Assessment of genetic diversity and petroleum hydrocarbon degrading ability of soil borne *Pseudomonas*

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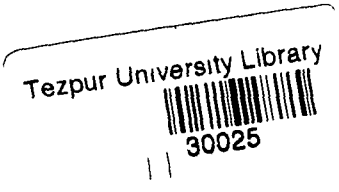

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CERTIFICATE BY THE SUPERVISOR

This is to certify that **Mr. Bhaben Tanti,** M.Sc. has carried out the work presented in the thesis entitled "Assessment of genetic diversity and petroleum hydrocarbon degrading ability of soil borne *Pseudomonas*" under my supervision and guidance. He has fulfilled the requirements of the regulations relating to the nature and prescribed period of research at Tezpur University. The thesis embodied accounts for his own findings and these have not been submitted previously anywhere for the award of any degree whatsoever either by him or anyone else.

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(Bhaben Tanti)

Place: Tezpur
Date: 30.05.2008

Declaration

I hereby declare that the subject matter of this thesis entitled "Assessment of genetic diversity and petroleum hydrocarbon degrading ability of soil borne *Pseudomonas*" is the record of work done by me, that the contents of this thesis did not form basis of any previous degree to me or to the best of my knowledge to anybody else, and that the thesis has not been submitted by me for any research degree in any other University/Institute.

This is being submitted to the Tezpur University for the award of the degree of Doctor of Philosophy in Molecular Biology and Biotechnology.

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Place: Tezpur

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List of Abbreviations

A	adenine
BSA	bovine serum albumin
C	cytosine
Cfu	colony forming unit
dATP	deoxyadenosine 5'-triphosphate
dCTP	deoxycytidine 5'-triphosphate
dGTP	deoxyguanosine 5'-triphosphate
DNA	de-oxy ribo-nucleic acid
dNTP	deoxynucleoside 5'-triphosphate
dTTP	deoxythymidine 5'-triphosphate
EDTA	ethylene diamine tetra acetic acid
EtBr	ethydium bromide
g	gram
G	guanine
GC-FID	gas Chromatography - Flame ion detector
h	hour
IR	infra-Red Spectrophotometer
ISR	intergenic spacer region
l	litre
lb	pound
mg	milligram
min	minutes
mM	milimolar
MSM	mineral salt medium
MWM	molecular weight marker
N	normal

NA	nutrient agar
NaCl	*sodium chloride*
NB	nutrient broth
nm	nanometer
O. D.	optical density
P.	*Pseudomonas*
PBS	phosphate buffer saline
PCR	polymerase chain reaction
PM	pico-mole
rDNA	ribosomal DNA
RFLP	restriction fragment length polymorphism
rpm	revolution per minute
rRNA	*ribosomal ribose nucleic acid*
SDS	sodium dodecyl sulphate
T	thiamine
T_m	*melting point*
Tris	tris(hydroxymethyl)aminomethane
UV/Vis	ultar violet/visible

Contents

Chapter I

Introduction

1.1. Microbial life in the sub- surface terrestrial environment

Microorganisms are present in a wide range of terrestrial subsurface environments, but are limited primarily by the availability of energy sources, pore space, and water. They obtain energy for growth and maintenance *via oxidation from rock-associated organic matter (heterotrophy), or from reduced* inorganic substrates such as H_2, CH_4, or S^{2-} (lithoautotrophy). The metabolic reactions involved in these processes include oxidation of organic matter to CO_2 and reduction of Fe(III) or Mn(IV) to Fe(II) or Mn(II), and of SO_4^{2-} to S^{2-}. Thus, such processes impart major changes in the geochemistry of the subsurface environments (Stevens and McKinley, 1995). Microbial populations in the terrestrial subsurface are generally characterized as being dispersed or present at relatively low population densities, heterogeneously distributed and with very low metabolic activities, even in comparison to extremely oligotrophic environments. There are, of course, exceptions to these situations where natural deposits of petroleum hydrocarbons or high concentrations of H_2 or CH_4 outgasing from the mantle or produced *in situ via* abiotic reactions in the earth's crust alter the environment. (Pedersen, 1998).

In spite of recent advances in scientific understanding of the microbiology of the terrestrial subsurface and of the biogeochemical processes, our knowledge about the details of the dynamics of the soil subsurface microbial community is not complete. Advantages offered by the robust molecular technologies and the powerful analytical approaches of geo and biochemistry have set the trend of new scientific investigations in the field of subsurface microbiology (Fredrickson and Fletcher, 2001).

Based on the constraints to microbial life in all environments and the understanding of the types of factors that have influences in the soil subsurface, estimates can be made for the key environmental parameters that are most likely to limit microbial life in the subsurface soil. Temperature, pressure, water availability, radiation, and available space are the primary physical conditions that determine distribution of microorganisms in subsurface environments (Brockman *et. al.,* 1995). The chemical constraints of an environment for survival of microbes depend upon concentrations of required electron donors, terminal electron acceptors, and micronutrients as well as the presence of toxic substances (Kieft, *et. al.,* 1993; Fredrickson *et. al.,* 1995).

Research on the microbial community structure in specific subsurface environments would lead to new findings related to microbial diversity and their evolution. Focus on understanding the distribution of microorganisms would provide direct information on where these organisms prefer to live with respect to the availability of various minerals in their habitat (Holman et al. 1995). A thorough understanding of the microbial community structure and their function is a crucial prerequisite for developing strategies in bioremediation of contaminated habitats.

1.2. Petroleum hydrocarbon degrading microbes

Petroleum hydrocarbon rich soil has a unique type of environment largely due to the presence of a variety of aliphatic and aromatic hydrocarbons creating a distinctive habitat for certain microbial communities. Petroleum is a complex oily mixture of aliphatic as well as aromatic hydrocarbons in their crude forms (Atlas, 1981). Members of microbial communities thriving in this kind of habitat have the potential to degrade and use some of the hydrocarbon compounds as the sources of carbon and energy. As hydrocarbons are

natural products as well as pollutants, it is not surprising that hydrocarbon-oxidizing bacteria are widely distributed in nature. Hydrocarbon oxidizers are ubiquitous, although with large variations in cell concentration (Balows, 1985). The varieties of hydrocarbon oxidizing bacteria in a particular ecosystem may change according to the time of sampling or the extent of oil pollution. Nutritional factors like availability of utilizable sources of nitrogen, phosphorus, the nature of hydrocarbon substrates and their effective concentrations as well as the presence of toxic substances in the petroleum product or in the environment influence the growth of hydrocarbon utilizing bacteria (Lovley and Chapelle, 1995). The location of hydrocarbon-oxidizing bacteria in natural environments has received considerable attention because of the possibility of utilizing their biodegradation potential in the treatment of the oil-contaminated sites. Compounds that are most susceptible to microbial metabolism occur naturally and have a simple molecular structure, are water-soluble, exhibit no sorptive tendencies, are non-toxic, and serve as a growth substrates for aerobic or anaerobic microorganisms (Balkwill, 1989). In contrast, those that are resistant to microbial metabolism exhibit properties such as a complex molecular structure, low water solubility, strong sorptive interactions, toxicity and which do not support the growth of microorganisms (Spain and Veld, 1983). Natural soil bacteria may be present in a dominant or slow-growing state, but when stimulated by optimum environmental conditions, they multiply rapidly and subsequently adapt to the new environment. Some of the common genera involved in biodegradation of oil products include *Nocardia, Pseudomonas, Acinetobacter, Flavobacterium, Micrococus, Arthrobacter, and Corynebacterium* (Atlas, 1977).

A common Gram-negative group of soil bacteria *Pseudomonas* in hydrocarbon rich soil has been well characterized (Chakrabarty, A. M. 1974). The genetic diversity amongst this important genus may be used to select suitable strains for construction of consortia for use in bioremediation of

habitats damaged during oil exploration, transportation or by accidental oil spills (Arnold *et. al.,* 1996)

1.3. Pollution by petroleum hydrocarbon

India's 15 oil refineries generate a huge amount of oily sludge, annually. The cumulative sludge, generated over the decades of existence of these refineries, is life threatening in its ecological impact. It takes years for even a few hundred tones of waste to degenerate naturally (Rosenberg *et. al.,* 1992). Moreover, this waste is supposed to be dumped in identified locations in secured pits. In the US and Europe these pits are provided with a leachate collection system and a polymer lining to prevent underground water contamination. However, the oil refineries in India do not find it a viable proposition to construct such storage pits. Moreover, storing the waste is not a sustainable approach to manage oily sludge, since it exposes the local habitats to dangerous levels of toxicity through air and water pollution (Churchill *et. al.,* 1995).

Besides the sludge from oil refineries, crude oil spills too are a cause of environmental degradation. Oil spills at port terminals are a frequent phenomenon, which invariably go unreported in the media. The Annual Report (1999) of the National Oil Spill Disaster Contingency Plan (India) has reported major oil spills at the port terminals of Vadinar, Kandla and Haldia amounting to 16 000 MT, 4000 MT and 5000 MT, respectively. Oil spills are common during oil explorations at the oil well drilling sites. Oil spills also occur at the oil collection centers, where oil is separated from water. Scientists are battling to come up with efficient and economical solutions to combat contamination of land and water caused by oil sludge and crude oil spills. All the emerging solutions indicate towards the use of natural (biological) processes to tackle the accompanying ecological threats.

1.4. Bioremediation of petroleum hydrocarbon polluted habitats

An understanding of how these toxigenic and mutagenic pollutants exert influences on microbial survival in the subsurface is of practical importance (Wang and Bartha, 1990). The success of *in situ* bioremediation processes generally hinges on the effectiveness of microorganisms whose physiological activity is required to accomplish the desired clean up task. Bioremediation is a technology that offers great promise in converting the toxigenic compounds to non-toxic products without further disruption to the local environment (Hutchins *et. al.*, 1998). Bioremediation is a popular approach of cleaning up petroleum hydrocarbons because it is simple to maintain, applicable over large areas, cost-effective and leads to the complete destruction of the contaminant. Strategies for inexpensive and clean *in situ* bioremediation of soil contaminated with crude oil include stimulation of the indigenous microorganisms by introducing nutrients and oxygen into the soil (Biostimulation) or through inoculation of an enriched mixed microbial consortium into soil (Bioaugmentation) (Wackett and Hershberger, 2001). Biostimulation is based on the assumption that, since microbes are ubiquitous, the indigenous microbes at the site will take care of the pollution and all that is necessary is the addition of fertilizers and nutrients to speed up the growth of that indigenous microbial population (Ogunseitan, 1996). Bioaugmentation on the other hand is a concept stemming from the fact that it is a way to clean up the pollution by inoculating the site with a consortium of specific targeted microbes in high densities. In both techniques, the environment must be carefully controlled and monitored for optimal microbial growth (Forsyth *et. al.*, 1995).

1.5. Problems due to high wax content of oil

Crude oil of the North - East regions of India contains about 11-18.8% of waxy substances (IOC Ltd., QC: TR/F-034, Repot No. 101.4/10, 2004) mainly paraffin wax of C_{18} to C_{30}. This huge amount of waxy substance creates considerable problems to the oil industry with respect to the followings:

- Paraffin wax deposits adjacent to production wells greatly reduce productivity by plugging fluid flow channels.
- Deposition of waxy crude oil also takes place in the perforations and production tubing effecting crude oil productivity.
- Deposition of waxy substances inside the pipeline is also a major problem that occurs during transportation of crude oil from drilling station to oil refinery.

A number of strategies based on mechanical and chemical approaches are being followed to overcome these problems arising out of the occurrences of wax in higher proportions in the crude oil. Some of these approaches are- removal of the waxy deposits from the production tubing by scrapping; hot oil washing to dissolve paraffin waxes on the perforations; washing with organic solvents such as xylene or toluene to remove paraffins from perforations and the formations; addition of amines to these solvents can aid solubilization of asphaltene deposits. These modes of removal of paraffin wax are very costly and at the same time these often create environmental pollutions (Concawe Report, 1999). Alternative strategies based primarily on the degradative properties of microbes offer more feasible options to address these issues. Understanding of the biochemical degradative pathways in the microbes is crucial for developing such strategies for bioremediation of systems impaired by the deposition of compounds like waxes.

1.6. Microbial community structure and bioremediation strategies

There is a strong reason to believe that proper understanding about the specific microbial population or community structure may be exploited for developing consortia for bioremediation of oil contaminated habitats and systems in an effective, rapid and inexpensive manner. Extensive as well as intensive studies on the microbial biodiversity of the oil rich habitats are prerequisites for exploring such possibilities. The widespread occurrence of hydrocarbon degrading bacteria in the petroleum oil contaminated sites and in the crude oil rich areas have focused interest on detection and differentiation of microbial strains based on phenotypic as well as molecular typing methods. Microbiological techniques and various molecular tools are generally employed for assessment of microbial diversity. Identification of bacteria with potentials to cause degradation of recalcitrant contaminating hydrocarbons therefore not only requires extensive microbiological studies with focus on elucidation of the degradative pathways, but also requires tools for their rapid detection and discrimination (Walker and Colwell, 1976; Van Hamme, *et. al.*, 2000).

Pseudomonas species have been the most intensively investigated bacteria owing to their ability to degrade many different contaminants. A large number of *Pseudomonas* species has been isolated which are capable of utilizing petroleum hydrocarbons. The diversity of *Pseudomonas* species in the petroliferous sub-surface soil of Assam *vis-à-vis* their petro-hydrocarbon degradation capabilities have not been reported as yet. So, there is a necessity for studying this important group of soil microbe for further exploration in constructing a consortium of bacteria for use in bioremediation of petroleum hydrocarbon contaminated habitat of Assam.

1.7. Biochemical and molecular approaches in microbial taxonomy

A phenotypic marker based approach that has been increasingly used in typing closely related bacterial genotypes is lectin typing. Lectins are proteins or glycoproteins, usually of plant origin but also of animal origin. These are non-immunoglobulin in nature, capable of specific recognition and reversible binding to carbohydrate moieties of complex glycol-conjugates without altering the covalent structure of any of the recognized glycosyl ligands. Lectins bind to sugar moieties in cell walls or membranes and thereby change the physiology of the membrane to cause agglutination other biochemical changes in the cell.

In Gram-negative bacteria, the outer membrane is made up of lipopolysaccharides (LPS) that contain many sugar residues. The LPS diversity among different bacterial isolates of the same as well as of different species is due to the presence of different sugar residues (Aspinall *et. al.,*1996). This diversity of LPS can be studied by lectin to differentiate bacteria belonging to the same genus or even species.

Traditional methods for the identification of some bacterial species can be time consuming and often necessitate the isolation of pure cultures before further characterization may be undertaken. Advances in molecular biology have allowed the identification of bacterial species by virtue of the unique nature of the genome of a species, often using methods based on the hybridization of the probes. Significant advances have been made in the use of molecular tools for tracing the phylogenetic relationship amongst allied bacterial species with great resolution. This has been facilitated by the finding that the sequences of highly conserved genes (most notably the ribosomal RNA (rRNA) genes), enable phylogenetic characterization of the microorganisms present in microbial communities (Pace *et al.*, 1997). This

was a boon to the field of bioremediation because it meant that by analysing rRNA sequences in contaminated environments, it was possible to determine definitively the phylogenetic placement of the microorganisms that are associated with bioremediation processes (Rogers *et al.*2003; Watanabe *et al.*, 2000). Coupling of the robust technology of polymerase chain reaction (PCR) with this molecular approach has revolutionized the entire field of microbial molecular taxonomy resulting in the emergence of related and very promising metagenomics approach (Handelsman *et. al.*, 2002).

PCR based techniques have enormous potential for detection of naturally occurring DNA polymorphism. Ribosomal RNA (rRNA) typing is a powerful tool for analysis of polymorphism at the genetic level among the microbes belonging to the same genus. Because of the pivotal role in translation, the rRNA genes are highly conserved in their structure and are functionally homologous in all organisms. In bacteria, the DNA encoding rRNA is arranged in an operon consisting of three genes, which represent 16S, 23S and 5S. These genes are separated by intergenic spacer regions (ISR), which exhibit a large degree of sequence and length variation at genus and species levels (Rodriguez-Valera, 2002). This hypervariable intergenic spacer region might be different among the same species. This polymorphism can be detected by scoring band presence versus absence in banding patterns that are generated by either restriction enzyme digestion or DNA amplification or both. However, all these methods for the detection of polymorphism in the DNA require relevant sequence information for the designing of appropriate primers or even the selection of suitable restriction endonucleases.

