which oil is spread). Dean (1968) reported that approximately two-thirds of Nigerian crude oil evaporates after a few days at sea, whereas only about 40% of Venezuelean crude evaporated under the same conditions. Fractions boiling under 350°C will evaporate within approximately one week. The importance of evaporation as a factor in affecting biodegradation of oil can be gauged from the fact that evaporation of the lighter components of oil leave the heavier more microbial recalcitrant molecules in the marine ecosystem.

The temperature of the water is another factor influencing the rate of microbial degradation of oil. A rise of 10°C will cause most enzymes (biological catalyst) to increase their reaction rates two to three fold. The relation between temperature and biodegradation is discussed by Linden (1978). Thus as a large percentage of seawater is near 4°C, biodegradation of the oil will be a very slow process and most organisms will be psychrophillic. Atlas and Bartha (1972a), however found out that the effect of

temperature on biodegradation also depends on the composition of oil. They suggested that low temperatures retard the rates of volatilization of low molecular weight hydrocarbons, some of which are toxic to microorganisms. Their presence is thought to delay the onset of biodegradation. However, Walker *et al.* (1974a) and Colwell *et al.* (1978) reported greater degradation of low molecular weight hydrocarbons at low temperatures than at higher temperatures. Thus although low temperatures affect the degradation of oil, its effects are interactive with other factors, such as the quality of oil and the composition of the microbial community.

Hydrocarbon-degrading microorganisms generally act at the oil-water interface. Hydrocarbon degrading microorganisms can be observed growing over the entire surface of an oil droplet, however, growth does not appear to occur within oil droplets in the absence of water. Availabilty of increased surface area is also thought to enhance biodegradation. It is suggested that bacteria and yeasts adhere to hydrocarbon droplets and the cell may synthesize some surfactant before growth commences (Atlas, 1981). In this way not only is the oil made more readily available to microorganisms, but movement of the emulsion droplet through the water column makes oxygen and nutrients more readily accessible to microorganisms (Atlas, 1981).

Another factor that plays an important role is the availability of nitrogen and phosphorous for degradation. Several investigators, Atlas and Bartha(1972b); Bartha and Atlas(1973); Floodgate(1979), have reported that concentrations of available nitrogen and phosphorous in seawater are severely limiting to microbial degradation. Other investigators however, have reached the opposite conclusion (Kinney *et al.*, 1969). When considering an oil slick, there is a mass of carbon available for microbial growth within a limited area. Since microorganisms require nitrogen and phosphorous

for incorporation into biomass, the availability of these nutrients is critical. Extensive mixing can occur in turbulent seas, but in many cases the supply of nitrogen and phosphorous is dependent on the diffusion of the slick. However, in the case of soluble hydrocarbons, nitrogen and phosphorous are probably not limiting since the solubilities of these hydrocarbons are so low as to preclude establishment of unfavourable C/N or C/P ratios. The results obtained on studies carried out on nitrogen and phosphorous supplementation by various workers have concluded that the rate of nutrient replenishment is generally inadequate to support biodegradation. Thus the addition of nitrogen and phosphorous containing fertilizers can be used to stimulate microbial degradation. However, it should be pointed out that if oil spills in a confined ecosystem such as a lake are treated with large amounts of nitrogen and phosphorous, then there may be a serious risk of eutrophication (Dart and Stretton, 1978).

The effects of other factors such as oxygen, salinity and pressure have also been studied with the general observation being that degradation proceeds at a slow rate at low oxygen concentration and high salinity and pressure (Schwarz *et al.*, 1974; Ward and Brock, 1978).

Thus petroleum when spilt in the marine environment initially forms a slick. As a consequence of the various abiotic and biotic factors, the oil will generally exist in four states, as films, in solution, as an emulsion, or as tar balls. Fig 1.5 shows the various states of oil in an event of an oil slick (International Petroleum Industry Environmental Conservation Association).

The biodegradation of oil has been considered and studied by several authors with a wide genera of hydrocarbon utilizers in the marine environment e.g. *Pseudomonas, Arthrobacter, Micrococcus, Nocardia, Vibrio, Acinetobacter, Brevibacterium,*

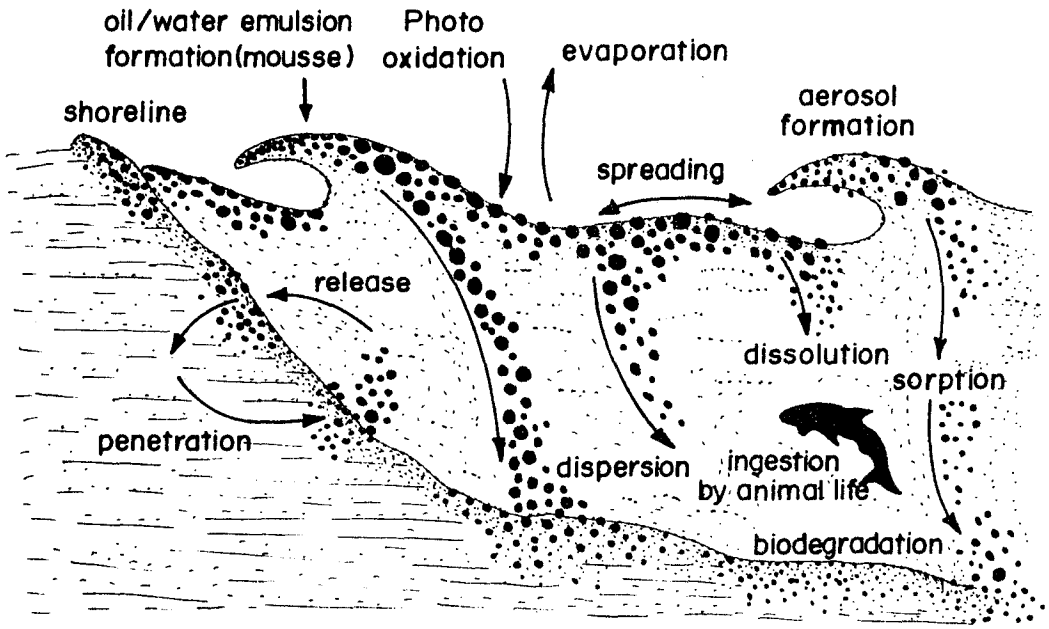


Fig. 1·5 The various states of oil in an event of an oil slick (International petroleum industry environmental conservation association)

Corynebacterium, Flavobacterium, Candida, Rhodotorula (Bartha and Atlas,1977; Leahy and Colwell,1990).

Various workers have reported that the populations of hydrocarbonoclastic microorganisms occurred in concentrations 10 to 100 times greater in the surface layers than at 10 cms depth (Crow *et al.*,1976). It is also clear from the various observations that population levels of hydrocarbon degraders and their proportions within the microbial community appear to be a sensitive index of environmental exposure to hydrocarbons. In unpolluted ecosystems, hydrocarbon utilizers generally constitute less than 0.15% of the viable microorganisms.

Several studies have been carried out to determine the metabolic pathways for the degradation of components of petroleum (Foster, 1962; Gibson, 1971; McKenna and Kallio, 1971). Hydrocarbons within the saturate fraction are degraded by a monoterminal attack, usually a primary alcohol is formed followed by an aldehyde and a monocarboxylic acid. Further degradation proceeds by β-oxidation with the subsequent formation of two-carbon units shorter fatty acids and acetyl CoA, with the eventual liberation of CO_2. Highly branched iosprenoid alkanes, such as pristanes, have been found to undergo omega oxidation, with the formation of dicarboxylic acids as the major degradative pathway (McKenna and Kallio, 1971; Pirnik,1977) (Fig. 1.6). The degradation of aromatic hydrocarbons on the other hand, involves the formation of a diol followed by cleavage and the formation of a di-acid such as cis,cis muconic acid (Rogoff, 1961; Gibson, 1971; Hooper,1978). In the case of asphaltic components, no uniform degradative pathway, comparable to the pathways established for aliphatic and aromatics, have yet emerged. However studies have shown that these NSO

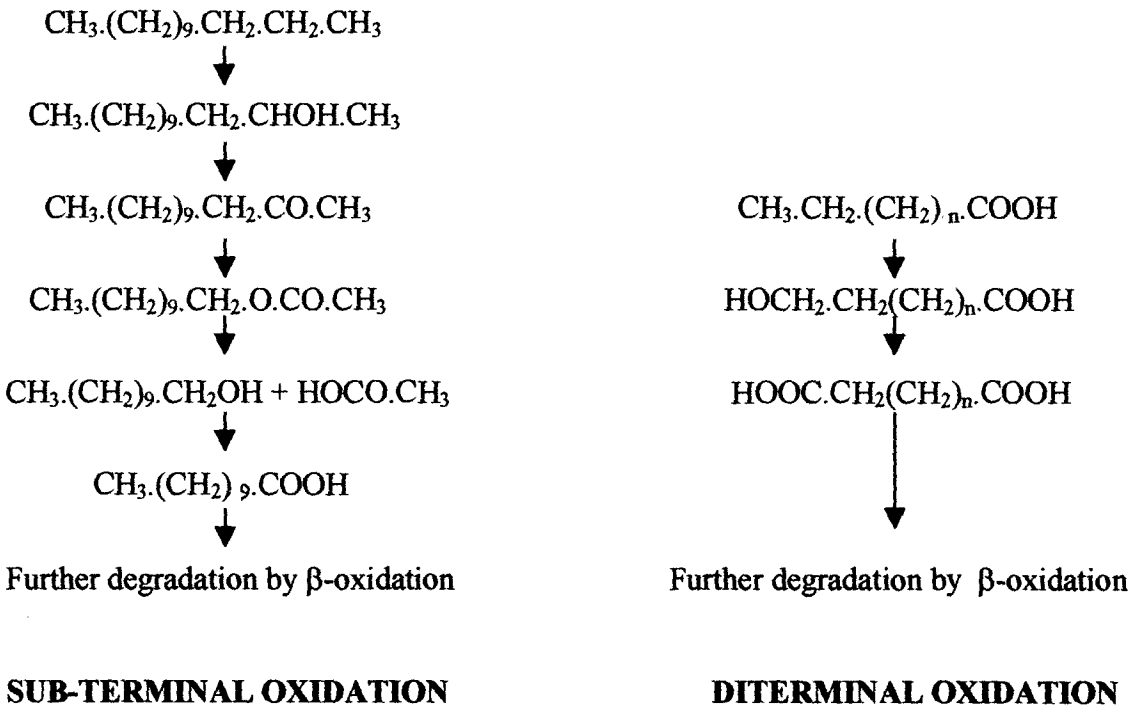


Fig. 1.6. Degradative Pathway of Alkanes (Ratledge, 1978)

compounds are initially converted into intermediates which are subsequently broken down via uniform degradative pathways.

Two more processess which need to be considered in the metabolism of petroleum hydrocarbons are sparing and co-oxidations. Both processes can occur within the context of an oil spillage. LePetitandTagger (1976) for example reported that acetate, an intermediate in hydrocarbon uitlization, reduced the utilization of hexadecane. A diauxic phenomenon was reported for the degradation of pristane,in which pristane was not degraded in the presence of hexadecane. The basis of the sparing effect is not well defined, it however, does not alter the metabolic pathway, but rather determines whether the enzymes necessary for metabolic attack of a particular hydrocarbon are produced or active (Atlas,1981).

The phenomenon of co-oxidation is another factor in which compounds which otherwise would not be degraded can be enzymatically attacked within the petroleum mixture due to the ability of the microorganism to grow on other hydrocarbons within oil. Thus a petroleum hydrocarbon mixture, with its multitude of potential primary substrates, provides an excellent chemical environment in which co-oxidations can occur. However, assessing the role of co-oxidations in the natural environment is difficult since multiple microbial populations are present. Their synergism could be an alternative hypothesis to explain similar results.

The rates of biodegradation of hydrocarbon from oil spills appear to be highly dependent on localized conditions. The fate of many components of petroleum, the degradative pathways which are active in the environment, the importance of co-oxidations in natural ecosystems, and the role of microorganisms in forming persistent contaminants from hydrocarbons such as the compounds found in tar balls are unknown

and require future research. With an understanding of the microbial degradation of hydrocarbons in the environment, it should be possible to develop models for predicting the fate of oil and to develop strategies for utilizing microbial hydrocarbon degraders for the removal of hydrocarbons in contaminated ecosystems.

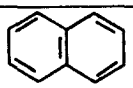
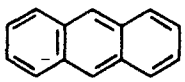
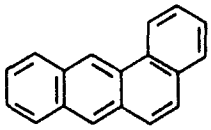
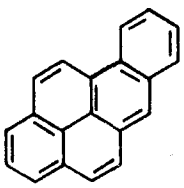
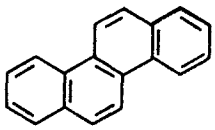
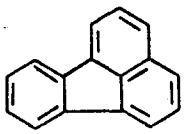
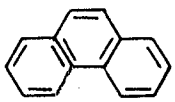
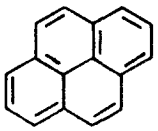
POLYCYCLIC AROMATIC HYDROCARBONS

Polycylclic aromatic hydrocarbons (PAH's) are compounds which are constantly produced and degraded in the environment. These are compounds containing carbon and hydrogen with the carbon atoms arranged in a series of adjoining six membered benzene rings. Some examples of polycyclic aromatic hydrocarbons are shown below in Table. 1.3 (Harrison *et al.*, 1975).

Besides, their presence in petroleum, polycyclic aromatic hydrocarbons are formed during incomplete combustion of almost any organic matter and have been isolated from air, water, soil, fossil and vegetable samples. Other common sources include cigarette smoke, chimney soot, oil pollution and industrial processes (Cerniglia, 1981).

Interest in the structure and physiological properties of PAH's was mainly due to the discovery that many of these compounds possess carcinogenic activity. The first reported case of chemical carcinogenicity was in 1761, by Dr John Hill (Gibson and Subramanium, 1984) but it was only hundred years later that its carcinogenicity was established due to the relationship between skin cancer and skin contact with tar and oil. Subsequent studies led to the isolation of benzo(a)pyrene from coal tar (Gibson and Subranium,1984). In the last decade,there has been a virtual explosion in terms of scientific reports on the mammalian oxidation of benzo(a)pyrene and other carcinogenic

Table 1·3 . List of some polycyclic aromatic hydrocarbons (PAH's) present in crude oil .

Structure	Mol. Wt.	Solubility (μgl^{-1}) at 25°C	Nature
 Naphthalene	128·18	12,500	Toxic
 Anthracene	178	75	Toxic
 Benzo (a) anthracene	228	10	Carcinogenic
 Benzo (a) pyrene	252	-	Carcinogenic
 Chrysene	228	6	Carcinogenic
 Fluoranthene	202	265	Toxic
 Phenanthrene	178	1600	Toxic
 Pyrene	202	175	Toxic

compounds (Miller, 1978). These have been occasioned, in no small part, by the growing realization that 60-90% of human cancers are caused by exposure to such type of environmental chemicals (Higginson and Muir, 1973).

Studies on the environmental distribution of polycyclic aromatic hydrocarbons (PAH's) were initiated in 1947 when Kern reported the presence of chrysene in soil samples. However, the advent of new and improved analytical techniques such as gas chromatography / mass spectroscopy and improved methods for the extraction and isolation of PAH's have led to the realization that a single sediment sample can contain thousands of aromatic compounds (Gibson and Subramanium, 1984). The elucidation of the individual structures of individual PAH's in soil, sediment and water samples is almost impossible, and even in crude oil, where large amounts of PAH's are available. In crude oil, PAH's exist in the form of complex side chains.

In the marine environments, the presence of PAH's is mainly anthropogenic, given that they are present in crude oil. However, PAH's in the form of petroleum hydrocarbons in the marine environments seem to be increasing every year with yearly estimates to be at least one million tonnes (Blumer *et al.*, 1970) and as high as ten million tonnes(Atlas and Bartha, 1972b). The main portion of this form of pollution is thought to originate not from major disasters but from daily influxes (Edwards, 1969). As might be anticipated from their high molecular weight and low polarity, the solubility of PAH's in water is of a very low order. Table 1.3 lists the solubility of some PAH's in water (Calder and Lader, 1976). Their toxicity coupled with their persistence makes these compounds very dangerous as environmental contaminants.

MICROBIAL DEGRADATION OF POLYCYCLIC AROMATIC HYDROCARBONS

Polycyclic aromatic hydrocarbons (PAH's) occur in many different environmental situations. They are deposited by a variety of mechanisms and are also derived from many initial carbon sources. The discovery that many possess carcinogenic activity has resulted in more interest in their toxicology and their behaviour in various natural environments.

It has now become evident that polycyclic aromatic hydrocarbons undergo metabolic activation that induces toxic, mutagenic and carcinogenic properties (Heidelberg, 1975; Sims & Grover, 1974,1981). The first sequence of biochemical reactions is usually the removal of any side chain which may be present (reaction A & B, Fig. 1.7) although there are a number of exceptions to this as shown in reaction 1 and 2 (Fig. 1.7)

The next step is the hydroxylation of the aromatic ring with the oxygen introduced into the molecule at this stage being derived from molecular oxygen. This hydroxylation is carried out by a group of enzymes known as oxygenases, which occur at a number of points in the degradative pathway and can be split into two types.

The first group are known by several names, mono-oxygenases, hydroxylases or mixed function oxidases. These enzymes add one atom of oxygen to a substrate molecule and reduce the second atom of oxygen to water by means of a reduced enzymic co-factor as shown in reaction 1,3 and 4 (Fig. 1.7).

The second group of oxygenases is known as the dioxygenases which add two atoms of oxygen to a substrate molecule and although a reduced coenzyme may be used, it is not with the concomitant formation of water. Examples of this in monocyclic are the dihydroxylation of benzoic acid to catechol or also the ring cleavage of naphthalene or

phenanthrene to form dihydiols (reaction 5 & 6, Fig. 1.7) and the *ortho* or *meta* cleavage of catechol (reaction 7, Fig. 1.7). The mode of hydroxylation of aromatic compounds may vary from one type of cell to another. For example benzene is converted to catechol by both prokaryotic and eukaryotic cells but there are important differences. The bacterial system is shown below (reaction 7, Fig. 1.7).

In contrast, the mammalian system which has also been shown to occur in yeasts involves a microsomal monooxygenase (mixed function oxygenase) dependent on cytochrome P-450 which leads to the formation of an epoxide which can then undergo enzymatic hydrolysis and subsequent oxidation to catechol (Cerniglia, 1981).

The difference between the two systems is more than academic as the arene oxides formed from polycyclic aromatic hydrocarbons are active carcinogens, and if these reactions are widespread amongst eukaryotic organisms, then these might be concentrated by phytoplankton and zooplankon at the lower end of the food chain. The aromatic nucleus (catechol) once formed is then split by *ortho* and *meta* cleavage (Fig.1.8). *Ortho* cleavage results in the production of β-keto adipic acid which is ultimately split to acetyl CoA and succinyl CoA whereas *meta*-cleavage gives rise to 2-hydroxy semi-aldehyde with the ultimate products being acetaldehyde and pyruvic acid (Dagley and Gibson, 1965) (Fig. 1.8).

Studies on the degradation of polycyclic aromatic hydrocabons have reported trans-dihydrodiols to be intermediates in the metabolism of PAH's(Walker and Wiltshire, 1953). Recent evidence suggests that a dioxygenase reaction is the principal mechanism for the initial oxygenation attack on aromatic hydrocarbons by prokaryotes, leading to the formation of *cis*-dihydroxydihydro compounds as metabolic intermediates (Cripps

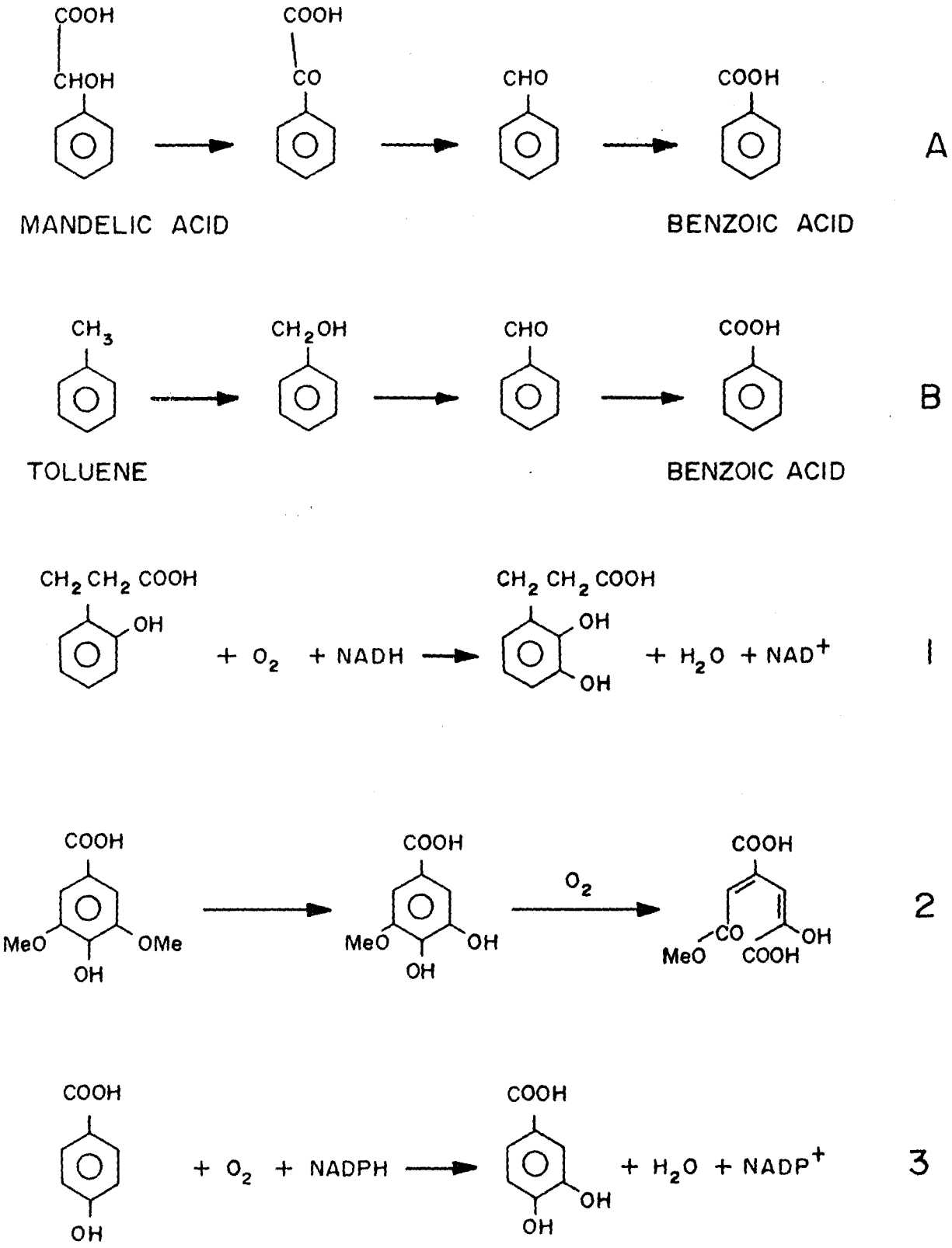


Fig. 1·7 Reactions involved in the degradation of aromatic compounds.

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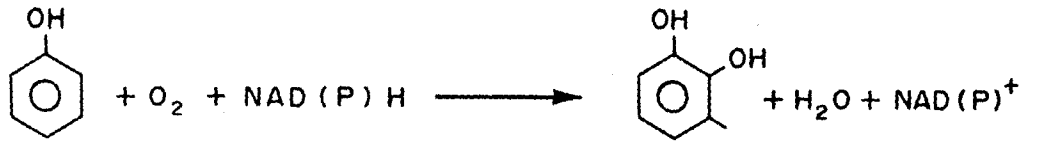

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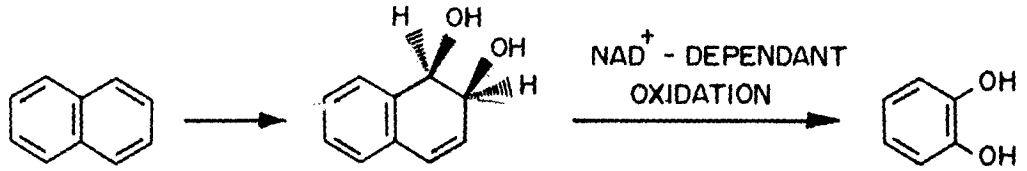
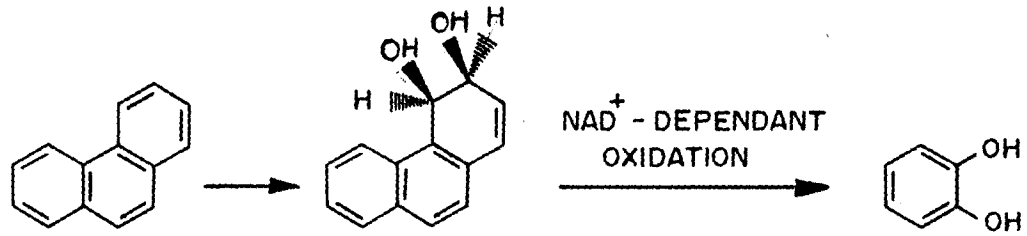


5

NAPHTHALENE

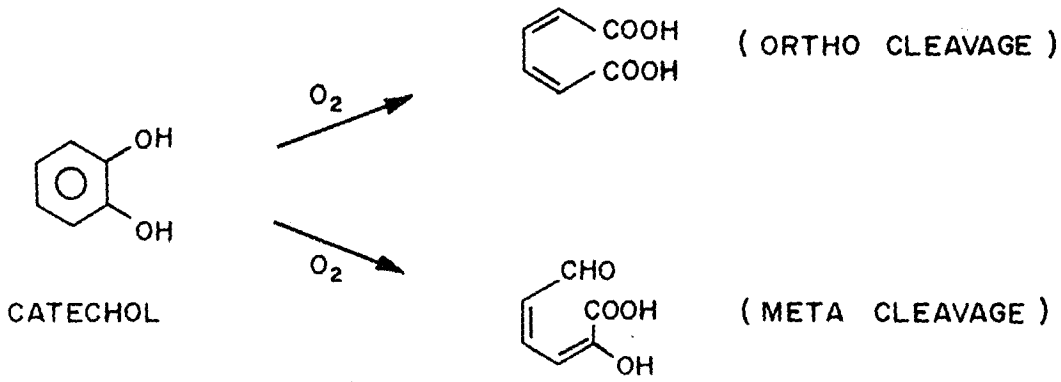
NAPHTHALENE
CIS-1,2-Dihydrodiol

CATECHOL



6

PHENANTHRENE



CATECHOL

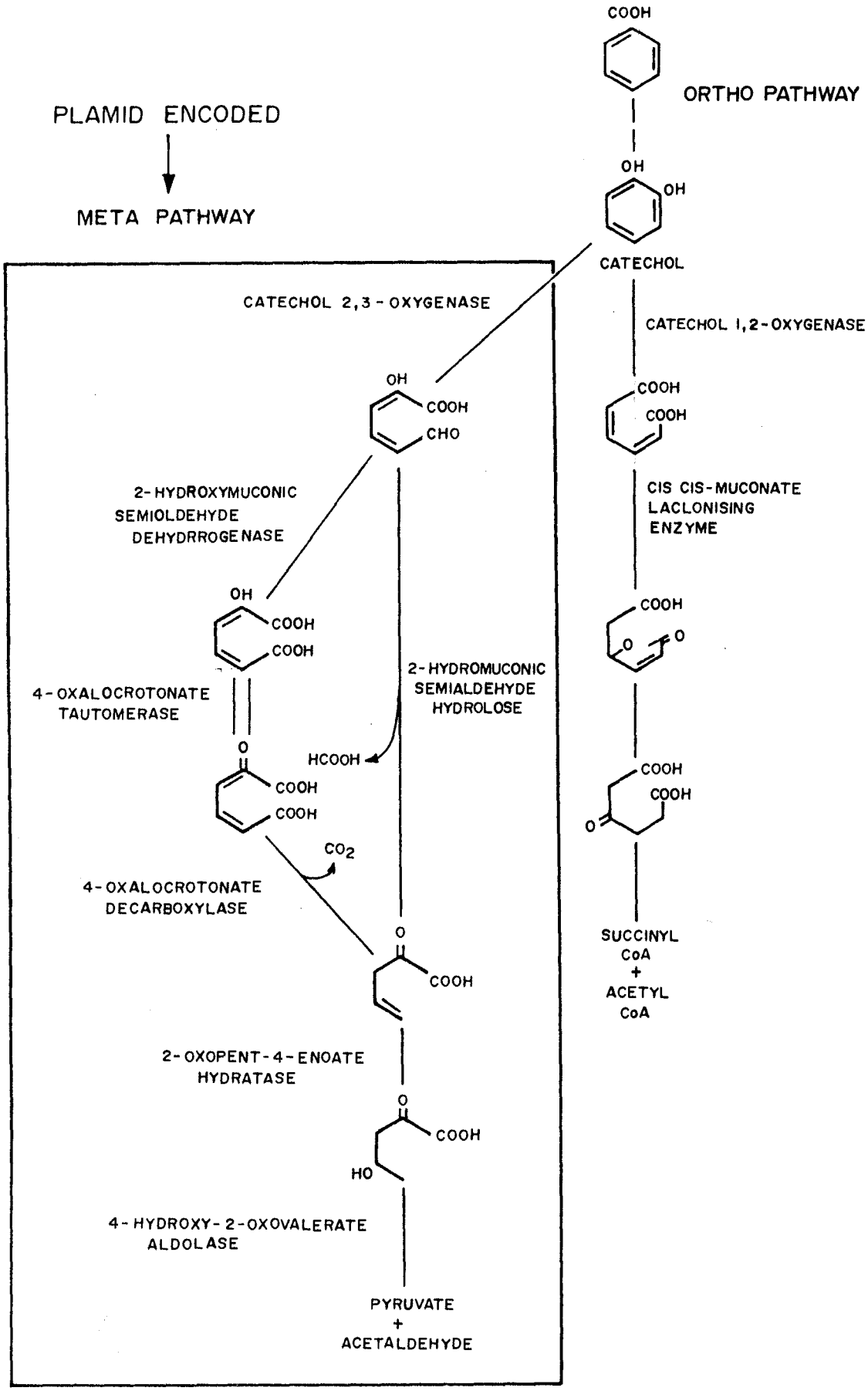


Fig. 1·8 Breakdown of catechol by ortho and meta cleavage

and Watkinson, 1978). In all cases, the stereospecificity of the dihydrodiol has been rigorously established as the *cis*-configuration.

The diol on subsequent nucleotide dependent oxidation was converted into catechol which could then be metabolized via ortho or meta cleavage (Fig. 1.8). The pathways discussed here are also applicable to a large number of natural and synthetic aromatic polcyclic hydrocarbons.

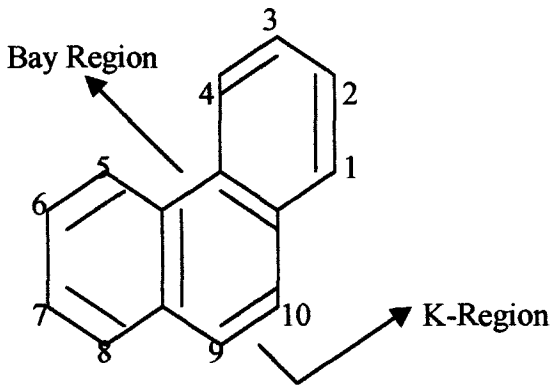
The breakdown of hydrocarbons also differs from organism to organism with certain organisms having the ability to utilize catechol via both *ortho* and *meta* cleavage. Davies and Evans (1964) showed that one strain of *Pseudomonas putida* was capable of splitting catechol derived from benzoate by the *ortho* pathway, whereas catechol derived from naphthalene was split by the *meta* pathway. This is obviously a problem of enzyme control, though it must be noted that some research work has shown that two separate pathways may co-exist for the degradation of a single compound (Jigami *et al.*; 1979).

The study of the degradation of aromatic and polycyclic hydrocarbons has shown that the breakdown of many of these compounds is subjected to tightly controlled regulation which may take place by either enzymatic induction or repression (Dagley, 1971; Clarke and Ornstein,1975). Various repression systems have shown that broadly speaking an organism will use the most easily available source of energy first, that is lactate or succinate. This point is of obvious importance in the study of hydrocarbon degradation in the environment, which may have access to a large number of substrates. Thus in conclusion we can say that polycyclic aromatic hydrocarbon degradation follows a similar pattern of degradation as that of aromatics with the initial attack being

by means of a dioxygenase to form a dihydrodiol which is subsequently converted to catechol. Metabolism of catechol then proceeds via *ortho* or *meta* cleavage.

PHENANTHRENE: STRUCTURE AND DEGRADATION

Phenanthrene is the simplest polycyclic aromatic hydrocarbon that contains a "Bay Region" and its structure resembles those found in carcinogenic PAH's, such as benzo(a) pyrene and benzo(a) anthracene. The " Bay Region" is the region between an angular benzo ring and the rest of the molecule.



Structure of Phenanthrene

Mechanical calculations have predicted that dihydrodiol-epoxides with the epoxy groups situated at this region are highly chemically and biologically reactive. Dihydrodiol-epoxides formed in this region are highly mutagenic and tumorogenic and suspected to be ultimate carcinogens.

However, phenanthrene has been shown to be inactive or weakly mutagenic in a salomonella -mammalian microsomal assay system (Oesch *et al.*, 1981). The lack of carcinogenicity for phenanthrene may be due to the fact that the bay region 1,2-dihydrodiol-3,4- epoxides have low biological activity (Jerina *et al.*,1977). Even though

phenanthrene is considered to be a non-carcinogen , there has been a considerable amount of information on the microbial metabolism of this hydrocarbon since it is widely distributed throughout the environment as a result of pyrolytic processes and as a minor contaminant in wastewater effluents from coal gasification and liquefication processes.

The microbial degradation of phenanthrene has been extensively studied. A wide variety of bacteria belonging to the genera *Pseudomonas* (Evans *et al.*,1965), *Aeromonas* (Kiyohara and Nagao,1976;1978), *Alcaligenes* (Kiyohara *et al.*,1982), *Micrococcus* (Ghosh and Mishra,1983), *Beijerinckia* (Kiyohara *et al.*,.1983), *Nocardia* (Peczynska-Czoch and Mordarski,1984), *Vibrio* and *Flavobacterium* (Cerniglia,1984) have been shown to degrade phenanthrene and other PAH's.

Bacteria initially oxidize phenanthrene in the 3,4 position to form *cis*-3,4-dihydroxy-3,4-dihydrophenanthrene (Fig1.9). Patel and Gibson (1974) later showed that cells of *Pseudomonas putida*, *Pseudomonas sp.* NCIB 9816 and a nicotinamide adenine dinucleotide dehydrogenase oxidized *cis*-3,4-dihydroxy-3,4-dihydrophenanthrene presumably to 3,4,-dihydroxyphenanthrene (Fig.1.9). This was further confirmed by Jerina *et al.*, (1971) who showed that mutant strains deficient in dihydrodiol dehydrogenase activity accumulated *cis*-3,4-dihydroxy-3,4-dihydrophenanthrene. Evans and his colleagues (1965) later reported that 3,4,-dihydroxyphenanthrene by an oxygenative attack was converted into *cis*-4-(1'-hydroxynapth-2'-yl)-2-oxobut-3-enoic acid. This meta-cleavage product was metabolized to 1-hydroxy-2-napthoic acid which was subsequently oxidatively decarboxylated to 1,2 -dihydroxynaphthalene(Fig.1.9) which was further degraded through salicyclate and catechol by enzymes of the

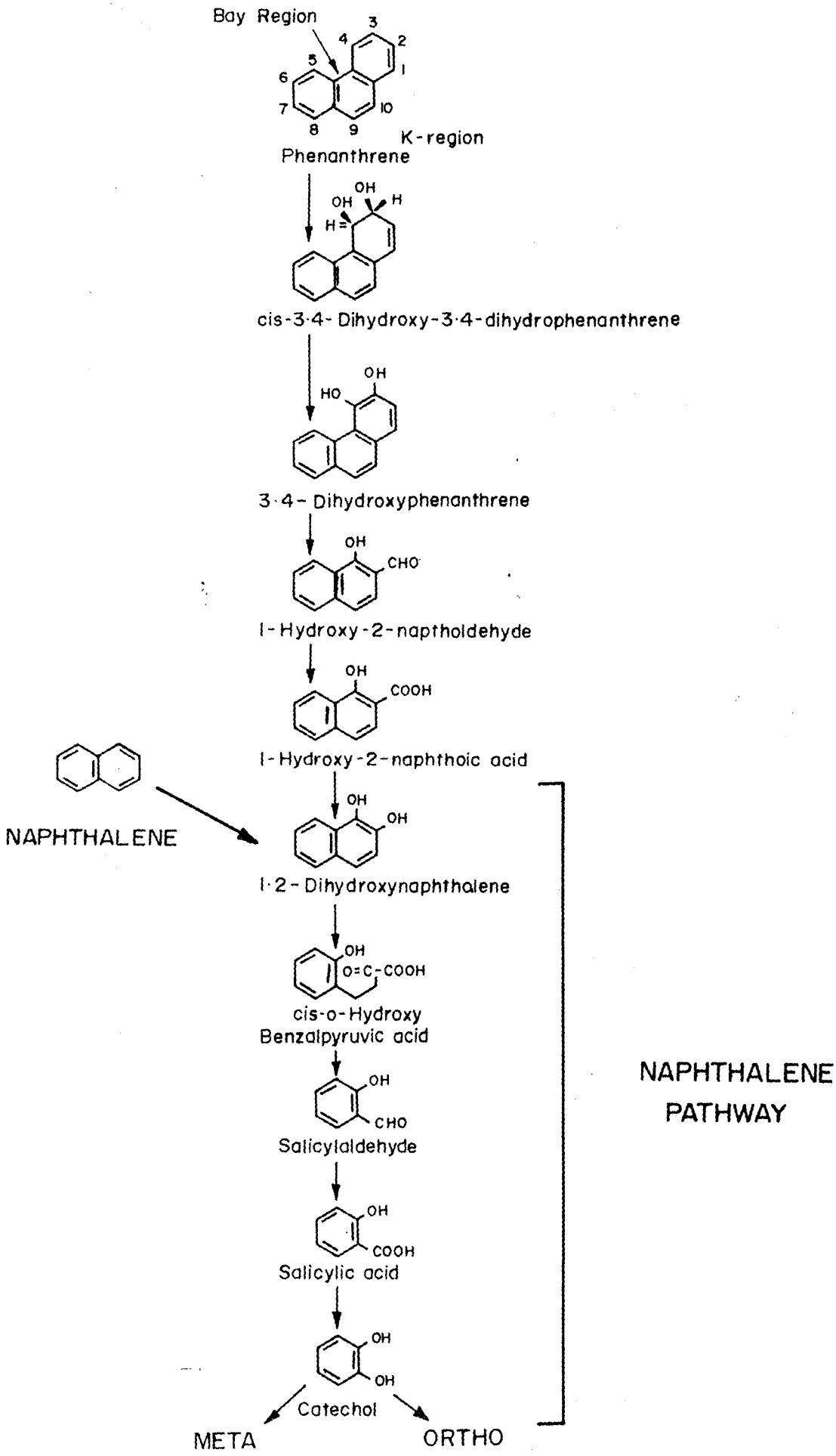


Fig. 1.9. Degradation of phenanthrene via the naphthalene pathway (Evans

naphthalene pathway. The early studies seem to indicate that the degradation of phenanthrene proceeded via the naphthalene pathway.

However, Kiyohara and Nagao (1978) showed that an *Aeromonas sp.* as well as various *Pseuodomona's*, *Vibrio's* and unidentified bacteria metabolized phenanthrene by an alternate pathway. They demonstrated that although all the strains oxidized phenanthrene through 1-hydroxy-2-naphthoic acid, none of the strains they isolated could catalyse the decarboxylation of napthoate to 1,2-dihydroxynaphthalene, a product of the naphthalene pathway. Infact they observed, that the *Aeromonas sp.* cleaved the bond between the carbon atoms of the hydroxyl and carboxyl groups of 1-hydroxy-2-naphthoic acid to form o-phthallic acid. This is then hydroxylated and decarboxylated to protocatechuic which undergoes either *ortho* or *meta* cleavage depending on the organism. Fig 1.10 shows the alternate or phthalate pathway for phenanthrene degradation.

In recent years, with the new techniques such as GCMS, HPLC, IR, NMR emerging the focus of phenanthrene biodegradation was mainly towards isolation of the degradation products of both these pathways. Genetic studies on phenanthrene degradation have always shown the NAH plasmid to mediate in phenanthrene metabolism (Menn *et al.*, 1993). There have been no reports of specific genes responsible for the degradation of phenanthrene.

