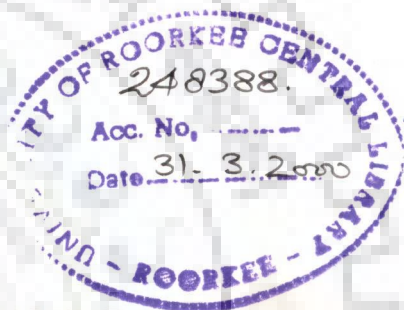# STUDIES ON ESTUARINE OIL SPILL MANAGEMENT

A THESIS

Submitted in fulfilment of the
requirements for the award of the degree
of

DOCTOR OF PHILOSOPHY

By

SUNEEL CHHATRE

DEPARTMENT OF BIOSCIENCES AND BIOTECHNOLOGY
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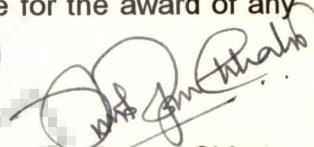ROORKEE-247 667 (INDIA)

JULY, 1998

Candidate's Declaration

I hereby certify that the work which is being presented in this thesis entitled **"Studies on Estuarine Oil Spill Management"** in the fulfillment of the requirement for the award of the degree of DOCTOR OF PHILOSOPHY, submitted in the Department of Bioscience and Biotechnology of the University of Roorkee is an authentic record of my work carried out during a period from April, 1995 to JULY 1998 under the supervision of Dr. P. Khanna, Director, National Environmental Engineering Research Institute (NEERI), Nagpur; Prof. (Dr.) G.S. Randhawa, Department of Bioscience and Biotechnology, University of Roorkee and Dr. H.J. Purohit, Scientist, Director's Research Division, NEERI, Nagpur.

The matter presented in this thesis has not been submitted by me for the award of any other degree of this or any other university.
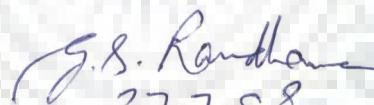
Date : 27.7.98

Suneel Chhatre

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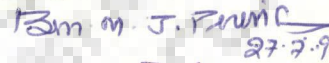
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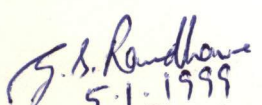
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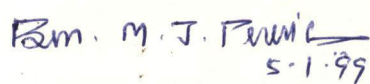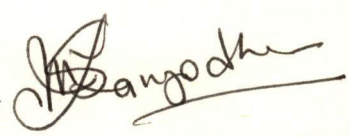The Ph.D. Viva-Voce examination of **Mr. Suneel Chhatre**
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Abstract

Preamble

Oil exploration, refining and transport activities have considerably increased over the past few decades due to increasing demand for petroleum crude world wide. This enhanced activity poses the problems related to oil pollution which are difficult to manage inview of recalcitrant and insoluble nature of oil. Accidental oil spills receive much attention and evoke public concern. Although oil spills occur rarely, they invariably lead to significant contamination of ocean and shoreline environment. Traditionally the oil spills are dealt with physical and chemical methods, which are well established [Ollis, 1995]. However, the biological methods, which are preferable due to ecological reasons, require considerable research endeavour [Prince, 1993]. A number of different technologies may fall into the category of biological methods including the use of straw or plant material as adsorbent for oil, addition of nutrients to enhance biodegradation of crude oil with indigenous microorganisms and addition of allochthonous organisms to the site (bioremediation). The last two procedures fall under programmed bioremediation [Swanell, 1996].

Exxon Valdez oil spill focused attention to alternative clean up methodologies [Pritchard & Costa, 1991]. For example, in physical methods, the use of sorbants is advisable as it facilitates a change of pollutant phase from liquid to semi-solid, which is easy to contain. While hydrophobicity and oleophilicity are primary determinants of successful sorbants, other important factors include retention of oil over time, amount of oil sorbed per unit weight of sorbent, and available specific surface area for sorption [Chol, 1992]. Among synthetic products, polypropylene and polyurethane foam are the most widely used sorbents in oil spill cleanup due to their high oleophilic and hydrophobic properties.

Several reports are available on the degradability of hydrocarbon components of crude oil through biological methods. In *Exxon Valdez* oil spill, nitrogen and phosphorus containing fertilizers were applied in order to accelerate the biodegradation of the oil by indigenous

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microorganisms. Most of the bioremediation techniques currently in use are based on this principle of enhancing oil degrading capabilities of indigenous microbes by adding nutrients [Prince, 1993]. However, a substantial lag period is always observed in microbial utilization of crude oil. Although the utilization of microorganisms specialized in degradation of recalcitrant compounds has been proposed by several researchers, successful applications of exogenous microorganisms to clean up pollutants have not yet been reported [Swanell, 1996]. Since, crude oil is a multisubstrate system of more than 300 compounds, it is not amenable to complete biodegradation by a mono culture system. Mixed culture approach has been reported by several researchers which provides fairly good degradation. However, mixed culture approach has several disadvantages as a process particularly as the rate of biodegradation is a crucial factor in oil spill clean up. Since a multispecies system, like mixed culture, is not fully characterized, the organisms being present in varying ratios undergo uncontrolled selective population growth and ultimately inhibit the degradative activity leading to accumulation of various components of the multisubstrate crude oil system. Another pre-requisite to biotreatment of oil spills in on-site emulsification as the microorganisms effectively attack solubilized components. Further, the biotreatment of marine spills requires that the organisms are augmented with ability to tolerate high salinity.

The present study explores the possibility of use of a two step treatment process for oil spill remediation, where in the first step a cost-effective sorbant is used to absorb crude oil, followed by biodegradation of remaining crude oil by a designed and adapted bacterial consortium. Unlike the mixed population, the constituent of consortia have been synchronized to specific physiological conditions leading to effective biodegradation of crude oil. This biodegradation strategy could also be applied to the "seeding" of biotreatment processes for petrochemical wastewaters/ refinery wastewaters or hydrocarbon contaminated soils.

The present study has the following objectives :

- Physical removal of crude oil using alkali treated sawdust

- Isolation and screening of hydrocarbonoclastic organisms from contaminated site(s)

- Design of consortium for degradation of crude oil and model petroleum/aromatic mixture

- Role of biosurfactant in crude oil degradation

- Determination of the impact of various inducer compounds on the adaption of consortia in favour of degradation of aromatic fraction of crude oil

Physical Skimming of Crude Oil

Hydrophobicity and buoyancy are the main characteristics of successful sorbents. In present study, a biological matrix sawdust has been used as sorbent after alkali treatment. Raw sawdust has a tendency to sink when it is applied on water body. To increase the buoyancy property and specific surface area, sawdust was treated with 1% sodium hydroxide under high temperature (120°C) and pressure (15 lb). The treatment increased the specific area of sawdust from 42 to 96 m^2/g and resulted in pore formation, which was confirmed by scanning electron micrographs. This was further supported by mercury porosimeter studies. The application of alkali treated sawdust yielded 90-95% removal of floating crude oil from water surface.

Isolation and Screening of Hydrocarbonoclastic Organisms for Design of Consortium

A semicontinuous batch fed reactor was used to enrich 5 lt. of activated sludge with crude oil. After six months the chemical oxygen demand of the effluent was reduced to 220 mg/L from 572 mg/L of initial value. At this stage, organisms were isolated by serial dilution plating and subjected to a three tier screening process. Primary screening was based on the morphological characteristics of organisms (colony appearance, optimal temperatur, incubation time etc.). Antibiotic sensitivity was the criterion for seconary screening. Six antibiotics were used for the

test. Tertiary screening involved hydrocarbon utilization potential of organisms. Five representative hydrocarbons (catechol, phenanthrene, dodecane, eicosane and octacosane) were used for final screening. on the basis of degradative charcteristics three bacterial isolates were selected alongwithn *P.putida* MTCC˙ 102 in the design of consortium. All the three selected isolates were subjected to a battery of biochemical tests to characterize their metabolic potential. Presence of certain key dissimilatory enzymes (phenol monooxygenase, catechol 2,3 dioxygenase, alk B) was checked by PCR.

Bacterial Consortium

On the basis of three tier screening; three isolates, viz. NCC.DSS$_3$, GSS$_6$, DSS$_8$ and a procured strain of *Pseudomonas putida* MTCC˙ 102 were used in the design of the consortium for crude oil degradation

NCC.GSS$_3$: gram negative, oxidase and catalase positive, utilises catechol

and phenanthrene efficiently

NCC.DSS$_6$: gram negative, oxidase and catalase positive, produces a biosurfactant

NCC.DSS$_8$: gram negative, oxidase and catalase positive, capable of

utilising long chain aliphatics (octacosane and tetracosane)

P.putida MTCC˙102 : Capable of consuming down stream metabolites formed in

aromatic degradation processes.

The designed consortium was adapted on catechol/ benzoate/ salicylate for 48 hours, and then used for degradation of a model petroleum. Gas chromatographic analysis demonstrated that catechol adapted consortium performed better. Therefore, for crude oil, catechol was selected for preculturing. Capillary gas chromatographic and gravimetric analysis have shown that 65-70% of crude oil is degraded by the designed consortium in 72 hours. Consortium grown on nutrient rich media (Luria Bertani Broth) could not degrade crude oil as efficiently as the adapted one. Similarly the individual organisms also did not exhibit the dissimilation property as

they demonstrated when inoculated together. The efficacy of consortium for crude oil degradation in soil was also tested where black cotton soil was spiked with crude oil. IOCL, Mathura soil, contaminated and weathered, was also subjected to bioremediation successfully. The aromatics present in soil were identified by GC-MS.

Osmotolerance & Hydrocarbon Degrading Potential

Though the designed consortium had metabolic flexibility to attack crude oil components effectively in normal conditions, these soil isolates were unable to tolerate high salt conditions. Therefore the consortium members were imparted with 'U' operon, subcloned in a broad host range vector (pMMB 206). The expression of pro'U' operon in selected transformants was evident from their ability to tolerate and grow under high salt concentration. Since the ptac, a strong promoter, is used in the pMMB 206 to express the operon, its basal expression is sufficient to provide desired level of osmo-protection. The degradation potential of genetically engineered consortium was tested with model petroleum (a mixture of representative molecules present in crude oil) and crude oil under 0.7 M NaCl (4% w/v) condition. It was observed that the addition of salt tolerance phenotype did not hamper the hydrocarbon utilizing capability of the members of the consortium.

Biosurfactant Production by DSS₆ and its Characterization

Biosurfactants are amphibolic molecules synthesized by many oil-degrading bacteria and include glycolipids, lipopetides and polysaccharide-protein complexes [Fiechter, 1992]. NCC. DSS_6 a member of consortium produced a biosurfactant, which was identified as rhamnolipid by Infrared Spectroscopy. The produced biosurfactant lowered the surface tension of liquid media from 72 to 37 dynes/cm in 96 hours. NCC. DSS_6 harbours a plasmid which was proved by alkaline lysis, boiling prep and Qiagen Column methods. The 20 kb plasmid was subjected to restriction digestion to confirm the size. To study the role of plasmid in biosurfactant production,

the plasmid was cured by acridine orange. The cured NCC. DSS_6 could not lower the surface tension of liquid media. The same plasmid was mobilized into *Pseudomonas putida* MTCC 102 by electroporation where it could not impart the property of surfactant production. Retransformation of the cured NCC. DSS_6 with the same plasmid again brought back the biosurfactant production property. These experiments prove that DSS_6 plasmid carries certain essential genetic determinants for biosurfactant operon, while rest of the genetic information is the on chromosome of NCC. DSS_6 .

Crude oil is a mixture of several hydrophobic compounds. For effective biodegradation, availability of these compounds to bacteria is an essential prerequisite. Biosurfactants lower the surface tension of water, and facilitate the formation of oil-in-water emulsion which is followed by crude oil biodegradation process. It was observed that without NCC. DSS_6, a biosurfactant producing organism, the designed and pre-adapted consortium lost the emulsifying potential and could not degrade oil rapidly.

Utilization of Various Fractions of Crude Oil

Crude oil is a heterogeneous mixture of unidentified compounds. Its components are broadly grouped into four classes according to their differential solubility in organic solvents, viz. the saturates (n- and branched-chain alkanes and cycloparaffins); the aromatics (mono-, di-, and polynuclear aromatic compounds containing side chains and/or fused cycloparaffinic rings), the resins (aggregates with a multitude of building blocks such as pyridines, aminolines, carbazoles, thiophenes), and the asphaltenes (aggregate of extended polyaromatics, naphthenic acids, sulphides, polyhydric phenols) [Leahy & Colwell, 1990].

The saturates present in crude oil are more amenable to biodegradation, and degrade rapidly while aromatic and polynuclear aromatic hydrocarbons are less susceptible. The resins and asphaltenes are known to be recalcitrant. To study the utilization of various fractions degraded by the designed consortium, the experimental samples were subjected to silica gel

column chromatography. Consumption of saturates/ aliphatic fraction was 81%, aromatic degradation 63%, while asphalt fraction did not achieve any substantial degradation. The results were confirmed by gas chromatographic profile of saturates and aromatic fraction before and after degradation.

Role of Inducer/ Adapter on Aromatic Degradation

The aromatic and polynuclear aromatic hydrocarbons (PAHs) are less biodegradable in nature. In the experiments with crude oil, the designed consortium degraded aromatic fraction but to a lesser extent. To increase the rate and extent of aromatic degradation, *Pseudomonas putida* ppG7 harbouring NAH plasmid was substituted in the consortium instead of *P. putida* MTCC 102 as the former is capable of degrading napthalene and similar compounds. Adaption on different intermediate/ inducer molecules other than catechol (or cross acclimation) was tried. Benzoate and salicylate were used in the experiments which are known inducers for NAH operon. A standard mixture of eleven aromatic compounds, representing different classes of aromatics (monocyclic, dicyclic, hetero cyclic etc.), was used as substrate. Again it was observed that catechol adapted consortia yielded better results compared to salicylate and benzoate as in case of model petroleum. The rationale behind the use of salicylate/ benzoate/ catechol as inducer was that, in multisubstrate-multispecies system like the consortium and aromatic mixture, the lower molecular weight aromatics would be degraded at a higher rate, and that eventually the down stream metabolites of these compounds in turn activate other operons required for the degradation of the consistuents of crude oil. This cross acclimation was observed in experiments with model petroleum and model aromatic mixture. The consortium was grown on citrate for control experiments. The protein profiles were prepared using SDS-PAGE from the consortium members grown on the inducers (catechol, benzoate, salicylate) and citrate. However, two-dimensional gels did not show any significant change.

Conclusion

Oil spill remediation has been attempted with various physico-chemical methodologies resulting in phase transfer of the hydrocarbons. The decontamination has been addressed with the supply of the nutrients, resulting in eutrophication or bioaugmentation as shown in *Exxon valdez* sea shore pollution studies. The present study deals with the problem through a combination of physico-chemical and biological methods. In the first step, the alkali treated sawdust removes 90-95% of crude oil from the water surface. The remaining oil is subjected to biodegradation by a designed bacterial consortium. The pre-adapted bacterial consortium has specialized organisms to perform different physiological functions for crude oil degradation, and synthetic petroleum wastewater treatment. The genetic modification for osmotolerance (imparting Pro '*U*' operon) extends the use of the consortium to high salt conditions. Biosurfactant induced emulsification enhances the flexibility of the consortium to attack various hydrophobic contaminants. The results from the study suggest that there is a need to evaluate the role of inducer(s) in aromatic degradation and the specificity of certain key enzymes. This may enable complete mineralization of the crude oil fractions to the final end product, viz. Carbondioxide.

Acknowledgements

July 27, 1998

SUNEEL CHHATRE

List of Publications & Presentations

Publications

1. Bacterial Consortium for Oil Spill Remediation

    Suneel Chhatre, Hemant Purohit, Rishi Shanker and Purushottam Khanna.

    Water Science & Technology, 1996, Vol 34, No. 10, p 187-196

2. Oil Spill Remediation : Use of Alkali Treated Sawdust as An Adsorbant

    Suneel Chhatre, Hemant Purohit, Rishi Shanker and Purushottam Khanna.

    In the proceedings of 3rd International Conference on Environmental Protection and Management, p 424-426

3. Microbial Consortia: An Approach for Oil Spill Management

    Hemant Purohit, **Suneel Chhatre**, Atya Kapley and Purushottam Khanna

    ENFO News, Vol 27, March 1997, p 2-3

4. Osmotolerance and Hydrocarbon Degradation of a Genetically Engineered Consortium

    Atya Kapley, **Suneel Chhatre**, Hemant J. Purohit, Rishi Shanker and Purushottam Khanna

    Bioresource Technology (In press)

5. Application of Allochthonous Hydrocarbonoclastic Bacteria in Bioremediation of Weathered PAH Contaminated Soil

    Suneel Chhatre, Hemant J. Purohit, G. S .Randhawa and Purushottam Khanna

    (Manuscript under preparation)

Chapters in Books

1. Biodegradation of Various Fractions of Crude Oil Using a Bacterial Consortium

    S.A. Chhatre, S. Das, H.J. Purohit, R. Shanker, T. Chakrabarti and P. Khanna.

    In Perspectives in Microbiology, edited by Dr. Kahlon

Presentations in National Conferences

1. Biodegradation of Various Fractions of Crude Oil Using a Bacterial Consortium

    S.A. Chhatre, S. Das, H.J. Purohit, R. Shanker, T. Chakrabarti and P. Khanna.

    Abs. No. EM-38, 34th Annual Conference of AMI, Ludhiana, Feb. 9-11, 1994

2. A Modified Strategy for Isolation and Screening of Hydrocarbon Utilizing Bacteria

    S. Das, **S.A. Chhatre**, H.J. Purohit, R. Shanker, T. Chakrabarti and P. Khanna.

    Abs. No. EM-38, 34th Annual Conference of AMI, Ludhiana, Feb. 9-11, 1994.

3. Degradation of Crude Oil in Hypersaline Condition by Genetically Engineered Microorganisms

    S.A. Chhatre, S. Das, H.J. Purohit, R. Shanker and P. Khanna.

    Abs. No. 23, National Seminar on Recent Trends in Biological Science, Raipur, 1994.

4. Degradation of Polycyclic Aromatic Hydrocarbons by Marine Bacteria : Effect of Inducers

    S.A. Chhatre, S. Das, H.J. Purohit, R. Shanker and P. Khanna.

    Abs. No. 1.14, National Symposium on Electrochemistry in Marine Environment, Madras, 1995.

5. Degradation of Aromatics

    Hemant Purohit, **Suneel Chhatre** and Purushottam Khanna.

    37th Annual Conference of AMI, Madras, December 4-6, 1996.

Presentations in International Conferences :

1. Oil Spill Remediation : Use of Alkali Treated Sawdust as An Adsorbant

    Suneel Chhatre, Hemant Purohit, Rishi Shanker and Purushottam Khanna.

    3rd International Conference on Environmental Protection and Management, 22-25th Feb., 1995, VRCE, Nagpur.

2. Bacterial Consortium for Oil Spill Remediation

    Suneel Chhatre, Hemant Purohit, Rishi Shanker and Purushottam Khanna.

18th Biennial Conference of International Association of Water Quality (IAWQ), 23-27 June, 1996, Singapore.

3. Oil Spill Remediation: Role of Biosurfactant

    Suneel Chhatre, Hemant J. Purohit, G. S .Randhawa, Rishi Shanker and Purushottam Khanna

    International Symposium on Bacterial Genetics and Pathway Engineering, Nagpur, 1997

4. Bioavailability-A key in Bioremediation of Crude Oil Spills & Pollution

    Hemant J. Purohit, **Suneel Chhatre,** G.S. Randhawa and Puroshottam Khanna

    To be presented at The Queen's University of Belfast (Ireland), ISEB'98 Symposium, June 22-27,1997

Patents Filed

1. An improved process for skimming oil from marine and fresh water, Patent No.1517/DEL/94.

2. Bacterial Consortium for Oil Spill Remediation & Pollution Control (US Patent Filed).

Abbreviations

ATSD - Alkali Treated Sawdust

BH - Bushnell-Haas

BHC - Bombay High Crude

CGC - Capillary Gas Chromatography

COD - Chemical Oxygen Demand

DTT - Dithiothreitol

EDTA - Ethylene diamene tetra acetate

FID - Flame Ionization Detector

GC - Gas Chromatography

GC-MS - Gas Chromatography-Mass Spectroscopy

GHC - Gulf High Crude

IR - Infra-red Spectroscopy

L - Liter

LB - Luria Bertani

M - Molar

mg - Milligram

ml - Milliliter

mM - Milimolar

mm - Millimeter

nm - Nanometer

PAGE - Polyacrylamide Gel Electrophoresis

PAHs - Polynuclear Aromatic Hydrocarbons

PCR - Polymerase Chain Reaction

SDS - Sodium dodecyl sulphate

ST - Surface Tension

TE - Tris-EDTA

USEPA - United States Environment Protection Agency

USTs - Underground Storage Tanks

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Chapter 1

Introduction

Introduction

Oil fuels our modern industrial society. Petroleum and petroleum products are key energy options in the modernization process. Due to this compelling need, world energy consumption has grown fifteen folds since 1900 and so has grown the production of petroleum. The impact of resultant activity has made petroleum a leading contaminant in both prevalence and quantity in environment.

Most of the oil is transported by sea during its life cycle between production and final consumption. Despite the best effort of petroleum industry and consumers, some oil is inevitably spilled [Prince,1993]. While accidental releases may contribute to only a small percentage of the oil released into the marine environment, large accidental oil spills receive much attention and summon considerable public concern. Beginning in 1960s and thereafter, several massive spill incidents were reported such as the grounding of the tank vessel *Torey Canyon* off Lands Ends, England in 1967, spilling 38 million gallons; grounding of the *Amoco Cadiz* in 1978 off the cost of France, spilling 76 million gallons; and the most publicized *Exxon Valdez* spill in Prince William Sound, Alaska in 1989, losing 10.8 million gallons [US Congress,1980; Hunt & Park,1997]. More recently, on March 18, 1996, the tank barge *Buffalo 292* spilled approximately 0.126 million gallons into the water of Galveston Bay, Gulf of Mexico [Clark *et al.*,1997]. Oil spilled on water tends to form a slick, which moves due to the tidal waves and in the direction of winds in the area. *Buffalo 292* spill (0.126 million gallons) moved 35 nautical miles within the first day of the spill, whereas *Exxon Valdez* (10.8 million gallons) spill drenched 1200 miles of coastline disrupting many wildlife and fishery resources [Environment Reporter,1991 ; Hunt & Parks,1997].

The traditional methods to cope with spills are confined to physical containment and collection using floating booms, dispersal by detergents and incineration [Ollis,1995]. However,

these methodologies lead only to phase transfer of the pollutants. For instance, *in-situ* combustion often leads to production of benzopyrene and its derivatives, which result in an increase in the toxicity due to residues, generated due to incomplete combustion. Use of chemical detergents to disperse oil slick has been banned in several countries due to its toxic effects. The discussion on traditional oil cleanup techniques would not be complete without its economic aspects. The requirement to maintain a large preparedness in terms of manpower, equipment and materials with their use exclusively restricted to intermittent emergency basis, renders such operations extremely cost-ineffective.

The limitations, side effects and high expense on traditional clean up techniques have stimulated interest in unconventional alternatives. *In-situ* bioremediation can be regarded as an option with extension of the role of microorganisms of breaking down of complex human, animal and plant wastes so that life can continue from one generation to the next. Without the activity of microorganisms in nature, the Earth would be buried in wastes, and the nutrient necessary for the continuation of life would be locked up in detritus. Nature maintains this equilibrium by using microbial capacities. The microbial world is characterized by an incredible metabolic and physiological versatility that permits microorganisms to inhabit hostile ecological niches and to exploit, as carbon and energy sources, compounds unpalatable for higher organisms. This metabolic versatility has led to the notion of microbial infallibility- their ability to degrade and to grow at the expense of any organic material- and is also the basis of the recycling of recalcitrant organic matter in the biosphere. It is therefore natural and logical that the enormous metabolic potential be harnessed to develop effective biotechnological processes for the elimination of petroleum hydrocarbons. Bioremediation is a rapidly developing technology for environmental restoration, utilizing natural microbial activity to combat hydrocarbon contamination due to oil spills and by being least intrusive and environmentally most sound.

Biotechnological processes to destroy toxic wastes offer many advantages over physico-chemical processes. When successfully operated, the microbial technologies are non-polluting because of the complete destruction of organic wastes, i.e. conversion of toxic organic compounds to innocuous C1 compounds and also offer the possibility of *in situ* treatment. Biotechnological processes are based on natural activity of microorganisms and constitute variations on classical domestic waste treatment processes, therefore public acceptability is higher than incineration or dispersal through synthetic detergents, which are increasingly difficult to site owing to resistance extended by affected communities.

The largest field bioremediation test for oil spill cleanup ever attempted and followed scientifically was conducted by the US Environmental Protection Agency (EPA) and the Exxon Corporation on the shoreline of Prince William Sound, Alaska following the spill from the supertanker *Exxon Valdez* in March 1989. In this three year program, nutrients (nitrogen and phosphorus were applied to increase the biodegradative capacity of indigenous population. Now bioremediation implies more than mere addition of nutrients. Cultures and culturable products have been added to different environments to stimulate or enhance biological removal of contaminants. Some of the investigators have demonstrated enhancement of biodegradation by bioaugmentation, while others failed to demonstrate such enhancement. Despite earlier setbacks, the concept of 'seeding' seems to be still attracting attention of researchers. Next step in bioremediation/bioaugmentation will demand strong support of molecular biology tools to assess the biodegradative capacity and tracking the inoculated strains. The maintenance of the desired density of the active catabolic population can only be assessed with high sensitivity tools like gene probes and polymerase chain reaction.

Chapter II

Literature Review

Literature Review

For several millennia, humans have used petroleum, first obtaining it from natural seeps and hand dug wells, and later from deep drilled wells using sophisticated extraction and processing methods. Commercial wells were first drilled around 1859. Since then, global energy use has grown approximately 4-5% per year with petroleum constituting one half of the fuel supply. The growing petroleum and petrochemical industry has made crude oil/hydrocarbons mixtures a leading contaminant, especially in the ocean.

The following literature review includes sources and occurrences of oil pollution, traditional means to combat oil pollution, microbial potential to degrade different components of crude oil and approaches for bioremediation of oil spills (by both indigenous and exogenous microbes).

2.1 Sources and Occurrences of Oil Pollution

2.1.1 Inland

Petroleum hydrocarbons enter fresh water environment in a variety of ways including land run-off, shoreline facilities, leaks and spills from vessels, pipelines and underground storage tanks (USTs). Often the discharges are small especially from non-point sources. However, the cumulative effect of these discharges is apparent in a large number of environments. The USA records approximately 10,000 spills per year and estimates that 10-30% of the USTs are leaking.

2.1.2 Marine

The major sources of oil entering marine environments include offshore production, marine transportation, atmosphere, and land based. Just less than half of all marine petroleum pollution is associated with transportation activities. In 1989, The US Energy Administration estimated that worldwide consumption of petroleum hydrocarbons was of the order of 10^{12} US gallons per year (3.8×10^{12} L per year). Much of this oil travels by water at some time between

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production and final consumption, and unfortunately, despite the best efforts of the petroleum industry and consumers, some of it is inevitably spilled. If we define major spills as those releasing > 10,000 US gallons, the Persian gulf war made 1991 the worst year for catastrophic oil releasing since 1979, with the Persian gulf spill of approximately 240×10^6 US gallons accounting for approximately 86% of the total [Welch & Yando,1993]. 1979 was the previous worst year, due to *IXTOC 1* blow out in Ciudad del carmen, Mexico releasing some 140×10^6 US gallons. These spills dwarf those from tankers, for example the largest tanker spill was probably that from *Amoco Cadiz* off Brittany, France in 1978, which lost about 67×10^6 US gallons [Bellier & Massart,1979].

2.2 Remediation

Traditional methods to cope with oil pollution/spill include physical containment using floating booms, adsorption by applying suitable adsorbents, dispersion by chemical detergents, incineration, etc.[Ollis,1995]. In this review the emphasis is on oil spill remediation in aquatic environment.

2.2.1 Physical Skimming of Crude Oil

Physical collection of the oil with booms and skimmers is generally the first priority of responders, but this is rarely easy and it is not usually completely effective after a large spill. Therefore, several sorbents have been tried for cleanup.

Oil sorbent material can be categorized into three major classes:

- inorganic mineral products
- organic synthetic products
- organic vegetable products

2.2.1.1 Mineral Products

Mineral products include perlite, vermiculites, sorbent clay and diatomite [Melvold *et al.*,1988; Schatzberg,1971]. These material do not show adequate buoyancy retention and their oil sorption capacity is generally low [Chol,1992].

2.2.1.2 Synthetic products

There has been a surge in the use of synthetic resins as sorbents for the removal of organic compounds from water. Advantages of their application are that they can be tailor made for specific situations and can be regenerated in situ [Johnson *et al.*,1973]. Most resin sorbents are polysterene-divinylbenzene copolymers or polymethacrylates cross-linked with an appropriate non-aromatic material [Kobayashi *et al.*,1977]. These sorbents commonly termed macroreticular resins, are characterized by being highly porous and having a large surface area. The pore size distribution is controlled during manufacturing by varying the level of cross-linking. Another important feature is the absence of any ionic functional groups in their structure. Therefore, they can serve as hydrophobic adsorbents capable of removing hydrophobic solutes from solution via van der Waals interactions. They are used also for removing chlorinated pesticides and dyes from industrial wastes.

Among synthetic products, polypropylene and polyurethane foam are the most widely used sorbents in oil spill clean up because of their high oleophilic and hydrophobic properties [Chol,1992]. A disadvantage of these materials is that they are not amenable to biodegradation and after the use they persist in the environment.

2.2.1.3 Organic Vegetable Products

It has been reported that organic vegetable products such as straw, corn cab and wood fibre showed poor buoyancy characteristics, relatively low oil sorption capacity and low hydrophobicity [Schatzberg,1971]. On the other hand, Johnson *et al.* [1973] later demonstrated the potential of cotton fibre to clean up oil spill. More detailed information is needed, however, before any further application of cotton fibre can be made in oil spill clean up. Kobayashi *et al.* [1977] examined a hollow cellulosic kapok fiber. According to their results, the oil sorption of kapok fiber used in a mat, block, band or screen was approximately 1.5-2.0 times greater than that of polypropylene mat, which sorbs 11.1g of B-heavy oil and 7.8g of machine oil in water. Later Chol [1992] demonstrated the oil absorbency of milkweed floss (*Asclepias*) which is also a hollow cellulosic material [Knudsen,1990]. Milkweed floss is expected to have hydrophobic

properties due to the surface waxes. It was reported that milkweed sorbed 40 g of crude oil/g of fiber at room temperature.

These studies show that natural sorbents can replace synthetic sorbents with an added advantage of being biodegradable. Plant material, which has cellulose, hemicellulose and lignin as chief building material, can be treated to increase the specific surface area, which is a crucial factor affecting oil sorption.

2.2.2 Use of Dispersants in Oil Spill Remediation

Oil spilled in water tends to spread and form a slick. As a result of wind and wave action, oil-in-water or water-in-oil (mousse) emulsion are formed [Leahy & Colwell,1990]. Dispersion of hydrocarbons in water column in the form of oil-in-water emulsion increases the surface area of oil and thus its availability for microbial attack. Therefore dispersants are used mainly to promote the natural biodegradation process by making hydrocarbons available to existing hydrocarbon degrading population. Artificial dispersants have been studied as an option to increase the surface area and hence the biodegradability of slicks. Dispersion formulations used in 1960s were highly toxic, and their application to oiled intertidal areas following the *Torrey Canyon* spill resulted in widespread mortality of flora and fauna [Colwell,1971]. More recently developed dispersants such as Corexit, are considerably less toxic but still have been shown to inhibit microbial processes [Griffiths *et al.*,1981]. The effectiveness of dispersants in enhancing the biodegradation of oil has been shown to be extremely variable and to be dependent on the chemical formulation of dispersant, its concentration and the dispersant/oil ratio. The widely accepted theory is that, chemical detergents/dispersants could lead to cell wall lysis or instant release of otherwise insoluble hydrocarbons, thereby inhibiting the bacterial growth.

2.2.3 Bioremediation of Oil Spills

Bioremediation is a rapidly developing field of environmental restoration, utilizing natural microbial activity to reduce the concentration and /or toxicity of various chemical substances. Oil spill bioremediation methods aim at providing favorable conditions of oxygen, temperature and nutrients to maximize biological hydrocarbon breakdown [Korda *et al.*,1997]. The core of

bioremediation lies in the amenability of pollutant molecules to biodegradation. Considering that, almost all components of petroleum are degradable, bioremediation promises to be more effective than other conventional methods.

2.2.3.1 Microbial Degradation of Hydrocarbons

The dissimilation of petroleum hydrocarbons by microbes has been known for a long time. The microbes ultimately consume oil accidentally released in the environment. Hydrocarbon metabolizing bacteria have been found almost everywhere they have been diligently sought. Bioremediation aims to simulate this consumption or complete destruction of hydrocarbons. Crude oil contains hydrocarbons ranging from methane to molecules with molecular weights in millions. A majority of these molecules are indeed composed solely of carbon and hydrogen, but most oil contains a small percentage of organic sulfur and traces of organic nitrogen [Prince,1993]. The myriad different molecules in petroleum products are usually grouped into fractions depending on their physicochemical properties. The most widely known and accepted classification is based on their differential solubility in organic solvents, which categorizes heterogeneous mixture of crude oil into four major classes : aromatics, saturates, resins and asphaltenes [Harayama,1996]. Most, if not all, of the hydrocarbons present in crude oil is amenable to biodegradation that is the basic core of bioremediation processes.

2.2.3.1.1 Biodegradation of Saturates

Hydrocarbons within the saturate fraction include n-alkanes, branched alkanes and cycloalkanes (naphthenes). The n-alkanes are generally considered the most readily degraded components in a mixture [Atlas, 1981]. Biodegradation of n-alkanes with molecular weights upto n-C_{44} have been demonstrated [Haines & Alexander, 1974]. Lal and Khanna [1996] also reported similar results where *Acinetobacter calcoaceticus* and *Alcaligenes odorans* were used. Both the strains grew well on pure n-alkanes upto C_{33} (tricontane). Organisms also utilized branched-chained alkanes (pristane) but they could not grow on cyclo-alkanes [Lal & Khanna, 1996]. Cycloalkanes are particularly resistant to microbial attack [Perry,1979]. Complex alicyclic

compounds such as hopanes (tripentacyclic compounds) are among the most persistent components of petroleum spills in the environment.

The degradation of n-alkanes normally proceeds by a monoterminal attack ; usually a primary alcohol is formed followed by an aldehyde and a monocarboxylic acid. Further degradation of the carboxylic acid proceeds by β-oxidation with subsequent formation of two carbon unit shorter fatty acids and acetyl coenzyme A, with eventual liberation of CO_2 [Atlas,1981].

2.2.3.1.2 Biodegradation of Aromatics

Crude oil contains numerous aromatic hydrocarbons and cyclic compounds, which range in size from monocyclic to fused ring structures. When oil is spilled in the environment, low molecular weight compounds may be lost by evaporation. The larger polycyclic aromatic hydrocarbons (PAHs) and heterocycles remain, and their ultimate environmental fate may be determined by microbial degradation [Foght & Westlake,1988]. Perhaps the most effort has been expended on the aromatic hydrocarbons due to their genotoxicity and the biodegradation of aromatic these compounds has been extensively reviewed by Gibson and others [Gibson,1968 & 1971]. Ability of bacteria to metabolize small aromatic hydrocarbons such as toluene, xylenes and naphthalene and also with more than four rings has been reported [Leahy & Colwell, 1990]]. Foght and Westlake [1988] reported the constitutive degradation capability of one isolate, Pseudomonas HL7b. The organism was isolated from an enrichment culture growing on aromatic fraction of crude oil. It was found to be capable of mineralizing and/or degrading a wide range of PAHs, S-,N- and O- heterocyclic analogues and alkyl polycyclic aromatic hydrocarbons but not aliphatic hydrocarbons. Lal and Khanna also reported degradation of anthracene, phenanthrene, dibenzothiophene, fluorene, fluoranthene, pyrene and chrysene by isolate *Alcaligenes odorans* P20 [Lal & Khanna,1996]. Among the PAHs, the biodegradability decreases with increase in the number of benzene rings [Herbes & Schwall,1978]. One significant aspect of aromatic

degradation is the use of mixed culture as proven by Sugiura *et al.* [1997]. An isolate *Acinetobacter* sp. T4 and a microbial consortium called SM8 were examined in the study.
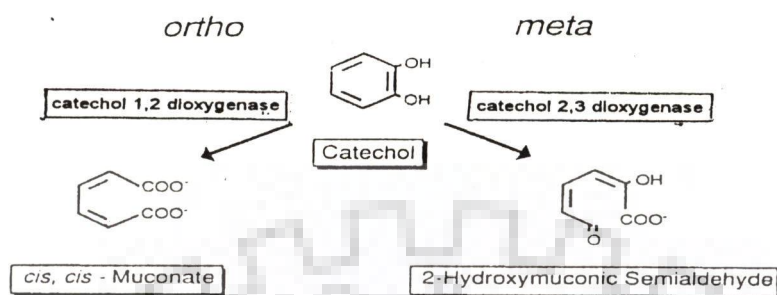
Fig.2.1 : Introduction of molecular oxygen into catechol by two alternative mechanisms
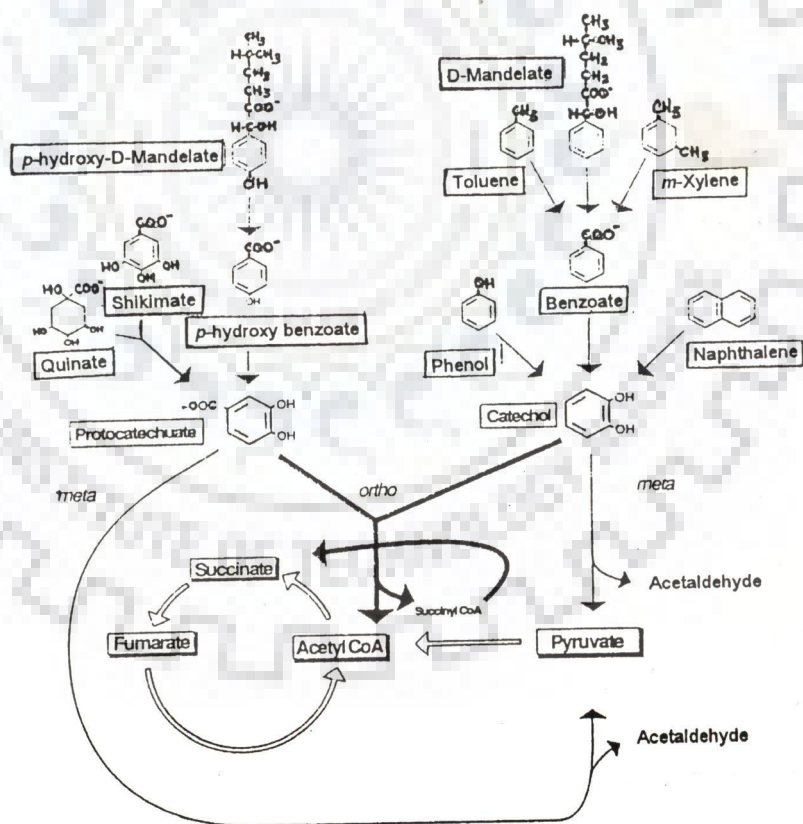
Fig.2.2 : Convergent aromatic catabolism-dissimilation of a variety of aromatics into Krebs'cycle channeled through few "key" intermediates

Acinetobacter could not degrade PAHs of crude oil. In contrast, SM8 degraded the polycyclic aromatic compounds [Sugiura *et al.*,1997].

As far as the biochemical interpretation of aromatic metabolism is concerned, few terms have more definitive meaning than ortho- and meta- cleavage of dioxygenated aromatic ring structure (Fig.2.1).

The dissimilatory pathway for several aromatic compounds such as naphthalene, phenanthrene, anthracene etc. are known and it has been established that few catabolites like catechol and benzoate are common in several known pathways (Fig.2.2) which further can be cleaved by either ortho- or meta-pathway enzymes. With regards to aromatic hydrocarbon metabolism, the ultimate destination of these peripheral degradative pathways is one of the more central metabolic process, the Kreb's cycle.

2.2.3.1.3 Biodegradation of Asphaltenes/Resins

Microorganisms that biodegrade a wide variety of saturates, aromatics and PAHs have been documented [reviews : Atlas,1981; Leahy & Colwell, 1990; Prince,1993]. However, resins and asphaltenes are considered as recalcitrant to biodegradation. After primary degradation, residual oil contains a greater proportion of resins and asphaltenes. Asphaltic components are complex structures viz. aggregates with a multitude of building blocks such as pyridines, quinolines, carbazoles, thiophenes, sulfoxides and amides and/or aggregates of extended polyaromatics, naphthenic acids, sulfides, polyhydric phenols, fatty acids and metalloporphyrins [Leahy & Colwell,1990; Sugiura *et al.*,1997]. Earlier, it was difficult to analyze these structure with then existing methodologies [Atlas,1981]. However, the elucidation of biochemical fate of asphaltic petroleum compounds has become possible due to the advanced analytical instrumentation techniques such as mass spectrometry, NMR spectrometry etc. Still, no uniform degradative pathway, comparable to those established for aliphatic and aromatic hydrocarbons, has yet emerged so far for these compounds. As far as the microbial potential for asphaltene

degradation is concerned, though Rontani *et al*,. [1985] reported the bacteria that degraded asphaltene/resins by cometabolism, no single organism was reported to utilize resin as a sole source of carbon and energy till 1995 when Venkateswaran and coworkers reported a *Pseudomonas* sp. UN3, isolated from a mixed population, that degraded 30% of resins. This strain also degraded saturates and aromatics (30%) present in crude oil (5,000 ppm). Though the cometabolism of resin was reported earlier by other groups [Cundell & Traxler, 1974; Rontani *et al*.,1985], this is the first report of direct utilization of these compounds as a sole source of carbon and energy by a single microorganism. In earlier studies, the presence of saturated hydrocarbons (C_{12}-C_{18} n-paraffins) was a pre-requisite for the degradation of resins and asphaltenes.