The proposed work envisages undertaking a comprehensive study of a common soil bacteria - *Pseudomonas* from the subsurface soil of certain petroleum polluted sites and oil fields of Assam with special emphasis on the

use of molecular techniques along with the conventional biochemical approaches. The objectives of the present study are presented below.

1.8. Aim and objectives

1. Isolation and identification of hydrocarbon degrading *Pseudomonas* species from oil fields and oil contaminated soil of Assam.
2. Assessment of the degradative potential of some of the selected *Pseudomonas* species with respect to degradation of paraffin wax.
3. Differentiation of *Pseudomonas* species using biochemical and molecular tools.
4. Establishment of the phylogenetic relatedness amongst the identified *Pseudomonas* species.

To achieve these objectives, the following approach shall be adopted:

1. Enrichment culture technique shall be used for isolation of hydrocarbon degrading bacteria from the environmental samples.
2. Various biochemical tests shall be done for identification of the *Pseudomonas* species.
3. Biodegradation of paraffin wax will be studied in the laboratory condition using FT-IR and GC analyses of the degraded products.
4. Differentiation of *Pseudomonas* strains into their different sublevels shall be done by using biochemical markers like lectins.
5. PCR based amplification of 16S-23S intergenic spacer regions shall be done.
6. Restriction digestion of the amplified products with appropriate restriction endonucleases shall be done for RFLP analyses.
7. Correlation among the *Pseudomonas* strains will be studied through construction of phylogenetic trees using statistical software.

Chapter II

Review of Literature

The integration of microbiology, geology, geochemistry and biotechnology has significantly enhanced our understanding of subsurface microorganisms and their role in biogeochemical processes (Anderson, 1997). Most early investigations of subsurface ecosystems relied heavily on cultivation techniques, enrichment cultures, and mesocosm studies using varied electron donors and acceptors to isolate, characterize, and identify subsurface microorganisms and metabolic processes (Albrechtsen and Winding, 1992; Amy, 1997; Balkwill, 1989; Bock et al.' 1994; Balkwill and Boone, 1997). Soil chemistry and the physico-chemical properties of the soil exert a deterministic influence in defining the soil microbial community structure. Amongst the myriad organic compounds present in the subsurface soil ecosystem, hydrocarbon compounds are not uncommon. Hydrocarbons are a ubiquitous class of natural compounds (Bossert and Bartha, 1984). Not only are they found in petroleum-polluted areas, but also significant quantities of aliphatic and aromatic hydrocarbons are found in most soils and sediments. Anthropogenic factors play a critical role in the contamination of soil with these compounds. Petroleum hydrocarbon being the only major source of energy utilized today, its exploration and refinement to various downstream products have been found to be responsible for serious ecological problems in various parts of the world including the North East India. These problems in general relate to habitat destruction caused by hydrocarbon pollution, pollution of agricultural fields (mainly, paddy fields), contamination of water bodies *etc.* There are also instances where serious operational problems in the oil industry itself arise due to the accumulation or deposition of some of the petroleum hydrocarbon compounds like wax inside the oil transporting conduits. The advent of biotechnology has opened up possibilities for employing microbes and soil microbial community structures for reclamation and restoration of the damaged

ecosystems as well as industrial operational systems. Bioremediation of polluted habitats has emerged as very cost- effective and ecologically sensible approach in the realm of environmental biotechnology.

2.1 Occurrence and composition of crude oil

Widespread occurrence of various hydrocarbons in varying proportions in soil has been known. The most probable origin of the low concentrations of widely distributed hydrocarbons in soil may be attributed to biosynthesis of these compounds by certain plants and microorganisms. Other sources of hydrocarbons may be traced to the natural seepage from the ocean floor and to the unburnt fuel oil. Crude oil is a major source of hydrocarbons. The complex mixture of compounds that comprises crude oil contains hundreds of thousands of hydrocarbons (Cooney, 1980; Cerniglia, 1984). These hydrocarbons are divided into three general classes – saturated hydrocarbons, aromatic hydrocarbons and polar organic compounds (Huesemann and Moore, 1993). Saturated hydrocarbons are further resolved into straight chain and branched alkane as well as cyclic alkanes with varying numbers of saturated rings and side chains (Bahl, *et. al.,* 1995). Aromatic hydrocarbons present in the crude oil contain one or more aromatic rings ranging from simple mono-aromatic compounds such as, benzene and toluene to poly aromatic compounds such as pyrene. The polar fraction comprises of compounds that contain polar heteroatoms like nitrogen, sulphur and oxygen (NSO compound) (Roberts, 1998). Refined oils and petroleum products are equally complex and contain many different hydrocarbon (Sharply, 1964).

2.2 Petroleum hydrocarbon contamination

Petroleum oil both in crude form and its various refined products cause pollution problem of varying magnitudes. Isolation, identification, biochemical and molecular characterization of bacteria having the genetic

capability to degrade such compounds are of great significance both from the academic as well as application points of view. Assam in the North-East region of India has rich petroleum hydrocarbon deposits on which the renowned oil industry has come up in the state. Pollution caused by petroleum hydrocarbons during the various processes of exploration, transportation, refining and downstream processing have resulted in considerable pollution of the soil and water. Environmental problems caused by petroleum hydrocarbons have emerged as a major concern for general public, government and the oil industries. The American Petroleum Institute (API) developed a petroleum decision framework to facilitate decision making for investigation and cleanup of petroleum contaminating soil and ground water (API, 1990). Economical and environmental considerations have compelled the petrochemical industries to reduce substantially the amount of hydrocarbon waste materials through modifications of the operational procedures (Milne *et. al.,* 1998). Polycyclic aromatic hydrocarbons (PAH) an important fraction of the petroleum hydrocarbons are considered among the major contaminants of soil and water because many of these compounds are reported to be cytotoxic, mutagenic and potential carcinogenic (Tsai *et. al.,* 2001). PAH contaminants are exceedingly recalcitrant to degradation due to their inhibitory nature and very low aqueous solubility (Wilson and Jones, 1993; Guieysse *et. al.,* 2001). There is a strong focus for developing suitable R & D solutions for this problem.

2.3 Distribution of hydrocarbon utilizing microorganisms in petroleum rich subsurface soil environment

Hydrocarbon-degrading bacteria and fungi are widely distributed in marine, freshwater and soil habitats. For microorganisms to biodegrade petroleum hydrocarbons, thousands of different compounds must be metabolized. Many different enzymes are presumably necessary to biodegrade the vast diversity of petroleum hydrocarbons. The distribution of microorganisms in

the subsurface soil depends upon their physiological capabilities and the stresses they encounter. Much of our understanding about the distribution of bacteria is based on laboratory studies, and only recently it has been realized that (a) many microorganisms cannot be successfully cultured in the laboratory (b) laboratory-based measurements of microbial activity can be orders of magnitude greater or smaller than *in situ* activities. The variations in bacterial populations isolated by enrichment culture depend largely on the hydrocarbon substrates used in the enrichment culture, the culture conditions, and the source of the inocula. Most hydrocarbon-utilizing bacteria in terrestrial environments belong to the genera *Pseudomonas, Achromobacter, Micrococcus, Acenetobacter, Acetobacter, Bacillus Flavobacterium, Corynebacterium, Nocardia, Arthrobacter, Candida, Rhodotorula* etc. Several thermophilic hydrocarbon-utilizing bacteria have been isolated, that include species of *Thermomicrobium* and others. Both Gram-negative and Gram-positive thermophilic bacteria have been found to be capable of utilizing hydrocarbon. Some isolated thermophiles are obligate hydrocarbon utilizers and cannot grow on other carbon sources (Merkel *et. al.*, 1978).

Based on the known constraints to microbial life in all environments and the understanding of the different factors that prevail in the subsurface, estimates can be made for the key environmental parameters that are most likely to limit microbial distribution in the subsurface. Certainly, at some depths within the earth, temperature is the lone factor limiting microbial survival. Therefore, the geothermal gradient establishes the innermost possible boundary to microbial distribution. The flux of electron acceptors, electron donors, other nutrients, waste products, and toxic compounds can also play critical roles effecting microbial life at subsurface environment. Knowledge about the other factors that control microbial distribution has been minimal.

Although a wide phylogenetic diversity of microorganisms exists in the petroleum rich environment (Anderson *et. al.*, 1997; Wackett *et. al.*, 2001),

Pseudomonas species have been the most intensively investigated bacteria owing to their ability to degrade many different contaminants. A large number of *Pseudomonas* species has been isolated which are capable of utilizing petroleum hydrocarbons. The genetics and enzymology of hydrocarbon degrading *Pseudomonas* species have been extensively studied (Chakrabarty *et. al.,* 1972, 1973; Dunn *et. al.,* 1973; Williams *et. al.,* 1978; Singer and Finnerty, 1984; Shapiro *et. al.,* 1984). Even, whole genome sequencing of *Pseudomonas putida* was also completed (Nelson, 2002).

2.4 Bioremediation

Bioremediation is defined as the process whereby organic wastes are biologically degraded under controlled conditions to an innocuous state, or to levels below concentration limits established by regulatory authorities (Mueller *et al.* 1996). The process involves the use of living organisms, primarily microorganisms, to degrade the environmental contaminants. The degradative potentials of naturally occurring bacteria and fungi or plants are exploited to detoxify substances hazardous to human health and/or the environment (Chhatre *et. al.,* 1996). The microorganisms may be indigenous to a contaminated area or they may be isolated from elsewhere and brought to the contaminated site. Biodegradation of a compound is often a result of the actions of multiple organisms.

There are two forms of bioremediation: the microbiological approach and the microbial ecology approach (Piotrowski, 1991). The microbiological approach involves supplying microorganisms that have been conditioned to degrade target compounds, along with appropriate nutrients, to the subsurface. These organisms could be developed through the recombinant DNA technology (prepackaged "superbugs"), which are strains engineered in the laboratory and transferred to the contaminated area, or they could be site-specific superbugs, which have been isolated

from the affected area itself and reintroduced at higher concentrations. This process often referred to as *bioaugmentation* has two major limitations namely, (a) nonindigenous cultures rarely compete well enough with an indigenous population to develop and sustain useful population levels and (b) most soils with long-term exposure to biodegradable waste have indigenous microorganisms that are effective degraders if the treatment unit is well managed (Mishra *et. al.*, 2001). The microbial ecology approach, also known as *biostimulation* on the other hand, involves altering the environment of the indigenous organisms to optimize their biodegradation of the contaminants. Although the microorganisms are present in contaminated soil, their population density is inadequate for bioremediation. The usually involves the addition of nutrients and oxygen at the contaminated sites in order to bring the population of the indigenous microorganisms to the effective level (Vidali, 2001).

Bioremediation can also be expressed as being engineered or intrinsic. Any modification of the bioremediation process is considered engineered bioremediation, and the lack of intervention is intrinsic bioremediation, or natural attenuation (Hart, 1996). Intrinsic remediation results from several natural processes, such as biodegradation, abiotic transformation, mechanical dispersion, sorption, and dilution that reduce contaminant concentrations in the environment (Morin, 1997). *In situ* bioremediation offers the possibility of a rapid and cost-effective solution to contamination of the environment by hazardous organic compounds (Catallo and Portier, 1992).

A major bioremediation project to decontaminate oil contaminated sites of ONGC, at Geleki and Rudrasagarh, CTF, Assam, India covering around 22,500 sq.m. area has been recently undertaken by The Energy Research Institute, New Delhi.

2.4.1 Biodegradation of petroleum hydrocarbon

Petroleum is an extremely complex mixture of hydrocarbons for which biodegradation of petroleum is an inherently slow process (Berry *et. al.*, 1987). Due to toxicity, molecular recalcitrance *e.g.*, polycyclic aromatic hydrocarbons, or the insolubility of petroleum hydrocarbons in water, soil microorganisms are not capable to mineralize these organic compounds (Neilson, 1990). For microorganisms to biodegrade petroleum hydrocarbons completely, thousands of different compounds must be metabolized. Many different enzymes are presumably required for biodegradation of these compounds (Alexander, 1980). The major biochemical reactions associated with the microbial metabolism of xenobiotics like petroleum hydrocarbons include acylation, alkylation, dealkylation, dehelogenation, amide or ester hydrolysis, oxidation, reduction, aromatic ring hydroxylation, ring cleavage and condensate or conjugate formation (Kaufman and Plimmer, 1972). Microbial action is initiated at metal, sulfur and other functional group sites, which subsequently proceeds to breakage of C-C bonds, even to biocracking of heavy petroleum (Premuzic *et. al.*, 1993). Bioremediation is being increasingly seen as an effective, environmentally benign treatment for soil and water contaminated as a result of oil spills (Benedik *et. al.*, 1998). Despite a relatively long history of research on oil-spill bioremediation, it remains an essentially empirical technology and many of the factors that control bioremediation have yet to be adequately understood (Bartha *et. al.*, 1986). A number of factors control the rates of biodegradation of petroleum hydrocarbons, including (a) the type of petroleum hydrocarbon mixture; (b) site physical and geochemical properties such as permeability, redox status, content of reduced minerals that can be oxidized by oxygen, and availability of microbial inorganic nutrients; (c) the length of time since contamination, which impacts the population density of degraders and the chemical form as bioavailability of the contaminant; and (d) toxicity from

high-petroleum hydrocarbon concentrations, partially or entirely filling pore spaces at many sites (Mulkins-Phillips and Stewart, 1974).

There is an exhaustive literature on biochemical pathways and the genetics of petroleum hydrocarbon biodegradation. Work with a variety of degradative pathways in *Pseudomonas* has shown that such functions can be encoded either on plasmids or on the chromosome. For example the ***tod*** gene for the degradation of toluene resides in the chromosome in *Pseudomonas putida* F1, whereas the ***nah*** genes responsible for naphthalene and salicylate degradation are found on plasmid **NAH7** (Cerniglia, 1984; Friello, *et. al.,* 1976). The **TOL** plasmid of *Pseudomonas putida* is the most extensively characterized catabolic plasmid; it encodes enzymes for the mineralization of toluene, m-xylene, m-ethyltoluene and 1, 3, 4-trimethyl benzene (Nelson, K. E. 2002). A novel *Pseudomonas anaerooleophile,* which is resistant to aliphatic, alicyclic and aromatic carbohydrates or a mixture thereof has been found capable of growing in a medium containing *n*-teradecane, toluene, cyclohexane and petroleum (Imanaka and Morikawa, 1993). Novotny (1992) characterized some thermophilic microorganisms that can metabolize recalcitrant substrates.

A database has been developed which provides rapid and reliable information on biodegradability of soil pollutants and on possible bioremedial action (Gleim *et. al.,* 1995). The database includes information on polycyclic aromatic hydrocarbons, helogenated compounds-their degradation in soil and use of pure and mixed cultures.

2.4.2 Role of oxygenase in biodegradation of hydrocarbons

Dioxygenases and monooxygenases are two of the primary enzymes employed by aerobic organisms during transformation and mineralization of petroleum hydrocarbons compounds. Incorporation of oxygen to the petroleum hydrocarbons during degradation requires the action of

oxygenase enzymes (Bausum and Taylor, 1986). The oxygenase required for the initial attack on hydrocarbons is typically a class of inducible enzyme, although induction is sometimes accomplished by molecules other than the substrates being oxidized (LePetit and Tagger, 1976). Inspite of the perplexing variety in structure and composition of a very great number of petroleum constituents, the number of different hydrocarbon oxygenating enzymes in bacteria is relatively small. One of the reasons for the relatively small number of oxygenases evolved by hydrocarbon degrading bacteria is because of broad substrate specificity *i.e.,* one enzyme can deal with more than one substrate, which is most unusual for enzymatic reactions. The vast degradation potential of microorganisms is because of the fact that microbes evolved a perplexing array of metabolic versatilities to convert most petroleum hydrocarbon oxidation products into substrates for their growth. Hydrocarbon oxidation can proceed in the absence of atmospheric oxygen.