Thus we can safely conclude that polycyclic aromatic hydrocarbons (PAH's) are ubiquitous in the natural environment with many of them possesing toxic, carcinogenic or teratogenic properties. In our study we have selected phenanthrene as a model compound for the degradation of PAH's. Although it is not mutagenic or carcinogenic to humans, it has been shown to be toxic to aquatic organsims (Savino and

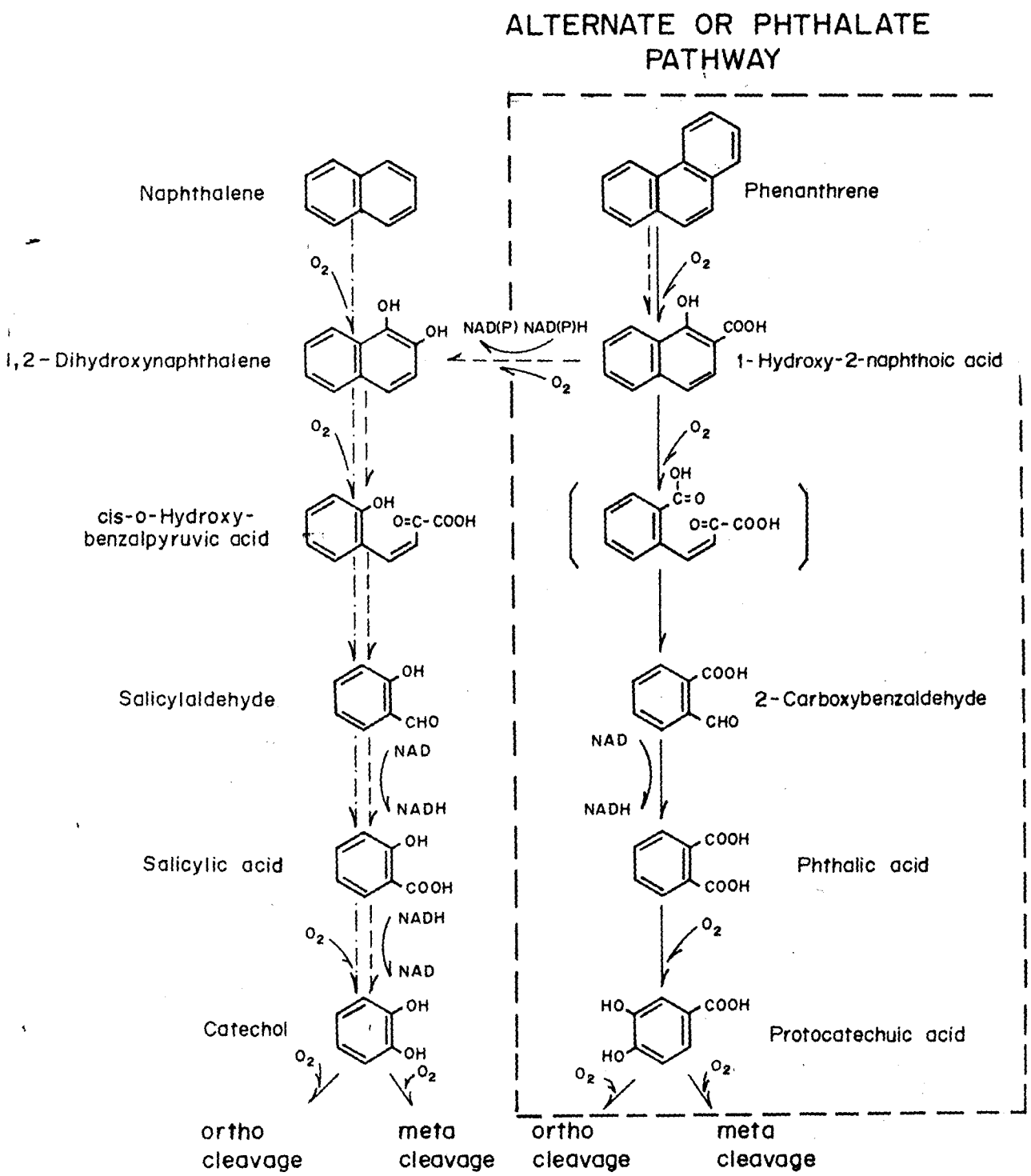


Fig. 1-10 Alternate or phthalate pathway for phenanthrene degradation (Kiyohara and Nagao, 1978).

Tanabe,1989). Furthermore, it is found to be present in high concentrations in PAH contaminated sites and many PAH's containing a phenanthrene moiety are known to be carcinogenic (Bezalel *et al.*, 1996).

AIM AND SCOPE OF WORK

In this study, we have selected marine biodegradative organisms for our cryopreservation studies. The ability of these marine microorganisms in removing toxic polluants such as pesticides and components of crude oil is well documented. At the same time these organisms tend to lose their biodegradative ability very easily making them difficult for preservation.

The study initially deals with optimizing the conditions that are most suitable for preserving single and mixed marine degradative microorganisms. The successful preservation at -70°C and subsequent amplification of a mixed marine bacterial consortium (possessing hydrocarbon degradative capacity) after prolonged storage enabled us to test the efficacy of the preserved consortium in an artificial microcosm containing crude oil/weathered oil. The basic aim was to establish a long-term storage protocol for the preservation of single and mixed degradative microorganisms keeping in mind the criteria of preservation i.e. ability to reproduce and maintenance of functional and genetic properties. The degradation of a PAH, phenanthrene, by a marine microorganism was also studied and the role of such an organism in degradation of other PAH's and crude oil was determined.

Chapter II

CRYOPRESERVATION OF NATURAL MARINE MICROORGANISMS

Despite the use of cryopreservation in stabilizing living material, it can stress cells. Therefore care must be taken to ensure that the preserved material remains unchanged after preservation. However, for a preservation method to be effective, factors such as the age of the organism, type of organism, type of media used, rate of freezing and thawing, types of cryoprotectants need to be studied. It is not only these factors which play an important role but also the ability to reproduce, maintenance of genetic and functional properties after freezing which play an important role in determining the efficiency of the preservation method. Only when these conditions are established can a good preservation protocol be set combining effective characterization and cataloging.

In this study, we have selected a marine strain GU101, identified as *Pseudomonas stutzeri*, a *meta*-toluic acid degrader isolated from the Goan Coast as a model for cryopreservation. The chapter discusses the optimum conditions such as the type of cryoprotectant, age of the strain, type of media used, the rate of freezing and thawing which go into making an effective method for preservation of the marine strain GU101.

MATERIALS AND METHODS

Media and chemicals

Artificial seawater (ASW) of pH 7.2 was prepared according to Chakravarthy (1993) and contained (per litre) 6.05g Tris-HCl; 17.52g NaCl; 0.74g KCl; 12.32g $MgSO_4$; 0.13g $(NH_4)_2HPO_4$; 0.14g $CaCl_2$. The Tris salt was prepared separately by dissolving it in 500ml of D/W and subsequently adjusting the pH to 7.2 with 1N HCl. The remaining components of the medium were then added and the volume made upto 1 litre. For solid media, agar at a concentration of 1.8% was used. m-toluic acid at a concentration of 5mM was used as a sole source of carbon. Stock solutions of m-toluic acid, 1M(Sisco Chemicals) was prepared by dissolving 3.41 g of m-toluic acid in 10 ml of 5N NaOH and the final volume was made upto 25ml with distilled water. m-toluic acid was added to the medium before autoclaving.

Media formulations used for growth of the culture included ASW supplemented with nutrient broth (1.3%); ASW supplemented with m-toluic acid (5mM) and nutrient broth (1.3%) in distilled water.

For total counts, marine nutrient agar (MNA) and ASW Agar supplemented with m-toluic acid plates were used. Marine Nutrient Agar contained (per liter) 1.30g nutrient broth, 0.50g yeast extract, 1.75g NaCl, 0.07g KCl, 1.32 g $MgSO_4$.

For cryopreservation studies,a temperature controlled -70°C deep freezer (Heraeus Septatech) and a Revco -20°C deep freezer were used. Screw-capped plastic vials (Corning) were used for storage of cells at ultra low temperatures. Glycerol was used as a cryoprotectant and prepared by diluting with fresh growth medium to a concentration of about 15%. The glycerol solution was sterilized by autoclaving at 121°C and 15 psi

and stored in dark. All cryopreservation experiments were carried out according to the seed lot system to preserve early passage material.

Estimation of growth

Culture growth was monitored by measuring the absorbance of the cell suspension at 560nm with a spectrophotometer (Spectronic 1201), and by viable counts on marine nutrient agar and ASW-agar supplemented with m-toluic acid (5mM).

Preservation of strains at room temperature and at 5°C

The culture was inoculated into two flasks each containing ASW supplemented with the appropriate substrate.The culture was grown for about 48 hours. One of the flasks was stored at 5°C while the other was kept at room temperature. At the same time 0.1ml of the culture broth was removed from one of the flasks and added to a sterile cryovial containing 0.4ml nutrient broth in ASW and 0.5ml 30% glycerol (final concentration being 15%). The cryovial was stored at 5°C. The total cell counts of the flasks and the cryovial were obtained at zero time and at various time intervals over a period of seven days. The cell counts were obtained by plating the culture on ASW agar supplemented with m-toluic acid plates. Colonies were checked for purity by plating on ASW supplemented with m-toluic acid (5mM) to detect the presence of a yellow colour.

Determination of optimum conditions for preservation of the marine strain

ASW medium (1ml) supplemented with nutrient broth were dispensed into a series of cryovials (around 5 ,) and autoclaved. Each of these vials was inoculated with a pure colony and incubated on a rotary shaker (110 strokes/minute) at 30°C. Vials were

removed after every 48 hours and sterile 30% glycerol (1ml) was added to each vial and quickly frozen to –20°C in the deep-freezer. After 12 days of storage at -20°C, the vials were removed and kept at room temperature for 10-15 minutes and the viable counts of the cells were determined.

The same protocol was repeated with nutrient broth in distilled water and ASW medium supplemented with 5mM substrate. Colonies were checked for purity by plating on agar plates contining ASW supplemented with m-toluic acid.

For slow freezing the vials were kept either at –20°C (slow freezing at 1°C/min) or at –70°C (slow freezing at 1°C/min). For rapid freezing the vials were dropped in an alcohol ice bath kept at –70°C. The alcohol ice bath was kept at –70°C for three days prior to starting of the experiment.

Determination of catechol 2,3 dioxygenase release from the medium from strain GU101 after cryopreservation

The culture was grown for around 48hours in ASW supplemented with m-toluic acid or sodium benzoate. An aliquot of the culture (2ml) was taken and dispensed into a series of vials (2 sets of five cryovials each). Both sets were kept at –20°C with one set being supplemented with nutrient broth in ASW and glycerol as per the preservation protocol. At various time intervals, one vial from each set was removed and the enzyme catechol 2,3 dioxygenase was assayed from the supernatant after centrifuging the cells. Bacterial counts were also determined from each of the vials.

The enzyme assay was done according to the method described by Nozaki (1970). Catechol 2,3 dioxygenase (C230) enzyme activity was assayed spectrophometricaly (Spectronic 1201,Milton Roy company) by measuring the increase in absorbance at

375nm due to the formation of 2-hydroxymuconic semialdehyde (HMS) which is a yellow product formed from catechol. The reaction mixture consisted of 2.8ml of 50mM phosphate buffer (pH 7.0) containing 10% acetone, 100mM catechol and 100µl of crude enzyme, the total volume being 3ml. One unit of enzyme corresponds to an absorbance change of 14.7 absorbance units (AU)/ minute (Nozaki, 1970).

RESULTS AND DISCUSSION

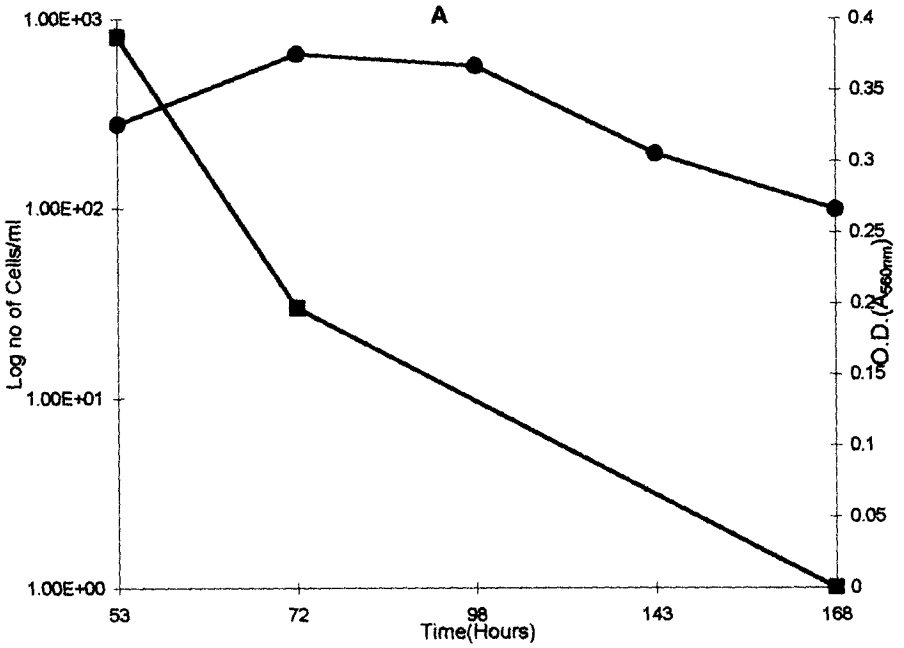
Preservation of microorganisms is a very complex process as a number of factors come into play such as the type of strain, age of the strain, temperature, rate of freezing (if low temperatures are used) etc (Rank, 1973; Butterfield *et al.*,1974; Nyiendo *et al.*,1974). Each cell appears to exhibit its own set of optimal conditions which renders maximum survival for that strain. A marine strain, isolated off the Goan Coast capable of degrading m-toluic acid was used as a model for cryopreservation studies. The strain was identified in the laboratory as *Pseudomonas stutzeri*, strain GU101 (Chakravarthy, 1993).

Stability of strain GU101 at room temperature, at 5°C and at 5°C with a cryprotectant

In reviewing preservation studies, it has been observed that some organisms show maximum survival at room temperature or 5°C whereas some show maximum survival when preserved at ultralow temperatures. However, most natural microorganisms often show a drastic loss of viability when stored at low temperatures. It was thus imperative for us to study the stability of a marine strain GU101 at room temperature,5°C and at 5°C supplemented with glycerol, because if it did survive at these temperatures we need not go into low temperature freezing as this would be uneconomical and could damage the cell.

In this study, strain GU101 was grown in ASW medium supplemented with m-toluic acid kept at room temperature, 5°C,and at 5°C supplemented with 15% glycerol.

As can be seen from Fig 2.1A and Fig 2.1B , strain GU101 showed a fairly low stability at room temperature, although the cell density remained high. By contrast,



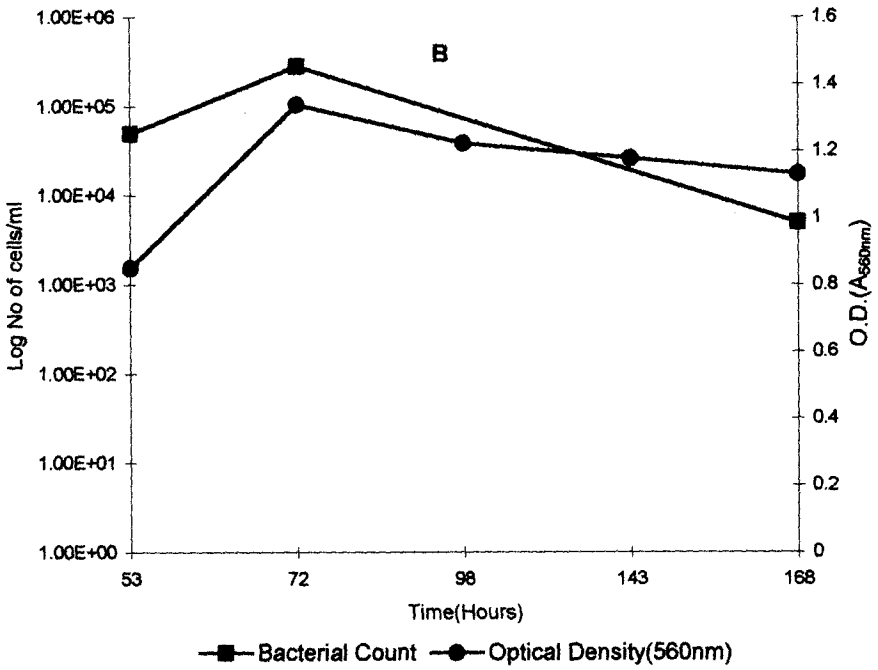


Fig. 2.1 Stability of Strain GU101 at 5°C (A) and at room temperature(B).

there was a distinct decrease in bacterial counts over a 169 hour time period when the culture was stored at 5°C. The addition of glycerol to cells that were subsequently stored at 5°C seemed to give little protective effect (Fig.2.2). What is surprising though is the rapid decrease in bacterial counts at 5°C for strain GU101 (Fig. 2.1A), as microorganisms are generally preserved near 5°C. This once again emphasizes the fact that the preservation of microorganisms is a poorly understood process. Each type of strain exhibits its own set of optimal conditions. Another disadvantage of preservation at these temperatures (5°C) is that these cells are not in an inanimate state, they are prone to slow growth or turnover. This renders them genetically unstable, being prone to loss of vigor.

Optimum conditions for preservation of marine strains

In determining the optimum conditions for preservation of marine strains, the physiological condition of the organism, the type of cryoprotectant and the medium used for preservation are some of the factors that have been considered by many investigators to be a factor in determining their ability to survive stress such as low temperature freezing (Heckly, 1978).

Glycerol, dimethylsulfoxide (DMSO) or ficoll, at a concentration range of around 5-10% are generally used as cryoprotectants. Glycerol has the advantage of being less toxic than dimethylsulfoxide(DMSO), although DMSO is more penetrating and therefore offers the cells greater cryoresistance (Simione, 1998).

The purpose of this study serves to establish two objectives, to determine the optimum age of cells for preservation and to identify the type of medium best suited for preservation of strain GU101. The type of media selected in our study seem to play an

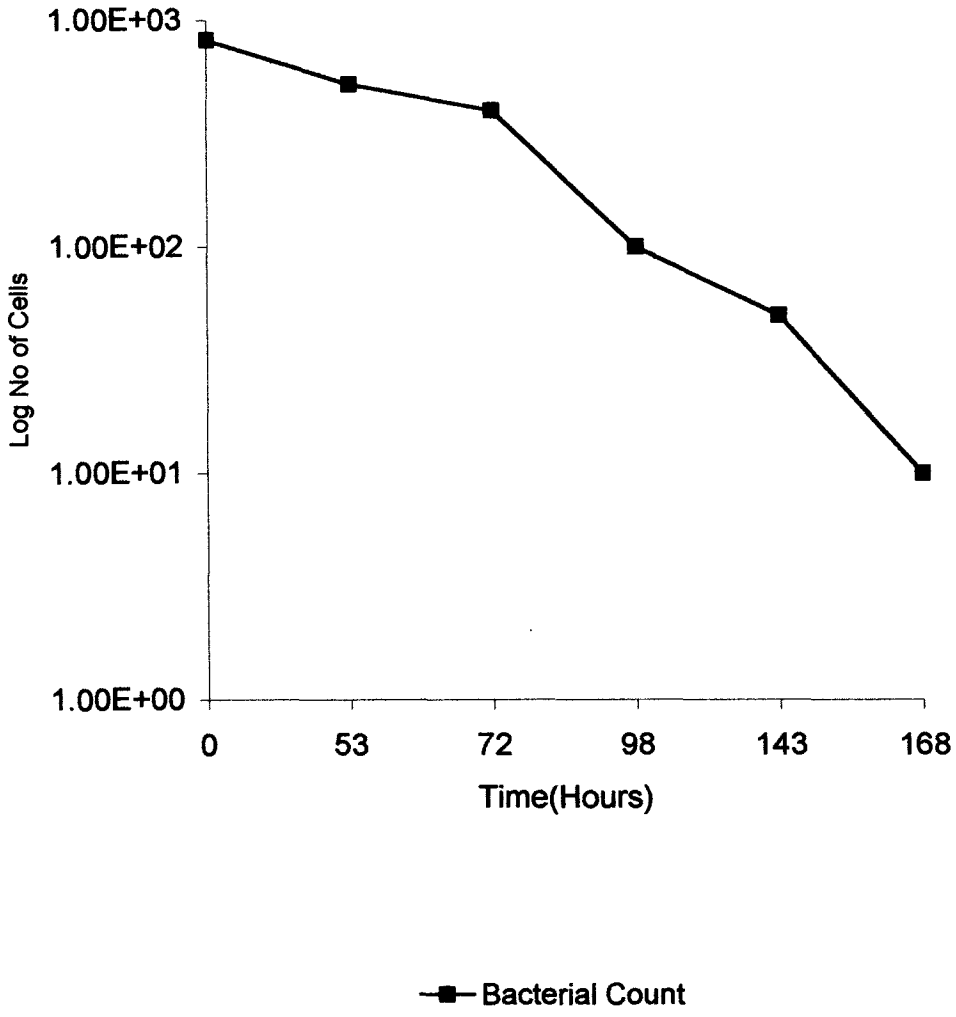


Fig.2.2 Stability of strain GU101 when preserved at 5°C with glycerol as a cryoprotectant.

important role in preservation. In the case of nutrient broth in distilled water, the role of nutrient broth in preserving organisms is sought to be studied. However, the addition of distilled water serves to establish whether the marine strain GU101 survives well in distilled water or artificial seawater which contains a high salt concentration. It naturally follows that the second medium selected was nutrient broth in artificial seawater due to marine nature of the strain. The third medium used was artificial seawater and m-toluic acid (medium which contains the substrate which strain GU101 utilizes). If it could survive well in this medium, it would make the preservation more economical and simpler. The use of glycerol along with additives such as 5%glucose, lactose or mannose or certain amino acids in preservation medium has been well studied (Daily and Higgens,1973). In our study, we used nutrient broth as an additive along with glycerol.

Three different media were used; namely nutrient broth in ASW, nutrient broth in distilled water and m-toluic in ASW for growth of the strain GU101 for preservation. Strain GU101 was grown in each of these media in 250ml flasks. A 1ml aliquot of the culture broth was removed at different time intervals and preserved at –20°C after adding 1ml of glycerol to give a final concentration of 15 %. The seed lot system was used for all cryopreservation studies (Fig.2.3). In this method, while preparing the first lot of the culture, a portion of the lot is set aside as seed material. The vials designated as seed material are maintained separately from the working stocks to ensure that they remain unused and are not handled during retrieval operations. When the first working stock is depleted, a vial is retrieved from the seed lot and used to prepare a second working stock. This continues until all the seed vials except one have been depleted. The last seed vial is then used to prepare a second seed lot. In this way the

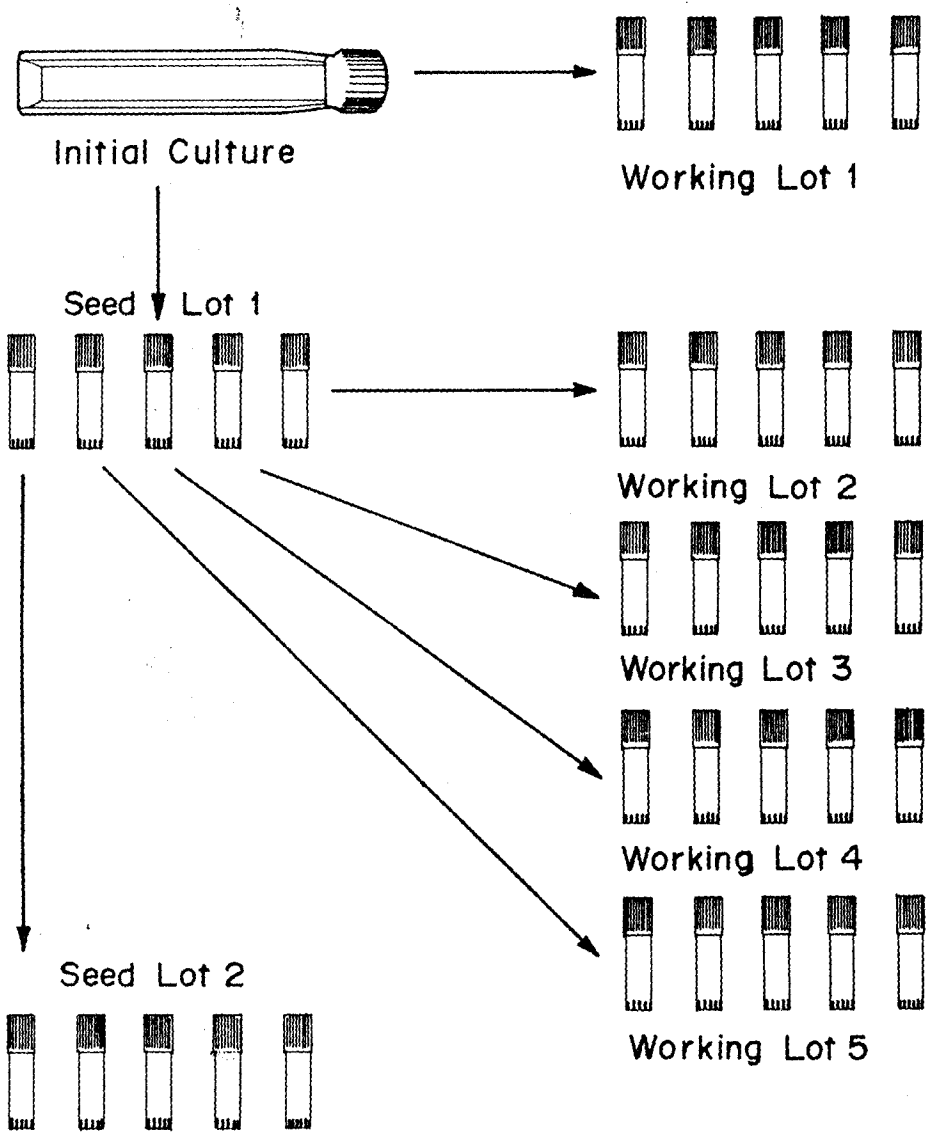


Fig. 2·3 The seedlot system for preservation of microorganisms. (Simione, 1998)

second seed lot remains only one or two passages from the original material, but may be seperated by many years if the lots are adequately sized.

The vials were removed after 12 days storage, thawed for 15 minutes at room temperature and the viable count of the culture was obtained.

The results of our study indicate that there is considerable loss of viability of cells which are still in the exponential phase of growth. However, there is a smaller loss in stationary phase cultures. Remarkably, 48 hours grown cells of strain GU101 appear to be best preserved in artificial seawater supplemented with nutrient broth in addition to 15% glycerol (Fig.2.4A). The other growth media also showed some preservation efficiency but not as effective as nutrient broth in ASW (Fig. 2.4B & C). As can be seen from Fig 2.4A, the strain reaches the beginning of stationary phase after 48 hours growth on ASW supplemented with nutrient broth. A number of workers in this field have shown that cultures at the beginning or middle stationary phase generally show maximum survival (Heckly,1978). A similar observation was seen in our study on strain GU101.

The lack of preservation efficiency in the other two media could be due to the fact that distilled water was used instead of the ASW, which could cause an osmotic imbalance subjecting the strain to an additional stress. In the case of ASW supplemented with m-toluic acid, the accumulation of toxic metabolites of m-toluic acid degradation may be decreasing preservation efficiency.

The addition of nutrient broth is also an important factor in increasing preservation efficiency. The study indicates that it is not glycerol per se which is offering protection against freezing because if that were the case it would have offered the same protection in the other two media. Rather, what seems to be taking place is that a large number of

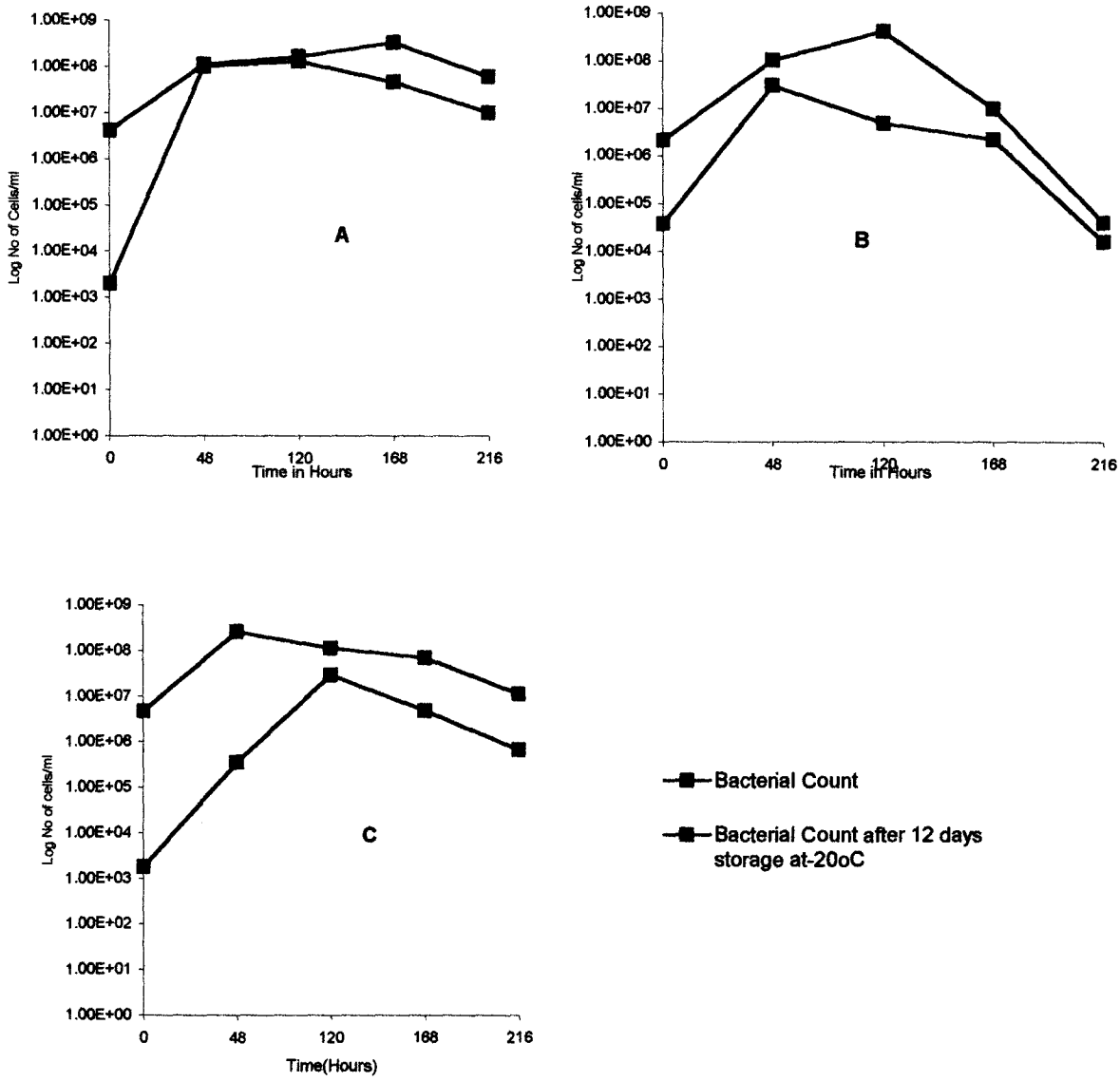


Fig.2.4. Determination of optimum age of cells for preservation of strain GU101 grown on nutrient broth in artificial seawater(A),nutrient broth in distilled water(B) and m-toluic acid in artificial seawater(C).

cells are actually reversibly damaged during the freezing process and the addition of nutrient broth serves to help in repairing this damage thereby increasing preservation efficiency. A number of workers have reported similar observations where the addition of a particular compound be it a sugar, amino acid etc., seems to repair the damage caused by freezing (MacLeod *et al.*, 1966; Moss and Speck, 1966 ; Kuo and MacLeod, 1969). This was further demonstrated by the fact that the strain GU101 grown in ASW and m-toluic acid to early stationary phase, was preserved well when 0.1ml culture, 0.4ml nutrient broth in ASW and 0.5ml 30% glycerol were mixed in a cryovial and stored at -20°C. The vials preserved under these conditions showed a viability of 80% of cells indicating that strain GU101 could be preserved well under these conditions (Fig. 2.5).

Effect of rapid and slow freezing on the cryopreservation of strain GU 101

Another important factor which affects the cryopreservation of microorganisms is the rate of freezing. It was thus imperative for us to study the effect of the rate of freezing on strain GU101, given the fact that the optimal conditions such as physiological age, medium and temperature were already established. For this, strain GU101 was grown in three sets (5-7 cryovials each) in nutrient broth in ASW for 48 hours. After addition of sterile glycerol to obtain a final concentration of around 15%, the vials were divided into three sets. The first and second set was cooled slowly to -20°C and -70°C at a rate of around 1-2°C/min, while the third set was cooled rapidly in an alcohol ice-bath which was previously cooled at -70°C. After preserving the cells for a two year period, the viability of cells in each vial was determined.

As can be seen from the results (Fig. 2.6A & B) both slow and rapid freezing gave good

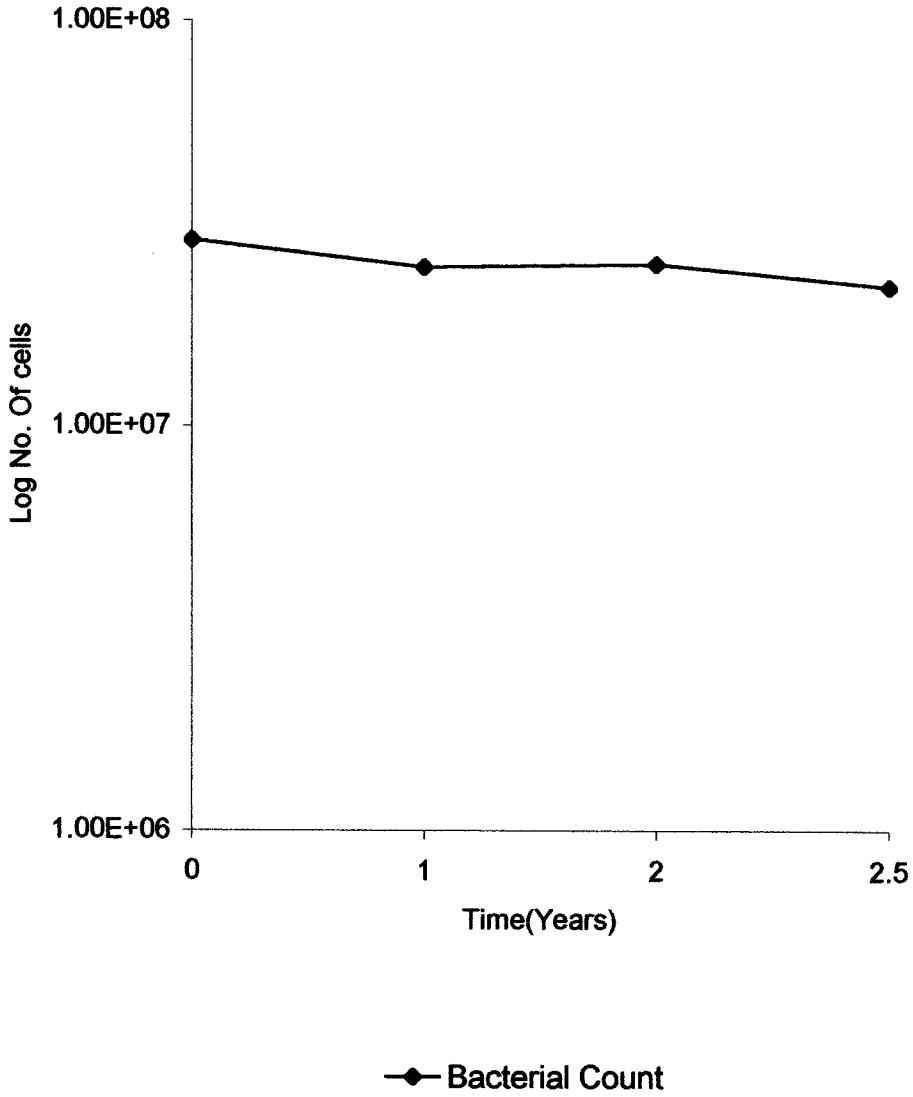


Fig. 2.5. Cryopreservation of strain GU101 at -20°C without growing the strain in nutrient broth.

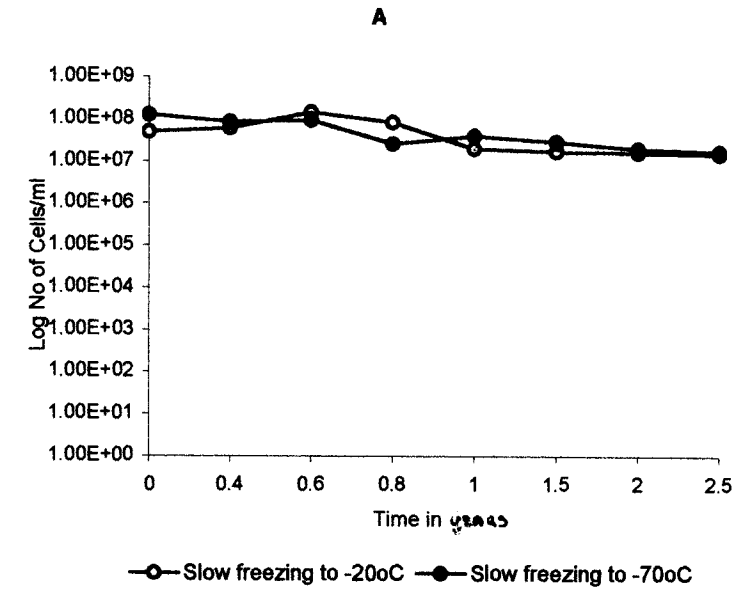
preservation efficiencies for strain GU101, although the efficiency in the case of rapid freezing was much lower. The importance of the rate of freezing is mainly explained by the fact that when cells are subjected to subzero temperatures, they "supercool". The manner in which they regain equilibrum depends chiefly on the rate at which they are cooled and on the permeability to water. If they are cooled slowly, or if their permeability to water is high, they will equilibrate by transfer of intracellular water to external ice (therefore solutes increase), in other words by dehydration (solution effects). But, if they are cooled rapidly or if their permeability to water is low, they will equilibrate atleast in part by intracellular freezing. Furthermore rapid freezing not only produces intracellular ice crystals, it also produces small crystals, which are likely to enlarge during warming because of their high surface free energies (Mazur, 1970).

Thus what we seem to have achieved in our study as indicated by Fig 2.6A & B is an optimum rate of cooling for preservation of strain GU101 i.e., a rate that is slow enough to prevent production of intracellular ice yet rapid enough to minimize the length of time cells are exposed to solution effects.

Release of a periplasmic enzyme C230 after freezing of strain GU101

When bacteria are frozen and thawed, survival is dependent on many parameters including cooling rate and the freezing menstrum. Calcott and MacLeod (1975) have presented evidence that the permeability barrier of the organism was damaged after freezing and thawing and that viability was related to the extent of damage to cells.

Our studies on the release of the periplasmic enzyme catechol 2,3 dioxygenase have clearly showed maximum release of enzyme in the medium when strain GU101 was preserved without protectant (Table 2.1). Minimum release of enzyme was also



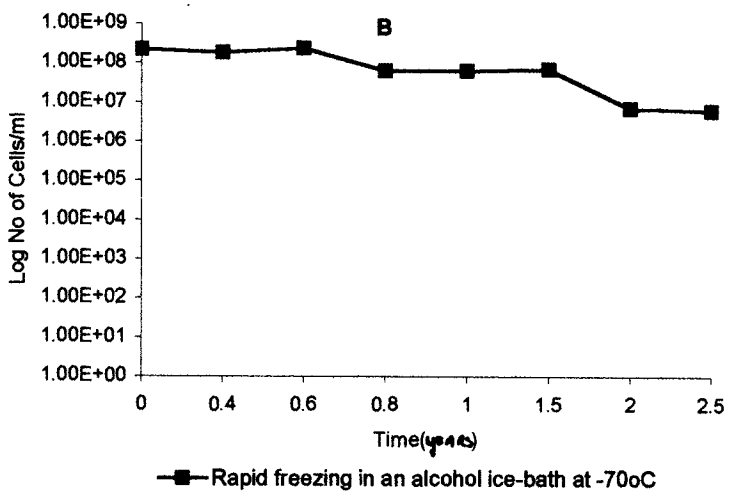


Fig. 2.6. Cryopreservation of Strain GU101 by A) slow freezing to -20°C & -70°C and B) rapid freezing in an alcohol ice-bath at -70°C.

observed when strain GU101 was preserved with glycerol and glycerol supplemented with nutrient broth indicating some membrane damage (Table 2.1). The bacterial counts on marine nutrient agar clearly showed a decrease when strain GU101 was preserved without protectants. This decrease was however, not so sharp as observed as seen in Fig. 2.1A. This could be due to the fact that the plating medium was ASW-agar supplemented with m-toluic acid in that experiment whereas the plating medium used here was marine nutrient agar clearly indicating the role of nutrient in enhancing preserving efficiency.

It thus appears that membrane damage is an important lesion associated with loss of viability. The addition of nutrient broth to the freezing menstrum clearly increases the bacterial counts. Many bacterial species appear to develop increased nutritional requirements after freezing. This has been noted particularly with various species of gram-negative bacteria (Straka and Stokes, 1959). Clearly, nutrient broth supplemented with glycerol seems to be effective in protecting strain GU101 from possible membrane and wall damage, thereby increasing viability.