2.2.3.2 Role of βiosurfactants in βiodegradation

The physical state of petroleum hydrocarbons has a marked effect on their biodegradation. At very low concentrations hydrocarbons are soluble in water, but most oil spill incidents release petroleum hydrocarbons in concentration far in excess of solubility limits (section 2.1, Sources & Occurrences). Wodzinsky and LaRocca [1977] found that liquid aromatic hydrocarbons were utilized by bacteria at the water-hydrocarbon interface but solid hydrocarbons were not metabolized. At 30^0C diphenylmethane is a liquid and could be degraded, but at 20^0C the solid form of diphenylmethane could not be metabolized by a *Pseudomonas* sp. They also found that naphthalene could not be utilized in the solid form but could be utilized if dissolved in a liquid hydrocarbon [Wodzinsky & LaRocca,1977]. Microbial consumption of water soluble substrates seems to present no problem but how microbes interact with materials that are inherently insoluble or sparingly soluble, is the question of utmost importance if the bacteria are to be exploited for pollution abatement. Three mechanisms are often invoked, and different bacterial species may use one or more of them : interaction with the low level of dissolved material, direct contact with drops or surfaces of the insoluble phase, and interaction with hydrocarbon "solubilized" by interaction with surfactants [Prince,1993]. Biosurfactants play an important role in latter two mechanisms, both attached to the cell surface to enhance attachment of the organisms to the oil, and excreted into the medium to enhance solubility. There is extensive literature on

these quite diverse compounds [Zajic & Mahomedy,1984 ; Zhang & Miller,1992 ; Bury & Miller,1993 ; Rocha & Infante,1997]. Zajic and coworkers [1974] had characterized the emulsifying agents produced by strains of *Pseudomonas* and *Corynebacterium*. In the coming years, the biosurfactants attracted lot of attention and several studies were conducted to determine their chemical nature and to asses their applicability in various industrial activities including their role in bioremediation. Generally, it can be said that biosurfactants are surface-active compounds produced by certain bacteria, yeast and fungi. These include a wide variety of chemical structures such as glycolipids, lipopeptides, polysaccharide protein complexes, phospholipids, fatty acids and neutral lipids [Iqbal *et al.*,1995 ; MacDonald *et al.*,1981 ; Persson *et al.*,1988]. Biosurfactants offer potential for dealing with oil spills by changing the interfacial properties of the oil-water interface, thereby increasing the dispersion and solubilization of organic compounds having limited water solubility. Moreover, these are ideally suited for environmental purposes because of their possibility of *in situ* production and being biodegradable and non-toxic [Kossaric *et al.*,1987]. Synthetic detergents could also be used for the dispersion of hydrophobic compounds [Tiehm,1994]. It was shown that certain non-ionic detergents and sodium dodecyl sulphate increased the solubility of PAHs in water phase. However, the degradation of PAHs was inhibited by sodium dodecyl sulphate because the detergent was preferred as a growth substrate over PAHs [Tiehm,1994]. Moreover, the use of synthetic dispersants has been hampered by USEPA guidelines and other countries are also following the same. Biosurfactants are biodegradable and can be produced on renewable substrates [Juwarkar *et al.*,1995] and thus have the potential to replace chemically synthesized surfactants, provided that the physiology, genetics and biochemistry of the biosurfactant producing organisms are better understood [Fiechter,1992].

2.2.3.3 Biodegradation of Crude Oil : A Multisubstrate-Multispecies Phenomenon

Effective biodegradation of crude oil is the key to successful bioremediation program. A number of organisms have been isolated and tested for their capability to degrade crude oil. Despite the hugely acclaimed versatility of microbial potential to degrade petroleum

hydrocarbons, no single species of microorganisms can degrade all the components of a given oil [Office of Technology Assessment,1991 ; Korda *et al.*,1997]. Currently several organisms are known, each capable of degrading usually one or, at best, a few petroleum components at a time. Heterogeneity of crude oil requires simultaneous action of different organisms. Therefore associations of different organisms are tested for oil degradation. In one of the studies, two types of Indian crude oil (Bombay High and Gujarat) were tested for their biodegradability by *Acinetobacter calcoaceticus* and *Alkaligenes odorans* [Lal & Khanna,1996]. *Acinetobacter calcoaceticus* S30 and *Alkaligenes odorans* P20 degraded Bombay High crude oil by 50% and 45%, while only 29% and 37% Gujarat crude oil (Heavy crude oil) was degraded by these isolates, respectively. In combination, they degraded 58% and 40% of Bombay High and Gujarat crude oil. The oil was quantified by gas chromatography and gravimetry in the experiments. Thin layer chromatography in combination with flame ionization detector (FID) was used to quantify crude oil by Harayama and coworkers [Harayama *et al.*,1996] and a mixed population of marine bacteria was investigated for crude oil degradation. 50% of saturated and 18% of aromatic fraction were degraded by the mixed population in 30 days. While there is considerable information on the microbial utilization of chemically defined hydrocarbons, there have been very few studies concerned with the relationship between biodegradability and physicochemical properties of petroleum. The biodegradation of four different crude oil samples, namely, Arabian light, Dubai, Maya and Shengli by *Acinetobacter* sp. T4 and by a microbial consortium called SM8 was examined in a study [Sugiura *et al.*, 1997]. The crude oil samples with higher API gravity were found more susceptible to biodegradation.

2.3 Bioremediation Approaches for Oil Spill

Since the microorganisms have the potential to utilize crude oil components as a carbon source, it could be exploited to cleanup oil spills. The stimulation of biodegradation or fastening the process of natural biodegradation is bioremediation. Bioremediation may be defined as the process of encouraging the natural process of biodegradation to clean up spills of all kind

[Prince,1993]. It may involve aiding and abetting the indigenous population in an affected environment; adding exogenous microbes from other sites; or even adding engineered strains with particularly desirable traits under controlled conditions.

2.3.1 Aiding and Abetting the Indigenous Population

The oil degrading organisms seem to be ubiquitous, with their number typically limited by the hydrocarbon supply. An oil spill removes this limitation and the oil degrading population undergoes a population explosion [Walker & Colwell,1976]. Where once the growth of hydrocarbon degrading organisms was limited by the hydrocarbon availability, it is now limited by other factors. In aerobic environments, the most likely limiting nutrients are nitrogen and phosphorus [Atlas & Bartha,1973] followed by iron [Dibble & Bartha,1976]. Therefore the most commonly used approach has been the application of fertilizers.

There have been numerous proposals for stimulating biodegradation. For example, Table 2.1 lists some of the patents that have been issued over the years, and it is by no means an exhaustive list. More than 40 companies offer bioremediation products and services in the 13th edition of the *International Oil Spill Control Directory* [1993]. All seek to increase the natural biodegradation process, but by several different mechanisms. Perhaps the simplest are those that aim to enhance the natural biodegradation process by adding nitrogen and phosphorous nutrients that will stay with the oil rather than wash away into the marine environment (Table 2.1). An appealing suggestion, although perhaps at first glance counter-intuitive, is to add a readily degradable carbon source with, or containing, the nitrogen and phosphorus to stimulate microbial growth and nutrient uptake [Olivieri *et al.*,1983; Ladousse & Tramier, 1991; Basseres *et al.*, 1993; Salomone, 1973]. Others have aimed to stimulate biodegradation by increasing the surface area of the oil so that there is a larger area available for microbial attack, for example, by adding surfactants [Foght *et al.*, 1989] or liposomes [Gatt *et al.*, 1991]. Other proposed approaches combine microorganisms [Mohan *et al.*, 1979] or nutrients [Lepain and Bronchart, 1984; Broanchart *et al.*, 1985] into an oil-dispersant package to stimulate the degradation of the dispersed oil.

Table 2.1 : Representative U.S. Patents on Oil Spill Bioremediation

U.S. Patent No.	Year	Authors	Title	Principal claim
3,728,279	1973	G.H.Salomone	Method and composition for the emulsification and degradation of pertoleum products and fertilizers thus obtained	Carbohydrate or protein supplement
3,769,164	1973	E.N.Azarowicz	Microbial degradation of petroleum	16 microbial strains
3,843,517	1974	R.W.McKinney, and R.L.Jordan	Methods for elimination of oil slicks	Freeze-dried microbes in microporous carrier
3,883,397	1975	P.M.Townsley	Material for biological degradation of petroleum	Nutrients encapsulated in fatty acid salts
3,959,127	1976	R.Bartha and R.M.Atlas	Biodegradation of oil on water surfaces	Oleophillic nitrogen and phosphorous fertilizers
4,087,356	1978	W.Marconi, R.Oliviere, L.Degen, and A.Robertiello	Method for depolluting fresh and seawater from petroleum products	Lipophilic and floating slow-release nitrogen fertilizers
4,136,024	1979	K.Bisa and T.Bisa	Aerosol dispersion of microorganisms to eliminate oil slicks	Aerosol of microorganism and nutrient
4,146,470	1979	R.R.Mohan, M.L.Robbins, A.I.Laskin, and L.A.Naslund	Use of microorganisms in combination with surface active agents to synergistically disperse oil slicks	Microorganisms plus dispersants
4,259,444	1981	A.M.Chakrabarty	Microorganisms having multiple compatible degradative energy-generating plasmids, and preparation thereof	Genetically engineered microorganisms
4,415,661	1983	M.J.Narsimhan,Jr. and M.J.Thirumalachar	Fungus to degrade petroleum oil spillages; pollution control	Novel oil-degrading fungus
4,460,692	1984	J.Tellier, A.Sirvins, J.C.Gautier and B.Tramier	Microemulsion of nutrient substances	Microemulsion of aqueous nutrients in an oil phase
4,462,910	1984	A.Lepain and R.D.E.Bronchart	Oil slick dispersants using diguanidinium salt	Nitrogen-containing dispersants
4,512,914	1985	A.Lepain and R.D.E.Bronchart	Composition and method for treating oil slicks	Monoalkyl guanidinium fertilizers
Reproduced from Prince, 1993				

2.3.2 Adding Exogenous Organisms (Seeding)

Seeding involves the introduction of exogenous microorganisms into the natural environment for the purpose of increasing the rate or extent, or both, of biodegradation of pollutants. The rationale for this approach is that the autochthonous microbial populations may not be capable of degrading the wide range of potential substrates present in complex mixtures such as petroleum.

The criteria to be met by effective seed organisms have been reviewed by Atlas [1977] and include the ability to degrade most petroleum components, genetic stability, rapid growth following storage, a high degree of enzymatic activity and growth in the environment, the ability to compete with indigenous microorganisms, nonpathogenecity, and the inability to produce toxic metabolites. Mixed cultures have been most commonly used as inocula for seeding because of the relative ease with which microorganisms with different and complementary hydrocarbon degrading capabilities can be isolated.

Seeding of petroleum-contaminated aquatic environments has been attempted, with mixed results. Tagger *et al.* [1983] observed no increase in petroleum degradation in seawater inoculated with a mixed culture of hydrocarbon-degrading bacteria. Atlas and Busdosh [1976] reported increased degradation of oil in a saline Arctic pond after inoculation with an oil-degrading *Pseudomonas* sp., but no improvement in a freshwater pond. Horowitz and Atlas [1980] found that greater losses of oil in seawater in an open flow-through system occured when octadecane-coated bacteria were applied two weeks after the addition of an oleophilic fertilizer to the system than when the fertilizer alone was added. In the same study, no significant increase in the loss of gasoline from freshwater sediment was produced by seeding. Rosenberg *et al.* [1975] were able to demonstrate removal of residual oil from the ballast tanks of oil tankers by microbial seeding, although removal appeared to result primarily from bioemulsification rather than biodegradation.

Terrestrial ecosystems differ from aquatic ecosystems in that soils contain higher concentrations of organic and inorganic matter and, generally, larger numbers of microorganisms and are more variable in terms of physical and chemical conditions [Bossert and Bartha, 1984].

The microbial community of soils usually includes a significant hydrocarbon-utilizing population, which readily increases in response to hydrocarbon contamination [Atlas *et al.*, 1981; Jensen, 1975; Llanos and Kjoller, 1976; Pinholt *et al.*, 1979]. The presence of indigenous microbial populations which are highly adapted to a particular soil environment would be expected to influence negatively the ability to seed microorganisms to compete successfully and survive; for this reason, soils are not widely considered to be amenable to improvements in rates of biodegradation through seeding alone [Atlas, 1977; Bossert and Bartha, 1984].

2.3.2.1 Use of GEMS

Genetically engineered microorganisms (GEMs) that possess novel metabolic pathways may be exploited to degrade specific anthropogenic pollutants. However, petroleum hydrocarbons have been shown to be amenable to biodegradation by the naturally occurring bacteria [Leahy and Colwell, 1990]. Only asphaltene fraction is known to be comprised of recalcitrant molecules. Nevertheless, several studies have been directed at the development of genetically engineered microorganisms for oil spill cleanup due to its heterogeneous nature. A.M.Chakrabarti and colleagues developed the first 'superbug', a multiplasmid *Pseudomonas*, in 1976. Since then, intense research efforts have been directed at the construction via recombinant DNA methods of bacteria with utility in seeding for bioremediation. One common approach has included the cloning of oxygenases and dioxygenases with the desired metabolic potential from various organisms. However, there is considerable controversy surrounding the release of such gems into the environment, and field testing of these organisms must therefore be delayed until the issue of safety, containment and the potential for ecological damage are resolved [Susmann, 1988].

2.3.2. 2 Adaptation of Microorganisms

Prior exposure of a microbial community to hydrocarbons, either from anthropogenic sources such as accidental oil spills, petroleum exploration and transportation activities, and waste oil disposal, or from natural sources such as seeps and plant-derived hydrocarbons [Bartha and Bossert, 1984; National Academy of Sciences, 1985], is important in determining how rapidly

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subsequent hydrocarbon inputs can be biodegraded. This phenomenon, which results from increases in the hydrocarbon-oxidizing potential of the community, is known as adaptation [Spain et al., 1980]. The three interrelated mechanisms by which adaptation can occur are:

(I)   Induction and/or depression of specific enzymes

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

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

Adaptation of microbial communities to hydrocarbons, i.e., increases in rates of transformation of hydrocarbons associated with oil-contaminated environments, has been reported in several studies. Walker et al., observed greater degradation of a mixed-hydrocarbon substrate [1975] and South Louisiana crude oil [1976] by sediment bacteria from an oil-polluted harbor than by bacteria from a relatively unpolluted environment. Uptake and mineralization rates of [^{14}C] hexadecane were also higher for planktonic bacteria from the polluted site [Walker and Colwell, 1975] supporting the adaptation strategy.

Bauer and Capone [1988] and Kerr and Capone [1988] provided evidence for 'cross-acclimation' of sediment microbial communities to PAHs, in which exposure to one compound, such as phenanthrene, effects an increase in metabolism rates of a compound of similar structure, such as naphthalene. The occurrence of this phenomenon was attributed to the broad specificity of selected microbial populations for PAHs and/or the existence of common pathways for PAH catabolism [Bauer and Capone, 1988]. The increase in transformation rates of naphthalene, phenanthrene, and benzo[α]pyrene with increasing ambient concentrations of PAHs, reported by Shiaris [1989] for sediments of a polluted estuary, probably represents another example of cross-acclimation. The capacity of bacteria for adaptive change could be exploited for seeding purpose.

2.3.2.2.1 Adaptation by Alteration of the Genetic Composition of the Microbial Community

Of the three mechanisms for adaptation of microbial communities to chemical contaminants, induction and derepression of enzymes, genetic changes, and selective enrichment, only the third has been examined in detail, as discussed in the previous section. This has been primarily a result of limitations imposed by available methods, which have, until recently, restricted the study of adaptation of microbial communities to the phenomenon of selective enrichment, a phenomenon which the numbers or proportion of microorganisms that can utilize the compound of interest increase within the community and can be enumerated by their ability to grow on a medium containing the compound as the sole carbon source.

The primary genetic mechanism for the adaptation of the microbial community is the amplification, by means of selective enrichment and gene transfer and mutation, of genes which are involved in the metabolism of the chemical contaminants [Barkey and Pritchard, 1988; Spain et al., 1983]. Direct monitoring of this process with respect to adaptation to hydrocarbons has recently been made possible by the development of DNA probes specific for the genes encoding hydrocarbon-catabolic pathways [Trevors, 1985]. Sayler et al., [1985], for example, using the colony hybridization technique, showed a correlation between the enhanced rates of PAH mineralization in oil-contaminated sediments and an increase in the number of colonies containing DNA sequences which hybridized to TOL and NAH plasmid probes. The colony hybridization procedure, however, has the disadvantage of requiring the growth of organisms on laboratory media, which limits sensitivity and does not allow detection of DNA sequences in viable but nonculturable microorganisms [Roszak and Colwell, 1987]. Dot blot hybridization, in which DNA is extracted from environmental samples and then probed [Holben et al., 1988; Ogram et al., 1987], can be used to detect specific DNA sequences in the environment without the need for isolation and culture of microorganisms. The newly described polymerase chain reaction technique can improve the sensitivity of the dot blot method by three orders of magnitude, permitting the detection of 1 cell per g of sediment sample [Steffan and Atlas, 1988].

2.3.2.2.2. Plasmid Mediated Adaptive Evolution in Microbial Community

Plasmid DNA may play a particularly important role in genetic adaptation in that it represents a highly mobile form of DNA which can be transferred via conjugation or transformation and can impart novel phenotypes, including hydrocarbon-oxidizing ability, to recipient organisms. The pathways for the metabolism of naphthalene, salicylate, camphor, octane, xylene, and toluene have been shown to be encoded on plasmids in *Pseudomonas* sp. [Chakrabarti, 1976]. Table 2.2 reviews some of the plasmids carrying genes involved in catabolism of naturally occurring organic compounds, some of them are present in crude oil. Exposure of natural microbial populations to oil or other hydrocarbons may impose a selective advantage to strains possessing plasmids encoding enzymes for hydrocarbon catabolism, resulting in an overall increase in the plasmid frequency in the community.

Table 2.2 : Properties of plasmids encoding for the degradation of naturally occurring organic compounds

Strain	Plasmid	Substrates	Size (kb)
Pseudomonas putida PpG1	CAM	Camphor	500
Pseudomonas oleovorans PpG6	OCT	Octane, Decane	500
Pseudomonas putida R1	SAL1	Salicylate	85
Pseudomonas putida PpG7	NAH	Naphthalene	83
Pseudomonas putida PaW1	TOL	Xylene, toluene	115
Acinetobacter calcoaceticus RJE74	pWW174	Benzene	200
Pseudomonas convexa Pcl	NIC	Nicotine, Nicotinic acid	-
Pseudomonas putida NCIB9869	pRA500	3,5-Xylenol	500
Pseudomonas sp. CIT1	pCIT1	Aniline	100
Pseudomonas putida CINNP	pCINNP	Cinnamic acid	75
Pseudomonas sp. CF600	pV1150	Phenol	-

-, data not available

Reproduced from Mergeay *et al.*, 1983.

Whereas the enzymology of many plasmid-encoded pathways has been elucidated, only recently, with the advent of molecular genetic and recombinant DNA techniques, have the many

similarities in enzymology, organization, regulation and evolution of degradative genes become evident. Application of rapid DNA sequence determination methods and computerized DNA sequence data base homology searches has further shown that some plasmids contain very similar genes although they are involved in degradation of apparently dissimilar substrates. For example, plasmid NAH7, encoding metabolism of naphthalene, and plasmid TOL pWWO, encoding toluene degradation, contain two different genes, *nahH* and *xylE*, both encoding catechol 2,3-dioxygenase, that are greater than 80% similar in DNA and protein sequence [Harayama *et al.*, 1987; Ghosal *et al.*, 1988]. It was further suggested that the isofunctional genes encoding chlorocatechol degradation are related to chromosomal genes for catechol degradation [Aldrich *et al.*, 1987; Frantz and Chakrabarty, 1987]. All of these data suggest that many degradative genes are related by common ancestry and that substantial genetic exchange and interaction has occured among various plasmids and the host chromosomes.

Recent evidence suggests that the regulatory genes for certain aromatic hydrocarbon degradation genes are also very similar to one another. Regulatory genes for degradation of naphthalene, chlorobenzoate, 2,4-dichlorophenoxyacetic acid, and catechol appear to be related, since they are members of the LysR family of positive transcriptional activators [Henikoff *et al.*,1988; Schell and Sukordhaman,1989].

2.4 Scope of Work

The literarure review indicates that the conventional methods of remediating oil spills mostly maintain crude oil as contaminant transferring it to another phase. Bioremediation provides cost effective, contaminant- and substrate-specific treatment with complete destruction of pollutant molecules present in crude oil constituents. Several studies have proved the microbial infallibility and biodegradation has been accepted as the ultimate fate of petroleum spilled in the environment. Bioremediation aims at the stimulation of this natural process by either adding nutrients to support and proliferate indigenous microbes or adding exogenous microbes. Nutrient-assisted bioremediation had been tried as a treatment method in *Exxon Valdez* spill. However,

the results of this approach are likely to be evident over a period of time due to the lag phase preceding the biodegradation process. Nutrient assisted bioremediation has been approached in the *Exxon Valdez* spill bioremediation program with limited results. A number of other laboratory scale studies propose 'seeding' or adding specialized microbial culture as an alternative. The microbial potential to metabolize various components of crude oil described in literature review could be exploited to remediate oil spills. Effective biodegradation of crude oil components is the key to successful bioremediation program. Petroleum contamination exists as a complex mixture of hydrocarbons. Despite the wide potential of microorganisms to degrade organic compounds under favorable conditions, no single species of microorganisms can degrade all the components of a given oil [OTA,1991]. Currently several organisms are known, each capable of degrading usually one or, at best, a few petroleum components at a time. Therefore effective bioremediation of crude oil requires a mixture of populations consisting of several different genera each capable of metabolizing the respective compounds. Use of mixed culture for preparing inocula is studied by several researchers and it has been established that the role of individual organisms in a mixed population depends on a number of known and unknown factors. Therefore there is a need to formulate a rapidly metabolizing population consisting of different organisms, providing desired flexibility, where role of each member is very well defined. The synchronous population may be defined as consortium which could be formulated depending on the composition of crude oil.

Further, the literature reveals that bioavailability of crude oil components is an essential pre-requisite in biodegradation. Hence, it is mandatory to understand the role of biosurfactant, which emulsifies crude oil, in oil degradation process, to maintain the bioavailability of substrate to the growing population. The second key factor is desired microbial physiology. The physiological state of bacteria at the time of inoculation may affect the lag period of degradation process which is the most crucial aspect of oil spill bioremediation. Adaptation is the phenomenon which is related to the rapid degradation of hydrocarbon by the bacteria already exposed to same or structurally similar compound [Spain,1980]. Key metabolites/intermediates of aromatic degradation pathway could be used to study their effect on crude oil degradation process which

could lead to adaptation therefore resulting in reduction in time required for the growth of culture. Where the concept of consortium widens the metabolic horizon for better degradation at species level, adaptation of individual organisms provides broad specificity or enzymatic flexibility to accommodate compounds of similar structures which are abundant in crude oil.

Another limitation in applying such microbial formulations in marine spills is hypersalinity. Osmotic stress could lead to decline in degradation rate and extent [Ward & Brock,1978]. Unlike marine environment, terrestrial spills pose the constraint of sorption of hydrophobic constituents of crude oil to the soil particles.

The effects of environmental parameters on the microbial degradation of hydrocarbons, the elucidation of metabolic pathways; genetic basis of hydrocarbon dissimilation by microorganisms and the effects of hydrocarbon contamination on microorganisms and microbial communities have been areas of intense interest and the subjects of several reviews [Leahy & Colwell,1990; Prince, 1993]. The intent of present study is the formulation of a predestined bacterial consortium for effective degradation of crude oil and enhancement in the biodegradative capacity of bacteria by adaptation (cross-acclimation) and by applying the emulsifying capacity to the consortium via biosurfactant. The formulation of bacterial consortium has been considered as the key to successful bioremediation program. The developed consortium could also be applied to control oil pollution in petroleum refinery and to treat refinery wastewater after little modification or using the existing biological treatment systems.

The application of bioremediation is still in its infancy and to make it more practical and effective, a physico-chemical method has been added for effective combat of marine oil spills, i.e. the use of a cost effective absorbent to skim crude oil physically, the remaining oil fractions subjected to bioremediation.

The objectives undertaken in this study are :
- Development of a suitable cost-effective sorbent for physical removal of crude oil

- Isolation of hydrocarbonoclastic organisms from contaminated sample(s) and their screening

- Formulation of bacterial consortium for crude oil degradation

- Characterization of selected isolates

- Utilization of various fractions of crude oil and effect of inducers

- Role of biosurfactant producing organism in the oil degradation process

- Imparting osmotolerance to the members of bacterial consortium

- Application of the designed consortium for bioremediation of oil contaminated soil.

Chapter III

Materials & Methods

Materials and Methods

The materials used and methods adopted for experimentation are described in detail in this chapter.

3.1   Materials

3.1.1   Chemicals and Glasswares

Analytical grade (AR) or Guaranteed reagent grade (GR) chemicals were used for the preparation of reagent solutions and media. For microbiological media, biochemicals of Hi-Media were used. For protein assay and fractionation, chemicals were purchased from Sigma Chemical Co. (St. Louis, USA), and Bio-Rad Laboratories, (Richmond, California). Molecular biology grade chemicals (free from RNase, DNase and proteases) used for plasmid screening and other DNA work were purchased from GIBCO BRL Inc. and Biorad (California). Borosilicate glasswares of Borosil or Vensil make were used.

3.1.2 Restriction Enzymes and Other Chemicals for Molecular Biology Experiments

All enzymes and buffers were purchased from GIBCO BRL Inc., Amersham Inc. and Boeringer Manneheim Co. (USA).

3.1.3 Crude Oil

Bombay High crude oil was procured from Oil & Natural Gas Commission (ONGC), Bombay for experimental work. Aliquots of crude oil were preserved at 4°C. Before addition, tubes were thawed at 37°C for 15 min. and required amount was drawn with a micropipette.

3.1.4 Standard Hydrocarbons

Individual hydrocarbons, dodecane, pentadecane, hexadecane, pristane, eicosane, octacosane, tetracosane, naphthalene, phenanthrene, acenaphthene, biphenyl, fluorene, fluoranthene and dibenzothiophene were purchased from Central Drug House, India; SRL, India;

S.D.Fine Chem.Ltd., India; and Merck Schuchardt, Germany. Hydrocarbons were used individually as a sole carbon source and in mixtures also as described.

3.1.4.1 Model Petroleum

A multisubstrate system simulating petroleum was designed containing 10 representative molecules present in crude oil. The composition of model petroleum is given in Table 3.1.

Table 3.1: Composition of Model Petroleum*

Category	Compound
Saturates	
C12	Dodecane (1.5 ml)
C15	Pentadecane (1.5 ml)
C16	Hexadecane (1.5 ml)
C20	Eicosane (140 mg)
C24	Tetracosane (140 mg)
C28	Octacosane (140 mg)
Aromatics	
Dicyclic	Naphthalene (140 mg)
Tricyclic	Phenanthrene (140 mg)
Heterocyclic	Dibenzothiophene (140 mg)

* *Pristane* was used as internal standard (1.5 ml), the total volume of above standard mixture was 6.37 ml

3.1.4.2 Model Aromatic Mixture

A model aromatic mixture was prepared to simulate aromatic fraction of crude oil containing representative hydrocarbons of different categories. The composition is given in Table 2.

Table 3.2: Composition of Aromatic Mixture

Category	Compound
Monocyclic	Toluene (1ml)
Monocyclic	*o,m,p* Xylene (1ml)
Dicyclic	Naphthalene (118mg)
Dicyclic	Biphenyl (141mg)
Dicyclic	Acenaphthene (141mg)
Dicyclic	Fluorene (152mg)
Tricyclic	Phenanthrene (163 mg)
Tetracyclic	Fluoranthene (186mg)
Heterocyclic	Dibenzothiophene (169 mg)

3.1.5 Antibiotics

Stock solutions of antibiotics were filter sterilized and stored at -20°C and when required, desired volumes were added to the autoclaved medium that had been allowed to cool to about 40°C.

3.1.6 Organic Solvents

Solvents for crude oil and hydrocarbon extraction and silica gel column chromatography (hexane, benzene, chloroform, methanol and dichloromethane) were of AR grade and purchased from E. Merck, Bombay.

3.1.7   Microbiological Media

3.1.7.1 Medium For Feeding the Batch Reactor

The reactor set to enrich hydrocarbon degrading organisms was supplemented with 0.01% glucose, 0.06% crude oil, 0.02% ammonium hydrogen phosphate and 0.04% triton-x 100.

3.1.7.2 Bushnell-Haas (BH) Medium

This medium was used for isolation of hydrocarbonoclastic organisms from reactor as well as in the degradation experiments throughout the study [Bushnell and Haas, 1941]. For isolation purpose, the medium was solidified with 2% agar (Hi-Media Agar Agar Type 1). It was sterilized

at 121°C temperature and 15 lbs pressure for 15 minutes. For degradation experiments, the medium was autoclaved as described above and substrate (crude-oil/hydrocarbon-mix/individual hydrocarbons) was added prior to inoculation. The composition of Bushnell-Haas medium was as follows:

Disodium hydrogen phosphate	:	2.5 g/L
Dihydrogen potassium phosphate	:	2.0 g/L
Magnesium sulphate	:	0.5 g/L
Ferrous sulphate	:	0.03 g/L
Calcium chloride	:	0.03 g/L
pH	:	7.0 ± 0.2

3.1.7.3 Nutrient Broth

Nutrient broth medium purchased from Hi-Media was used to grow the bacteria for biochemical characterization during identification of cultures, and for preparing freezer stocks of the cultures. The composition of the nutrient broth used was as follows:

Peptone	:	5g/L
Sodium Chloride	:	3.0g/L
Yeast Extract	:	3.0g/L
pH	:	7.4 ± 0.2

3.1.7.4 Nutrient Agar

Nutrient agar was prepared by adding 2% Agar Agar Type-I to the above mentioned composition of nutrient broth medium. Nutrient agar was used to grow the cultures during isolation and also for studying the colony characteristics during identification of cultures.

3.1.7.5 Luria Bertani (LB) Broth

Luria Bertani broth was used to grow the cultures for plasmid preparations, and to obtain initial inoculum of cells for experimental purposes. The composition of the Luria Bertani broth used was as follows:

Tryptone	:	10g/L
Yeast Extract	:	5g/L
Sodium Chloride	:	10g/L
pH	:	7.3 ± 0.2

3.1.7.6 Gluteraldehyde Phosphate Buffer

Gluteraldehyde phosphate buffer solution(2.5%) used for Scanning Electron Microscopy of bacterial cultures was prepared as follows:

Sodium dihydrogen phosphate	:	3.31 g/L
Disodium hydrogen phosphate	:	3.77 g/L
25% commercial gluteraldehyde	:	100mL
Double distilled water	:	925mL
pH of the final solution	:	7.3 ± 0.1.

3.1.7.7 Potassium Phosphate Buffer (pH 7)

1M stock solutions of dipotassium hydrogen phosphate and potassium dihydrogen phosphate were prepared. 0.1M potassium phosphate buffer of pH 7.0 was prepared as follows:

| 1M K_2HPO_4 | : | 61.5ml |
| 1M KH_2PO_4 | : | 38.5ml |

Both the solutions were mixed, diluted to 1000ml with distilled water and sterilized.

3.1.8 Strains Used

Several strains apart from the isolates were used for various purposes during the course of this investigation.

Pseudomonas putida MTCC*102

Pseudomonas putida KT 2443

Escherichia coli DH 5α

Pseudomonas putida G7

Apart from these standard strains, several bacteria were isolated from a crude oil fed semicontinuous reactor.

3.2 Methods

3.2.1 Pretreatment of Sawdust

Raw sawdust was treated with 1% sodium hydroxide under high temperature (120°C) and pressure (1.5 lb) conditions. A 10% slurry was prepared in conical flask (10 g saw dust in 100 ml of 1% alkali) and subjected to conditions described above. After an hour the sawdust was washed 10 times with tap water and twice with distilled water to remove the lignin content and dried at 50°C. This alkali treated sawdust (ATSD) was stored at room temperature and used for experiments.

3.2.1.1 Skimming of Crude Oil by ATSD

ATSD was tested for its oil sorbing capacity. 45g of crude oil were spread on an aquarium with water having an surface area of 500mm^2. Over the oil slick 15g of sawdust was sprinkled (3:1 ratio). A glass plate was used to mechanically skim out crude oil from crude oil sawdust mixture.

3.2.1.2 Increase in Specific Surface Area

The specific surface area was determined by methylene blue absorption studies. In brief, 0.5g of ATSD/raw sawdust was absorbed with 400 to 2400mg/L of methylene blue in Erlenmeyer flasks. The mixture was agitated at 100 rpm on a rotary shaker for 90 minutes in 50ml volume. At different time intervals the samples were drawn and the unabsorbed methylene blue was studied spectrophotometrically by measuring absorbance at 615nm. Methylene blue level was extrapolated using a standard graph.

3.2.1.3 Pore Formation

Scanning electron microscopic studies were carried out to see the changes on surface of saw dust particles after the alkali treatment. To compare the data, raw sawdust samples (without treatment) were also subjected to electron micrography. For pore size distribution, sawdust

samples were analyzed on mercury porosimeter (Poresizer 9320, V.100) by standard procedure. In brief, the treated and untreated sawdust samples were loaded in penetrometers separately and weighed on analytical balance. The samples were evacuated to get rid of gases and vapours before filling with mercury. The penetrometer were filled with mercury when vaccum reached to 50µm of Hg and transferred to high pressure port for automatic data collection.

3.2.2 Microbiological Methods

3.2.2.1 Sterilization of Media and Other Solutions

The basal salts medium, nutrient broth, nutrient agar, basal salts agar medium, buffers and salt solutions were sterilized by autoclaving at 121^0C temperature and 15 lbs psi pressure for 15 minutes. Sugar solutons were filter-sterilized by passing through 0.22µm membrane filter (Millipore filter).

The stock solutions of all substrates used (catechol, salicylate, benzoate, citrate, model petroleum, model aromatic mixture, glucose, crude oil, diesel, and petrol) were filter-sterilized and added to the sterile medium in the required concentration.

3.2.2.2 Enrichment of Hydrocarbonoclastic Organisms

5L of sludge from an oxidation ditch treating industrial waste and contaminated soil from refinery premises was enriched in a semicontinuous batch reactor. The daily feed is described in section 3.1.7.1. Every 48 hrs, the effluent was subjected to chemical oxidation demand (COD) analysis.

3.2.2.2.1 Isolation of Bacteria

After 5 months, a 10ml sample was drawn from the reactor and bacteria were isolated by conventional serial dilution plating method.

3.2.2.2.2 Screening of Hydrocarbon Degraders

The isolates were subjected to a three tier screening including antibiotic sensitivity test and hydrocarbon utilization range.

3.2.2.2.2.1 Primary Screening

The isolates were grown overnight in LB medium to study the optimal temperature and incubation time for growth and the colony characteristics.

3.2.2.2.2.2 Secondary Screening

LB grown cultures were subjected to antibiotic sensitivity tests in secondary screening. Cultures were inoculated on nutrient agar plates by pour plate method along with antibiotic discs and incubated at 30°C. Six antibiotics viz. ampicillin, penicillin, kanamycin, tetracycline, gentamicin and chloramphenicol.

3.2.2.2.2.3 Tertiary Screening

Selected isolates were tested for hydrocarbon utilization. Five hydrocarbons representing different fractions of crude oil were used individually as single source of carbon and energy. Catechol (monocyclic) and phenanthrene (tricyclic) were selected to represent aromatics and eicosane (C20), tetracosane (C24) and octacosane (C28) to represent aliphatics. Overnight LB grown cultures pelleted, washed and were inoculated in BH medium with above mentioned substrates.

3.2.2.3 Identification of the Bacterial Cultures by Biochemical Approach

Morphological characterization was carried out by gram staining and the colony characteristics on the nutrient agar plates. Motility was observed by hanging drop method. The spore staining method of Norris and Swain [1971] was adopted to detect the presence of spores in the bacterial isolates.

For the biochemical characterization of the microorganisms the different media used were as follows:

- Sugar fermentation tests were carried out using peptone water with different sugars at 0.5% concentration

- Tryptone broth was used for the production of Indole

- Glucose peptone broth was used for Methyl Red (MR) and Voges-Prausker (VP) tests

- Koser's citrate medium was used to study the utilization of citrate as carbon source

- Gelatin medium for gelatin liquefaction by gelatinase enzyme

- Christensen's medium for the production of Urease (Christensen, 1946)

- Nutrient broth with potassium nitrate (nitrate medium) to study nitrate reduction (Lanyi, 1987)

- Nutrient agar plates for catalase test

- Nutrient agar with 10% milk for caseinase test

- Starch medium for starch hydrolysis

- Tween 80 medium for lipolytic activity

The different media employed for the biochemical characterization were prepared as per the composition given in Difco manual [1984]. The procedures adopted for the biochemical tests were as stated in 'Laboratory Techniques in Bacteriology' [Levine, 1959] and the identification was carried out by referring to Prokaryotes [1981] and 'Bergey's Manual of Systematic bacteriology' [1984].

3.2.2.4 Optical Density

Optical density of the bacterial culture broth was measured at 620nm wavelength using Spectronic 21 (Bausch and Lomb) spectrophotometer. Aliquots were drawn at regular intervals for determining the optical density. The optical density was measured immediately at 620nm against the control (Growth medium without culture). The quartz cuvettes(1cm path length, 3ml capacity) used in this measurement were cleaned with dilute hydrochloric acid followed by double distilled water till absorbance of the two cuvettes was observed to be the same. For protein estimation plastic cuvettes of 1cm path length and 1ml capacity were used.

3.2.2.5 Scanning Electron Microscopy

For morphological analysis of pure bacterial isolates, Scanning Electron Microscope Model STEREOSCAN - 250 MKB from Cambridge Instruments having magnification power upto 11000 times was used. Scanning electron microscopy of the bacterial cultures was carried out after fixing the cultures as follows:

An 18 hour grown culture from LB broth was smeared on a coverslip and heat fixed. The smear was later fixed up with gluteraldehyde phosphate buffer for 4-5 hours. The samples were dehydrated sequentially with 20%, 30%, 50%, 70% and 90% ethyl alcohol, 15 minutes in each. The sample was dehydrated in 100% alcohol overnight. The alcohol was decanted and the smear on the coverslip was allowed to air dry. The cultures were coated with osmium tetrachloride and then subjected to scanning electron microscopy.

3.2.2.6 Preparation of Seed Culture of Consortium Members for Degradation Experiments

The cells of selected isolates (members of the consortium) were grown in LB medium (section 2.1.7.5) and harvested in the stationary phase after 18 hours of incubation at $30^{\circ}C$ with continuous shaking at 150 rpm. The cell pellets were obtained by centrifugation at 6000 rpm for 5 min., washed with autoclaved BH medium (section 2.1.7.2) and recentrifuged at 6000 rpm for 5 min. The washed pellets were inoculated into BH medium amended with 100 ppm of catechol (or citrate/salicylate/benzoate) and incubated at 150 rpm. Cells were harvested in late log or early stationary phase and inoculated in BH medium amended with hydrocarbon substrates (crude oil/model petroleum/model aromatic mixture) as required.

3.2.2.7 Degradation Experiments Using Designed Consortium

Seed cultures were prepared as described in section 3.2.2.6 individually and inoculated in fresh BH medium to give the final optical density of 0.01/ml at 620 nm. Various substrates viz. crude oil, model petroleum, model aromatic mixture were used as carbon source. The culture tubes/flasks were incubated at $30^{\circ}C$ with continuous shaking at 150 rpm. Periodically the whole flask was extracted with hexane and analyzed by COD or gas chromatography (section 3.2.4).

3.2.2.8 Production and Extraction of Biosurfactant

The surfactant production property of NCC. DSS_6 was studied by culturing the organism in glucose medium which was deficient in phosphates (45mg/L of KH_2PO_4) as reported earlier [Janiyani et al., 1992]. The culture flasks were incubated at $30^{\circ}C$ with continuous shaking at 150 rpm. Samples were withdrawn at time intervals of 48, 72 and 96 hrs for surface tension

determination. Biosurfactant from the culture medium was extracted by the method of Itoh *et al.* [1972]. Inbrief, cell free broth was acidified to pH 5.0 with 1N HCl and extracted twice with 0.5 volume of ethyl acetate. The combined extract was then concentrated on a rotary flash evaporator. The residue was dissolved in 10ml of 0.05M sodium bicarbonate at pH 4.0 adjusted with 1N HCl and again extracted using diethyl ether (1:1). The ether extract was concentrated on rotary flash evaporator to remove ether completely, prified by silica gel column chromatography and the biosurfactant was stored at 4°C.

3.2.3 Molecular Biological Methods

3.2.3.1 Screening the Potential Isolate for Presence of Plasmids

The isolates were screened for presence of plasmids using the methods for small scale preparation of plasmid DNA as given in Molecular Cloning - A Laboratory Manual [Sambrook, Fritsch & Maniatis, 1989].

3.2.3.1.1 Harvesting and Lysis of Bacteria

- A single bacterial colony of culture from nutrient agar plate was transferred into Luria Broth medium. The culture was incubated overnight at 30°C with continuous shaking at 150 rpm.

- 1.5ml of each culture was poured into a microcentrifuge tube and centrifuged at 12,000g for 3 minutes at 4°C in a cooling centrifuge.

- The supernatant medium was removed by aspiration leaving the cell pellet as dry as possible.

3.2.3.1.2 Bacterial Cell Lysis

The following three methods were used for lysing the bacterial cells:

    I.   Rapid alkaline extraction method of Birinboim and Doly [1979]

    II. Boiling preparation Method of Holmes and Quigley [1981]

    III. Large and small plasmid extraction method of Kado and Liu [1981]

I. Alkaline Lysis Extraction Method of Plasmid DNA [Birinboim and Doly, 1979]

This protocol is a modification of the methods of Birinboim & Doly [1979]. The following solutions were used:

Solution I: Glucose Tris EDTA Solution

Glucose	:	50mM
Tris HCl (pH 8.0)	:	25mM
Sodium Salt of EDTA (pH 8.0)	:	10mM

The solution was autoclaved for 15 minutes at 10lbs psi pressure and stored at 4^0C to avoid contamination.

Solution II: Alkaline SDS Solution

NaOH (freshly diluted from sterile 10N stock)	:	0.2 N
Sodium Dodecyl Sulphate	:	1.0 %

Solution III: Potassium Acetate Solution

5M Potassium Acetate	:	60.0 ml
Glacial Acetic Acid	:	11.5 ml
Distilled Water	:	28.5 ml

Thus, the resulting solution is 3M with respect to potassium and 5M with respect to acetate. The solution was sterilized by membrane filtration.

Procedure

- The bacterial pellet (obtained in step 3 above) was resuspended in 100 ml of ice-cold Solution I by vigorous vortexing.

- 200ml of freshly prepared Solution II was added, closed the tube tightly and the contents mixed by inverting the tube rapidly five times. The tube was then stored on ice for 5 minutes.

- 150ml of Solution III was added to the contents in the tube, closed the tube tightly and vortexed gently for 10 seconds. The tube was then stored on ice for 5 minutes.

- Contents of the tube were then centrifuged at 12,000g for 5 minutes at 4^0C and the supernatant transferred to fresh tube.

- Equal volume of phenol:chloroform was added to the supernatant and mixed by vortexing. After centrifuging at 12,000g for 5 minutes at 4^{0}C the supernatant was transferred to fresh tube.

- 400ml of chloroform was added to the supernatant and centrifuged at 12,000g for 5 minutes. The upper layer was transferred to fresh tube.

- The double stranded DNA was precipitated with two volumes of ethanol at room temperature and mixed by vortexing. The mixture was allowed to stand at room temperature for 5 minutes and then centrifuged at 12,000g for 5 minutes at 4^{0}C.

- The supernatant was removed by gentle aspiration and the tube was allowed to stand in an inverted position on tissue paper to allow all the fluid to drain away.

- The pellet of double stranded DNA was rinsed with 1ml of 70% ethanol at 4^{0}C, centrifuged at 12,000g for 5 minutes and supernatant removed completely by gentle aspiration. The pellet of nucleic acids was allowed to dry in air for 10 minutes.

- The pellet of nucleic acids was redissolved in 50 ml of TE buffer (pH 8.0) containing DNAase-free pancreatic RNAase (20 mg/ml), vortexed briefly and stored at -20^{0}C.