2.4.3 Metabolic pathways of biodegradation of hydrocarbon

The general degradation pathway for an alkane involves sequential formation of an alcohol, an aldehyde, and a fatty acid. The fatty acid is then cleaved (decarboxylated), releasing CO_2 and forming a new fatty acid, which is two carbon units shorter than the parent molecule. This process is known as β-oxidation (Rehm and Reiff, 1981) (Fig.2.1). The initial enzymatic attack involves the class of enzymes called *monooxygenases.*

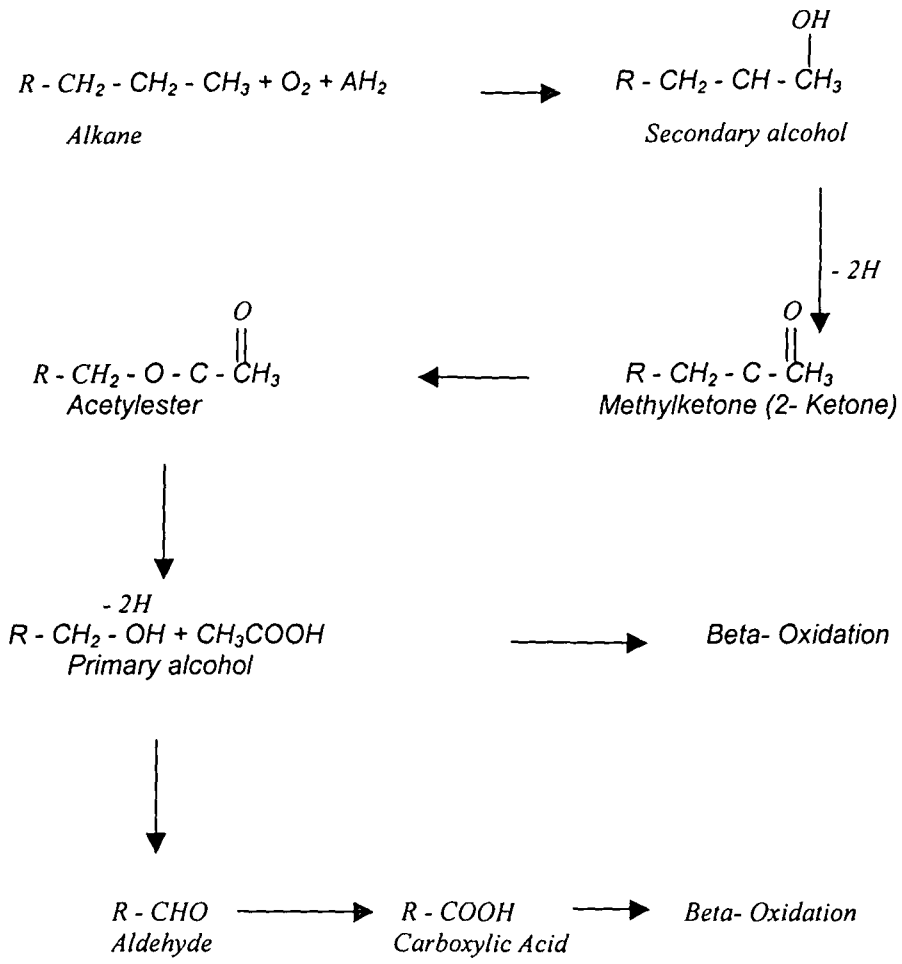


Fig.2.1 Activities of monooxygenase

Microorganisms have evolved catabolic enzyme systems for metabolism of naturally occurring aromatic compounds (Perry, 1977; Gibson, 1982). In the oxidation of aromatic hydrocarbons, oxygen is the key to the hydroxylation and fission of the aromatic ring. Bacteria incorporate two atoms of oxygen into the hydrocarbons to form dihydrodiol intermediates. The hydroxyl groups are *cis*-dihydrodiols. Oxidation of the dihydrodioles leads to the formation of catechols, which are substrates for enzymatic cleavage by oxygenases, forming a dicarboxylic acid, *e.g.,* muconic acid. In contrast, certain strains of fungi and higher organisms (eukaryotes) incorporate one atom of molecular oxygen into aromatic hydrocarbons to form aren oxides, which can undergo the enzymatic addition of water to yield *trans*-dihydrodiols. The class of enzyme involves in this process is *dioxygenase* (Foster, 1962) (Fig. 2.2).

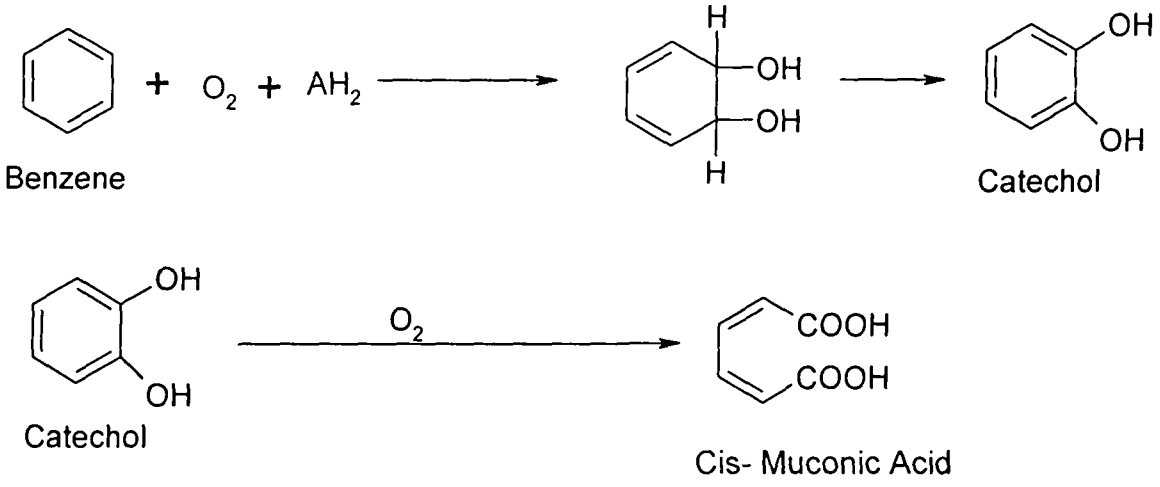


Fig.2.2. Activities of Catechol into *cis*- Muconic Acid

2.5 Paraffin wax and its biodegradation

Petrolatum waxes are complex mixtures of hydrocarbons (predominantly higher molecular weight alkanes), with carbon numbers in the range C_{12} to C_{85} (waxes) and C_{20} to C_{85} (petrolatums). The assimilation of paraffin waxes has been described for a diverse range of microorganisms following laboratory studies; a wide range of bacteria, yeasts and fungi have been shown to grow on paraffin wax using it as a sole carbon source under aerobic conditions. Initial decomposition of wax has been suggested to be similar to the degradation of short chain alkanes. Assimilation of a highly refined paraffin wax containing 91% of normal paraffins from C_{25} to C_{37} (mainly C_{28} to C_{32}) has been found in many species of bacteria and yeast (Yamada and Yogo, 1970). Yeast cultures grown on paraffin wax (Miller and Johnson, 1966) have resulted in typical cell yields of 70% although incomplete utilisation of paraffin wax occurred (Eyk *et al.*, 1968; Eyk *et. al.*, 1970).

One of the major aliphatic compounds present in crude oil is petroleum wax. Petroleum wax occurs in relatively higher proportions (11-18%) in the crude oil of Assam (IOC, 2004. Q. C. Laboratory, DR: QC: TR/F-034,

Repot No. 101.4/10). This is responsible for a number of operational and environmental problems for the oil industry. The wax present in the crude oil is deposited inside the oil pipelines forming a layer during the transportation of the crude oil from the drilling sites to the refineries. Formation of the layer of petroleum wax inside the oil transporting conduits, affect the transportation process.

Huge amounts of slack wax and petroleum tar (slack wax treated with acid) are generated in course of the crude oil refining processes. These result in considerable pollution of soil and water at the dumping sites. The problem is accentuated by the immobility of the slack wax and the tar and also by their insolubility in water (Concawe Report, 1999).

Biodegradation of wax assumes special significance in the context of its presence in Assam oil with relatively higher proportion, which is responsible for operational problems in the oil industries. No report on the biodegradation of wax using bacteria from the petroliferous soil of Assam is found.

2.6 Biochemical and molecular approaches in assessing the bacterial diversity in petroleum rich subsurface soil

Even though microorganisms are relatively much less conspicuous in natural environment, it is estimated that about half the biomass on earth is microbial (Whitman *et. al.,* 1998). Microbial life is widely distributed as microorganisms are found in all ecosystems. The long evolutionary history of microbial life has been the major driving factor in determining the vast diversity of microbial life (Woese, 1998). Amongst all microbes, bacteria are remarkable in their ability to live in all environments that are hospitable for life and to use the greatest array of energy sources and oxygen. Their early evolution and exploitation of chemical resources in diverse environment have played major roles in determining their unparalleled biodiversity (Staley, 2002).

There have been constant efforts to place bacteria in to useful groups where distinctions can be made. The Gram stain, biochemical tests *etc.,* are landmarks in this kind of efforts in making perfect assessment of the bacterial diversity and in defining the phylogenetic relationships amongst this diversity. The advent of molecular biology has enabled definitive classification of bacterial species based on their 16S rRNA sequence homology. Finer resolutions at the phenotypic and the genotypic levels have been achieved through the use of phenotypic methods such as the one that is based on differences of the lipopolysaccharides (LPS) in the bacterial cell wall.

2.6.1 Lectin typing of bacteria

Much of our understanding of bacterial interactions with subsurface soil is derived from laboratory studies. Many such investigations have attempted to identify the factors that control adhesion by manipulating and measuring variables relating to substratum characteristics, bacterial surface charge or hydrophobicity, or solution chemistry. A diversity of polymers and structures is involved in the adhesion of different bacterial types. Extracellular polysaccharides are produced by attached bacteria (Allison and Sutherland, 1987) and apparently strengthen their binding to surfaces and to other organisms. Lipopolysaccharide (LPS) structure apparently can influence adhesion of Pseudomonads in some cases. Alterations in length of the polysaccharide chain (or O-antigen) on the LPS can affect adhesion properties, either because it plays a primary role in adhesive interactions or it makes groups near the membrane, such as phosphate groups in the core-lipid A region (Makin and Beveridge, 1996) or outer membrane proteins (Arredondo et al., 1994). Because of the extreme complexity in bacterial surface chemistry and the difficulty in identifying specific polymers that act as adhesives, attempts have been made to characterize bacterial surface properties according to parameters that approximate gross characteristics, such as surface charge, hydrophobicity or surface free energy (Cisar, *et. al.,* 1995). For example, attachment to soil and sediment particles has been shown to be positively related to bacterial surface hydrophobicity. In this connection, the characterization of polysaccharide moieties in the bacterial cell wall is an important factor from the application point of view.

The LPS is found on the outer membrane of all Gram negative bacteria. Its molecular architecture has three regions, a lipid region anchoring the molecule of the surface, called Lipid A and a polysaccharide portion divided into a core region and a more variable O specific side chain which

is the most distal part of the molecule from the bacterium's surface. Microbial surfaces bear many of the sugar residues capable of interacting with lectins (Nikaido and Nakae, 1979). Carbohydrate moieties present on the bacterial cell envelop varies interspecifically or intraspecifically (Poxton, 1993). Indeed, any surface-exposed sugar is a potential lectin-binding site. The ability of lectins to react with microbial glycoconjugates means that it is possible to employ them as sorbents for whole cells, and this feature together with their extreme specificity makes then useful tools for differentiation of bacteria. The use of lectin is a simple, reproducible, and sensitive typing system that would be extremely valuable for bacterial typing.

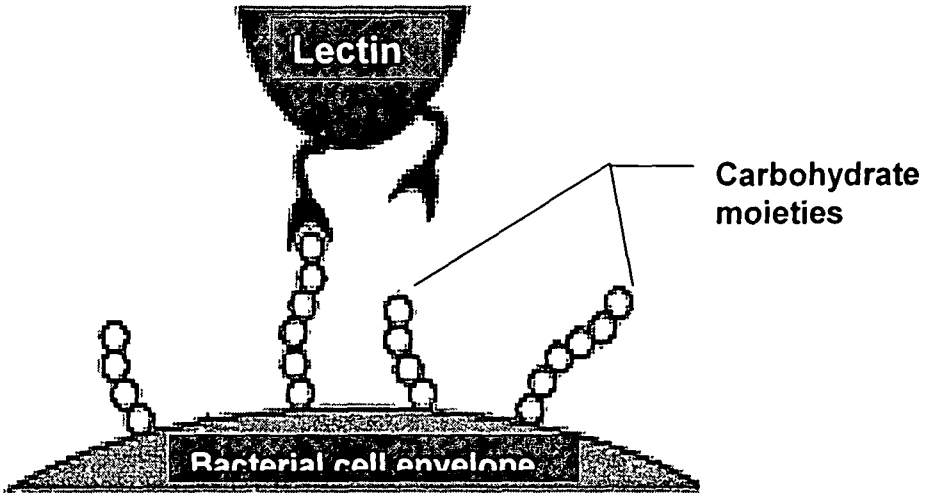


Fig. 2.3 A schematic representation of lectin-carbohydrate interaction

A lectin can be considered as a protein containing a key that fits into a certain type of lock. This lock is a specific type of carbohydrate. All life forms - plant and animal, insect and fungus have cell membranes that contain carbohydrates that remain within and project from the membrane. If a lectin with the right key comes in contact with one of those '*locks*' on the gut wall or artery or gland or organ it '*opens the lock*', *i.e.,* it disrupts the membrane and damages the cell and may initiate a cascade of immune and autoimmune events leading to cell death (Fig. 2.3).

Lectins were first described in 1888 by Stillmark working with castor bean extracts. Lectins are attractive reagents for the laboratory users because of their diverse specificity, their commercial availability, their high specific activities, their wide range of molecular weights, and their stability in standard buffers, and the fact that they can be readily freeze-dried for storage. Lectins can be reacted for use in genetics, failure of immune factors for protection, viral infection, bacterial infection or gut permeability induced by medication or infection. A numbers of lectins are commercially available in purified forms from various plants like soy, peanut and other beans, wheat germ and wheat, milk, peanut oil, nightshades etc. and also from animals as well as microorganisms. In lectin typing assay, a panel of lectins can be used based on their specificity for carbohydrates for differentiation among various bacteria inetrspecifically or intraspecifically.

Many members of the lectinic protein family also have been used to characterize agglutination of red blood cells (Matsubara *et. al.,* 1994). Typing of lectins in epidemiological studies has been found to be a most useful tool for discriminating strains of related species (Charland, 1995). Lectins have been used in typing schemes for numerous species including *Campylobacter spp.* (O' Sullivan *et. al.,* 1990; Wong *et. al.,* 1986; Fogg, *et. al.,* 1995), *Staphylococcus aureus* (Munoz *et. al.,* 1994; Munoz *et. al.,* 1999); *Streptococci spp* (Slifkin and Gil, 1983); *Bacillus spp.* (Cole *et. al.,* 1984) etc. Extensive work on 309 samples of *Helicobacter pylori* from 18

the hybridization with the probes. These methods, however, are time consuming. The development of the robust technique of Polymerase Chain Reaction (PCR) has accelerated the development of new approaches in bacterial identification and assessment of bacterial diversity (Saiki *et. al.,* 1988).

The rRNA genetic locus, *rrn*, is a genetic unit of broad evolutionary interest since it is found in both prokaryotic and eukaryotic organisms (Borneman *et. al.,* 1997). Ribosomal RNA is a particularly good marker for phylogenetic studies involving microorganisms for several reasons. First, the ribosomes, an integral part of protein synthesis containing rRNA are found universally in all organisms (Kiss, *et. al.* 1977). Second, the rRNA is highly conserved because large changes in sequence affect the normal functioning of the critical and complex process of protein synthesis. The concept of microbial diversity has been changed by the growth of available sequence data from ribosomal 16S rDNA (Barry *et. al.,* 1999). In fact, there is sufficient conservation within this locus for it to be used in a universal organization of evolutionary relationships (Cedergren *et. al.* 1993*)*. The utility of the rRNA sequence as a taxonomic tool has been demonstrated in bacteria, where 16S rRNA sequence analyses have completely redefined earlier phylogenetic relationships that had been dependent too much on cellular metabolism (Fox *et. al.* 1980; Woese *et. al.* 1987). In addition to highly conserved areas that have been used to study the relationships among distant taxa, the 16S sequence contains more variable regions, which are useful in the differentiation of genera and species. Several reports are available for use of direct PCR of 16S rRNA gene on *Pseudomonas* species (Widmer *et. al.*1998). However, within the rRNA genes there are regions of conserved sequence among species interspersed with regions of sequence variation (Weefs, *et. al.* 1990). The constant and variable region of the microorganisms can be compared to each other for homology and can be used to establish evolutionary relationships and draw phylogenetic trees (Fox, *et. al.* 1985, 1987).