In conclusion, the study demonstrates that strain GU101 could be cryopreserved efficiently when grown for 48 hours in nutrient broth and artificial seawater and subsequently supplemented with glycerol to final concentration of around 15%. Alternatively, strain GU101 could also be grown in artificial seawater and m-toluic acid to early stationary phase and preserved by the addition of fresh nutrient broth coupled with 15% sterile glycerol. Thus the protective effect of glycerol and possible repairing effect of nutrient broth on strain GU101 was demonstrated in this study. The establishment of these optimum conditions for cryopreservation of strain GU101 gave us scope to determine the preservation of mixed marine degradative bacteria (consortia).

Table. 2.1 Effect of protectants on release of the periplasmic enzyme, Catechol 2,3 dioxygenase(C230) into the medium and bacterial counts of strain GU101 after freezing at –20°C.

Test	Total enzyme activity (IU/ml) on complete disruption and total bacterial count/ml.	Release of C-23O enzyme(IU/ml) and bacterial counts/ml after freezing at-20°C for 24hours.		
		Culture	Culture + 15% Glycerol	Culture + Nutrient broth + 15% Glycerol
Bacterial Counts(per ml)	4.2×10^8	1.4×10^5	2.38×10^6	4.9×10^8
Catechol 2,3 dioxygenase (IU/ml)	0.211	0.100	0.041	0.051

Successful cryopreservation of marine degradative consortia would allow us the possibility of using these consortia for bioremediation studies.

Chapter III

CRYOPRESERVATION AND AMPLIFICATION OF MIXED MARINE MICROORGANISMS DEGRADING AROMATIC COMPOUNDS

As described in the previous chapter, the cryopreservation of microorganisms is a complex process involving a number of criteria that need to be satisfied in order to determine the efficiency of the preservation technique. Obviously working with single cultures has the advantage whereby the criteria of preservation can be individually studied. The problem often arises when preserving mixed cultures as each culture has its own optimum characteristics thereby making the cryopreservation process difficult to establish.

The importance of preservation of mixed cultures is so relevant today particularly in the field of bioremediation of complex organic pollutants such as crude oil, pesticides etc. This is because of the fact that in nature, organic compounds are not present singly, but as components of a mixture of compounds. Degradation and bioremediation of these compounds is brought about by the concerted action of microbes which form a community as no single species in a mixture contains the genetic and therefore enzymatic complement of the mixed culture. Thus it becomes imperative for us to establish a preservation method for mixed marine consortia whereby the equilibrium and growth characteristics of the strains are maintained at low temperatures with a high degree of efficiency and stability.

Industrial processes and microbial degradation technologies involving microorganisms have been used by man for a long time. While industrial processes aim to synthesize a specific product using a selected strain of an organism, microbial degradative technologies try to render harmless those substances which generally are mixed together in large numbers as waste, and cannot be used profitably. In the case of degradative technologies, the use of pure cultures though effective is impeded by the quality and complex nature of the wastes whereas inherently enriched mixed populations

accomplish these decompositions easily. To fit into the economic and other constraints of the process, which define that the mixture / consortia should grow actively in a highly reproducible manner, the consortium has to be maintained in a state that allows very rapid growth once dormancy has been perturbed. This is where the cryopreservation of mixed cultures (be it for industry of degradative technologies) plays an important role because if they can survive the freezing process, they can be stored at low temperatures for a long time and can be used as and when required. In the previous chapter, the optimization of a preservation technique for a single microbial strain GU101 was discussed. Chapter II deals with the cryopreservation of two consortia namely Consortium I which consists of strain GU101, a meta-toluic acid marine degrader, and GU100, a marine agar degrader whereas Consortium II consists of four bacterial strains; GU102, a naphthalene degrader, GU103, a xylene degrader, GU104, a quinoline degrader and GU 105, a pyridine degrader. The dynamics of the preserved mixed consortium as well as the amplification of consortium II when checked over a two and a half year preservation period are also discussed.

MATERIALS AND METHODS

Media and chemicals

Tris-Artificial Seawater(ASW) of pH 7.2 was prepared and meta-toluic acid was prepared according to **Materials and Methods**(Chapter I).

Marine Salts Phosphates(MSP) of pH 6.8 was prepared with modifications according to Gherna and Pienta(1989) and contained (per liter) 17.52g NaCl, 5.0g $MgCl_2$, 3.0g $MgSO_4.7H_2O$, 0.5g $CaCl_2$, 1.0g KCl, 1.0mg $FeSO_4$, 1.0 g $(NH_4)_2SO_4$, 0.5 g KH_2PO_4 and 1.1g K_2HPO_4. The phosphate salts were prepared separately in double strength, autoclaved separately and added to a double strength autoclaved solution of the remaining salts. For solid media agar was used at a concentration of 1.8% w/v.

In the case of GU100 (agar degrader), agar (HiMedia Chemicals, Mumbai) was added to Tris-ASW medium to a final concentration of around 0.05%. The substrates used in the study mainly included quinoline(Aldrich Chemicals., USA), pyridine(S.D. Fine Chemicals,), naphthalene(BDH) and xylene(S.D. Fine Chemicals). All chemicals were of analytical grade.

Media formulations for consortium I included; Tris-ASW supplemented with 0.05% agar and m-toluic acid. For total counts, marine nutrient agar(MNA), Tris-ASW agar and Tris-ASW -meta-toluic acid agar plates were used.

Media formulations for consortium II included Marine Salts Phosphates(MSP) supplemented with quinoline(0.02%), xylene(0.04%), naphthalene(0.04%) and pyridine(0.04%). For cryopreservation studies, these formulations were used separately or as a mixture where all the four substrates were mixed together, the final concentrations being as indicated above. For total counts, the above media supplemented with 1.8 % w/v agar was used.

For cryopreservation studies, instruments and chemicals were as per materials and methods (chapter I). All cryopreservation experiments were carried out according to the seed lot system (Fig.2.3) to preserve early passage material.

Estimation of growth

Culture growth was monitored by measuring the absorbance of the cell suspension at 560nm in a spectrophotometer (Spectronic 1201) and a Klett Summersons Photoelectric colorimeter (Arthur Thomas Company, USA) and by viable counts on various media as indicated earlier.

Cryopreservation of a mixed marine cultures at -70°C

Pure colonies of each of the marine cultures were picked and grown in 1ml of ASW/MSP with the appropriate substrate. 1ml of ASW/MSP supplemented with nutrient broth was dispensed into a series of cryovials which were appropriately labeled. Each of the cryovial was inoculated with the appropriate consortium and incubated on a rotary shaker at 110 strokes/min. After 48 hours of incubation, all the cryovials were removed and 1ml of sterile glycerol (30%) was added to each of them to obtain a final concentration of 15%. The cryovials were quickly cooled to –70°C in an ethanol bath and stored at –70°C. At zero time, the first set was taken out and the count obtained on their respective media. The remaining vials were stored at –70°C and were analyzed periodically at various time intervals over a two year time period.

An alternate method was also employed whereby the strains in a consortium were grown separately on their respective substrates until the beginning of the stationary phase. 1ml of culture broth from each strain was taken and mixed in a sterile tube. 0.1

ml of this mixture was then taken in cryovial, to which was added 0.4ml of nutrient broth in ASW/MSP and 0.5ml of 30% glycerol. Analysis of the cryovials was done in a similar manner as per previous method.

Activation of the preserved cultures

Whenever required, the frozen vials were thawed at 30°C for 5 min and a loopful of culture was inoculated in ASW or MSP medium with the appropriate substrates. Growth was monitored by measuring an increase in turbidity as compared to controls.

Determination of viability of preserved cultures

Whenever required, viability in terms of bacterial counts was obtained by immediately thawing frozen vials and plating 0.1ml of appropriately diluted sample on ASW/MSP medium containing the appropriate substrate.

Preservation and amplification of cultures

Marine degradative strains were grown separately on their respective substrates until they reach the beginning of stationary phase. 1ml of each strain was taken and mixed in a sterile tube. An aliquot of this mixture was inoculated in ASW/MSP medium containing all the substrates. Growth was followed by measuring an increase in turbidity and at various growth phases bacterial counts of the individual strains were obtained by plating an appropriately diluted sample on agar plates containing their respective substrates. After obtaining the count an aliquot was taken and preserved as per the alternate method for preservation of marine cultures as described in chapter I. Frozen cryovials at each growth phase were amplified by rapidly thawing the vial and

inoculating its contents in ASW/MSP medium containing the appropriate substrates. Growth was monitored by turbidity and bacterial counts were obtained by plating on ASW/MSP agar plates containing the appropriate substrate.

RESULTS AND DISCUSSION

When microorganisms are frozen and thawed, the survival that the population exhibits is dependent on a number of factors such as physiological age, nutrition during growth, rate of freezing etc. (Ray and Speck, 1973; MacLeod and Calcott, 1976). The situation becomes more complicated when mixed cultures are used as it becomes difficult to study the effect of freezing on each strain individually. While the results of microbial, biological and genetic research on biodegradation of pollutants by marine organisms are accumulating , the development of practical processes such as the cryopreservation of degradative microbes and their applications on an environmental scale does not appear to be given the prominence it deserves. This is inspite of the fact that this early stage in an industrial or biodegradative process can have a dramatic impact on the process as a whole.

Two consortia were used in our study on the cryopreservation of mixed cultures. Consortium I mainly consisted of GU101, a meta-toluic acid degrader and GU100, a agar degrader, whereas consortium II constituted GU102, GU103, GU104 and GU105. All the strains were isolated from the coastal and high seas off the Goan Coast. In order to determine the efficiency of preservation of mixed marine bacterial strains, it is necessary to establish the very same criteria that are used for the preservation of single strains. However, given the fact that a preservation method was already established for single marine strains(Chapter 1), the same protocol was used for preserving mixed culture consortia whereby the viable and functional properties were checked before and after freezing.

Cryopreservation of consortium I at -70°C using nutrient broth in artificial seawater with glycerol as a cryoprotectant.

Consortium I mainly consisted of a binary mixture of strains namely GU101, a meta-toluic acid degrader and GU100, a agar digester. Our results indicate that strain GU101 and GU100 could be preserved well at –70°C using glycerol as a cryoprotectant after 48 hours growth in nutrient broth in artificial seawater. Viability was determined as in Materials and Methods. The consortium showed a minimum loss of its viability when preserved over a two and a half year period (Fig. 3.1). This once again emphasizes the role of nutrient broth in artificial seawater supplemented with 15% glycerol in maintaining the consortium's functional properties. Such nutritional requirements like the presence of an amino acid or sugar after freezing were observed for a number of microorganisms (Arpai,1962; Nakamura and Dawson, 1962; MacLeod *et al.*, 1966; Moss and Speck, 1966). This has been noted particularly with species of gram negative bacteria where suspensions of cells which gave a high plate count on a particular medium showed a reduced count on the same media after freezing. Those cells converted by freezing to dependance on an enriched medium for growth have been referred to as being metabolically injured (Straka and Stokes, 1959).

Cryopreservation of marine consortium II at -70°C.

Consortium II is a mixture of four marine bacterial strains comprising GU102, GU103, GU104, GU105 utilizing naphthalene, xylene, quinoline and pyridine, respectively. In this study, the four degradative marine microorganisms were isolated on different substrates. The strains were grown to the beginning of the stationary phase and cryopreserved as a mixture at –70°C using nutrient broth in MSP and 15% glycerol

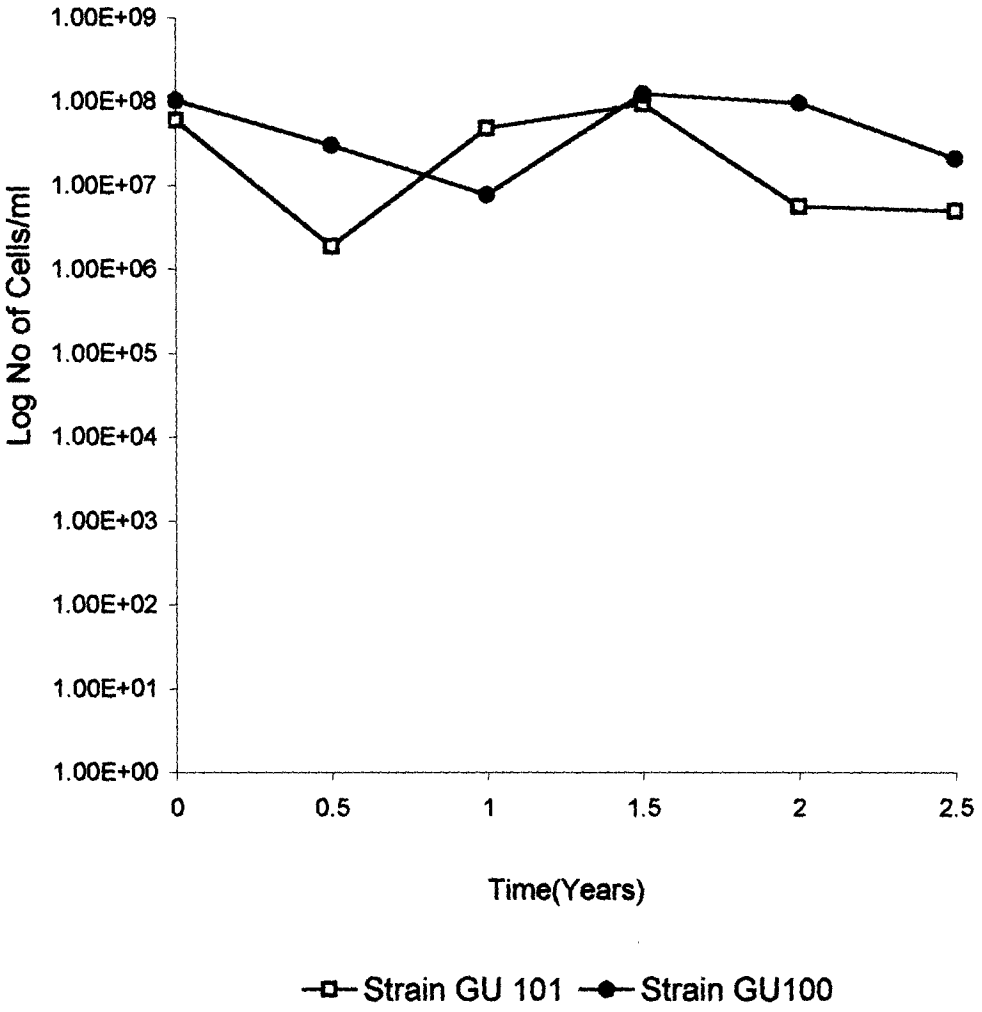


Fig. 3.1. Cryopreservation of a mixed culture (Consortium I) by freezing at -70°C. Viability was determined ASW-agar and ASW-agar containing m-Toluic acid.

The effectiveness of preservation was assessed by checking the consortium's functional and viable properties over a two and a half year time-period. The successful preservation of this mixed consortium would allow us the possibility of expanding our preservation technique to mixed cultures which could be preserved and amplified in a minimum period of time.

As can be seen from Fig. 3.2, the effectiveness of nutrient broth in MSP and 15% glycerol in preserving consortium II is well demonstrated when checked over a two year time period. Viability of individual strains was determined on media with respective substrates as sole source of carbon. The appearance of colonies on their respective substrate containing agar plate was quite rapid even after one year of storage at -70°C. However, an increased incubation period was required for the appearance of colonies after one and a half year storage. The importance of growing the strains in the consortium to early stationary phase or mid-stationary phase has also been continuously the criteria for preservation of these types of strains. Similar observations have been reported for a number of microorganisms (Heckly,1978).

Effect of temperature of preservation on the viability of mixed cultures (Consortium II)

For a number of years it was considered essential to freeze organisms rapidly to obtain high survival (Heckly,1978). However, viability is mainly affected due to intracellular freezing and subsequent recrystallization on warming particularly if warming is slow. On the other hand, slow cooling mainly suffers from the disadvantages of solution effects. In this study Consortium II was preserved and stored at -70°C as per the protocol mentioned earlier. After about three months storage at -70°C, some of the vial

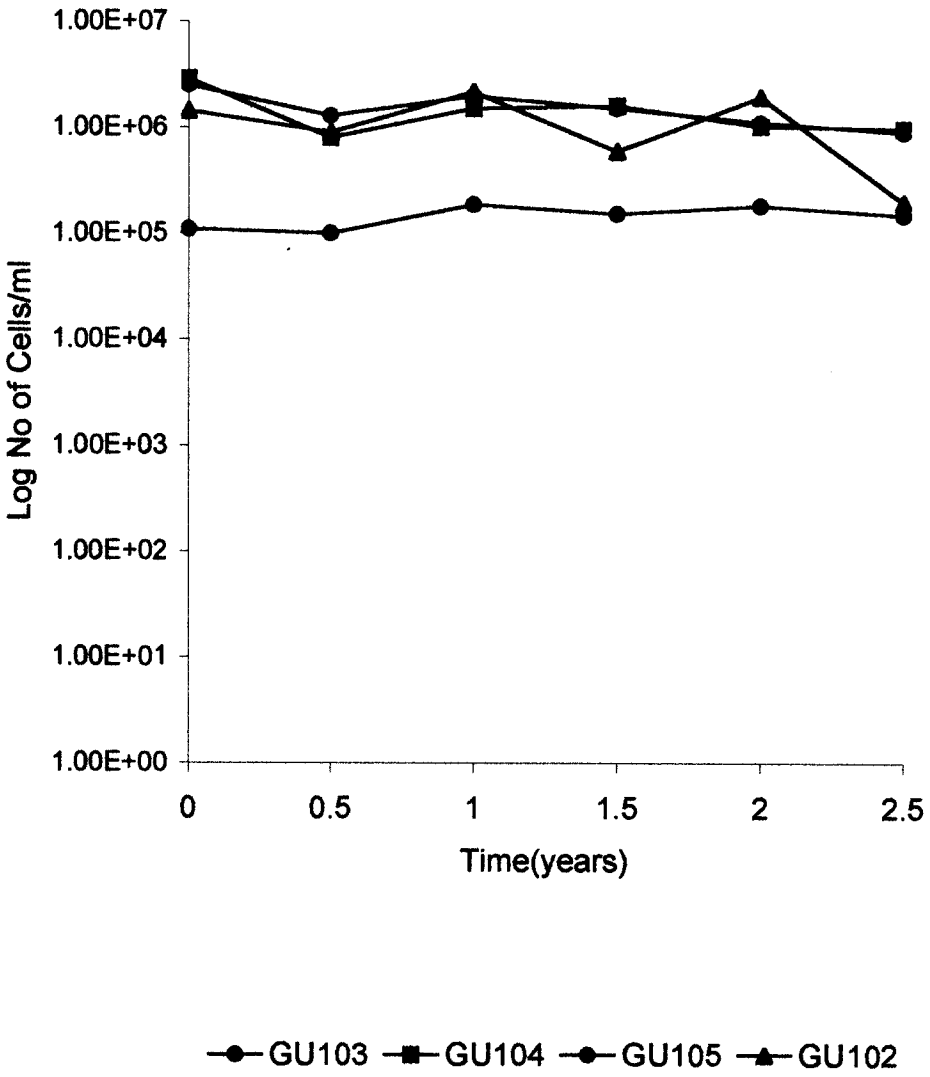


Fig. 3.2. Viability of a mixed culture(Consortium II) at -70°C. Viable number of cells of strain GU102, strain GU103, strain GU104 and strain GU105 were determined on ASW-agar containing naphthalene, xylene, quinoline and pyridine, respectively.

were transferred to -20°C. At zero time, i.e. the point of transfer of the cryovial to -20°C, the count was obtained by plating on their respective substrates. The counts were then obtained for each of the strains in consortium II stored at -20°C for a two and half year time period.

As can be seen from Fig. 3.3, the consortium was preserved efficiently with minimum loss of viability even after two years of storage after transfer from -70°C to -20°C indicating the effectiveness of the freezing media and cryoprotectant in protecting against rapid fluctuations in temperature.

Dynamics of members of the mixed culture (Consortium II) on mixed substrates

One of the aims in preserving mixed cultures is to ascertain that the proper equilibrium of the mixed cultures is maintained at all times. To understand this fact, strain GU102, 103, 104 and 105 was inoculated in a flask with MSP containing all the four substrates naphthalene(0.04%), xylene(0.04%), quinoline(0.02%) and pyridine(0.04%) as carbon sources. The control flask did not contain the susbtrates.. Growth of consortium II in the control and test flasks was monitored by absorbance measurements at 560nm and plate counts on their respective media at various time-intervals over a 17 day time-period. Fig. 3.4 shows the growth curve of consortium II on a mixed substrate. The dynamics of the bacterial counts of the test and control are also seen in Fig.3.5A & 3.5B. The growth curve showed a sharp increase in optical density between 70 to 90 hours of growth which was also accompanied by an increase in bacterial counts of all the four strains.(Fig. 3.4, 3.5A). Strain GU102 grew rapidly with a high count as compared to other strains. The bacterial count in the control flask which was devoid of any carbon sources showed a complete disturbance in the equilibrium between the four strains as

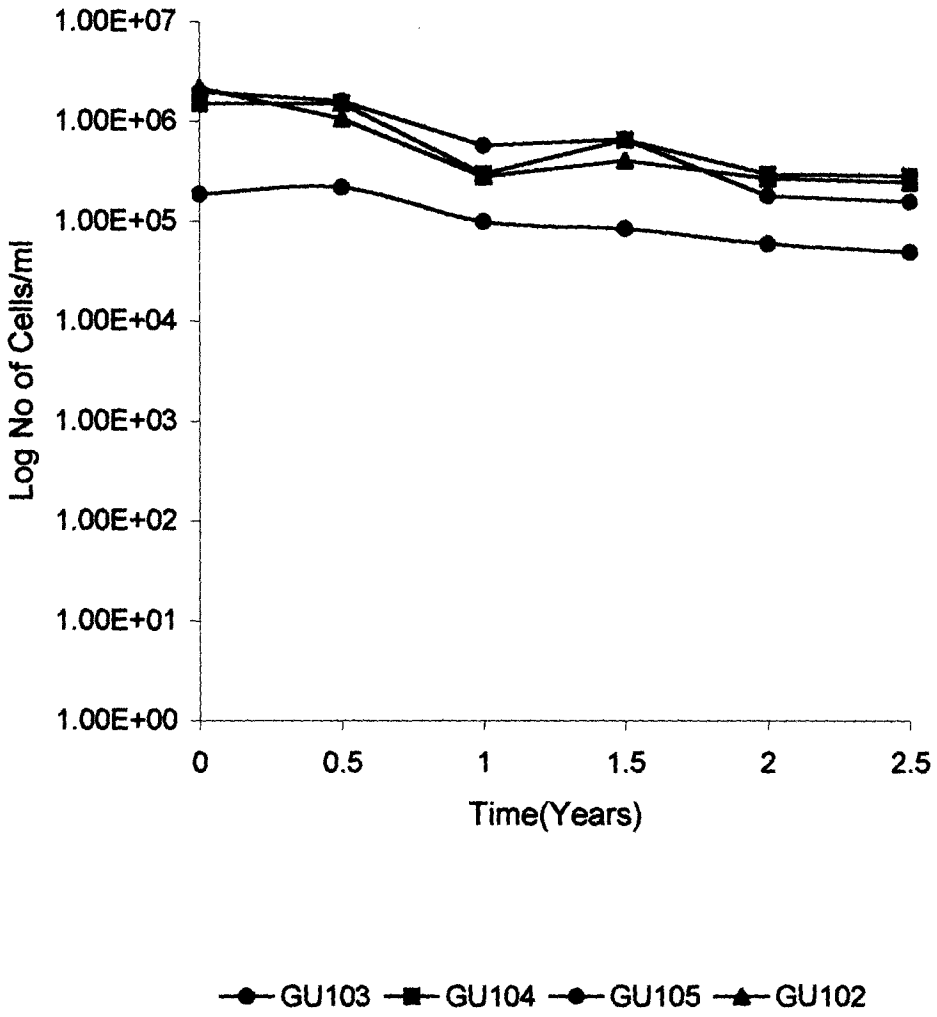


Fig. 3.3. Viablity of mixed cultures at -20°C. Viable number of cells of strain GU102, strain GU103, strain GU103 and strain GU105 were determined on ASW-agar medium containing naphthalene, xylene, quinoline and pyridine, respectively.

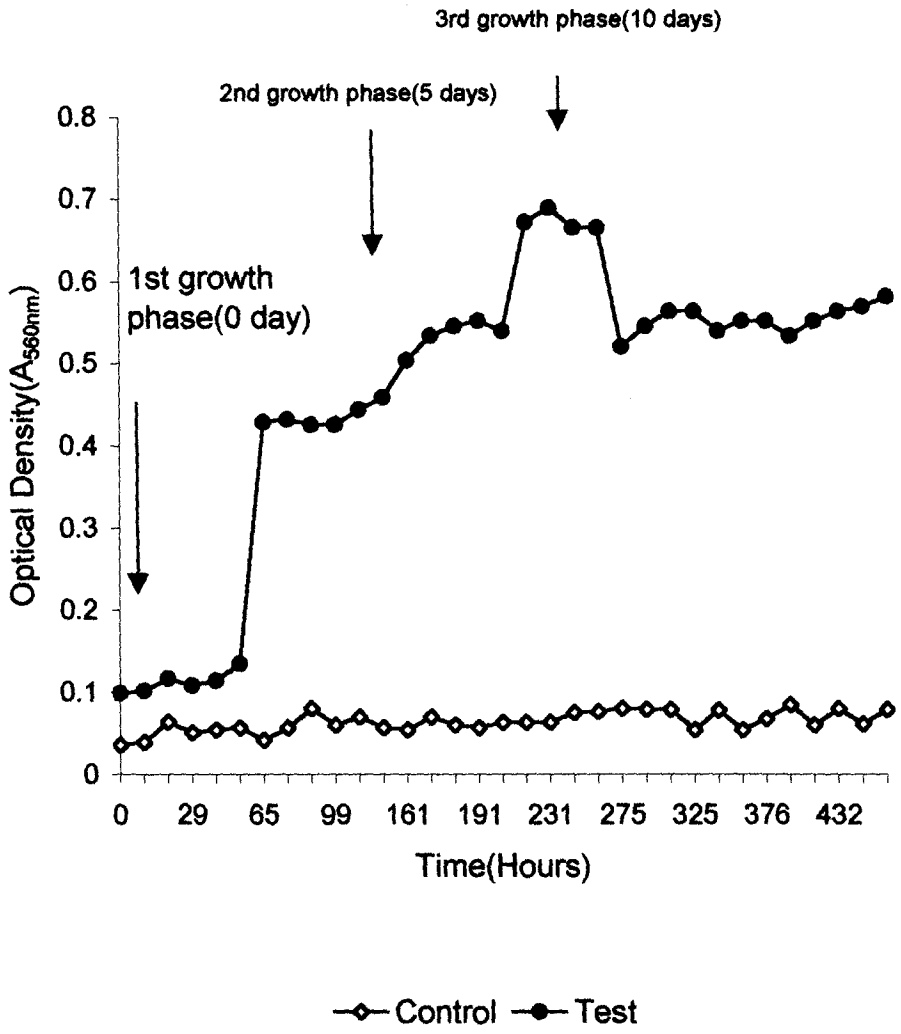


Fig. 3.4. Growth of a mixed marine consortium (Consortium II) on four substrates. Arrows indicate the various growth phases at which the mixed culture was preserved.

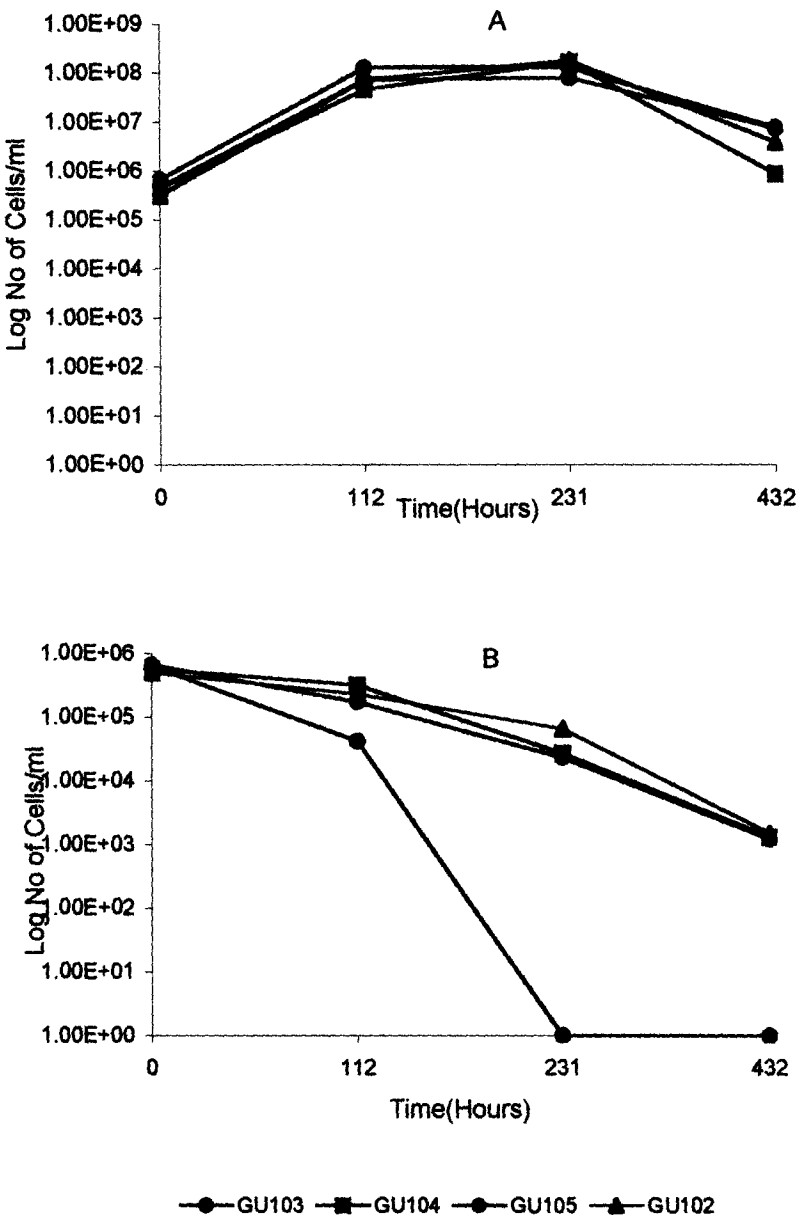


Fig. 3.5 . Growth of the mixed marine consortium (Consortium II) in ASW medium with A) substrates and B) without substrates. Viable number of cells of strain GU102, GU103, GU104 and GU105 were determined on ASW-agar medium containing naph, xyl, quin, pyr.

seen in Fig. 3.5B. However, the strains survived for almost three days under starved conditions indicating that these strains could survive extended starvation periods which is characteristic of microorganisms in the marine environment. The results of our study indicate that Consortium II grew efficiently when challenged with a mixed substrate with all the four strains growing effectively.

Cryopreservation and amplification of a growing mixed marine degradative consortium (Consortium II) at –70°C

To find out whether the mixed cultures could be amplified in a minimum period of time, the mixed culture was frozen at different time interval during growth of Consortium II. At different time intervals during growth of consortium II (zero time, five and ten days) (Fig. 3.4), 0.1ml of the grown mixture was taken in a cryovial to which was added 0.4ml nutrient broth in marine salts phosphates(MSP) and 0.5ml glycerol (30%). A series of such cryovials was prepared. The cryovials were subsequently stored at -70°C. The cryovials stored at -70°C at different growth phases were amplified and monitored as per previously mentioned protocol. The preserved cryovials were monitored over a two and a half year time period. The control flasks were without substrates.

Amplification of the first growth phase of consortium II : Our results on the amplification of the first growth phase of consortium II indicate that the consortium was able to grow rapidly after three months storage at –70°C (Fig. 3.6A). However, after two years storage, growth of the consortium was characterized by an increase in the lag phase (Fig. 3.6B). Bacterial counts indicate that the equilibrium between the strains was maintained both after three months and two year storage at –70°C (Fig. 3.7),

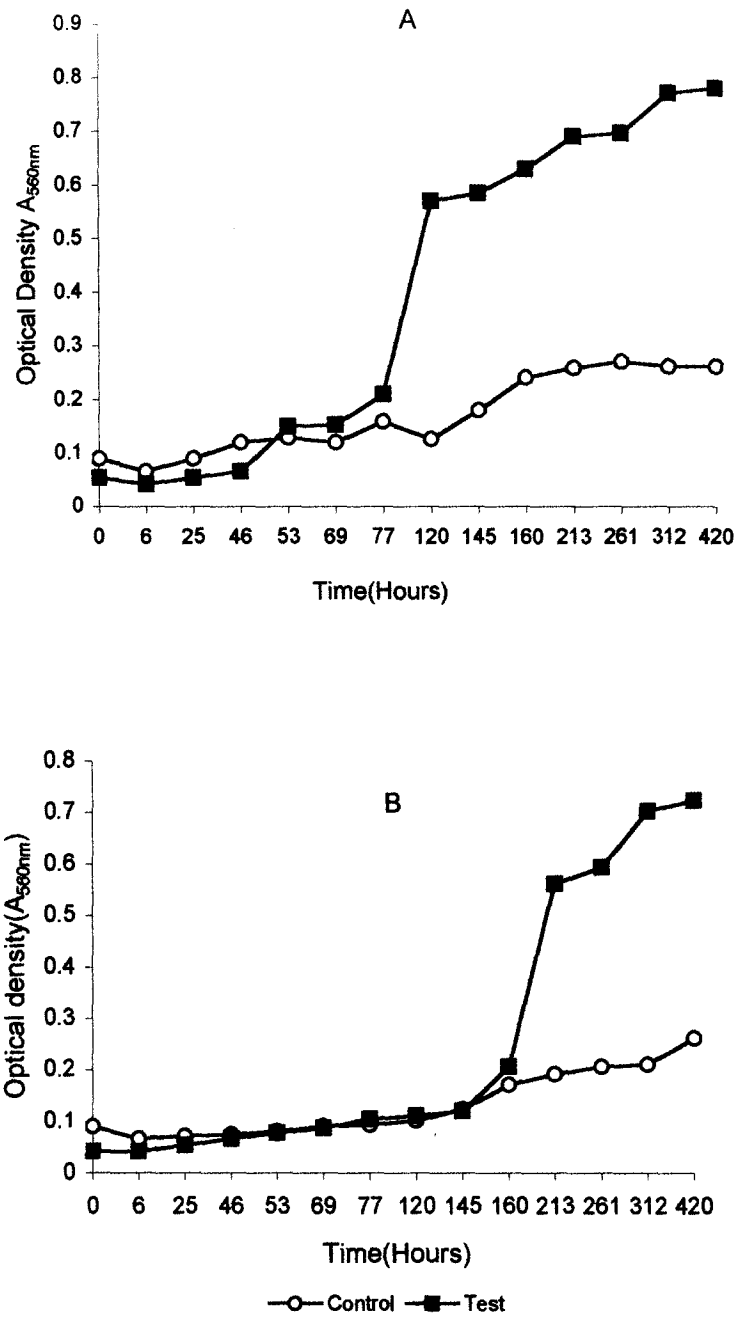


Fig. 3.6. Amplification of the First Growth phase of Consortium II after A) Three months B) Two years storage at -70°C. Control flask contained no substrates.

although this equilibrium between the four strains in consortium II was attained after a prolonged time period during normal growth as observed earlier in Fig. 3.5A. This prolonged time-lag for consortium II could be explained by the fact that consortium II was preserved at a very early stage of growth. It is a well known fact that the physiological age of the organism can have a profound effect on the survival of the microorganism after freezing. A well nourished cell in a culture at the beginning of the early stationary phase is usually the most resistant cell (Heckly, 1978). Thus the mixed culture in consortium II may be exhibiting some freezing injury which is indicated by low initial bacterial counts (Fig.3.7). This type of injury is usually manifested by a time-dependent inability to perform a particular function such as ability to form a colony, degrade a particular substance etc, but given a nonstressful environment or the addition of a protectant/additive most cells will repair this injury (MacLeod and Calcott, 1976). A similar result seems to be observed with consortium II with an early stage of growth not being suitable for preservation, however the addition of nutrient broth and glycerol in the freezing medium allows the strains in consortium II to recover and subsequently be amplified after a prolonged time-lag.

Amplification of the second growth phase of Consortium II: Amplification of the second growth phase of consortium II shows efficient and rapid amplification, both after three months and two years storage at –70°C as indicated by Fig. 3.8. A sharp increase in optical density was observed after 53-60 hours of growth of consortium II which was very similar to control (Fig. 3.4). The equilibrium between the strains was maintained efficiently after both three months and two years storage at –70°C although strain GU104 shows a lower growth as compared to control(Fig. 3.9, 3.5A). Similar bacterial counts were observed after three months and two year storage at –70°C when compared

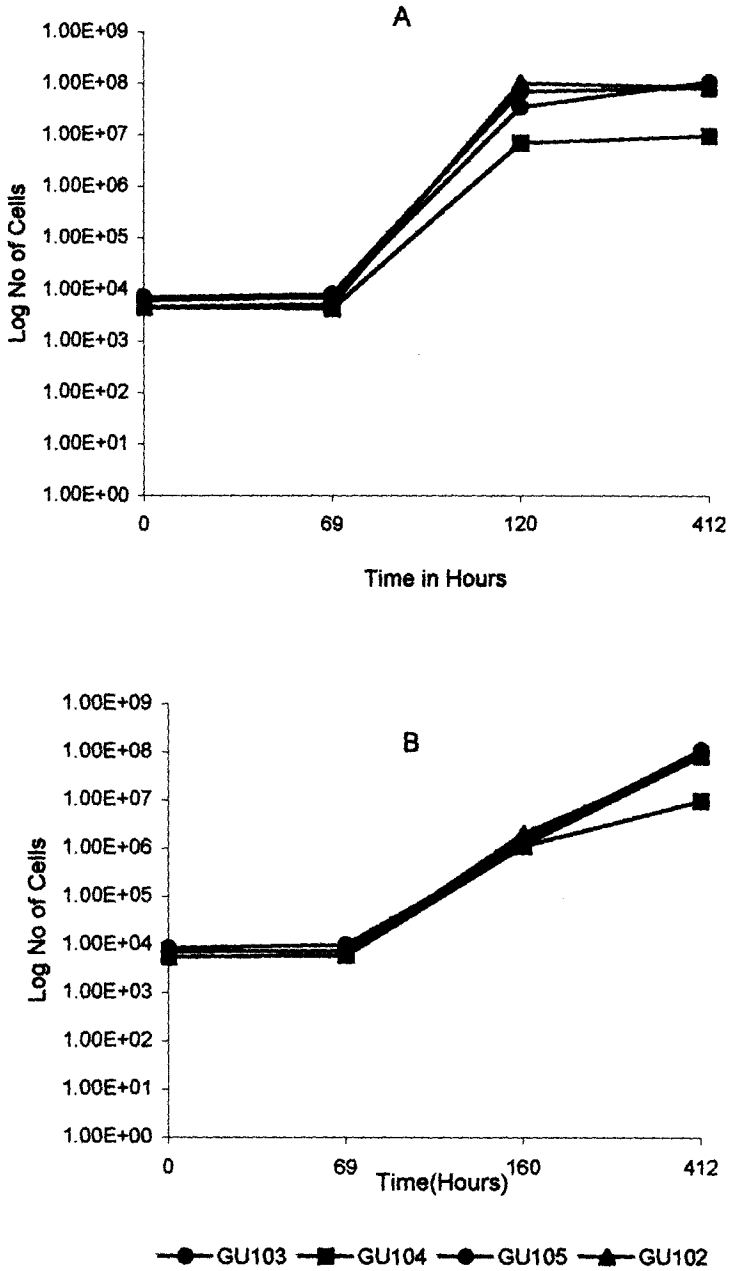


Fig. 3.7. Amplification of the First Growth Phase of consortium II after A) Three months B) two years storage at -70°C. Viable counts of cells were determined on ASW-agar medium containing nah, xyl, quin, pyr.