II. Lysis by Boiling Preparation Method

This method is adapted from Holmes and Quigley [1981]. The following solutions were used:

Solution I (STET)

NaCl	:	0.1M
Tris.Cl (pH 8.0)	:	10mM
EDTA (pH 8.0)	:	1mM
Triton X-100	:	5%

Solution II (Lysozyme)

10mg/ml of lysozyme dissolved in 10mM Tris.Cl (pH 8.0).

Procedure

The following procedure was adopted for lysis of bacterial cells using Boiling Preparation Method:

- The bacterial pellet (obtained in step three of harvesting of bacteria) was resuspended in 350ml of STET.

- 25ml of freshly prepared solution of lysozyme (Solution II) was added and mixed by vortexing for 5 seconds.

- The tubes were placed in a boiling water bath for exactly 40 seconds.

- The bacterial lysate was centrifuged at 12,000g for 10 minutes at room temperature.

- The pellet of bacterial debris was removed from the microfuge tube with a sterile toothpick.

- 40ml of 2.5M sodium acetate (pH 5.2) and 420ml of isopropanol were added to the supernatant obtained, and mixed by vortexing. The tubes were incubated for 5 minutes at room temperature.

- The pellet of nucleic acids was recovered by centrifugation at 12,000g for 5 minutes at 4^0C.

- The supernatant was removed by gentle aspiration. The tubes were allowed to stand in an inverted position on a paper towel to allow all of the fluid to drain away.

- 1ml of 70% ethanol was added to the pellet and recentrifuged at 12,000g for 5 minutes at 4^0C.

- The supernatant was removed by gentle aspiration and the open tubes were stored at room temperature until the ethanol had evaporated completely (2-5 minutes).

- The nucleic acids were redissolved in 50ml of TE (pH 8.0) containing DNAase-free pancreatic RNAase (20mg/ml), vortexed briefly and the DNA was stored at -20^0C.

III. Large and Small Plasmid Extraction Method of Kado and Liu [1981]

This method is adapted from Kado and Liu [1981]. The following solutions were used:

Solution I (E Buffer)

40mM Tris acetate

2mM Sodium salt of EDTA

pH 7.9 (adjusted with glacial acetic acid)

Solution II (Lysing Solution)

3% SDS in 50mM Tris (pH 12.6)

pH adjusted by adding 1.6ml of 2N NaOH

Procedure

The following procedure was adopted for lysis of bacterial cells using Kado and Liu's Method:

. The bacterial pellet (obtained in step three of harvesting of bacteria) was resuspended in 100ml of E buffer.

. The cells were lysed by adding 200ml of lysing solution and incubated at 65^0C for 40 minutes.

. The chromosomal DNA was extracted with two volumes of phenol-chloroform mixture.

. The solution was emulsified by brief shaking.

. The emulsion thus formed was centrifuged in cold at 6000rpm for 10 minutes.

. The supernatant was carefully withdrawn in sterile eppendorf tubes and ethanol precipitated, as mentioned in alkaline lysis, before subjecting to agarose gel electrophoresis for screening of plasmids.

3.2.3.1.3 Equilibration of Phenol

Before use, phenol was equilibrated to a pH > 7.8 because DNA partitions into the organic phase at acid pH.

Procedure

. Liquefied phenol stored at -20^0C was allowed to warm to room temperature, and then melted at 68^0C. Hydroxyquinoline was added to a final concentration of 0.1%, as this compound is an antioxidant, a partial inhibitor of RNAase and a weak chelator of metal ions [Kirby, 1956]. In addition, its yellow colour provides a convenient way to identify the organic phase.

An equal volume of 0.5M Tris.Cl buffer (pH 8.0), at room temperature, was added to the melted phenol and the mixture was stirred on a magnetic stirrer for 15 minutes. After stirring, when the two phases separated, the upper (aqueous) phase was aspirated as much as possible using a glass pipette.

An equal volume of 0.1M Tris.Cl (pH 8.0) was added to the phenol. The mixture was stirred on a magnetic stirrer for 15 minutes and then the upper aqueous phase removed as in step 2. The extraction was repeated until the pH of the phenolic phase was greater than 7.8 (as measured with pH paper).

After the phenol was equilibrated and the final aqueous phase removed, 0.1 volume of 0.1M Tris.Cl (pH 8.0) containing 0.2% b-mercaptoethanol was added. The phenol solution was then stored in this form under 100mM Tris.Cl (pH 8.0) in a light-tight bottle at 4^0C

3.2.3.2 Plasmid DNA Preparation by Qiagen Columns

Several vectors and standard plasmids were used in the study. Purified plasmid DNA for such experiments was prepared by Q-500 or Q-100 columns(Qiagen Inc. USA), according to the manufacturer's protocol.

The bacteria were grown in LB medium (100 ml) with appropriate antibiotic and harvested after 18 hours.

The bacterial pellet was resuspended in 4ml of buffer P1.

4ml of buffer P2 was added, mixed and the tube was incubated at room temperature for 5 minutes.

After 5 minutes, 4ml of buffer P3 was added and mixed carefully by inverse motion. The mix was incubated on ice for 15 minutes.

The reaction mix was centrifuged at 12000 rpm for 30 minutes. The step was repeated again in case of turbid supernatant.

The supernatant was dispensed in 1.5ml eppendorf tubes and centrifuged at 12,000 rpm for 30 minutes.

- A Q-100 column was equilibrated with 4ml of buffer QBT.

- The supernatant from step 7 was applied onto the Q-100 column and allowed to move downwards.

- Once the supernatant had passed through column, the column was washed with 10ml of buffer QC twice.

- After washing the DNA was eluted with 5ml of buffer QF.

- The elute was dispensed in eppendorf tubes (0.8ml in each tube) and 0.7 volumes of isopropanol was added to it and incubated at room temperature overnight.

- The DNA was precipitated by centrifugation at 12,000 rpm for 30 minutes at 4°C.

- DNA pellet was washed with 70% ethanol, airdried for 5 minutes, redissolved in TE buffer and stored at -70°C.

3.2.3.3 Extraction of total DNA from bacterial cell

DNA of the isolate was extracted by a modified method of Marmur [1961]. Following reagents were used:

Saline EDTA

NaCl	:	0.15M
EDTA	:	0.1M
pH	:	8.0

Saline Citrate

NaCl	:	0.15M
Trisodium citrate	:	0.015M
pH	:	8.0

Sodium Lauryl Sulphate (25% Solution w/v)

Chloroform-Isoamyl Alcohol Mixture (24:1 v/v)

Ethyl Alcohol (95%)

Ribonuclease (RNase)

2ml of crystalline Rnase (10 mg/ml stock solution) was added to 10mM Tris HCl (pH 7.5) and 15mM NaOH solution. The solution was then heated at 100^0C for 15 minutes to inactivate every contaminating DNase.

Procedure

- Bacterial cells (5 gm) were harvested at logarithmic growth phase, and washed twice with 30ml of acetone followed by 30ml of ether. The washed cells were recovered each time by centrifugation at 15,000 x g, at 20^0C for 20 minutes.

- The cell pellet was suspended in 2.5ml of saline EDTA solution containing lysozyme (10mg/ml) and 0.25ml of sodium lauryl sulphate was added dropwise. The suspension was incubated at 30^0C for 15-20 minutes for complete cell lysis.

- To the suspension, 30ml of saline EDTA and 3ml of sodium lauryl sulphate were added and incubated in a water bath at 60^0C for 30 minutes and then centrifuged at 15,000 x g for 20 minutes to remove the cell debris.

- The supernatant was collected and deproteinized by shaking vigorously with an equal volume of chloroform-isoamyl alcohol mixture.

- The resulting emulsion was separated into two layers by centrifugation for 10 minutes at 15,000 x g. The upper aqueous phase containing nucleic acids was carefully removed and precipitated gently by adding approximately two volumes of chilled ethanol.

- The layers were gently mixed with a stirring rod and the nucleic acids were pooled as a thread like precipitate. The precipitate was drained to remove ethanol and suspended in 2 to 3ml of diluted saline citrate.

- This suspension was further deproteinized by adding equal volumes of chloroform-isoamyl alcohol mixture. The mixture was shaken for 15 minutes and centrifuged at 15,000 x g for 10 minutes.

The supernatant containing the nucleic acids was removed, precipitated and deproteinized as described above until the protein was completely removed. The supernatant thus obtained was precipitated with ethyl alcohol and dissolved in saline citrate buffer.

Ribonuclease was added to a final concentration of 50 mg/ml and the mixture was incubated for 30 minutes at 37^0C for the removal of RNA. The digest was again subjected to deproteinization repeatedly until little or no denatured protein was visible at the interface after centrifugation.

The supernatant was once again precipitated with ethanol and the drained nucleic acid was dissolved in 2.25ml of saline citrate.

3.2.3.4 Restriction Digestion

The plasmid as well as total DNA was subjected to restriction digestion in several experiments for different purposes. The restriction endonucleases used in experiments were procured from GIBCO BRL and Boeringer Mannheim. Appropriate buffers were used according to the manufacturer's instructions. All digestion were conducted at 37^oC for 3 hours.

3.2.3.5 Polymerase Chain Reaction

Hydrocarbon degrading isolates selected for designing of consortium were screened by PCR for the presence of catabolic genes involved in known bacterial biodegradative pathways for saturates (*alkB*) and aromatics (*xylE* and *dmpN*).Oligonucleotide primers used for PCR were derived from the published sequences of these catabolic genes. Total DNA of the isolates (prepared by Marmur's protocol, Section 2.2.2.3) was used as template DNA for PCR. DNA was quantified spectrophotometrically and 1mg of this was used for PCR.

3.2.3.5.1 Reaction Volume

For PCR amplification, 5ml of DNA extract (=1mg) was added to a final volume of 50ml of reaction mixture which contained :

Distilled Water: 32.5ml

Buffer   :   5.0ml

dXTPs	:	2.5ml
Primers	:	5.0ml
Total	:	50ml

3.2.3.5.2 Operating Conditions

PCR was conducted on a PCR system (*Perkin Elmer* GeneAmp PCR System 9600) using following PCR parameters :

5 min.	94°C	
1 min.	94°C (denaturation)	
1 min.	55°C (annealing)	40 cycles
1 min.	72°C (extension)	
3 min.	72°C	

PCR products were analyzed by agarose gel electrophoresis (1.2%).

3.2.3.6 Agarose Gel Electrophoresis

Detection of plasmid DNA, total DNA, PCR (polymerase chain reaction) products and digested DNA fragments was carried out by agarose gel electrophoresis in a horizontal gel electrophoresis system having dimensions of 23 x 15 x 7 cm (Biotech Analytical Model).

3.2.3.6.1 Preparation of Tank Buffer for Agarose Gel Electrophoresis

The tank buffer employed for agarose gel electrophoresis of plasmid DNA consisted of a low salt buffer system which avoids overheating of buffer and is capable of resolving high molecular weight plasmid DNA [Mickel *et al.*, 1977]. The composition of the tank buffer was as follows:

Tris acetate	:	40mM
Sodium salt of EDTA	:	2mM
pH adjusted with glacial acetic acid	:	7.9

The buffer was sterilized by autoclaving at 15 lbs pressure and 121°C temperature for 15 minutes.

3.2.3.6.2 Preparation of Gel

For preparation of gel, 0.8% agarose was used. Agarose was melted in tank buffer and poured into a sealed gel platform of size 130 x 65 mm. Sample wells were made by using a comb which consisted of 8 teeth, each having dimensions of 1.5 x 5 mm and spaced apart by 2mm.

3.2.3.6.3 Preparation of Loading Dye

Bromocresol purple	:	0.25%
Tris acetate	:	0.05M
Glycerol	:	50.0%

The solution, after adjusting its pH to 7.9, was sterilized by membrane filtration [Kado and Liu, 1981].

The DNA samples were dissolved in 20ml of TE (pH) and mixed with 10ml of loading dye and subjected to agarose gel electrophoresis along with the standard plasmid marker puc18 and 1kb ladder.

3.2.3.6.4 Accessories and Conditions Required for Agarose Gel Electrophoresis

The agarose gel electrophoresis was carried out in a horizontal gel electrophoresis system. The power supply was regulated by Electrophoresis Power Pack (Biotech Analytical Model). The agarose gel electrophoresis was carried out at 120 V and 60 mA for 2 hours until the loading dye reached the bottom of the gel.

3.2.3.6.5 Staining of Gel

After completion of electrophoresis, the gel was stained in an aqueous solution of 0.5 mg/ml ethidium bromide for 30 minutes and destained with sterile distilled water for 30 minutes.

3.2.3.6.6 Visualization of Plasmid DNA

The plasmid DNA was visualized in Vilber Lourmat UV-transilluminator Kaiser RA-1, and photographed by solid state camera attached to the transilluminator.

3.2.3.7 Purification of Digested DNA Fragments

Desired DNA was resolved on low melt agarose. The DNA bands were cut from the gel and purified by GlassMax™ DNA isolation Matrix System (GIBCO BRL Inc.) according to the manufacturer's instructions.

Procedure

- Per 0.1g of agarose slice, 300ml of binding solution was added and incubated at 50°C for 7 min.

- 10ml of glassbead solution was added to the mixture after vortexing.

- Mixture was incubated at room temperature for 1 hour with constant shaking.

- Reaction mixture was centrifuged at 13000 rpm for 20 sec.

- Pellet was resuspended in 500ml of cold wash buffer, recentrifuged. This step was repeated twice more.

- The pellet was air dried, resuspended in 20ml TE, incubated at 50°C for 3 min., centrifuged at 13000 rpm for 1 min. Supernatant was saved, checked on agarose gel and stored at -20°C.

3.2.3.8 Protein Profile of Consortium Members on Different Inducers

Procedure

- The isolates were grown in BH Medium with catechol (or citrate/salicylate/benzoate) as substrate and harvested in the late log phase after 15 hours of incubation at 30°C with continuous shaking at 150 rpm.

- The cell pellet obtained by centrifugation at 6000 rpm for 10 minutes, was washed with 0.1M phosphate buffer (pH 7.0), recentrifuged at 6000 rpm for 5 minutes and resuspended in lysis buffer in 1:10 dilution of cells (wet weight).

- The cells were lysed by freeze and thaw method using liquid nitrogen and 65°C water bath, thrice. After third cycle, the cell suspension was sonicated on a Sonicator XL from Heat Systems USA, with a microtip (20 sec pulse; thrice) and again subjected to centrifugation at 10,000 rpm for 10 minutes at 5°C temperature.

The supernatant obtained was subjected to ultracentrifugation (Beckman's Optima™ TLX Ultracentrifuge, USA) at 45,000 rpm for 30 minutes. The pellet was discarded and in supernatant, the protein contents were measured spectrophotometrically by Bradford method (Section 2.2.3.3). The protein samples were subjected to SDS-PAGE.

3.2.3.9 SDS - Polyacrylamide Gel Electrophoresis (SDS-PAGE)

The following solutions were prepared:

Solution I: Acrylamide and N'N'-methylenebisacrylamide

Acrylamide	:	29% (w/v)
N'N'-methylenebisacrylamide	:	1% (w/v)
pH	:	< 7

Prepared in deionized water (warm), and stored in dark bottle at room temperature.

Solution II: Sodium Dodecyl Sulphate (SDS)

10% (w/v) stock solution prepared in deionized water and stored at room temperature.

Solution III: Tris Buffers for Preparation of Resolving and Stacking Gels:

Tris base dissolved in deionized water and pH adjusted with 1N HCl, as follows:

1.5M Tris (pH 8.8)	:	For resolving gel
1.0M Tris (pH 6.8)	:	For stacking gel

Solution IV: TEMED (N,N,N',N'-tetramethylethylenediamine)

The commercially available TEMED solution (procured from BioRad, California) was directly used.

Solution V: Ammonium Persulphate

10% (w/v) stock solution prepared in deionized water and stored at 4^{0}C.

Solution VI: Tris Glycine Electrophoresis Buffer

25mM Tris Base

250mM Glycine (electrophoresis grade)

0.1% SDS

pH 8.3

A 5x stock solution was prepared as follows:

Tris Base	:	15.1g
Glycine (in 900ml deionized water)	:	94g
10% (w/v) SDS stock solution	:	50ml

Volume adjusted to 1000ml with deionized water.

Solution VII: 1x SDS Gel Loading Buffer

50mM Tris.Cl (pH 6.8)

100mM Dithiothreitol (DTT)

2% SDS (electrophoresis grade)

0.1% Bromophenol Blue

10% Glycerol

*DTT was added just before the buffer is used, from a 1M stock

Solution VIII: Fixing Solution

Glacial Acetic Acid	:	10%
Methanol	:	20%
Deionized Water	:	70%

It is necessary to fix the gel at room temperature in 5-10 volumes of the fixing solution before staining. The bromophenol blue will turn yellow as the acidic fixing solution diffuses into the gel. Fixation was continued for 5 minutes, after all the blue color had disappeared, the gel washed briefly in deionized water.

Solution IX: Coomassie Brilliant Blue R-250 Staining Solution

Coomassie Brilliant Blue R-250	:	0.25g
Methanol	:	45ml
Deionized Water	:	45ml
Glacial Acetic Acid	:	10ml

The solution was filtered through Whatman No.1 filter paper to remove any particulate matter.

This staining solution was used in the initial experiments and later replaced by a dilute composition as described by Chen *et al.,* [1993], as follows:

Coomassie Brilliant Blue R-250	:	3mg
Methanol	:	45ml
Deionized Water	:	45ml
Glacial Acetic Acid	:	10ml

The gel was stained the gel by soaking it in the above solution for 1hr to overnight (no need to prefix the gel).

Solution X: Destaining Solution I

Methanol	:	45ml
Deionized Water	:	45ml
Glacial Acetic Acid	:	10ml

Solution XI: Destaining Solution II

Methanol	:	5%
Glacial Acetic Acid	:	7%
Deionized Water	:	Make upto 100ml

Composition of Resolving Gel with 10% Acrylamide(10ml):

Deionized Water	:	4.0ml
30% Acrylamide mixture	:	3.3ml
1.5M Tris (pH 8.8)	:	2.5ml
10% SDS	:	0.1ml
10% Ammonium Persulphate	:	0.1ml
TEMED	:	0.004ml

Composition of 5% Stacking Gel (2ml):

Deionized Water	:	1.4ml
30% Acrylamide mixture	:	0.33ml
1.0M Tris (pH 6.8)	:	.0.25ml

10% SDS	:	0.02ml
10% Ammonium Persulphate	:	0.02ml
TEMED	:	0.002ml

Procedure

SDS-polyacrylamide gel electrophoresis was carried out according to the procedure mentioned in Molecular Cloning - A Laboratory manual [Sambrook, Fritsch & Maniatis, 1989]). The procedure is as follows:

- The glass plates were assembled as per instructions.

- The volume of the gel mold was determined and the desired volume of resolving gel prepared.

- The acrylamide solution was poured into the gaps between the glass plates leaving sufficient space for the stacking gel. The acrylamide solution was carefully overlaid with isobutanol . The gel was placed in a vertical position at room temperature.

- After polymerization was complete (30 minutes), the overlay was poured off and the top of the gel washed several times with deionized water to remove any unploymerized acrylamide. All traces of water after draining off, were removed with a paper towel.

- The stacking gel solution was prepared and poured directly onto the surface of the polymerized resolving gel. A clean teflon comb immediately inserted into the stacking gel solution to avoid trapping air bubbles. More stacking gel solution was added to fill the spaces of the comb completely. The gel was placed in a vertical position at room temperature.

- While the stacking gel polymerized, the samples were prepared by heating them to $100^{0}C$ for 3 minutes in 1x SDS gel loading buffer to denature the proteins.

- After polymerization was complete (30 minutes) the teflon comb was removed carefully, and the gel mounted on the mini gel electrophoresis apparatus. Tris glycine electrophoresis buffer was added to the reservoir tank of the mini gel electrophoresis apparatus.

- 15ml of each sample was loaded in a predetermined order into the bottom of the wells. An equal volume of 1x SDS gel loading buffer was loaded into wells that were unused.

- The electrophoresis apparatus was attached to an electric power supply and a voltage of 8V/cm was applied to the gel. After the dye front moved into the resolving gel, the voltage was increased to 15v/cm and the gel run until the bromophenol blue reached the bottom of the resolving gel.

- The glass plates were then removed from the electrophoresis apparatus and placed on a paper towel. Using a spatula the plates were separated and the orientation of the gel marked by cutting a corner from the bottom of the gel that is closest to the leftmost well (slot 10).

The gel was then subjected to staining with Coomassie Brilliant Blue after fixing it in the fixing solution.

3.2.3.9.1 Staining of SDS-Polyacrylamide Gel with Coomassie Brilliant Blue

Procedure I

- The gel was placed in the fixing solution for 1 hour till the bromophenol blue turned yellow and finally diffused out of the gel into the solution and all the blue colour disappeared. The gel was washed briefly in deionized water.

- The gel was then immersed in five volumes of staining solution and placed on a slowly rotating platform for 2 hours at room temperature.

- The staining solution was drained off after 2 hours and the gel destained by soaking it in destaining solution I for 2 hours on a rocking platform, with two changes of the destaining solution I.

- After 2 hours the gel was destained in destaining solution II for another 4 hours on a rocking platform, with three changes of the destaining solution II.

- After destaining, the gel was stored in deionized water containing 20% glycerol, and later photographed for permanent record.

This staining solution was used in the initial experiments and later replaced by a dilute composition as described by Chen *et al.,* [1993], as follows:

Procedure II

. The gel was stained by soaking it in the dilute Coomassie Brilliant Blue R-250 solution for 6 hours (no need to prefix the gel).

. After 6 hours the gel was destained in destaining solution II for another 3 hours on a rocking platform, with three changes of the destaining solution II.

After destaining, the gel was stored in deionized water containing 20% glycerol, and later photographed for permanent record.

3.2.4 Analytical Methods

Sampling and sample storage was accomplished by using standard operating procedures [U.S. Environmental Protection Agency (EPA),1995 ; Korda *et al.,*1997]. No plastic surface was used for sampling. Samples of suitable volume were drawn appropriate to the estimation procedures. Samples were generally analyzed immediately or they were preserved according to the procedures given for different parameters in the Standard Methods for the Examination of Water and Wastewater (APHA, AWWA, WPCF, 17th Edition, 1989) and analyzed later.

Depending upon the hydrocarbon being estimated, samples to be analyzed chromatographically were either acidified and used as aqueous samples or extracted in suitable organic solvent, before injecting into the Gas Chromatograph. The different analytical methods for the estimation of pure hydrocarbons, mixtures and crude oil as adopted from Standard Methods for the Examination of Water and Wastewater (APHA, AWWA, WPCF, 1989), are described in this section.

3.2.4.1 Chemical Oxygen Demand

The chemical oxygen demand was measured using the dichromate reflux method. The samples were refluxed in strongly acidic solution with a known excess of potassium dichromate. After digestion, the remaining unreduced $K_2Cr_2O_7$ was titrated with ferrous ammonium sulphate to

determine the amount of $K_2Cr_2O_7$ consumed and the oxidizable organic matter was calculated in terms of oxygen equivalent. Samples were filtered through 0.45mm poresize filters before determining their chemical oxygen demand.

3.2.4.2 Gas Liquid Chromatography

Total and remaining hydrocarbons were also monitored by gas chromatography. Three gas chromatographs were used for the purpose based on the nature of substrate used in the experiment.

3.2.4.2.1 Packed Column Gas Liquid Chromatography

The routine crude oil samples (control and experimental) were analyzed by packed column gas chromatography. Perkin Elmer Model Sigma 3B fitted with Dexil-400 column (*Kalpvruksha*), dual FID Chromatograph with Omniscribe Recorder from Digital Electronics Ltd., was used for analysis of crude oil samples before and after degradation. Samples were acidified to pH 4.0 and extracted with hexane (1:1; v/v). 2ml of the extracts were injected. The operating conditions were maintained as follows: Injector and detector port maintained at 300°C; Oven temperature gradient programming - initial 80°C for 5 min, raised to 130°C @ 30°C/min, 130°C to 270°C @ 5°C/min and kept isothermal at 270°C for 5 min. The carrier gas was nitrogen at 4.5 psi. Hydrogen-Air mixture was used to ignite flame for flame ionization detector (FID).

3.2.4.2.2 Capillary Gas Chromatography

The simulated model petroleum and model aromatic mixture were monitored before and after degradation by capillary gas chromatography. Perkin Elmer Autosystem Gas Chromatogaph, with software LC 1002 and fitted with FID was used for analysis of individual as well as mixtures of hydrocarbons. It was attached with Okidata microline 320, 9 pin printer. Acidified samples (pH 4.0) were extracted with hexane (1:1,v/v). The extracted samples (organic phase) were injected into the Capillary column CP-Sil 8cb (*ChromPack*) column for analysis of various hydrocarbons that were separated by the temperature gradient programme. The operating conditions for model petroleum were maintained as follows: Injector and detector

temperatures 300^{0}C. Oven temperature gradient programming- 100^{0}C at the start of the programme, held for 6 minutes, then raised at the rate of 30^{0}C/min, to 130^{0}C and 130 to 270^{0}C @ 12^{0}C and kept isothermal at 270°C for 5 minutes. Operating conditions for model aromatic mixture were maintained as follows: Injector and detector temperatures 300^{0}C; Oven temperature gradient programming- 45^{0}C at the start of the programme, held for 5 minutes, then raised at the rate of 30^{0}C/min, to 120^{0}C and 120 to 270^{0}C @ 10^{0}C and kept isothermal at 270°C for 2 minutes. The carrier gas was hydrogen at 4.5 psi. Crude oil extracts were also analyzed by capillary gas chromatography. The conditions for crude oil analysis were same as described for packed column gas chromatography.

3.2.4.2.3 Gas Chromatography Mass Spectroscopy

Contaminants present in the soil no.2 (collected from IOCL premises, Mathura) were analyzed on a Varian Saturn-3 gas chromatograph fitted with DB-5 (25 M long) column and identified by a mass-spectroscope coupled with it. The injector port and detector temperature was 280°C. The oven programme was : 130°C initially, held for 5 min., raised @ 10°C/min. to 170°C, raised @ 4°C/min. to 270°C and held at 270°C for 2 min.

3.2.4.3 Protein Estimation

Protein estimation during purification of enzymes was carried out using the method described by Bradford [1976]. This method involves the binding of Coomassie Brilliant Blue G-250 to the protein. The binding of the dye to protein causes a shift in the absorption maximum of the dye from 465nm to 595nm, and it is the increase in the absorption at 595nm which is monitored. Absorbance at 595nm was measured using 1ml capacity plastic cuvettes against a reagent blank prepared from appropriate buffer and protein reagent. Standard curve was prepared using bovine serum albumin.

3.2.4.4 Silica Gel Column Chromatography

The crude oil is a heterogenous mixture of several hydrocarbons which can be categorized into four major classes : aromatics, saturates, resins and asphaltenes [Harayama, 1994]. The categorization is based on their differential solubility in organic solvents. The crude oil used in this

study, Bombay High Crude, was fractionated by column chromatography as described by Hong-vu Song et al.[1990] before and after degradation.

In brief, a 30 x 15 mm glass column was packed with activated silica gel. The sample was prepared by suspending 0.5 g of crude oil in 2 ml chloroform. The suspension was adsorbed on 2 g of silica gel and chloroform was allowed to evaporate. A layer of anhydrous sodium sulphate was placed over the sample to adsorb any water and minimize the interference of solvent with crude oil. The saturated, aromatic and asphaltene fractions of crude oil were eluted with 120 ml of hexane, benzene and chloroform : methanol (1:1, v/v), respectively.

3.2.4.5 Infra-Red Specrtoscopy

Biosurfactant produced by an isolate was characterized by infra-red spectroscopy (IR) using a Perkin-Elmer spectroscope model 9538. The partially purified biosurfactant was dissolved in carbon tetrachloride scanned

3.2.5 Presentation of Data

The values presented in the tables for each parameter in the ensuing chapters are the means of atleast six observations collected from each experiment. The results of batch experiments shown in figures are the average of at least three sets of observations.

Chapter IV

Results

Results

4.1 Physical Skimming of Crude Oil Using Alkali Treated Sawdust (ATSD)

It has been reported that plant products and other natural absorbents demonstrate poor buoyancy characteristics, relatively low oil sorption capacity and low hydrophobicity [Schatzberg,1972]. This has also been observed with raw sawdust when applied as absorbent on water surface. In this study, saw dust was subjected to alkali treatment under high temperature and pressure condition which causes deliginification. Release of lignin could be seen as the reaction mixture turned black in colour. The surface phenomenon was studied after washing out the lignin content with distilled water. This delignified sawdust was tested for its absorption capacity by methylene blue. Increase in specific surface area was determined by methylene blue absorption isotherms.

4.1.1 Increase in Specific Surface Area of Sawdust Particles

The standard curve for methylene blue is depicted in Fig. 4.1 while Fig. 4.2 demonstrates the amount of methylene blue absorbed by treated and untreated sawdust.

Methylene blue concentration (ppm)
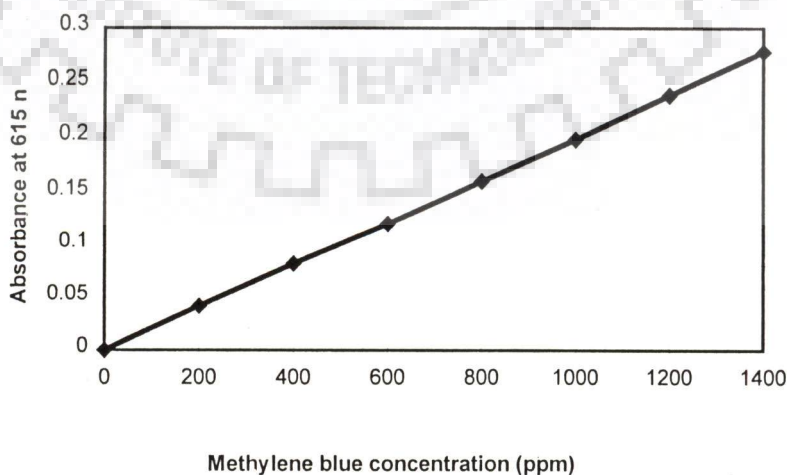
Fig. 4.1 : Standard curve for methylene blue
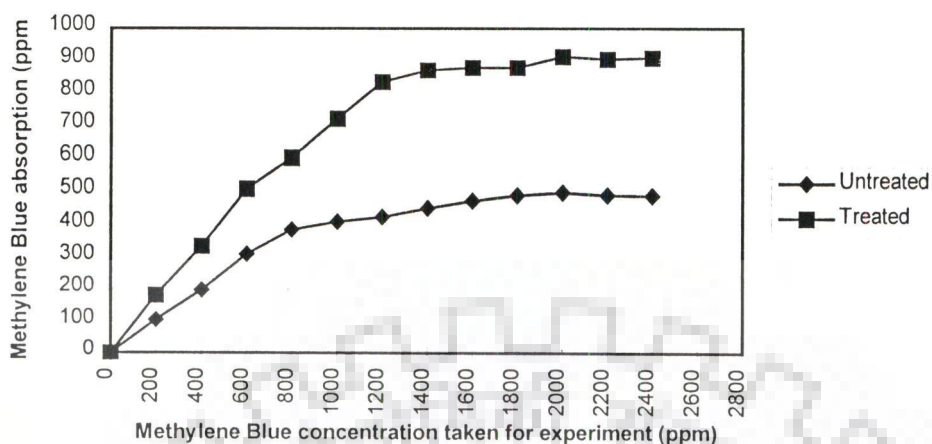
Fig. 4.2 : Methylene Blue absorption isotherm for treated and untreated sawdust

Specific surface area was calculated using the relation [Johnson,1994] :

Surface area = $6.02 \times 10^{-2} M_f A_m$ $(m^2 g^{-1})$

where

M_f = amount of methylene blue (ppm) absorbed per gram of sawdust (treated/untreated)

A_m = area per molecule (in A^{o2}) on the surface

Under the condition of optimum flocculation the area per absorbed molecule is taken as $130A^o2$ which corresponds to the molecule lying flat on the absorbent surface. The value of Mf was obtained from inflexion of standard curve of methylene blue. For untreated sawdust, the surface area was 42 m^2/gm. Delignification due to alkali treatment resulted in the increase of surface area (96 m^2/gm). Pore size distribution analysis by mercury intrusion method revealed that pores being formed due to the alkali treatment were of bottle neck shape and also the presence of narrow necked inter particles voids (Fig 4.3a & b) which justifies the high oil sorbing capacity of ATSD. The formation of pore in ATSD is also further confirmed by scanning electron microscopy (Plate 4.1(a) and (b)). Both treated and untreated saw dust were subjected to scanning electron microscopy. While untreated saw dust showed only rough surface, the treated sample

LOG DIFFERENTIAL INTRUSION Vs DIAMETER
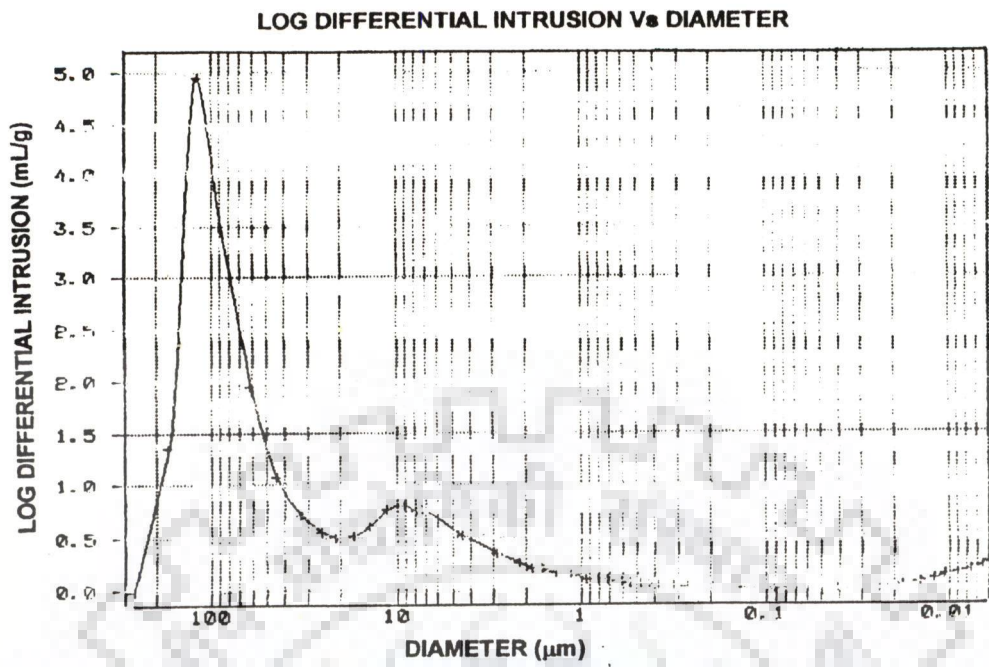
Fig.4.3(a) : Mercury Intrusion Porosimetry- Sawdust Before Treatment

LOG DIFFERENTIAL INTRUSION Vs DIAMETER
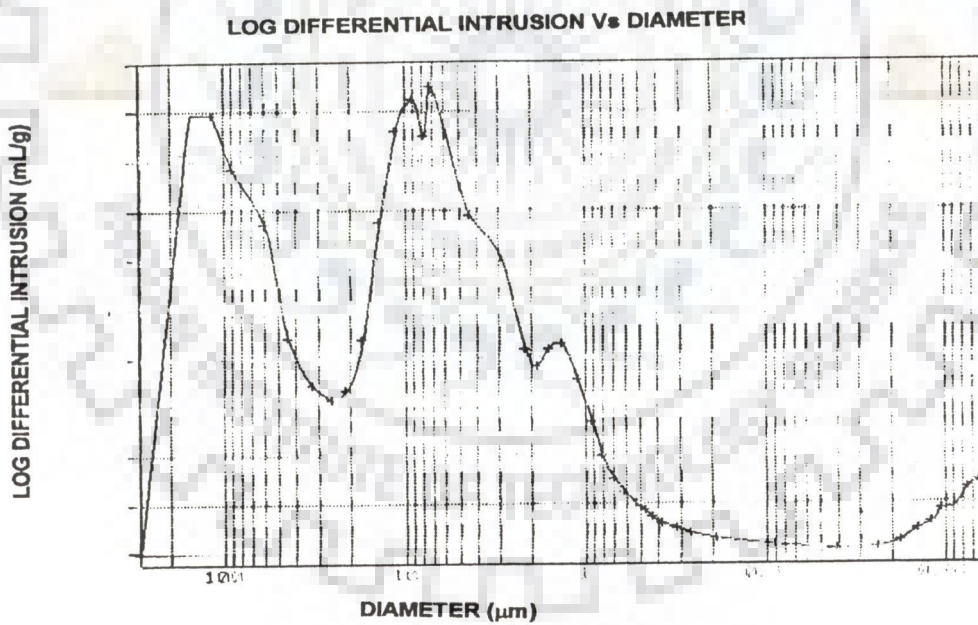
Fig.4.3(b) : Mercury Intrusion Porosimetry- Sawdust After Alkali Treatment

demonstrated vacuoles, formed probably because of lignin removal leaving spaces between the cellulose and hemicellullose network (Plates 4.1(a) and (b)).

4.1.2 Removal of Crude Oil Using Alkali Treated Sawdust

These observations that alkali treatment gives the more absorption capacity to this matrix was successfully applied for removal of spilled oil over waterbody. ATSD demonstrates high buoyancy and relatively high oil sorbing capacity as it removed 90-95% of crude oil from water surface as evident from the Plate 4.2

4.2 Isolation and Screening of Hydrocarbon Degrading Bacteria

4.2.1 Enrichment of Microorganisms in Semicontinuous Reactor

Every 24 hours, the sludge in the reactor was allowed to settle down and effluent samples were withdrawn followed by refilling the reactor with fresh distilled water. Samples were subjected to Chemical Oxygen Demand (COD) analysis [APHA,1992] to monitor the utilization of Gulf High Crude which would be the barometer for enrichment of hydrocarbon degraders. At the initial stage, the COD of the system was 572 mg/L as the degradation was negligible. With time, the reactor got stabilized and the COD was reduced to 220 mg/L. In 5 months time, the COD reduction reached upto 60% within 24 hours (Fig. 4.4) which indicates the abundance of hydrocarbon degrading organisms ultimately resulting in decrease in COD.

Fig. 4.4 : COD Reduction in the Effluent of Oil-fed Semicontinuous Reactor
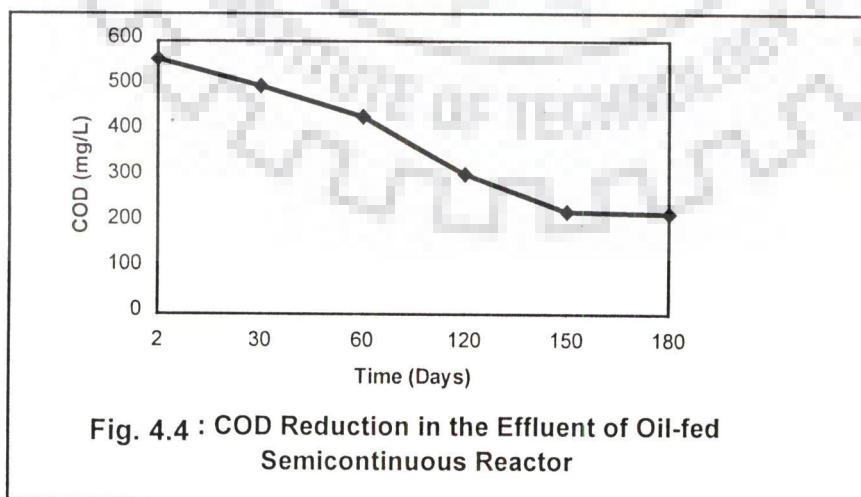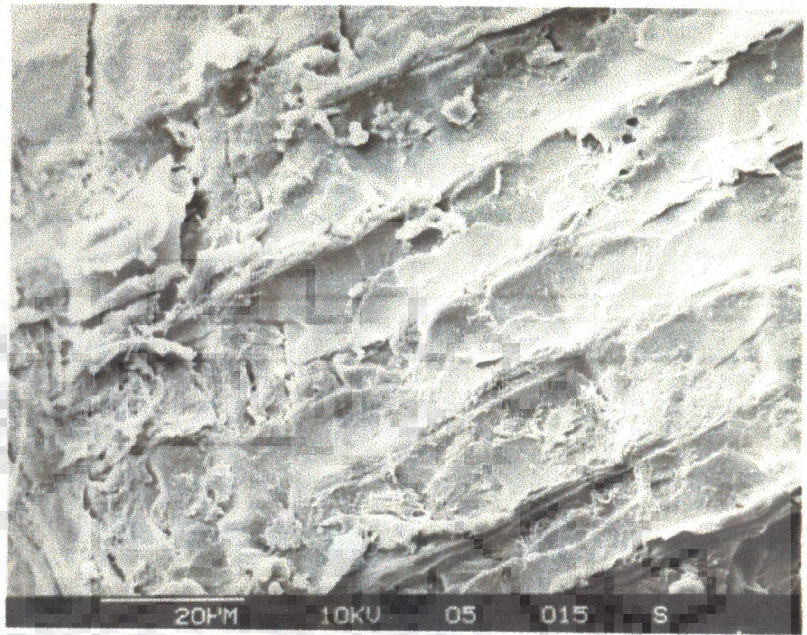
Plate 4.1(a) : Scanning Electron Microscopy - Sawdust Before Treatment
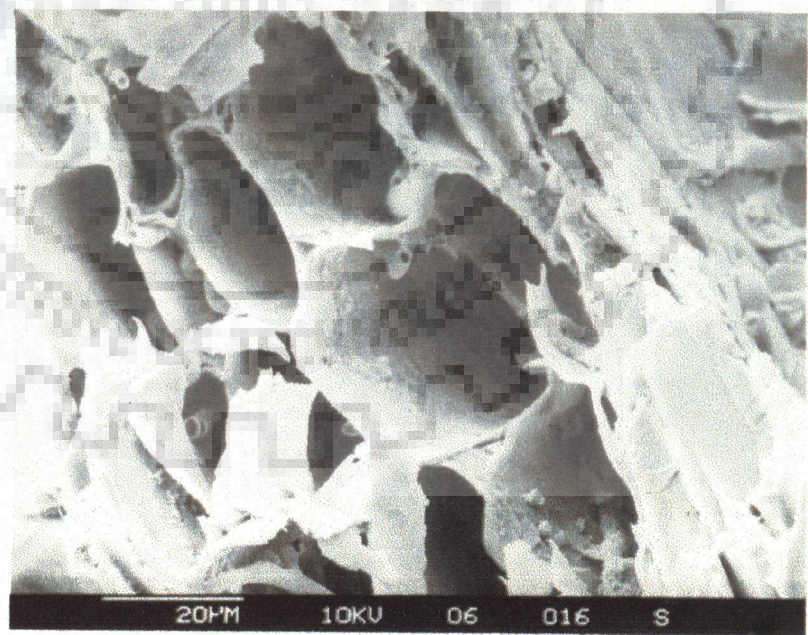
Plate 4.1(b) : Scanning Electron Microscopy - Sawdust After Alkali Treatment
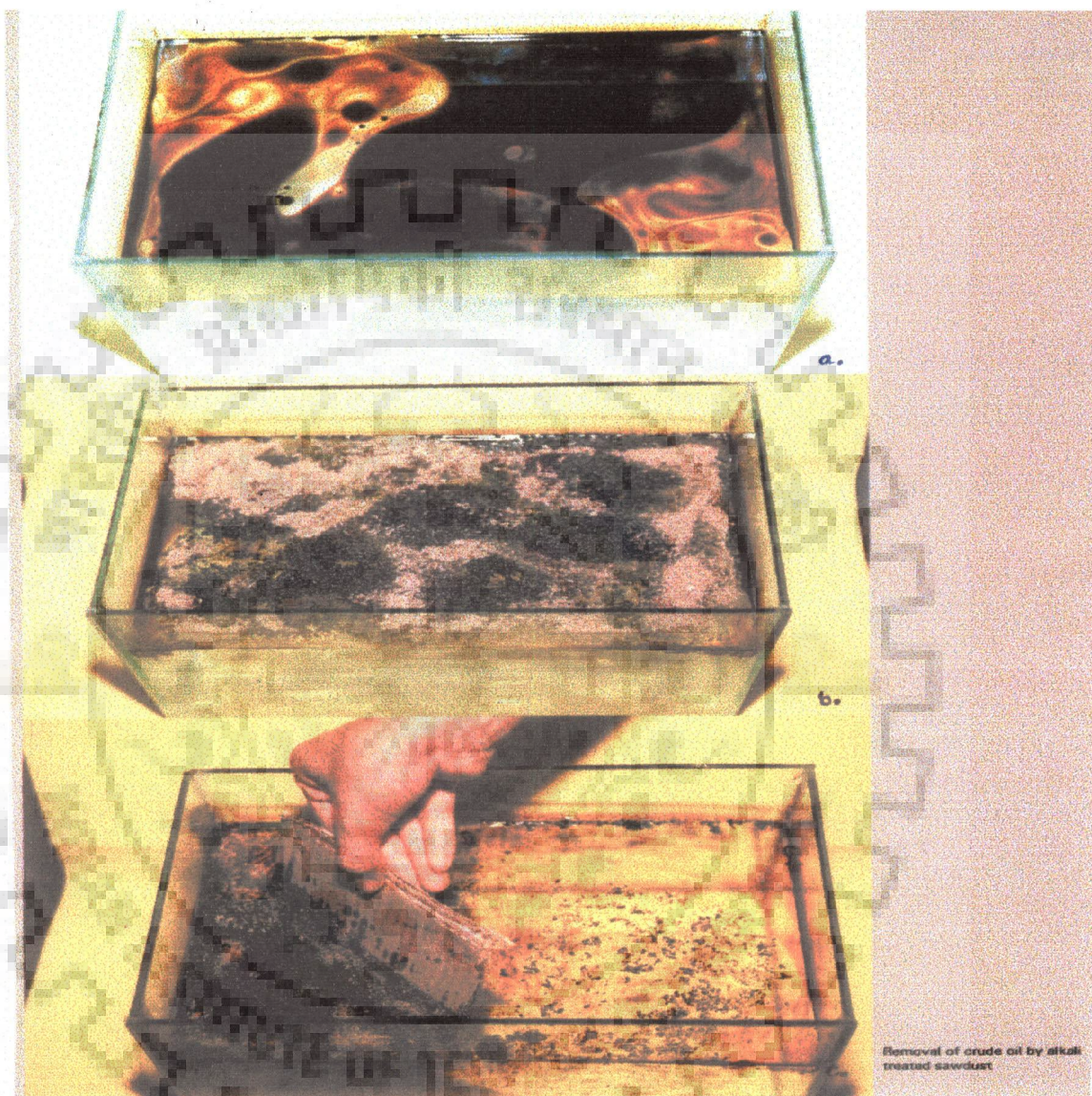
Plate 4.2 : Removal of spilled oil from an experimental aquarium using alkali treated sawdust
    (a) oil spilled over an water surface
    (b) treated sawdust has been applied over the oil slick
    (c) skimming the sawdust-oil mix

4.2.2 Isolation of Pure Bacterial Cultures

After having achieved the COD reduction of 60% (in 24 hr.), 10 ml of sludge was withdrawn from reactor and its serial dilution was used for the isolation of bacteria.