The pioneering work of Woese (1990) has revolutionized our perceptions of biological evolution. The sequence data of 16S and 18S rRNA from a broad spectrum of organisms were utilized to construct a scientifically based tree of life Neefs, *et. al.,* 1990). The universal tree of life shows that three domains of life exist: Bacteria, Archaea and Eucarya (Woese *et. al.,* 1990).

2.6.3 Ribotyping of bacteria

A general strategy is one in which primers for PCR derived from the conserved regions of the 16SrRNA genes are used for their amplification and subsequent sequencing across the intervening variable regions (Eisenach, *et. al.,* 1988). Using this strategy, variable (V) regions can be amplified from any Eubacteriaum using universal primers form the neighboring conserved regions. Homology alignments to rRNA sequences in the Genebank and European Molecular Biology Laboratory (EMBL) databases can also be carried out using available sequence analysis software packages, which facilitate the construction of phylogenetic trees.

Another strategy involves the amplification of the variable intergenic spacer regions (ISRs) between the 16S and 23S genes, using primers from the constant 3' region of the 16S and the 5' region of the 23S genes respectively (Fig. 2.4A-C) (Graham, *et. al.,* 1997). The sizes of the spacer regions vary between genera and even species, although most species of the same genus have similar sized fragments. Because spacer regions have no known structural function, our rational is that these regions are under less selective pressure and ought to be more variable. This diversity is due to variations in the number and type of tRNA sequences found within the spacers (Li, 1997). Recently, it has been shown that length and sequence polymorphisms in the spacer regions within the *rrn* loci can be used to discriminate between different species of prokaryotes (Bintrim, 1997).

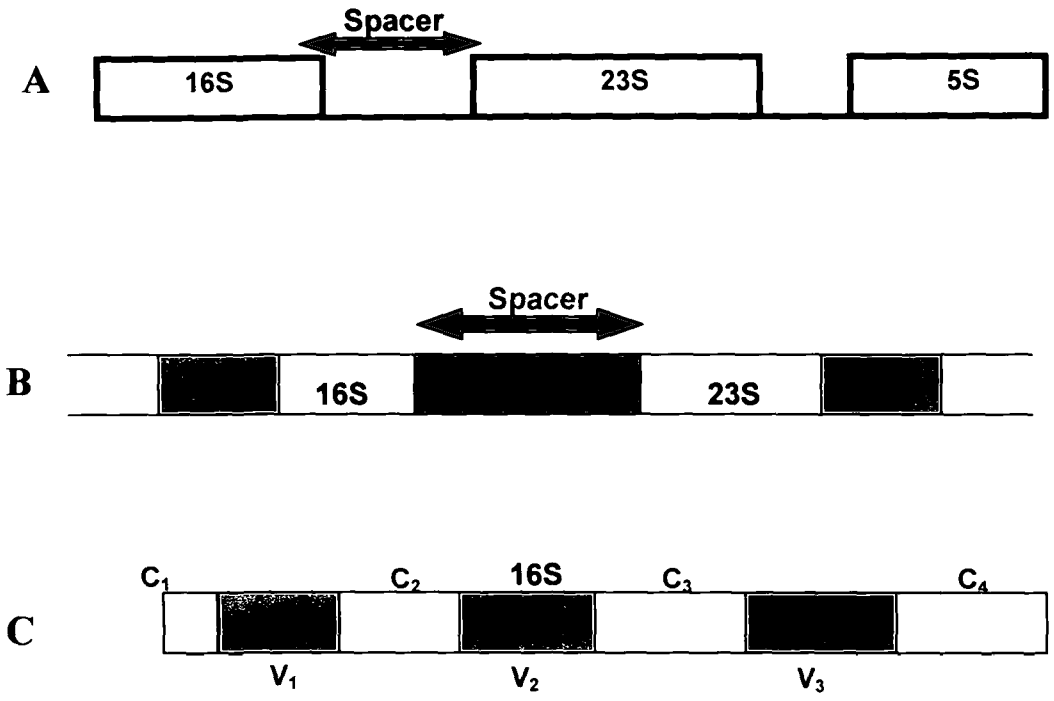


Fig.2.4 A. Schematic representation of a prokaryotic ribosomal operon, each rRNA operon consists of three genes, 16S, 23S and 5S genes respectively.

B. The position of the intergenic or spacer region in between 16S – 23S rRNA genes.

C. The 16S gene showing the conserved (C) and variable (V) regions.

Differentiation of *Xanthomonas* species based on analysis of the 16S-23S rDNA intergenic spacer sequences have been reported (Goncalves *et. al.* 2002). Differentiation of *Helicobacter pylori* strains was conducted directly from gastric biopsy specimens by PCR based restriction fragment length polymorphism analysis (.Li *et. al,* 1997). The most direct and certainly the most rapid method to visualize the polymorphic character of ISR is to carry out PCR amplification of the spacer regions by using primers from highly conserved flanking sequences Bruce *et. al.,* 1992). The length and sequence polymorphisms present in the PCR product can then be used for the recognition of genera and species. Additional information inherent in the polymorphic character of the amplified product is accessible by several means. The PCR product can be digested with specific restriction endonuclease (PCR-RFLP), and the resulting fragments can be resolved electrophoretically for study of their restriction length polymorphic patterns (Jensen, *et. al.,* 1993; Mishra *et. al.,* 2001). If the PCR product contains the restriction endonuclease recognition sequence at unique locations, then the resultant fragment size pattern can be indicative of a particular species (Pace, 1997). Restriction fragment length profiles obtained by the digestion of genomic DNA and PCR amplified products have helped in characterization of a number of organisms (Cancilla *et. al.,* 1992). There are several reports on the use of PCR-RFLP approaches for detection of DNA sequence diversity in a number of bacteria *e.g., Helicobacter pylori* (Akopyanz *et. al.,* 1992), Carnobacterium (Kabadjova *et. al.,* 2002). A higher level of resolution was obtained in discriminating 17 species of *Xanthomonas* using 16S-23S rDNA intergenic spacer sequences (ITS) (Goncalves *et. al.,* 2002).

2.7. Molecular phylogeny of bacteria

The recent exciting developments in biological evolution have come about through advances in molecular phylogeny and macromolecular sequencing. Until recently, it was not possible to scientifically evaluate the

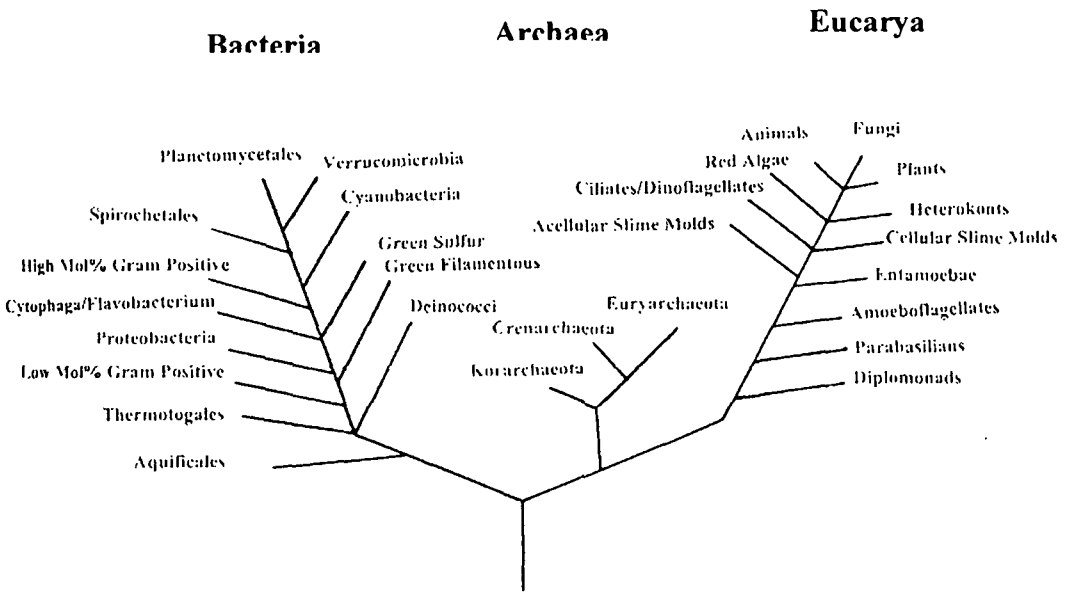


Fig. 2.5 The Universal Tree of Life showing the major phyla Bacteria, Archaea and Eucarya based on small subunit rRNA sequences (Woese *et. al.*, 1990)

evolution of bacteria and other microorganisms (Cedergren *et. al.*, 1993). Unlike plants and animals, these organisms are structurally too simple to enable their scant fossil record to be useful for identifying species and activities. However, it became apparent that the sequence of the subunits of macromolecules, such as proteins and nucleic acids, retained the evolutionary history of organisms (Woese, *et. al.*, 1985; Woese, 1987).

All life forms are related by descent to one another. Closely related organisms are descended from more recent common ancestors than are distantly related ones. The purposes of phylogenetic studies are (1) to reconstruct the correct genealogical ties between organisms and (2) to estimate the time of divergence between organisms since they last shared a common ancestor.

In phylogenetic studies, the evolutionary relationships among a group of organisms are illustrated by means of a phylogenetic tree. A phylogenetic tree is a graph composed of nodes and branches, in which only one branch connects any two adjacent nodes. The nodes represent the taxonomic units, and the branches define the relationships among the units in terms of descent and ancestry. The branching pattern of a tree is called the topology. The branch length usually represents the number of changes that have occurred in that branch. The taxonomic units represented by the nodes can be species, populations, individuals, or genes (Li. 1997). Nodes can be divided into external and internal nodes. The former represent the extant taxonomic units under comparison and latter as operational taxonomic units (OTUs). Internal nodes represent ancestral units.

Among the various methods that are available for reconstruction of phylogenetic trees like distance matrix methods, maximum parsimony methods, maximum likelihood methods and methods of invariants, unweighted pair-group method with arithmetic mean (UPGMA) is the most common method that have been extensively used because of its simplicity for tree reconstruction. UPGMA was originally developed for constructing taxonomic phenograms, *i.e.,* trees that reflact the phenotypic similarities between OTUs (Sokal and Michener, 1958). However, it can also be used to construct phylogenetic trees if the rates of evolution are approximately linear and there exists relation between evolutionary distance and divergence time (Nei, 1975).

2.7 Molecular approach to *Pseudomonas* taxonomy

Pseudomonads are a very large and important group of Gram-negative bacteria. Members of this group comprise a substantial proportion of the microflora of free-living saprophytes in soils, fresh water, marine environments, and many other natural habitats. They range from species

pathogenic to humans (*P. aeruginosa*) and plants (e.g., *P. syringae* and *P. solanacearum*), to species capable of degrading organic and recalcitrant compounds (*P. aeruginosa, P. cepacia* and *P. putida*), to those that promote agricultural growth (*P. fluorescence*). Recent developments in the use of rRNA homology groups and conserved features of biosynthetic pathway have made phylogeny-based classification possible. Most *Pseudomonas* species can be assigned to one of the five natural clusters called "RNA similarity groups" (Gupta *et. at*,1980) (Table-2.1)

Table-2.1 Species of *Pseudomonas* assigned to the various rRNA similarity Groups

rRNA similarity group	Species
I	*P. aeruginosa, P. fluorescence* (several biovars), *P. putida, P. chlororaphis, P. syringe* (many pathovars), *P. cichorii, P. stutzeri, P. mendocina, P. alcaligenes, P. pseudoalcaligenes, P. agarici, P. angulata, P. fragi, P. synxantha, P. taetrolens, P. mucidolens, P. oleovorans, P. resinovorans*
II	*P. cepacia, P. gladioli, P. caryophylli, P. pseudomallei, P. mallei, P. solanacearum, P. pickettii, P. pyrrocinia, P. andropogonis*
III	*P. acidovorans, P. testosterone, P. saccharophila, P. facilis, P. delafieldii, P. alboprecipitans, P. palleronii*
IV	*P. diminuta, P. vesicularis*
V	*P. geniculata, P. gardneri* and the members of *Xanthomonas*

Chapter III

Materials and Methods

3.1 Materials

3.1.1 Collection of environmental samples

Soil samples contaminated with crude petroleum oil were collected from various locations of oil fields, *viz.,* Borholla, Rudrasagarh, Lakowa, Galekey under Oil and Natural Gas Corporation Ltd. and Naharkotia, Jaipur, Digboi under Oil India Ltd. of Assam. Additionally, soil samples contaminated with slack wax and petroleum tar were also collected from Digboi Oil Refinery.

Soil samples were collected from 3″ - 4″ depths from the surface. These were brought to the laboratory in polythene bags and kept at 4°C.

3.1.2 Bacterial strain

Hydrocarbon degrading bacterial strain *Pseudomonas aeruginosa* MTCC1034 was obtained from Institute of Microbial Technology (IMTECH) Chandigarh, which was used as a reference strain in the lectin typing and PCR-RFLP analyses.

3.1.3 Chemicals and reagents

Components of Mineral Salt Media (MSM) and media for biochemical tests were procured from Hi Media Laboratory Pvt. Ltd., India. Hydrocarbons used in the study were procured from Merck, Germany. For comparing the GC analyses in terms of retention time, authentic standards of hydrocarbons were obtained from Sigma (USA). Lectins for lectin typing

assay and Proteinase K were obtained from Sigma (USA). Primers for PCR-RFLP of *Pseudomonas* isolates were purchased from Bioserve India Ltd. (Hyderabad, India). PCR kits for amplification of 16S-23S rRNA intergenic spacer region (ISRs) of *Pseudomonas* and marker DNA were obtained from Sigma-Aldrich India Ltd. (Bangalore, India). Restriction endonucleases were procured from Bangalore Genei, Bangalore and Boehringer Mannheim, (USA).

3.2 Isolation and preservation of hydrocarbon degrading bacteria

3.2.1 Isolation of bacteria by enrichment culture

One gm of each soil sample was added to 100 ml of mineral salt medium (MSM) with 2% (v/v) of each of n-hexadecane, benzene, and 2% (w/v) of paraffin wax, anthracene, naphthalene, pyrene, flourene, phenanthrene and carbazole separately as a sole source of carbon and incubated at 30^0C for 7 days with a shaking of 200 rpm. The composition of the MSM (Balows, 1985) was, (g/l) of NaCl 28.4; K_2HPO_4 4.7; KH_2PO_4 0.56; $MgSO_4$ 0.5; $CaCl_2$ 0.1; NH_4NO_3 2.5 and trace elements solution 1 ml/l containing (g/l) H_3BO_3 0.3, $CoCl_2$. $6H_2O$ 0.2, $ZnSO_4$ $7H_2O$ 0.1, $MnCl_2$. $4H_2O$ 0.03, Na_2MoO_4. H_2O 0.03, $NiCl_2$ $6H_2O$ 0.02, $CuSO_4$ $7H_2O$ 0.01, adjusted to pH 7.1. After 7 days, 1 ml inoculum was transferred to the same (fresh) medium and incubation was carried out for another 7 days under identical condition. The process was repeated three times and finally 1 ml inoculum from the third enrichment culture was appropriately serially diluted from which 100 µl was plated on nutrient agar plates. Isolates exhibiting distinct colonies and cellular morphologies were isolated by repeated subculturing onto NA plates. Isolated pure colonies were picked up and preserved in glycerol stock at -20° C Ultra Low Freezer (New Brunswick Scientific, USA).

3.2.2 Routine maintenance and preservation of microorganisms

Pure cultures of bacteria were preserved at 4^0C in nutrient agar slants and transferred to fresh slants at an interval of one month. Isolates were also stored in 15% (v/v) glycerol in nutrient broth and kept at -20^0C for long time storage.

3.3 Taxonomic identification of *Pseudomonas* species

The structure and morphology of the colonies, their pigmentation, staining reactions, physiological and biochemical characteristics were examined by standard methods as detailed bellow:

3.3.1 Morphological Characters And Staining Reactions

3.3.1.1 External structure of the colonies

The size, shape, colour, texture, surface nature, elevation, type of margin, consistency, translucency or opaqueness, rate of growth, pigment production (if any) were noted.

3.3.1.2 Motility

Motility of the organisms was noted by the "hanging drop" method (Bradbury, 1982).