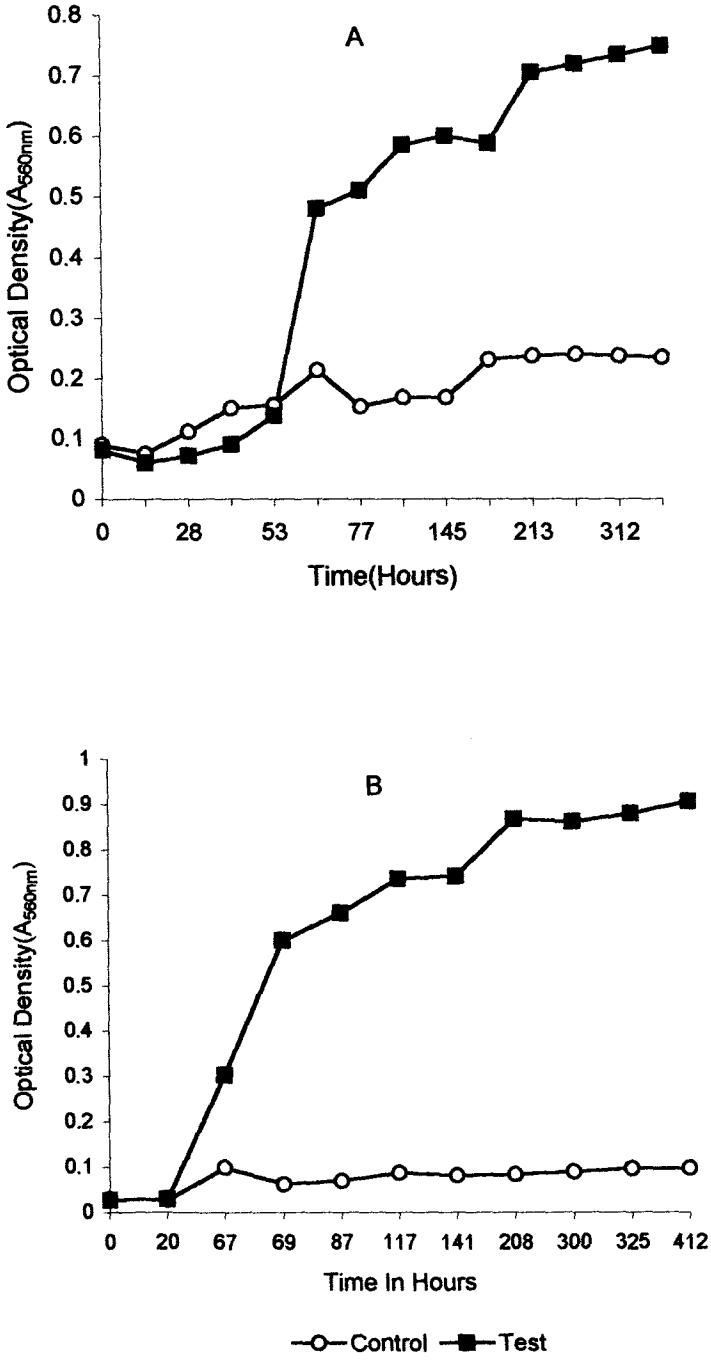


Fig. 3.8. Amplification of the second growth phase of consortium II after A) three months B) two year

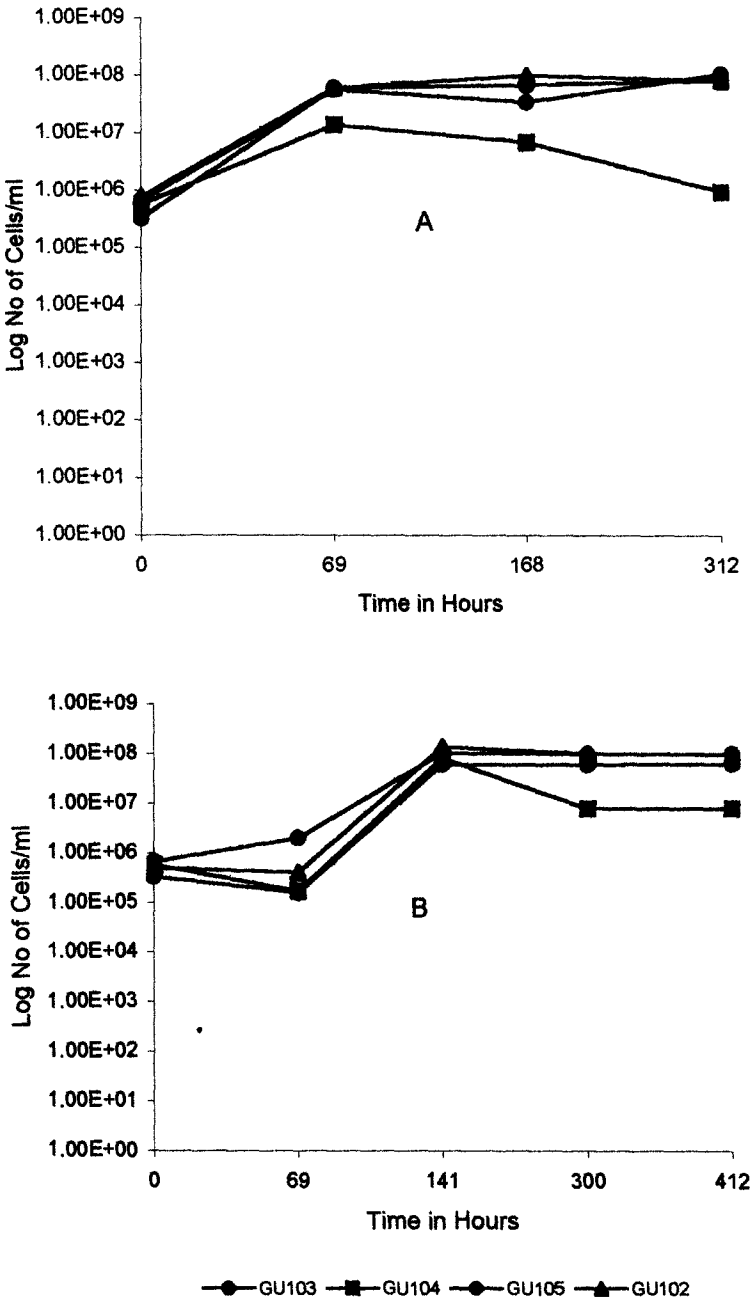


Fig. 3.9. Amplification of the second growth phase of consortium II after A) Three months B) two years storage at -70°C.Viable number of cells were determined on ASW medium containing naph, xyl,quin,pyr.

to control, indicating that minimum damage to consortium II occurred when preserved at this stage to growth. This once again emphasizes the fact that freezing and thawing of mixed cultures in consortia subjects bacterial strains to damage which depends on a number of factors such as physiological age, freezing medium, etc. Our results indicate that consortium II was preserved and amplified within a short period of time when preserved at this stage of growth.

Amplification of the third growth phase of Consortium II: Our results on the amplification of the third growth phase after three months and two year storage at –70°C indicate that consortiumII showed an enhanced time lag as compared to the first and second growth phases. A sharp increase in optical density was observed after 77-87 hours of growth as compared to control (Fig. 3.10) which showed this increase after around 55-60 hours. Bacterial counts of consortium II indicate lower number of bacterial cells as compared to control(Fig. 3.11) indicating some freezing damage at this stage of growth. The equilibrium between the strains also appears to be disturbed as seen in Fig.3.10. Thus, late stationary phase of consortium II indicates some freezing damage thereby showing a prolonged amplification time. These results are in contrast to results obtained by Heckly (1978), who observed that young cells were more sensitive to freezing as compared to stationary phase cultures. This once again emphasizes the fact that careful treatment of cells is essential to maximize the recovery of a given population. It appears that each type of cell appears to exhibit its own set of optimal conditions.

Thus consortium II is best preserved and amplified at growth phase II, i.e. after five days of growth. The importance of rapid amplification is mainly judged from the fact that most degradative technologies have been criticized for its slowness in the initiation

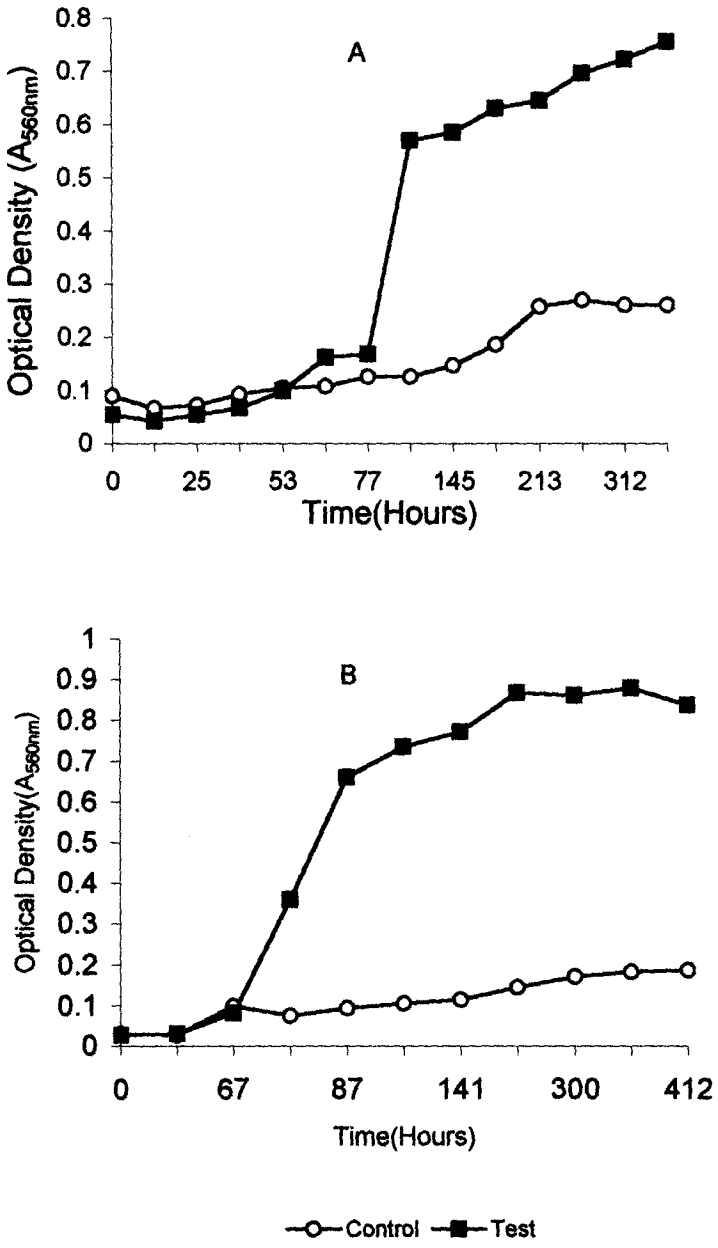


Fig. 3.10. Amplification of third growth phase of Consortium II after A) three months B) two years storage at -70°C.Control flask contained no substrates.

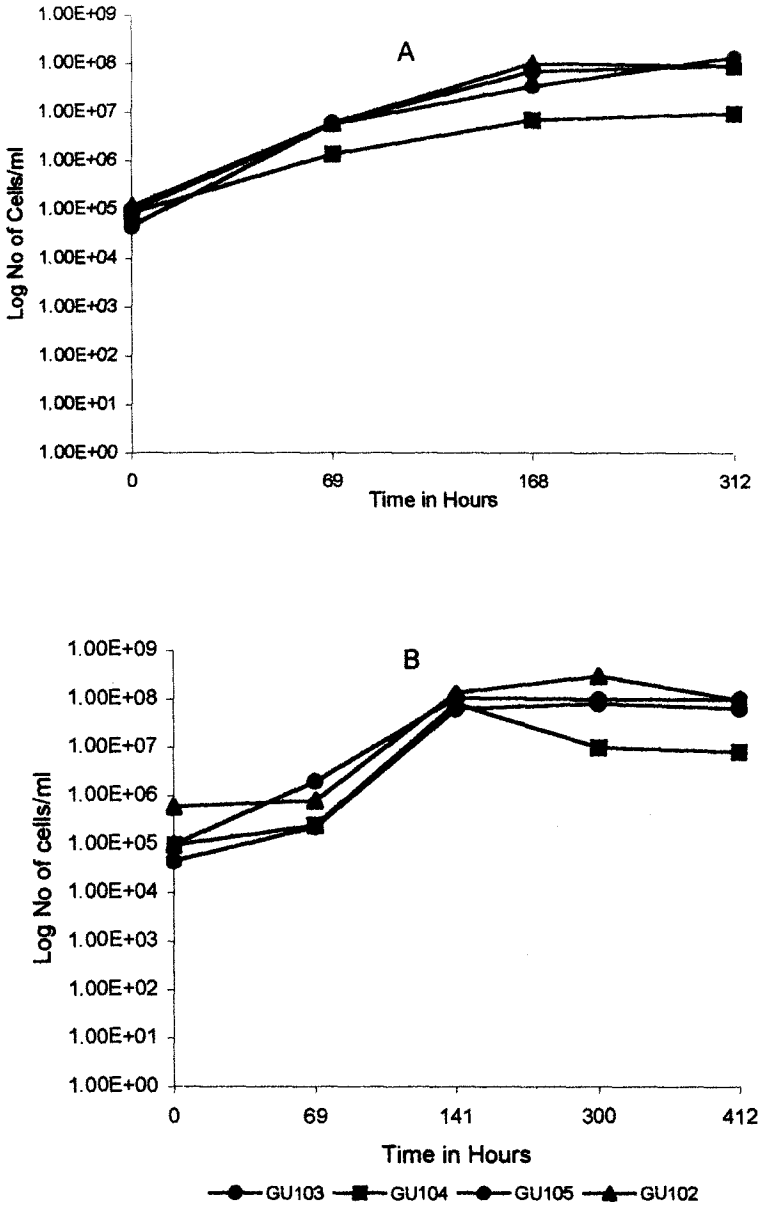


Fig. 3.11. Amplification of the third growth phase of consortium II after a) three months b) two years storage at -70°C. Viable number of cells were determined on ASW-agar medium containing naph, xyl, quin, and pyr.

of the bioremediation process. The successful preservation and amplification of consortium II without any loss of its functional properties within a minimum period of time, suggests that consortium II could be used as a potential degradative consortium for oil spill clean up in the marine environment. Moreover, similar preservation methods could be employed for marine degradative microorganisms used in microbial degradative technology which would allow rapid amplification of the organisms after accidental release of pollutants such as crude oil/ hydrocarbons.

In conclusion, our cryopreservation studies have successfully demonstrated the preservation of single and mixed marine degradative strains for a considerable length of time with minimum loss of viability. The important findings of the cryopreservation process suggest that:

1) The cultures form an early stationary phase were most resistant to freezing. A number of investigators have also shown that cells from the stationary phase are more resistant to damage by freezing and thawing than cells from early or mid-log phase(Heckly,1978). They suggested that an increase in cell density coupled with the possibility that lysed cells could yield cryoprotective substances result in an increase in cryopreservation efficiency. Our results seem to follow a similar pattern.
2) Our studies have shown that nutrient broth in ASW/MSP supplemented with 15% glycerol has been effective in preservation of single and mixed marine strains. A number of workers have studied the effect of a cryoprotectant like glycerol supplemented with an additive on the preservation of microorganisms (Daily and Higgens.1973). They suggested that bacterial species appear to develop increased nutritional requirements after freezing. Cell suspensions which show a similar count on minimal and enriched media before freezing show a reduced viability after

freezing but gave a higher count when an enriched medium was used. These cells converted by freezing to a dependence on the enriched media for growth have to be referred to as being metabolically injured. The marine bacterial strains used in our study also seem to be injured in a similar way as indicated by a low viability when preserved without nutrient broth. This could be due to the fact that microorganisms which have been frozen , leak intracellular solutes and become more penetrable by solutes in the suspending medium due to damage to the cytoplasmic and cell membrane. The capacity of nutrient broth and other rich compounds such as sugars, amino acids etc. to protect microorganisms from freezing could be due to two possible explanations. One is that the damage to the membrane causes the intracellular pool of certain components to leak from the cell. If a critical concentration of a certain component is required inside the cell for the cell to grow and divide, growth would be expected to occur if the necessary component at the appropriate concentration was supplied in the medium. Alternatively, some component, be it a sugar or amino acid may be required specifically to repair damage caused by freezing. The use of nutrient broth in our study seems to provide the suspending medium with the right components for preservation of single and mixed marine strains. The role of glycerol in preserving microorganisms from freezing is well documented (Simione,1998). This is mainly due its low toxicity and its ability to protect against solution effects. These type of substances penetrate cells and obviate freezing damage probably because of their ability to buffer "salt concentrations" by their colligative properties.

3) In our study, a rate of 1°C/ min was optimum for cryopreservation of single and mixed marine strains. The importance of the rate of freezing can be gauged from the

fact if cells are cooled rapidly they suffer damage from intracellular freezing whereas if they are cooled slowly, "solution effects" affect the preservation efficiency. The use of a rate of a 1°C/min in our study seems to indicate a fine balance between these two factors. This optimum rate coupled with the use of nutrient broth in ASW/MSP supplemented glycerol gives the best preservation efficiency for marine degradative strains used in our study.

The studies on cryopreservation of marine degradative strains serve to establish a method for long-term storage of marine microorganisms. Keeping the various results obtained in our study, a protocol was established for long-term storage and amplification of single cultures and mixed marine strains (Fig. 3.12). The protocol could serve as a reference guide for preserving single and mixed marine strains.

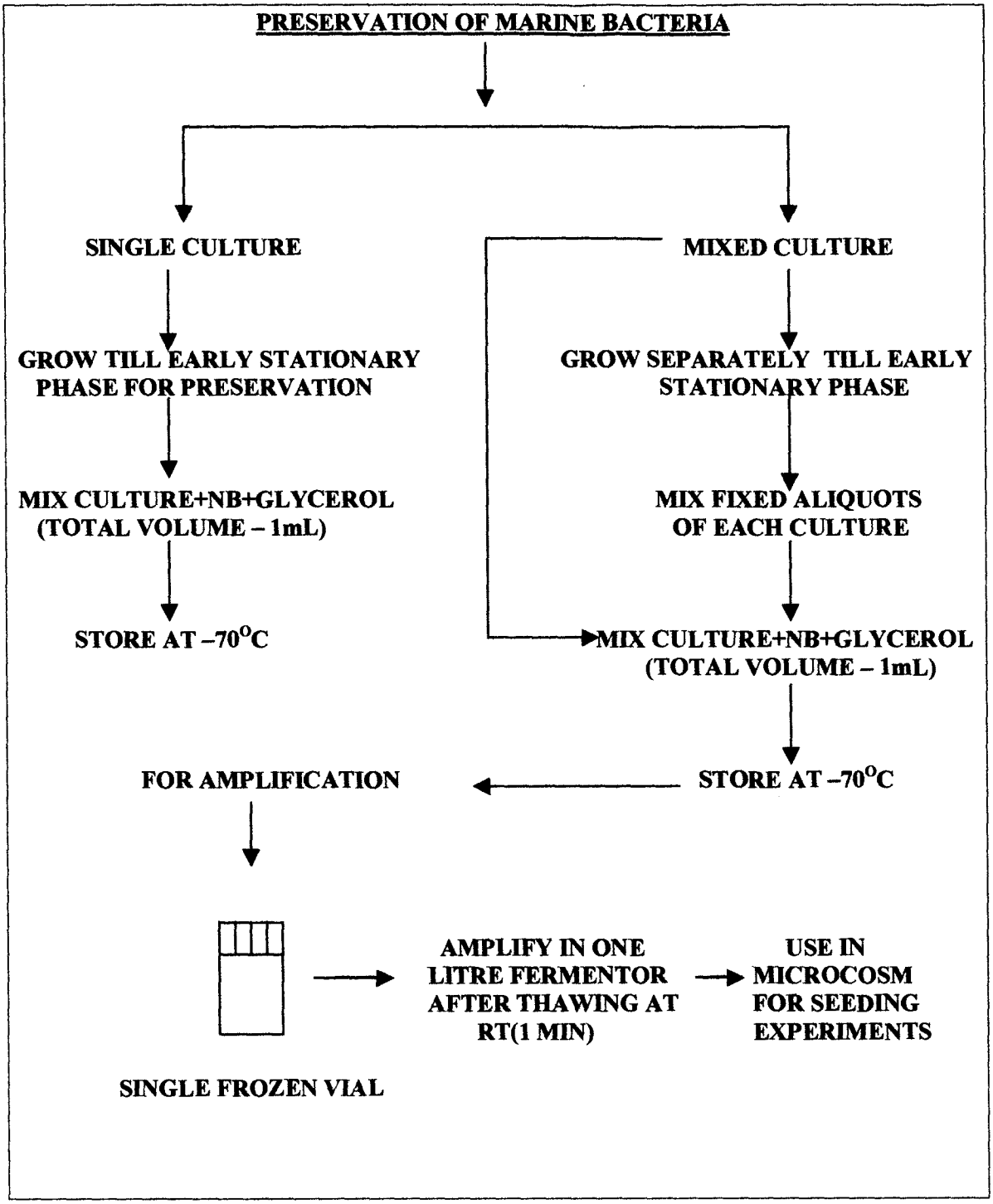


Fig. 3.12. Protocol for preservation and amplification of single and mixed marine bacterial strains.

Chapter IV

ISOLATION OF A MARINE BACTERIUM RESPONSIBLE FOR THE MINERALIZATION OF POLYCYCLIC AROMATIC HYDROCARBONS WITH SPECIAL REFERENCE TO PHENANTHRENE AND CRUDE OIL

Hydrocarbons are ubiquitous in the marine environment. They are derived from many sources, the largest fraction probably coming from marine organisms such as zooplankton and phytoplankton (Blumer,1968). In addition, petroleum hydrocarbons which consist of aromatic and heterocyclic compounds are introduced into the sea in amounts estimated to be atleast one million tonnes and as high as ten million tonnes per year (Blumer *et al.*, 1970). As a consequence of this large influx of petroleum hydrocarbons, many genera of organisms have been exposed to petroleum or its constituents. Of these, bacteria have been considered as oil degraders rather than organisms which could be adversely affected by petroleum. A variety of bacteria belonging to the genera *Pseudomonas*, *Aeromonas*, *Alcaligenes*, *Nocardia* etc have been shown to degrade petroleum hydrocarbons offering us the possibility of bioremediation of contaminated sites (Leahy and Colwell, 1990).

Of importance is a class of petroleum hydrocarbons known as the polycyclic aromatic hydrocarbons (PAH's). PAH's exhibit toxic, carcinogenic or terotogenic properties (Guerin and Jones,1998). Furthermore, PAH's show very low solubility in aqueous solutions and are often associated with particle surfaces. For this reason, their environmental distribution is mildly affected by natural processes such as volatilization, photolysis and degradation making PAH's to be recalcitrant under many natural conditions. Their toxicity coupled with their chemical persistance makes these compounds very dangerous as environmental contaminants.

In this study, phenanthrene (Phe) has been used as a model compound for studying the biodegradation of PAH's since it is found in high concentrations in petroleum contaminated sites and many PAH's containing the phenanthrene moiety are carcinogenic.

This chapter deals with the isolation and characterization of a phenanthrene degrading marine bacterium. The biochemical pathway it utilizes, its role in crude oil and PAH degradation are also discussed. Furthermore, this chapter also deals with the distribution of marine hydrocarbon degraders in the North Arabian Sea.

MATERIALS AND METHODS

Marine salts phosphates (MSP) of pH 6.8 was prepared with modifications, according to Gherna & Pienta (1989) and contained per litre 17.52g NaCl; 5.0g $MgCl_2$; 3.0g $MgSO_4.7H20$; 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 1.8%(w/v). Phenanthrene at a concentration of 0.08%(w/v) was used as a sole source of carbon. Phenanthrene and the products of the pathway were obtained from Sigma Chemical Co, USA. Bombay High crude oil was obtained from the Oil and Natural Gas Commision (ONGC), Mumbai, India. Crude oil was used at a concentration of 0.1%(v/v). Secondary carbon sources such as pyruvate, lactate and succinate were used at a concentration of 0.2%(w/v).

Model hydrocarbon mixture was prepared as per the table given below.

Composition of Model Hydrocarbon Mixture

Substrate	Concentration
Hexadecane	0.1%(v/v)
Naphthalene	0.01%(w/v)
Phenanthrene	0.08%(w/v)
Dibenzothiophene	0.01%(w/v)
Fluorene	0.01%(w/v)

Culture growth was monitored by measuring the absorbance of the cell suspension on the model hydrocarbon mixture at 560nm in a spectrophotometer (Spectronic 1201). Gas Chromatography analysis was performed by extracting the flask contents in an equal amount of ethyl acetate. The ethyl acetate extract was passed over anhydrous sodium sulphate and evaporated to dryness. The dried extract was dissolved in minimum amount of ethyl acetate and analyzed by gas chromatography, Netel Chromatographs, Omega QC^{++} using a SE-30 packed stainless steel column attached to a flame ionization detector (FID). The temperature program was set for an initial temperature of 60°C which increased to 300°C at a program rate of 6°C/min. Nitrogen was used as a carrier gas at a flow rate of 25ml/min.

Processing of water samples on board the scientific vessel R.V. Gaveshni

Water samples (500ml) were collected and filtered through a 0.22μm millipore membrane. Total viable counts (TVC) were determined on marine nutrient agar (MNA) before incubating the millipore filter in 5ml of marine salts phosphates(MSP) containing 0.08%(v/v) sodium benzoate at room temperature (Fig. 4.1). Growth was followed by monitoring an increase in turbidity in the liquid marine salts phosphates (MSP). Tubes which turned yellow were scored as strains possessing *meta* and / or *ortho* cleavage ability.

Detection of polycyclic aromatic hydrocarbon (PAH's) degrading bacterial strains

Analysis after enrichment of cultures was carried out by inoculating a small amount of cultures from surface waters on MSP containing separately anthracene{Anth) (0.08%w/v), phenanthrene{Phe}(0.08%w/v), naphthalene{Nah}(0.04%w/v),

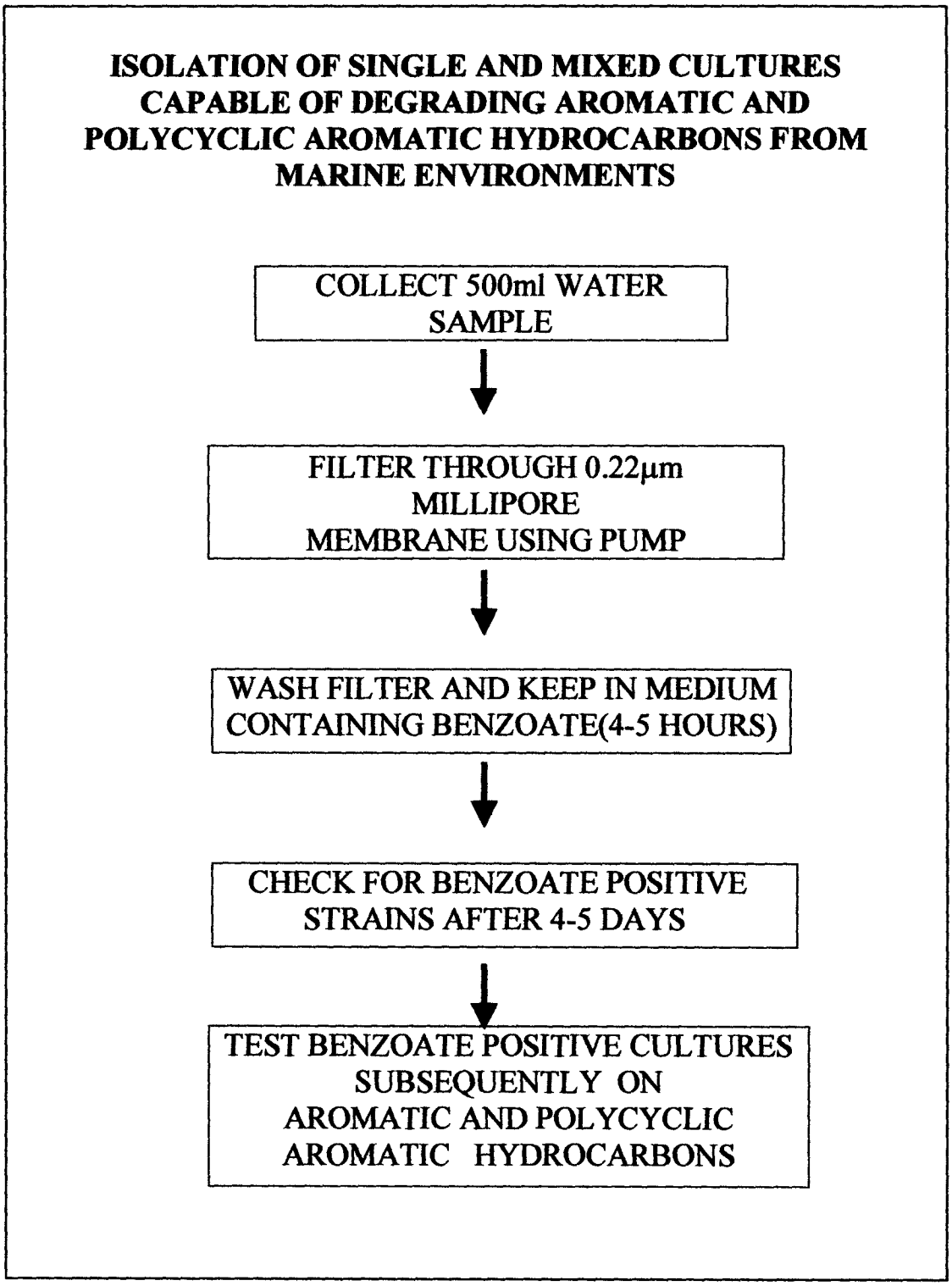


Fig. 4.1. Flow chart showing the benzoate enrichment technique.

pyridine{Pyr}(0.04%v/v), Fluorene{Flo}(0.08%w/v), Xylene{Xyl}(0.04%v/v) and m-toluic acid (5mM).

Strain identification

All tests for identification of this isolate were done according to the Bergeys Manual of Determinative Bacteriology (Kreig and Holt, 1984). Tests for *ortho* and *meta* cleavage were done according to the method of Stanier *et al.* (1966). Tests for pigment formation (King *et al.*, 1954) were also performed. Antibiotic sensitivity was determined by placing 5mm antibiotic (HiMedia Chemicals Co) discs of Whatman filters with the appropriate concentration of antibiotic on plates seeded with the culture and detecting the zone of inhibition. The culture was also sent to the Institute of Microbial Technology (IMTECH), Chandigarh, for identification and deposition in their culture collection.

Estimation of growth of strain GU109

Culture growth was monitored by measuring the absorbance of the cell suspension at 560nm in a spectrophotometer (Spectronic 1201), by viable counts on MSP-agar supplemented with 0.08%(v/v) sodium benzoate. The dry weight of strain GU109 growing in MSP supplemented with Phe was determined by centrifuging a 5ml aliquot of the culture at 10,000 rpm for 15 minutes. The pellet was washed twice in distilled water. The weight of the biomass was taken using a balance after drying the pellet to a constant weight by heating at 80°C.

Growth on various aromatic substrates, singly and in mixtures

Carboxybenzaldehyde(0.02%w/v), salicyclic acid(0.01%w/v), salicylaldehyde(0.02%v/v), catechol(0.01%w/v), o-phthallic acid(0.01%w/v), 1-hydroxy-2-naphthoic acid(0.02%w/v), sodium benzoate(0.08%v/v), sodium lactate(0.2%v/v), pyridine(0.02%v/v), quinoline(0.02%v/v), xylene(0.02%v/v), m-toluic acid(5mM), dibenzothiophene(0.02%w/v), BHCO crude oil(0.1%v/v). anthracene(0.02%), naphthalene(0.04%w/v) and phenanthrene(0.08%w/v) were all tested as single growth substrates.

Growth was monitored spectrophometrically and the depletion of substrate and appearance of products was determined by GC on binary mixtures containing A) Phe and DBT B) Phe and Flo C) Phe and Nah and D) Phe alone.

For Gas Chromatographic analysis, the entire contents of GU109 growth flasks (25ml) containing phenanthrene were extracted at various time intervals using ethyl acetate (25ml) so as to determine the appearance of products and depletion of phenanthrene. The ethyl acetate layer was concentrated to 1ml and 0.7µl was injected into the GC (Netel Chromatographs) having a stainless steel packed column(SE-30) with a flame ionization detector(FID). GC conditions were as follows: Column initial temperature – 60°C , Column final temperature – 300°C, Program rate – 6°C/min, Carrier gas(N_2) flow rate – 25ml/min, Detector temperature – 300°C, Injector temperature – 300°C.

In the case of binary mixtures, 1ml of the culture supernatant was extracted with an equal amount of ethyl acetate. The ethyl acetate layer was concentrated to 0.5ml and 0.5µl was injected into the GC. The conditions were the same as above, except the column initial temperature which was 100°C.

Extraction and analysis by thin layer chromatography(TLC)

Cells of strain GU109 were grown at 30°C in MSP containing 0.08%(w/v) phenanthrene till sufficient turbidity was obtained. They were then pelleted by centrifugation at around 10,000 rpm for 20 minutes at 4°C and resuspended in 2ml of MSP. One milliliter of cell suspension was supplemented with 10ul of a 200 mM solution of 1-hydroxy-2-naphthoate, and the mixture was incubated for three minutes at room temperature. After acidification by the addition of 100µl of 10N HCl, the suspension was centrifuged at 10,000 rpm for 5 minutes at 5°C. The resulting supernatant was extracted with three volumes of ethyl acetate. The ethyl acetate phase was recovered and evaporated. The dried sample was then redissolved in 100ul of ethyl acetate and applied to a TLC plate(silica gel , Merck, F-254) and developed with a solvent system of ethyl acetate - 2-propanol(2:1v/v). 1-hydroxy-2-naphthoic acid, 2-carboxybenzaldehyde, protocatechuate, salicylaldehyde, phthalate and salicyclate were dissolved in diethyl ether and applied as standards (Iwabuchi *et al.*, 1998).

Extraction and assay of catechol 2,3-dioxygenase(C230)

At the end of log phase of growth, 100ml of culture was pelleted in a refrigerated centrifuge (Sorvall RC 5C) at 15,000 rpm for 10 minutes. The pellets were washed twice with 0.85% KCl and centrifuged. Washed cells were then resuspended in 3ml of 50mM of phosphate buffer (pH 7.0) containing 10% acetone. The cells were lysed by sonication for 75 seconds (15 sec of sonication alternating with a 45 sec break) using a Vibra cell ultrasonic disintegrator. The cell lysates were centrifuged at 15000 rpm for 30min at 4°C in a Sorvall SS-34 fixed angle rotor. The supernatants were collected and

used for the assay of catechol 2,3-dioxygenase (C230) activity. The assay was carried out as per method in Chapter 2.

Oxygen uptake analysis

Exponentially growing cells of GU109 were harvested by centrifuging at 8000 rpm for 10 min at 4°C(Sorvall RC5C centrifuge). The pellets were washed in 50mM phosphate buffer (pH 7.0) and resuspended in the same buffer to give a final absorbance of 5.0 at 560nm. The cells were maintained at 4^0C at all times by immersing in an ice-bath. Rates of uptake by such resting cells of strain GU109 in the presence of various aromatic and other substrates were measured on a Gilson 5/6 oxygraph at 30°C with a Clarke electrode. Rates of oxygen uptake were corrected for endogenous respiration of cells and expressed as nmoles of oxygen/min/mg cells.

Fractionation of crude oil by column chromatography

After drying, the residual samples were fractionated into the aliphatic , aromatic and NSO or polar components by an adaptation of the method described by the Oil and Natural Commission (ONGC, Dehradun) using column chromatography.

A 45cm X 2.0cm glass column was packed with activated silica gel(60-120 mesh) and alumina in an equal ratio to a height of 15cms. The residual oil from the control and the test flask was adsorbed on 0.5 gms of activated silica gel and overlayered on the column. The saturate, aromatic and polar fractions were eluted sequentially with 80ml each of petroleum ether, benzene and methanol respectively.

Gas chromatographic analysis of crude oil fractions.

The crude oil saturate and aromatic fractions were monitored before and after degradation by injection into a Netel Omega QC Gas Chromatograph (Windows based software) fitted with a FID and stainless steel packed column (SE-30). The injector and detector were maintained at 300°C and the oven temperature was programmed to rise from 60°C to 300°C at the rate of 6°C/min and then held at 300°C for 15mins.

Gravimetric measurement of mineralization of crude oil.

After elution, the saturate, aromatic and NSO fractions, from both the control and the test flasks were evaporated at 60°C and the residues were transferred to pre-weighed vials and weighed on a Mettler balance. The difference in weight between the control and the test for each fraction was determined and expressed as % degradation.

RESULTS AND DISCUSSION

Crude oil contains numerous aromatic hydrocarbons and heterocyclic compounds which range in size from monocyclic to large fused ring structures. Thus when it enters the marine environment it provides a wide variety of substrates for the indigenous microbial populations. Inspite of this large degradative capacity, the isolation of hydrocarbon degraders particularly PAH degraders is rare not only because of dilution in the vast marine environment but also because of the fact that these pure PAH substrates are acutely toxic to bacteria on initial isolation (Sepic *et al.*, 1997). Phenanthrene, a representative of polycyclic aromatic hydrocarbons, was used in our study as a sole carbon and energy source.

Isolation and distribution of PAH degraders in the North Arabian Sea.

Hydrocarbons in the environment are biodegraded primarily by bacteria and fungi. The fraction of hydrocarbon utilizing bacteria and fungi range from 0.003% to 100% for marine bacteria (Mulkins-Phillips and Stewart,1974; Holloway et al.,1980).

In order to examine the extent of the marine degradative potential in the North Arabian Sea, a cruise aboard the scientific vessel *R.V. Gaveshni* (Cruise No. 243) was undertaken during the months of Febuary and March 1995. Surface waters were collected from 12 sites in the North Arabian Sea using a Niskin Sampler (Fig 4.2). Water samples collected were processed as per procedures given in **Materials and Methods** of this study. Table. 4.1 shows the physical parameters such as pH, salinity, depth and total viable count (TVC) of the surface water samples. The sites are characteristic in having constant physical parameters and a considerable presence of bacterial populations. Although none of the physical parameters alone limit the

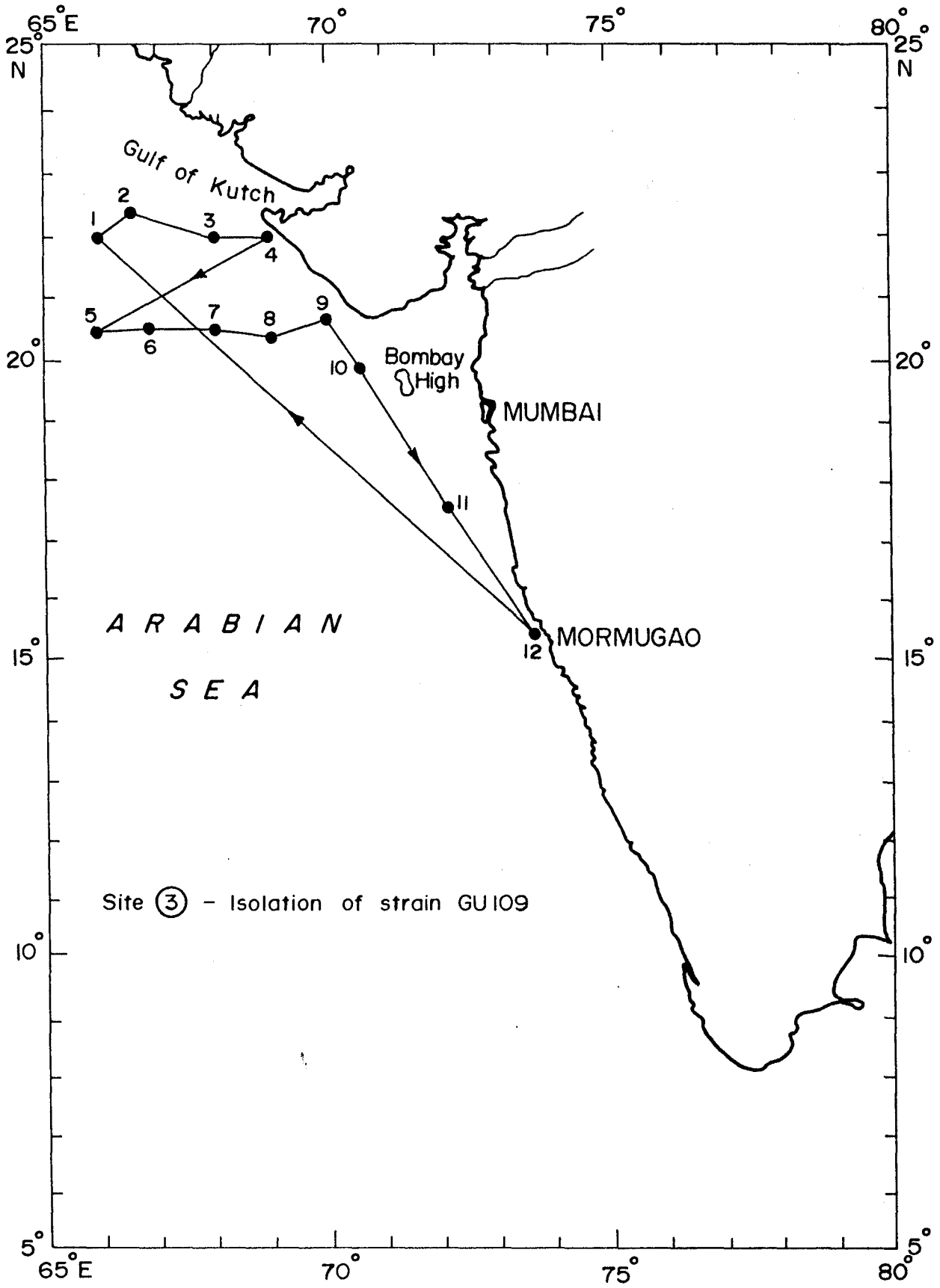


Fig. 4·2 Cruise track of R. V. Gaveshani (Cruise No.243) showing sampling sites.

Table 4.1 : Characteristics Of Sites Sampled Off the Gulf of the Kutch, North Arabian Sea.