Thirty five different isolates were obtained from the three different kind of substrates (hydrocarbon) plates. Four isolates were obtained from diesel, fifteen from gulf high crude and sixteen from petrol. The isolates obtained on these substrates have been summarized in Table 4.1.

Table 4.1: Isolates Obtained on Different Substrates Amended to BH Medium

Substrate Used	Isolates Obtained
Diesel	4
Gulf High Crude Oil	15
Petrol	16

4.2.3 Screening and Identification of the Isolated Cultures based on the Three Tier Screening Strategy

Pure cultures of bacteria were screened for their capability to degrade hydrocarbon compounds using a three tier screening procedure as follows.

4.2.3.1 Primary Screening

This included characterization of the isolated cultures on the basis of their morphology, incubation time and temperature and colony characteristics. The results are presented in Table 4.2.

4.2.3.2 Secondary Screening

Antibiotic sensitivity pattern of the isolates was the criterion for secondary screening. The drug resistance marker genes are usually carried by the extrachromosomal DNA, which is also known to harbor the hydrocarbon degradative operon(s) (Tol, Sal, Nah, etc.) [Chakrabarty,1976 ; Sayler,1990]. To differentiate the isolates genetically, six antibiotics were used to carry out

Table 4. 2: Primary Screening of the Isolates

S.No.	Isolate	Incubation Time (h)	Temperature (^{0}C)	Surface/ Submerged	Morphology
1.	DSS$_2$	24	37	Surface	Rods
2.	GSS$_{13}$				
3.	GSS$_{14}$				
4.	PSS$_{18}$				
5.	PSS$_3$	48	37	Surface	Rods
6.	GSS$_{10}$				
7.	GSS$_{11}$				
8.	GSS$_1$	24	30	Surface	Long Rods
9.	GSS$_7$	72	37	Surface	Short Rods
10.	GSS$_{15}$				
11.	DSS$_8$	144	30	Surface	Short Rods
12.	PSS$_{13}$				
13.	GSS$_{12}$	144	30	Surface	Long Rods
14.	DSS$_6$	72	30	Surface	Rods
15.	PSS$_4$				
16.	PSS$_5$				
17.	PSS$_6$				
18.	PSS$_{15}$				
19.	PSS$_{17}$				
20.	GSS$_2$	24	30	Surface	Rods
21.	GSS$_9$				
22.	PSS$_1$				
23.	GSS$_3$	48	30	Surface	Rods
24.	GSS$_4$				
25.	PSS$_2$				
26.	PSS$_{14}$				
27.	PSS$_{16}$				
28.	DSS$_7$	24	30	Submerged	Rods
29.	GSS$_5$				
30.	GSS$_6$	24	30	Submerged	Long Rods
31.	GSS$_7$	24	30	Submerged	Short Rods
32.	GSS$_8$				
33.	PSS$_8$				
34.	PSS$_9$				
35.	PSS$_{10}$				

Table 4. 3: Secondary Screening of the Isolates

Isolate	Degree of Sensitivity*			
	Tetracycline	Gentamicin	Chloremphenicol	Kanamycin
Diesel				
DSS$_2$	b	a	R	b
DSS$_6$	b	a	R	a
DSS$_7$	b	a	R	b
DSS$_8$	b	a	R	b
Petrol				
PSS$_1$	b	a	b	a
PSS$_2$	b	a	R	b
PSS$_3$	b	a	b	a
PSS$_4$	b	a	a	a
PSS$_5$	b	a	R	a
PSS$_6$	b	a	R	b
PSS$_7$	b	a	R	b
PSS$_8$	b	a	R	b
PSS$_9$	b	a	R	b
PSS$_{13}$	b	b	R	R
PSS$_{14}$	b	a	R	R
PSS$_{15}$	b	a	R	b
PSS$_{16}$	c	b	R	b
PSS$_{17}$	c	b	R	b
PSS$_{10}$	c	b	b	b
Gulf High Crude				
GSS$_1$	b	a	R	a
GSS$_2$	b	a	R	a
GSS$_3$	c	a	a	b
GSS$_4$	d	R	a	a
GSS$_5$	b	a	a	a
GSS$_6$	a	a	R	a
GSS$_7$	a	a	R	a
GSS$_8$	b	a	R	a
GSS$_{10}$	b	a	a	a
GSS$_{12}$	c	a	R	b

All isolates were found to be resistant to Ampicillin (10µg/disc) and Penicillin-G (10µg/disc)

* a < b < c < d   where a,b,c,d represent zone of inhibition of 1,2,3, or 4 cms, respectively

Table 4. 4: Tertiary Screening of the Isolates

No.	Isolate	Catechol (Monocyclic)	Phenanthrene (Tricyclic)	Dodecane (C_{12})	Eicosane (C_{20})	Octacosane (C_{28})
1.	DSS$_2$	+	++	+	++	+
2.	DSS$_5$	+	++	++	+	+++
3.	DSS$_6$	+++	++	+	+	+++
4.	DSS$_7$	+	+	+	++	++
5.	DSS$_8$	++	+++	+++	+++	++
6.	GSS$_1$	++	++	+++	++	++
7.	GSS$_3$	+++	+++	+	++	+
8.	GSS$_4$	++	+	+	+	+
9.	GSS$_5$	++	+	+	+	++
10.	GSS$_7$	++	++	+	+	+++
11.	GSS$_{10}$	++	+	+	+	+
12.	GSS$_{12}$	++	++	++	++	+++
13.	PSS$_1$	+	++	+	+	+
14.	PSS$_2$	+	++	++	++	++
15.	PSS$_4$	+	++	++	++	+
16.	PSS$_8$	++	+	+	+	++
17.	PSS$_{13}$	++	+	+	+	+
18.	PSS$_{14}$	+++	+	++	+	++
19.	PSS$_{15}$	+	++	+	++	++
20.	PSS$_{16}$	++	++	++	+++	++
21.	PSS$_{17}$	+	++	++	+	++
22.	PSS$_5$	+	++	+	+	+

Growth/Utilization monitored in terms of absorbance at 620 nm

+ : O.D.0.05-0.20

++ : O.D. 0.21-0.40

+++ :O.D. > 0.40

antibiotic sensitivity discs assay. The antibiotics tested and the results obtained are mentioned in the Table 4.3. The zone of inhibition was measured for each antibiotic. All organisms were found to be resistant to ampicillin (10μg/disc) and penicillin (10μg/disc) while only 12 isolates were resistant to chloramphenicol (30μg/disc). The isolates also showed varying degree of sensitivity to tetracycline, gentamicin and kanamicin as seen from the data presented in Table 4.3.

4.2.3.3 Tertiary Screening

The organisms were finally segregated on the basis of their capability to utilize hydrocarbons. Five representative hydrocarbon molecules were provided to twenty organisms individually in BH medium. Catechol and phenanthrene (50mg/L) were used as representative aromatics and eicosane, octacosane and dodecane (100 mg/L) were chosen to represent aliphatics. Growth was monitored by measuring absorbance of culture broth at 620 nm. The results are depicted in Table 4.4.

Only two organisms, NCC.DSS$_8$ and NCC.GSS$_3$ grew well on phenanthrene, a dicyclic aromatic. Similarly only two organisms, NCC.DSS$_6$ and NCC.DSS$_8$ utilized long chain aliphatics, eicosane (C$_{20}$) and octacosane (C$_{28}$), very effectively. For further studies, these three organisms, NCC.DSS$_6$, NCC.DSS$_8$ and NCC.GSS$_3$ were selected rationally as their combined efforts would lead to effective degradation of various fractions of crude oil.

4.2.4 Characterization of Selected Isolates

On the basis of growth characteristics at the final level of screening, three organisms were selected for further studies.

4.2.4.1 Colony Characteristics

The three isolates were inoculated on nutrient agar and *Pseudomonas* isolation agar using the spread plate technique and incubated at 30^{0}C to study the colony characteristics of the isolates on these agar media. The results obtained are summarized in Table 4.5, one significant observation being that the NCC.GSS$_3$ and NCC.DSS$_8$ isolates did not grow on the *Pseudomonas*

isolation agar while the NCC.DSS$_6$ isolate gave diffused, circular blue green colonies on this agar. Plate 4.3, 4.5 and 4.7 show the characteristic colonies of the three isolates as appearing on nutrient agar plates.

Table 4.5: Colony Characteristics of the Selected Isolates on Various Media

Colony Characteristics	Isolate NCC.DSS$_6$	Isolate NCC.DSS$_8$	Isolate NCC.GSS$_3$
Nutrient Agar	Circular, translucent, smooth surface, entire margin, large convex colonies with moist appearance	Circular, Translucent, Convex colonies with smooth margin	Small circular colonies, smooth surface and margin
Pseudomonas **Isolation Agar**	Circular, small, smooth, blue green moist colonies,	No growth observed	No growth observed

4.2.4.2 Morphological Characterization

The three bacterial isolates NCC.DSS$_6$, NCC.DSS$_8$ and NCC.GSS$_3$, were inoculated in nutrient broth and incubated at 30^{0}C for 18 hours to obtain active growth of the organisms. The gram reaction of the isolates was determined by differential staining. All the three isolates gave a gram negative reaction. All the three cultures were observed to be motile under the hanging drop method. Spore staining method of Norris and Swain [1971] suggested that the isolates were nonsporulating. Pigment production by the bacterial isolates was studied on King's A medium [King *et al.*, 1954]. No pigment production was observed with isolate NCC.DSS$_8$ and NCC.GSS$_3$ while NCC.DSS$_6$ produced blue green pigment. The morphological characteristics of the isolates are depicted in Table 4.6.

Plate 4.3 : Colony Characteristics on Nutrient Agar Plates - Isolate NCC.DSS$_6$
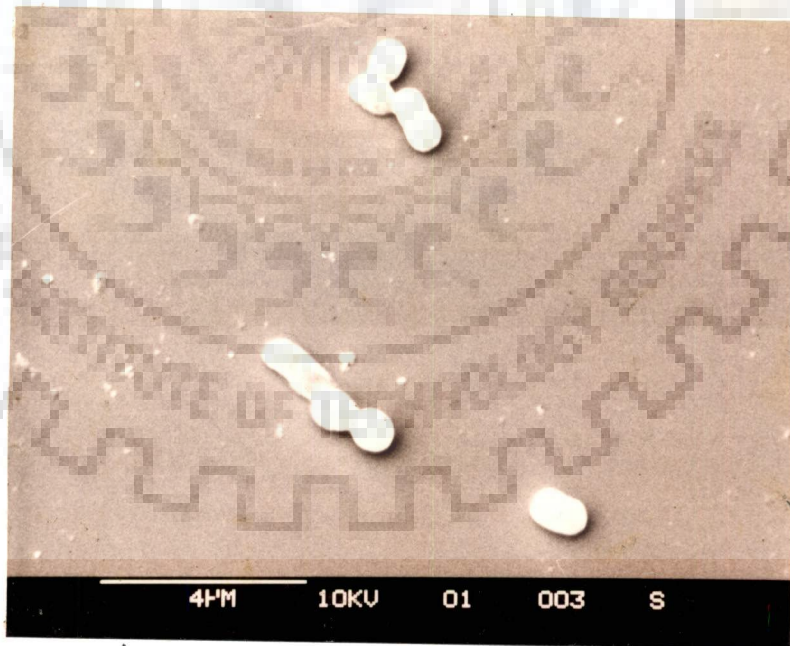

Plate 4.4 : Scanning Electron Micrograph - Isolate NCC.DSS$_6$

Plate 4.5 : Colony Characteristics on Nutrient Agar Plates - Isolate NCC.DSS$_8$
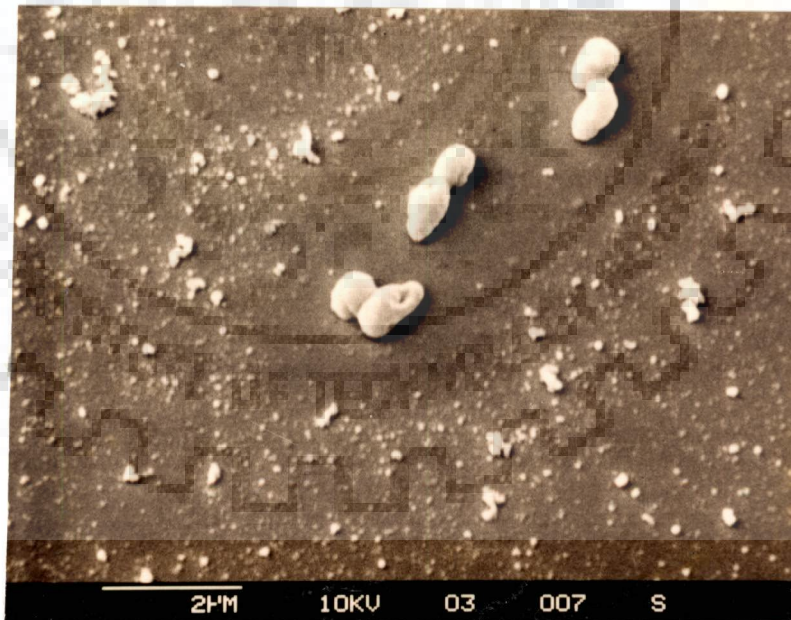

Plate 4.6 : Scanning Electron Micrograph - Isolate NCC.DSS$_8$

Plate 4.7 : Colony Characteristics on Nutrient Agar Plates - Isolate NCC.GSS$_3$
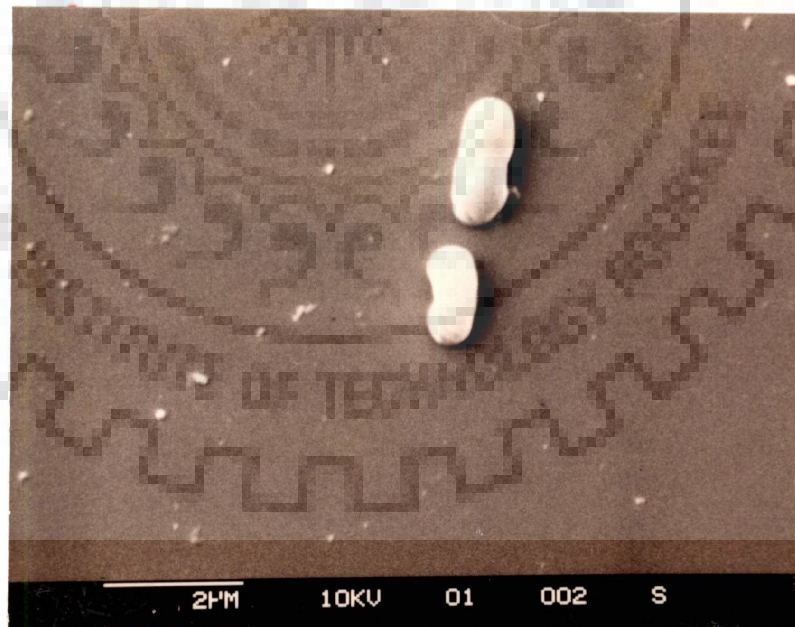
Plate 4.8 : Scanning Electron Micrograph - Isolate NCC.GSS$_3$

Table 4.6: Morphological Characteristics of the Isolates

Morphological Characteristics	NCC.DSS$_6$	NCC.DSS$_8$	NCC.GSS$_3$
Gram Staining **Shape** **Motility** **Spores**	Gram negative Short rods Actively motile Absent	Gram negative Short rods Motile Absent	Gram negative Short rods Motile Absent
Pigment Production on King's A Medium	Blue Green Pigment	No pigment produced	No pigment Produced

The scanning electron microscopy of the bacterial isolates was carried out by fixing the cultures with gluteraldehyde phosphate buffer, dehydration with isopropyl alcohol and then coating with osmium tetraoxide. The scanning electron micrographs of the isolates are shown in Plate 4.4, 4.6 and 4.8. Isolate NCC.DSS$_6$ appeared as short rods with rounded ends and the NCC.GSS$_3$ and NCC.DSS$_8$ rods with sharp edges.

4.2.4.3 Biochemical Characterization

The three tier screening strategy employed for the selection of prospective cultures, comprises a part of the identification studies i.e. it completes the substrate specificity, drug resistance patterns and morphological characterization of the isolated cultures. This was followed by the physiological and biochemical characterization, based on the methods mentioned in "The Bergey's Manual of Systematic Bacteriology" and "Prokaryotes".

4.2.4.3.1 Biochemical Tests for Identification

Biochemical characterization of the cultures was carried out by performing a series of tests, beginning with the IMViC test, followed by the oxferm test, the enzymatic profiles of the cultures, and their ability to utilize various sugars and other carbon sources as single substrates for growth. With the IMViC test it was found that all the three isolates could not produce indole, gave a negative methyl red test, could utilize citrate, and gave a positive Voges-Proskeur test. When tested for H$_2$S production, all isolates gave a positive response. The ability of the isolates to oxidize or ferment sugars with and without acid or gas production was determined using

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glucose oxidation fermentation (OF) medium [Lanyi,1987]. Replicate tubes were incubated under aerobic and anaerobic conditions at 30°C. The tested organisms were capable of oxidizing as well as fermenting the sugar.

The results for the enzymatic reactions reveal that all the three isolates were highly aerobic as evident from their high oxidase and catalase activity. The NCC.GSS$_3$ isolate also possesses lipase and arginine dihydrolase enzymes and is also capable of reducing nitrate, while the NCC.DSS$_6$ and NCC.DSS$_8$ isolates show a negative response for lipase. Table 4.7 gives the biochemical characterization of the isolates.

Carbohydrate utilization was studied in a medium containing peptone, sodium chloride and a single carbohydrate substrate (pentoses, hexoses). The results of the sugar fermentation tests are depicted in Table 4.8.

Based on the results mentioned in Tables 4.1 to 4.8 and those observed from Plate 4.3 to 4.8, the three isolates have been identified as *Pseudomonas sp.*

Table 4.8: Utilization of Various Sugars by the Isolates

Sugar	Isolates		
	NCC.GSS$_3$	NCC.DSS$_6$	NCC.DSS$_8$
L-Arabinose	Positive	Negative	Positive
D-Fructose	Positive	Positive	Positive
D-Galactose	Positive	Negative	Negative
D-Glucose	Positive	Positive	Positive
Inositol	Positive	Negative	Negative
Lactose	Positive	Negative	Negative
Maltose	Positive	Positive	Positive
D-Mannitol	Positive	Negative	Negative
D-Mannose	Positive	Negative	Positive
Sarbose	Negative	Negative	Negative
D-Sorbitol	Positive	Negative	Negative
Sucrose	Positive	Positive	Positive
Xylose	Positive	Negative	Negative

Table 4.7: Biochemical Characterization of the Isolates

Biochemical Test	NCC.DSS$_6$	NCC.DSS$_8$	NCC.GSS$_3$
IMViC Test			
Indole Production	Negative	Negative	Negative
Methyl Red Test	Negative	Negative	Negative
Voges Proskeur Test	Positive	Positive	Positive
Citrate Utilization	Positive	Positive	Positive
H$_2$S Production	Positive	Positive	Positive
Oxferm Test			
Oxidation	Positive	Positive	Positive
Fermentation	Positive	Positive	Positive
Aerobic/Anaerobic	Facultative aerobe	Facultative aerobe	Facultative aerobe
Starch hydrolysis (Amylase)	Positive	Positive	Positive
Casein hydrolysis (Caseinase)	Negative	Positive	Positive
Tween 80 hydrolysis (Lipase)	Positive	Negative	Positive
Gelatin liquefication (Gelatinase)	Positive	Positive	Negative
Arginine dihydrolase	Positive	Negative	Positive
Urease production	Positive	Negative	Negative
Oxidase test	Positive	Positive	Positive
Catalase test	Positive	Positive	Positive

4.2.4.3.2 Ring Cleavage Mechanism in Isolates

The type of breakdown of aromatic ring (ortho- or meta- cleavage) is a significant characteristic of Genus *Pseudomonads* [Stanier,1966]. The selected organisms were found to be growing on catechol which is a cyclic compound (Table 4.4). To study the mechanism of ring fission, isolates were grown on catechol, cells harvested and tested for Rothera reaction. A positive reaction exhibits deep purple colour. All the isolates (NCC.DSS$_6$, NCC.DSS$_8$ and NCC.GSS$_3$) gave deep purple colour in Rothera test while one negative control, an *E. coli* clone containing *xyl E* from TOL plasmid [Stein, 1990], gave yellow color for meta- cleavage reaction.

4.2.4.4 Characterization of Isolates at Genetic Level

4.2.4.4.1 Screening the Isolates for Presence of Plasmids

The hydrocarbon degrading capabilities are often associated with catabolic plasmid(s), especially in *Pseudomonas* sp. There are several traits known to be of plasmid origin [Sayler *et*

*al.,*1990]. Hence the selected isolates were screened for the presence of plasmid(s). The plasmid screening was carried out using the methods for small scale preparation of plasmid DNA as mentioned in Molecular Cloning - A Laboratory Manual (Editors: Sambrook, Fritsch & Maniatis). Three methods as mentioned in materials and methods (section 3.2.3.1) were used for lysing the bacterial cells. Only NCC.DSS$_6$ demonstrated a supercoiled circular plasmid plasmid band in agarose gel electrophoresis (Plate 4.9) which was further confirmed as plasmid by restriction digestion and transformation to wild type *Pseudomonas putida* MTCC*102.

4.2.4.4.2 Characterization of Isolates on the Basis of Catabolic Pathway Using PCR

The isolates were further characterized on the basis of their genotypes for the presence of key enzymes of hydrocarbon dissimilation pathways using polymerase chain reaction (PCR) [Whyte *et al.*,1995]. The characterization was based on the presence of catabolic genes from known bacterial biodegradative pathways using oligonucleotide primers derived from those genes. Three primers were used for the experiments (listed in Table 4.9). The genomic DNA of the isolates was used as template in PCR reactions. The genomic DNA was prepared according to Marmur's protocol, measured spectrophotometrically at 260 nm and stored at -70°C. 1µg of this DNA was used as template. The PCR products were resolved on a 1.5% agarose gel by electrophoresis.

The PCR analysis of NCC.DSS$_8$ resulted in the amplification of DNA homologous to *alk B* gene (Table 4.9), *Pseudomonas oleovorans'* total DNA was used as the control [van Belien *et al.* ; Whyte *et al.*, 1995]. The Isolate NCC.GSS$_3$ gave positive signals with primer of catechol 2,3 dioxygenase and *dmpN* primers (Plate 4.10-12). The positive control for *dmpn* was *Pseudomonas putida* Pp27 [Shingler *et al.,*1995] while for catechol 2,3 dioxygenase, *xyl E* of the TOL operon (pWWO) was used as the positive control [Stein, 1990].

Table 4.9: Oligonucleotide Primers Used for PCR Amplification of Catabolic Genes

Catabolic Gene	Primer sequence	PCR fragment size (bp)	Microorganism
alkB Forward	5'-TGGCCGGCTACTCCGATGATCGG AATCTGG-3'	870	*P.oleovorans* ATCC 29347
Reverse	5'-CGCGTGGTGATCCGAGTGCCGCT GAAGGTG-3'		
xylE Forward	5'-GTGCAAGCTGCGTGTATGGACAT GAGCAAG-3'	834	*E.coli* from Stein [1990]
Reverse	5'-GCCCAGCTGGTCGGTGGTCCAGG TCACCGG-3'		
dmpN Forward	5'-CATGACTTCGCCCATATGTACGA CC-3'	550	*P.putida* pp27 [Shingler,1995]
Reverse	5'-GTATTTCGGCGGCCGCATGCCATA GC-3'		

Plate 4.9 : Agarose Gel Electrophoresis - plasmid detection in NCC.DSS$_6$ showing a low molecular weight

plasmid

Lane1 : Uncut Plasmid (NCC.DSS$_6$) prepared by Qiagen-500

Lane2 : Digested with *AccI*

Lane3 : Digested with *HindIII*

Lane4 : 1Kb ladder (molecular marker)

Plate 4.10 : Agarose Gel Electrophoresis- PCR amplification of total DNA of selected isolates using *alkB*
primers

Lane1 : NCC.GSS$_3$ DNA

Lane2 : NCC.DSS$_6$ DNA

Lane3 : NCC.DSS$_8$ DNA

Lane4 : *P. putida**102 DNA

Lane5 : Positive control (total DNA of *P. oleovorans*)

Lane6 : 1Kb ladder

Plate 4.11 : Agarose Gel Electrophoresis- PCR amplification of total DNA of selected isolates using *dmpN*
primers

Lane1 : Positive control (total DNA of *P. putida* pp27)

Lane2 : NCC.GSS$_3$ DNA

Lane3 : NCC.DSS$_6$ DNA

Lane4 : NCC.DSS$_8$ DNA

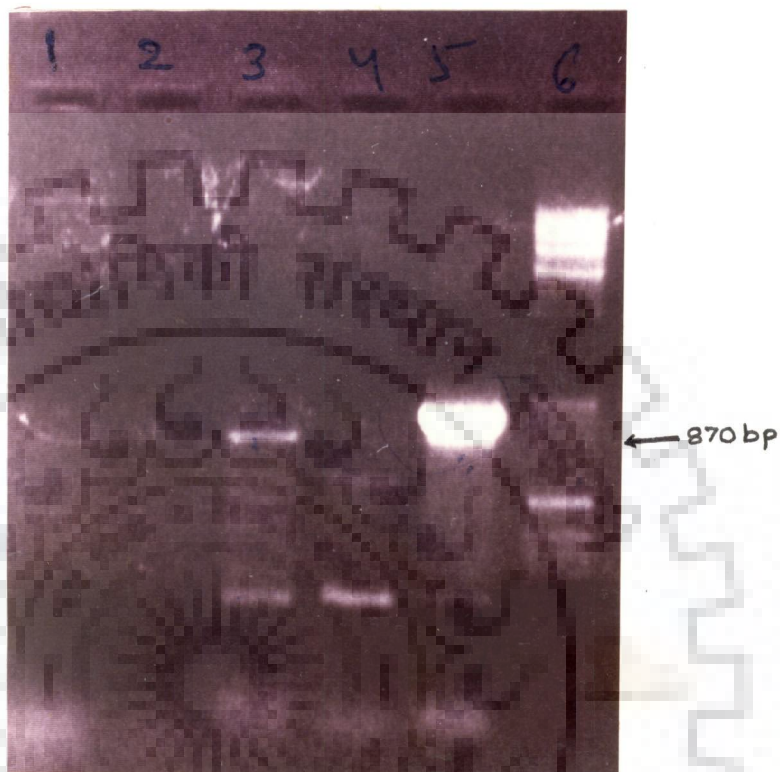Lane5 : *P. putida**102 DNA

Lane6 : 1Kb ladder

Plate 4.12 : Agarose Gel Electrophoresis-PCR amplification of total DNA of selected isolates using *xylE*
primers

Lane1 : NCC.GSS$_3$ DNA

Lane2 : NCC.DSS$_6$ DNA

Lane3 : NCC.DSS$_8$ DNA

Lane4 : *P. putida**102 DNA

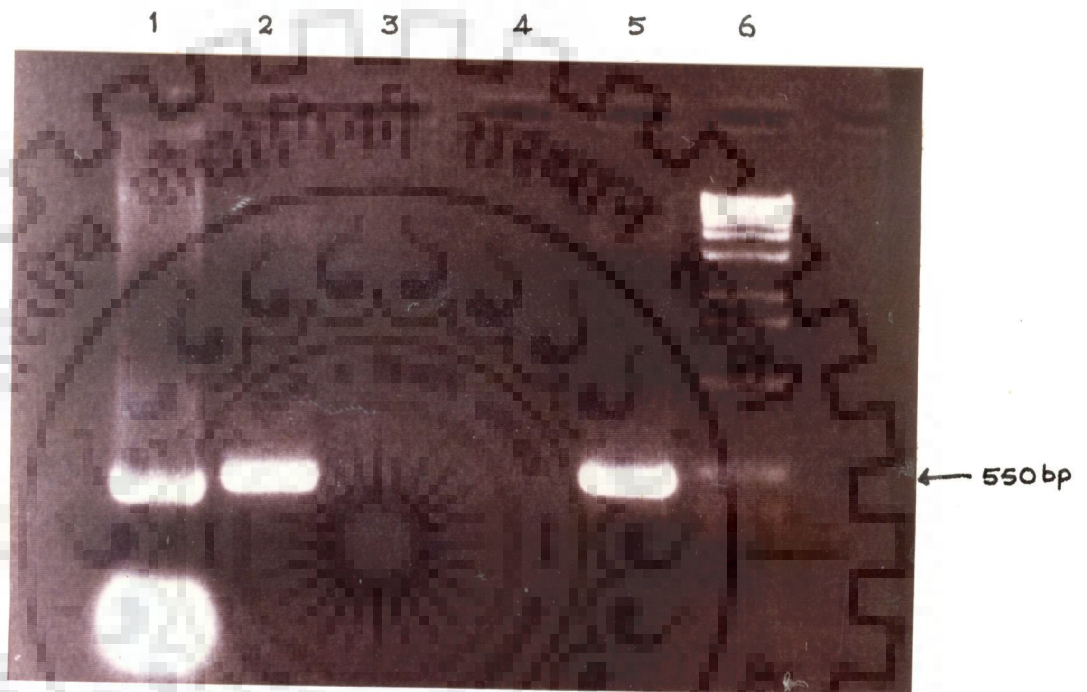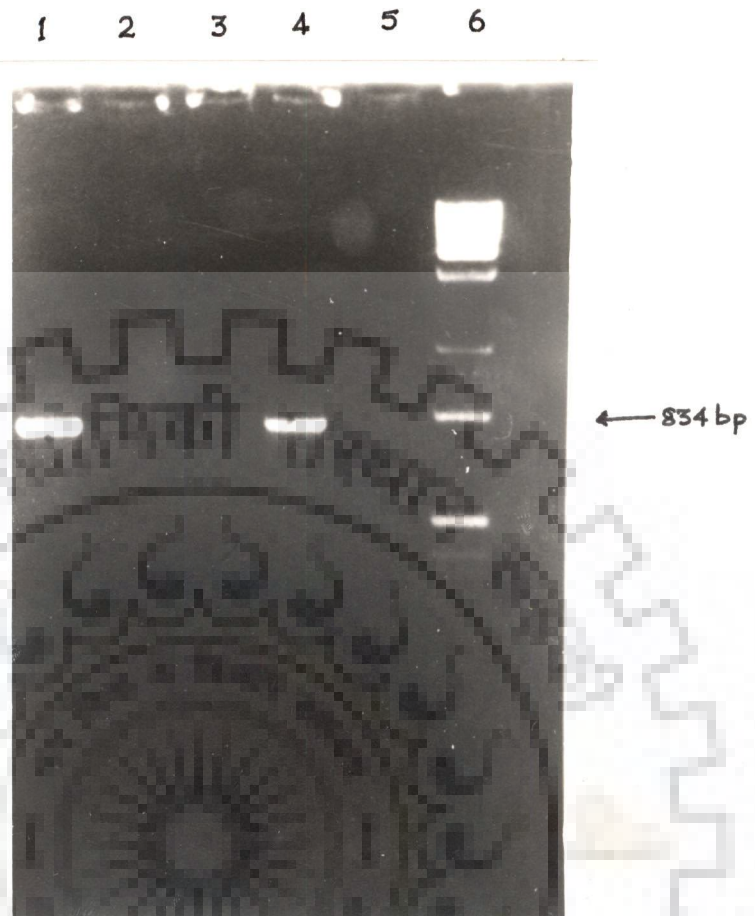Lane5 : Positive control (total DNA of *P. oleovorans*)

Lane6 : 1Kb ladder

4.3 Hydrocarbon Degradation Potential of Bacterial Consortium

The degradation experiments were conducted by designing a consortium having three different bacterial isolates and *P. putida* MTCC*102. The efficacy of the consortium for crude oil degradation was assessed in this study. The role of individual organism in the process was also studied.

4.3.1 Selection of Inducer For Seed Culturing

Seed culture inoculum was prepared by adapting all the four members of consortium in BH medium amended with catechol/salicylate or benzoate (100mg/L) as the sole source of carbon. A heterogeneous mixture of representative hydrocarbons (model petroleum) was used as substrate at the concentration of 100ppm (composition given in Table 3.1). The gas chromatographic profile of model petroleum was obtained after extraction of the culture medium after 72 hours of incubation with 10 ml hexane (1:1 v/v). It was observed that catechol adapted consortium degraded model petroleum most efficiently (Fig. 4.5 b). Citrate grown consortium exhibited low extent and rate of degradation while benzoate and salicylate, the known inducer for upper and lower pathway of NAH operon [Schell, 1990] demonstrated fairly good degradation but not as much as catechol (Fig.4.6 a&b). Therefore catechol was selected for pre-adaptation of consortium prior to oil degradation.

4.3.2 Biodegradation of Crude Oil by Catechol Adapted Consortium

The seed cultures of the consortium members were prepared on catechol as described in section 4.3.1. Bombay High Crude oil was used as a sole source of carbon and energy in the degradation experiments at the concentration of 1% in 25 ml BH medium. The culture flasks were incubated at 30^0C at rotary shaker at 150 rpm for 84 hours. Citrate grown consortium was also used in the same manner to study the difference between the degradative capacities of catechol adapted and unadapted consortium. Consortium members were inoculated individually also in one set of experiment. Samples were drawn every 12 hours for estimation of biomass through the measurement of absorbance of culture medium at 620 nm to study the utilization of petroleum

Fig. 4.5 : Effect of various inducers on the degradation of model petroleum by the designed consortium
{a. Chemical Control, b. Catechol Grown Consortium }
Peak1-dodecane Peak2-naphthalene Peak3-pentadecane Peak4-hexadecane Peak5-pristane Peak6-dibenzothiophene Peak7-phenanthrene Peak9-eicosane Peak8-tetracosane Peak10-octacosane
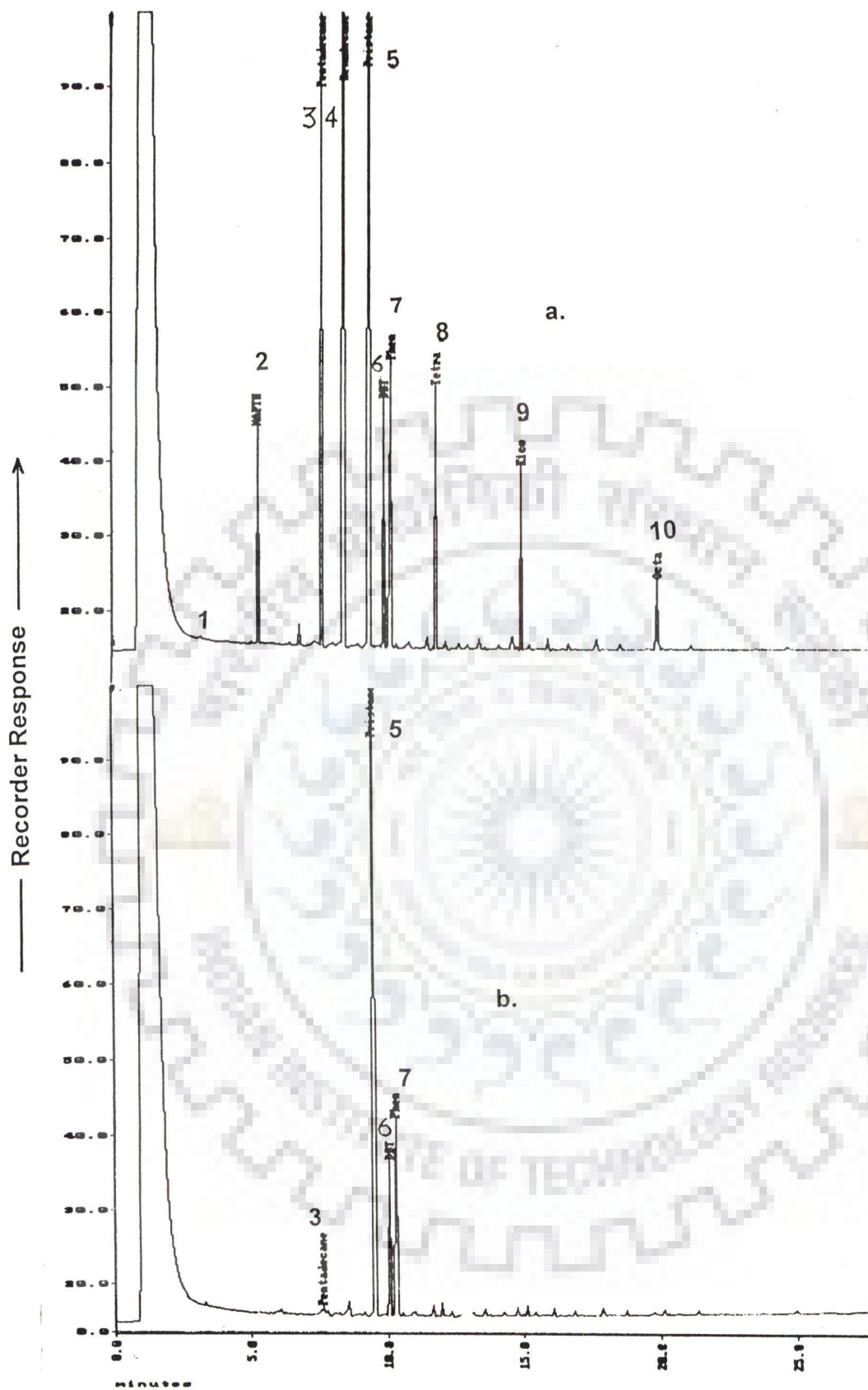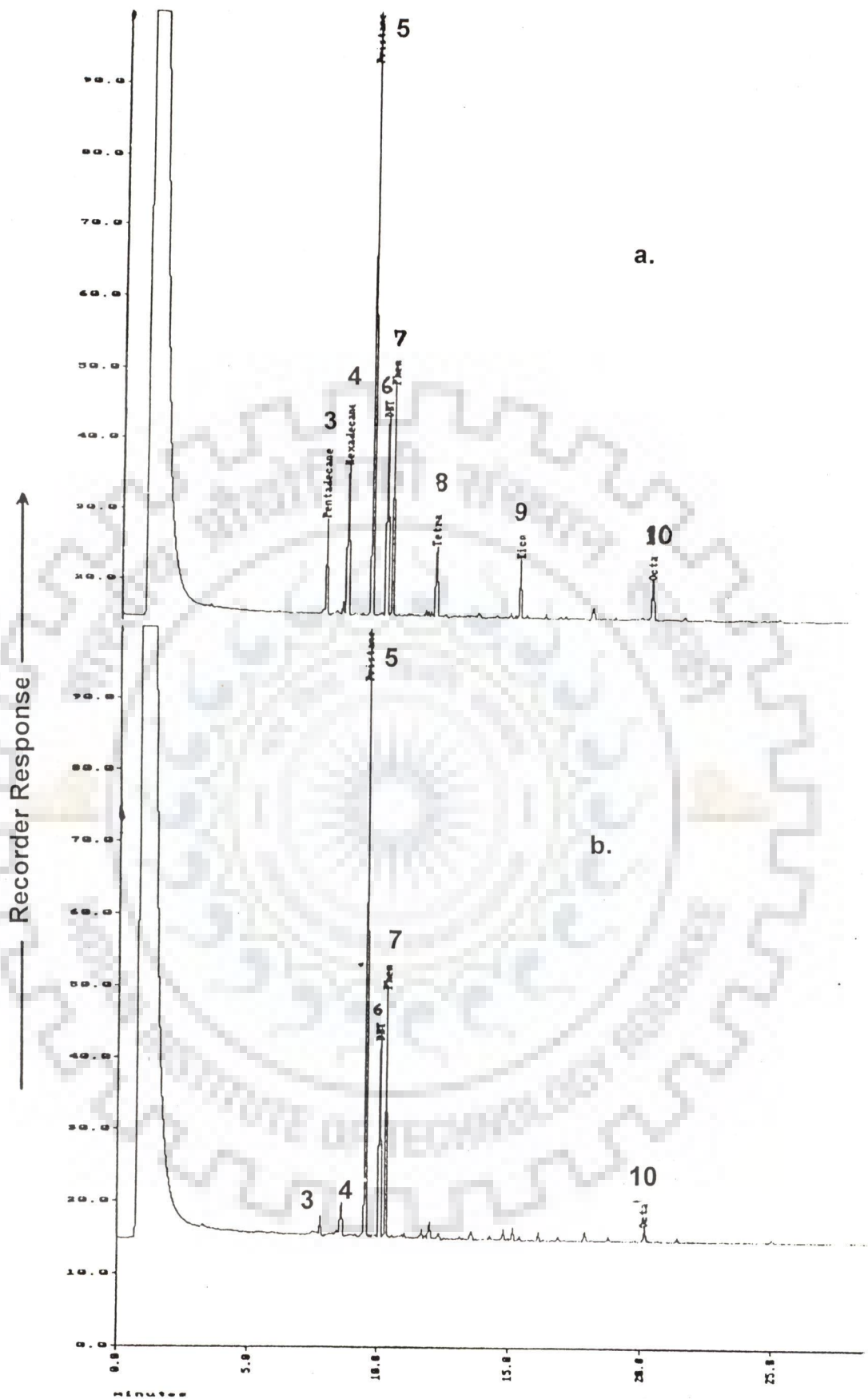
Fig. 4.6 : Effect of various inducers on the degradation of model petroleum by the designed consortium

{a. Salicylate Grown Consortium, b. Benzoate Grown Consortium}

Peak1-dodecane Peak2-naphthalene Peak3-pentadecane Peak4-hexadecane Peak5-pristane Peak6-dibenzothiophene Peak7-phenanthrene Peak9-eicosane Peak8-tetracosane Peak10-octacosane

hydrocarbons, one set of the culture flasks was extracted with hexane and extracts were analyzed by packed column gas chromatography. For COD estimation, hexane was evaporated on rotary flash evaporator at room temperature and the residue was used [APHA,1989]. Considering the chemical complexity of the hexane extracts, the concentrations of various fractions of crude oil resolvable by GC/FID were accounted as total area of all peaks having a height of 10% or more of the detector response. All treatments were in replicate and the average values with standard deviation of 5% or less were taken into consideration for the interpretation of results. the final analysis was done on a capillary gas chromatograph (Perkin Elmer, Autosystem equipped with 1022 GC plus).