3.3.1.3 Gram staining

Jenson's method modified by Cruickshank (1965) and recommended by Bradbury (1982) was followed for detection of the Gram positive and Gram negative nature of the organisms. Size and shape of the cells and their arrangement was also noted.

3.3.2 Physiological Characteristics

3.3.2.1 Optimum temperature for the growth

20 ml of freshly prepared bacterial suspensions in nutrient broth were taken in capped tubes and incubated at different temperatures (4°, 15°, 30° and 41° C) for 48 hours. The survival of the colonies at different temperatures was noted.

3.3.2.2 Optimum pH for the growth

The isolates were grown on nutrient agar (NA) or in nutrient broth at different pH (4, 5, 7, 8 and 9) and the growth was noted after 48 hours of incubation at 30° C.

3.3.3 Biochemical Characteristics

3.3.3.1 Catalase test (Holding and Collee, 1971)

The enzyme breaks hydrogen peroxide liberating oxygen and water.

$$H_2O_2 \rightarrow H_2O + \tfrac{1}{2}O_2$$

The test organisms were grown on Nutrient Agar tubes at 30° C for 48 hours. 1 ml of 30% H_2O_2 was poured over the culture. Formation of effervescence of gas bubbles indicates positive assay for catalase.

3.3.3.2 Methyl red and Voges – Proskauer (MR-VP) test

The methyl red test determines whether the production of acid from glucose lowers the pH and holds it at or near 4.2 or less. The Voges-Proskauer test determines whether the acid formed or MR-VP medium is converted into acetoin (or acetyl methyl carbinol) or not. In alkaline pH regime and under aerobic conditions acetoin is oxidised to diacetyl, which reacts with peptone to form a pink colour.

The above experiment was done by the method of Holding and Collee,(1971) (g/l) Glucose 5.0, K_2HPO_4 5.0 and Peptone 5.0 in which 8 ml of the MR-VP medium was inoculated with the bacterial culture and incubated at 30° C for 48 hours for observation.

3.3.3.2.1 Methyl red test

A few drops of methyl red (MR) reagent was added to 4 ml of the above medium. If the reagent turns red, it indicates positive reaction due to the accumulation of acidic products of glucose fermentation.

3.3.3.2.2 Voges-Proskauer test

The remaining 4 ml of culture was added to 5% Naphthol in absolute alcohol and to this was added 0.5 ml of the 40% KOH solution. The tubes were shaken thoroughly and allowed to stand for 5-30 minutes. The appearance of a pink red colour indicates positive reaction due to the formation of acetoin.

3.3.3.2.3 MR reagent (Methyl red indicator)

For this 0.1 g of methyl red was dissolved in 300 ml of 95% ethanol and the volume raised to 500 ml in a volumetric flask with distilled water.

3.3.3.3 Oxidative – Fermentation (OF) test (Hugh & Leifson, 1953)

Hayward (1964) modified the Hugh and Leifson's (1953) Oxidation – Fermentation (OF) test. One - percent separately sterilised D-glucose, L-arabinose, xylose and mannitol solution were added separately in aseptic condition to the modified Hugh and Leifson's medium (g/l) Peptone 2.0, Nacl 5.0, K_2HPO_4 0.2, Agar 3.0, carbohydrate (glucose, arabinose, xylose and mannitol) 1.0% and Bromothymol Blue (1%) 3.0 ml), pH adjusted to 7.1 ±0.5. A pair of tubes was stab inoculated by each organism. To one of the tubes was added sterile paraffin oil to provide anaerobic condition. If the test organism oxidises carbohydrate aerobically and produced acid, then the medium in the tube, which doesn't have the paraffin oil as the top layer, would show a yellow coloration from the top downwards. If the carbohydrate were fermented in anaerobic condition then both tubes would show the yellow colour.

3.3.3.4 Starch hydrolysis

The starch hydrolysis was examined according to the method described by Blazevic and Ederer (1975).The test organisms were spot inoculated in the media (g/l) Starch 20, $(NH_4)_2SO_4$ 1.0, Glucose 1.0, Nacl 1.0, $MgSO_4$ 0.8, KH_2PO_4 2.0, Sodium thiosulfate 1.0, Agar 15 and incubated at 30° C for 3 days. The plates were then flooded with Grams iodine. Development of a clear zone around growth against blue colour of the medium indicated hydrolysis of starch.

3.3.3.5 Gelatin hydrolysis

The method of Frazier (1962) was used for the assay of gelatinase. The media contains (g/l) Gelatin 40.0, Tryptone 17.0, Peptone 3.0, Nacl 5.0 and Dipotassium phosphate 2.5.

All the ingredients except gelatin were dissolved in 500 ml of distilled water. Gelatin was added to 400 ml distilled water separately and boiled. Both components were mixed and the volume was made up to 1000 ml. All the tubes containing the above media were inoculated and incubated for 4 to 7 days at 30° C. After incubation the tubes were cooled at 4° C for 15 minutes.

Tubes where the media remained liquefied produce gelatinase and showed positive test for gelatin hydrolysis and those tubes that remained solid demonstrated negative reaction for gelatin hydrolysis.

3.3.3.6 Casein hydrolysis

Casein hydrolysis test can be done by supplementing nutrient agar medium with milk. The medium is opaque due to the casein in colloidal suspension. Formation of a clear zone adjacent to the bacterial growth, after inoculation and incubation of agar plate cultures, is an evidence of casein hydrolysis. The skim milk agar media consists of g/l of skim milk powder 100.0, peptone 5.0, and agar 15.0, pH to be adjusted at 7.2.

3.3.3.7 Lipid hydrolysis

To ability of microorganisms to hydrolyze lipid is accomplished with an extracellular enzyme lipase triacylglycerol acylhydrolase. Lipase production test is based on the principle that lipolytic microorganisms will show a zone of lipolysis *i.e.,* a clear zone surrounding the bacterial growth when inoculated on nutrient agar medium supplemented with the lipid substrate such as triglyceride tributyrin. The loss of opacity of the agar medium is due to the hydrolytic reaction yielding soluble glycerol and fatty acids.

3.3.3.8 Reduction of nitrates

Test for reduction of nitrates was carried out by inoculation of bacteria in the medium containing (g/l) KNO_3 1.0, Beef extract 3.0 and Peptone 5.0.Tubes were incubated until good growth occurred. The presence of

nitrite was detected by adding 1 ml of Griess III Osvy's reagent [a mixture of (i) 2 ml HCl + 0.6 g sulfonilic acid + 70 ml distilled water = 100 ml, (ii) 0.6 g α-Napthylamine + 10 ml distilled water + 1 ml HCl = 100 ml with distilled water and (iii) 16.4 g Sodium acetate in 100 ml distilled water] into the cultures. Formation of the pink colour indicated nitrate positive.

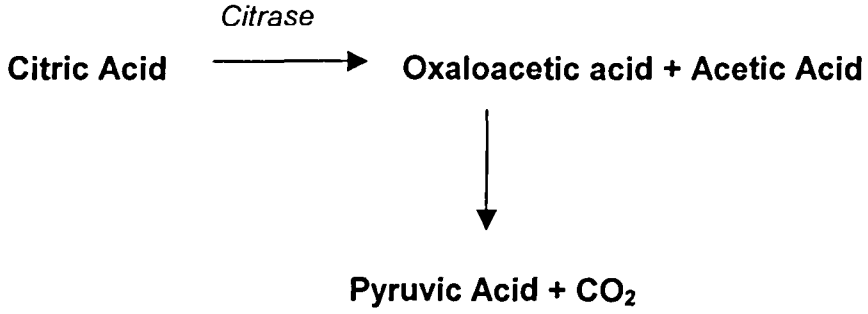
3.3.3.9 Indole production test

Kovac's method (1928) was followed for detecting indole production by the organisms. Tubes containing 5 ml of 1% aqueous solution of tryptophan in Nutrient broth were inoculated with the test organisms and incubated for 28-84 hours 30° C.

Then 0.5 ml of Kovac's reagent (10.0 g 0f para-dimethylaminobenzaldehyde +15 ml of isoamyl alcohol + 50 ml of conc. HCl) was added to each tube and mixed the appearance of a deep red colour within minutes indicated production of indole by the organisms.

3.3.3.10 Citrate production test

Citrate test is used to differentiate among enteric bacteria on the basis of their ability to utilise citrate as the sole carbon source. The utilisation of citrate depends on the presence of an enzyme *citrase* produced by the organism that breaks down the citrate to oxaloacetic acid and acetic acid. These products are later converted to pyruvic acid and carbon dioxide enzymatically as shown below:

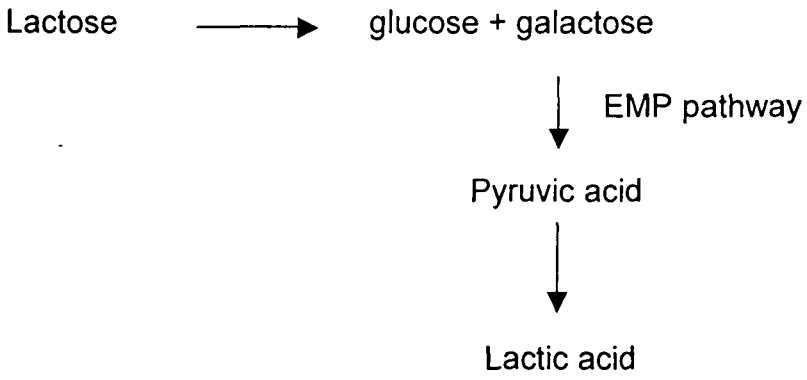


The citrate test is performed by inoculating the microorganisms into an organic synthesis medium, Simmon's citrate agar containing (g/l) Ammonium dihydrogen phosphate 1.0, Dipotasium phosphate 1.0, Sodium chloride 5.0, Sodium citrate 2.0, Magnesium sulphate 0.2, Agar 15.0, Bromothymol blue 0.8), where sodium citrate is the only source of carbon and energy. Bromothymol blue is used as an indicator. When the citric acid is metabolized, the CO_2 generated combines with sodium and water to form sodium carbonate an alkaline product, which changes the colour of the indicator from green to blue and this constitutes a positive test.

Brothymol blue is green when acidic (pH 6.8 and below) and blue when alkaline (pH 7.6 and higher).

3.3.3.11 Litmus milk reaction

Organisms capable of using lactose as a carbon source for energy production utilize the inducible enzyme b - galactosidase and degrade lactose as follows:



The presence of lactic acid is easily detected because litmus is purple at a neutral pH and turns pink when the medium is acidified to an approximate

pH 4.0. The media consists of (g/l) Skim milk powder 100.0 and litmus 0.075.

3.3.3.12 Oxidase test

Oxidase enzymes play a vital role in the operation of the electron transport system during aerobic respiration. Cytochrom oxidase catalyzes the oxidation of reduced cytochrome by molecular oxygen, resulting in the fermentation of H_2O or H_2O_2 The ability of bacteria to produce cytochrom oxidase can be determined by the addition of test reagent, *p*-aminodimethylanaline oxalate, to colonies grown on a plate medium. In this test *p*-aminodimethylaniline oxolate (Difco) was used.

After adding two or three drops of the *p*-aminodimethylanaline oxalate to the surface of the of each test organisms, if the color changes from pink to maroon and finally purple is the positive reaction and no change of color is the negative reaction.

The test for poly-b-hydroxybutyrate and other fluorescence and nonfluorescence pigments were conducted by following standard protocols.

3.3.3.13 G+ C content

Isolation of bacterial DNA was done following the protocol of Murmur, 1961 and determination of their G + C content were done as described by Mandel and Murmur, (1968).

3.3.3.14 Utilization of growth organic factors

Utilization of Trehalose, 2-Ketogluconate, *meso*-Inositol, Geraniol, L-Valine, L-Arginine, □-Alanine test and also organic growth factors like

Pantothenate, Biotine, Methionine, Cystine, Cyanocobalimin, Arginine dehydrolase were conducted by following the standard protocol. as described by Cappacino and Sherman (1999).

3.4 Biodegradation of paraffin wax

3.4.1 Screening of paraffin wax degrading bacteria

Paraffin wax degrading bacteria were selected based on their ability to utilize paraffin wax. Isolated pure cultures from the soil samples collected from slack wax and petroleum tar contaminated sites were grown in duplicate for thrice in mineral salt media supplemented with 1% paraffin wax as sole source of carbon and incubated at 30^0C for 72 hrs. The pure colonies were then selected for further studies.

3.4.2 Preparation of media and supply of paraffin wax

The ability of the bacteria for utilization of paraffin wax under laboratory condition was tested. The mineral salt medium (MSM) used throughout the study was that described by Balows, (1985). Biodegradation assays were carried out in 250-ml Ehrlenmeyer flasks containing 50 ml of MSM with 1% paraffin wax. Cells from enrichment cultures subcultured in Luria-Bertain medium (LB) were used as inocula. After overnight incubation, 1ml culture from the LB broth was washed with sterile water, which was used to inoculate the MSM containing 1% paraffin wax. Each paraffin wax degrading bacteria was inoculated separately. Mixed cultures were set up with all isolates in two sets with and without Tween-80 as synthetic surfactant respectively. Each inoculum contained approximately 10^7 cfu/ml bacterial cells. Uninoculated MSM containing paraffin wax was used as the control. Cultures were incubated for 30 days at 30° C with rotary shaking at 120 rpm.

3.4.3 Growth kinetics of paraffin wax degrading bacteria

Growth of bacteria in the cultures was measured by optical density in a UV-Vis Spectrophotometer (Hitachi, Model U-2001) at 595 nm at 24 hours intervals.

3.4.4 Extraction of residual paraffin wax from culture media

The residual paraffin wax from cultures and control were extracted with hexane. The extracts were dried in with Na_2SO_4 and concentrated in a rotary evaporator.

3.4.5 FT-IR analysis

The extracted residual paraffin wax was grounded and FT-IR spectra were recorded using KBr pellet in a Nicolet Impact 410 spectrophotometer in order to confirm the intermediate metabolites of paraffin wax degradation. The substrate under investigation should be absolutely dry as water absorbs strongly at about 3710 cm^{-1} and also near 1630 cm^{-1}.

3.4.6 GC Analysis

GC-MS analyses were performed with a Varian Saturn GC/MS (CP-3800/2200). Compounds were separated by CP-Sil 8 CB low bleed capillary column (30m X 0.25mm diameter); and helium as the carrier gas. The injector and detector were maintained at 300° C and the oven temperature was programmed to rise from 80° C to 240° C in 5° C/min increments. The fractions were determined by matching the retention time with authentic standards (Sigma, USA) and by determining area count of the chromatographic peaks for identification.

3.5 Lectin agglutination assay

3.5.1 Preparation of bacterial biomass

Bacterial biomass was harvested from nutrient agar plates in 0.01 M sodium phosphate buffer (pH 7.2), centrifuged at 3000 X g for 10 min and resuspended in 10 ml of PBS (Phosphate buffer saline, pH 7.2). Samples were washed once in PBS, resuspended in 5 ml of PBS (pH 4.0) and incubated at 20°C for 30 min to induce autolysis of the cells and protein release. Subsequently, treated cells were washed twice in PBS, resuspended in 5 ml of PBS containing 0.1 mg of Proteinase K per ml, incubated at 60°C for 1 hour and then heated to denature the enzyme at 100°C for 5 min. Each suspension was centrifuged at 5000 X g for 15 min and the resultant pellet of the cell debris was resuspended in PBS to an OD_{590} of 0.9.

3.5.2 Preparation of lectin solution

A panel of nine lectins (Table-3.1) (Sigma Aldrich Ltd.) was used in the lectin assay. Lectins were prepared by dissolving the lectins at a concentration of 0.5 mg/ml in PBS containing 0.02% $CaCl_2$ and 0.02% $MgCl_2$ following the protocol of Hynes *et al.*, (1999).

3.5.3 Lectin Typing

Bacterial samples (40 µl) were mixed with 10 µl of lectin solution in U-shaped microtiter wells or alternatively, with 10 µl of PBS as negative control and allowed to settle overnight, undisturbed, at room temperature. After overnight incubation of the lectins with bacterial biomasses at room temperature, reaction patterns were observed and scored. Results were

read by visual inspection and scored under microscope (Leica MPS 30). A positive reaction was evidenced by a carpet of aggregated cells at the bottom of the wells and a negative reaction was evidenced by a dot of cellular material at the bottom of the wells. Negative results were confirmed by tilting wells at an angle > 45° and observing the movement of cellular material.