St No	Location		Viable Count Surface waters	Depth of Sediment	Temp of Surface Waters	Avg pH	Avg Salinity(ppt)
	Lat N	Long E					
1	22°	66°	3.2×10^4	2540mts	25.00°C	8.0	36.40
2	22° 0'28"	66°59'33"	1.3×10^3	1690mts	25.44°C	8.8	36.40
3	21°59'8"	68° 0'8"	8.1×10^3	120mts	25.19°C	8.7	36.25
4	21°59'8"	69° 0'4"	1.54×10^3	42mts	24.20°C	8.8	36.18
5	20°18'38"	66°08'26"	ND	2900mts	25.10°C	8.7	36.46
6	20°25'12"	66°58'31"	6.80×10^2	2600mts	25.82°C	8.7	36.45
7	20°21'43"	68°03'49"	1.46×10^5	2970mts	26.21°C	8.7	36.13
8	20°20'5"	69°06'3"	8.60×10^4	1324mts	26.00°C	8.8	36.11
9	20°29'9"	70°03'2"	8.70×10^5	75mts	25.32°C	8.7	35.88
10	19°52'38"	70°36'32"	1.15×10^5	83mts	26.30°C	8.8	35.90
11	17°31'28"	72°16'15"	ND	93mts	27.40°C	8.7	35.86
12	15°24'19"	73°41'19"	ND	25mts	26.10°C	8.8	35.45

distribution of bacterial populations in the North Arabian Sea, the presence of benzoate degraders in all sites except site number 3, indicates a constant activity to mineralize aromatic compounds (Table. 4.2). The incubation of the filtered water sample resulted in an enrichment of a consortium of degradative heterotrophs, the number of members of benzoate degraders in the consortium being a maximum of three. All the cultures isolated were typically gram negative with some of them showing the presence of *meta*-cleavage pathway for aromatic hydrocarbon degradation indicated by the accumulation of a yellow colored hydroxymuconic semialdehyde when sprayed with catechol (Table. 4.2). The presence of *meta*-cleavage pathway in turn indicates that the heterotrophs are engaged in the mineralization of substituted aromatic compounds including PAH's. The extent of the degradative potential on substituted aromatics is depicted in Table. 4.2.

The data suggests that the whole of the North Arabian Sea is constantly exposed to various aromatic compounds resulting in isolation of PAH degraders in almost all the sites (Fernandes *et al.*, 1994). This could be due to the close proximity of the sampled sites to the oil tanker routes used for the transportation of oil (Sengupta and Kurieshy,1981). The presence of PAH degraders at sites 10,11 and 12 indicates the extent of oil pollution as a fallout of shipping activity due to the close proximity of the sites to Mormugoa Harbour, a major port in India (Fig. 4.2).

Thus in conclusion, the study demonstrated that

a) The North Arabian Sea is rich in microorganisms that degrade complex polycyclic aromatic hydrocarbons, and
b) The technique of using very simple aromatic compounds like benzoate for initial enrichment has proved to be useful in isolating microorganisms bearing exceptional traits of mineralization of complex molecules like PAH's and substituted aromatic

Table 4.2 :Enrichment analysis of marine surface water samples on benzoate and growth on PAH's and substituted aromatic hydrocarbons.

St No	No of Strains degrading benzoate	Prominent mode of ring cleavage	PAH degraders	Substituted Aromatic degraders
1	3	Meta	Naph	Xylene/Tol
2	None	None	None	None
3	2	Meta	Naph/Phe	Xylene/Tol
4	1	Meta	None	Xylene/Tol
5	2	Ortho & Meta	Naph/Phe	None
6	1	Ortho & Meta	Naph/Phe	Xylene/Tol
7	2	Ortho & Meta	Phe	None
8	3	Ortho & Meta	Phe	Tol acid
9	2	Meta	Phe	Xylene/Tol
10	1	Ortho & Meta	Naph/Phe	Tol
11	2	Ortho & Meta	Phe	None
12	2	Ortho & Meta	Naph/Phe	Xylene/Tol

Naph – Naphthalene

Phe - Phenanthrene

Tol - Toluic acid

compounds in the deep-sea.

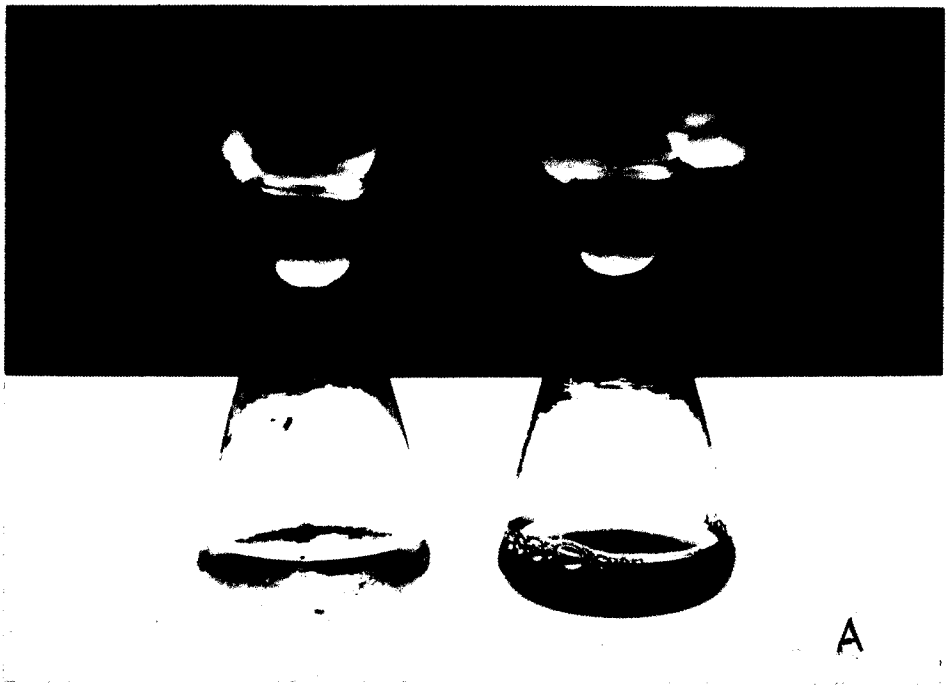
Isolation and characterization of a marine bacterium utilizing phenanthrene

Enrichment of marine bacteria from seawater samples using the benzoate enrichment technique resulted in the isolation of a number of PAH degraders. An enriched sample from site 3 was incubated in MSP containing 0.08%(w/v) phenanthrene. The subsequent incubation resulted in the isolation of a single strain degrading phenanthrene which produces a brown/buff coloration in the medium. In the MSP medium with phenanthrene as a sole carbon source, the entire medium turned light brown with no increase in turbidity. The color of the medium slowly darkened with a corresponding increase in turbidity (Fig.4.3A).

On a medium incorporated with carbon sources other than phenanthrene, this strain showed characteristic exopigment formation. On Kings medium B, the strain produced a yellow visible pigment which fluoresced green under ultraviolet light. The strain also produced a yellowish green pigment in liquid medium containing sodium benzoate (0.08%v/v) and a greenish blue pigment on sodium lactate (0.2%v/v) as a sole carbon and energy source.

On phenanthrene containing MSP-agar plate, the colonies appeared pin point whereas on marine nutrient agar, the colonies were oval with a convex elevation and cream in color. The strain was a gram negative rod (Fig. 4.3B), motile and aerobic and we temporarily termed it as GU109 till further identification. A series of tests were conducted in accordance with the Bergeys Manual and the strain showed a similarity with the *Pseudomonas sp.* (Table 4.3).

The results were similar with those carried out at the Institute of Microbial Technology,



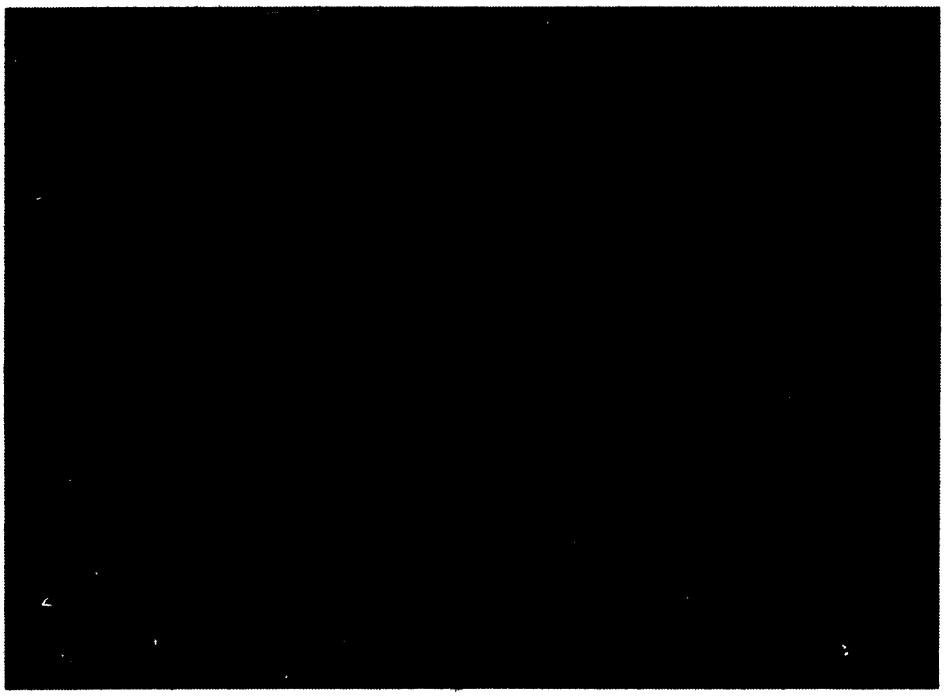


Fig. 4.3A. Photographs showing brown coloration of the medium after growt
strain GU109 and B) photomicrograph showing gram negative character of s
GU109.

TABLE NO. 4.3 : Morphological, physiological and biochemical tests performed on the marine bacterial isolate, strain GU109.

Tests	Results
Colony Morphology	
Margin	Entire
Elevation	Convex
Color	Cream
Size	1-2mm
Consistency	Butyrous
Shape	Oval
Grams Reaction	Gram negative
Motility	Motile
Biochemical Tests	
Indole production	-
Methyl red test	-
Voges Proskauer test	-
Citrate utilization	+
Urea hydrolysis	-
Casein hydrolysis	-
Starch hydrolysis	+
Tween 80 hydrolysis	+
Catalase test	+
Gelatin liquefaction	-
Arginine dihydrolase	+
Oxidase test	-
Acid Production from carbohydrates	
Glucose	+
Lactose	+
Maltose	+
Mannitol	+
Sucrose	-
Frcutose	+
Sorbitol	+
Antibiotic Sensitivity	
Streptomycin (25µg/disc)	Sensitive
Chloramphenicol (10µg/disc)	Sensitive
Tetracyline (10µg/disc)	Resistant
Penicillin (10µg/disc)	Resistant
Kanamycin (30µg/disc)	Sensitive

Chandigarh (Table 4.4). The strain GU109 was designated as *P. putida* and given the deposition number MTCC 3316.

Growth characteristics of *P. putida* strain GU109 (MTCC 3316)

A great number of marine bacteria are capable of a wide variety of metabolic reactions and can consequently utilize a wide variety of nutrients (Rheinheumer,1976). MacLeod (1965) believes that the ability to live in the sea is the only characteristic which clearly distinguishes marine microorganisms from other bacteria and it also assures that a few mutational changes are needed to change a marine form into one which will survive in a non-marine environment.

Our results on the salt requirement for *P. putida* strain GU109 (MTCC 3316) indicate that the strain grows optimally on peptone medium with 3% sodium chloride (NaCl). On MSP with varying concentrations of sodium chloride (NaCl) and phenanthrene as a sole source of carbon and energy, strain GU109 showed optimal mineralization of phenanthrene at 3% NaCl concentration (Fig. 4.4). Growth gradually decreased with increasing salt concentrations. However, slow mineralization of phenanthrene by strain GU109 did occur in MSP medium without salt indicating the adaptability of strain GU109. Thus although strain GU109 was isolated from the marine habitat, it could still grow in a medium lacking sodium chloride.

Growth of *P. putida* strain GU109 (MTCC 3316) on 0.08%(w/v) phenanthrene resulted in an initial brown coloration of the medium with no increase in turbidity. The turbidity subsequently increased with an increase in bacterial counts (Fig. 4.5). The increase in brown coloration continued to increase as indicated by an increase in optical density with no increase in bacterial counts. It is not clear why phenanthrene

TABLE. 4.4. MORPHOLOGICAL, PHYSIOLOGICAL AND BIOCHEMICAL TESTS PERFORMED ON THE MARINE BACTERIAL ISOLATE, STRAIN GU109 AT IMTECH, CHANDIGARH

MORPHOLOGICAL TESTS

TESTS	RESULTS
Grams Reaction	Negative
Shape	SR
Motility	+
Fluorescence(UV)	Negative

PHYSIOLOGICAL TESTS

GROWTH AT TEMPERATURES	**RESULT**
4°C	--
10°C	ND
15°C	+
22°C	+
26°C	+
30°C	+
37°C	Weak
42°C	--
55°C	--
65°C	--
GROWTH AT pH	
pH 5.0	+
pH 5.7	+
pH 6.8	+
pH 8.0	+
pH 9.0	+
pH 11.0	+
GROWTH ON NaCl (%)	
2.5	+
5.0	+
7.0	_
8.5	_
9.0	_
10.0	_
GROWTH UNDER ANAEROBIC CONDITION	_

TABLE NO. 4.4.(contd) Biochemical tests

Tests	Result
Growth on MacConkey agar: a) Lac fermentor	+
Indole Test	–
Methyl Red Test	–
Voges Proskauer Test	–
Citrate Utilization	+
Casein Hydrolysis	–
Starch Hydrolysis	–
Urea Hydrolysis	+
Nitrate Reduction	–
Nitrite Reduction	–
H_2S Production	+
Cytochrome Oxidase Test	+
Catalase Test	+
Oxidation/Fermentation(O/F)	–
Gelatin Liquefaction	–

Acid Production From Carbohydrates	Result
Adonitol	+
Arabinose	+
Cellobiose	+
Dextrose	+
Dulcitol	+
Fructose	+
Galactose	+
Inositol	+
Inulin	+
Lactose	–
Maltose	+
Mannitol	+
Mannose	+
Melobiose	–
Raffinose	–
Rhamnose	+
Salicin	–
Sorbitol	+
Sucrose	+
Trehalose	+
Xylose	+

(ND = not detected)

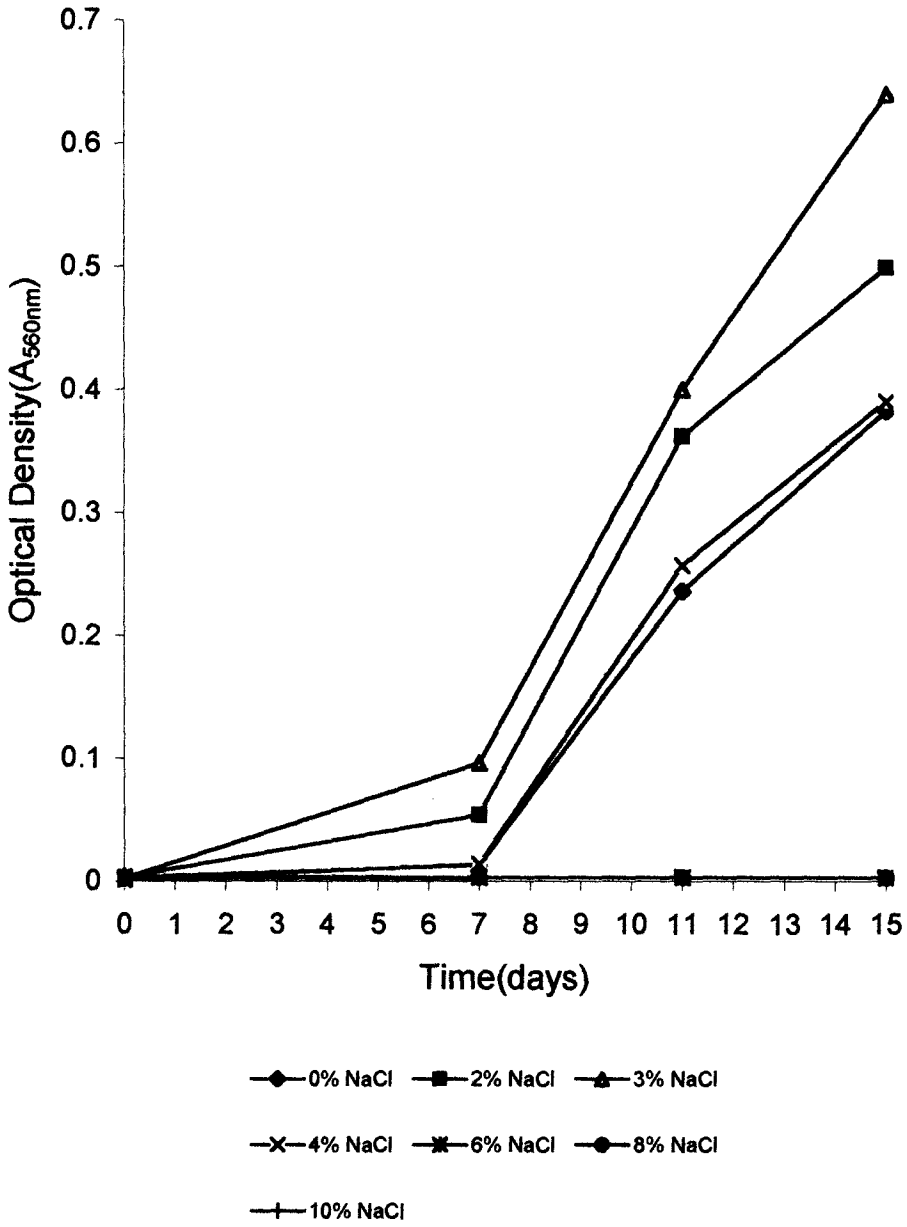


Fig. 4.4. Mineralization of phenanthrene by strain GU109 in different concentrations of sodium chloride.

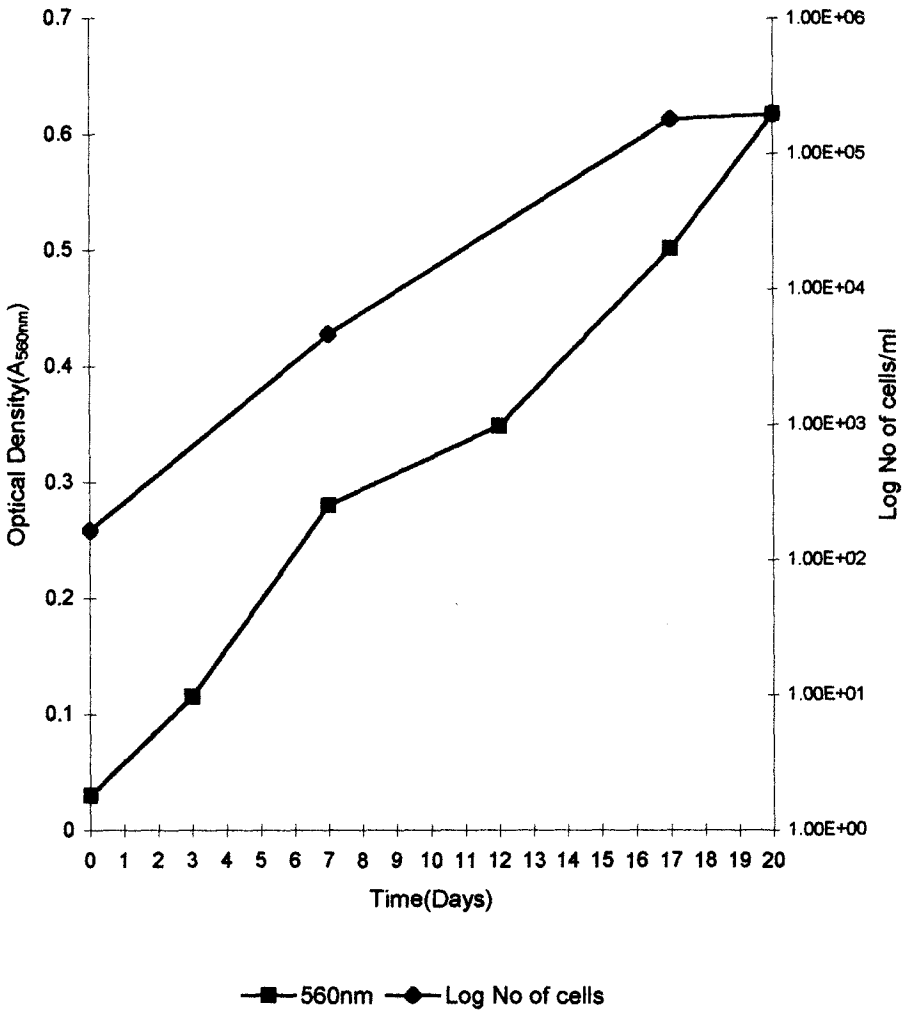


Fig. 4.5. Growth of strain GU109 in MSP supplemented with phenanthrene.

degradation excretes a large amount of intermediates. One possibility is that some intermediates in the phenanthrene and naphthalene degradative pathway are chemically unstable and dead-end products are formed spontaneously from the intermediates and secreted into the medium. Another possibility is that PAH degrading pathways are evolutionary primitive, and unbalanced activities of the pathway enzymes could provoke the accumulation of some pathway intermediates (Iwabuchi *et al.*,1994).

A dry wt of 0.1mg, 0.3mg and 0.5mg corresponded to an optical density of 0.1, 0.5 and 1 at 560nm, indicative of a low growth yield for strain GU109. Gas chromatographic profiles of growth flasks of strain GU109 on 0.08%(w/v) phenanthrene extracted with ethyl acetate indicated a low mineralization of phenanthrene (Fig. 4.6) probably due to the substrate being in excess. The ethyl acetate extracts also showed the appearance of a peak at retention time 20.19min which corresponds to that of carboxybenzaldehyde in the medium after 4-5days of growth (Fig. 4.6). The presence of carboxybenzaldehyde in the medium is indicative of the fact that strain GU109 degrades phenanthrene via the phthalate pathway. Thus *P. putida* strain GU109 (MTCC3316) appears to be attacking the crystal thereby increasing its solubility in the medium. This results in phenanthrene being made more available to strain GU109 which subsquently results in an increase in growth and turbidity. The adherence of strain GU109 to phenanthrene crystals was observed microscopically (Fig 4.7).

Screening for the degradative ability of *P. putida* strain GU109 (MTCC 3316)

Crude oil contains numerous aromatic hydrocarbons and heterocyclic compounds which range in size from monocyclic to large fused ring structures. Thus when oil is

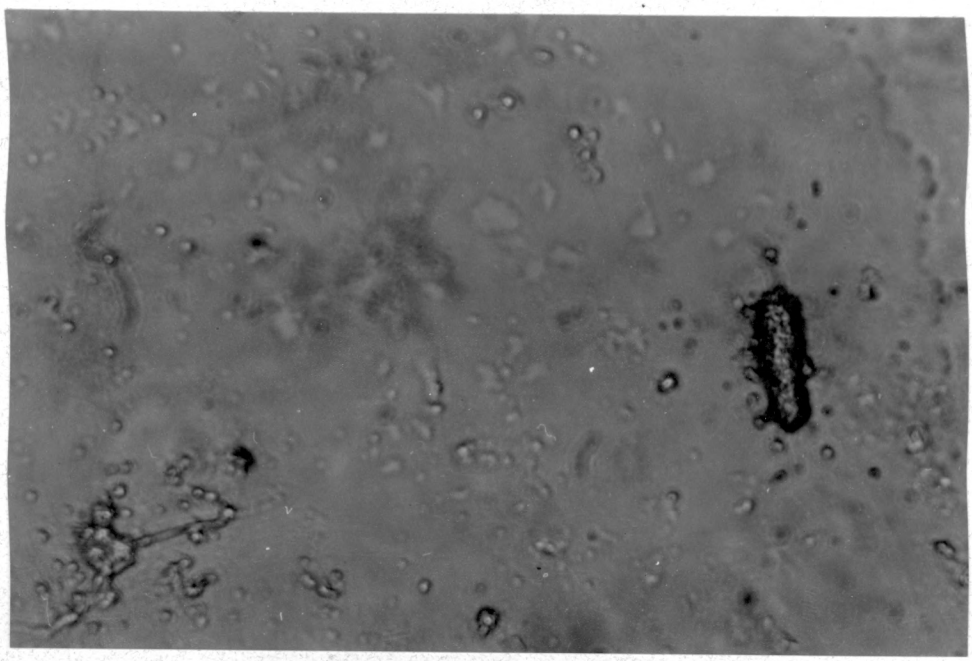


Fig. 4.7. Photomicrograph showing adherence of strain GU109 to phenanthrene crystals.

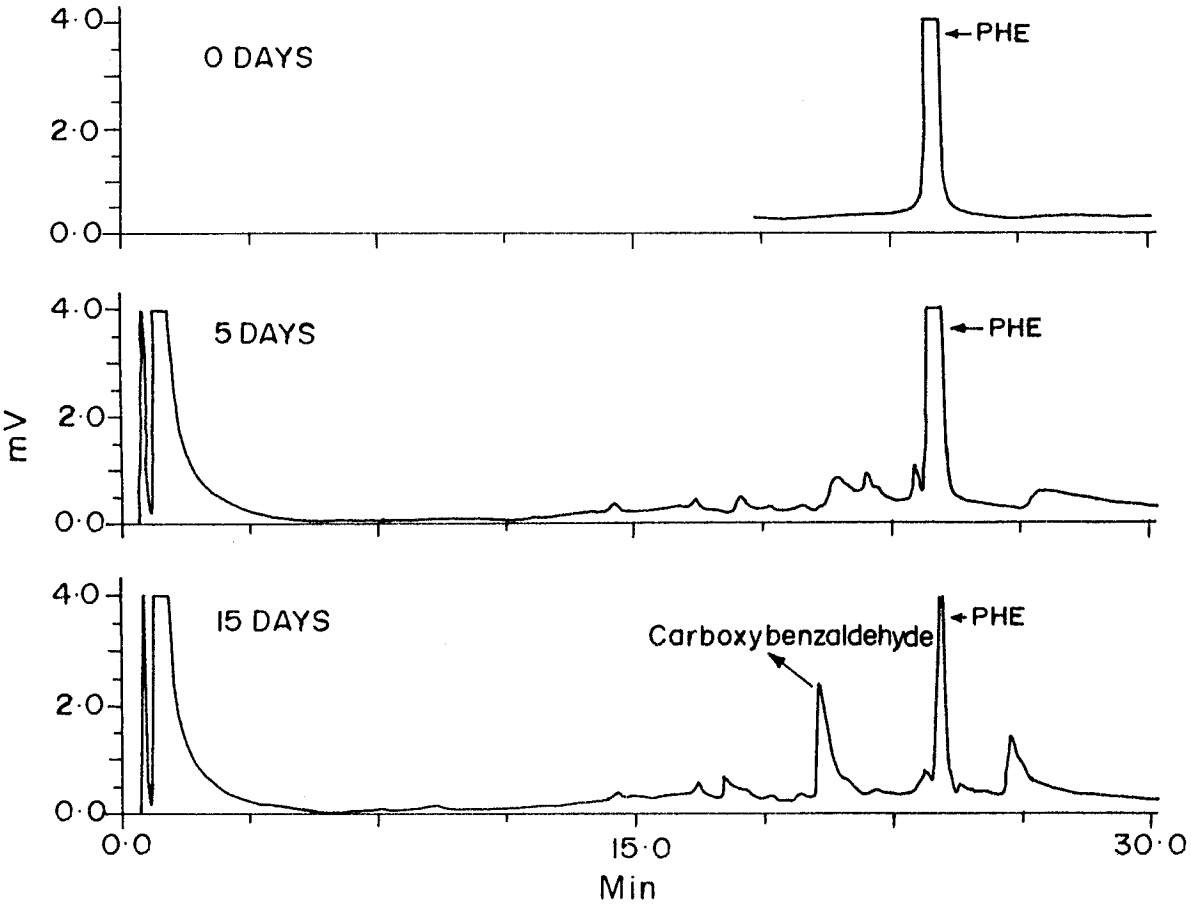


Fig. 4·6. Gas Chromatographic profiles of ethyl acetate extracts of flasks showing the depletion of phenanthrene and the appearance of carboxybenzaldehyde in the medium.

spilled in the environment, low molecular weight compounds may be lost by volatilization, however the large PAH's and heterocycles remain, and their ultimate environmental fate may be determined by microbial degradation.

Substrates utilized by *P. putida* strain GU109 (MTCC 3316)

Our studies on the degradative ability of *P. putida* strain GU109 (MTCC 3316) indicate a very broad degradative ability (Table. 4.5). Growth of *P. putida*, strain GU109 on benzoate resulted in the formation of a yellow coloration whereas growth on lactate resulted in the production of a green quinone like pigment which lost color on reduction with potasium metabisulphite. On napthalene as a sole carbon source, strain GU109 produced an orange quinone-like pigment (λ_{max} – 437nm) which is again oxygen dependent with no appreciable increase in the optical density. This characteristic of pigment production is prominent amongst marine bacteria which are often characterized on this basis.

Growth on carboxybenzaldhyde, 1-hydroxy-2-naphthoic acid, phthalic acid and protocatechuic acid gave us the first positive indication that *P. putida* strain GU109 (MTCC3316) utilizes the alternate pathway or phthalate for phenanthrene mineralization (Kiyohara and Nagao, 1978). Strain GU109 however does not grow on NSO compounds like DBT, quinoline and pyridine.

When grown on binary mixtures, *P. putida* GU109 (MTCC 3316) showed positive mineralization on Phe and DBT, Phe and Nah and Phe and Flo (Fig. 4.8). Low turbidities for strain GU109 were however obtained for Phe and Nah and Phe and Flo growth flasks. Both the flasks showed accumulation of quinone like compounds (orange in Phe and Nah and yellow in Phe and Flo). Stringfellow and Aitken (1995) also

Table. 4.5. Substrate utilization by strain GU109.

SUBSTRATE	GROWTH AS TURBIDITY
Carboxybenzaldehyde	Faint turbidity
Salicyclic Acid	Negative
Salicylaldehyde	Negative
Catechol	Faint Turbidity
O-Phthalic Acid	Positive
1-Hydroxy-2-Napthoic Acid	Positive
Protocatechuic Acid	Positive
Sodium Benzoate	Positive(Yellow Coloration)
Sodium Lactate	Positive(Green Coloration)
Pyridine	Negative
Quinoline	Negative
Xylene	Negative
M-Toluic Acid	Positive
Dibenzothiophene	Negative
Crude Oil (BHCO)	Positive
Napthalene	Positive(Orange coloration)
Anthracene	Negative
Fluorene	Negative

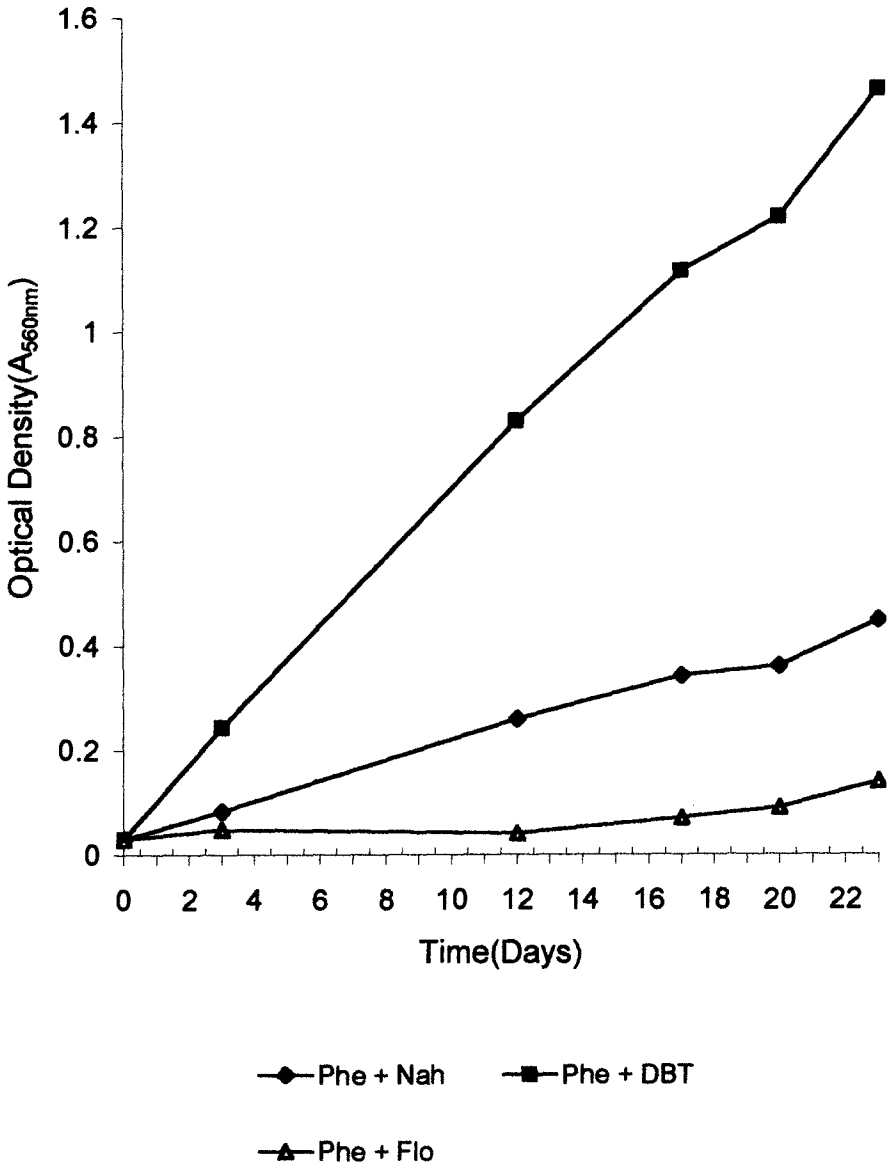


Fig. 4.8. Growth of strain GU109 on binary mixtures.

observed the formation of a yellow metabolite as a consequence of fluorene degradation. HPLC and GC-MS analysis established it as 1-fluorenone.

What is surprising though is the ability of *P. putida* strain GU109 (MTCC 3316) to mineralize DBT and fluorene only in the presence of phenanthrene. Growth on Phe and Flo however, results in the inhibition of degradation of phenathrene by strain GU109. Growth on Phe and DBT showed an increase in optical density and formation of red colored metabolites similar to an *Alcaligenes sp.* strain GU110 as reported by Rodrigues, J (personal communication). In hydrocarbon degradation, two processes need to be considered, co-oxidation and sparing. Both these processes can occur within the context of a petroleum spillage. *P. putida* strain GU109 (MTCC 3316) may be showing a phenomenon of co-oxidation where compounds which are not otherwise degraded can be enzymaticaly attacked within a hydrocarbon mixture due to the ability of the individual hydrocarbon to grow on other hydrocarbons within a hydrocarbon mixture. Gas chromatographic profiles of cell free supernatants of strain GU109 on binary mixtures revealed a common profile, suggestive of interlinks in the pathway for degradation (Fig. 4.9).

Determination of catechol 2,3-dioxygenase (C23O) activity from *P. putida* strain GU109 (MTCC 3316)

Meta-cleavage dioxygenases that cleave aromatic ring structures are key enzymes in the catabolism of aromatic compounds. This is particularly true of substituted monoaromatic compounds such as alkyl benzenes and PAH's, that cannot be degraded by conventional *ortho*-cleavage pathways that normally require formation and isomerization of a lactone intermediate (Gibson and Subramanium, 1984). Major

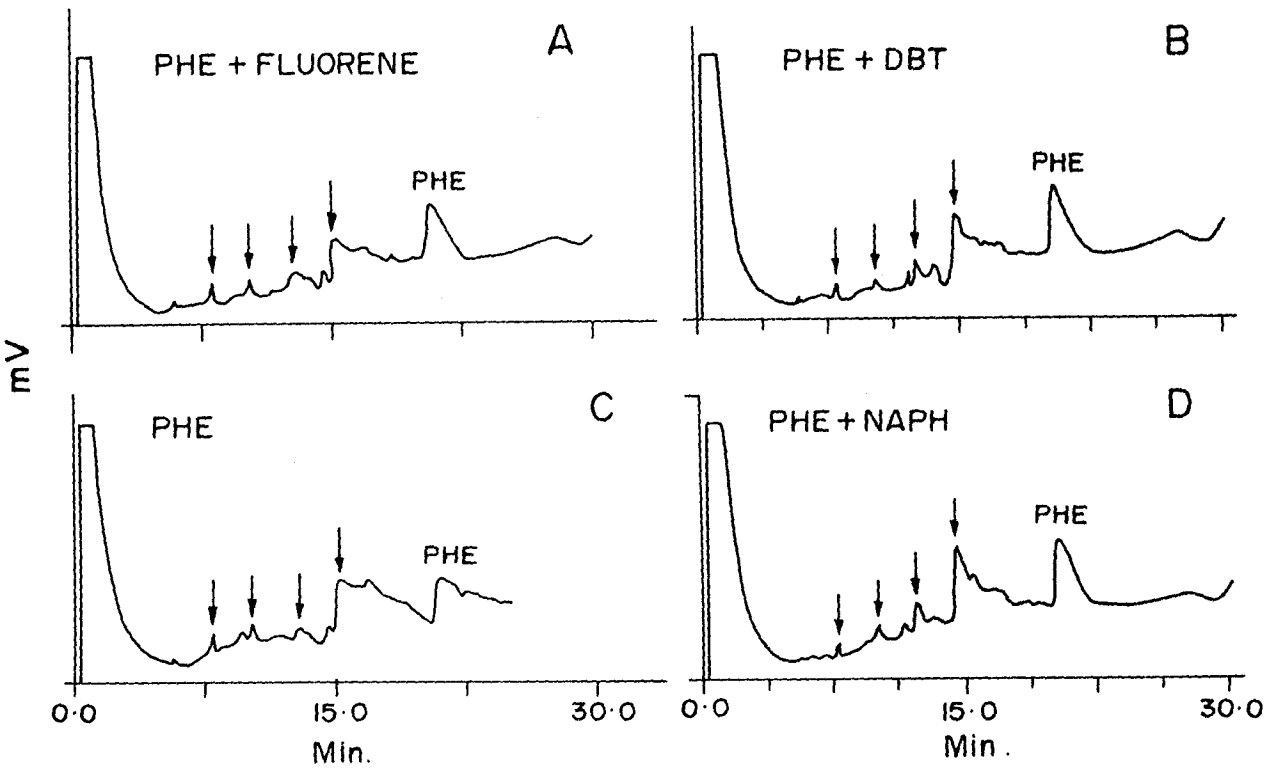


Fig. 4·9. Gas Chromatographic profiles of ethyl acetate extracts of cell free supernatants of strain GU 109 grown on A) Phenanthrene and Fluorene B) Phenanthrene and DBT C) Phenanthrene and D) Phenanthrene and Napthalene for 12 days.

Note: The similarity of peaks at retention times is depicted by arrows.

oxidative pathways have shown that most aromatic compounds are degraded to catecholic intermediates and they are metabolized further by two possible routes 1) a meta-(extradiol-) cleavage(EDO) pathway or 2) an ortho-(intradiol-) cleavage(IDO). The best characterized EDO is the enzyme catechol 2,3-dioxygenase (C230). On account of the key position of the C230 enzyme in PAH degradation, the enzyme was assayed form *P. putida* strain GU109 (MTCC 3316).

The Rothera's test (Stainier *et al.*, 1966) a preliminary screening for *meta* or *ortho* cleavage of catechol revealed the development of a pale yellow colour, which is indicative of *meta*-cleavage. *ortho*-cleavage was not detected. The extraction and assay of C230 enzyme from GU109 cells grown on different substrates revealed a similar level of C230 activity for all the substrates tested (Table 4.6). The results indicate that *meta*-cleavage of catechol was the most common mode of degradation for the lower pathway of PAH degradation in *P. putida* strain GU109 (MTCC 3316). The presence of C230 enzyme is a very common feature in P*seuodmonas sp.* and *sphingomonas sp.* which utilize PAH's as sole carbon and energy source (Meyer *et al.*, 1999).

Oxygen uptake by *P. putida* strain GU109 (MTCC 3316) on different aromatic substrates

Two metabolic pathways have been described for the metabolism of phenanthrene (Evans *et al.*, 1965; Kiyohara and Nagao, 1978). Both pathways involve the formation of 1-hydroxy-2-naphthoic acid which is then channeled via the phthalate or naphthalene pathway. The measurement of oxygen uptake by strain GU109 showed oxygen uptake with intermediates of both the phthalate and naphthalene pathway (Table. 4.7). On

Table. 4.6 : Catechol 2,3-dioxygenase activity of strain GU109.

Growth substrates	Activity enzyme units (IU/ml)	Specific activity (Units/mg protein)
Phenanthrene (0.08%)	0.119	1.19
Benzoate (0.08%)	0.145	1.115

Table No. 4.7 : Oxygen uptake rates of strain GU109 cells grown on lactate, benzoate and phenanthrene.