It was observed that the catechol adapted designed bacterial consortium degraded crude oil effectively. Gas chromatography (Fig. 4.8) and COD analysis (Fig.4.7) showed that in 72 hrs, 65-70% degradation was achieved. COD indicates the mineralization capacity of the consortium while gas chromatography entails the exhaustive changes in hydrocarbon profile of crude oil due to diversified biotransformation capability of consortium, *in toto*.

Figure 4.7: COD Reduction of Crude Oil after Degradation by the Designed Consortium (after 72 hours)
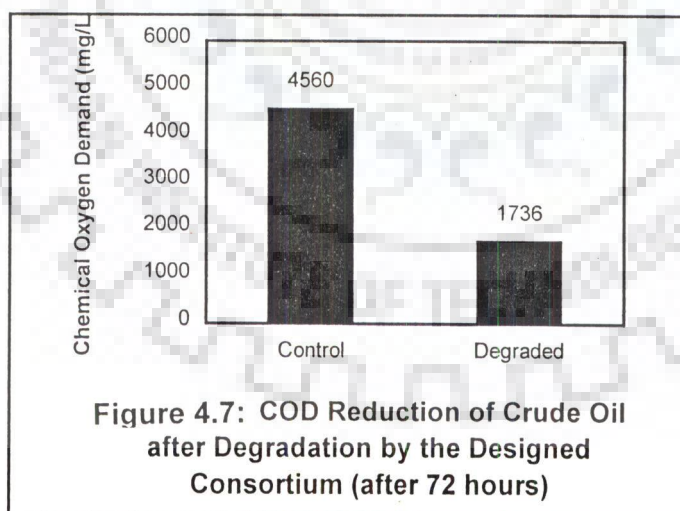
The final samples were analyzed on a capillary GC and similar results were obtained (Fig. 4.9). The unidentified peaks of crude oil components were quantified by the LC1022 integrator, attached with Autosystem (PE), measuring the peak area under each peak. When the catechol

Fig. 4.8 : Degradation of Bombay High Crude (1%) by the designed consortium adapted on catechol (analyzed on Sigma 300, FID)

A. Chemical Control- abiotic loss in 72 hr.

B. Experimental- degradation by consortium in 72 hr.
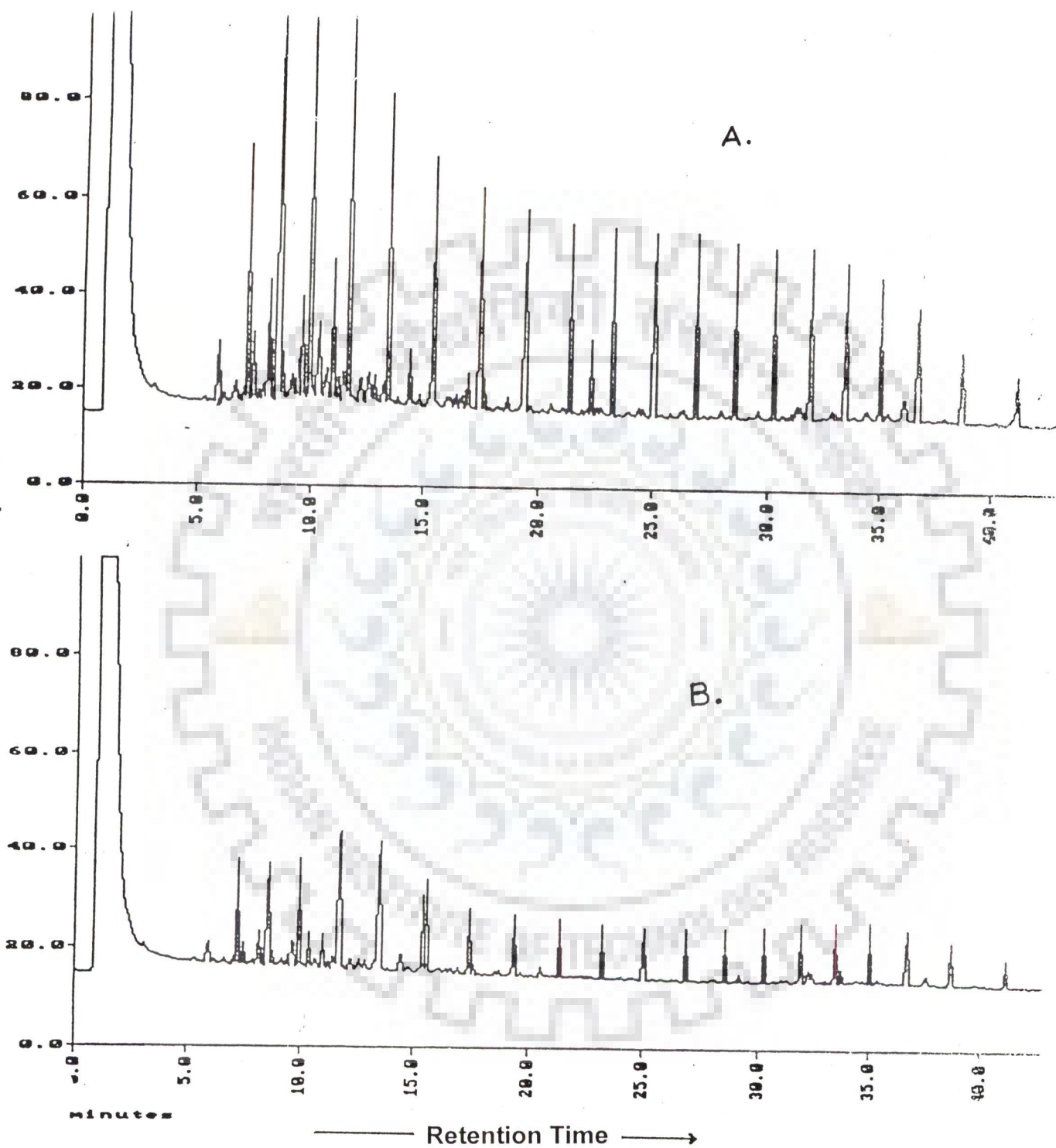
Fig. 4.9 : Degradation of Bombay High Crude (1%) by the designed consortium adapted on catechol (analyzed on PE Auto System, FID)

A. Chemical Control- abiotic loss in 72 hr.

B. Experimental- degradation by consortium in 72 hr.

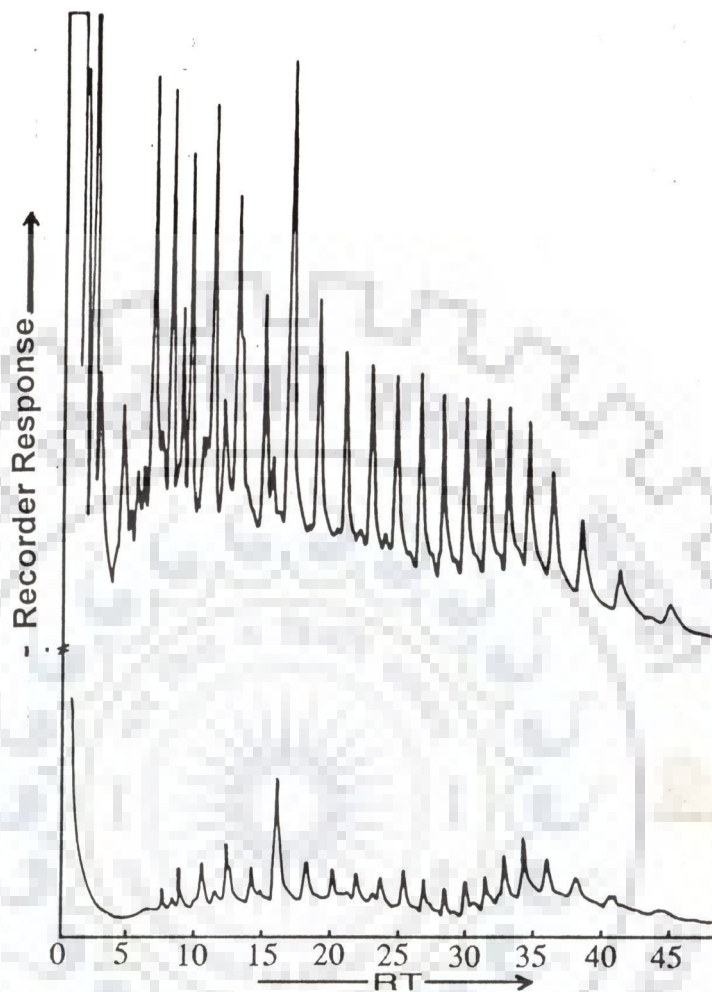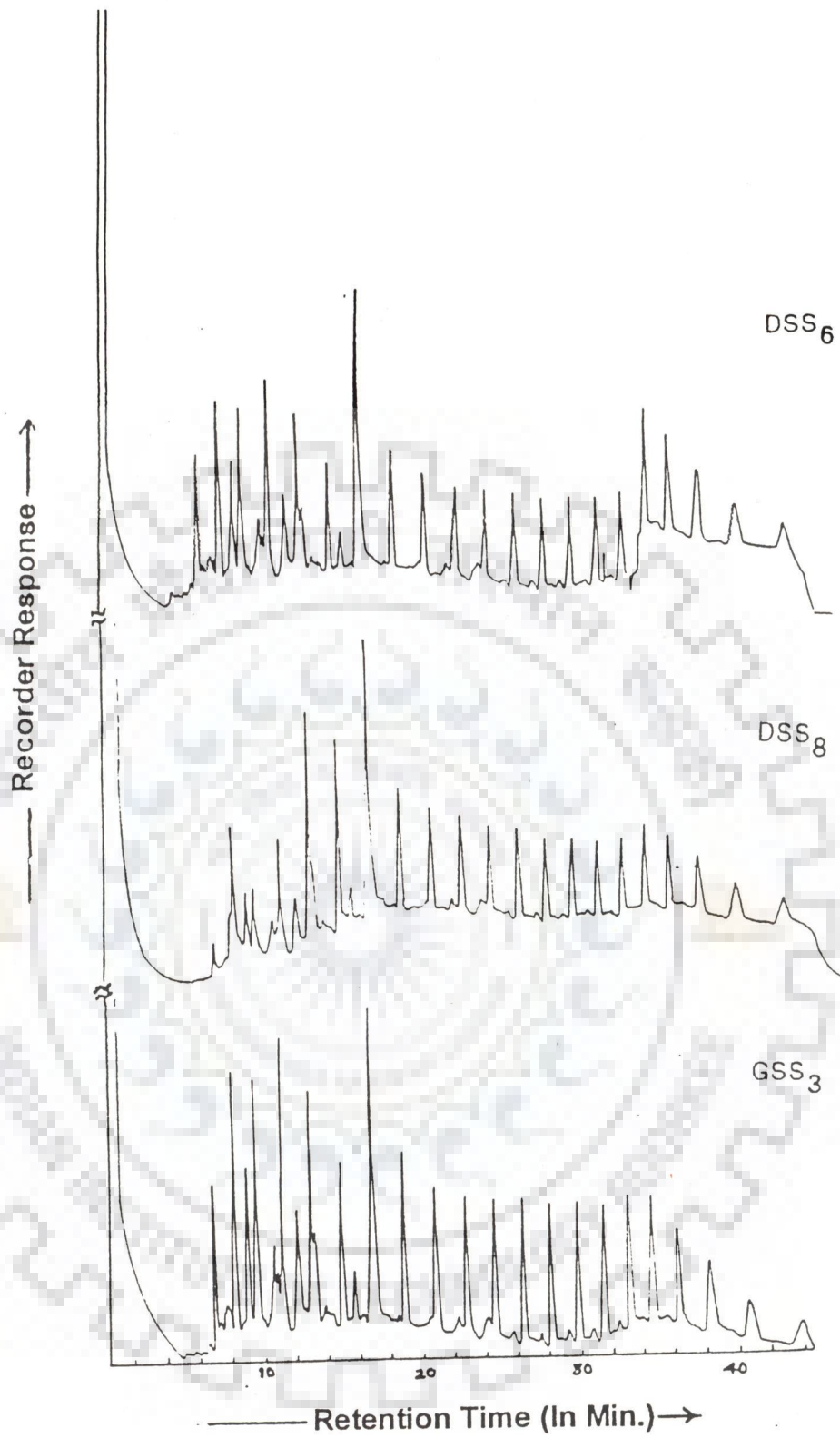Fig. 4.10 : Degradation of Bombay High Crude (1%) by individual consortium members adapted on catechol (analyzed on Sigma 300, FID)

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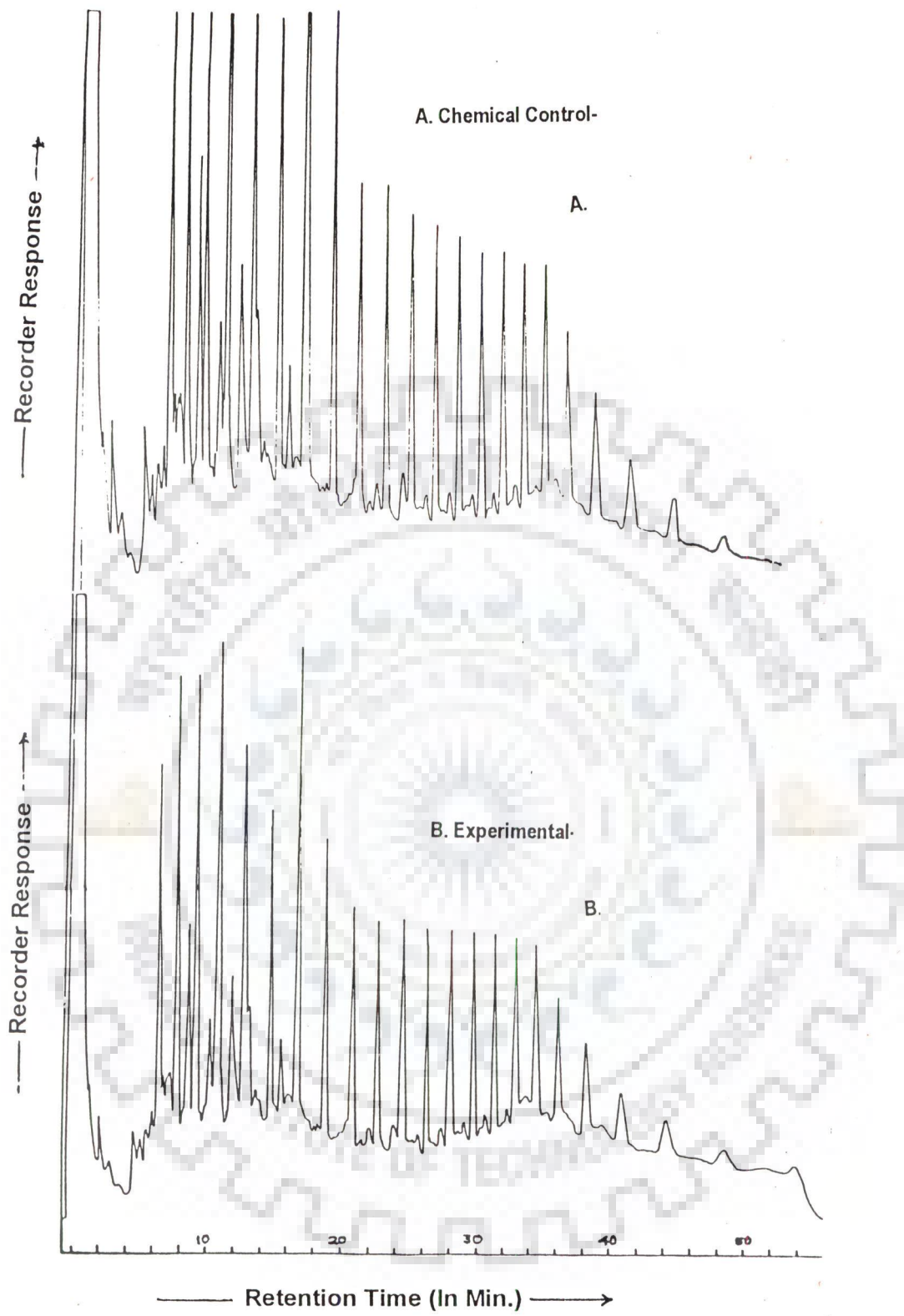
Fig. 4.11 : Degradation of Bombay High Crude (1%) by the designed consortium grown on citrate (analyzed on Sigma 300, FID)

A. Control

B. Degraded by consortium in 72 hr

grown consortium members were inoculated individually, the degradation was substantially reduced (Fig. 4.10). This indicates that though the organisms have capacities to attack various components of crude oil individually, their simultaneous actions are required for effective degradation. The citrate grown consortium could not degrade crude oil as efficiently as catechol grown (Fig. 4.11). The biomass determination also supported the results as the growth on crude oil was found approximately 0.9-1.1 OD/ml after 84 hours (Fig. 4.12).

Figure 4.12. Growth of the designed consortium on Crude Oil
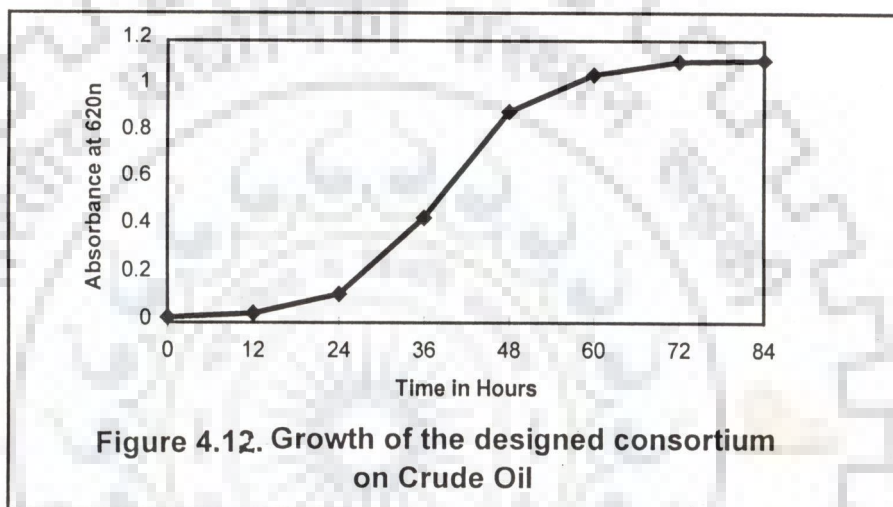
4.4 Biosurfactant and Crude Oil Degradation

4.4.1 Production Extraction and Characterization of Biosurfactant

Biosurfactants are surface active molecules produced by certain bacteria, yeast and fungi. These include a variety of chemical structures such as glycolipids, lipopeptides, polysaccharides, protein complexes, fatty acids and neutral lipids [Iqbal et al.,1995 ; MacDonald et al.,1987].

NCC.DSS$_6$, a member of the oil degrading consortium was found to be producing a biosurfactant. The characterization of the surfactant was done by culturing the organism in a defined medium which was deficient in phosphates (45 mg/L KH$_2$ PO$_4$) as reported earlier [Janiyani et al., 1992]. The culture flasks were incubated at 30°C on a rotary shaker. Samples were drawn at time intervals of 24,48,72 and 96 hours, cells pelleted and the cell free broth was used for surface tension determination.

The biosurfactant produced by isolate NCC.DSS$_6$ lowered the surface tension of liquid media to 37 dynes/cm^2 (Fig. 4.13) in 96 hrs against the control 72 dynes/cm^2(distilled water).

Figure 4.13:Surface tension redution by NCC.DSS6 biosurfactant
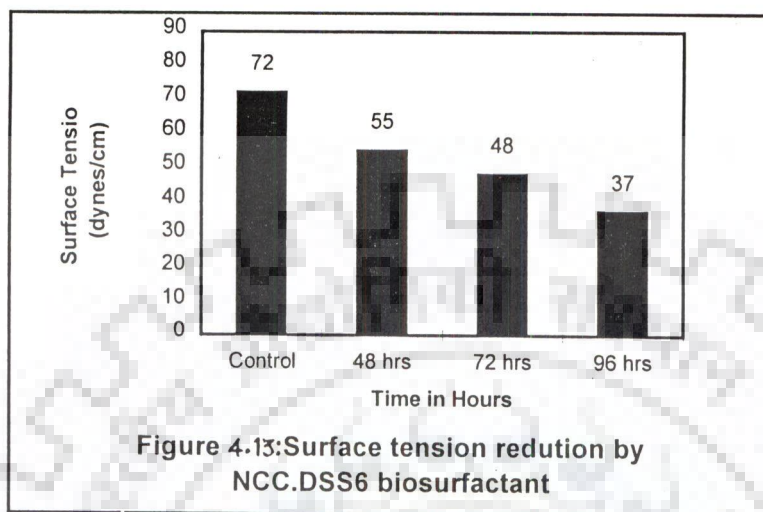
The surfactant was extracted from cell free broth and subjected to IR analysis (Fig. 4.14 b) which demonstrated close resemblance to the reported IR spectra of rhamnolipid produced by *P.aeruginosa* (Fig. 4.14 a)[Janiyani *et al.*, 1992; Itoh *et al.*, 1971]. Moreover, the isolate NCC.DSS6 which has been identified as *Pseudomonas* sp., shares many common features with *Psudomonas aeruginosa*, one of them is pigment production.

4.4.2 Plasmid Mediated Biosurfactant Production in NCC.DSS6

The isolate NCC.DSS$_6$ was found to be harboring a plasmid, which has been characterized in this study for its role in biosurfactant synthesis. The plasmid DNA was prepared by Qiagen column (Q-500) maxi-protocol and subjected to restriction digestion to determine it's size which was found to be of 20 kb (approximately). Plate 4.13 exhibits the restriction digestion pattern of DSS6 plasmid with two restriction endonucleases, *Acc I* and *EcoR V*. Lane 1 shows the uncut plasmid, prepared by Qiagen column (Q-500). Lane 2 and 3 demonstrated the restriction digestion pattern of of plasmid with *Acc I* and *EcoR V*. 1kb molecular marker (ladder) was loaded in lane 5.
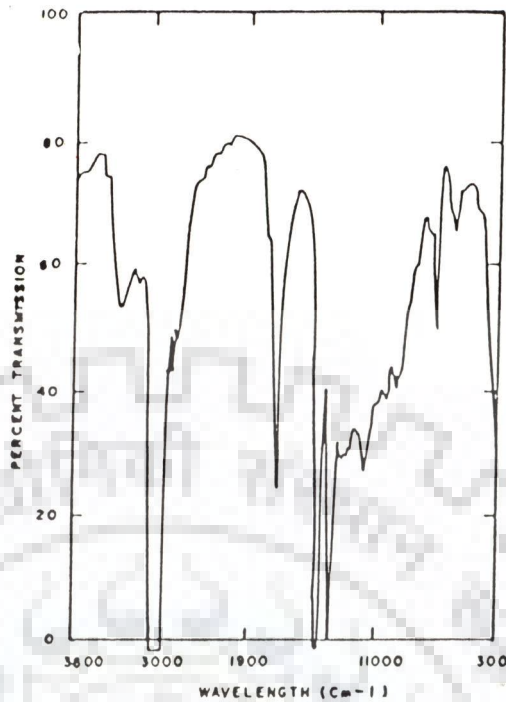
Fig. 4.14 a : Infrared Spectrum-rhamnolipid produced by *Pseudomonas aeruginosa* (reproduced from Janiyani *et al.*,1992)
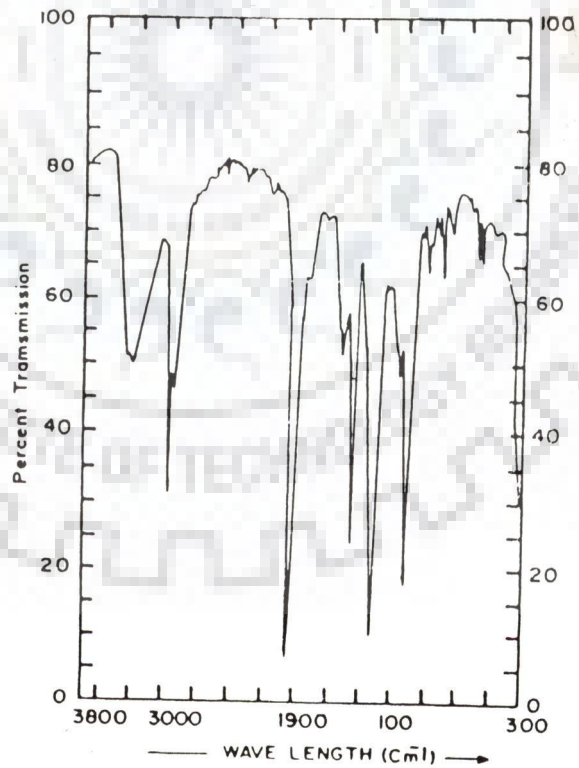
Fig. 4.14 b : Infrared Spectrum- biosurfactant produced by isolate NCC.DSS$_6$ showing close resemblance with rhamnolipid produced by *Pseudomonas aeruginosa*
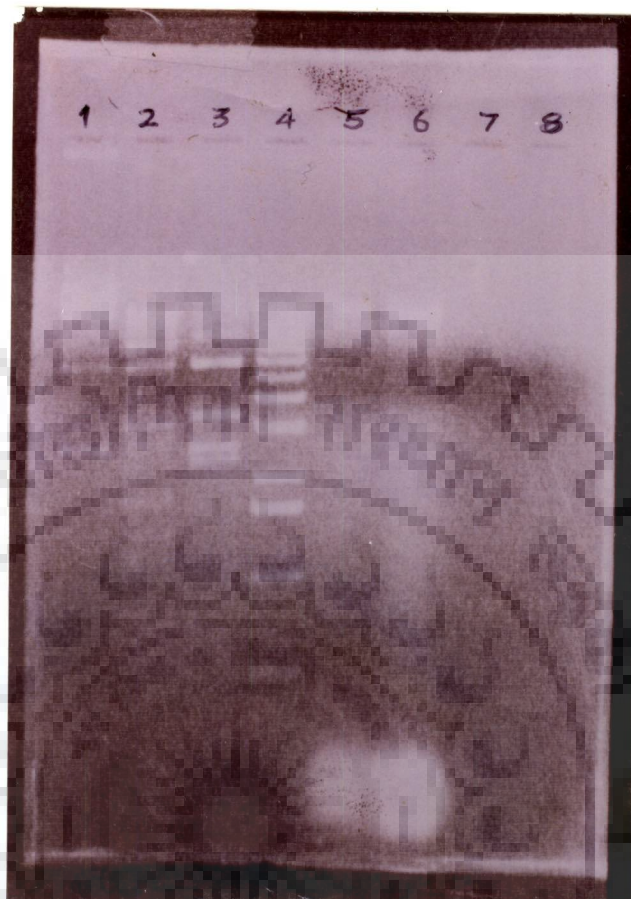
Plate 4.13 : Agarose Gel Electrophoresis- Plasmid Profile of NCC.DSS$_6$ demonstrating restriction digestion

pattern, successful curing and retransformation of the plasmid

Lane1-uncut NCC.DSS$_6$ plasmid prepared by Qiagen-500 column

Lane2-digested with *AccI*

Lane3-digested with *HindIII*

Lane4-1Kb ladder

Lane5-mobilization in cured NCC.DSS$_6$

Lane6-mobilization in *Pseudomonas putida* MTCC*102

Lane7-wild *Pseudomonas putida* MTCC*102

Lane8-cured NCC.DSS$_6$

The plasmid was cured by acridine orange method. After 5 successive transfers in LB medium amended with acridine orange (25μg to 100μg) the curing was accomplished. A number of mutants were picked up and checked for the presence of plasmid by alkaline lysis. The cured organism did not show the plasmid band (Plate 4.13 ; Lane 8). The same plasmid was mobilized into the cured NCC.DSS$_6$ and *Pseudomonas putida* MTCC*102 by electroporation. The mobilization was confirmed by plasmid DNA preparation. Both the cultures demonstrated plasmid band of same size (Plate 4.13, Lane 5 and 6). The cured and retransformed NCC.DSS$_6$ was grown on glucose and subjected to surface tension determination. It was observed that surface tension of cell free broth was reduced to 42 dynes/cm^2 in 96 hours (Fig. 4.15) showing that the biosurfactant synthesis capability in NCC.DSS$_6$ was regained by the successful mobilization of the plasmid which suggested the possible role of plasmid in biosurfactant synthesis in isolate NCC.DSS$_6$.

Figure 4.15: Surface Tension Reduction by cured NCC.DSS6 after 96 hrs of incubation
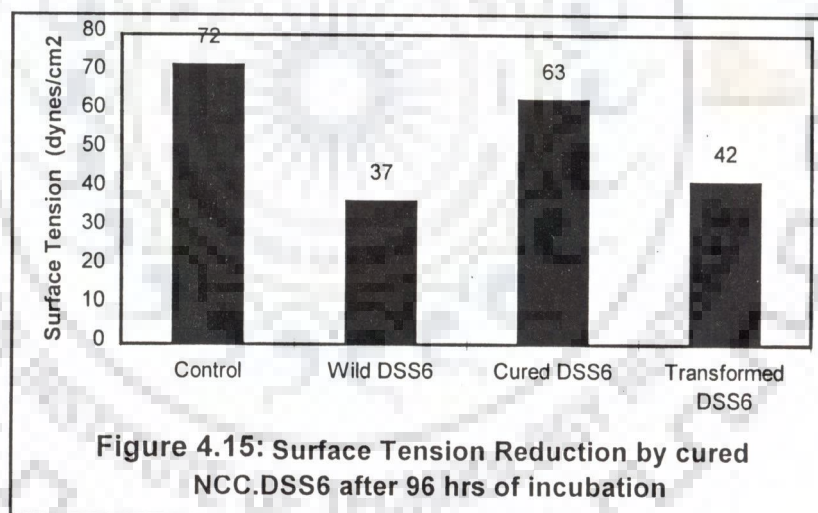
Transformed *P. putida* MTCC*102, grown on glucose, when subjected to surface tension determination did not lower the surface tension of liquid medium. The surface tension of cell free broth of *P. putida* MTCC*102 wild was recorded 62 against 72 dynes/cm^2 of control (distilled water) after 96 hours while transformed *P. putida* MTCC*102 gave 57 dynes/cm^2 (Fig. 4.16) suggesting that the plasmid is not solely responsible for biosurfactant production capability of NCC.DSS$_6$.

Figure 4.16: Surface Tension Reduction by *P. putida* MTCC*102
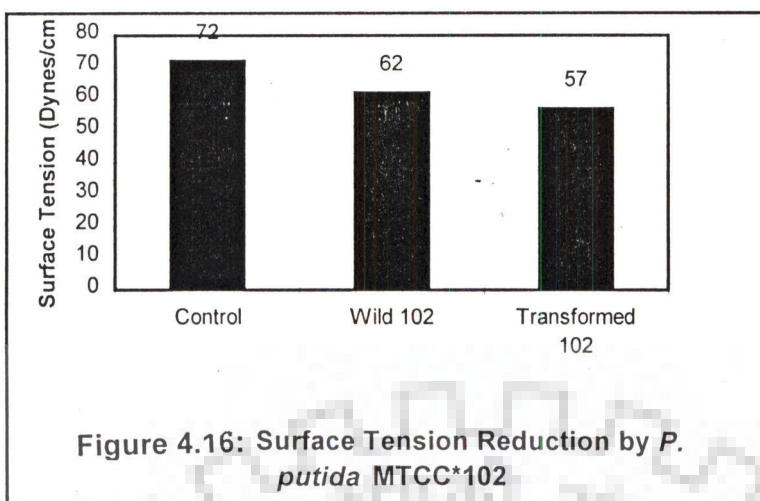
4.4.3 Role of Biosurfactant in Oil Degradation Process

Emulsification of crude oil is an essential pre-requisite for effective oil degradation process as only 0.02% of total crude oil is soluble in water. Biosurfactant produced by several hydrocarbon degraders is the natural alternative to synthetic detergents. Isolate NCC.DSS$_6$ is such an organism, which produces rhamnolipid, a biosurfactant, thus bringing about the surface tension of liquid medium to 37 dynes/cm2 (Fig. 4.13). The effect of exclusion of NCC.DSS$_6$ from the oil degrading consortium was studied in an experiment. The other consortium members were grown as described in section 4.3 and subjected to crude oil degradation without NCC.DSS$_6$. The hexane extracts of 48 hours grown culture were analyzed on gas chromatograph as described before. Chromatograms showed that without NCC.DSS6, the degradation of Bombay High Crude was not effective (Fig. 4.17 b) as compared to the regular four membered consortium (Fig. 4.17 c). Fig. 4.17 (a) is the control of Bombay High Crude showing abiotic loss which was found negligible in due course of time (48 hours).
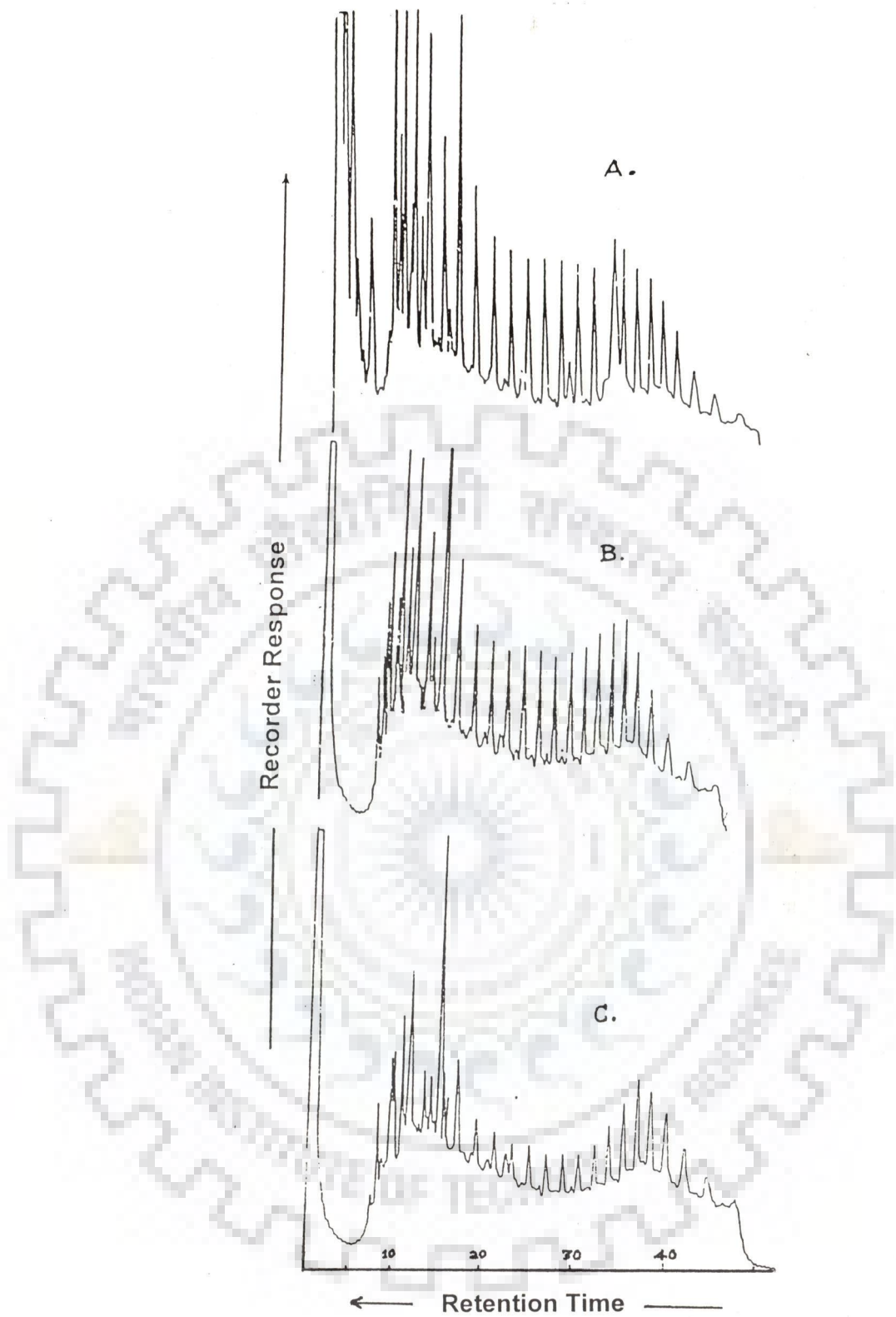
Fig. 4.17 : Effect of biosurfactant on degradation of Bombay High Crude (1%) by the designed consortium adapted on catechol (analyzed on Sigma 300, FID)

A. Control

B. degradation of Bombay High Crude by the consortium without NCC.DSS$_6$ in 48 hr.

C. degradation of Bombay High Crude by the consortium with NCC.DSS$_6$ in 48 hr.

4.5 Efficacy of the Consortium for Utilization of Various Fractions of Crude Oil

The crude oil is a heterogeneous mixture of several hydrocarbons which can be categorized into four major classes : aromatics, saturates, resins and asphaltenes [Harayama, 1994]. The categorization is based on their differential solubility in organic solvents. The crude oil used in this study, Bombay High Crude, was fractionated by column chromatography as described by Hong-vu Song et al.[1990] before and after degradation.

4.5.1 Fractionation of Crude Oil

The protocol adapted from Hong Vu et al. [1990] worked with Bombay High Crude and the fractions eluted with solvents are depicted in Table 4.10.

Table 4.10: Gravimetric analysis of Bombay High Crude Oil

Fraction of Crude Oil	Weight (mg/ml)	Eluted with
Aliphatic	752	Hexane
Aromatic	77.6	Benzene
Asphaltene	38.2	Chloroform : Methanol

4.5.2 Spiking

To check the efficacy of fractionation protocol, the crude oil samples were spiked with representative aliphatic (octacosane, 3mg/500mg of crude oil) and aromatic (phenanthrene, 2mg/500mg of crude oil). The spiking resulted in the elution of representative aromatic, phenanthrene with benzene (aromatic fraction) and representative aliphatic, octacosane with hexane (saturate fraction) as shown in gas chromatograms (Fig.4.18 & 19). Phenanthrene gave a peak at 27.8 min. and octacosane at 36.0. The elution of both the compounds with their respective solvents proves the efficacy of the fractionation protocol.