Table 3.1 Panel of lectin used for lectin agglutination assay

Sl. No	Lectin	**Specific carbohydrate**
1	Lectins from *Helix pomatia* (HPA)	N-acetyl-α- galactosaminyl
2	Lectins from *Limulus* polyphemus (LPA)	N-acetylated D-hexosamines
3	Concanavalin A (ConA)	a-manose
4	Lectins from *Bandeiraea simplicifolia* (BSL)	α-D-galactosyl
5	Lectins from *Triticum vulgaris* (WGA)	N-acetyl -β-D glucosaminyl
6	Lectins from *Lens culinaris* (LcH)	α-glucose
7	Lectins from *Solanum tuberosum* (STA)	N-acetyl-β-D-glucosamine
8	Lectins from *Phaseolus vulgaris* (PHA)	α-D-glucosyl residues
9	Lectins from *Glycine max* (SBA)	β-N-acetylgalactosamine

3.5.4 Data analysis

The results of lectin typing were converted into binary matrices assigning 1 for positive and 0 for negative response in the agglutination reaction by lectins. Similarity matrices were constructed by using SPSS (Version-11.0, Lignam University) software followed by Jaccard similarity coefficient analysis. A dendrogram was constructed by using UPGMA (unweighted pair group method with average) and 100% similarity with respect to lectin reactions were clustered together.

3.6 Amplification of Intergenic Spacer Regions (ISR) of 16S-23S rRNA of *Pseudomonas*

Intergenic spacer regions (ISRs) of 16S-23S rRNA of 43 *Pseudomonas* species were subjected to amplification using Polymerase Chain Reaction. This is an *in vitro* method of generation of multiple copies of the DNA fragment of interest thus facilitating a detailed and better analysis of the same. PCR involves selective amplification of the region of interest, flanked by a primer on either side. The millions of copies generated are a result of repetitive cycles involving denaturation of the double stranded DNA template, primer annealing and elongation by thermostable *Taq* Polymerase.

3.6.1 Synthesis of primers for ISR amplification

PCR amplification was performed for the 16S-23S *rrn* operon of nuclear DNA (Fig.3.1). Oligonucleotide primers were designed to be complementary to conserved regions near the 3′ end of the 16S and 5′ end of the 23S regions of the ribosomal RNA operons of *Pseudomonas aeruginosa* based on a computer alignment of ribosomal RNA sequences

downloaded from GenBank (*Pseudomonas aeruginosa* numbering; **GenBank Accession Number PA0668.1**).

The ISRs were amplified with primers 16S/p1 and 23S/p2, which anneal to positions 1406 to 1426 of the 16S rRNA gene and to positions 84 to 104 of the 23S rRNA gene respectively. The resultant PCR products contained the complete 16S-23S ribosomal ISR and parts of the flanking rDNAs (ca. 130 bp of 16S rDNA and 104 bp of 23S rDNA) (Table 3.2).

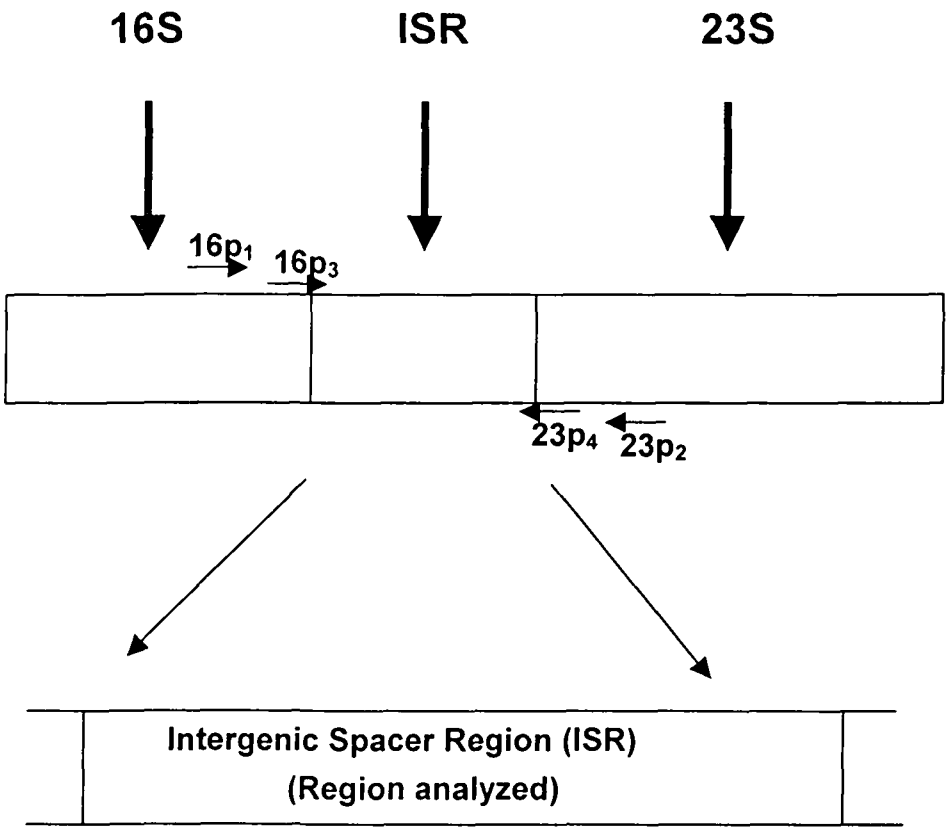


Fig.3.1 A schematic representation of 16S-23S rRNA ISR showing the positions of PCR and nested PCR primers.

3.6.2 Nested PCR

In order to confirm the specificity of 16S-23S ISR PCR amplification process, the PCR products were used as templates in a second PCR with primers 16S/p3 and 23S/p4, which anneal to positions 1498 to 1518 of the 16S rRNA gene and positions 76 to 56 of the 23S rRNA gene respectively. The DNA and parts of the flanking rDNAs (ca. 38bp of 16S rDNA and 76 bp of 23S rDNA). All the primers were designed by using Fast-PCR software (*www.biocenter.helsinki.fi/bi/bare-1_html/oligos.htm*). Primers were procured from Bioserve India Ltd, Hyderabad. The PCR products obtained after amplification were used for ISR-RFLP analysis.

Table-3.2 List of primers for PCR amplification

Serial No.	Lab. No.	Sequence
01	16S/p_1	5′ ATGGGAGTGGGTTGCTCCAG 3′
02	23S/p_2	5′ GGATCAAAGTCTGTTTGCCGAC 3′
03	16S/p_3	5′ GTAGCCGTAGGGGAACCTGC 3′
04	23S/p_4	5′ GCTTTTCGCAGGCTACCACG 3′

3.6.3 Reaction Mixtures for PCR amplification

The reaction mixture for PCR amplification was prepared under aseptic conditions under a UV hood. Prior to preparing the mix the hood was cleaned using 70% alcohol and then given a 20 minutes exposure to UV light. In thin walled PCR tubes a 25 µl mix was prepared for amplification. The reaction mix contained 2 µl of each of the primers from a 2.5 pM stock, 2.5 µl of 10X PCR buffer with $MgCl_2$, 2µl of 2.5 mM dNTP's mix, 0.5 µl of *Taq* Polymerase (3 units/µl) and the rest of the volume was made up with sterile deionised MQ water. Each amplification run contained a control with all ingredients of the PCR mix without template DNA.

Reaction set up (for 25 µl):

2.5 pM/µl Forward Primer	-	2.0 µl
2.5 pM/µl Backward Primer	-	2.0 µl
2.5 mM/µl dNTP's	-	2.0 µl
10XPCR buffer with $MgCl_2$	-	2.5 µl
(3 unit/µl) *Taq* Polymerase	-	0.5 µl
Deionised H_2O	-	16.0 µl

After PCR amplification, the products were run on a 1.0% agarose gel and the results were documented in a Gel-Doc System (Model 2000, BioRad, USA).

3.6.4 Template DNA for PCR amplification

A method was developed based on the protocol of H. Grunenwald (2000), where a pinch of cell from bacterial colony was added to the PCR reaction mixture directly using the fine end of a yellow tip in place of separately isolated DNA to avoid time and cost. As negative control, instead of bacterial cells, 1 µl MQ water was used in the reaction mixtures.

3.6.5 PCR programming

All PCR reactions were carried out in 25 µl volume contained 2.5 µl of 10X PCR buffer with $MgCl_2$, 2 µl of a mixture containing each dNTP's at a concentration of 2.5 mM, each primer at a final concentration of 5.0 pM and 1 U of *Taq* DNA polymerase.

The amplification was run for thirty cycles and each cycle comprised of 30 seconds of denaturation at 94^0 C, 30 seconds of annealing at 51^0 C, and a 2 minutes of extension step at 72^0 C. This was followed by an extension time of 5.00 minutes at 72^0 C at the end of the run. The first cycle was proceeded by incubation for 7 min at 95^0 C. After 30 cycles, there was a final 5 min extension at 72^0 C. The PCR amplifications were performed using a Perkin Elmer Thermal Cycler (GeneAmp PCR2400, Perkin Elmer, USA).

The annealing temperature for the different primer pairs varied. The annealing temperature was calculated using the following formula

$$\text{Annealing Temperature } (T_m) = [4\ (G+C) + 2\ (A+T)] - 4]$$

In cases where the annealing temperature of the two primers differed, amplification was done at the lower annealing temperature. However, this

was not a rule in all cases and the annealing temperature was modified, *i.e.,* decreased or increased depending upon the results obtained. In cases where a lot of non-specific amplification occurred, the stringency conditions were increased, by increasing the annealing temperature. Lower annealing temperatures were tried when it was difficult to get amplification.

3.6.6 Primer stock solution

The primers used for amplification were synthesised at M/S Bioserve India Ltd. (Hydarabad). In lyophilised condition, desalted and often PAGE purified primers were procured. These were then dissolved separately in sterile MQ water to make 2.5 pM/μl stock solution respectively. The stock solutions were stored in a deep freezer at -20^0C for future use.

3.6.7 Deoxyribonucleotide (dNTP) solutions

dNTP's were acquired from Bangalore Genei, India. Individual 10mM stock solutions of all the four dNTPs were obtained. For making 2.5 mM of working solution, equal volumes of all four dNTP's (10 mM stock) were added. This mixture had a final concentration of 2.5 mM of each dNTP's.

3.6.8 *Taq* DNA Polymerase and PCR Buffer

Taq DNA Polymerase, which is a 94 KD thermostable enzyme, isolated from the bacterium *Thermus aquaticus*, was used for amplification of DNA. This enzyme lacks 3' to 5' exonuclease activity (proof reading) but has inherent 5' to 3' exonuclease activity. This enzyme and the PCR buffer [10 mM Tris-HCl (pH-9.0), 1.5 mM $MgCl_2$, 50 mM KCl and 0.01% gelatin] were procured from Bangalore Genei. The stock was stored at -20^0C.

3.6.9 Agarose Gel Electrophoresis

The PCR products were subjected to agarose gel electrophoresis. Depending upon the size of DNA to be visualized, the strength of the gel was decided. The molecular weight of the DNA is inversely proportional to the gel percentage. When a big fragment was to be visualised a low percent gel (w/v) was used and *vice-versa* in case of a smaller band. The agarose gel was made in 1X TBE along with Ethidium Bromide solution (0.5 μg/ml) and the buffer in the electrophoresis tank was also of 1X concentration. For running PCR products, a 1.5% gel was used; 10 μl of PCR product was mixed with loading buffer and loaded onto the gel. During electrophoresis, a DNA molecular weight marker (100 bp ladder procured form Bangalore Genei) was added to one of the wells. In case of PCR-RFLP analysis, 2.0% agarose gel was used keeping all the conditions constant. Electrophoresis was carried out at 100 volts for 1 hour, visualized and then documented on the Gel Doc-2000 (BioRad) (Maniatis, *et. al.,* 1989).

3.6.10 Purification of the PCR products

PCR products were purified by following manufacture's protocol with QIA-quick PCR purification kit obtained from Qiagen, USA.

3.6.11 Restriction digestion of amplified 16S-23S ISR (PCR-RFLP)

The purified amplicons produced for the 16S-23S intergenic spacer regions were digested using two different restriction endonucleases. A list of the enzymes used, their sites and reaction conditions are given in Table-3.3. The restriction digestion was done in microcentrifuge tubes in which a 20 μl mix was prepared. 5-8 μl of amplicon (containing 200 ng DNA) was digested with 2 units of enzyme. 2 μl (1X) of the appropriate buffer (10X) was added and the remaining volume was made up with

sterile MQ water. The tube was sealed with parafilm and incubated for overnight at the optimal temperature as per manufacturer's direction. To stop the digestion, 2µl of the gel loading buffer (Bromophenol Blue) was added. The samples were the electrophoresed on a 2.0% agarose gel at 50 volt for four hours. The gel was stained with ethidium bromide and visualized and documented in the Gel Doc2000.

Table: 3.3 LIST OF RESTRICTION ENZYMES USED

Enzyme	Source	Target Sequence	Buffer	Incubation Temperature
*Mbo*1	*Moraxella bovis*	↓GATC CTAG↑	Buffer B*	37^{0}C
*Alu*1	*Arthrobacter luteus*	AG↓CT TC↑GA	Buffer L**	37^{0}C

*- Bangalore Genei; **- Boehringer Mannheim

3.6.12 Construction of dendrogram

The banding pattern generated by PCR-RFLP was converted into binary matrices assigning 1 for presence and 0 for absence of a designed band. Similarity matrices were constructed by using SPSS version 11.0 software (Information Technology Service Centre, Lingnan University, 2002) followed by Jaccard similarity coefficient analysis. To construct the dendrogram, levels of similarity among the banding profiles were calculated by using the band-matching Dice coefficient (S_D) and the cluster analysis of similarity matrices was calculated with the unweighted pair group method with arithmetic averages (UPGMA). Isolates were assigned to a different type when any band differences were observed.

Chapter IV

Isolation and identification of hydrocarbon degrading microbes

4.1 Results and Discussion

Hydrocarbon rich petroliferous soil has distinct physico-chemical features, which is most likely to create unique ecological habitat for microbial communities in the oil contaminating sites. Assam, in the North East region of India has extensive areas having rich oil and natural gas deposits. The present study relates to an assessment of the diversity of one of the most common Gram negative soil bacterium, *Pseudomonas* in the oil and natural gas rich sites of Upper Assam. Bacterial strains growing on specific hydrocarbons were isolated by enrichment culture. Soil samples were collected from six oil exploration sites in the Upper Assam regions.

4.1.1 Isolation of microbes capable of utilizing hydrocarbons

Ninety-four bacterial isolates were obtained from different environmental samples, collected from the petroliferous sub surface soil using aliphatic (n-hexadecane, paraffin wax) and aromatic hydrocarbons (benzene, anthracene, naphthalene, pyrene, flourene, phenanthrene and carbazole) as the sole source of carbon and energy. Each of these isolates has distinctive morphological features like shape, size, colony shape and color, opacity, and texture. As *Pseudomonas* is well known for their ability to degrade a diverse range of hydrocarbons, *Pseudomonas* specific tests (Table-4.1) were carried out to establish their identity at the genus and the species level (Table-4.2) (Pallaroni, 1986). Forty-three isolates (~ 45%) of the total isolates obtained in the pure cultures were identified as *Pseudomonas*.