	Growth substrates(Oxygen uptake in nanomoles/min/mg dry weight)		
Substrates oxidized	Lactate	Benzoate	Phenanthrene
1-hydroxy-2-napthoic acid	46.96	104.28	16.456
Phenanthrene	44.54	66.31	11.481
Naphthalene	0	107.74	86.866
2-Carboxybenzaldehyde	38.52	0	13.715
O-phthalic acid	0	18.831	3.944
Protocatechuic acid	35.71	18.781	49.875
Salicyclic acid	ND	13.01	19.451
Salicylaldehyde	0.00319	ND	ND

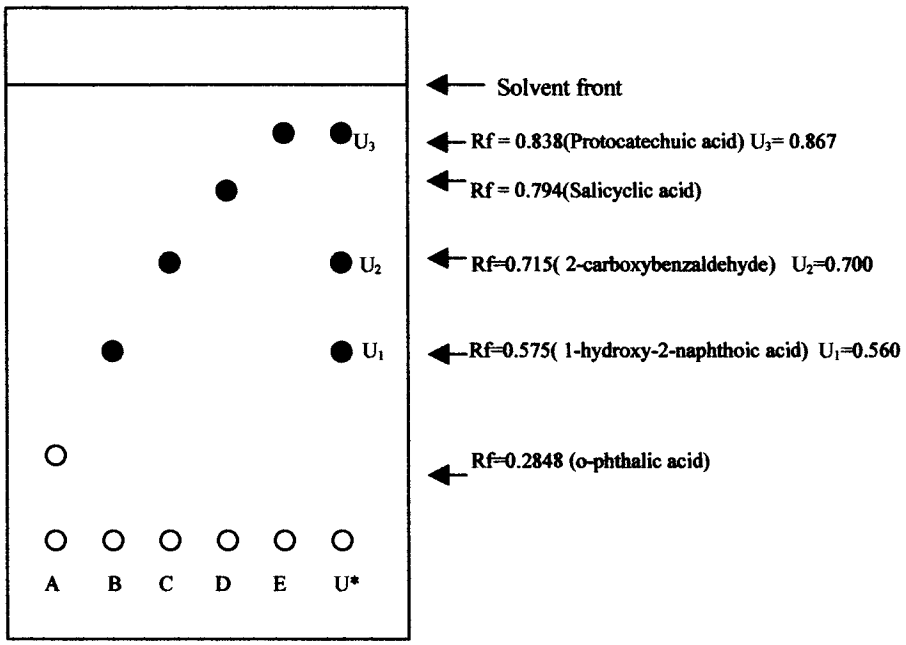
ND – Not detected

benzoate, strain GU109 showed a much higher oxygen uptake as compared to phenanthrene as substrate. However, on lactate, no oxygen uptake on naphthalene and its intermediates was detected. The data suggests that the phthalate pathway seems to be preferred mode of phenanthrene degradation by strain GU109. Similar results were reported by Kiyohara and Nagao(1978) on thirteen strains of bacteria. They also observed that in all $phe^{+}nah^{+}$ strains, the pathways for the degradation of phenanthrene and naphthalene seem to be independent for each other. Strain GU109 seems to have a similar mode of degradation.

Analysis of metabolites of phenanthrene degradation produced by *P. putida* strain GU109 (MTCC 3316)

Two routes of degradation of phenanthrene by bacteria have been reported (Kiyohara and Nagao, 1978) . With both routes, phenanthrene is initially oxidized to 1-hydroxy-2-naphthoic acid , which is subsequently channeled into one or two branches, the naphthalene pathway or the phthalate pathway (Fig. 1.10). To identify the route for phenanthrene degradation by strain GU109, metabolites of 1-hydroxy-2-naphthoic acid produced by intact cells of *P. putida*, strain GU109 (MTCC 3316) were identified by thin-layer chromatography (TLC).

Three spots corresponding to 1-hydroxy-2-naphthoic acid, 2-carboxybenzaldehyde and protocatechuic acid were detected after 3 min (Fig. 4.10). No spot corresponding to salicyclic acid or salicylaldehyde was observed. The results suggest that *P. putida* strain GU109 (MTCC 3316) degraded phenanthrene via the o-phthalate pathway (Iwabuchi *et al.*, 1998). Presence of carboxybenzaldehyde in the GC extracts seem to confirm this observation (Fig.4.6).



U* - 1-hydroxy-2-naphthoic acid incubated with GU109 cells for 5 minutes.

Solvent system : Ethyl Acetate – 2-propanol (2 : 1)		
Rf	Metabolite	Name
0.560	U_1	1-hydroxy-2-naphthoic acid
0.700	U_2	2-carboxybenzaldehyde
0.867	U_3	Protocatechuic acid

Fig. 4.10 : Thin layer chromatography of degradative products of phenanthrene by strain GU 109.

Degradation of a model hydrocarbon mixture and crude oil by *P. putida* strain GU109 (MTCC 3316)

Recent oil spills due to tanker accidents and similar occurrences all over the world have drawn attention to the problem of hydrocarbon contamination in the environment. Biodegradation of hydrocarbons by bacterial consortia is one of the primary mechanisms by which one can clean up the hydrocarbon pollutants from the environment. Studies concerning the relationship between chemical composition and biodegradation of crude oil have shown that oil containing a higher concentration of alkanes was more susceptible to microbial attack (Westlake *et al.*, 1974b). On the other hand, polycyclic aromatic hydrocarbon (PAH) components of crude oil are considered most toxic and resistant to biodegradation. Thus in order to evaluate the potential of strain GU109 in oil degradation, an artificial model hydrocarbon mixture was constituted. The model hydrocarbon mixture used was designed to represent the major classes of hydrocarbons in petroleum.

The use of model hydrocarbon mixtures to estimate the microbial potential of degrading hydrocarbons by GC offers a reasonable, quick and reliable alternative to methods like column chromatography followed by GC and mass spectroscopy (Walker and Colwell, 1974 b).

The results of our study on model hydrocarbon mixtures indicate that *P. putida*, GU109 (MTCC 3316) was able to partially degrade all the components within the model hydrocarbon mixture (Fig. 4.11 and 4.12). Although the phenanthrene peak appears to be the same, there is almost a 10% decrease in its peak area. The study once again demonstrates the co-oxidation effect whereby the presence of phenanthrene helps in degrading other components which would not otherwise be degraded individually.

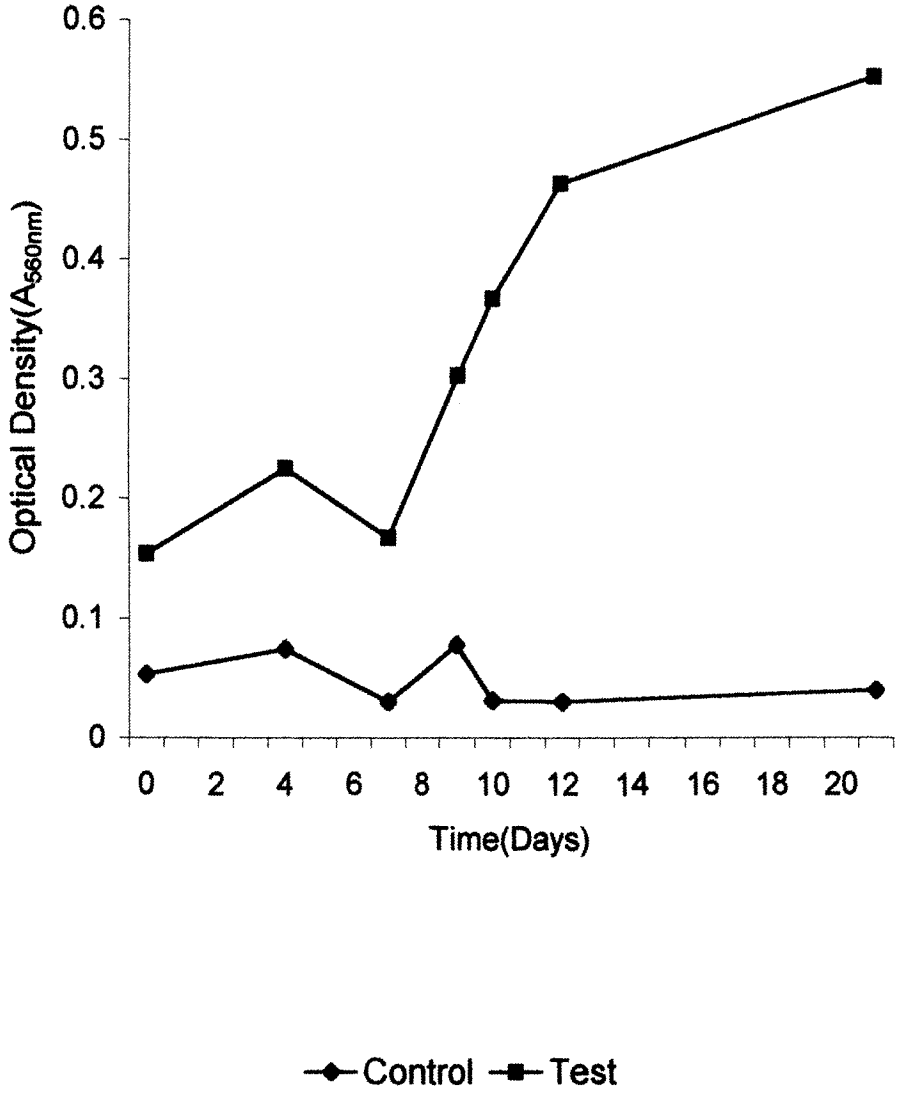


Fig. 4.11. Growth of strain GU109 on a model hydrocarbon mixture.Control flask consisted of substrates and was devoid of inoculum.

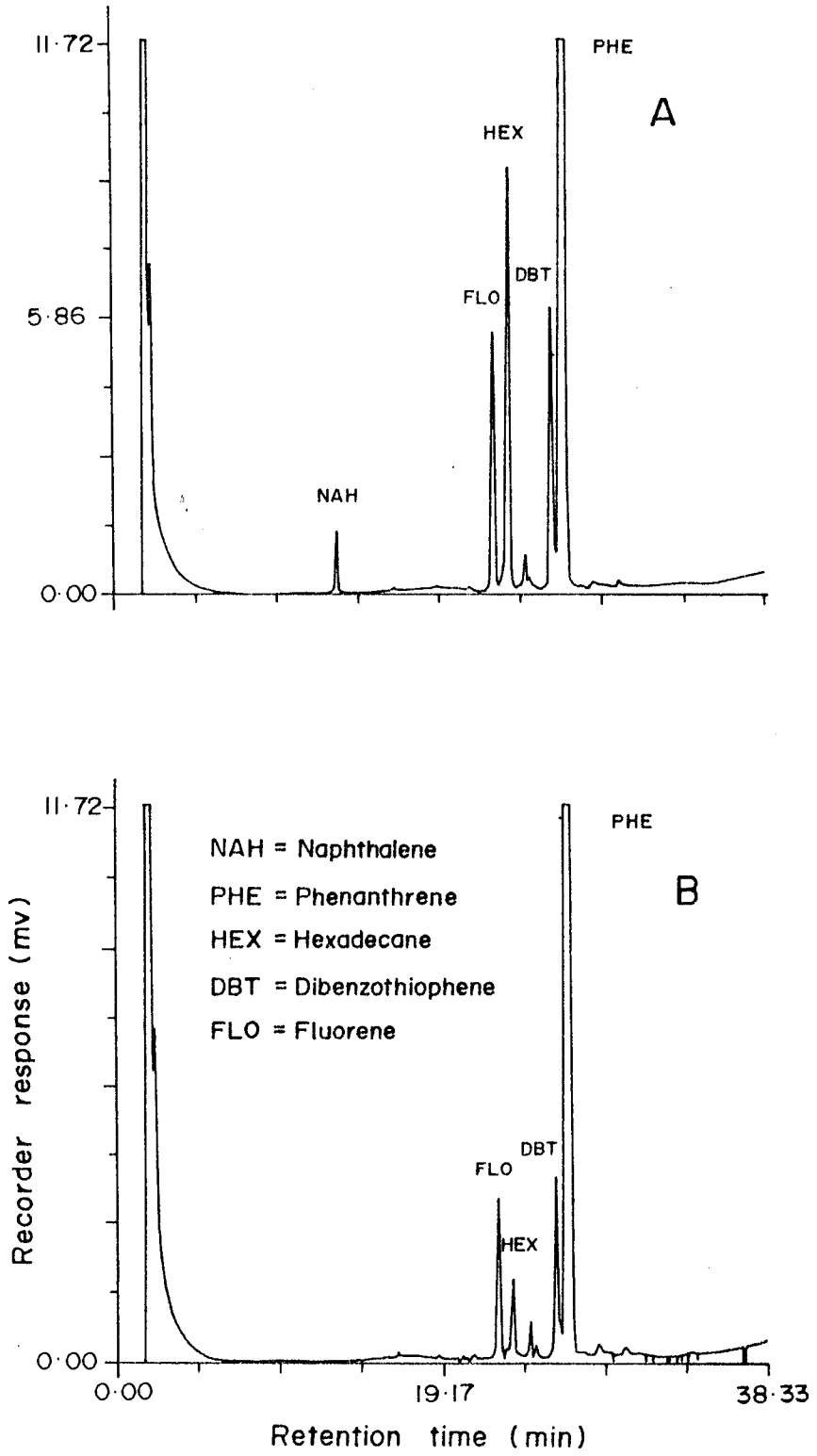


Fig. 4·12 Gas chromatographic profiles of a model hydrocarbon mixture A) Un-inoculated sterile control B) Test: after growth of strain GU109 (15 days)

Such representative hydrocarbons in model petroleum serve to permit comparison with "natural" petroleum. Furthermore , model hydrocarbon mixtures are ideal substrates for studies involving questions of sequential or preferential utilization. Both these phenomena; co-oxidation and preferential degradation seem to be observed with strain GU109. It appears to require phenanthrene to grow on other substrates yet attacks it slowly when present in a mixture as model petroleum.

P. putida, strain GU109 (1%v/v) culture from maintenance flasks was inoculated in 50mls of MSP with BHCO (0.1%v/v) as a sole carbon source and also with crude oil supplemented with phenanthrene (0.08% w/v) in 500ml conical flasks and incubated at room temperature on a shaker at 100 rpm/min. Appropriate uninoculated controls were also kept. Growth was monitored at 560nm. The entire flask contents after growth of GU109 on crude oil , were extracted with chloroform, and dried over anhydrous sodium sulfate and the solvent removed by evaporation at 60°C.

In crude oil and crude oil supplemented with phenanthrene, growth of *P. putida* GU109 (MTCC3316) was observed as an increase in turbidity (Fig. 4.13 & 4.14). Visual examination of both the flasks, revealed a common phenomenon, whereby after growth, the physical nature of the oil changes, where in the oil tends to get dispersed as fine fibre like threads, followed by an increase in turbidity of the aqueous medium. In case of crude oil supplemented with phenanthrene, turbidity was accompanied by the formation of brown colored intermediates indicative of the fact that *P. putida* was able to degrade both phenanthrene and crude oil (Fig. 4.15).

P. putida, strain GU109 was capable of degrading both the alkane and aromatic fractions of Bombay High crude oil (BHCO) with preferential and considerable degradation of the alkane fraction as compared to the aromatic

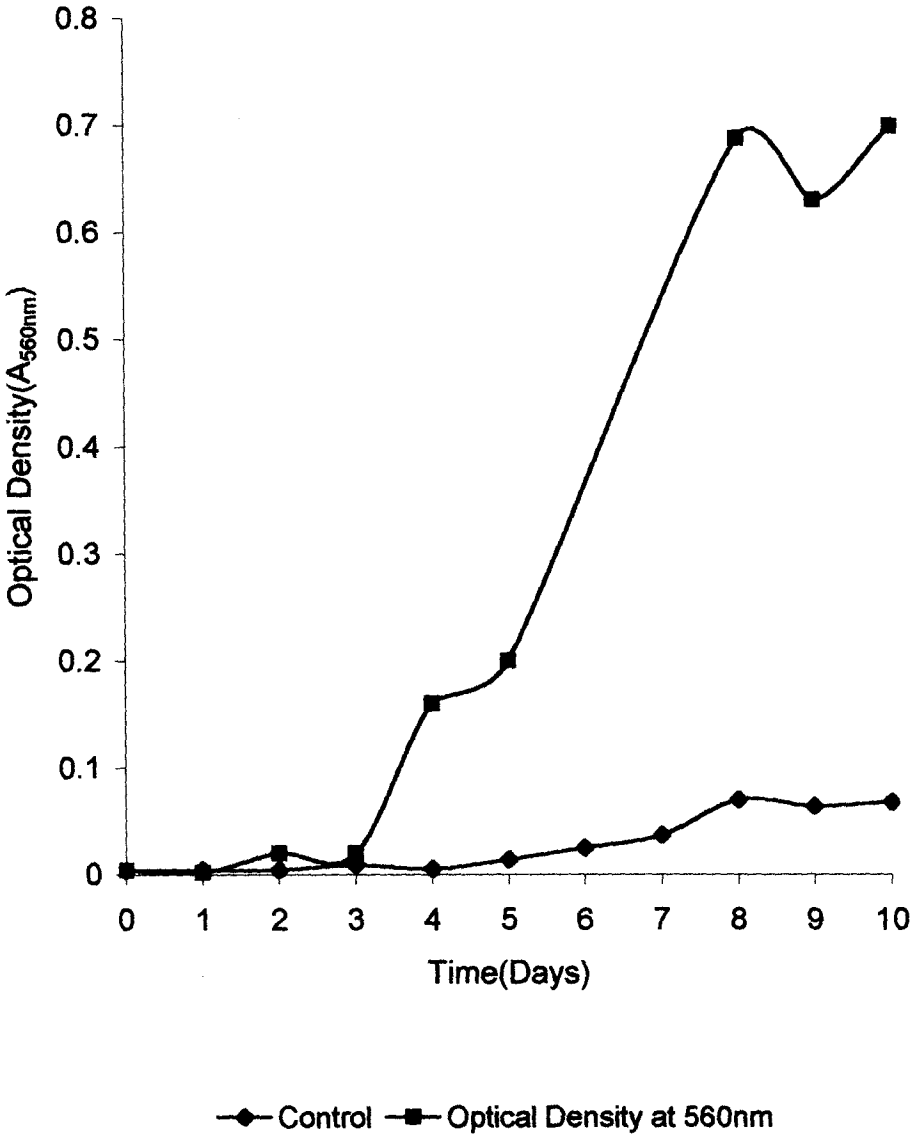


Fig.4.13. Growth of strain GU109 on crude oil. Control flask consisted of substrates and was devoid of inoculum.

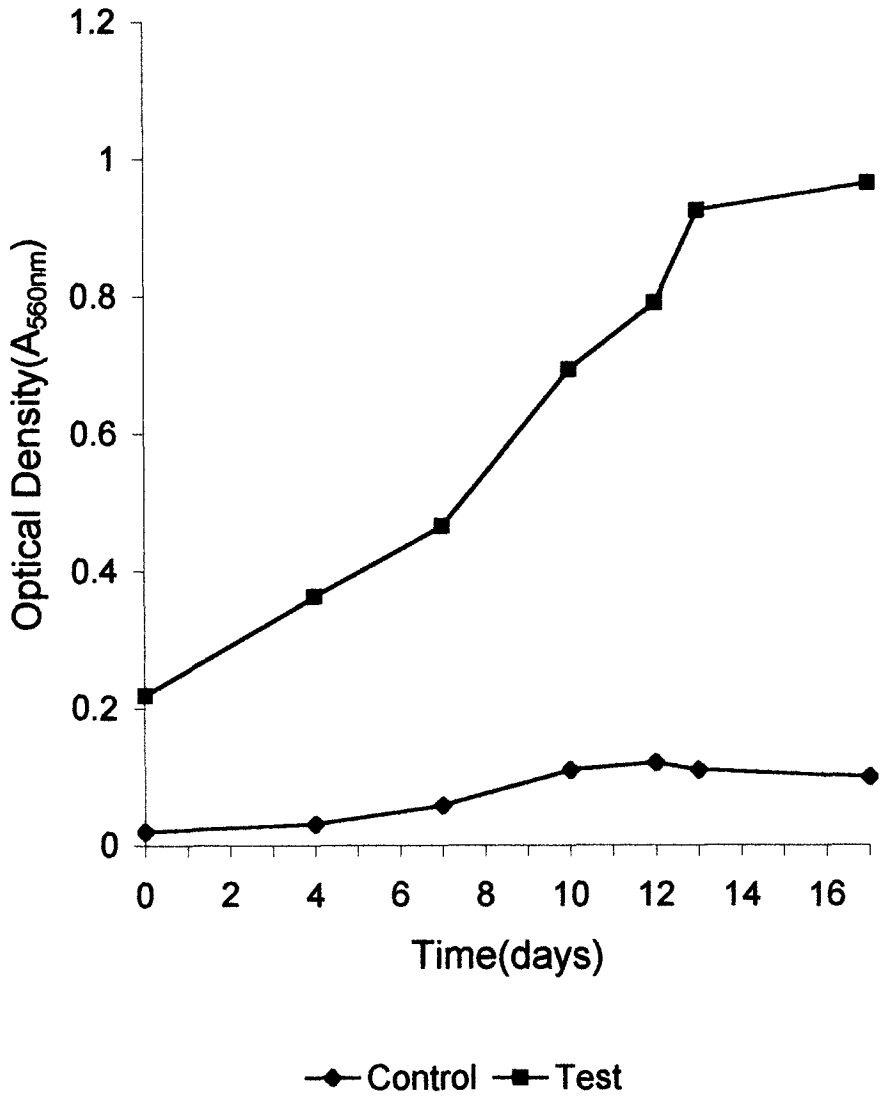


Fig.4.14. Growth of strain GU109 on crude oil and phenanthrene. Control flask consisted of substrates and was devoid of inoculum.

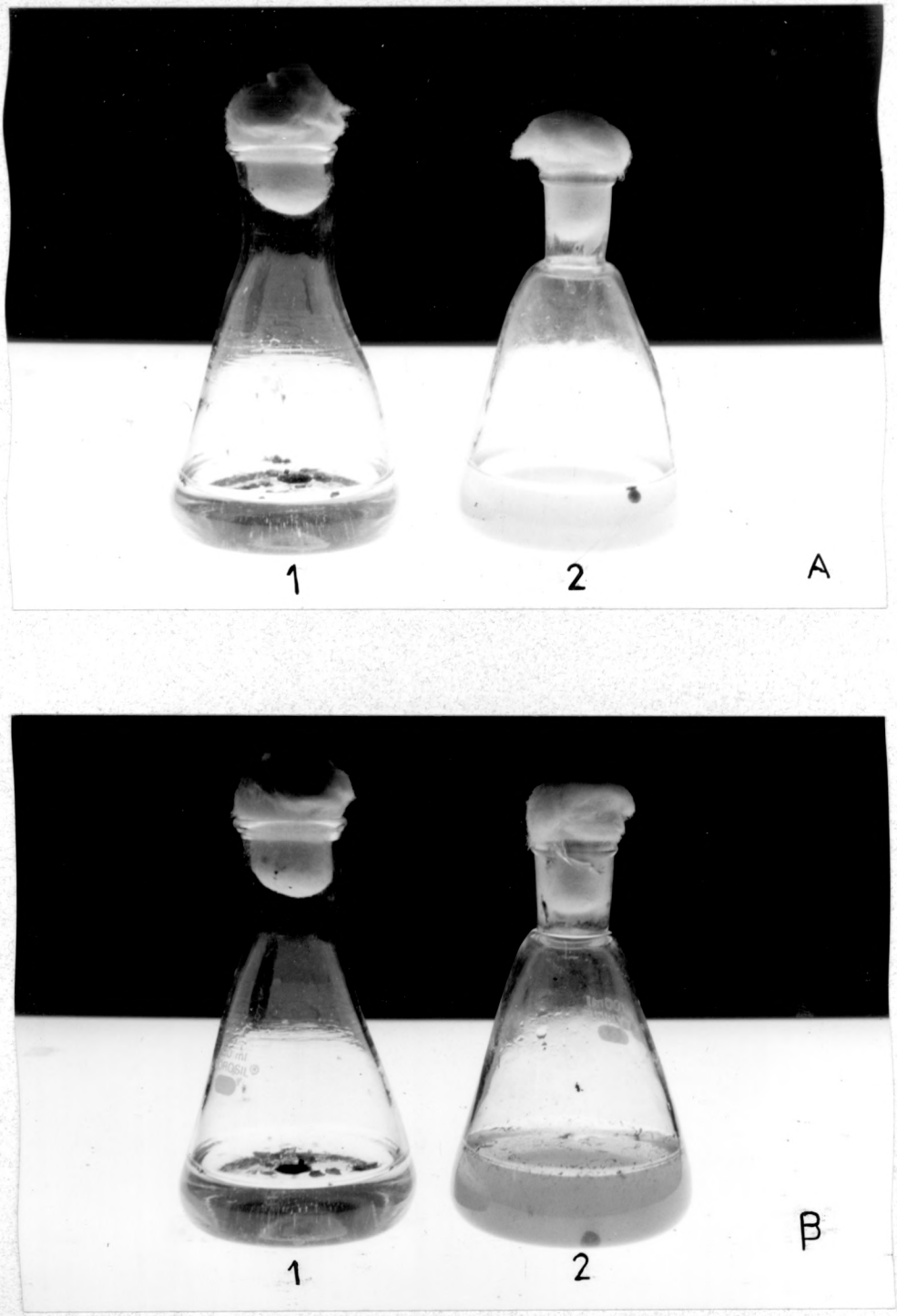


Fig. 4.15. Photograph showing growth of strain GU109 on A) crude oil and B) crude oil supplemented with phenanthrene.

1) Control : Uninoculated MSP with crude oil / crude oil supplemented with Phe.
2) Test : Inoculated with strain GU109.

fraction(Table 4.8, Fig 4.16 & Fig. 4.17). Similar results were obtained by Lal and Khanna (1996). However on crude oil supplemented with phenanthrene, there seems to be a general increase in the degradation of the alkane, aromatic and NSO fraction as compared to crude oil alone (Table 4.8, Fig.4.18 & Fig 4.19). These results seem to be consistent with the observations obtained earlier that the presence of phenanthrene in the medium enhances the strains capability, thereby enabling it to degrade other components as indicated by its growth on model petroleum.

The results suggest that, *P. putida* strain GU109 (MTCC3316) utilizes the phthalate pathway for the degradation of phenanthrene. It is also apparent that the strain has a broad degradative capability for oxidizing or degrading PAH and aromatic heterocycles encompassing tricyclic, bicyclic and S-heterocycles (DBT) as well. Growth on crude oil and crude oil supplemented with phenanthrene also confirms this observation. Thus it is possible that broad specificity oxygenase(s) rather than a suite of substrate specific oxygenase(s) are responsible for the range of aromatic compounds oxidized. In addition to the oxygenase(s), evidence showing the presence of ring cleavage enzymes such as C230 also support the broad degradative capability of strain GU109.

Thus it may be possible to use *P. putida* strain GU109 (MTCC 3316) either singly or as part of a consortium for seeding marine environments where a recalcitrant compound or a mixture of such compounds has been spilled. The broad specificity and oxidative capablity of *P. putida* strain GU109 (MTCC3316) could facilitate the removal of recalcitrant oil components.

Table 4.8 : Degradation of Bombay High crude oil by strain GU109

Fraction	Amount remaining in			
	Control sample*		Degraded sample	
	mg	%	mg	%
Culture grown on crude oil				
Alkanes	15	100	8.6	42.6
Aromatics	11	100	7.3	33.63
NSO	9	100	11	122
Culture grown on crude oil and phenanthrene				
Alkanes	13	100	6.5	50
Aromatics	10	100	5.9	41
NSO	8	100	7	12.5

* Control flask consisted of substrates and was devoid of inoculum.

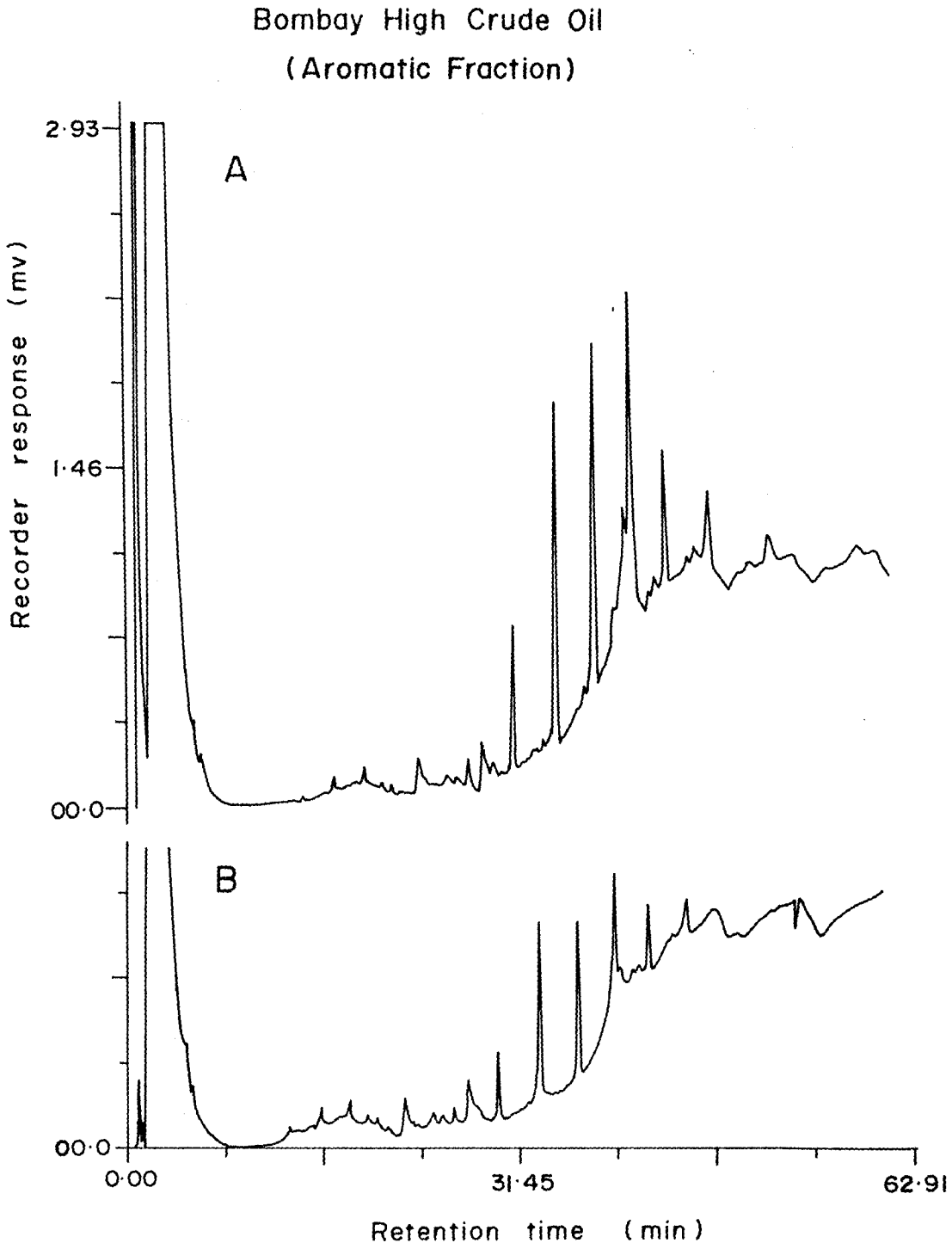


Fig. 4·17 GC profiles of aromatic fraction of BHCO

A) Un inoculated sterile control

B) Test : after growth of strain GU 109 (10 days)

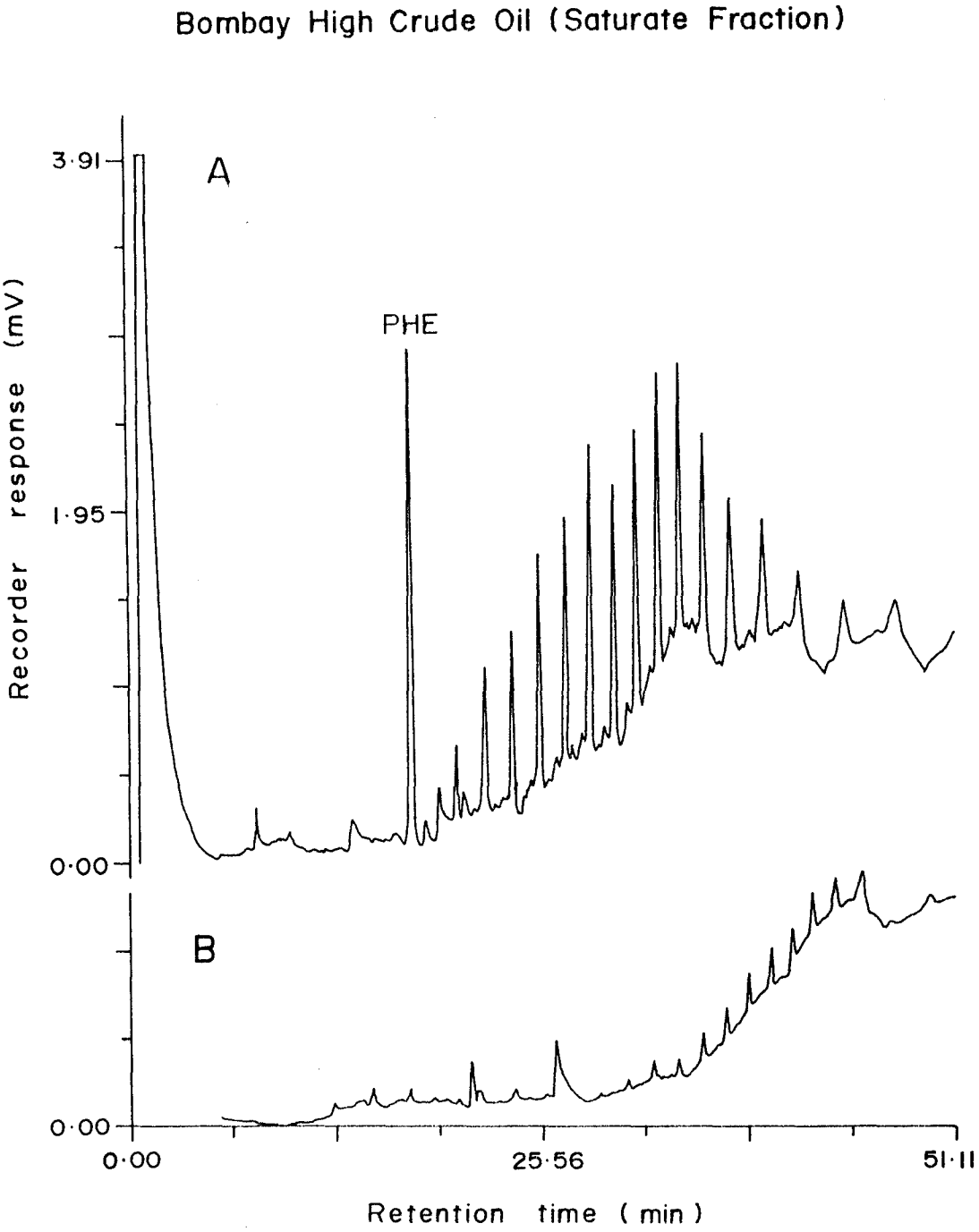


Fig. 4·18 GC profiles of saturate fraction after growth of strain GU 109 on BHCO supplemented with PHE

A) Un inoculated sterile control

B) Test: after growth of strain GU 109 (17 days)

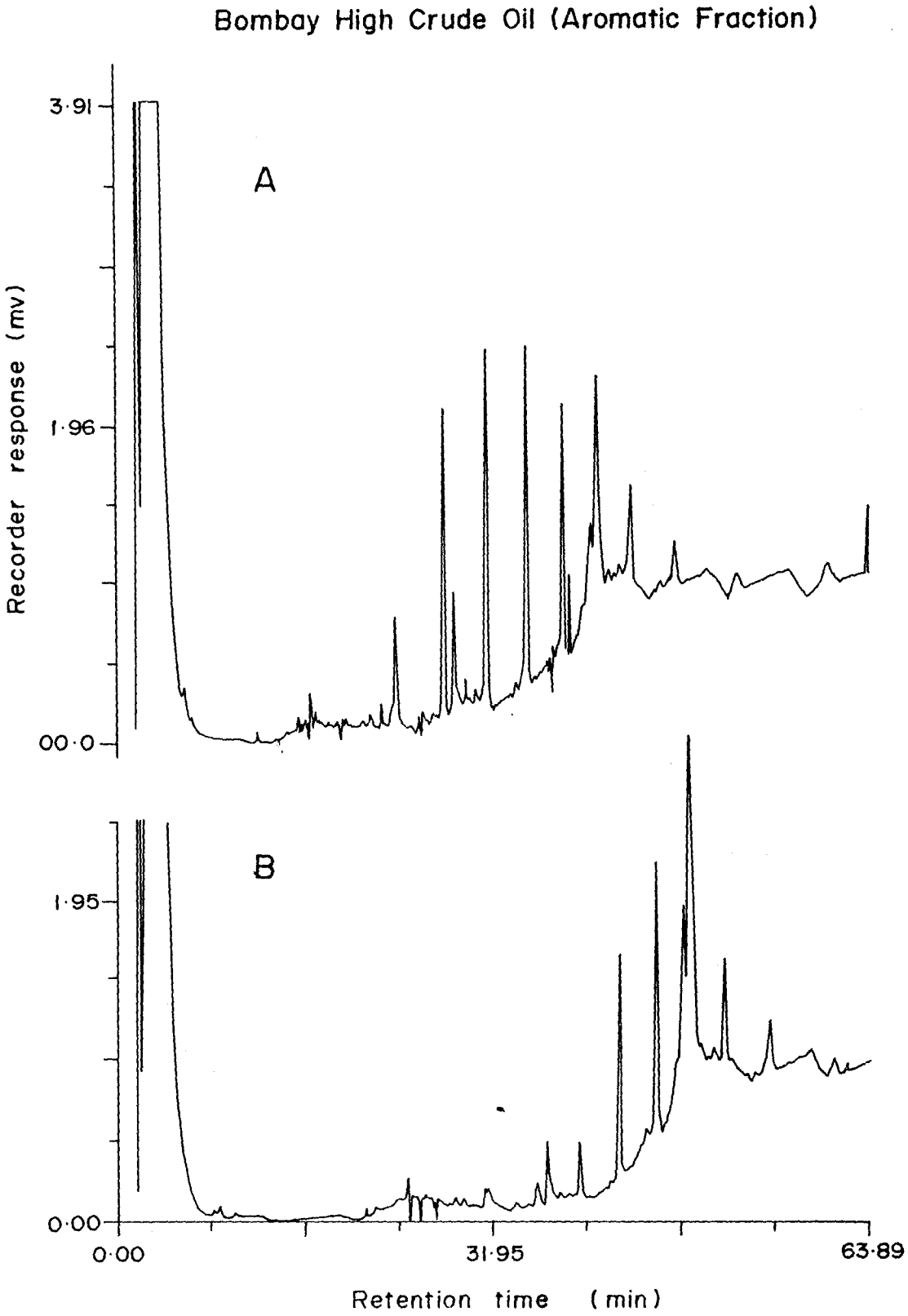


Fig. 4·19 GC profiles of aromatic fraction after growth of strain GU 109 on BHCO supplemented with PHE
A) Un inoculated sterile control
B) Test: after growth of strain GU 109 (17 days)

Chapter V

EFFICACY OF PRESERVED MIXED BACTERIAL CULTURES IN MINERALIZATION OF CRUDE OIL AND WEATHERED OIL

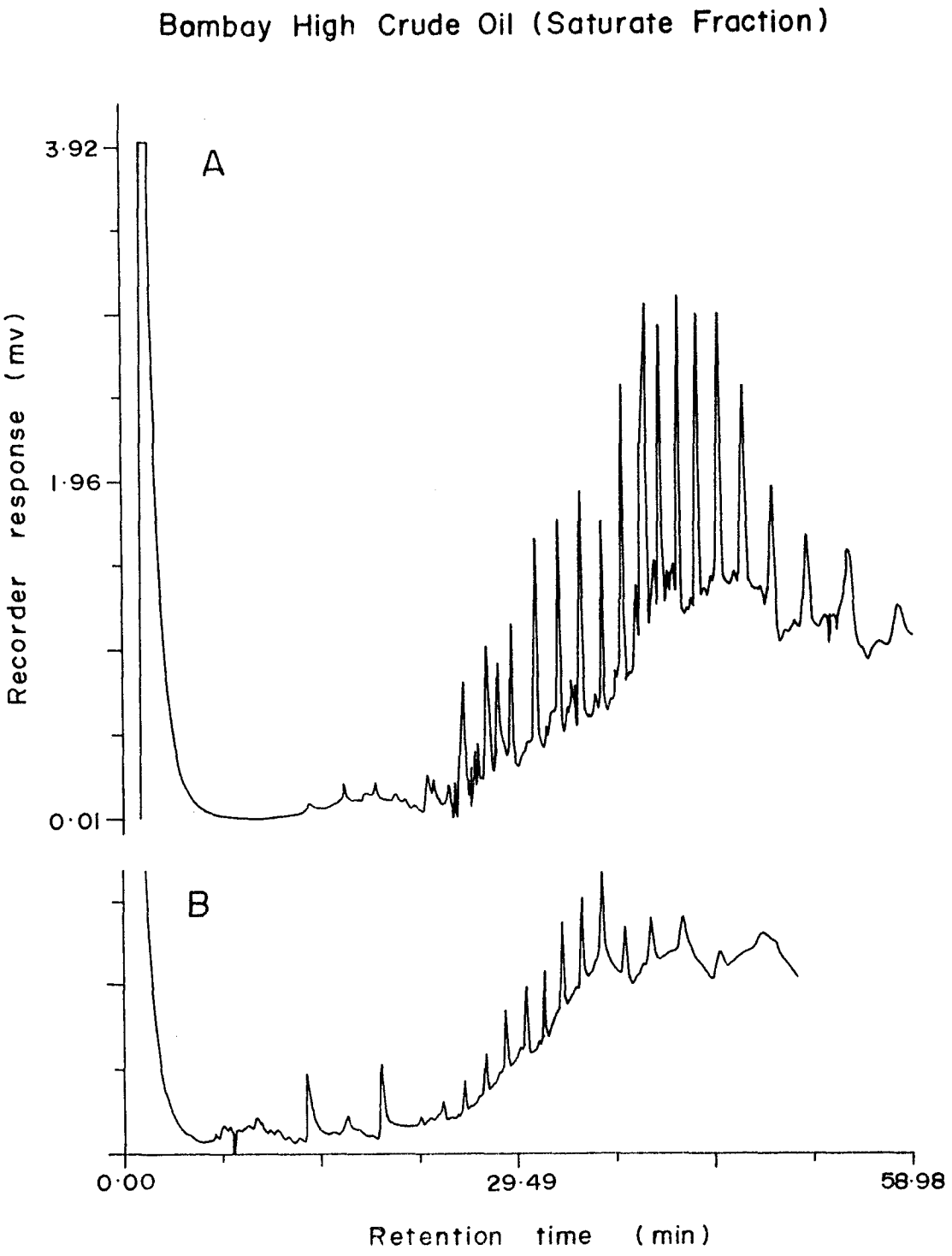


Fig. 4·16 GC profiles of saturate fraction of Bombay High crude oil (BHCO) A) Un inoculated sterile control B) Test: after growth of strain GU 109 (10 days)

Bioremediation as a technological process is being widely used today whereby biological systems are harnessed to effect the cleanup of environmental pollutants. Currently microbial systems are most widely used in bioremediation programmes, generally in the treatment of waters contaminated with organic pollutants such as pesticides, crude oil etc. Consortia of bacteria are considered to be more effective than single cultures given the complex nature of environmental pollutants.