4.5.3 Utilization of Various Fractions of Crude Oil

The remaining crude oil, after degradation was also subjected to column chromatography. After the optimum time for degradation, the medium was extracted with hexane (1:1 v/v), solvent evaporated and the residue dissolved in chloroform to prepare the sample as described above. Apart from the fractionation of crude oil, the utilization of fractions of crude oil and the degradative

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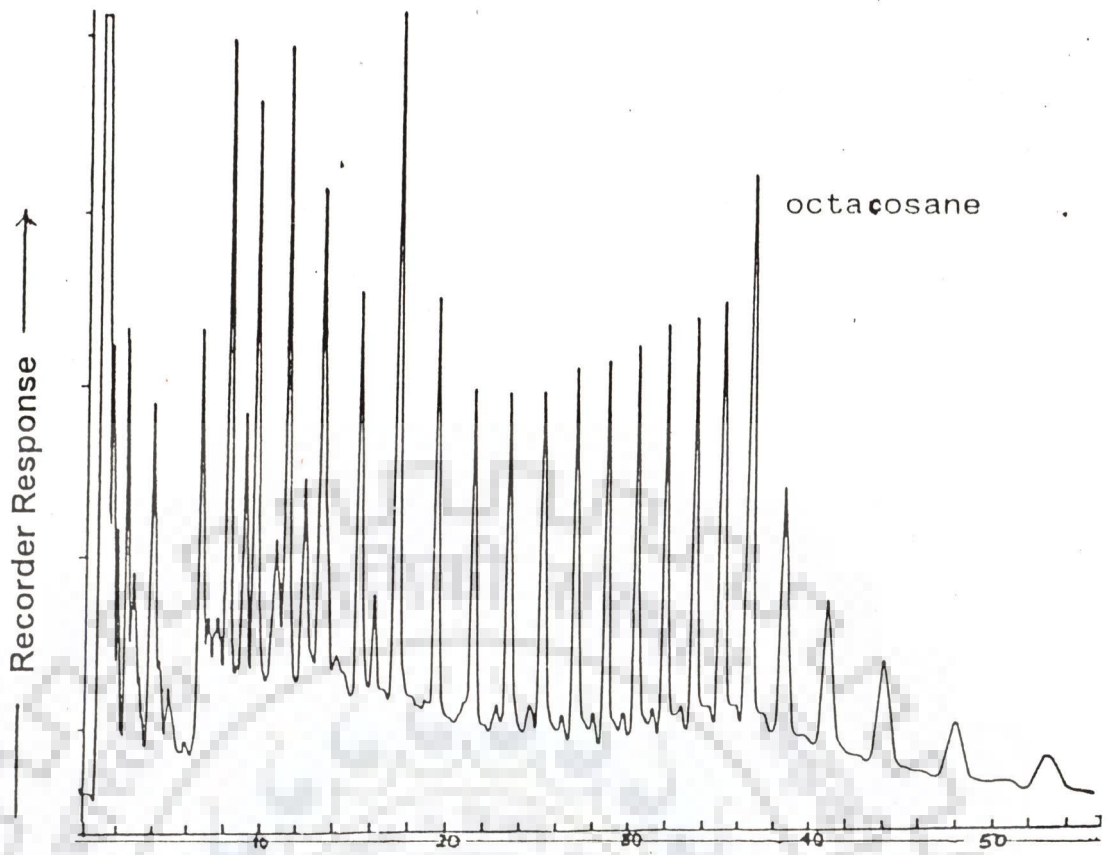
Fig. 4.18 a : Gas Chromatograph - aliphatic fraction (hexane elute) of Bombay High Crude after silica gel column chromatography showing octacosane peak (RT 36.0)
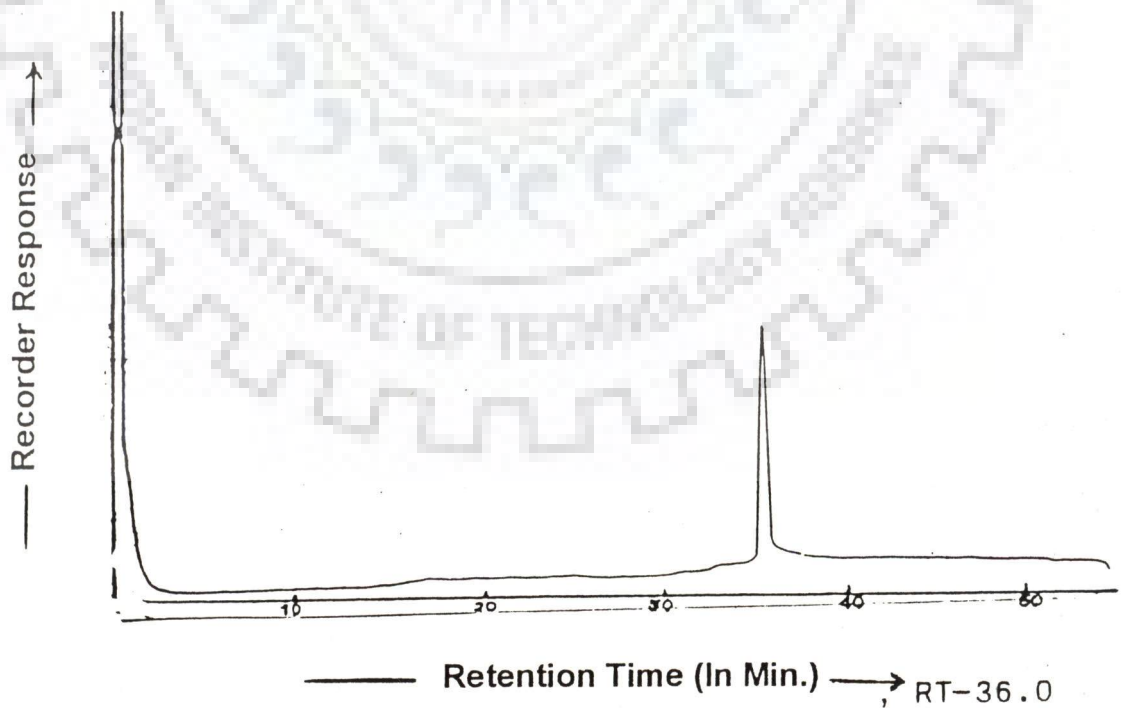
Fig. 4.18 b : Gas Chromatograph - standard of octacosane (RT 36.0)
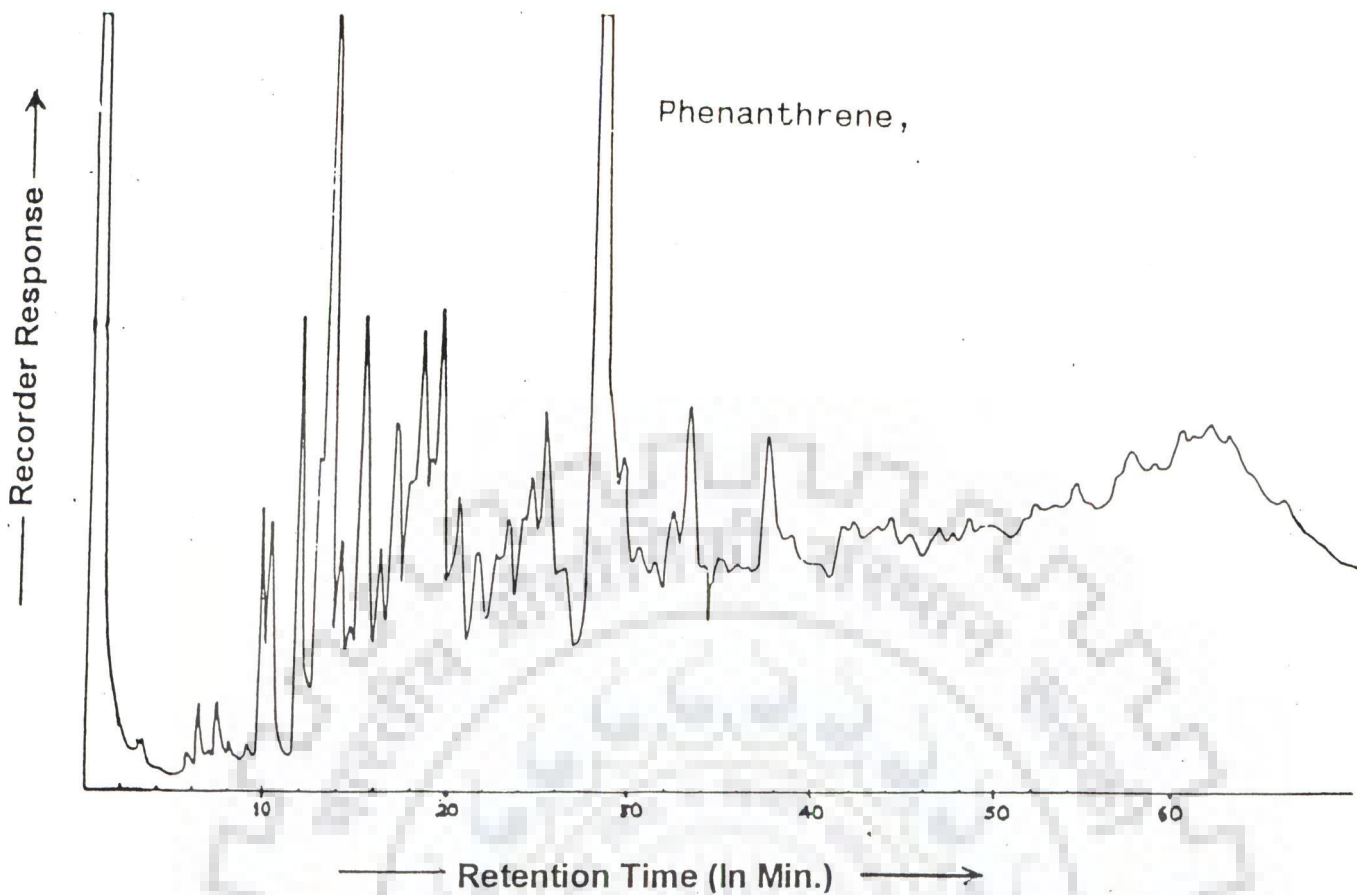
Phenanthrene,

Fig. 4.19 a : Gas Chromatograph - aromatic fraction (benzene elute) of Bombay High Crude after silica gel column chromatography showing phenanthrene peak (RT 27.8)
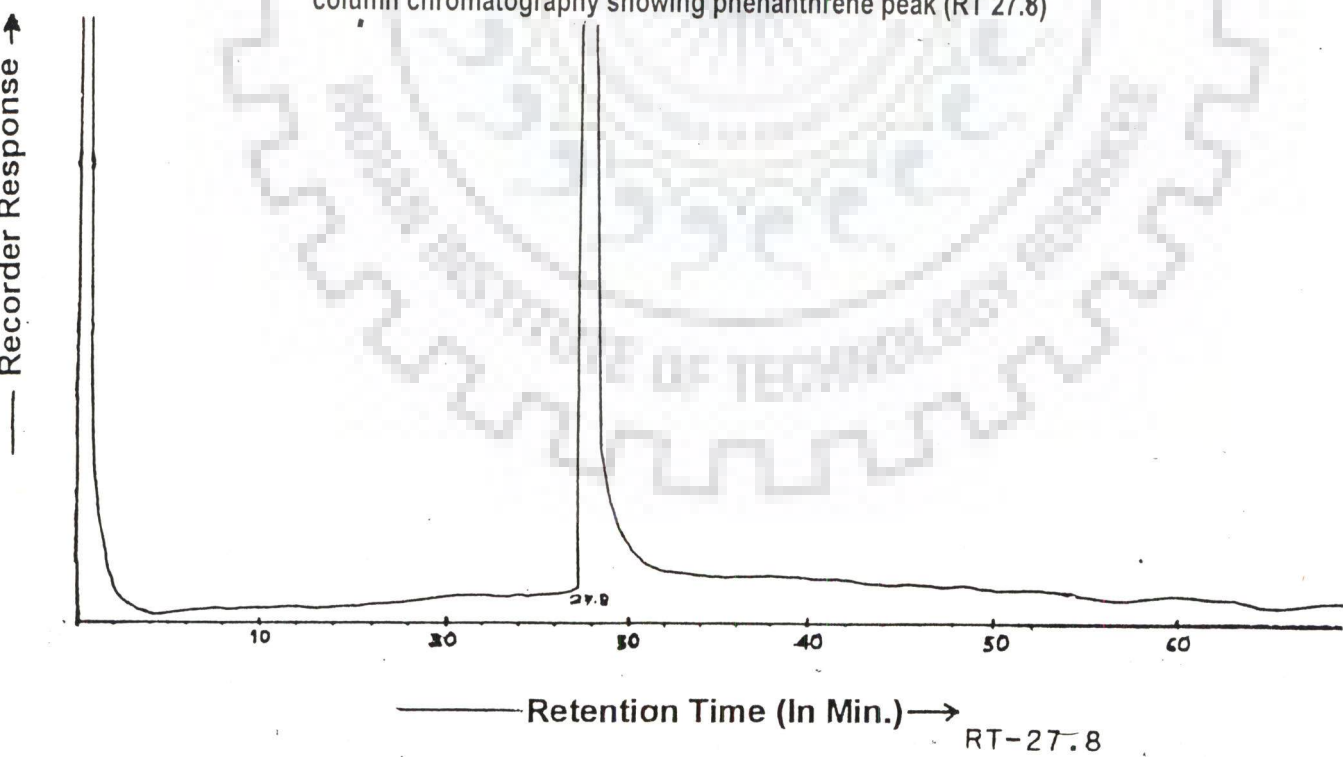
RT-27.8

Fig. 4.19 b : Gas Chromatograph - standard of phenanthrene (RT 27.8)

capabilities of the designed bacterial consortium were studied by designing a model petroleum, mixture of representative hydrocarbon molecules [Janiyani, 1994] and it's degradation by the consortium. The composition of model petroleum is given in Table 3.1. The experiments conducted were similar as in case of crude oil. The preparation of seed culture of consortium members, experimental setup and analysis is described in section 4.3

The fractionation of degraded samples (by consortium) followed by gravimetric analysis revealed that 81.5% saturates and 65% aromatics were degraded (Fig. 4.20).

Figure 4.20: Utilization of Various Fractions of Crude Oil by the Designed Consortium
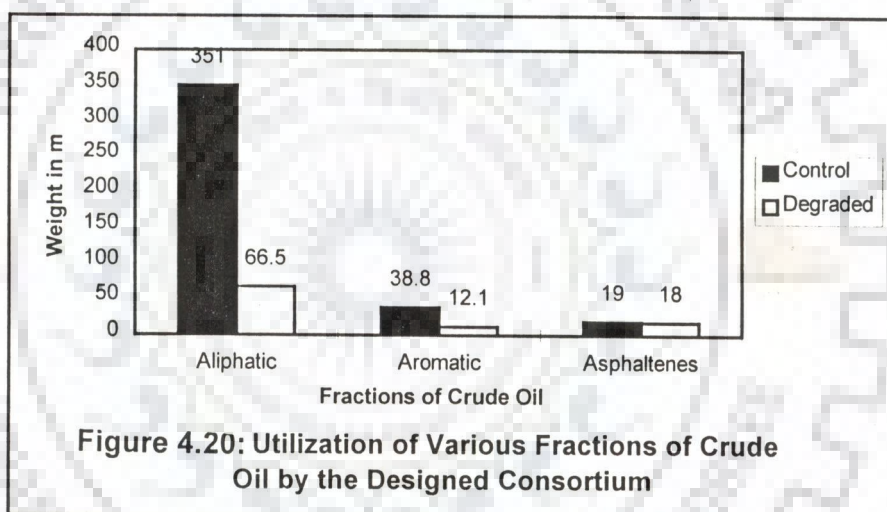
The resin and asphaltene fraction did not show much variation in gravimetric analysis before and after degradation. The samples were analyzed by gas chromatography also to supplement the gravimetric data. The saturate fraction demonstrated more reduction than aromatic as evident in the gas chromatograms (Fig. 4.21 & 4.22). With model petroleum also, the alkanes (C_{12}-C_{15}) were consumed rapidly (Fig. 4.23). Longer alkanes (C_{20}-C_{28}) and aromatics (tricyclic and heterocyclic) were degraded less and slowly. Other investigators have reported similar degradation pattern where saturates are degraded more easily than PAHs [Perry, 1992]. The results were confirmed by both packed and capillary gas chromatography.
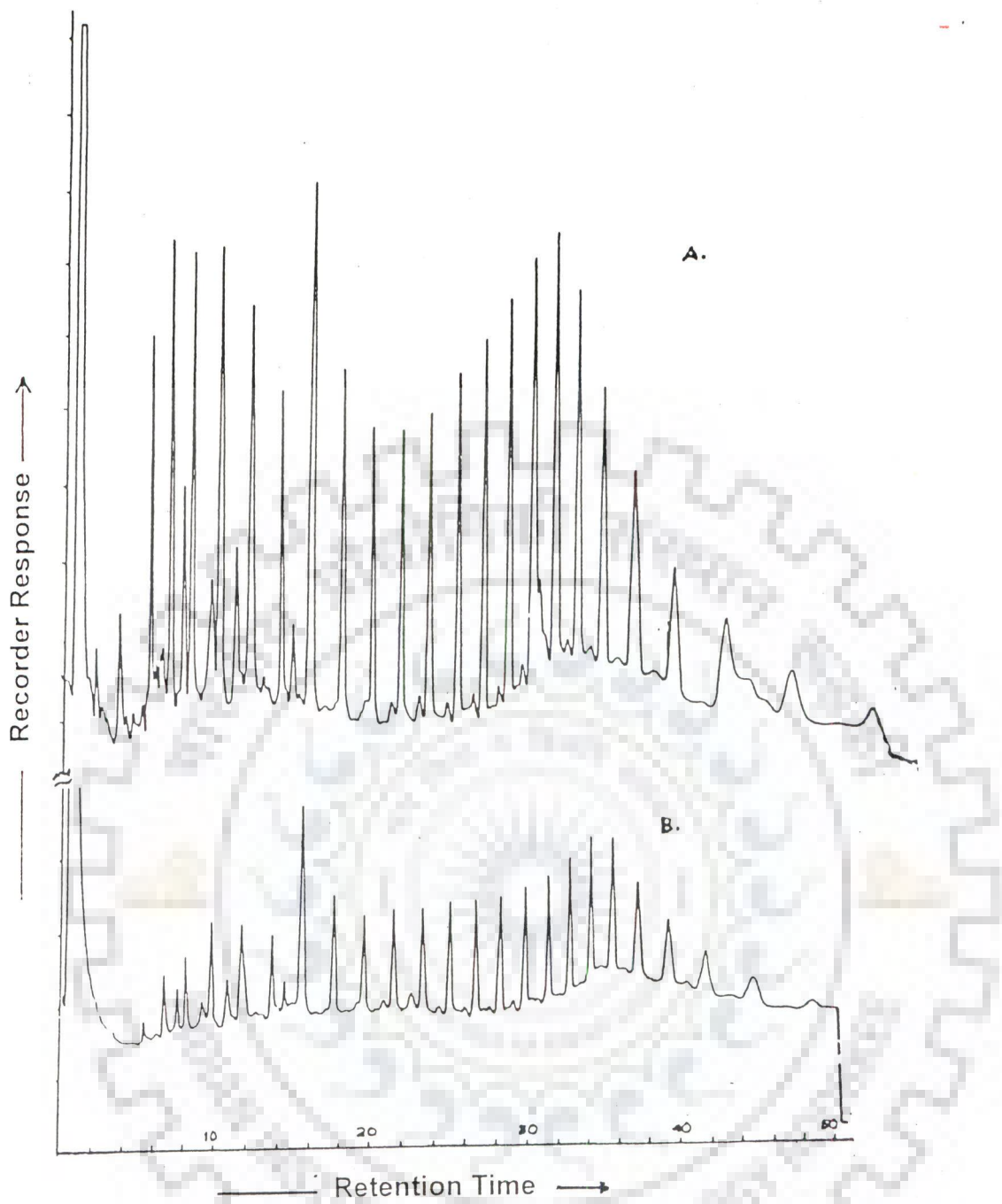
Fig. 4.21 : Gas Chromatograph - degradation of aliphatic fraction of Bombay High Crude (1%) by the designed consortium adapted on catechol (analyzed on Sigma 300, FID)

A. Aliphatic fraction before biodegradation

B. Experimental- degradation by consortium in 3 days
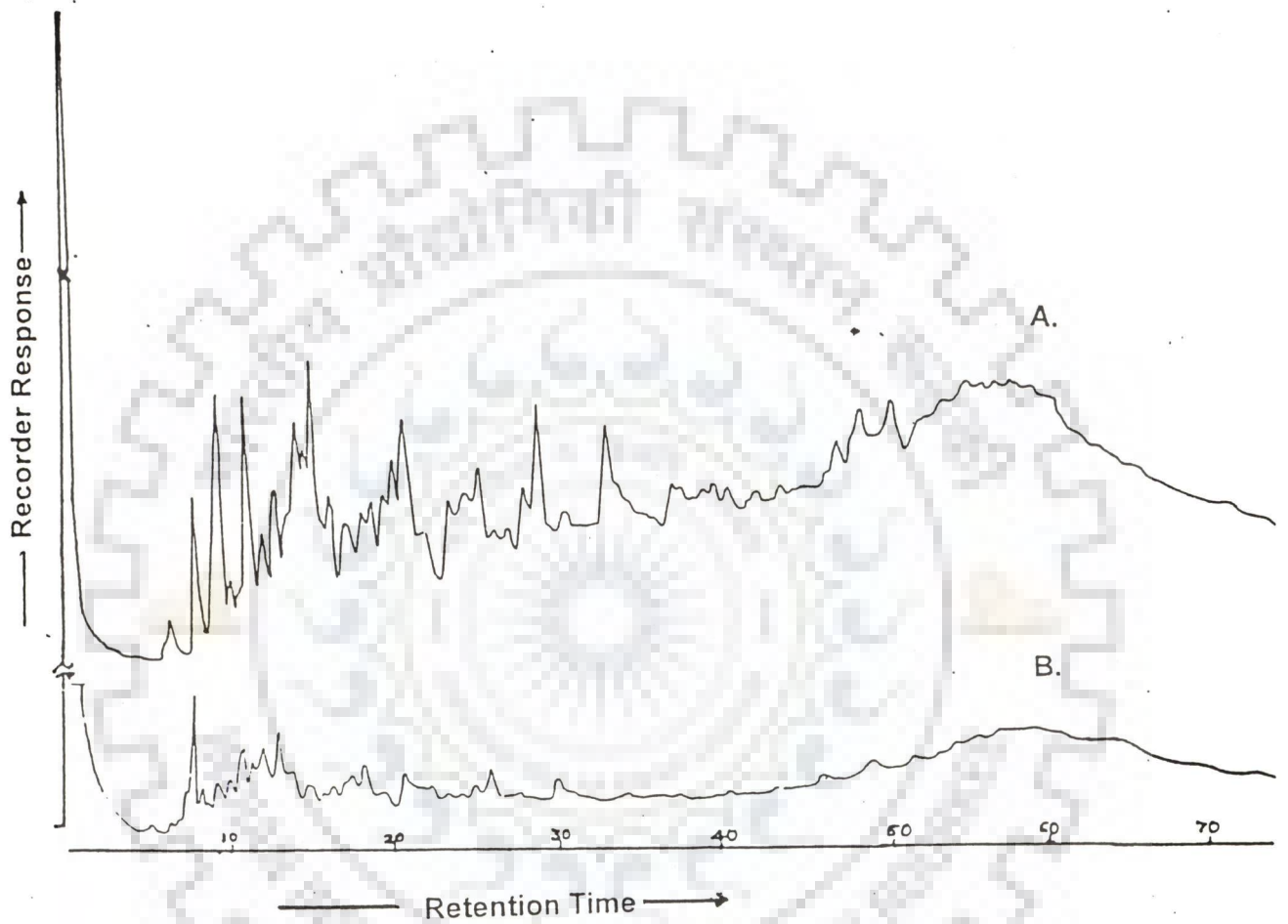
Fig. 4.22 : Gas Chromatograph - degradation of aromatic fraction of Bombay High Crude (1%) by the designed consortium adapted on catechol (analyzed on Sigma 300, FID)

A. Aromatic fraction before biodegradation

B. Experimental- degradation by consortium in 3 days
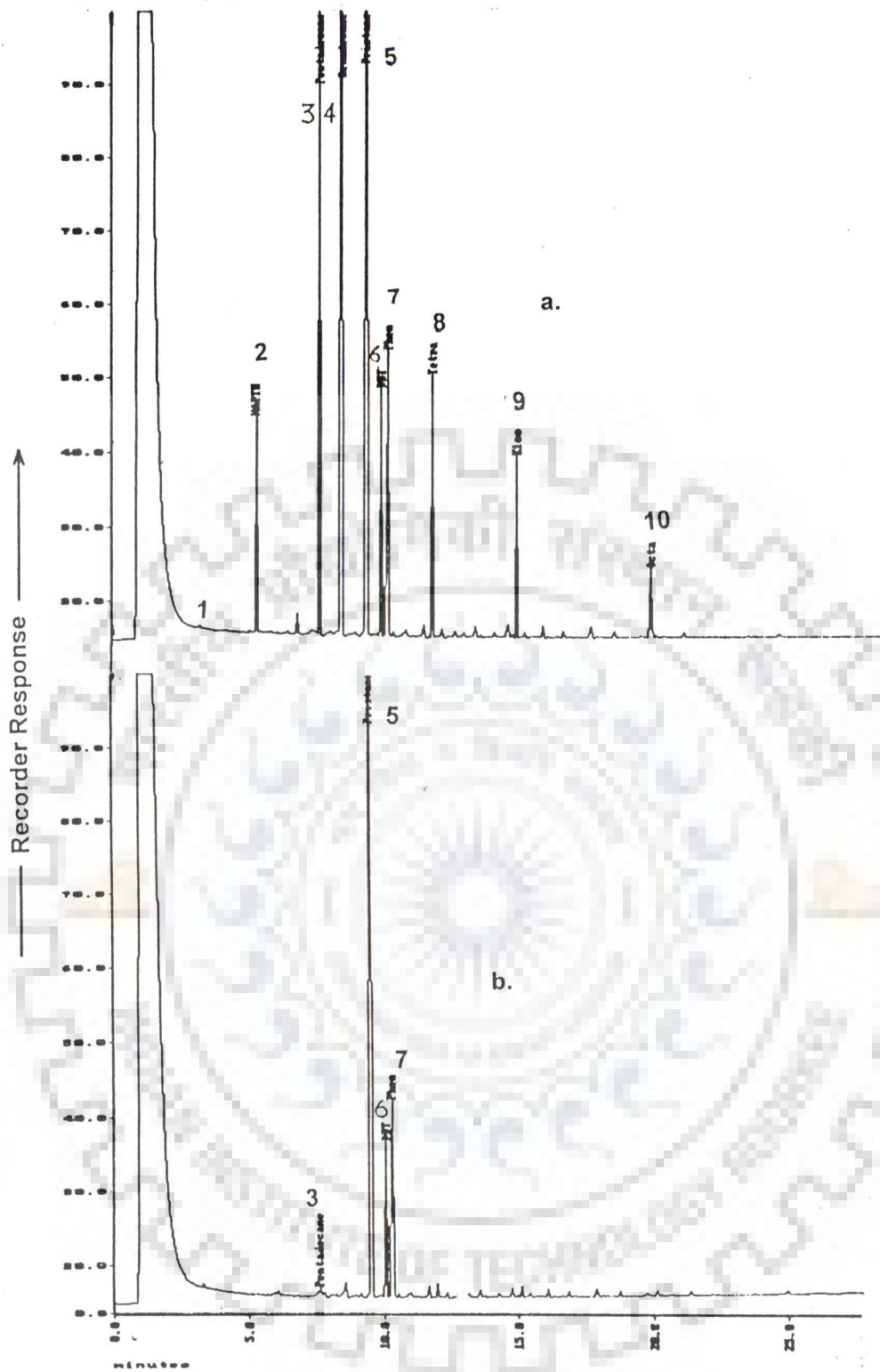
Fig. 4.23 : Gas Chromatograph – utilization of various compounds present in model petroleum (analyzed on PE Auto System, FID)

A. Control

B. Experimental

Peak1-dodecane Peak2-naphthalene Peak3-pentadecane Peak4-hexadecane Peak5-pristane Peak6-dibenzothiophene Peak7-phenanthrene Peak8-eicosane Peak9-tetracosane Peak10-octacosane

4.6 Degradation of Aromatics by the Consortium

In previous experiments, aromatic degradation was found less (Fig. 4.19-4.23) in comparison to aliphatic constituents of crude oil/model petroleum. Studies concerning the relationship of chemical composition and biodegradability of crude oil have shown that crude oil containing a higher concentration of alkanes was found more susceptible to microbial attack [Westlake et al. 1974; Walker et al. 1975]. Aromatics, particularly polycyclic aromatic hydrocarbons (PAH) are known to be less biodegradable and the biodegradability decreases with the increase in the number of benzene rings [Herbes and Schwall, 1978]. The aromatic degradation potential of the designed consortium was studied with a model aromatic mixture consisting of representative molecules of various categories of aromatics (Table 3.2). Pseudomonas putida G7, harboring NAH plasmid was included in the consortium in place of Pseudomonas putida MTCC*102 to provide additional dissimilatory capacity.

4.6.1 Growth of Consortium Members on Different Inducers

The seed culture of the consortium was prepared as described in section 4.3 with catechol/benzoate or salicylate as substrate (sole source of carbon). The consortium members exhibited normal growth pattern on substrates as was evident from the spectrophotometric analysis (Fig. 4.24-4.27). Salicylate which is a known inducer for NAH operon, was proved to be favoring growth of Pseudomonas putida PpG7 while others (NCC.DSS$_6$, NCC.DSS$_8$, NCC.GSS$_3$) demonstrated less growth on the expense of it (Fig. 4.25). With benzoate, the maximum optical density was measured in the range of 0.9 to 1.1 while on catechol it was little less (Fig. 4.24 & 4.26). One interesting finding while using catechol as a carbon source was photo-oxidation of catechol which turned the culture black thus inhibiting growth of microbes, especially at higher concentrations. Therefore, 100 ppm of substrates were used to grow the cultures.

Figure 4.24: Growth of the consortia members on Benzoate (100 ppm)

Figure 4.25: Growth of the consortia members on Citrate (0.2%)

Figure 4.26: Growth of consortium members on Catechol (100 ppm)

Figure 4.27: Growth of the consortia members on Salicylate (100 ppm)
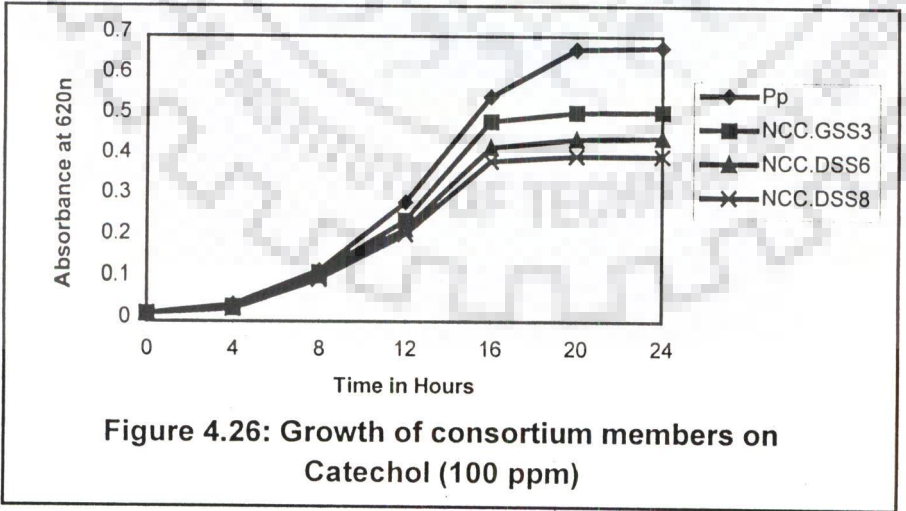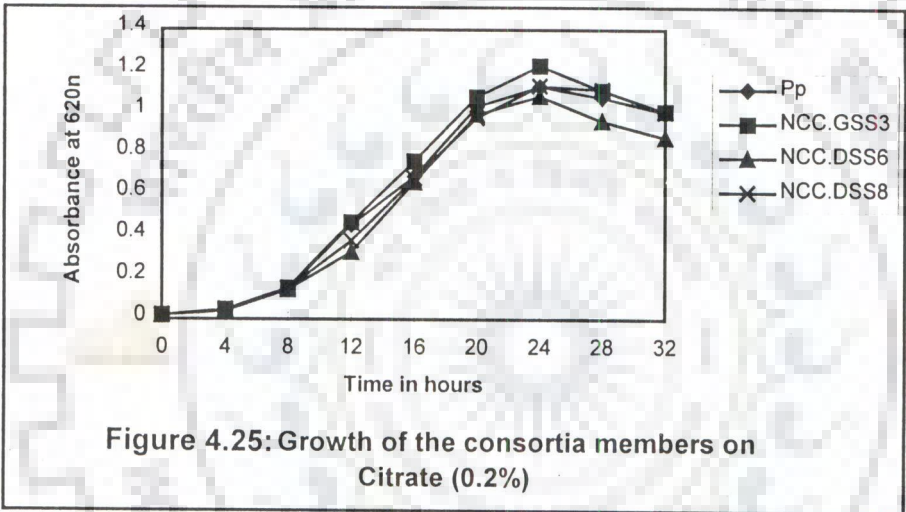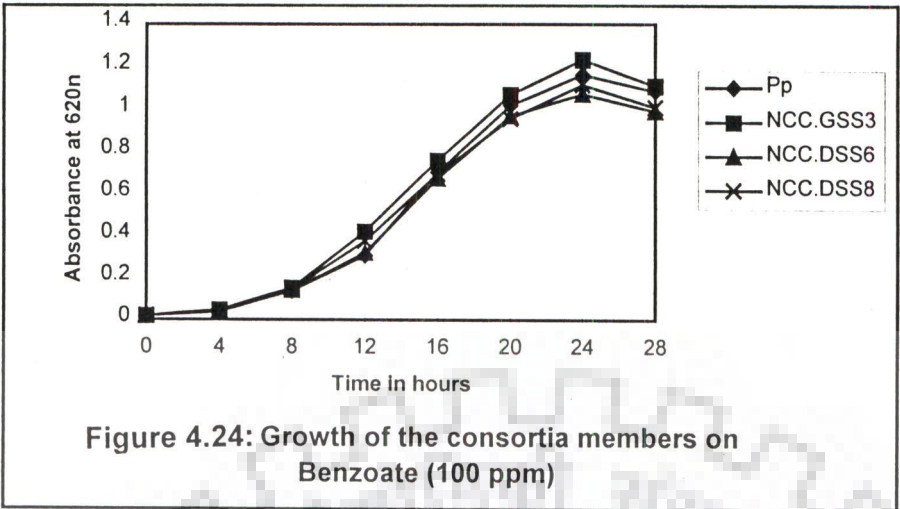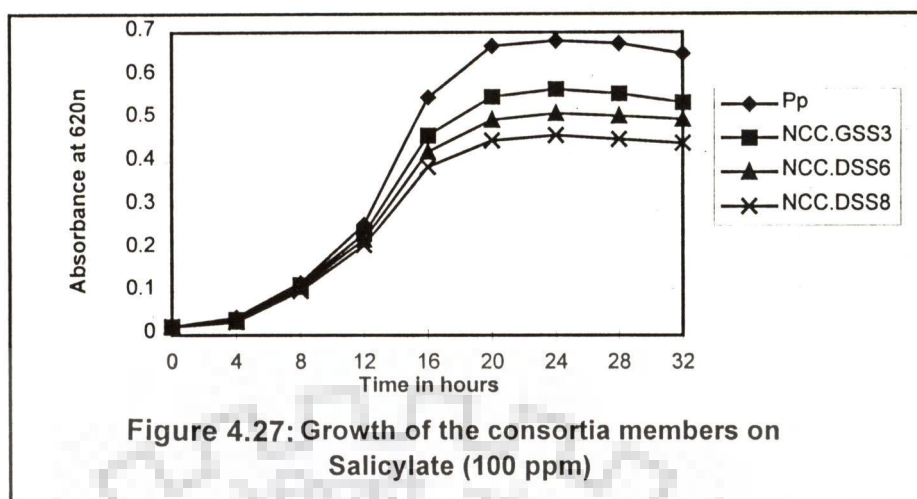
4.6.2 Degradation of Model Aromatic Mixture by Designed Consortium

The adapted organisms were subjected to degradation of a model aromatic mixture (Table 3.2) in the similar manner described above (section 4.3 & 4.5). The aromatic mixture was diluted with hexane to give final concentration of 100 ppm of total aromatics. The hexane-diluted mixture was added in 5 ml of warm medium prior to inoculation of consortium and left for 3 hours to allow the evaporation of solvent. After that the pre-adapted consortium was inoculated to give final OD of 0.02. The culture tubes were incubated at 30°C on a rotary shaker at 150 rpm.

The degradation of model aromatic mixture and the effect of various inducers was determined by analyzing the hexane extracts of culture broth after 8 days' incubation on capillary GC. The GC method was calibrated with appropriate standards and the controls were run parallel to the experiments.

Capillary gas chromatographs show that volatile monocyclic aromatic components of the mixture (o,m and p xylene and toluene) were evaporated in due course of time (Fig. 4.28 a & b). Naphthalene could not be detected after 7 days, however, other components were in detection range. Dicyclics (biphenyl, acenaphthene & fluorene) were degraded more than heterocyclic (dibenzothiophene), tricyclic (phenanthrene) and especially tetracyclic, represented by fluoranthene as reported by other authors also. Catechol adapted consortium exhibited better biodegradation potential than benzoate and salicylate grown (Fig. 4.28).
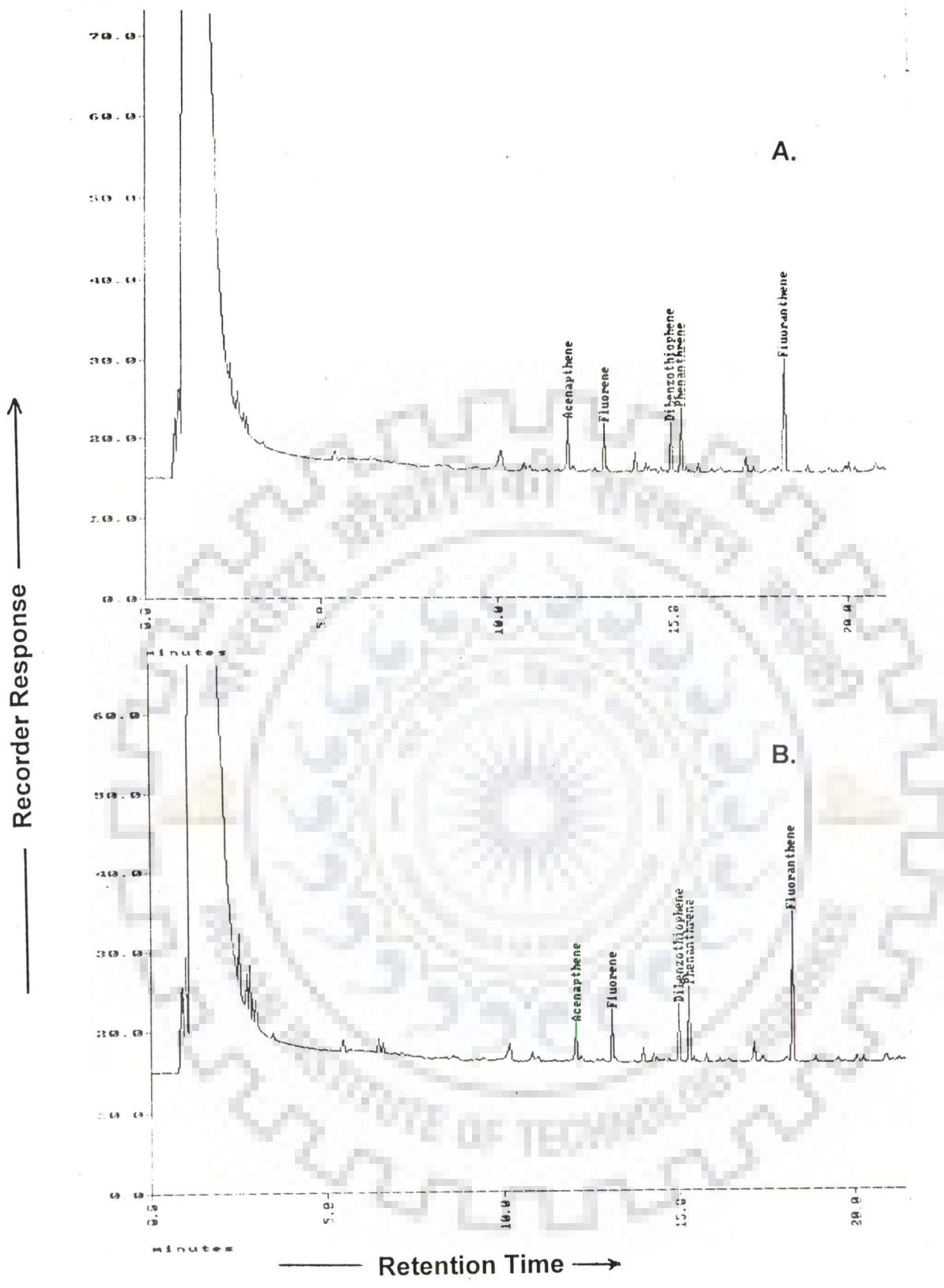
102

Fig. 4.28 : Capillary Gas Chromatograph - degradation of model aromatic mixture (100 ppm) by the modified

consortium adapted on different inducers after 8 days (analyzed on PE Auto System, FID)

A. Chemical Control

B. Catechol grown consortium
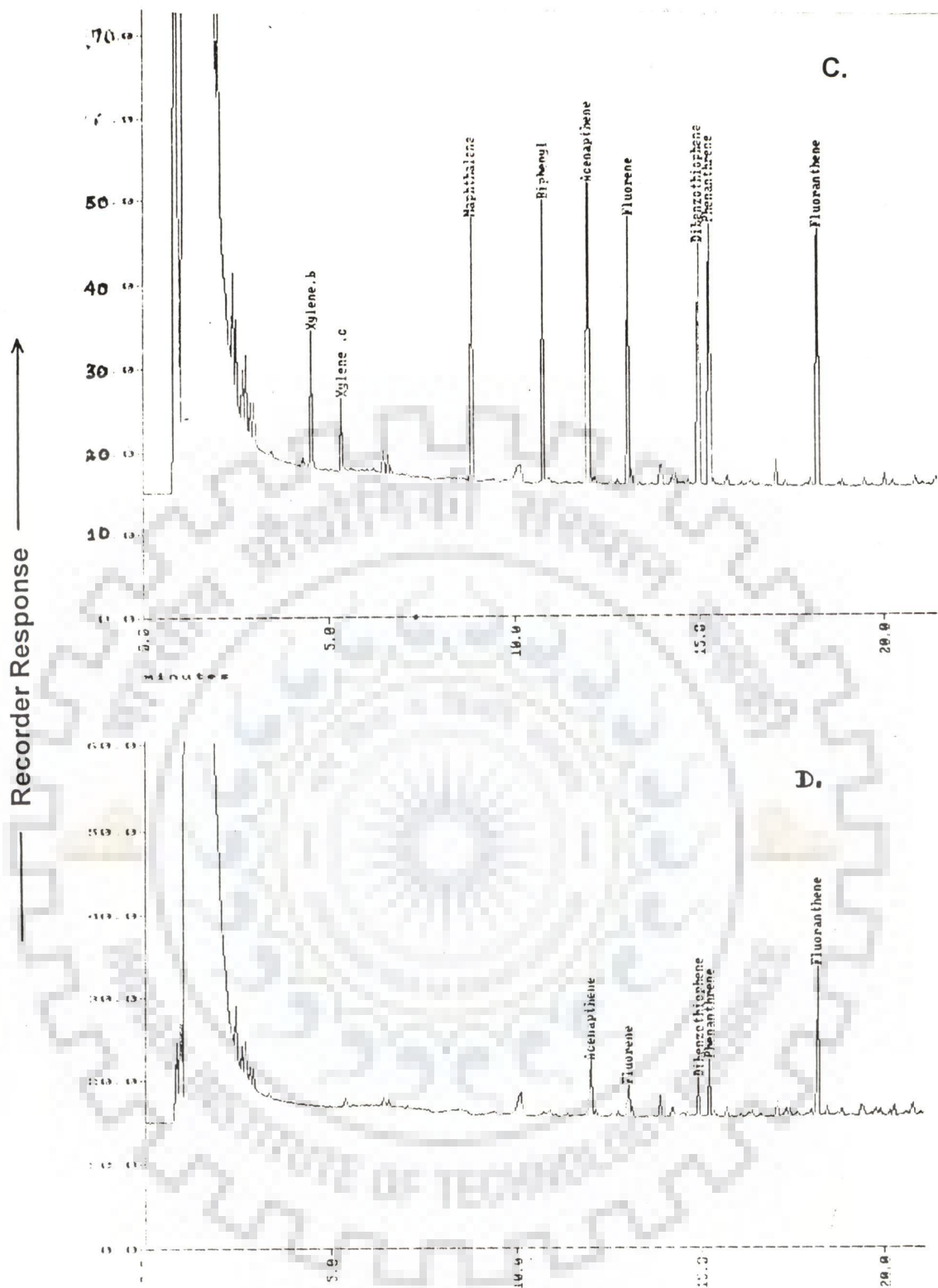
Fig. 4.28 : Capillary Gas Chromatograph - degradation of model aromatic mixture (100 ppm) by the modified consortium adapted on different inducers after 8 days (analyzed on PE Auto System, FID)

C. Benzoate grown consortium

D. Salicylate grown consortium

4.6.3 Protein Profile of Consortium Members

The consortium members were grown on citrate/catechol/salicylate and benzoate individually, the cells were harvested and subjected to sonication followed by subjected to SDS-Poly acrylamide gel electrophoresis (PAGE). The two dimensional gels demonstrated no significant change in the protein profile of consortium members on different substrates. Plate 4.14 to 4.17 are the gel photographs which demonstrate protein profile crude extract of individual organisms grown on various substrate and a protein marker.