Table 4.1 Biochemical and morphological tests of *Pseudomonas*. Results are the average of four independent experiments

Morphology:	Rod shaped with 1-2 μm in diameter, motile, Gram negative, border irregular, surface raised,
Growth:	
Agar	Abundant, pale yellow
Broth	Good growth, with sediment
pH	4.0 – 10.0, optimum at neutral pH
Temperature	Range 28-37^0 C, optimum at 30^0 C
Catalase	Positive
Voges-Proskauer Test	Negative
Methyl Red Test	Negative
Acid from	
D-Glucose	Positive
L-Arabinose	Positive
D-Xylose	Positive
D-Mannitol	Positive
Gas from glucose	Negative
Hydrolysis of	
Casein	Positive
Gelatin	Positive
Starch	Negative
Lipid	Positive
Utilization of Citrate	Negative
Formation of Indole	Negative
Nitrate reduction	Positive
Litmus milk reaction	Peptonized

Table 4.2 **Biochemical tests of *Pseudomonas for species level identification.*** Results are the average of four independent experiments

Characteristic	***P. aeruginosa***	***P. alcaligens***	***P. cepacia***	***P. putida***	***P. florescence***
G + C	~ 67 mole%	~ 60 mole%	~ 60 mole%	~ 61 mole%	~ 59-63 mole%
Diffusible non-fluorescent pigments	+ (blue-green)	-	+ (variou colors)	-	-
Poly-β-hydroxybutyrate test	-	-	-	-	-
Growth at 41^{0}C	+	+	-	-	-
Growth at 4 ^{0}C	-	-	-	-	-
Organic growth factors require (pantothenate, biotin, methionine or cystine, cyanocobalimin)	-	-	-	-	-
Arginine dihydrolase	+	+	-	+	+
Oxidase reaction	+	+	+	+	+
Denitrification	+	+	-	-	+
Gelatin hydrolysis	+	d	d	-	+
Starch hydrolysis	-	-	-	-	-
Utilization of: Glucose	+	-	+	+	+
Trehalose	-	-	+	-	+
2-Ketogluconate	+	-	+	+	+
meso-Inositol	-	-	+	-	+
Geraniol	+	-	-	-	-
L-Valine	d	-	d	+	+
□-Alanine	+	d	+	+	+
L-Arginine	+	+	+	+	+

D = doubtful result

Pseudomonas is a large genus, which comprises of a diverse range of species. To identify the species of these forty-three isolates, species-specific tests were carried out following Bergy's Manual for Determinative Bacteriology (Holt, 1994). On the basis of the biochemical data (Table-4.2) 43 *Pseudomonas* isolates were tentatively identified as *P. aeruginosa, P. cepacia, P. alcaligenes, P. putida* and *P. fluorescence* respectively. The individual isolates were designated as AB11, AB13, AB15 *etc.* (Table-4.3).

Table 4.3 List of *Pseudomonas* species and their designated codes

Assigned code	Species
AB11, AB13, AB15, AB23 AB25, AB37, AB41, AB43, AB46, AB48, AB49, AB53, AB54, AB89, AB91, AB92, AB93, AB94	*P. aeruginosa*
AB63, AB64, AB68, AB69 AB71, AB73, AB78, AB79, AB81, AB56, AB57, AB59, AB62	*P. fluorescence*
AB82, AB83, AB84, AB52	*P. cepacia*
AB55, AB58, AB65, AB67	*P. putida*
AB44, AB85, AB86, AB87	*P. alcaligenes*

4.1.2 Petroleum hydrocarbon degrading potentials of the *Pseudomonas* species

Pseudomonas isolates belonging to different species and their hydrocarbon utilization ability is presented in Table-4.4.

Results of the degradation studies in the enrichment cultures using a variety of aliphatic and aromatic petroleum hydrocarbons as sole sources of carbon and energy revealed that the different strains of the *Pseudomonas* belonging to five species have distinctive metabolic capabilities in assimilating various petroleum hydrocarbons. Of all the species, *P. aeruginosa* have been found to be versatile in having the potential to degrade both aliphatic and aromatic hydrocarbons *viz., n*-hexadecane, paraffin wax, benzene and pyrene. *P. putida* and *P. fluorescence* have been found to be capable of assimilating benzene and naphthalene while *P. putida* can additionally assimilate anthracene and *P. fluorescence pyrene. P.* alcaligenes is the only species capable of utilizing phenanthrene and carbazole. *P. cepacia* has the least assimilatory potential with capability to degrade only flourene.

P. aeruginosa was found out to be more in number and have a wider diversity in degrading petroleum hydrocarbons. Out of eighteen *P. aeruginosa,* eight isolates are able to grow on n-hexadecane, five are able to grow on paraffin wax, four are able to grow on benzene and one isolate is able to grow on pyrene. This suggests that *P. aeruginosa* can grow on both the aliphatic as well as aromatic hydrocarbons. All thirteen isolates of *P. fluorescence* are able utilize only aromatic hydrocarbons of which, eight are able to grow on naphthalene, three are benzene and 2 are able to degrade pyrene. All four isolates of *P. cepacia* grow on fluorene. Two of the isolates of *P. putida* can grow on anthracene; one each is able to grow on benzene and naphthalene separately. Out of the four *P. alcaligenes*, two each are able to grow on carbazole and phananthrene separately.

Table-4.4 *Pseudomonas* species and their hydrocarbon utilization ability.

Hydrocarbons	*P. aeruginosa*	*P. cepacia*	*P. alcaligenes*	*P. putida*	*P. fluorescence*
n-Hexadecane	08	-	-	-	-
Paraffin wax	05	-	-	-	-
Benzene	04	-	-	01	03
Anthracene	-	-	-	02	-
Naphthalene	-	-	-	01	08
Pyrene	01	-	-	-	02
Flourene	-	04	-	-	-
Phenanthrene	-	-	02	-	-
Carbazole	-	-	02	-	-

Pseudomonas isolates belonging to different species and their hydrocarbon utilization ability revealed that *Pseudomonas* isolates belong to the same species are capable of growing on different hydrocarbons and isolates belong to different species are able to grow on same type of hydrocarbons.

Chapter V

Biodegradation of paraffin wax

5.1 Results and Discussion

5.1.1 Screening for paraffin wax degrading *Pseudomonas* species

Out of 43 bacterial isolates, 5 isolates have been obtained from the soil samples collected from slack wax and petroleum tar contaminating sites at Digboi Refinery, Assam. The organisms isolated by enrichment culture with paraffin wax were identified as *Pseudomonas aeruginosa,* which were designated as *Pseudomonas* AB23, *Pseudomonas* AB25, *Pseudomonas* AB37, *Pseudomonas* AB41 and *Pseudomonas* AB43 respectively on the basis of microbiological and biochemical characterization following standard protocols of Bergey's Manual of Systematic Bacteriology.

5.1.2 Growth of individual isolates and consortia in paraffin wax containing media

Cultures incubated for 30 days in MSM containing 1% paraffin wax showed a higher degree of degradation than the corresponding replicates incubated for 10 and 20 days only.

The growth profiles of the bacterial strains individually and in consortia (mixed culture of all the species) as determined by the optical densities of the culture medium are shown in Fig.5.1. Almost all the cultures produced turbidity indicating an ability of each of the isolates to utilize paraffin wax. The consortium- 2 (mixed culture with 0.1% Tween-80) had the highest optical density followed by the consortium-1 (without Tween-80). Among the 5 *Pseudomonas* species, AB37 showed highest turbidity. Rest of the strains showed almost identical types of growth pattern.

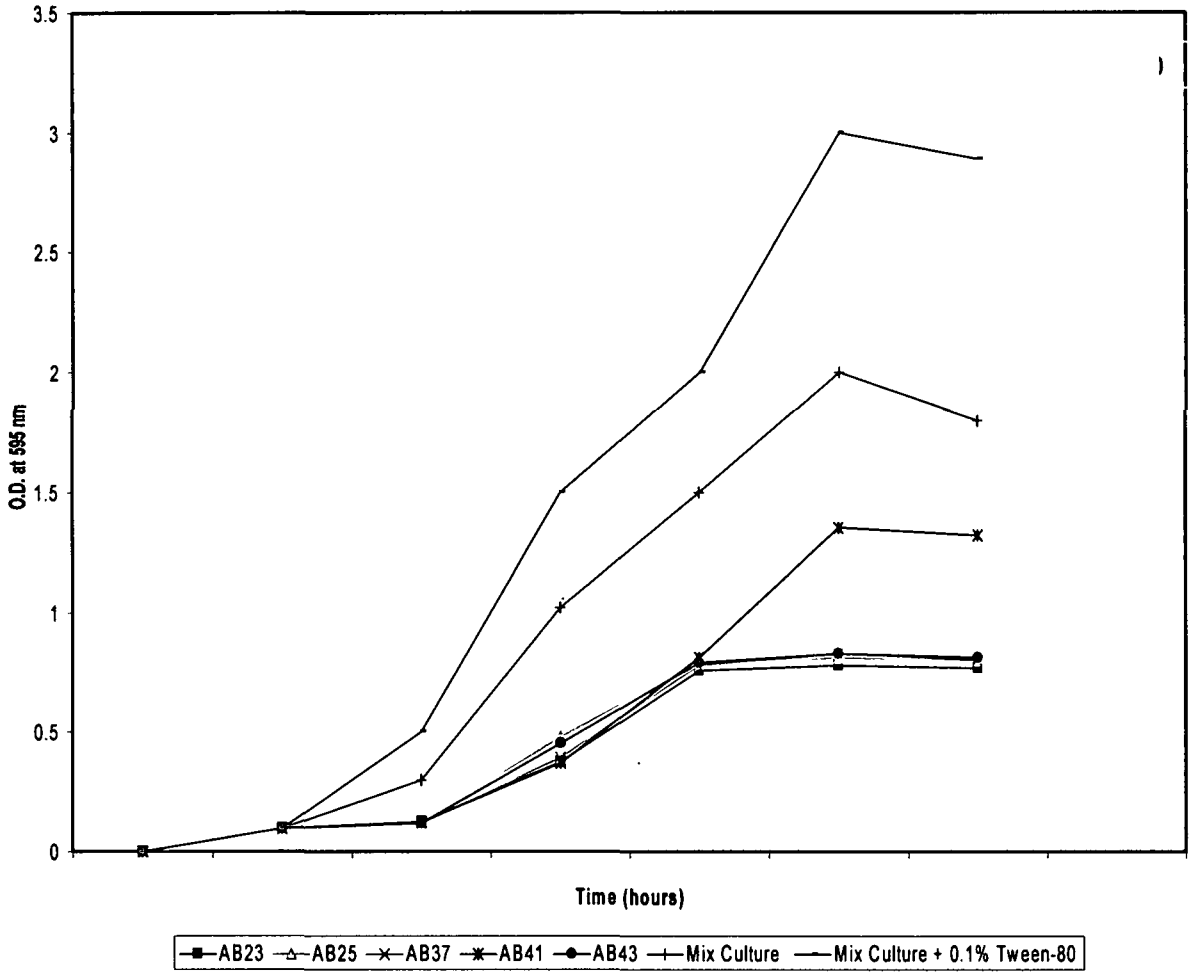


Fig.5.1 Growth kinetics of individual *Pseudomonas* species and two mixed cultures with and without Tween-80

5.1.3 FT-IR spectra of the residual paraffin wax

Isolation of microbial organisms with genetic potential to degrade petroleum hydrocarbons like paraffin wax is critical for solving the problem of retarded oil flow through the transporting pipelines due to petroleum wax deposition inside the conduits. Five strains belonging to *Pseudomonas aeruginosa* that occur in the hydrocarbon contaminated sites in the vicinity of oil fields of Assam have been found to be very efficient degrader of paraffin wax.

Fourier Transform Infra-Red (FT-IR) spectroscopy investigation has been carried out to determine biodegradation of paraffin wax by the selected *Pseudomonas* species. In the FTIR spectrum of all the cultures after degradation characteristics peaks around 3400 - 3500 cm^{-1} and at around 1100 -1150 cm^{-1} have been obtained, which may be due to degradation into alcohol or carboxylic - OH and alcoholic C - O stretching. Peaks are also observed around 1650 cm^{-1} and 2800 cm^{-1} which may be attributed to the presence of aldehydic C=O and C-H. Other peaks are also observed around 1650 cm^{-1}, 1350 - 1380 cm^{-1} and around 1450 cm^{-1} which is due to the carboxylic C =O stretching, C - O stretching and O - H bending vibration of carboxylic acids. The absence of any peak at these wave lengths except at 3600-3200 cm^{-1} (for –OH group) and at 1480-1440 cm^{-1} in the control (noninoculated culture) prove that paraffin wax present in the other cultures was biodegraded by the *Pseudomonas* species (Fig.5.2A-H).

FT-IR analyses of the culture extracts have clearly confirmed the biodegradation of paraffin wax by the isolates through the generation of intermediate compounds known to be involved in the degradation pathway of paraffin wax. It has been reported that the biodegradation of alkanes results in the formation of metabolites like alcohol, aldehyde and fatty

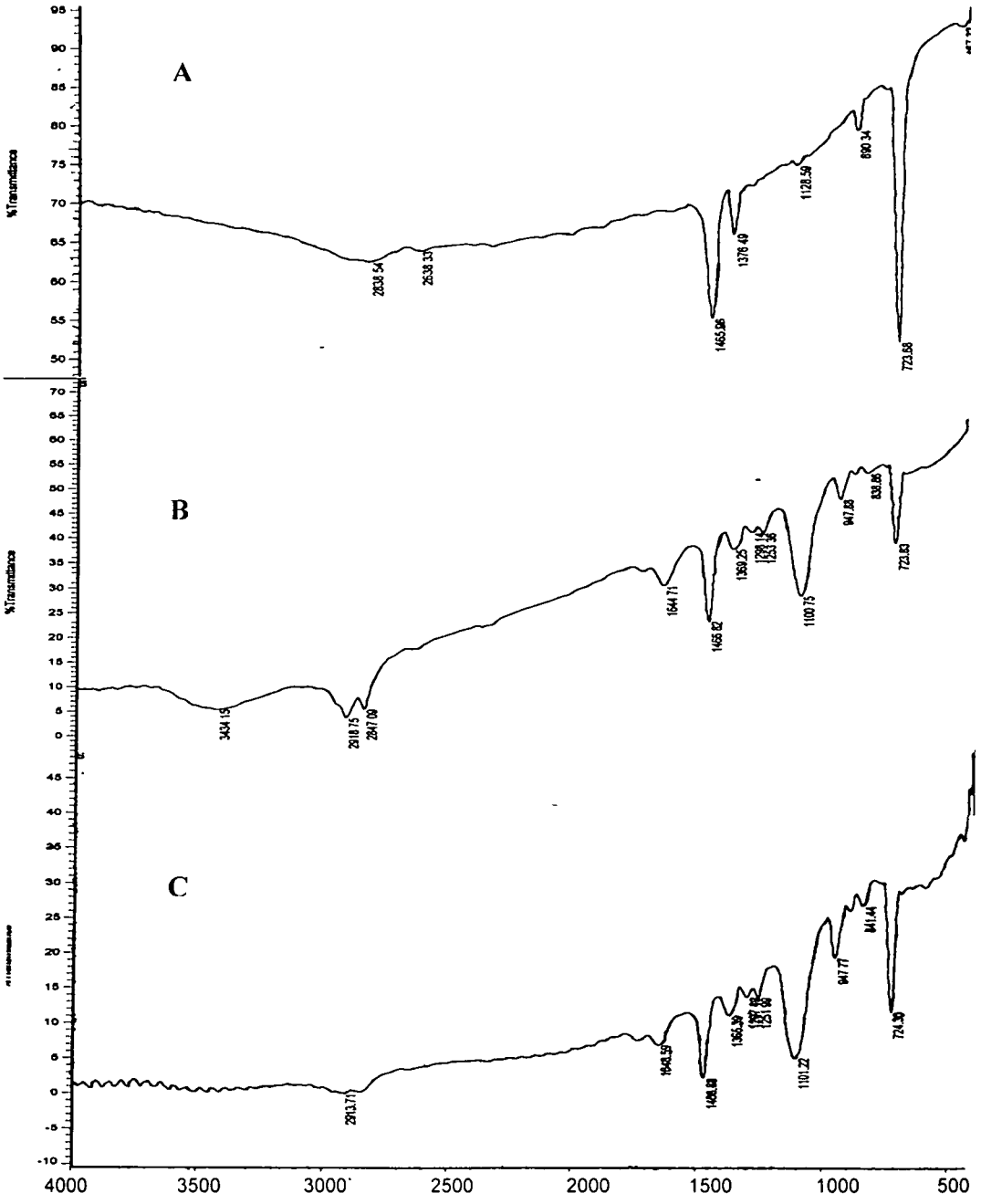


Fig.5.2 Fourier Transform infrared spectra (FT-IR) of the degraded paraffin wax. (A) FT-IR spectra of the standard fraction of Paraffin wax after 30 days of incubation in the controls; (B) cultures inoculated with *Pseudomonas aeruginosa* AB23; (C) cultures inoculated with *Pseudomonas aeruginosa* AB25.