However, bioremediation as process mainly suffers from the drawback of it being a slow degradative process as compared to physical methods which are rapid and the outcome generally predictable within a short time (Head, 1998). One of the prime requirements in hastening the biodegradative process is generation of an actively degrading biomass in a minimum period of time so as to provoke a rapid and high increase of pollutant degrading bacteria.

In this chapter , two marine bacterial consortia, A and B were used in testing the consortia efficacy in marine microcosms containing crude oil and weathered oil. respectively. A part of each of these consortia was cryopreserved and amplified based on the methods formulated in the chapter III so as to check the effectiveness of the preserved consortia in a marine micrcocosms. The study serves to demonstrate the efficacy of mixed marine microbial degraders in attacking crude oil/tar ball and rapid amplification coupled with effective degradation of cryopreserved strains in the event of an oil spill.

MATERIALS AND METHODS

Setting up of marine microcosms

In order to study the effectiveness of mixed consortia in cleaning up oil spills/ weathered oil , it was imperative to set up an artificial ecosystem/microcosm for experimental bioremediation experiments. Four glass tanks ,two 640 litres capacity and two of 324 litres capacity were filled with natural seawater. These tanks were filled with filtered natural seawater, and fitted with self contained aerators and mechanical stirrers for wave action. The aerators were linked to an independent aeration line. The temperature was controlled at 26°C. Crude oil and weathered oil was used as a substrate at concentration of 0.1% v/v and 0.15% w/v. A parallel control microcosm supplemented with nitrates(8mM $NaNO_3$) and Phosphates(5mM KH_2PO_4/K_2HPO_4) as additional source of nitrogen and phosphorous was also set up. Fig. 5.1 shows a diagram of the experimental microcosm.

Formulation of mixed marine consortia

Consortium A was established so as to determine its effectiveness in cleaning up oil spills. Consortium A constituted four strains, GU101, GU104, GU109 and GU110 (Table 5.1). Strains GU101, GU109 and GU110 were amplified in a 100ml flask containing their respective substrates. Strains GU109 and GU101 were cryopreserved strains at –20°C for around two years(Chap III). Strain GU104 was amplified separately as the presence of quinoline inhibited the growth of the other strains. The flasks were incubated at 30°C on a rotary shaker at 100 rpm. Consortium A was used as a 0.05%v/v inoculum containing crude oil (0.1%) as substrate.

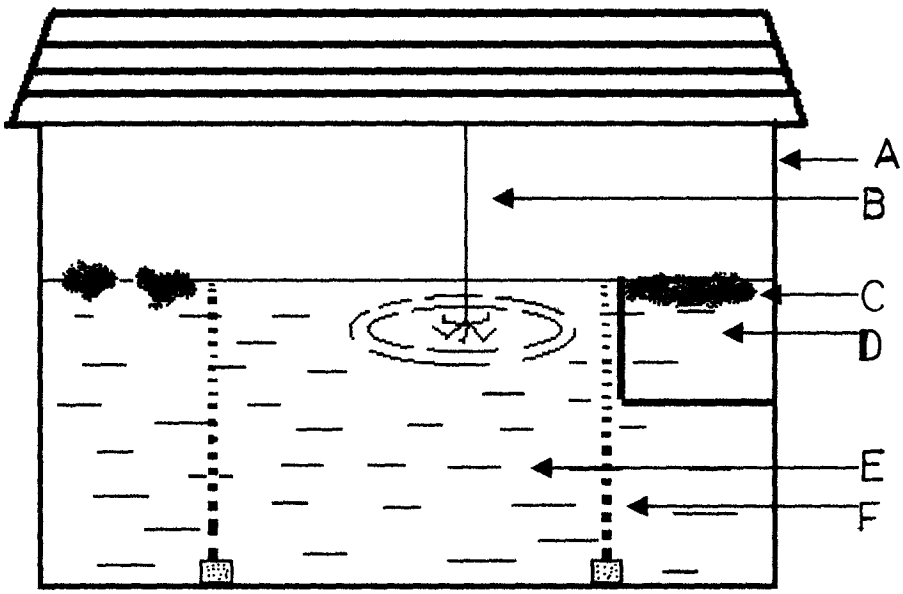


Fig. 5.1. Line diagram of an artificial microcosm used in this study. A) 324 litre glass tank, B) stirrer, C) tarballs /crude oil , D) sampling site E) filtered seawater, F) aerators.

Consortium B consisted of strains GU101(m-Tol), GU102(Naphthalene), GU 103(Xylene), GU104(Quinoline), GU105(Pyridine), GU109(Phenanthrene) and GU 110(Dibenzothiophene) (Table 5.2). Strains GU101, GU109 and GU110 were grown separately and 0.5ml of each pure culture was inoculated into 100ml of Artificial Seawater containing a mixture of their respective susbtrates at the appropriate concentrations (m-tol, 5mM; Phe, 0.08%; DBT, 0.02%). The other four strains GU102 to GU105 were preserved at –70°C as a consortium. These were amplified separately as indicated in chapter III. The flasks were incubated at 30°C on a rotary shaker at 100rpm. The two parts of consortium B were used as a 0.05%v/v inoculum in experimental microcosms containing tarball representing weathered oil (0.15%) as substrate.(Table 5.1 and 5.2).

Determination of growth and degradation.

50ml aliquots of samples were filtered using 0.22μm millipore filters which were washed in 5ml of artificial seawater medium. Viable counts of each of the components of the mixed culture were established by the most probable number(MPN) method. The individual components of the mixed culture were enumerated by serially diluting the washed sample tenfold in artificial seawater media containing their principal substrates. The highest dilution showing growth was taken as a measure of the viable count. Viable counts were also determined using benzoate(0.08%) as a substrate since it supports growth of all the members of consortia A and B which were used in the experimental microcosms.

For gas chromatographic analysis, degradation of crude oil was determined by extracting 50ml of water sample with an equal amount of chloroform. 0.5 to 1g of activated silica

Table 5.1 Consortium A : List of bacterial cultures used in oil spill simulation studies.

Place isolated	Strain No.	Principal Substrate	Strain identified as	Reference
Goa	GU 101	m-Toluic acid	*Pseudomonas stutzeri*	Chakravarthy (1993)
Vengurla	GU 104	Quinoline	*Ochromobactrum sp.*	Coelho (1998)
Gulf of Kutch	GU 109	Phenanthrene	*Pseudomonas.putida*	This study
Goa	GU 110	DBT	*Alcaligenes sp.*	Rodrigues (2000)

Table 5.2. Consortium B : List of bacterial cultures used in weathered oil simulation studies.

Place isolated	Strain No.	Principal Substrate	Strain identified as	Reference
Goa	GU 101	m-Toluic acid	Pseudomonas .stutzeri	Chakravarthy (1993)
Vengurla	GU 102	Naphthalene	Unidentified	Coelho *et al.*(1995)
Vengurla	GU 103	Xylene	Unidentified	Coelho *et al.* (1995)
Vengurla	GU 104	Quinoline	*Ochromobactrum sp.*	Coelho (1998)
Vengurla	GU 105	Pyridine	Unidentified	Coelho *et al.* (1995)
Gulf of Kutch	GU 109	Phenanthrene	Pseudomonas putida	This study
Goa	GU 110	DBT	*Alcaligenes sp.*	Rodrigues (2000)

gel was added and the extract was evaporated to dryness. The dried silica gel was loaded on a column containing equal amounts of silica gel and alumina and fractionated into the saturate, aromatic and NSO fractions as per **Materials and Methods**, (Chapter IV). The various fractions were analysed by injecting 0.4-0.5µl of extract in a Shimadzu gas chromatograph 14B. The gas chromatograph was equipped with a capillary column and a flame ionization detector (FID). The injector and detector were maintained at 320°C and the oven temperature was programmed to rise from 60°C to 300°C at the rate of 6°C/min. Each sample of 0.5µl injected corresponded to 100,000 times of the original sample.

To study degradation of tarball, 100mg of tarball from each tank was dissloved in 5ml of carbon tetrachloride and chromatograms obtained on a Shimadzu gas chromatograph 14B.

RESULTS AND DISCUSSION

Most of the bioremediation techniques currently utilized are based on the enhancement of the oil-degrading capabilities of indigenous microbes by adding nutrients such as nitrates and phosphates. (MacDonald and Rittman, 1993). Although the utilization of microorganisms for the degradation of recalcitrant compounds was proposed long ago, successful application of exogenous microorganisms to clean up pollutants has still not been very effective. (Venosa *et al.*,1992). Factors such as temperature, the addition of nitrates and phosphates, abiotic losses all seem to play critical roles in the bioremediation process. Under such circumstances, we were interested in developing techniques for the bioremediation of crude oil and naturally weathered oil (tarballs).

Bioremediation of an artificial oil slick.

The work describes the effect of a heavy innoculation of a marine bacterial community (consortium A), previously adapted to aromatic, polycyclic and NSO hydrocarbons to an artificial oil slick in natural seawater. To generate conditions of simulating a typical oil slick, marine microcosms were designated as follows.

Microcosm A : Seawater supplemented with crude oil/ tarball representing weathered oil.

Microcosm B : Seawater supplemented with crude oil/tarball and nitrogen & phosphorous.

Microcosm C : Seawater supplemented with crude oil/tarball and nitrogen & pho sphorus and inoculum.

Microcosm D : Seawater supplemented with crude oil/tarball and inoculum without nitrogen and phosphorous.

These microcosms were used for seeding with consortium A and B (Table 5.1 & 5.2). A part of each consortia A and B was preserved . The objective was to provoke a rapid and high increase of the petroleum degrading bacteria in the early stage of an oil spill.

Growth kinetics of consortium A in experimental microcosms containing natural seawater supplemented with crude oil.

Growth of Consortium A on crude oil shows that all the strains were maintained at reasonably high numbers after 30 days in the experimental microcosms. Growth of the strains on benzoate medium revealed a higher growth rate in microcosm C containing nitrogen and phosphorous as compared to microcosm D without nitrogen and phosphorous (Fig.5.2). Strain GU109 and strain GU 104 however showed the opposite trend (Fig.5.3) with strain GU101 and GU110 following the same trend as benzoate (Fig.5.4).

However, all the strains showed a sharp increase in bacterial numbers between the 5-11 day time period. This increase in counts is concominant with the decrease in pH and dissolved oxygen in the tanks with and without nitrogen and phosphorous indicating high microbial activity of consortium A (Fig. 5.5). The amount of oxygen utilized after the initial attack depends largely on the mineralization that occurs. For the complete oxidation of an alkane to CO_2 and H_20, 3-4 g of alkane carbon is required(Hughes and McKenzie,1975). Acetyl-CoA, fatty acids, formic acid, succinic acid are common intermediates which then enter the central metabolic pathway to be utilized as carbon precursors for growth and ATP generation. The decrease in pH and dissolved oxygen could be attributed to this process, although this process is self-regulatory and its effect is unlikely to be serious over a period of time with a gradual normalization of pH and

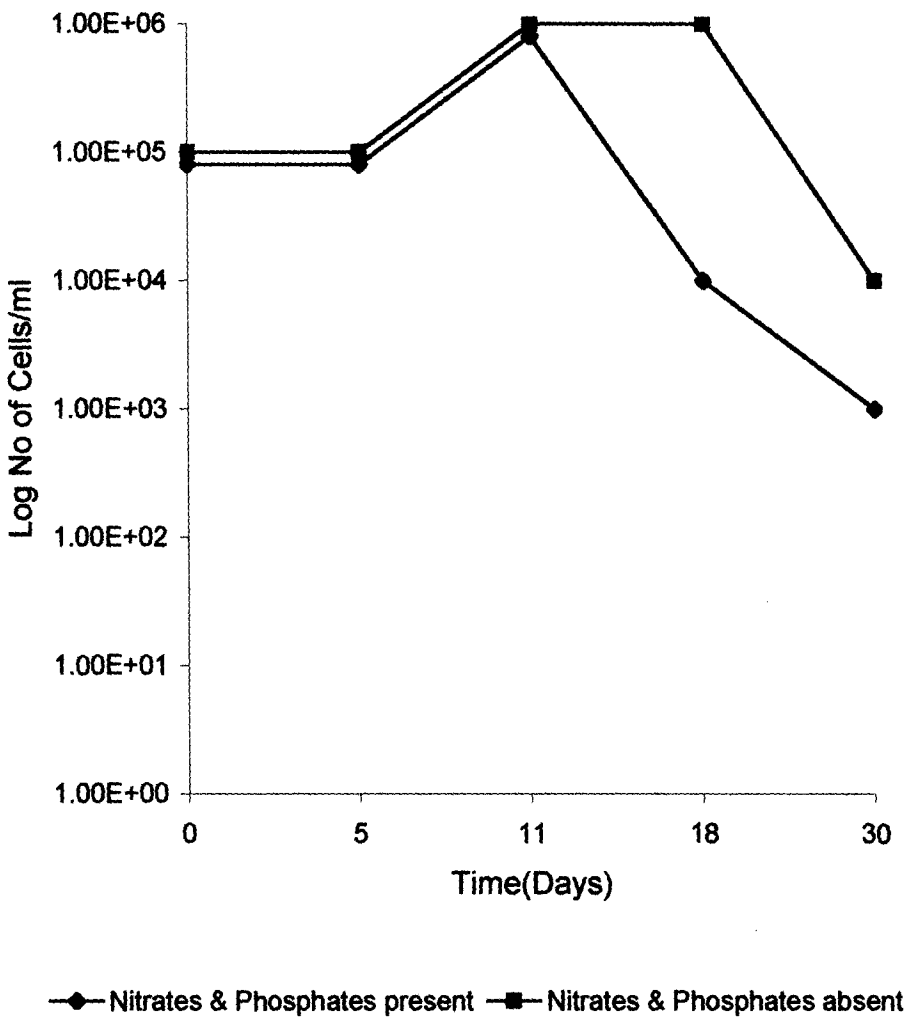
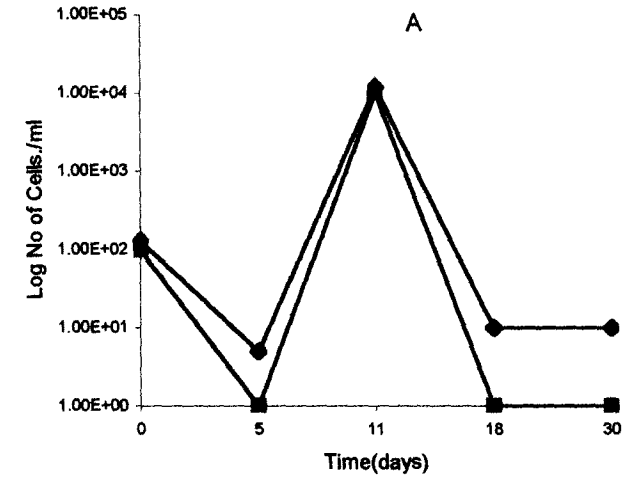


Fig.5.2. Growth kinetics of the four members of a mixed culture grown in microcosms containing crude oil in the presence and absense of nitrates and phosphates .The total counts were measured in benzoate medium since all the strains grew on benzoate.



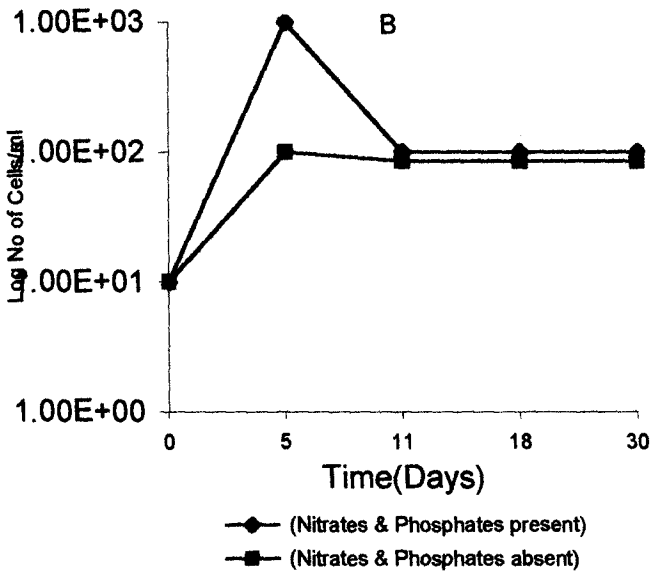
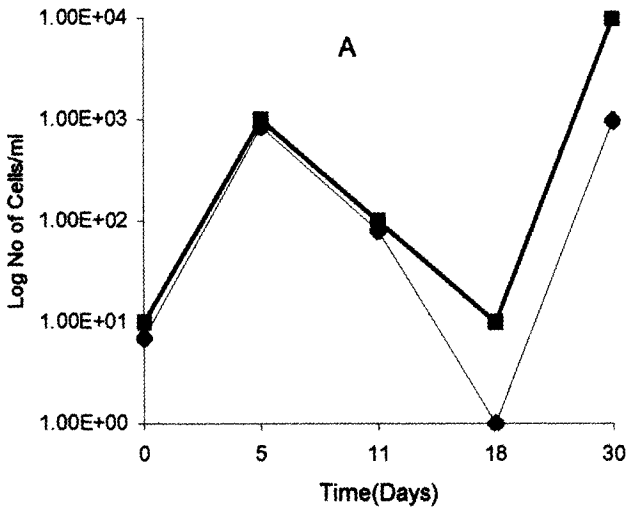


Fig. 5.3. Growth kinetics of strain GU104(A) and strain GU109(B) in microcosms maintained wih crude oil with and without nitrates and phosphates. Total counts of strain GU104 were determined on quinoline medium and strain GU109 on phenanthrene medium.



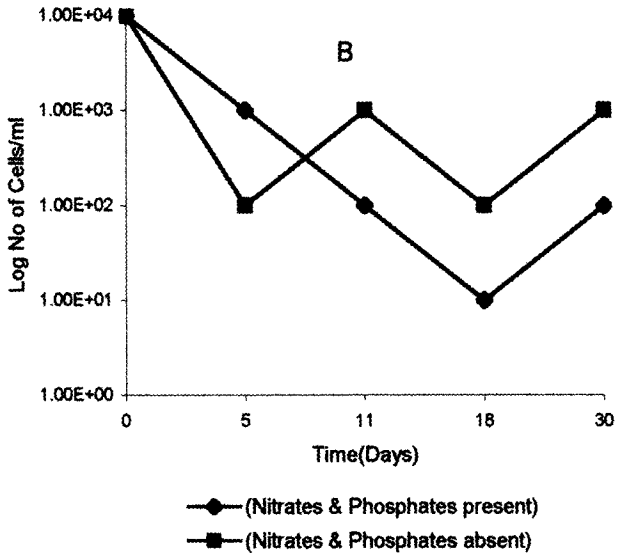
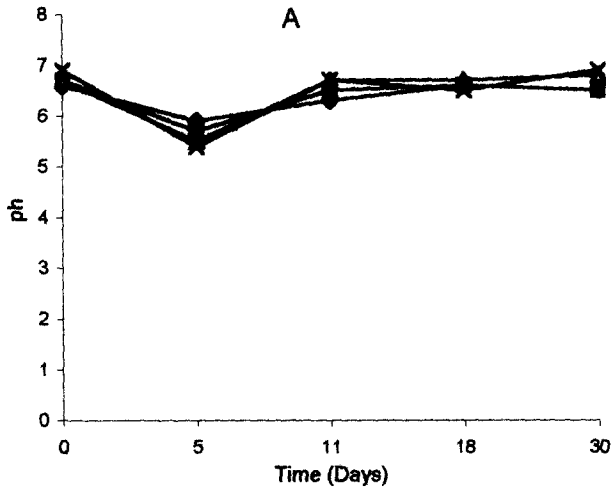


Fig. 5.4. Growth kinetics of strain GU101(A) and strain GU110(B) in microcosms maintained with crude oil with and without nitrates and phosphates.Total counts of strain GU101 were determined on m-Tol medium and of strain GU110 on DBT medium.



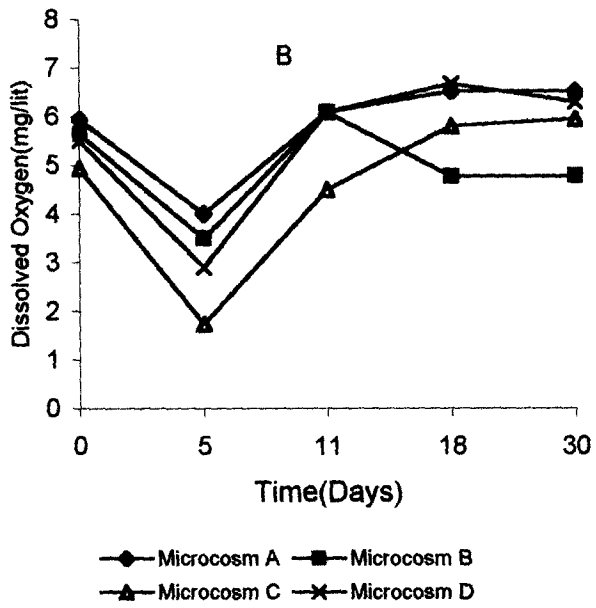


Fig 5.5. A) pH and B) dissolved oxygen profiles of experimetal and control microcosms.

dissolved oxygen values.

Thus, consortium A seems to play an active role in the mineralization of crude oil in experimental microcosms as indicated by an increase in bacterial counts. However, nitrogen and phosphorous do not seem to play a important role in enhancing biodegradation rates. This could be due to the adaptive response of the natural microbial population. In more polluted waters, where iron, nitrogen and phosphorous are in plenty, studies have shown that stimulation oil degradation by nitrogen and phosphorous is marginal as compared to relativelt cleaner waters (Dibble and Bartha, 1976). Thus although, consortium A was effective in growing in microcosms containing crude oil, we need to clearly understand the microbial ecology of petroleum degrading bacteria, as well as of the natural bacterial population of a given habitat, before microbiological processes can be taken in treating oil spills.

Gas chromatography of water samples taken from micrcocosms containing crude oil at various time intervals of growth of consortium A.

The water samples were extracted and separated using column chromatography into the saturate, aromatic and NSO fractions **(Materials & Methods, Chap IV).** These fractions were then analysed by gas chromatography.

Saturate fraction: Gas chromatography analysis of the saturate fraction of the water samples show the appearance of a large number of compounds between the 5-11 day grwoth period for microcosm C and micorcosm D (Fig. 5.6 & 5.7). The results are consistent with the increase in bacterial counts with a parallel decrease in pH and dissolved oxygen during the same time period indicative of a high degradative activity by consortium A with respect to the alkane fraction. Both microcosm C and microcosm

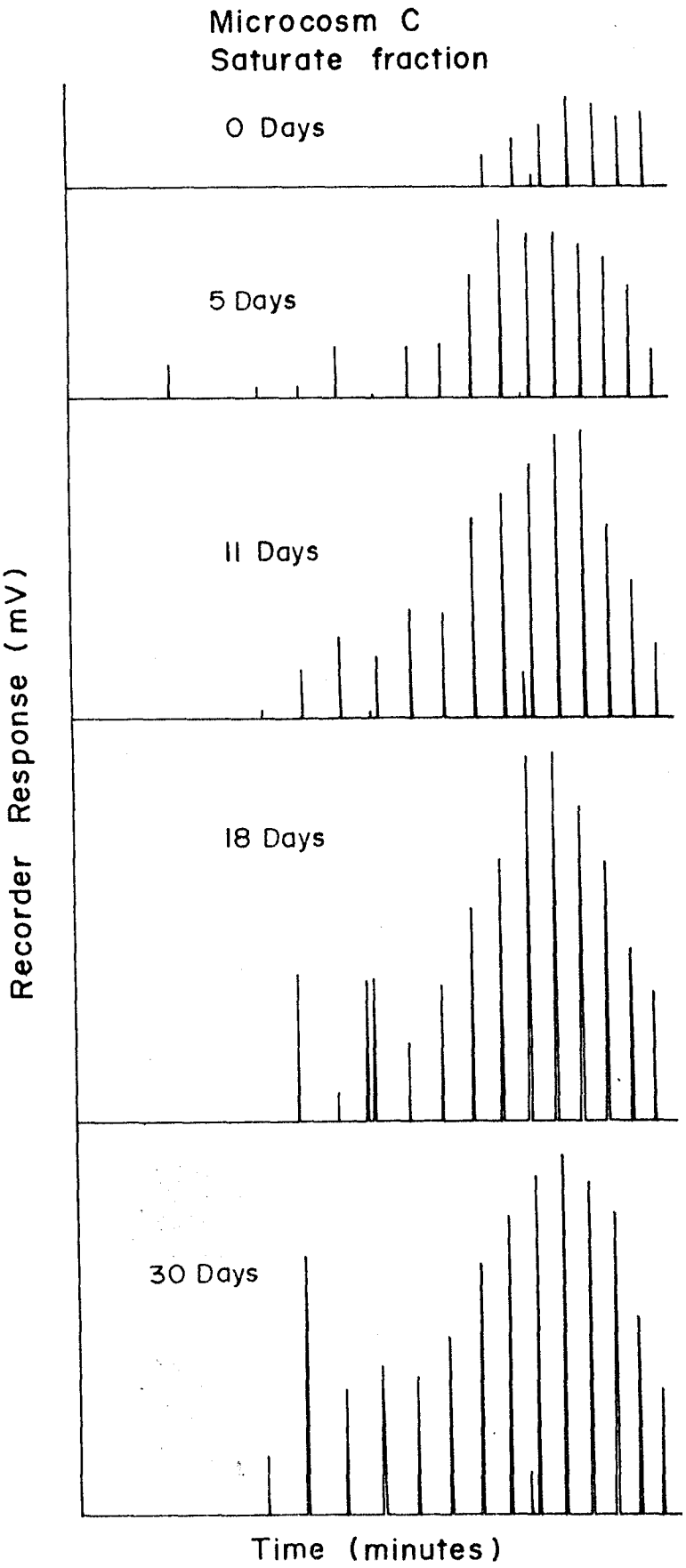


Fig. 5·6 Gas chromatographic profiles of the saturate fraction of crude oil in microcosm C which contains inoculum and nitrogen & phosphorous.

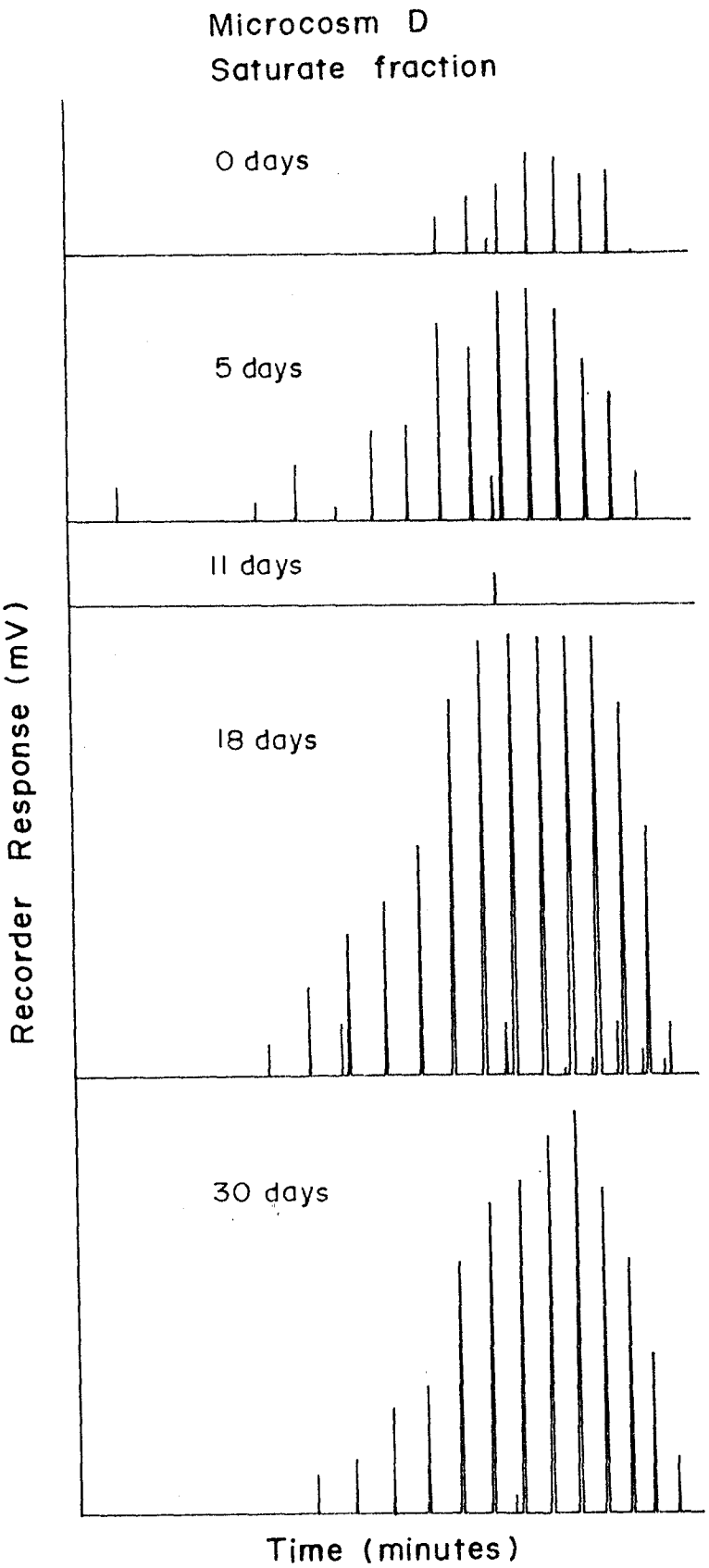


Fig.5·7 Gas chromatographic profiles of the saturate fraction of crude oil in microcosm D which contains only inoculum.

D showed a similar degradation profile over the 30 day experimental period with a large number of petroleum hydrocarbon compounds being released into the water after 30 days. Thus although the profiles show an increasing trend, comparison of these profiles with microcosm B profile clearly shows degradation of oil with a large number of peaks still persisting in microcosm B which does not contain any inoculum (Fig. 5.8).

A number of workers have reported rapid biodegradation of the saturate fraction in the environment or in flasks (Hughes and McKenzie, 1975; Gilbert and Higgins, 1978). However, most of them have extracted the entire flask contents rather than the surrounding medium. Strain GU109,strain GU104,(Coelho,1999) and strain GU110(Rodrigues, 2000) which form part of consortium A have also shown rapid degradation of the saturate fraction when the entire contents of the flask were extracted. Thus consortium A appears to be attacking the saturate fraction as indicated by the appearance of peaks in the water samples. It is possible that the capacity to degrade oil by consortium A is dependent on the capacity of the individual strains to solubilize and emulsify oil. This could explain the fact that there is a slow release of hydrocarbons initally with a sharp increase between 5-11 days which is accompanied by the decrease in pH and dissolved oxygen. Thus consortium A appears to be effective in attacking the saturate fraction of oil by means of slow release and subsequent degradation.

Aromatic fraction: Gas chromatographic profiles of the water samples show that the aromatic fraction appears in the micrococosms after the same period i.e 5-11 day time period indicative of a combined attack on both the saturate and aromatic fractions. However, these peaks were rapidly degraded after 30 days as observed by the decrease in the number of peaks over 30 days (Fig.5.9 and Fig. 5.10). The microcosm B also shows some part of the aromatic fraction after 10-15 days but these peaks persist after

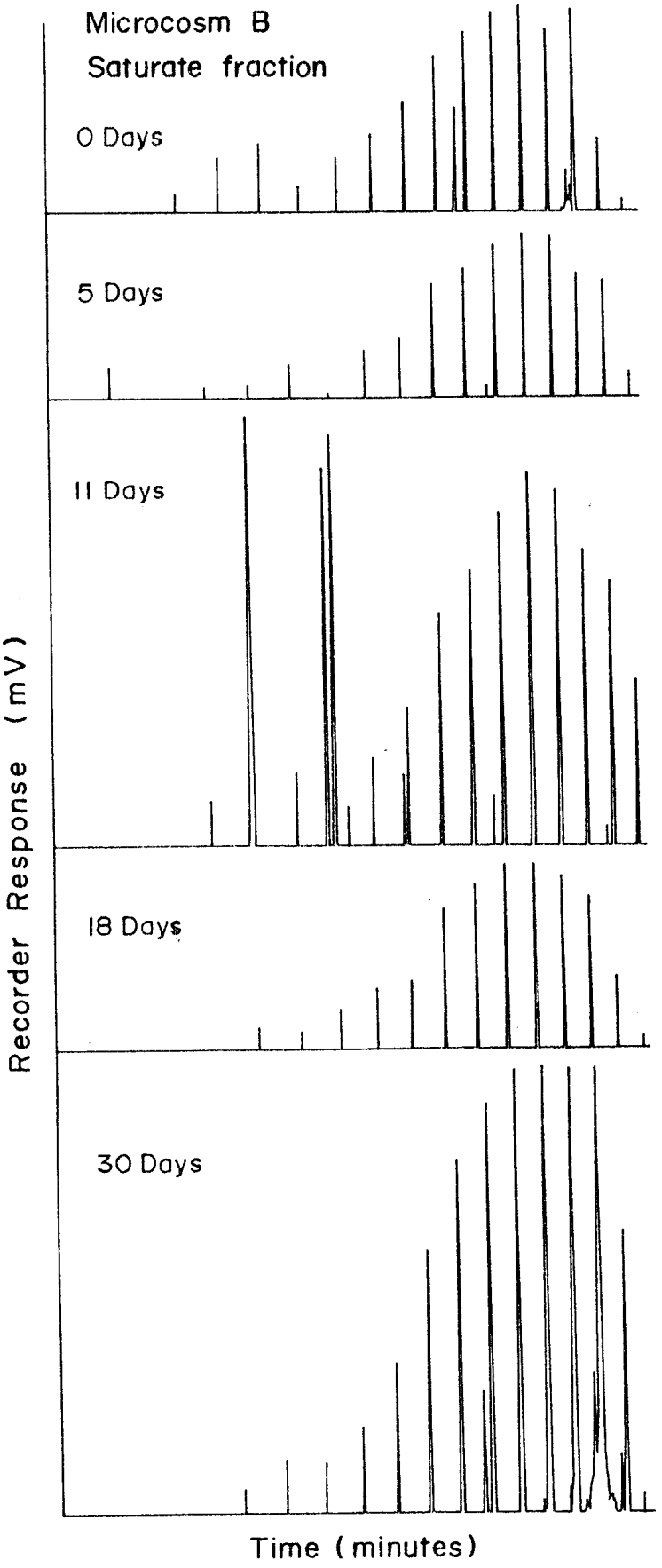


Fig. 5·8. Gas chromatographic profiles of the saturate fraction of crude oil in microcosm B which contains only nitrogen and phosphorous.

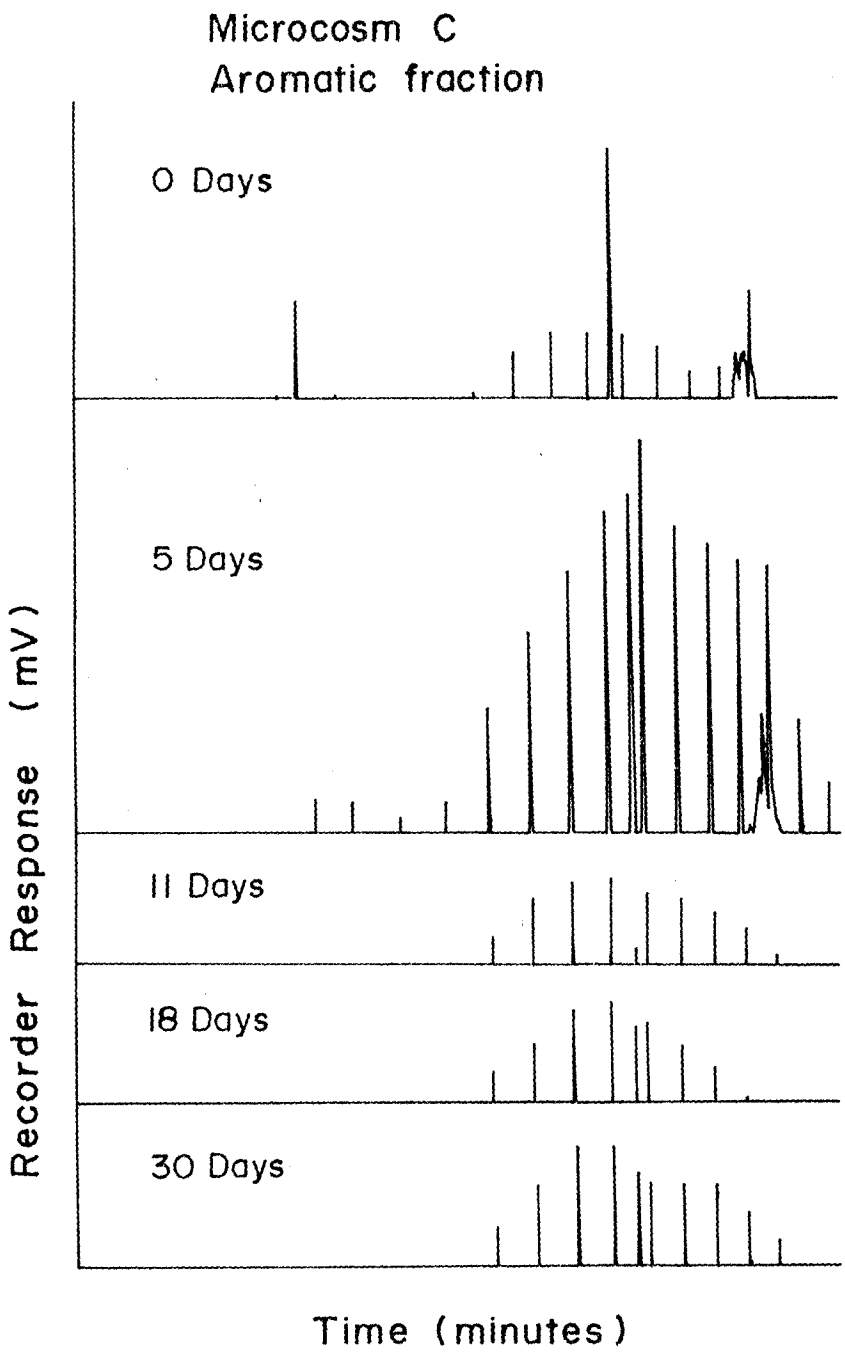


Fig. 5·9. Gas chromatographic profiles of the aromatic fraction of crude oil in microcosm C which contains inoculum and nitrogen and phosphorous

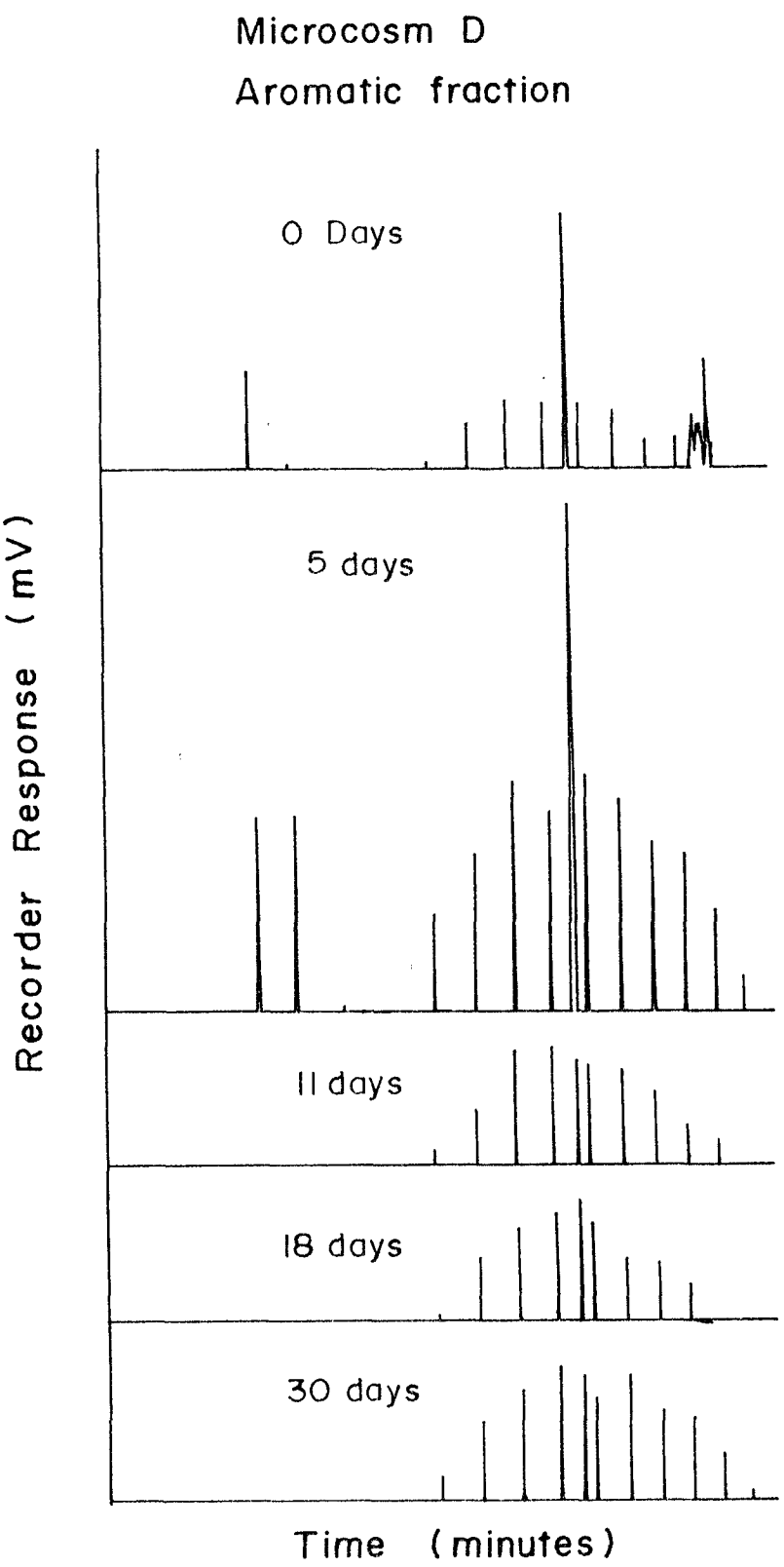


Fig. 5·10 Gas chromatographic profiles of the aromatic fraction of crude oil in microcosm D which contains only inoculum.