4.7 Hydrocarbon Degradation and Salinity

4.7.1 Imparting the Pro'U' operon to Members of the Oil Degrading Consortium

NCC.DSS$_6$, NCC.DSS$_8$, NCC.GSS$_3$ and *Pseudomonas putida* MTCC*102 were imparted with the phenotype of osmotolerance by transforming the organisms with a plasmid construct received as a gift from Dr. A. Kapley [Kapley *et al.*,1997]. The plasmid construct was developed by ligating *the E. coli pro 'U' operon* (*EcoRV-HindIII* fragment of size 4.2 kb) to a broad host range vector pMMB206 (size 9 Kb ; digested with *HindIII-SmaI*). The transformants were selected on LB plates with 20µg/ml chloramphenicol. The plasmid mobilization was confirmed by plasmid preparation by alkaline lysis and restriction digestion. The subcloned fragment (*Pro 'U'*) was digested with *EcoRV - Hind III* enzymes (Plate 4.18).

4.7.2 Expression of Pro 'U'

The salt tolerance capability of transformed organisms was studied by growing them in the LB medium amended with varying concentrations of NaCl (0.5 to 1M). The growth was measured spectrophotometrically after 24 hours at 620nm). It was observed that the transformed organisms demonstrated higher growth rate in high salt concentration as is evident from Fig. 4.29-4.32. The role of the osmotolerance phenotype was best observed in *Pseudomonas putida* MTCC*102 wherein the wild type organism does not grow at 1 M NaCl but the transformed does (Fig. 4.32).

200.0 kDa

97.4 K.Da

68.0 kDa

43.0 KDa

18.4 K Da
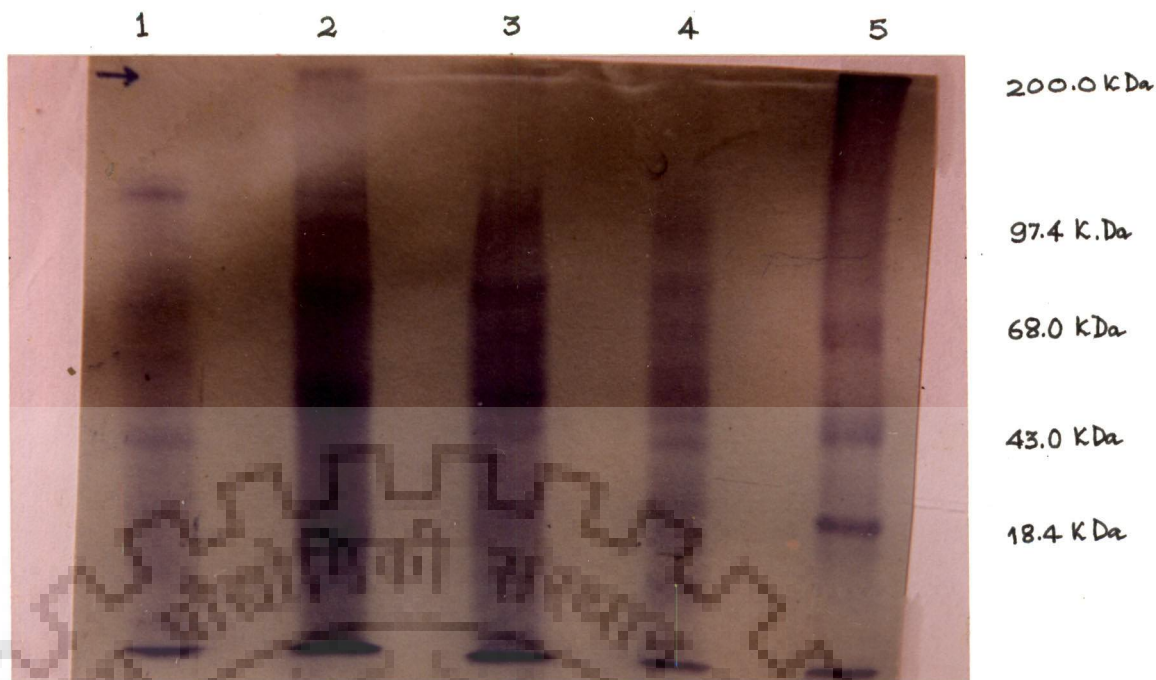
Plate 4.14 : Protein Profile of Consortium Member- NCC.GSS$_3$ analyzed by SDS-PAGE
Lane1-Salicylate grown; Lane2-Benzoate grown;
Lane3-Citrate grown; Lane4-Catechol grown;
Lane5-   Molecular marker
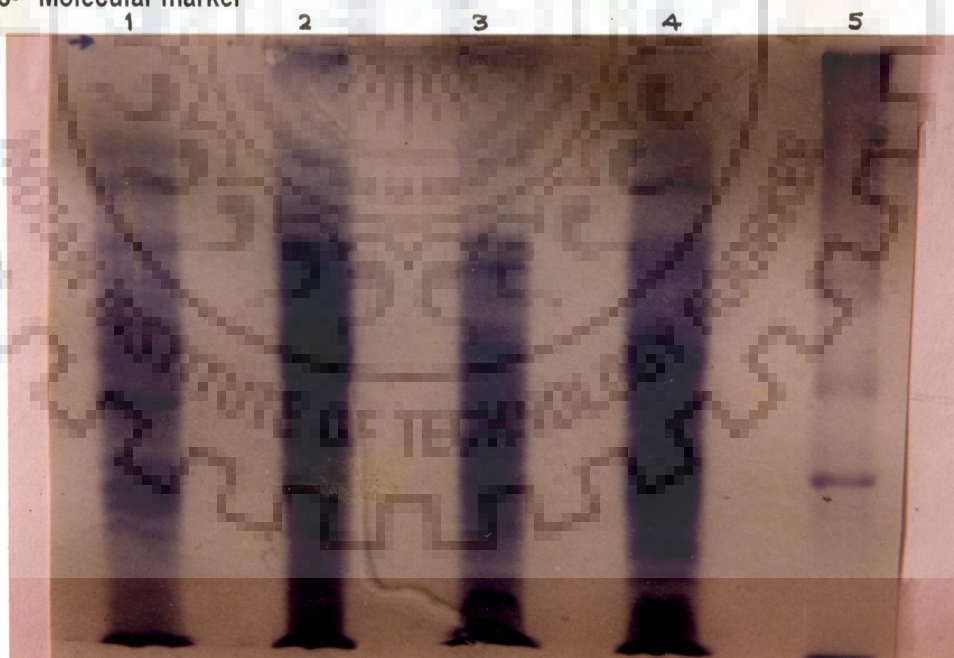
Plate 4.15 : Protein Profile of Consortium Member- NCC.DSS$_6$ analyzed by SDS-PAGE
Lane1-Salicylate grown; Lane2-Benzoate grown;
Lane3-Citrate grown; Lane4-Catechol grown;
Lane5-   Molecular marker

200.0 KDa

97.4 K.Da

68.0 KDa

43.0 KDa

18.4 KDa
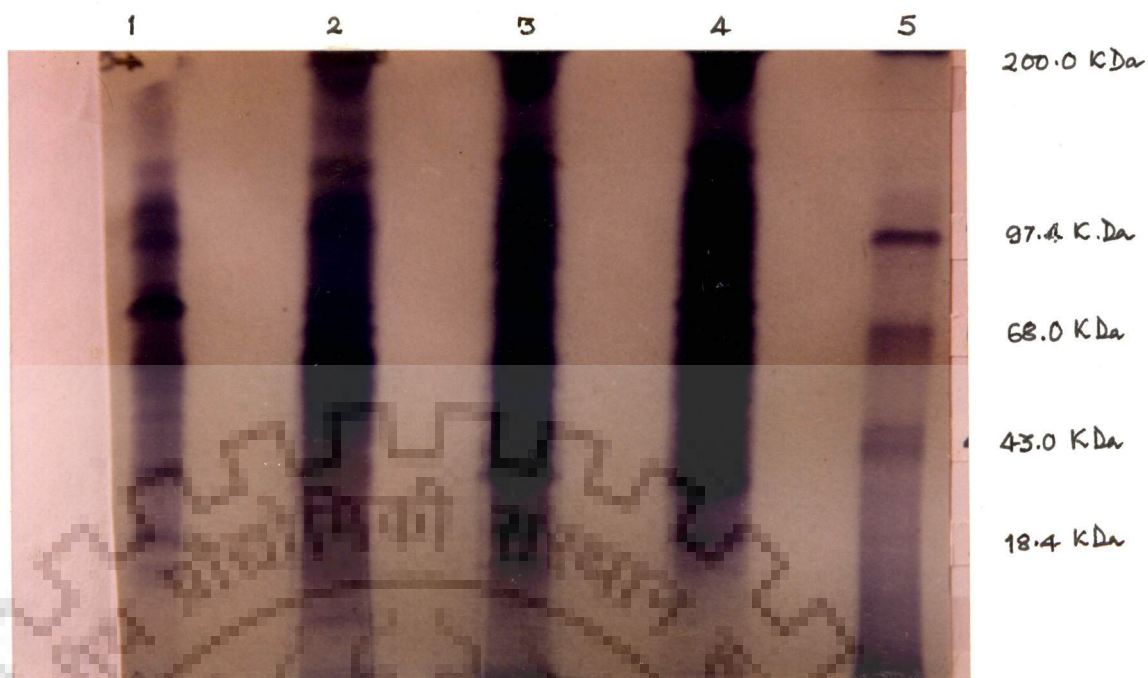
Plate 4.16 : Protein Profile of Consortium Member- NCC.DSS$_8$ analyzed by SDS-PAGE

Lane1-Salicylate grown; Lane2-Benzoate grown;

Lane3-Citrate grown; Lane4-Catechol grown;

Lane5- Molecular marker

Plate 4.17 : Protein Profile of Consortium Member-P. putida ppG7 analyzed by SDS-PAGE

Lane1-Salicylate grown; Lane2-Benzoate grown;

Lane3-Citrate grown; Lane4-Catechol grown;

Lane5- Molecular marker

Plate 4.18 : Agarose Gel Electrophoresis- Plasmid detection in consortium members demonstrating
successful transformation of the plasmid pMMB 206 containing pro*U* operon

Lane1-3- consortium members demonstrating 13.2 Kb band of construct

Lane4-construct uncut

Lane5-Pseudomonas putida MTCC*102

Figure 4.29: Growth of NCC.GSS3 in LB medium amended with various concentrations of NaCl

Figure 4.30: Growth of NCC.DSS6 in LB medium amended with various concentrations of NaCl

Figure 4.31: Growth of NCC.DSS8 in LB medium amended with various concentrations of NaCl

Figure 4.32: Growth of *P. putida* MTCC102 in LB medium amended with various concentrations of NaCl
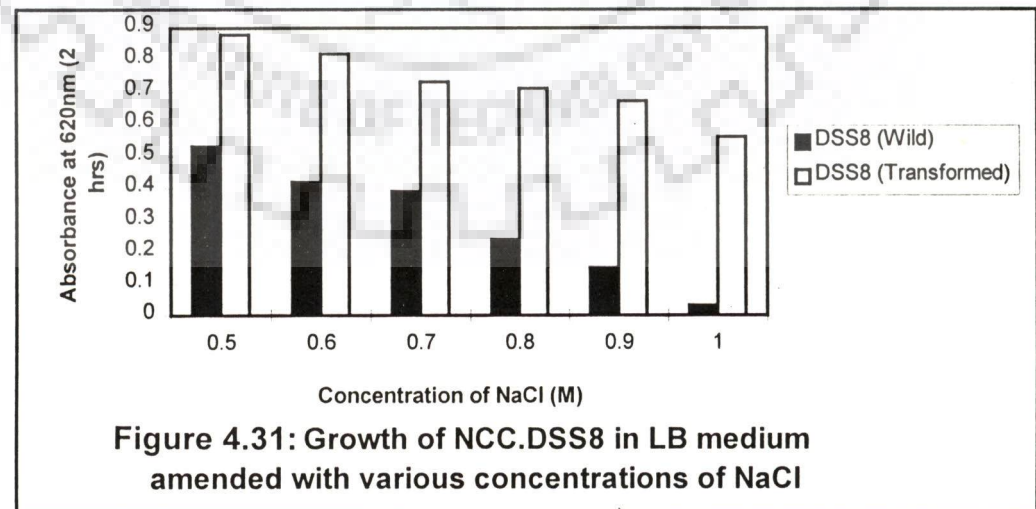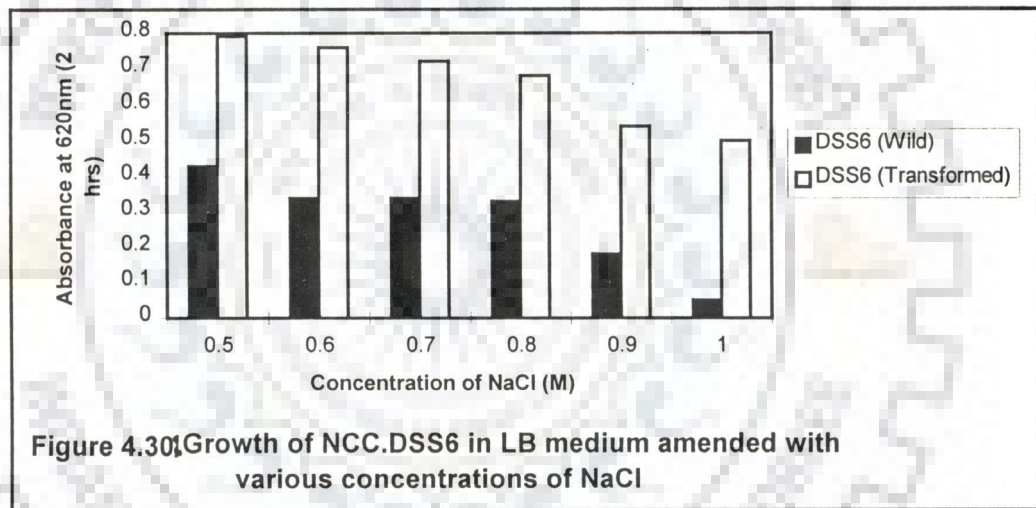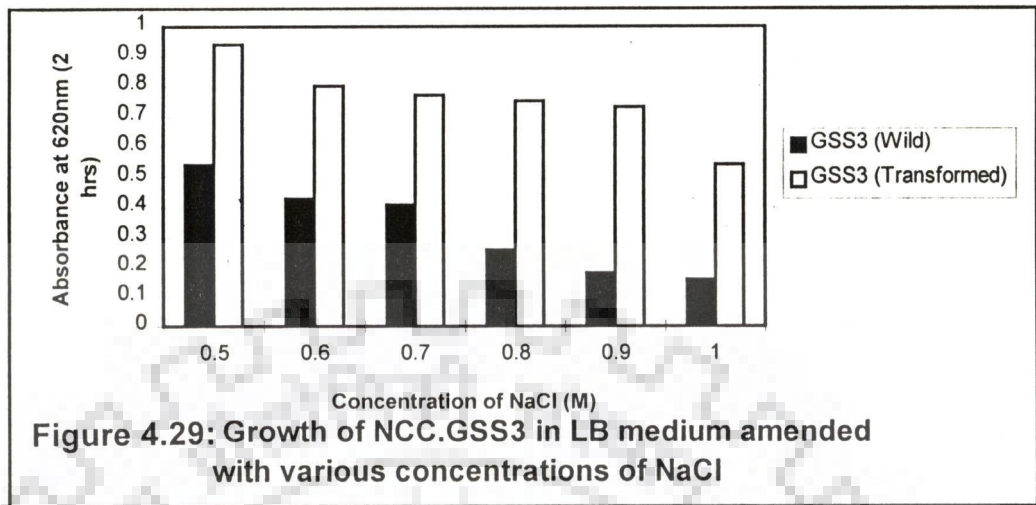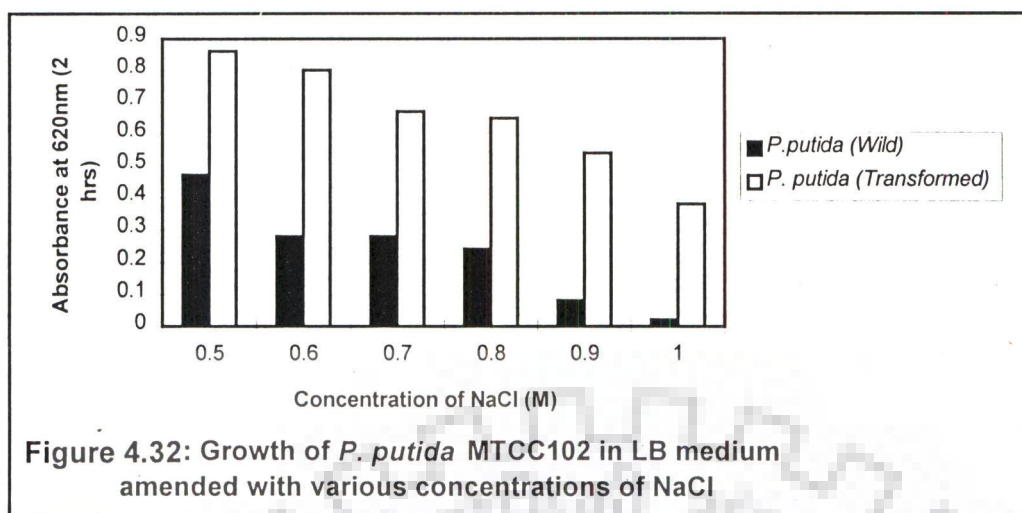
4.7.3 Degradation of Hydrocarbons by Transformed Consortium in 4% Saline Condition

The seed culture of transformed organisms was prepared in BH medium amended with catechol. The adapted organisms were inoculated in BH medium amended with 1% crude oil and model petroleum (Table 3.1) and incubated for 72 hours at 150rpm at 30^0C. The culture flasks were extracted with equal volume of hexane (1:1, v/v) and hexane extracts were analyzed by gas chromatography. The model petroleum samples were analyzed on capillary gas chromatograph and crude oil samples by packed column gas chromatography in the similar manner, under same conditions described before. It was observed that the hydrocarbon dissimilation property of consortium was not hampered in hypersaline condition with transformed organisms. The catechol adapted consortium degraded both model petroleum and crude oil efficiently as by the wild organism. Fig. 4.34 exhibits the gas chromatograph of model petroleum before and after degradation. As the figure shows lower alkanes (C_{12}-C_{16}, peaks 1,2 and 3) disappeared completely after 72 hours, while the aromatics (dibenzothiophene and phenanthrene) were degraded to 50%. Pristane (peak 4), serving as an internal standard remained unaffected in the culture medium. Higher alkanes eicosane, octacosane and tetracosane (C_{20}, C_{24}, C_{28}; peaks 7, 8, 9) were also consumed in due course of time. Fig. 4.33 demonstrates gas chromatographic profile of Bombay high crude oil before and after degradation.

Fig. 4.33 : Gas Chromatograph - degradation of Bombay High Crude (1%) in 4% NaCl by the designed
consortium adapted on catechol (analyzed on Sigma 300, FID)

A. Chemical Control- abiotic loss in 72 hr.

B. Experimental- degradation by consortium in 72 hr. in normal medium

C: Experimental- degradation by transformed consortium in 72 hr. in 4% NaCl
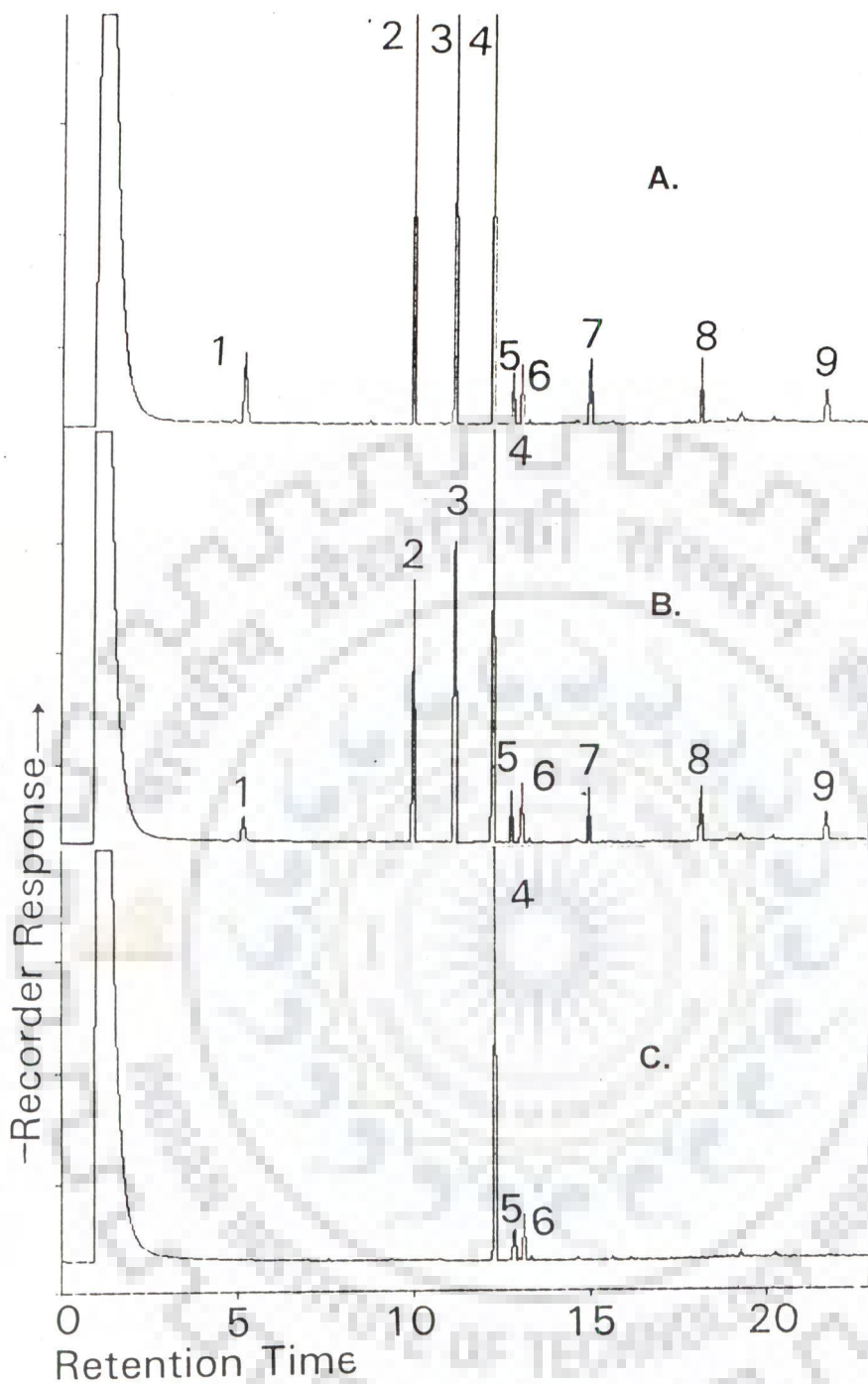
Fig. 4.34 : Capillary Gas Chromatograph - degradation of model petroleum (100ppm) by the designed and transformed consortium adapted on catechol (analyzed on PE Auto System, FID)

A. 0 hr.

B. Chemical Control (72 hr)

C. Experimental (72 hr)

Peak1-dodecane Peak2-naphthalene Peak3-pentadecane Peak4-hexadecane Peak5-pristane Peak6-dibenzothiophene Peak7-phenanthrene Peak8-eicosane Peak9-tetracosane Peak10-octacosane

Fig. 4.34 (a) is the control Bombay high crude, to assess the abiotic loss due to evaporation, which is negligible in the duration of the experiments, and Fig. 4.34 (b) is the degradation pattern after 72 hours. In 4% saline (0.7M NaCl), the degradation of Bombay High Crude was more or less similar when the transformed consortium was applied.

4.8 Bioremediation of Contaminated Soil

This study aims at the application of a designed and adapted bacterial consortium for effective bioremediation of hydrocarbon contaminated soil. The degradation of Bombay high crude as well as model petroleum (mixture of representative hydrocarbon molecules) was studied in a virgin soil (black cotton soil) and a PAH contaminated, weathered soil.

Black Cotton soil was collected from the institute premises (NEERI) having the pH of 6.8 and organic content 5.6%. Sieved and dried soil (5.0 g) was autoclaved thrice in glass tubes (15 cm.' 23 mm.) for 30 minutes before inoculation and spiking. Soil No. 2 was weathered soil collected from a petroleum refinery dump site. Soil samples were sieved through 1mm mesh. Wherever necessary, sieved and dried soil was autoclaved thrice. Two multi-substrate systems, Bombay high crude oil and model petroleum (Table 3.1) were used to spike virgin black cotton soil. 5 gms of soil samples were taken in glass tubes for experimentation. Black cotton soil (soil no. 1) was autoclaved three times as mentioned above and sterilization was confirmed by plating on nutrient rich medium. Sterile soil was spiked with 100^m of Bombay high crude or 10^m of model petroleum dissolved in 1ml chloroform which facilitated mixing of hydrocarbons in soil. After adding chloroform, the soil was mixed thoroughly. Solvent was allowed to evaporate prior to inoculation. Consortium members, individually grown on catechol, were inoculated to give the final optical density(A^{620}) of 1.0/ g of soil. After inoculation, the culture tubes were incubated at 30^{0}C in the dark. After 8 days, the oil was extracted with hexane (1:1 vol./wt) and subjected to gas chromatography. Experiments were carried out in triplicate and chemical controls were run in parallel. For soil no.2 (contaminated), the methodology remained same except spiking. Along with chemical controls, a biological control (autoclaved soil) was also run with soil no. 2.

After incubation period the remaining hydrocarbons in the soil were determined by gas chromatography. Crude oil was analyzed on packed column gas chromatograph while for model petroleum, capillary gas chromatograph was used.

Fig. 4.35 depicts the gas chromatographic profile of Bombay High Crude before and after biodegradation. The chemical controls run parallel to the study revealed that there was no significant loss due to volatilization in the experiment in due course of time (8 days) as the oil used was weathered. The removal of hydrocarbons could only be attributed to the catabolic capabilities of designed consortium as bioaugmentation was carried out after sterilization. Similar results were observed with model petroleum (Fig. 4.36) where dodecane (C_{12}) and naphthalene were degraded more than 90% while pentadecane (C_{15}) and hexadecane (C_{16}) degradation was 60%. Phenanthrene (tricyclic), dibenzothiophene (S containing heterocyclic), eicosane (C_{20}), tetracosane (C_{24}) and octacosane (C_{28}) were relatively less biodegradable (30-45%). Other investigators have reported similar degradation pattern where saturates are degraded more easily than PAHs [Perry, 1992] however, the adapted consortium reported here requires a relatively short lag period to grow on hydrocarbons. Soil no.2 collected from a refinery dump site was selected to study the efficacy of designed bacterial consortium in natural conditions. The soil contained various hydrocarbons of saturate and aromatic nature as well as organic compounds of plant origin. Some of the compounds have been identified by GC-MS and listed in Table 4.11. The microcosm experiments conducted with soil no.2 demonstrated that consortium effectively reduced contamination level (Fig. 4.37).The biosurfactant producing organism, NCC.DSS$_6$, played a key role in bioremediation of contaminated soil. Without NCC.DSS$_6$, the consortium could not attack hydrocarbons even though a chemical detergent, Tween-80 was provided (Fig. 4.37 c).
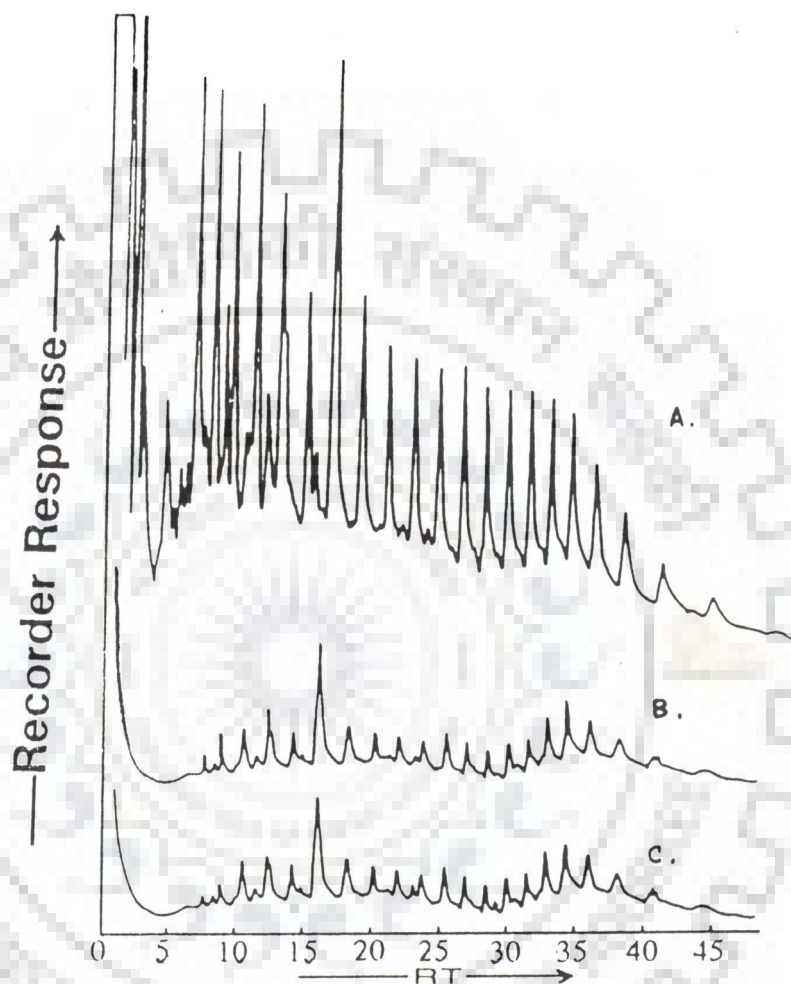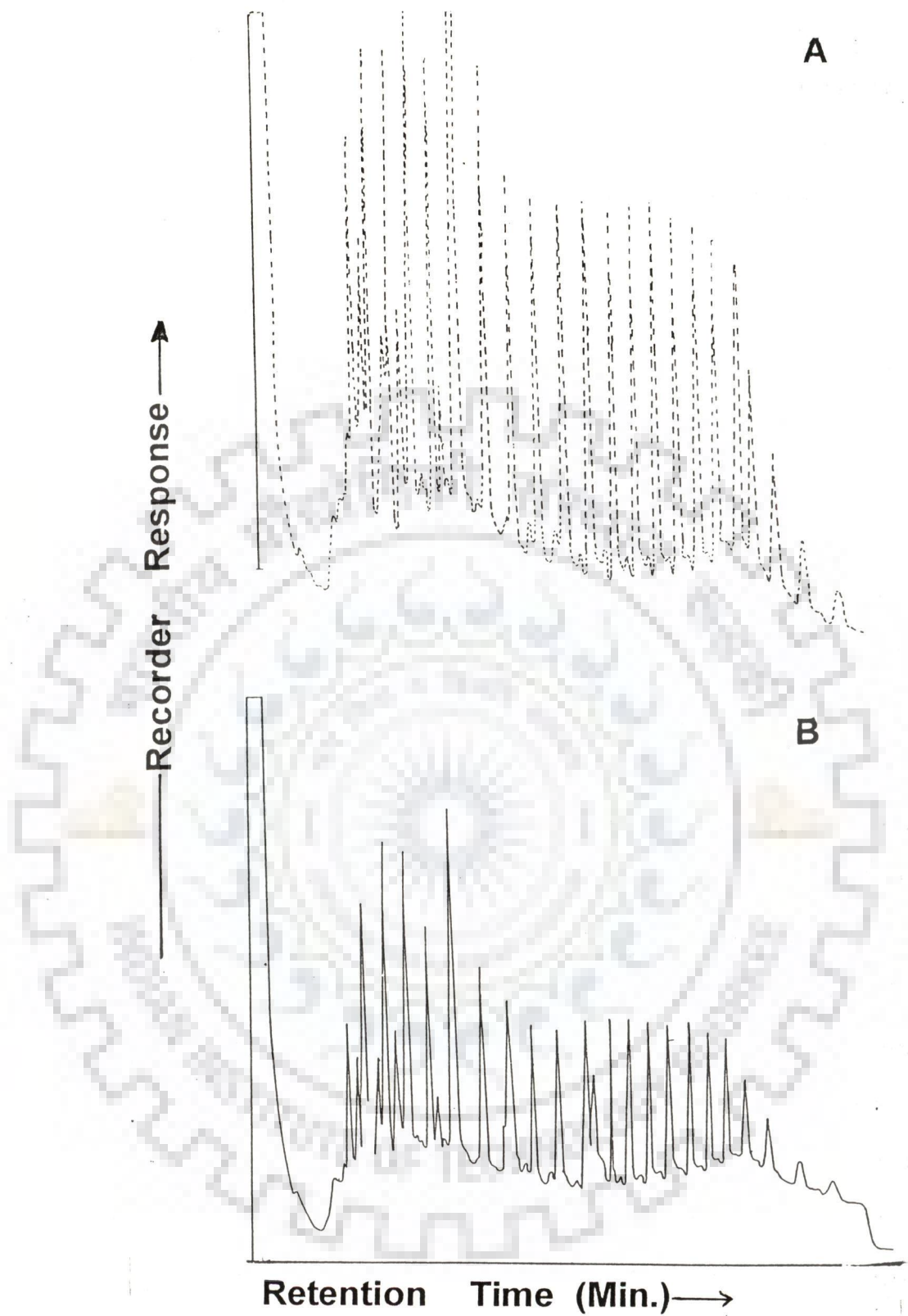
Fig. 4.35 : Gas Chromatograph - degradation of Bombay High Crude (1%) in virgin black cotton soil by the designed consortium adapted on catechol (analyzed on Sigma 300, FID)

A. Chemical Control- abiotic loss in 8 days

B. Experimental- degradation by consortium in 8 days
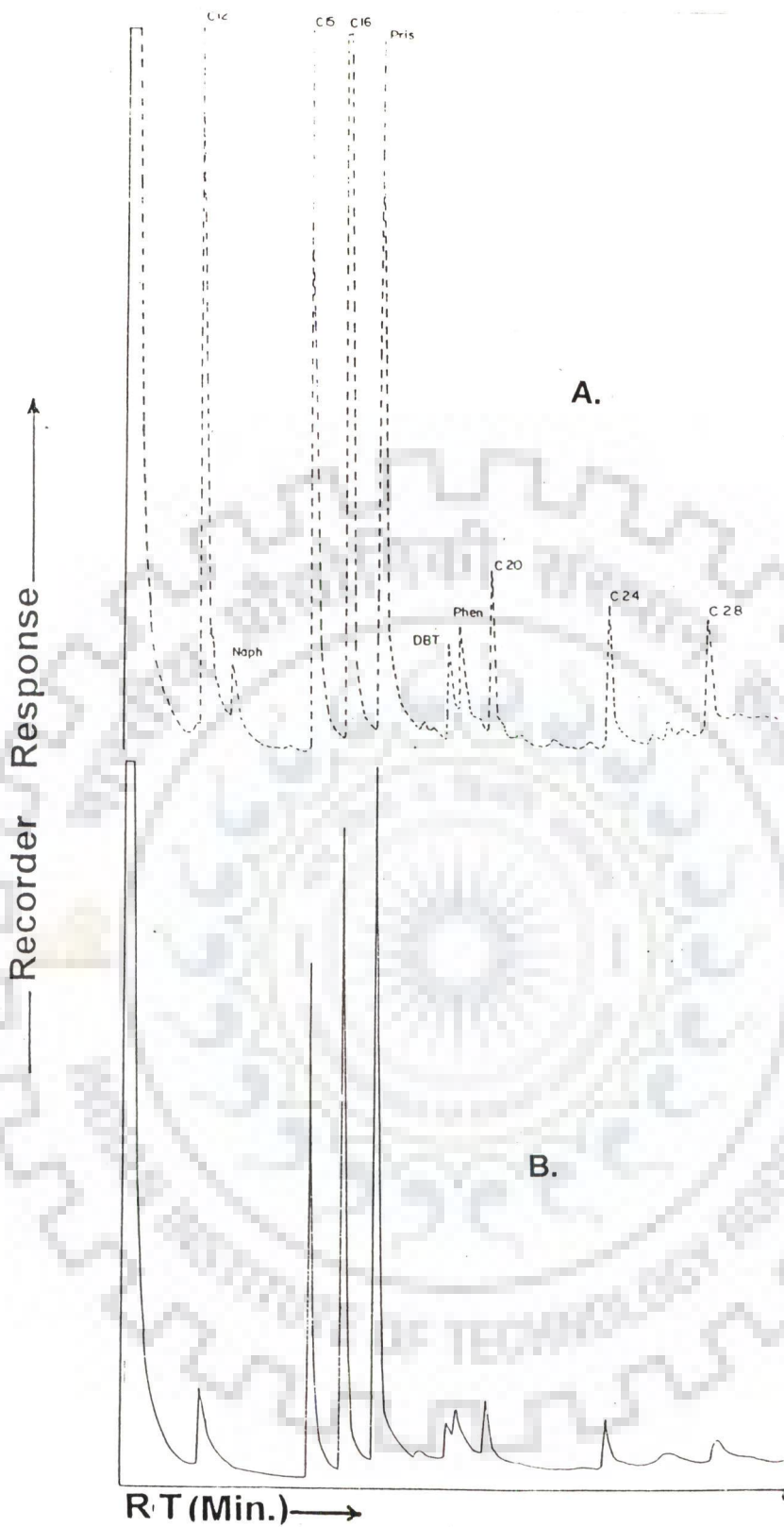
Fig. 4.36 : Gas Chromatograph - degradation of model petroleum in virgin black cotton soil by the designed consortium adapted on catechol (analyzed on Sigma 300, FID)

A. Chemical Control- abiotic loss in 8 days

B. Experimental- degradation by consortium in 8 days
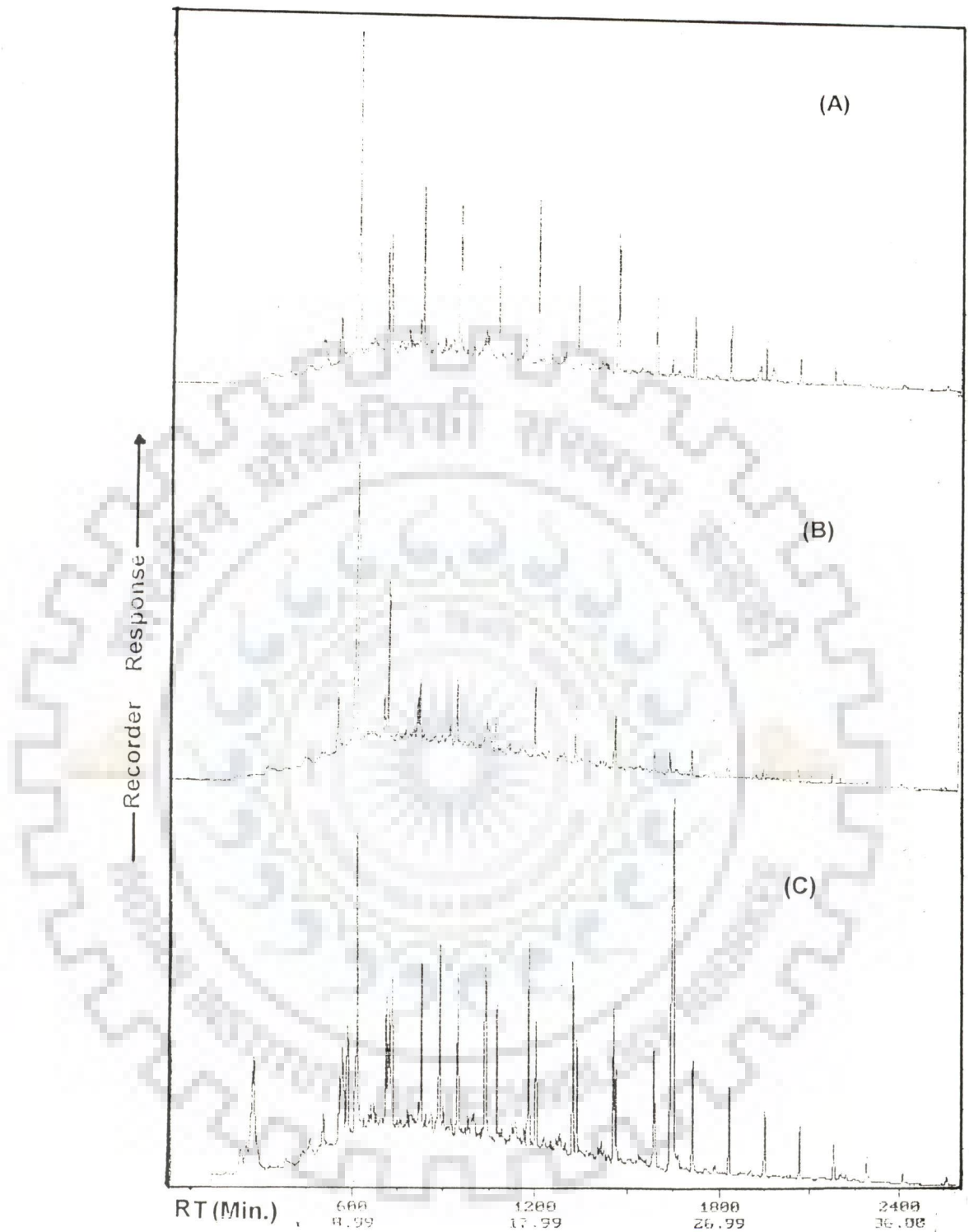
Fig. 4.37 : Gas Chromatograph - application of catechol adapted consortium in bioremediation of PAH contaminated and weathered soil (analyzed on Varian, *Saturn*)

A. Chemical Control- profile of contaminants present in weathered soil [Table 4.11)

B. Experimental- degradation by consortium in 8 days

C. NCC.DSS$_6$ is replaced with Tween-80

Table 4.11 : Contaminants Present in Weathered Refinery Soil

S. No.	Compounds present* in refinery soil
1.	1-methyl-7-(1-methyl ethylnaphthalene)
2.	2,3-dimethyl-phenanthrene
3.	2,3,5-trimethyl-phenanthrene
4.	n-heptadecylcyclohexane
5.	phytol
6.	9-dodecyltetradecahydro-anthracene
7.	2,7,7-trimethyl decane

* *Identified by GC-MS (Varanus, Saturn-3) on DB-5 Column*

Chapter V

Discussion

Discussion

5.1 Physical Skimming of Crude Oil Using Alkali Treated Saw Dust

It is evident that the treated sawdust could be used as a sorbent to cleanup spilled oil. Though some natural sorbents have been reported to have hydrophobic properties before any treatment due to their surfaces waxes e.g. milkweed floss (*Asclepias*) [Knudsen,1990] which is cultivated or naturally grown in some states in the United States. Kenaf is another cellulosic material [Chol,1992]. Nevertheless, no work has been reported on the oil sorption capacity of these materials. However, raw sawdust also does not show such properties. But after treating it with 1% alkali (NaOH), the saw dust particles exhibited property of oil absorption (Plate 4.2). The acquired oil sorbing capacity could be attributed to the delignification of sawdust caused by alkali treatment. In alkaline condition under high temperature and pressure, the lignin constituents of sawdust undergo hydrolysis and give the saw dust-alkali slurry a black color. The delignification of sawdust results in pore formation in the cellulose- hemicellulose network. Mercury porosimetry indicated the presence of bottleneck pores and narrow necked inter particle voids. Removal of lignin could also be a reason to the increased buoyancy characteristic which is a crucial parameter for the successful absorbents (Fig. 4.2-4.3 and Plate 4.1). Absorption of methylene blue confirmed the increase in surface area of sawdust. By using alkali treated sawdust, 90-95% crude oil could be removed physically in the first step of treatment (Fig. 4.2).

5.2 Enrichment and Isolation of Hydrocarbonoclastic Organisms

Microbial potential to degrade petroleum hydrocarbons has been known for essentially as long as microbial metabolism has been appreciated. Crude oil degrading organisms were enriched in a semicontinuous batch reactor fed with crude oil in this study as per the standard enrichment technique. The dissimilatory capacity for oil consumption was measured by Chemical

Oxygen Demand method [APHA, AWWA,WPCF,1992] after every 48 hrs. In five months time the COD reduction of the effluent reached to 59.82 % (Fig. 4.4) which indicates the mineralization capabilities of the existing microbial population in bioreactor. Initially, when the hydrocarbon degraders were less in number, the crude oil fed to the reactor was not degraded and the COD of the effluent was as high as 515 mg/L. Over the period of time, the number of hydrocarbonoclastic organisms increased and the daily feed of oil was consumed, reducing the COD of effluent. The selection was made from the reactor with efficiency of about 60% COD removal. At this stage, the organisms were isolated by conventional plating method and subjected to a three-tier screening. Out of 35 isolates, 16 were capable of growing on petrol, 4 on diesel and 15 on Gulf high crude. All the isolates were screened on the basis of their colony characteristics, drug resistance and hydrocarbon utilization range. Screening, essentially eliminates the probability of selecting the same organism more than once at different steps for all the selected gram -ve organisms. The secondary screening included the characterization on the basis of drug resistance pattern which can help in even understanding species variation thereby helping in strain characterization. On the other hand, the xenobiotic associated specialized physiology is encoded mostly on extrachromosomal DNA i.e. the plasmids [Sayler,1990] which are also known for carrying the additional drug resistance marker genes. These characteristics can be easily assessed and help in establishing the genotypic diversities amongst various isolates. The antibiotic sensitivity tests were accordingly carried out for the isolated organisms. Since the isolated organisms were gm-ve, all were resistant to penicillin-G while varying degrees of sensitivity was observed towards chloremphenicol, gentamycin, kanamycin and tetracycline and complete resistance to ampicillin as evidenced by the data in Table 4.3. Finally the organisms were segregated for their hydrocarbonoclastic capabilities using representative hydrocarbon molecules as sole source of carbon and energy. Five hydrocarbon substrates representing various classes of petroleum constituents were used individually to assess the metabolic potential of isolates. The tertiary screening led to the selection of isolates to design a bacterial consortium for crude oil degradation which was the ultimate aim of the study.

5.3 Criteria for the Selection of Bacteria to Design a Consortium

Crude oil is a heterogeneous mixture of several hydrocarbons, generally grouped into four classes according to their differential solubility in organic solvents : the saturates (n- and branched- chain alkanes and cycloparaffins), the aromatics (mono-, di-, and polynuclear aromatic compounds containing alkyl side chains and/or fused cycloparaffin ring), the resins (aggregates with a multitude of building blocks such as pyridines, quinolines, carbazoles, thiophenes, sulfoxides and amides) and the asphaltenes (aggregates of extended polyaromatics, naphthenic acid, sulfides, polyhydric phenols, fatty acid and metalloporphyrins)[Leahy & Colwell,1990]. Individual organisms can metabolize only a limited range of hydrocarbon substrates [Britton,1984]. Effective degradation of crude oil may require simultaneous action of several different microbes possessing different metabolic capacities. Several researchers have tried mixed culture approach [Horowitz et al.1976; Atlas,1981] but the process is slow as it encounters a multisubstrate-multispecies growth kinetics where simpler alkanes are consumed in the beginning followed by dominating biotransformation phase for most of the aromatics. The unstable selection and maintenance could lead to the extinction of some key member of mixed population which might be having important role to play in degradation process. Therefore a consortium, a defined mixture of pre-determined members with synchronous metabolic potential, is suggested in this study where all the members are well characterized metabolically and added in a certain ratio so that the population dynamics can be maintained relatively even in a multisubstrate system. The first objective of this study was to select different organisms having capabilities to attack various fraction of crude oil. The organisms isolated and selected in this study, for oil degrading consortium, were $NCC.DSS_6$, $NCC.DSS_8$ and $NCC.GSS_3$. Isolate $NCC.DSS_6$ consumed dodecane and phenanthrene very well (Table 4.4). Froth was observed in the $NCC.DSS_6$ culture tubes which indicated the biosurfactant activity. The organism was later characterized as biosurfactant producer and biosurfactants play a crucial role in oil degradation process [Juwarkar et al., 1990]. $NCC.DSS_8$ was found to be consuming saturates, eicosane (C_{20})

and octacosane (C_{28}), better than the other isolates and NCC.GSS$_3$ was selected due to its aromatic degradation capability (catechol and phenanthrene, Table 4.4). These three organisms were further characterized in the study.

5.4 Characterization of the Selected Isolates

5.4.1 Identification Based on Biochemical Tests

From the results obtained on morphological and biochemical characterization, the isolate NCC.GSS$_3$, NCC.DSS$_6$ and NCC.DSS$_8$ could be placed in the genus *Pseudomonas*, as the organisms are gm-ve, facultative, non-sporulating, highly motile, rod (Tables 4.6 & 4.7), growing well at 30 to 37^{0}C and a neutral pH. They derive energy from the hydroxylation of the various hydrocarbons (Table 4.4). Most *Pseudomonads* can grow in mineral medium with ammonium or nitrate ions and a single organic compounds as sole energy and carbon source without requiring the addition of any organic growth factor. Isolate NCC.DSS$_6$ and NCC.DSS$_8$ also grew on *Pseudomonas* isolation agar which contains acetamide, oftenly used by various *Pseudomonas* sp. All the three isolates were positive for amylase (starch hydrolysis), lipase (tween-80 hydrolysis) and gelatinase (gelatin liquification) activity. NCC.GSS$_3$ also gave positive results for arginine dihydrolase. NCC.DSS$_6$ demonstrated the pigment production capacity as the colonies on nutrient agar were blue-green which is a characteristic feature of *Pseudomonas aeruginosa*.

5.4.2 Screening for Plasmid(s)

Many of the *Pseudomonads* plasmids have been shown to carry genes for metabolism of compounds such as salicylate, camphor, naphthalene, toluene, xylene and 3-chlorobenzoate [Chakrabarty, 1976]. The genes for these dissimilatory pathways, unlike the biosynthetic genes on chromosomes, are tightly linked on the plasmids. Therefore, the isolates (inferred as *Pseudomonas* sp. based on the results of biochemical tests) were screened for the presence of plasmids. Only NCC.DSS$_6$ was found to be harboring a plasmid (Plates 4.13 & 4.9). Other isolates did not demonstrate any low molecular weight plasmid band on agarose gel electrophoresis. The DSS$_6$ plasmid was later characterized for its role in biosurfactant synthesis

in strain NCC.DSS$_6$. The methodologies used in this study for plasmid preparation are not meant for the isolation of megaplasmids like NAH, SAL or TOL, therefore it can not be said conclusively that the dissimilatory capacity of the selected isolates is not plasmid encoded, however, it eliminates the probability of isolates harboring plasmids of 1-30Kb size, except NCC.DSS$_6$. As these plasmids are often associated with drug resistance markers, the rationale behind the study of native plasmid(s), is the characterization of plasmid encoded antibiotic resistance and other markers which would be helpful in future in genetic manipulation for strain improvement.

5.4.3 Characterization on the Basis of Presence of Dissimilatory Pathway(s)

The oxidative degradation of some organic substrates or their intermediates by *Pseudomonads* involves the participation of oxygenases. Both mono and dioxygenases coupled to a variety of electron donors are well represented in various *Pseudomonas* sp. The mechanisms of ring fission (ortho- or meta-) could also be a criterion in differentiation of *Pseudomonads* across the genera. The meta- cleavage enzyme system is often coded on megaplasmids as in case of NAH or TOL and catechol 2,3 dioxygenase is the ring cleavage enzyme while ortho- fission is considered chromosomally coded and catechol 1,2 dioxygenase is the key enzyme [Harayama, 1994]. The three selected isolates could grow on catechol in mineral medium (Table 4.4) but did not harbor any dissimilatory plasmid except NCC.DSS$_6$ (Plate 4.13). It was assumed that the catechol ring was cleaved by ortho- pathway which was confirmed by subjecting the isolates to Rothera test [Stanier *et al.,* 1966]. All the three isolates gave deep purple colour indicating the presence of β-ketoadipate, the ring fission product in ortho- pathway. A negative control was used in the study which gave yellow colour of *cis, cis* muconate, the ring fission product of meta- pathway, as the organism was imparted *xyl E* of Tol operon which encodes for catechol 2,3 dioxygenase [Nakia *et al.*, 1987; Stein, 1990].

Some of the microbial catabolic pathways responsible for the degradation, including the *alk* (C$_5$-C$_{12}$ n-alkanes), *nah* (naphthalene), and *xyl* (toluene) pathways, have been extensively characterized and are generally found to be located on large catabolic plasmids in *Pseudomonas*

sp., for example the OCT, NAH, and TOL plamids [Sayler *et al.*,1990 ; Whyte *et al.*,1997]. A number of genes and respective enzymes have been thoroughly worked out. The isolates were also studied by PCR to understand the genetic determinants for the presence of some of these catabolic operons (Table 4.9). The target determinants selected were gene homologous to *alk B* from *Pseudomonas oleovorans*, coding for alkane monooxygenase, *xylE* from TOL operon (*P. putida* pWWO) encoding catechol 2,3 dioxygenase, the ring cleavage enzyme in meta- pathway and *dmpN* coding for phenol hydroxylase, originally from phenol degrading *P. putida* pp27 [Shingler *et al.,* 1995]. Isolate NCC.GSS$_3$ demonstrated positive signal with catechol 2,3 dioxygenase primer (Plate 4.12). The presence of catechol 2,3 dioxygenase by PCR in NCC.GSS$_3$ with positive Rothera test suggest that it may have both the pathways. There are very few reports describing and characterizing microorganisms that can catabolize both aliphatic and PAHs. For example, many of the 200 strains isolated by Foght *et al.,* [1990] could mineralize aliphatic or aromatic hydrocarbons but not both, suggesting that the alkane and PAH biodegradation may be mutually exclusive properties in bacteria. On the other hand, 36% of hydrocarbon degradative strains isolated from sediments contaminated with oil from the 1989 *Exxon Valdez* spill had both *alkB* [van Beilein *et al.*,1994] and *xylE* [Nakia *et al.*,1983] genes indicating that *alk* and *xyl* pathways for aliphatic and aromatic degradation can exist in the same organism [Sotsky *et al.*,1994]. Similarly in the presented study, NCC.DSS$_8$ which grew on catechol and demonstrated the presence of -ortho pathway, also had *alkB* (Plate 4.10). NCC.GSS$_3$ demonstrated presence of *dmpN* and *xyl* pathways (Plate 4.11 & 4.12). The very purpose of this consortium is effective degradation of crude oil which requires assembly of different metabolic activities. The selection of above mentioned three isolates may be justified on the basis of the difference in their metabolic potential. NCC.DSS$_8$ had *alkB* for the consumption of alkanes while NCC.DSS$_6$ and NCC.GSS$_3$ both had catechol 1,2 dioxygenase. Isolate NCC.GSS$_3$ which exhibited wide substrate utilization range as far as aromatics are concerned, also demonstrated presence of meta- fission enzyme which could be the reason for effective aromatic degradation. The added advantage of PCR based characterization is that it can be used to track

down these organisms in field. The genotypic differences of the isolates could be important in the tracking exercise.

5.5 Efficacy of Oil Degrading Bacterial Consortium

Microorganisms are known to attack specific saturates, aromatic and polar compounds [Sable, 1946] present in crude oil. Effective degradation of crude oil would require simultaneous action of several metabolically versatile microbes and is generally observed in the environment as an adaptive process. The mixed culture studies suggest that if metabolically active hydrocarbonoclastic organisms could be added to reduce the lag period before the indigenous population could respond. The necessity for seeding with complementary hydrocarbon degrading bacteria arises from the rationale that indigenous microbial populations may not be capable of degrading a wide range of potential substrates in a complex mixture such as crude oil. Atlas [1977] suggested that complex mixture of hydrocarbon degraders would be necessary in order to effectively degrade all of the hydrocarbons in a complex petroleum mixture. The role of mixed populations of microorganisms in degradation of recalcitrant xenobiotics is well recognized. However, in a mixed culture system, the growth of the organisms cannot be regulated due to the nutrient stress and competition. In this study the ability of the designed bacterial consortium with wide hydrocarbonoclastic capacity had been employed for degradation of various fractions of crude oil. The designed consortium had members with desired physiological capabilities. Three isolates (NCC.DSS$_6$, NCC.DSS$_8$ and NCC.GSS$_3$) were selected along with a strain of *P. putida* MTCC*102 to design a consortium. These genetically different isolates were selected on the basis of their substrate utilization range. In designing the consortium a key aspect taken into consideration was the solubility and accessibility of the compounds in crude oil to microorganisms. Since only 0.02% of crude oil is water-soluble there is a need for emulsification. Hence, an organism NCC.DSS$_6$, capable of producing a rhamnolipid (Fig. 4.14) by utilizing short chain aliphatics like dodecane, was included in the consortium as a member. The other two isolates NCC.DSS$_8$ and NCC.GSS$_3$ were selected due to their specialized metabolic potential of utilizing long chain aliphatics and aromatics, respectively. *P. putida* MTCC*102 known for

its capability to consume wide range of hydrocarbons including various downstream metabolites formed in the degradation was chosen as a member of the consortium.

In previous studies a significant lag period is observed in crude oil degradation process [Atlas & Bartha, 1973]. The lag period is essentially due to the time required for induction of enzymes for degradation of aliphatic and aromatic molecules in crude oil. In the present study, the organisms were pre-induced by culturing the members of consortium on catechol, a central metabolite and inducer in degradation of several benzenoid compounds via both ortho- and meta- pathways [Keith & Peter, 1976]. Further, catechol may serve as a cascade inducer as the degradation of certain aromatic compounds in crude oil leads to production of intermediates like benzoate, salicylate and others that then serve as inducers for certain other catabolic operons. This preconditioning of seed cultures was observed to be the major factor in reduction of the lag period in crude oil degradation when compared with non-preconditioned i.e. citrate grown consortium (Fig. 4.11). The efficacy of preconditioning with catechol was proven further by the observed rapid degradation of aromatic compounds present in model petroleum (Fig. 4.5)

The rapid degradation mediated by preconditioned oil degrading organisms overcomes the reported dilution of seeded organisms by indigenous dominant microbiota which requires several days to adapt to petroleum degradation [Tagger et al., 1983]. Further, even if the inoculated or seeded microorganisms are subsequently replaced by competition with indigenous hydrocarbon utilizers, there is the benefit of reducing lag time before indigenous populations get adapted to the presence of certain hydrocarbons. The microbial degradation of crude oil is a surface phenomenon and nutrient addition is unlikely to have dramatic effect specially where pooled oil is present. Hence, the use of preconditioned organisms as a consortium has an advantage over the 'seeding' of organisms with or without nutrients or surfactants as an oil-dispersant package as demonstrated by other workers [Felix,1995].

The increased catabolic potential by prior exposure of the organisms to catechol can be explained by the adaptation phenomenon [Spain et al.,1980]. The three interrelated mechanisms by which adaptation can occur are (I) induction and /or depression of specific enzymes (ii) genetic

changes which result in new metabolic capabilities and (iii) selective enrichment of organisms able to transform the compound or compounds of interest. This cross acclimation of consortium by using catechol as a substrate resulted in effective crude oil degradation. Bauer and Capone [1988] and Kerr and Capone [1983] have reported similar results where exposure to one compound, such as phenanthrene effected an increase in metabolism rate of a compound of similar structure, such as naphthalene. The occurrence of this phenomenon could be attributed to the broad specificity of selected microbial populations for PAHs and/or the existence of common pathways for PAH catabolism. However, the regulation in expressive induced potential via common key metabolites can not be ruled out.

The capacity of biodegradation of crude oil by consortium was proved by gas chromatographic data which was supported by chemical oxygen demand analysis [APHA,1987]. Biodegradation involves chemical changes in the parent molecule, in this case petroleum hydrocarbons, usually accompanied by a reduction of COD, but the products of biodegradation are not necessarily simple or harmless. However, mineralization implies a complete recycling to harmless inorganic end products (CO_2 and H_2O for hydrocarbons). COD of degraded samples in this study was measured 60% less than the control (Fig. 4.7) which indicates the extent of mineralization while gas chromatographic data imply biodegradation *in toto*.(Fig. 4.8 & 4.9).

5.6 Biosurfactant and Crude Oil Degradation

As discussed earlier, one key factor taken into consideration while designing the consortium was ability to emulsify oil. Isolate $NCC.DSS_6$ which was found to be producing rhamnolipid (Fig. 4.14) was included in the consortium. The efficacy of natural biosurfactants is well documented [Fiechter 1992 ; Koch *et al*., 1992 ; Bury and Miller 1993]. Extracellular rhamnolipid brings about the formation of oil-in-water emulsion thereby facilitating the hydrocarbon uptake which results in effective oil degradation. Bacterial consortium without $NCC.DSS_6$ could not degrade crude oil effectively (Fig. 4.17) which confirmed the necessity of biosurfactant in oil degradation process. $NCC.DSS_6$ was found to be harboring a 20kb plasmid (Plate 4.13) which was associated with

biosurfactant synthesis as the cured organism could not lower the surface tension of liquid medium unlike wild organism and after the successful mobilization of the same plasmid into cured organism, the biosurfactant production property was regained. *P. putida* MTCC*102 was also imparted the same plasmid but could not reduce the surface tension liquid medium. It could be concluded that the chromosome and plasmid shared the biosurfactant (rhamnolipid) synthesis pathway in isolate NCC.DSS$_6$.

The degradation data from the experiments described above, strongly recommends the advantage of applying the designed bacterial consortium instead of undefined mixed culture system. The additional studies are required to assess the degradative capacity of the consortium specifically for different fractions of crude oil.

5.7 Utilization of Various Fractions of Crude Oil by Catechol Adapted Consortium

Degradation of Bombay High crude oil by the designed and adapted bacterial consortium is a multisubstrate-multispecies phenomenon. The formulation of the consortium is based on the composition of crude oil and required metabolic potential so that all the components of crude oil would be consumed. In this study, the utilization of various fraction of crude is studied as the heterogeneity of crude oil as carbon source could be very crucial in bioremediation. The ultimate goal of consortium is the dissimilation of all constituents of crude. To achieve the required efficiency the consortia could be modified according to the need of the environment. The saturate fraction of crude oil is considered as the most biodegradable fraction of crude oil. Biodegradation of n-alkanes with molecular weights upto n-C_{44} have been demonstrated [Haines & Alexander,1974]. In this study also the degradation of short chain alkanes (C_{12}-C_{15}) was more profound than longer ones (C_{20}-C_{28}) as proved by gas chromatography (Fig. 4.21 & 4.23). The total consumption of saturate fraction of Bombay High crude oil was 81% (Fig. 4.20). The fractionation of remaining crude oil also reveals that aromatic degradation is less than aliphatic (65%). Gas chromatographic data supports the gravimetric analysis. The biodegradability of aromatics decreases with increase in benzene rings [Hebes & Schwall,1978]. The designed consortium degraded dicyclic naphthalene, tricyclic

phenanthrene and sulfur heterocycle dibenzothiophene in the same order (Fig. 4.23) but less in comparison to aliphatics. Rontani *et al.* [1985] reported the degradation of asphaltene fraction by cometabolism (in presence of paraffins), but in the experiments described above there was no considerable change in asphaltene fraction before and after degradation (Fig. 4.20). To verify the efficiency of fractionation protocol, silica gel column chromatography method adapted from Hong-vu Song *et al.* [1990], the Bombay High crude was spiked with representative aromatic (phenanthrene) and aliphatic (octacosane) hydrocarbons. The hydrocarbons were detected in the respective elutes i.e. phenanthrene in benzene elute and octacosane in hexane elute, by gas chromatography (Fig. 4.18 & 4.19) which confirmed the authenticity of fractionation protocol.

5.8 Adaptation of Consortium on Other Inducers and Degradation of Aromatic Mixture

The aromatics present in crude oil have been the major cause of concern due to their persistence in the environment. Though the designed and catechol adapted consortium degraded PAHs, the efficiency was lower (Fig. 4.20-23). Therefore, two amendments had been made. First, *Psudomonas putida* MTCC*102 was replaced with *Pseudomonas putida* G7 (harboring archetypal NAH7 plasmid known for catabolism of naphthalene) [Schwell,1986] and secondly the known inducers for upper and lower pathway of naphthalene degradation encoded in *nah* operon, were used to preculture the consortium members. As discussed above, adaptation plays a key role in bringing about the dissimilation of relatively persistent molecules and also enhances the rate and extent of turnover. Spain *et al.* [1980] reported the increase in biodegradation by a microbial community adapted on compounds of similar structures. Moreover both benzoate and salicylate are known to induce *nah* operon. In plasmid NAH7, the naphthalene catabolic genes are organized into two operons, *nah* and *sal*. The two catabolic operons are controlled by a positive regulator gene, *nahR,* that is located immediately upstream of the *nahG* gene. Induction of operons is not controlled by naphthalene but by its metabolite, salicylate [Yen & Gunsalus,1982]. Menn *et al.* [1993] reported for the first time the direct biochemical evidence that the naphthalene plasmid degradative enzyme

system was involved in the degradation of higher-molecular-weight PAH other than naphthalene (anthracene and phenanthrene) [Menn *et al.*,1993]. In the experiments described above, It was observed that all the consortium members utilized benzoate, catechol and salicylate as single source of carbon and energy and *Pseudomonas putida* G7 grew profoundly on salicylate than others (Fig. 4.27). The log phase harvested cells were used to study the degradation of a model aromatic mixture which simulated aromatic fraction of crude oil. Eleven compounds representing various classes of aromatics were mixed in a certain ratio to prepare this mixture (Table 3.2). As reported by several researchers, the biodegradation of aromatics decreases with the increase in the number of benzene rings, the capillary gas chromatography proved that lower aromatics were eliminated to a large extent while higher ones remained (Fig. 4.28). The degradation profile did not vary much with the inducer though catechol adapted consortium performed little well than benzoate and salicylate, probably due to the better growth of other consortium members on catechol (Fig. 4.26). Two-dimensional SDS-polyacrylamide gel electrophoresis could not differentiate the protein pattern of consortium members grown on different inducers (Plate 4.14-4.17).

The rationale behind including *Pseudomonas putida* ppG7 in the oil degrading consortium, is the prospective exploitation of flexible dioxygenases like naphthalene dioxygenase (*nah* operon) for dissimilation of PAHs other than naphthalene [Menn *et al.*,1993] thereby widening the range of dissimilation. Naphthalene dioxygenase is known for making diol derivatives of most of the poly/heterocyclic aromatic hydrocarbons. The diol derivatives are water-soluble intermediates which can be thus made available to other members of the consortium for further metabolism. The overall degradation of a model aromatic mixture containing 10 different aromatic molecules, was found little more when catechol was used for pre-culturing which is the key intermediate in all aromatic degradation pathways. The results emphasize the need to understand the balance in metabolic physiology of bacteria in adaptation which enables them to consume wide variety of hydrocarbons.

5.9 Hydrocarbon degradation and salinity

Hydrocarbon degradation is affected by a number of environmental factors. There are few published studies that deal with effects of salinity on the microbial degradation of hydrocarbons. Shiaris [1989] reported a generally positive correlation between salinity and rates of mineralization of phenanthrene and naphthalene in estuarine sediments. Kerr and Capone [1988] observed a relationship between the naphthalene mineralization rate and salinity in sediments of the Hudson river that was dependent upon the ambient salinity regime, with estuarine sites exhibiting a lack of inhibition of mineralization over a wider range of salinity than was the case for the less saline upstream site. In a study of hypersaline salt evaporation ponds, Ward and Brock [1978] showed that rates of hydrocarbon metabolism decreased with increasing salinity in the range 3.3 to 28.4% and attributed the results to a general reduction in microbial metabolic rates. The members of the bacterial consortium developed in this study were also unable to sustain their natural growth pattern when subjected to hypersaline condition (0.5 to 1 N NaCl) as evident in Fig. 4.29-4.32. Several osmotolerant bacteria respond to increased osmotic pressure by accumulating compatible solutes or osmolytes to high intracellular levels [Csonka,1989]. The accumulation of osmolytes raises the internal osmotic pressure, thus maintaining turgor. Among the most prominent compatible solutes are a relatively few amino acids (glutamate, glutamine, alanine and proline) and aminoacid derivatives such as glycine betaine (N,N,N-trimethylglycine). The *pro'U'* operon is associated with the accumulation of these osmoprotectants in the best-understood systems such as *E.coli* and *S. typhimurium* [Gowrishanker,1988]. Therefore, to make the members of consortium survive in the hypersaline environment, a broad host range vector (pMMB 206, Plate 4.18) having the insert of pro'U' was mobilized. It was observed that all the transformed organisms tolerated hypersalinity upto 1 M NaCl (Fig. 4.29-4.32). Further, the subcloning of the *pro'U'* operon against *tac* promoter provides the potential for induction by IPTG to increase expression level several folds above the basal expression as has been reported for *xylE* genes [Morales *et al.*,1991]. However, in the present study, the basal level expression of the

promoter is sufficient enough to provide required osmoprotection. The added genotype of osmotic tolerance did not hamper the ingenious dissimilatory capacity of oil degrading consortium and in both the test conditions (crude oil as well as model petroleum), the degradation pattern was similar to their wild type counterparts (Fig. 4.33-4.34).

5.10 Bioremediation of Hydrocarbon Contaminated Soil Using Designed Consortium

The immediate objective of this study was to evaluate the possibility of applying a designed bacterial consortium to remediate hydrocarbon-contaminated soil. The consortium designed and described in this study was successfully tested for crude oil degradation in shake flask cultures. The adaptation of consortium on catechol, an intermediate of aromatic degradation pathway, reduced the lag period required for crude oil degradation.

Crude oil is a heterogeneous mixture of several unidentified hydrocarbons of different nature. The degradation studies with crude oil are complex as far as the analysis is concerned. In a complicated system like soil where humic acid and several other impurities are abundant, the analysis becomes more difficult. To make the study simple while simulating real life situation, a model mixture of representative hydrocarbons was prepared. Short chain alkanes (dodecane (C_{12}), pentadecane (C_{15}) and hexadecane (C_{16}), long chain alkanes (eicosane (C_{20}), tetracosane (C_{24}) and octacosane (C_{28}), bicyclic aromatic (naphthalene), tricyclic aromatic (phenanthrene) and sulphur containing heterocyclic molecules (dibenzothiophene) were mixed to prepare a standard model petroleum (Table 2.1). Pristane was used as the internal standard. This multisubstrate system was used to spike a virgin soil to provide a known contamination level and the efficacy of consortium was checked. Hydrocarbons differ in their susceptibility to microbial attack and in the past, have generally been ranked in the following order of decreasing susceptibility: n-alkanes > branched alkanes > low molecular weight aromatics > cyclic alkanes [Perry, 1984]. Biodegradation rates have been shown to be highest for saturates, followed by the light aromatics, with high molecular weight aromatics and polar compounds exhibiting lower rates of

degradation. Like other investigators, it was found that short chain alkanes (dodecane) and low molecular weight aromatics (naphthalene) were degraded rapidly while phenanthrene and dibenzothiophene (PAHs) were relatively persistent in nature (Fig. 4.36). Virgin black cotton soil was also spiked with Bombay High Crude and subjected to biodegradation by the consortium. Gas chromatographic data demonstrated substantial loss of hydrocarbons (Fig. 4.35). Both the experiments suggest that the designed bacterial consortium is effective in bioremediation of contaminated soil.

Ogunstein et al. [1993], demonstrated the effect of 2-hydroxybenzoate, an intermediate of naphthalene degradation pathway on naphthalene mineralization by adding the metabolite directly in the soil, and observed the increase in the metabolic activity (nah AB of NAH operon) of indigenous population. However, in this study catechol, an intermediate in ortho and meta-pathway is used for adaptation of consortium members. The adapted consortium is then subjected to contaminated soil. Consortium grown on citrate could not degrade crude oil as efficiently. ,Catechol or substituted catechols are key intermediates in any aromatic degradation pathway resulting from oxidation of aromatic ring via hydroxylases or similar enzyme systems. It has been shown that these intermediates can directly react as regulatory molecules to turn on upper or lower pathway of a catabolic operon [Ogunstein et al. 1993] . Hence, the adaptation on catechol may be considered vital for the seeding.

The consortium has been designed on the rationale that, for effective crude oil degradation, simultaneous action of different bacteria is required. The consortium members were able to attack different fractions of crude oil. NCC.DSS$_6$, a member of consortium provided emulsifying potential to the consortium by utilizing aliphatic fraction which resulted in increase in bioavailability of hydrocarbons, usually insoluble or sparingly soluble in water. However, the synthetic detergent, Tween-80 used in the study, when added to the soil no.2, resulted in the inhibition of metabolic capacities of the consortium. Such inhibition may be attributed to several factors. The surfactant itself or its biodegradation products could have inhibited the growth of consortium members or the surfactant could have been used as preferential substrate or on the

other hand the mobilization of high concentration of hydrocarbons could have proven toxic to the micro flora. Similar results have been observed by Tiehm [1994] who has shown that the biodegradation of phenanthrene (200mg/L) by a bacterial consortium in the aqueous phase was inhibited by the presence of a chemical detergent, sodium dodecyl sulfate (SDS) although SDS is considered as a relatively non-toxic and biodegradable surfactant [Clarke *et al.* 1992]. Deschenes *et al.* [1992] suggested that the application of such surfactant might proliferate the population capable of biodegrading the surfactant in use only which could lead to reduction in the degradation of target molecules. The addition of Tween-80 facilitates the desorption of hydrocarbon molecules from soil surface as evident in the GC profile (Fig. 4.37) of Tween-80 containing soil studies. The data suggest that non-specific desorption due to Tween-80 raises the hydrocarbon level in soil immediately which might reach above the toxicity level for consortium. The bioavailability mediated via $NCC.DSS_6$ is at the cost of contaminating hydrocarbons. This results in sustained release of hydrocarbons from soil. Hence, at a given point of time, the hydrocarbon level would be well below the toxic level for consortium. This scenario provides a balanced availability of carbon source in favour of reduction of contamination levels in either of the conditions i.e. virgin soil spiked with crude oil or PAH contaminated and weathered soil (soil no.2).

Chapter VI

Conclusion

Conclusions

The microbial world is characterized by an incredible metabolic and physiological versatility that permits microorganisms to survive in hostile ecological niches. Bacteria can consume wide range of molecules, as carbon and energy source, which are unpalatable to higher organisms. The enormous metabolic potential of bacteria has also been exploited to remediate hydrocarbon contamination, an unavoidable aftermath of modernization process. *Exxon Valdez*, the first large scale experiment, advocated application of nutrients/fertilizers to provide nitrogen and phosphorus to indigenous population, as an approach to oil spill remediation. The present study proposes a two step treatment strategy to combat marine oil spills wherein the first step would be physical skimming of crude oil using alkali treated saw dust as an absorbent which would be followed by biodegradation of remaining crude oil by 'seeding' of a designed bacterial consortium. The seeding process could also be applied for abatement of oil pollution in refinery premises. The emphasis is on the development of exogenous microbes having specific degradative traits to formulate a consortium on the basis of substrate characteristics which results in reduction in lag phase of oil degradation process.

Prior to biodegradation, the spilled oil was physically skimmed by using a cost efficient sorbent alkali treated sawdust. The specific surface area of sawdust was enhanced by alkali treatment (1% NaOH) in high temperature and pressure condition, which caused delignification, ultimately resulting in an increase in the surface area which was measured by methylene blue and mercury porosimetry. The treated sawdust removed 90-95% of crude oil. The remaining crude oil was subjected to biodegradation making oil spill cleanup a two step treatment process. This study envisaged the use of designed bacterial consortium consisting of four organisms

(NCC.DSS$_6$, NCC.DSS$_8$, NCC.GSS$_3$ and *Pseudomonas putida* MTCC*102) to remediate crude oil spills.

Seeding requires highly versatile and metabolically diversified organisms as they face hostile ecological conditions. The hydrocarbonoclastic organisms used in this study have been isolated from a semicontinuous batch reactor where a stable population dynamics for 60% crude oil degradation was achieved by enrichment of sludge and contaminated soil. The isolates were characterized at biochemical and molecular levels through a three tier screening strategy including drug resistance and hydrocarbon utilization pattern. Three isolates were selected for designing a consortium along with a procured strain of *P. putida* MTCC*102. Consortium may be defined as synchronous population of different microbes complementing desired metabolic potential of each other where the role of each member is well designated. The consortium for crude oil degradation includes NCC.DSS$_6$ (biosurfactant producer), NCC.DSS$_8$ (aliphatic hydrocarbon degrader), NCC.GSS$_3$ (aromatic hydrocarbon degrader) and *P.putida* MTCC*102 (known for intermediate consumption). The composition of the consortium or the selection of isolates was based on the need of desired metabolic traits. For effective crude oil degradation, emulsification is an essential prerequisite, therefore, NCC.DSS$_6$ was included which produced rhamnolipid (a biosurfactant) and emulsified crude oil making its components available to aliphatic and aromatic degraders (NCC.DSS$_8$ & NCC.GSS$_3$). The role of *P.putida* MTCC*102 is consumption of downstream metabolites produced during the degradation process so that the population dynamics should not shift in favour of the metabolites. The consortium members were further characterized at genetic level by polymerase chain reaction using oligoprimers for known catabolic determinants (*alkB, dmpN, xylE*). Isolates were also screened for presence of plasmids and NCC.DSS$_6$, the biosurfactant producer, was found to be harboring a 20kb plasmid.

The efficacy of consortium for crude oil degradation was tested by gas chromatography and chemical oxygen demand analysis. Biodegradation involves chemical changes in the parent

molecule, in this case petroleum hydrocarbons, usually accompanied by a reduction in COD, but the products of biodegradation are not necessarily simple or harmless. However, mineralization implies a complete recycling of petroleum hydrocarbons to harmless inorganic end products (CO_2 and H_2O for hydrocarbons). COD of degraded samples in this study, was measured 60% less than the control which indicates the extent of mineralization while gas chromatographic data imply biodegradation *in toto*. With model petroleum, observed degradation was more pronounced as proved by capillary gas chromatography. The reduction of lag period in degradation process is due to the adaptation of consortium members on catechol, a key intermediate in aromatic degradation. Three metabolites viz., salicylate, benzoate and catechol were tested and it was observed that catechol played an important role in activating oil degradation. The results emphasize that immense potential for microorganisms does not depend solely on the wealth of catabolic enzymes that they possess, but also upon their capacity for adaptive change. Such a capacity is promoted by their inherent patterns of regulation, which allow for the coincidental induction of different catabolic pathways, and this in turn allows for a positive selection to novel patterns of biodegradation.

Bioavailability of crude oil components is an essential pre-requisite for effective crude oil degradation, which was addressed by the biosurfactant produced by NCC.DSS6, identified as rhamnolipid by infrared spectroscopy in this study. The biosurfactant producing capacity of NCC.DSS$_6$ was found to be encoded on a 20kb plasmid, as the curing of the plasmid resulted in the loss of surface tension reduction in cell free broth. Mobilization of same plasmid by electroporation into cured NCC.DSS$_6$ brought back the surface tension reduction capacity in cell free broth. However, *P.putida* MTCC*102 did not reduce surface tension when imparted with the same plasmid, which indicates that the biosurfactant synthesis operon in NCC.DSS$_6$ is shared by chromosome and the plasmid. There is a need to investigate the genetic determinants that lie on the plasmid for enhanced production of biosurfactant.

One of the major constraints in conducting bioremediation program in marine condition is the hypersalinity. Exposure of the cells to high osmotic pressure results in the rapid efflux of intracellular water, which leads to a decrease in cell volume and turgor pressure. The consortium members were imparted with the phenotype of osmotolerance by transforming the organisms with a plasmid construct containing *E.coli* Pro'U' (in pMMB206, a broad host range vector) operon against *tac* promoter. The additional phenotype of osmotolerance did not hamper the dissimilatory capacity of consortium in hypersaline medium as proved by capillary gas chromatography with a simulated model petroleum mixture. The transformed consortium was applied successfully in saline. The formulated consortium was also applied to remediate a weathered petroleum refinery soil contaminated with PAHs (identified by GC-MS), where the bioavailability was the major limitation. This was addressed by the biosurfactant producing strain NCC.DSS$_6$ which resulted in a controlled release of PAH below toxic level to consortium by synthesizing biosurfactant at the cost of hydrocarbons. The addition of synthetic detergent Tween-80 resulted in the immediate release of PAHs thereby inhibiting the degradation which was evident in gas chromatographs. 30-40% degradation was observed with four-member consortium.

This developed understanding in crude oil biodegradation provides valuable supplementary information for bioaugmentation under programmed and forced bioremediation scenarios. Where the concept of consortium widens the metabolic horizons for better degradation at species level, adaptation of individual organisms provides broad specificity or enzymatic flexibility to accommodate compounds of similar structures which are abundant in crude oil. Thus this study projects seeding of specialized hydrocarbon degrader strains as an effective tool for bioremediation purposes. Not only to combat marine oil spills, but the process could also be exploited to treat contaminated soil and refinery wastewater.

6.1 Future Research Needs

This study proposes seeding of allochthonous bacterial consortium to consume spilled oil. Before the release of such organisms in field, a reliable detection methodology or tracking protocol should be developed. The characterization of consortium members on the basis of certain catabolic traits (*xylE, dmpN, alkB*) using PCR could be exploited to develop a sensitive enumeration technique. Another interesting finding in this study is the reduction of lag phase required for degradation by cross-acclimation. Though catechol was found to be the best inducer, there is a need to study the level of ring cleavage enzymes (oxygenases) in the seed culture stage. The future studies should be directed towards the catabolic capacity of the population in microcosm with reference to balanced maintenance of different members and their degradative potential. The additional carbon source as an inducer, inhibitor or even in some instances to maintain the degradative population will play a key role in bioaugmentation programme. The system like crude oil, a multisubstrate option for bacteria, demands a strong regulatory decision making in cometabolism to sustain dynamically variable carbon stresses. These studies in cometabolism with individual substrate will generate the knowledge base for oxidative potential in bacterial systems and its operation in favour of different oxygenases with minimum or no lag phase in adapted conditions.
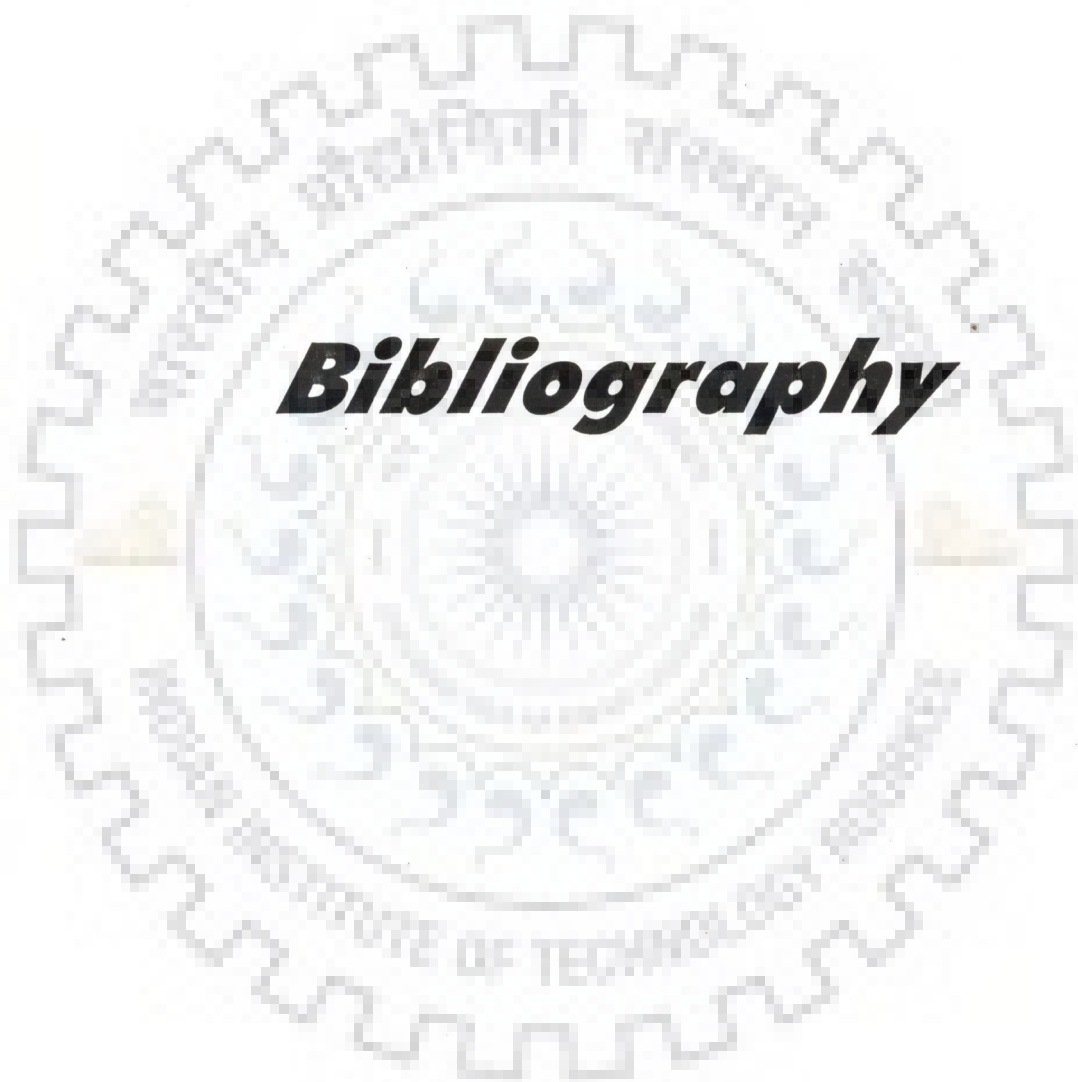
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