30 days as indicated in the gas chromatographic profile (Fig. 5.11). Thus consortium A appears to be clearly attacking the aromatic fraction as observed by the depletion in a number of peaks. This could be due to the fact that strain GU104, GU109, GU110 besides growth on their principal substrates could also grow on other aromatic compounds similar to an adaptive mechanism. Furthermore strain GU109 has shown a cometabolic property whereby the presence of phenanthrene in the medium enhances the degradation of other co-substrates(Chapter IV). GU104 has the ability to mineralize quinoline therby lifting the inhibition on other microorganisms(Coelho, 1999). Strain GU110 has the ability to attack the sulfur fraction of crude oil(Rodrigues, 2000). All these properties of the mixed culture coupled with genetic instability, a high and broad degree of enzymatic activity, ability to compete with the indigenous microflora makes this consortium a very effective mixture for seeding oil spills with a high aromatic content.

NSO fraction: Gas chromatography profiles of the NSO fraction of water samples in microcosms containing crude oils as substrates show the presence of peaks in both micrososm C & D. within the 5-11 day growth period. Comparison of the two profiles show similar degradation patterns with the presence of a large number of peaks on the 5^{th} day and a subsequent decrease in the number of peaks over a 30 day experimental period.(Fig. 5.12 & 5.13). Microcosm B shows a sharp iincrease in the number of peaks after 5 days which are subsequently degraded clearly indicating the role of the natural microbial population in the control tank (Fig. 5.14).

Studies carried out by Lal and Khanna (1996) have reported an increase in the asphaltic content after degradation in laboratory experiments. Similar results were obtained by a number of workers who have demonstrated the the NSO fraction is poorly degraded

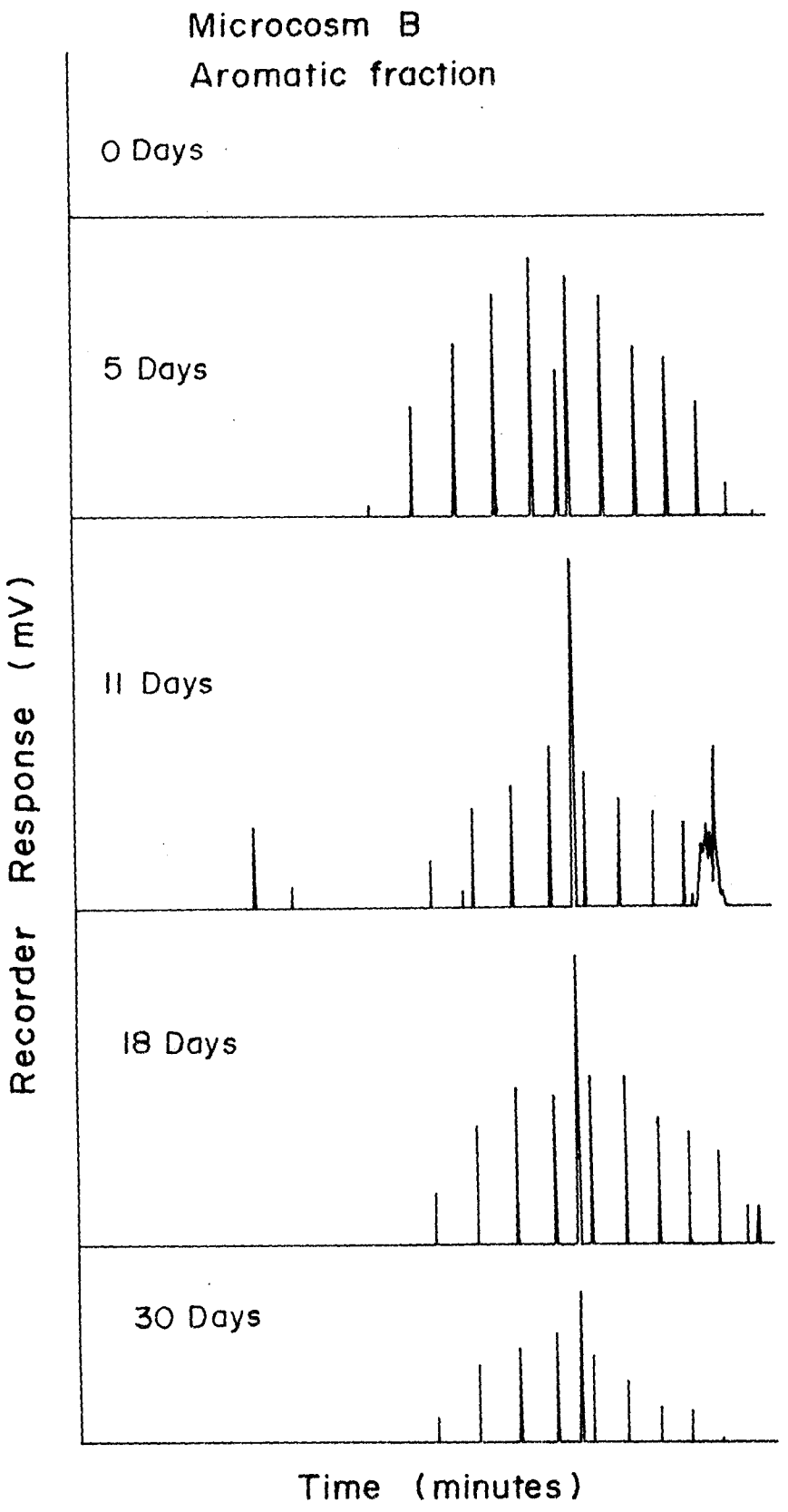


Fig. 5-11. Gas chromatographic profiles of the aromatic fraction of crude oil in microcosm B which contains only nitrogen and phosphorous.

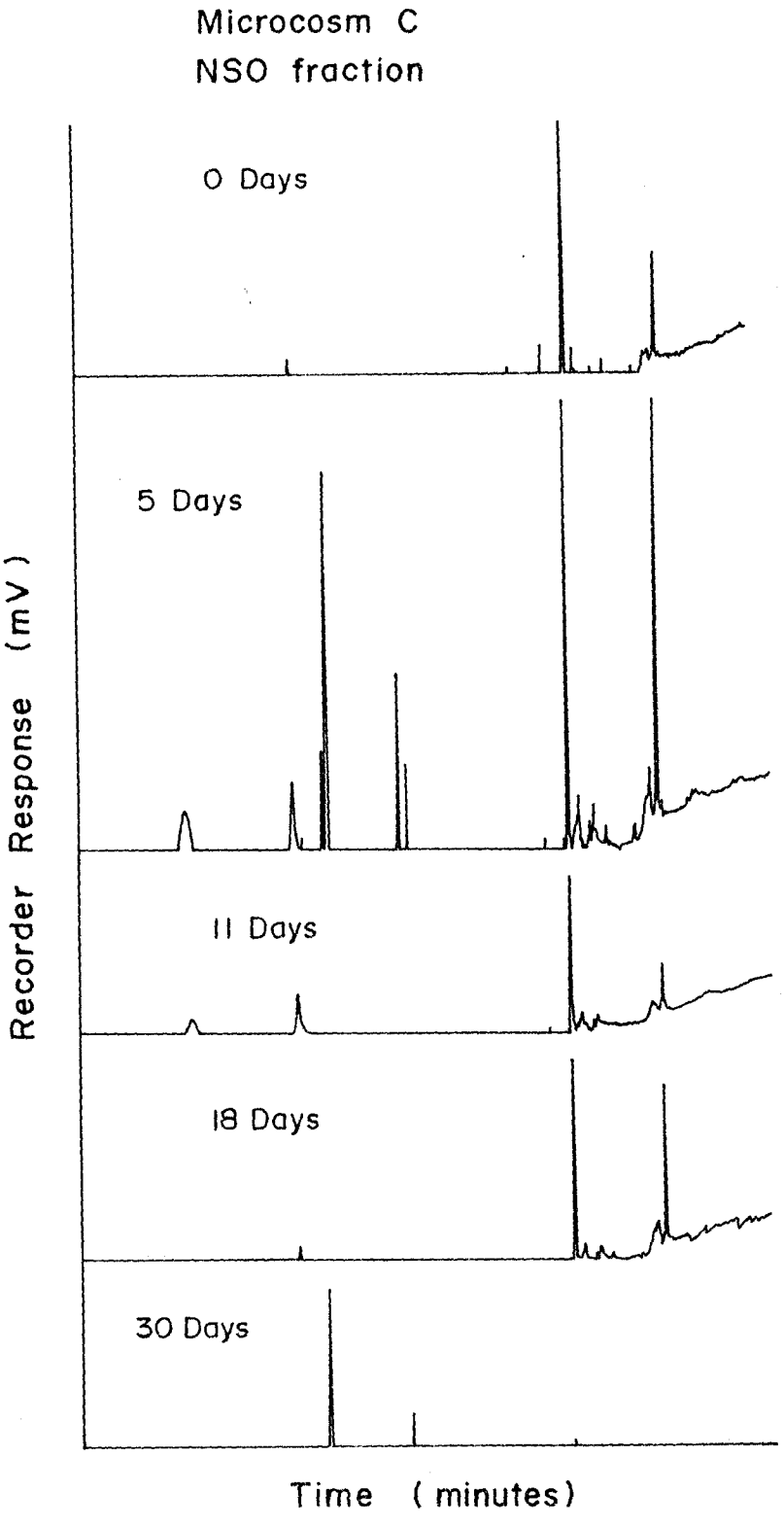


Fig. 5·12 Gas chromatographic profiles of the NSO fraction of crude oil in microcosm C which contains inoculum and nitrogen and phosphorous

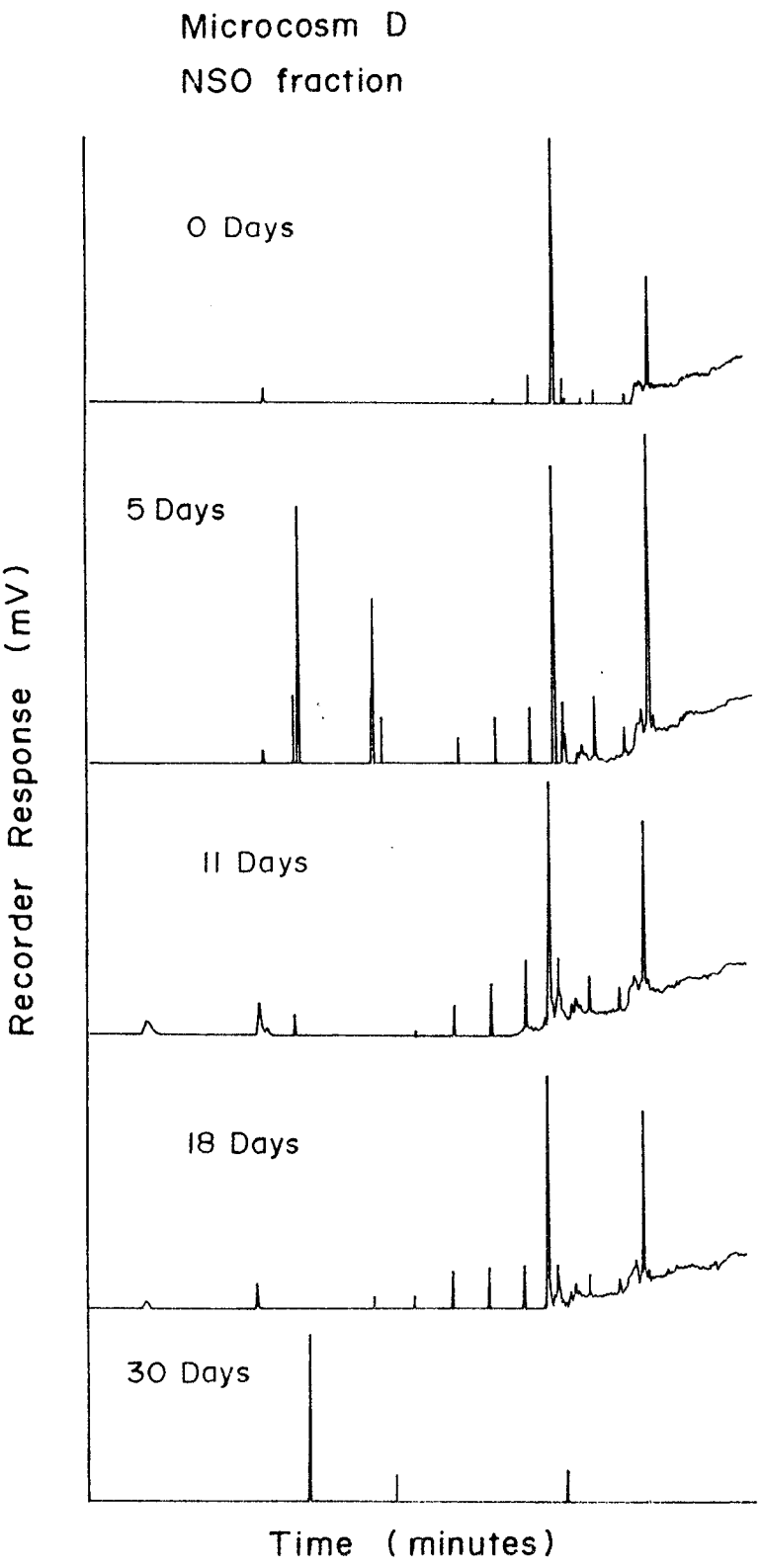


Fig. 5·13 Gas chromatographic profiles of the NSO fraction of crude oil in microcosm D which contains only inoculum.

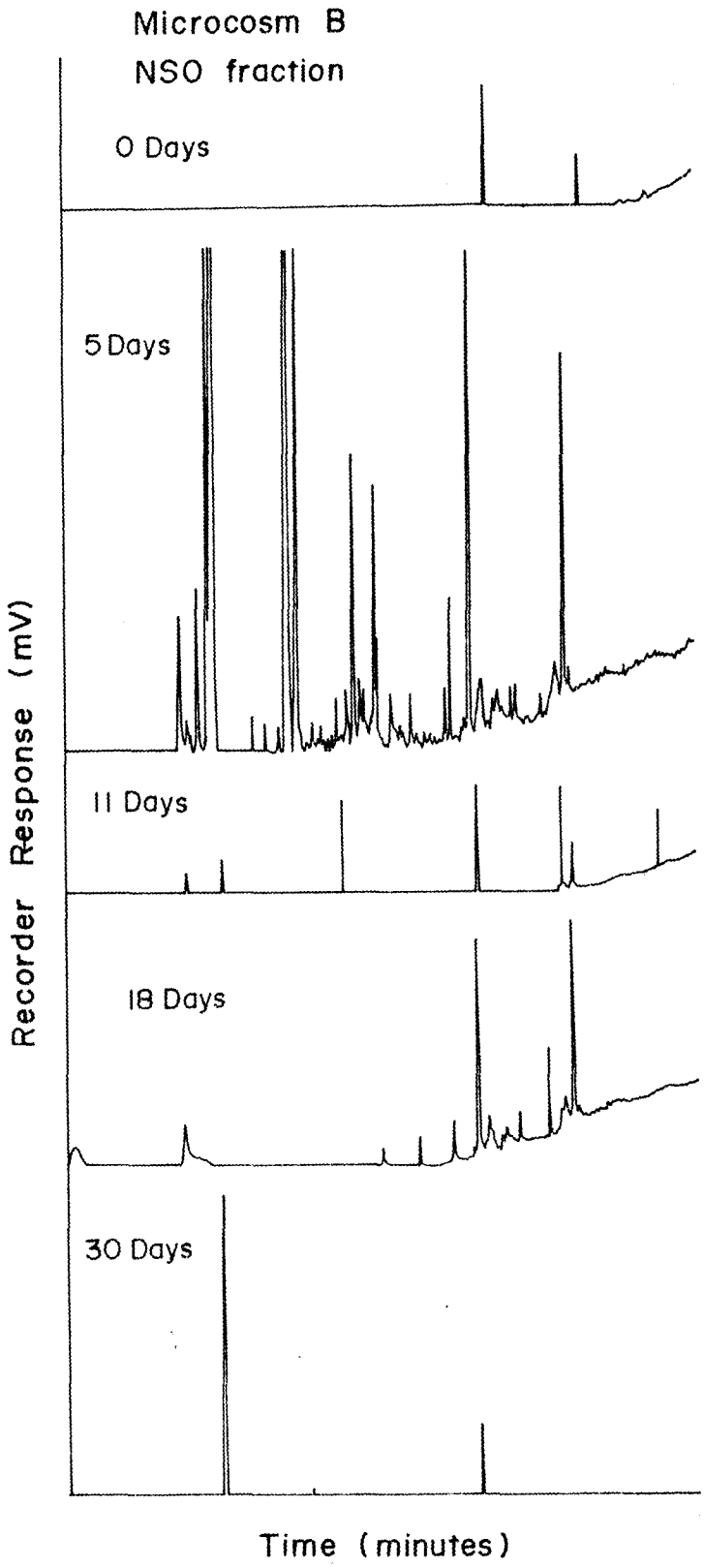


Fig. 5·14. Gas chromatographic profiles of the NSO fractic of crude oil in microcosm B which contains on nitrogen and phosphorous.

(Walker *et al.*,1975a,b.; Perry, 1984 ;Leahy and Colwell, 1990). Furthermore, it was observed that the NSO fraction appeared very concentrated but showed poor sepreation using gas chromatography, probably because of the fact that this fraction has boiling point range above 400°C. These factors make it difficult to make a reasonable assesment of the degradationof the NSO fraction.

Bioremediation of weathered crude oil(Tar balls)

Oil spilled in water tends to spread and form an oil slick which undergoes various processes of weathering(Fig. 1.5) to form tarballs which eventually get washed ashore. Tarballs aggregates of weathered and undegraded oil which restrict access to microorganisms.(Colwell *et al.*, 1978). Thus after the initial enrichment of saturate and aromatic degraders at sea, the microrganisms degrading more complex hydrocarbons need to employed for effective bioremdiation. Thus a seven membered mixed culture (consortium B) capable of attacking a wide variety of hydrocarbon moities was used.

Studies on the degradation of weathered oil by consortium B indicate that when benzoate was used as the principal substrate, growth kinetics of consortium B initially increased and the remained stationary after 50 days in microcosm C which was supplemented with nitrogen and phosphorous. In microcosm D , growth was much less and reached a lower equilibrium point as compared to microcosm C (Fig. 5.15). The growth kinetics of the other strains revealed that all the other strains grew efficiently and showed higher numbers in microcosm C as compared to microcosm D (Fig. 5.16 , 5.17, 5.18). It is a well established fact that availibility of nitrogen and phosphorous limits the microbial degradation of hydrocarbons in seawater (Dibble and Bartha, 1979).

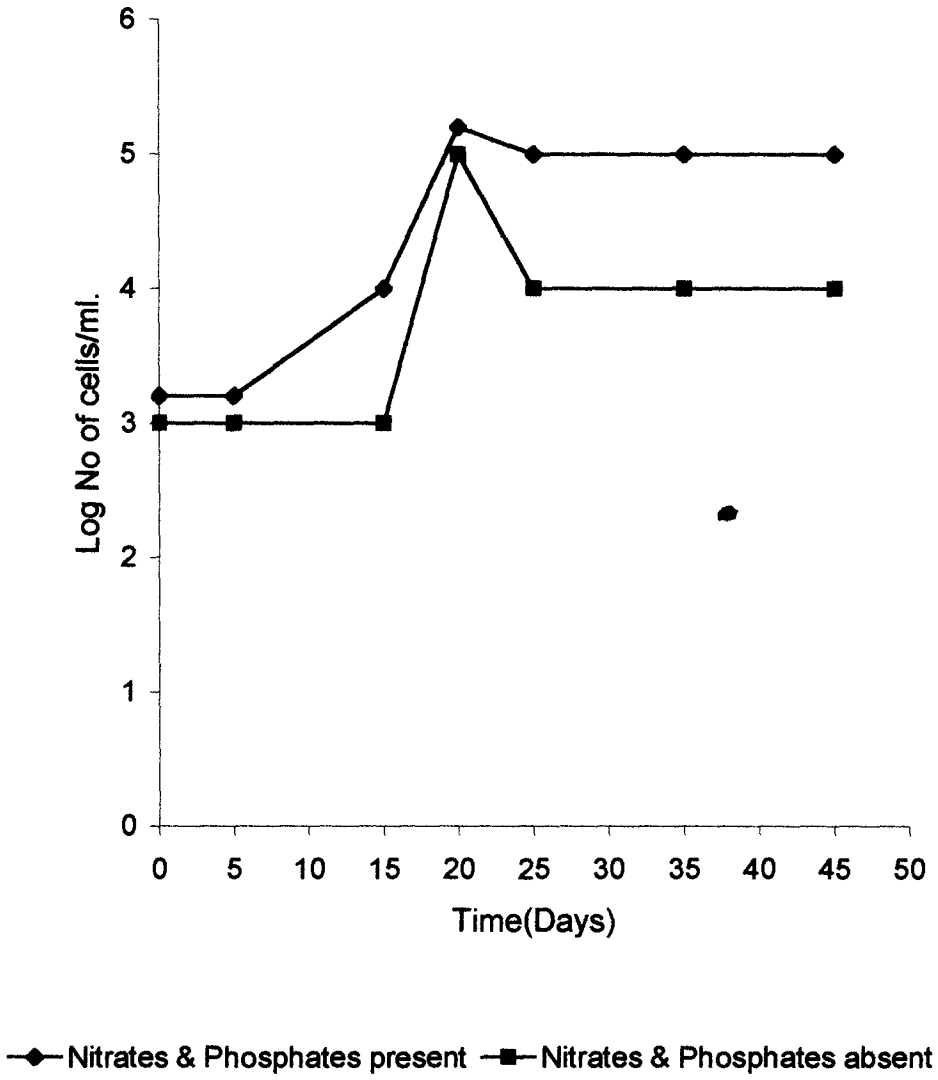


Fig. 5.15. Growth kinetics of seven members of the mixed culture (consortium B) grown in microcosms containing weathered oil with and without nitates and phosphates. The total counts were measured in benzoate since all the strains grew on benzoate medium.

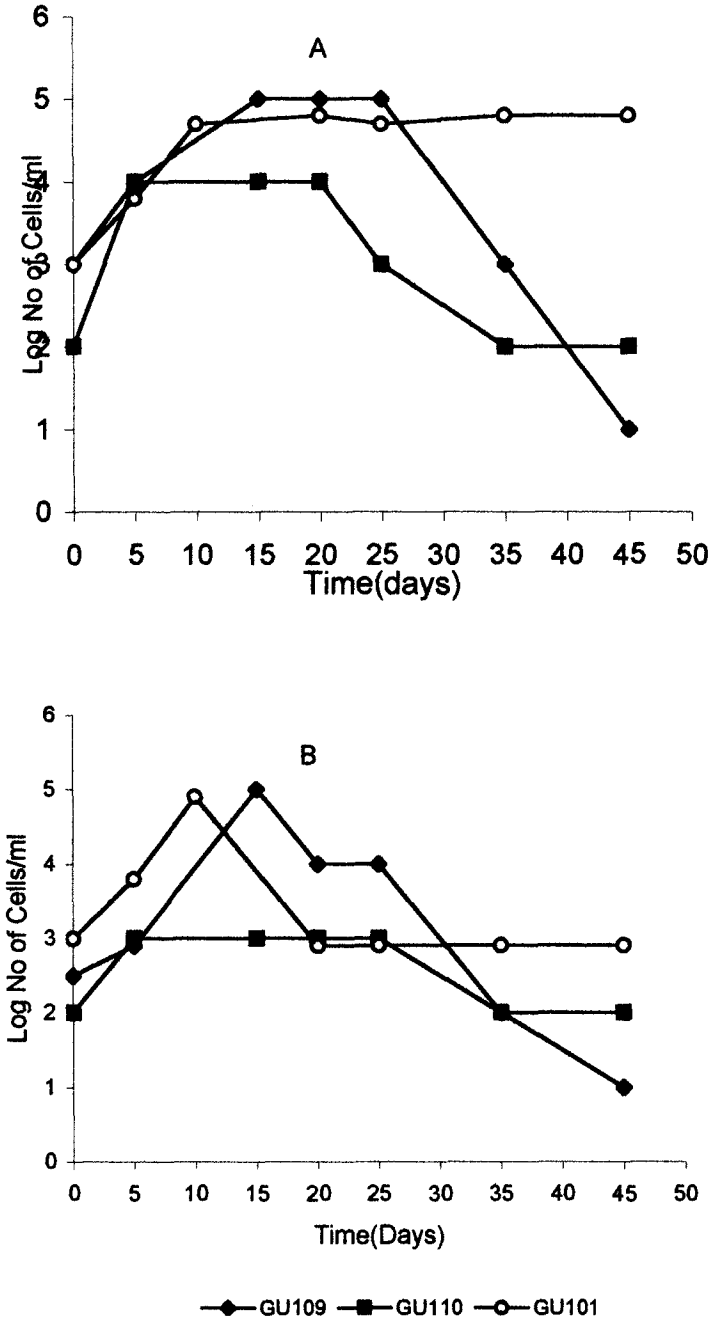


Fig. 5.16.Growth kinetics of strain GU101, GU109 and GU110 in a seven membered consortium B in microcosms containing (A)nitrogen and phosphorous and (B)without nitrogen and phosphorous.

Strain GU109 was able to grow and survive the entire 50 day experimental period as part of the consortium although the numbers slowly decreased nearing the end of the experimental period (Fig. 5.16). The consistency of the weathered oil observed at the end of exposure clearly revealed that the weathered oil gets disintegrated due to microbial action. Charecteriatic growth of the consortia on the surfaces and penetration of the core of the weathered oil was also noticed after analysing cross-sections(Coelho et al., 1995) indicating efficient degradation of weathered oil, which is considered to be extremely recalcitrant.

Growth kinetics of the preserved consortia as part of consortium B in experimental microcosms containing seawater supplemented with weathered oil (tarballs)

The importance of the growth kinetics of the preserved consortia namely GU102, GU103, GU104 and GU105 can be guaged from the fact that these strains were stored at -70°C for one and a half years. Successful amplification in an experimental microcosm would allows the advantage of using different preserved consortia as an when required. The data suggests that this consortium of preserved bacteria was succesfully amplified as part of consortium B in microcosms containing weathered oil as substrate. All the four strain showed reasonably high numbers even after 50 days with the tank supplemented with nitrogen and phosphates showing higher growth (Fig.5.17, Fig. 5.18). What is interesting to note is the significant increase in numbers of the preserved consortium after 5 days growth in microcosms. The data seems to be consistent with the results obtained in this study on the amplification of this consortium in small flasks(Chapter III), which showed a sharp increase after around three days. Thus the data clearly

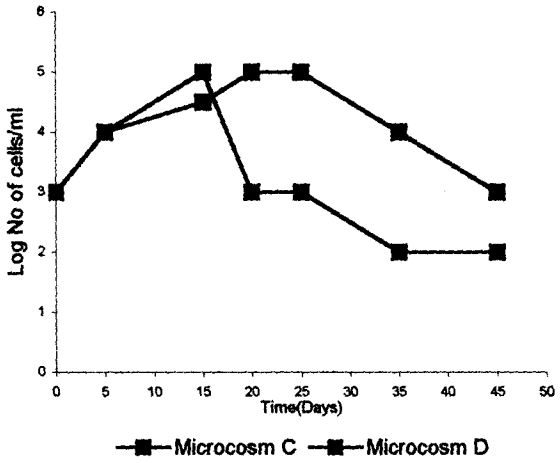


Fig.5.17A. Growth Kinetics of strain GU104, a quinoline degrader in a seven membered consortium grown in microcosms. Microcosm C contains nitrogen and phosphorous, micrososm B is without nitrogen and phosphorous.

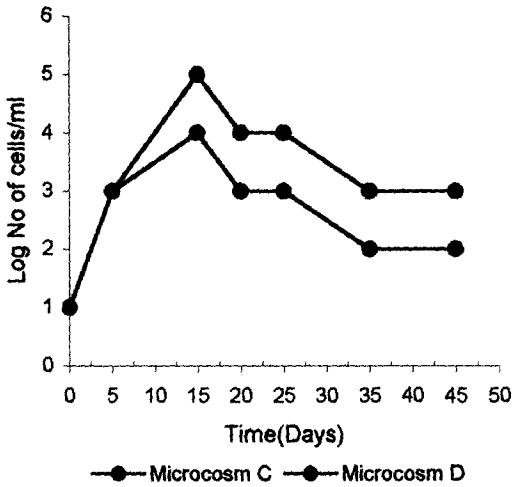


Fig. 5.17B. Growth kinetics of strain GU105, a pyridine degrader in a seven membered mixed culture grown in microcosms. Microcosm C contains nitrogen and phosphorous, microcosm D is without nitrogen and phosphorous.

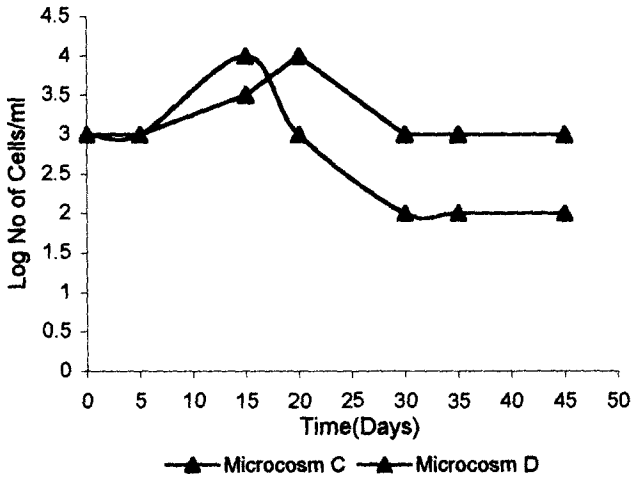


Fig. 5.18A. Growth kinetics of strain GU102, a naphthalene degrader in a seven membered mixed culture (consortium B)grown in microcosms. Microcosm C contains nitrogen and phosphorous, microcosm D is without nitrogen and phosphorous.

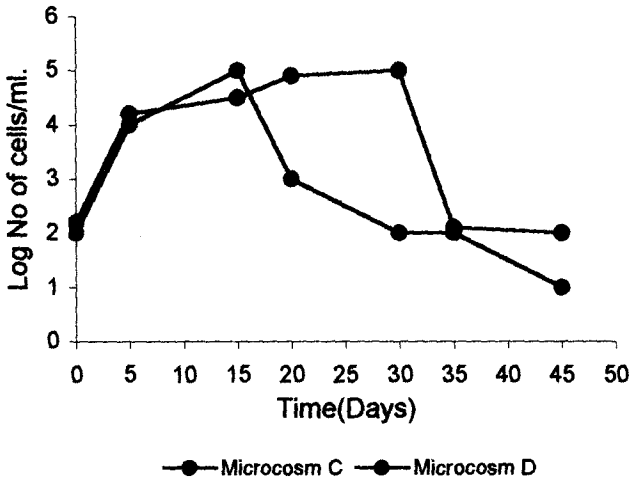


Fig.5.18B. Growth Kinetics of strain GU103, a xylene degrader in a seven membered mixed culture(consortium B) grown in microcosms. Microcosm C contains nitrogen and Phosphorous, microcosm D is without nitrogen and phosphorous.

suggests that the preserved consortium was succesfully amplified in experimental microcosms as observed by bacterial counts with all the strains maintaining a stable equilibrium.

Thus in conclusion consortium A was clearly effective in the degradation of the saturate and aromatic fractions. The 5-11 day time period seems to be the period which shows maximum degradative activity for both crude oil and tarball. However, the role of the natural population in limiting microbial degradation was clearly seen indicating the inportance of this fact when studying microbial populations.

Consortium B on the other hand clearly showed effective bioremediation of weathered oil. Eff ective cryopreservation and amplification of a preserved consortium of four strains as part of consortium B was also demonstrated when challenged with a recalcitrant substrate such as weathered oil. The four strains also maintained reasonably high numbers over the 50 day experimental period in microcosms.

Thus our simulation studies on crude oil and weathered oil(tarball) have clearly moved towards making bioremediation a more credible technology.

Chapter VI

SUMMARY

The degradation of organic pollutants by microorganisms has been studied for many decades resulting in a remarkable understanding of the biochemical pathways and molecular genetics involved in the catabolism of a number of pollutants by different types of microorganisms. It was thus assumed that with the the help of this knowledge the catabolic diversity of microorganisms could be harnessed to solve a wide range of pollution problems (Head, 1998). Despite this valuable basic knowledge, bioremediation is yet to be accepted as a routine technology because of factors such as viability during storage, genetic instability, prominent amongst which is the necessity to isolate and preserve natural marine hydrocarbon degradative strains.

Our studies have on the preservation of a natural marine degradative strain GU101, a m-toluic acid degrader have clearly demonstrated that this strain could be preserved at the beginning of stationary phase for around two and a half years when grown in nutrient broth in ASW and preserved with 15% (v/v) glycerol. Alternatively, GU101 could be grown on its principal substrate, m-toluic acid to early stationary phase and then preserved by the addition of glycerol (15%v/v) and fresh nutrient broth in ASW. The addition of glycerol and nutrient broth was clearly a prime requirement in protecting GU101 from the damage caused by freezing and thawing. The addition of glycerol supplemented with additives has been also employed by Daily and Higgins(1973). Optimal preservation of strains at stationary phase or late stationary phase was also reported by Bretz and Ambrosini (1966) and Heckly(1978). However, as reported by Calcott (1985), it should be noted that each type of strain exhibits its own set of optimal conditions which renders maximum survival.

When the same technique was applied to a mixed culture of four strains namely GU103, a xylene degrader, GU102, a naphthalene degrader,

GU104, a quinoline degrader and GU105, a pyridine degrader, all the strains showed remarkable survival over a two year time period. Growth and amplification of the consortium was observed in a minimum period of time when the consortia was preserved at the 2nd growth phase. Both the equilibrium between the strains and the functional properties were maintained over the entire two year period. The study clearly demonstrates the efficacy of the technique in preserving single and mixed marine strains. The successful preservation and amplification suggests that similar preservation techniques could be employed for marine degradative microrganisms in microbial degradation technologies which would allow rapid amplification of preserved cultures at polluted sites.

Our studies on the distribution of PAH's degraders in the North Arabian Sea clearly showed that this area is constantly exposed to various aromatic compounds resulting in the isolation of PAH's degraders in almost all the sites. What is more important though is that a very simple substrate such as benzaote has proved to be effective in isolating microorganisms capable of mineralizing complex molecules like PAH's and substituted aromatics.

Pseudomonas putida, strain GU109(MTCC 3316), a phenanthrene degrader is clearly a result obtained using the above mentioned technique. The strain clearly degrades phenanthrene via the o-phthalate pathway (Kiyohara and Nagao, 1978). The strain also grew on naphthalene with oxygraph studies showing loss of this ability when grown on lactate suggesting that the two pathways were independent of each other. It is interesting to notes that although strain GU109 could not utilize dibenzothiophene and and fluorene as a sole source of carbon it could degrade/utilize these substrates in the presence of phenanthrene. Clearly the findings suggest that multiple PAH's are being transformed by

a common enzyme system in whole cells, whether the competition is for oxidases, transport enzymes etc. is not clear. Similar results were obtained by Stringfellow and Aitken (1995).

When strain GU109 was grown on crude oil (as a sole source of carbon) it attacked the alkane fraction very rapidly with almost 45% degradation as compared to control. The presence of phenanthrene clearly enhanced the degradation of all fractions of crude oil and this was clearly demonstrated in the growth of strain GU109 on a model hydrocarbon mixture. The results clearly suggest that phenanthrene enhances the degradation of other PAH's and higher compounds by strain GU109. An understanding of the mechanisms involved can contribute to the development of a better approach for modelling and controlling PAH biodegradation within complex mixtures.

Amplification studies of the cryopreserved consortia rvealed that a mixed marine bacterial consortium consisting of strain GU102, a naphthalene degrader, GU103, a xylene degrader, GU104, a quinoline degrader, GU105 and GU105, a pyridine degrader could be amplified in a minimum period of time when preserved at an early stationary phase. The importance of the results in this study indicate that similar preservation methods could be employed for marine degradative microorganisms used in bioremediation technology after accidental release of pollutants such as crude oil/hydrocarbons.

The efficacy of the cryopreserved bacterial mixture as part of consortium A and B in artificial microcosms containing crude oil and weathered oil was clearly demonstrated. When crude oil was used as substrate, all the strains showed remarkable survival with maximum degradation and high microbial activity during the 5 to 11 day growth period as indicated by a drop in pH, dissolved oxygen and gas chromatography profiles of water

samples.

On weathered oil, consortium B showed effective degradation as indicated by changes in the consitency of oil and the survival os strains over the entire experimental period (Coelho *et al.*, 1995). The role of nitrogen and phosphorous clearly seems to enhance biodegradation of weathered oil, although the nature of the water sample and the type of natural populations that exist play an important role in determining nutrient addition (Dibble and Bartha, 1976).

From this study,it is clear that the success of bioiremediation is not limited by a lack of knowledge of pollutant catabolism, but rather a restricted understanding of the interplay between biotic and abiotic factors that determine the outcome of any particular bioremediation process that takes into account these interactions(Head, 1998).

The study has tried to ensure the success of this bioremediation technology by obtaining an easy and rapid method for isolation of natural marine PAH degraders using benzoate as an initial substrate; preservation of these natural microorganisms as single and mixed cultures at –20°C and –70°C uisng nutrient broth and glycerol and rapid amplification of these marine microbial consortia in artificial microcosms, to effectuate the degradation of crude oil/weathered oil. The results have clearly tried to ensure the effectiveness of these methods in bioremediation.

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