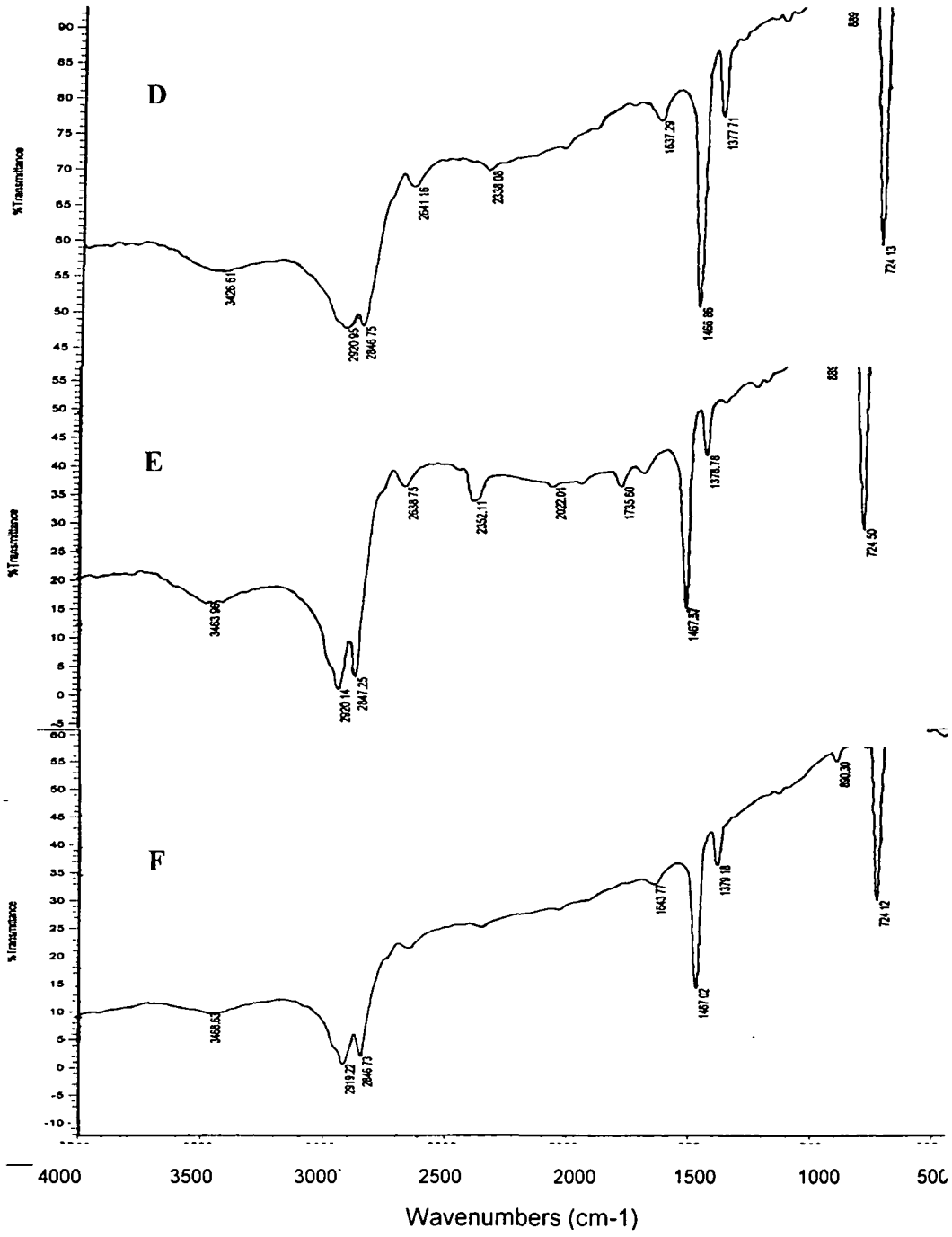


Fig.5.2 Fourier Transform infrared spectra (FT-IR) of the degraded paraffin wax. Cultures inoculated with *Pseudomonas aeruginosa* strains (D) AB37; (E) AB41 and (F) AB43

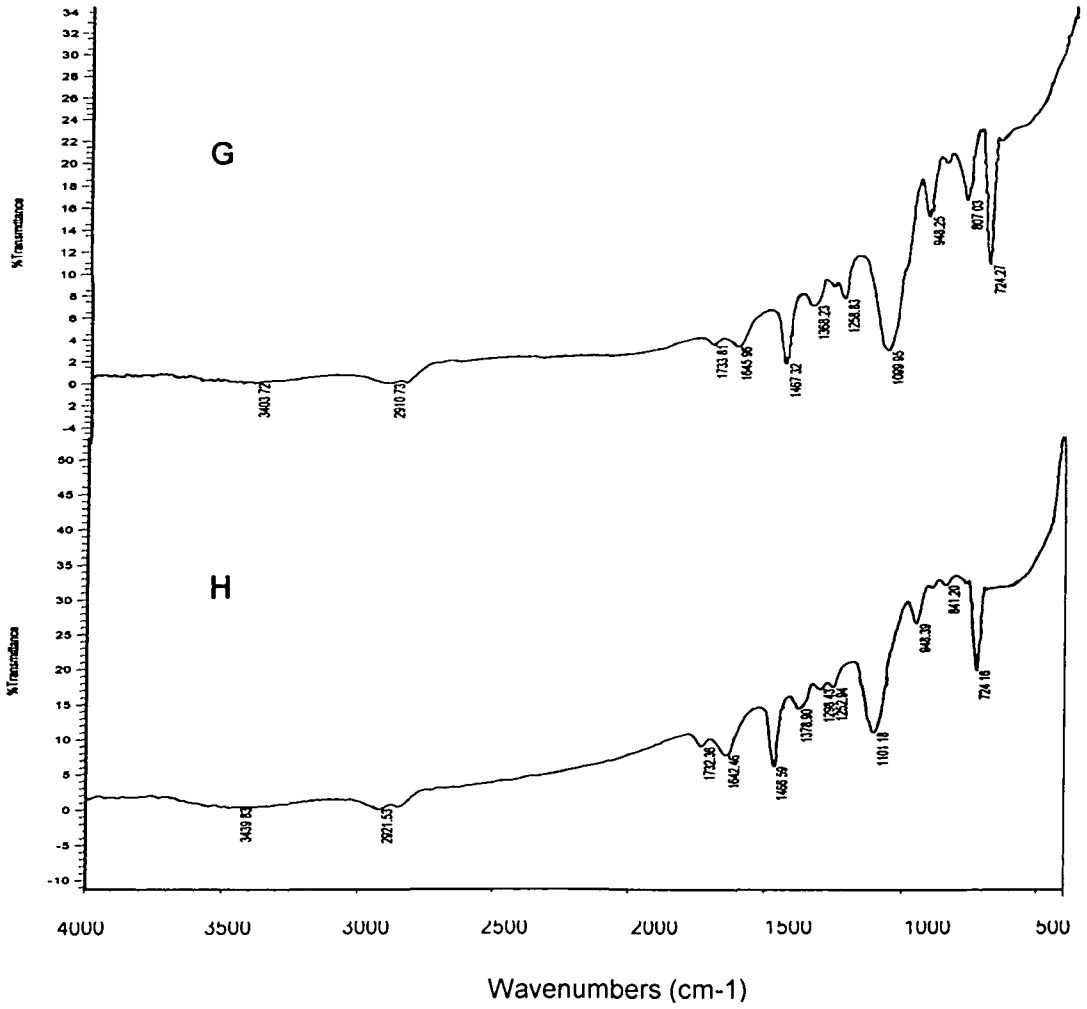


Fig.5.2 Fourier Transform infrared spectra (FT-IR) of the degraded paraffin wax. Cultures inoculated with consortium containing all the strains of *Pseudomonas aeruginosa* (G) without Tween-80 and (H) with Tween-80.

acids. The fatty acids thus formed undergoes β - oxidation and produces new fatty acids of shorter carbon length (Fish, 1982).

5.1. 4 GC-FID analyses

The biodegradation of paraffin wax in the cultures of selected *Pseudomonas aeruginosa* strains was also analyzed by GC-FID. Degradation of the paraffin wax evident from the GC analyses, which revealed the presence of various degraded residual fractions in the samples. From the area count of the chromatographic peaks [Fig. 3(B, C, D, E and F)], it was seen that the degradation rates of the strain AB23, AB25, and AB43 were almost similar showing 86% degradation. There was 84% degradation of the paraffin wax in the case of the culture containing *Pseudomonas* AB41 and 88% in case of *Pseudomonas* AB37.

In order to determine the influence of surfactant on the mobilization of paraffin wax, 0.1% Tween-80, a surfactant was added to the media inoculated with the consortium 2. GC analyses of the culture extracts from this consortium showed about 97% degradation of paraffin wax in comparison to that by Consortium 1 (91%) (Fig.5.3G & H).

From the comparison of the GC analyses of the culture extracts in terms of the retention time with the control (without any bacterial inoculum), it was clearly observed that the low molecular weight hydrocarbons (C_{12} – C_{18}) have been completely degraded. Further, the peak intensities observed in the extracts from the individual cultures of each of the isolates have been found to be much diminished indicating degradation of paraffin wax. Appearance of a number of additional peaks indicates the presence of intermediate metabolites arising out of the degradation of paraffin wax.

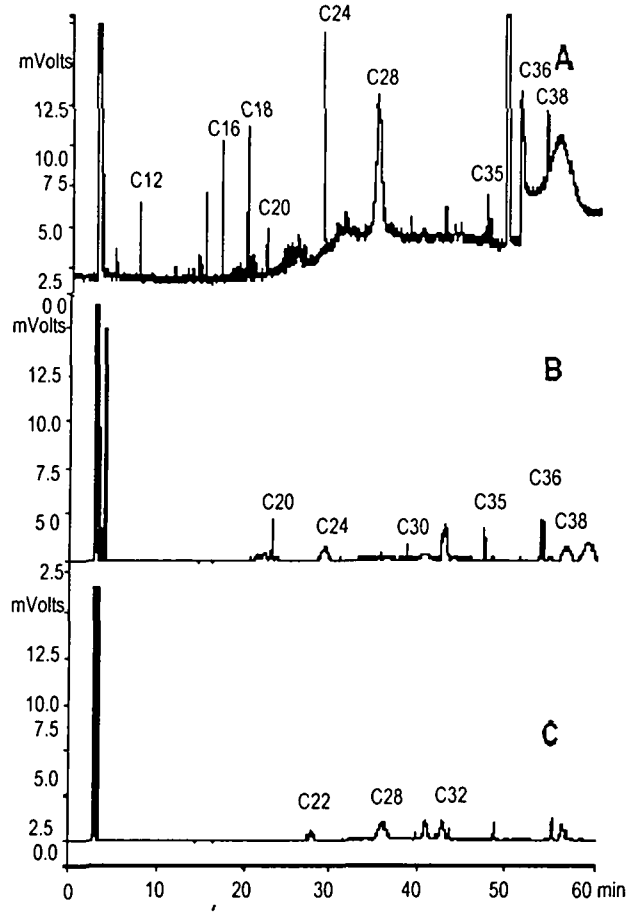


Fig.5.3 Chromatographic profiles (GC-FID) of the residual paraffin wax. (A) GC-FID analysis of the standard fraction of Paraffin wax after 30 days of incubation in uninoculated culture (control); (B) in cultures of *Pseudomonas aeruginosa* AB23; (C) in cultures of *Pseudomonas aeruginosa* AB25

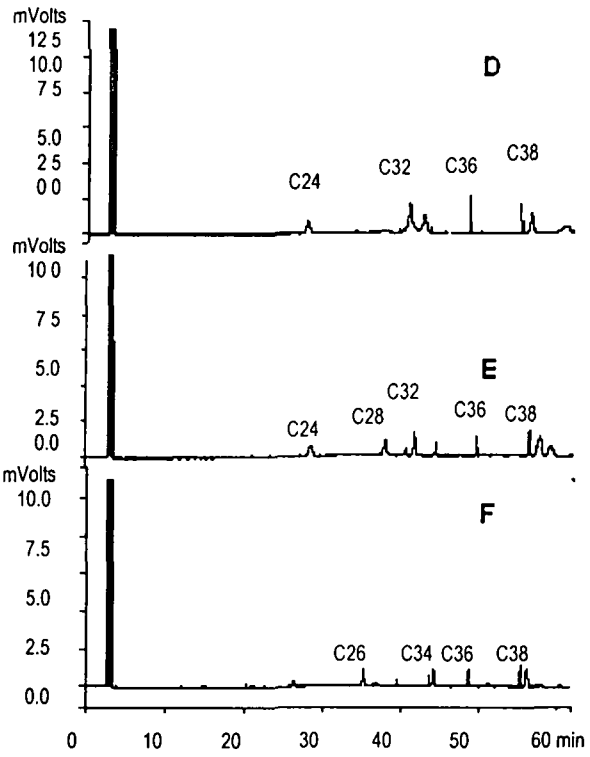


Fig. 5.3 Chromatographic profiles (GC-FID) of the residual paraffin wax. (D) in culture of *Pseudomonas aeruginosa* AB37;
(E) in cultures of *Pseudomonas aeruginosa* AB41
(F) in cultures of *Pseudomonas aeruginosa* AB43.

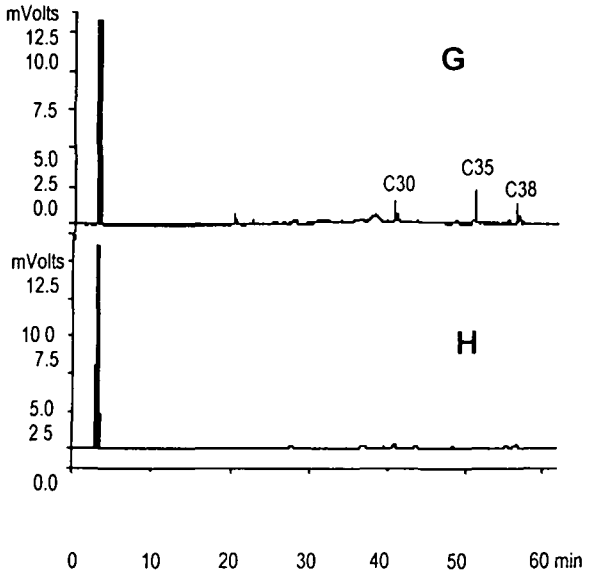


Fig. 5.3 Chromatographic profiles (GC-FID) of the residual paraffin wax.

(G) in cultures of consortium without Tween-80 and

(H) in culture of consortium with 0.1% Tween-80 as synthetic surfactant

The role of biosurfactant in enhancing the mobilization of hydrocarbons from the media to the bacterial cells had been reported (Churchill *et al.*, 1995; Foght *et al.*, 1989). In the present study, influence of surfactant on the mobilization of paraffin wax was determined which showed 97% degradation of paraffin wax in presence of 0.1% Tween-80 (a synthetic surfactant). This is an evidence of the fact that addition of surfactant has enhanced the process of biodegradation possibly through increased mobilization of paraffin wax into the bacterial cells. This validates the earlier report of Zhang *et al.*, (1997).

The five strains of *Pseudomonas aeruginosa* have been found to be relatively more effective in degrading paraffin wax when used in a consortium in contrast to the isolates individually. Similar result was obtained by Itoh and Suzuki, (1972) with respect to utilization of *n*-paraffin by mutants of *P. aeruginosa*. This may be due to some synergistic effect of the isolates in the consortium (Chhatre *et al.*, 1996).

The *Pseudomonas aeruginosa* strains can be used for exploring their efficacy in removal of the paraffin wax deposits inside the conducting oil pipelines and also as bioremediating agent at the slack wax and petroleum tar contaminated sites *in situ*.

Chapter VI

Lectin typing of *Pseudomonas* species

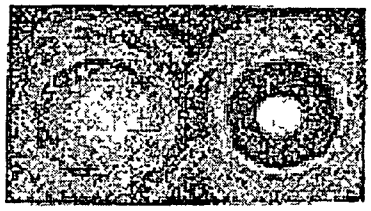
6.1 Results and Discussion

Cell wall chemistry of *Pseudomonas* isolates constitutes highly variable phenotype in terms of specific polysaccharide moieties present on the wall. This variation in the cell wall chemistry can be exploited as a reliable taxonomic criterion to distinguish various isolates. Use of the highly specific polysaccharide binding property of a particular lectin with respect to cell wall polysaccharide moieties in *Pseudomonas*, therefore, can be considered as a rapid, reliable yet easy method for typing.

6.1.1 Scoring of lectin reaction pattern

The binding of 35 *Pseudomonas* isolates to LPA confirmed the presence of N-acetylated D-hexosamines on the bacterial cell envelope in most of the *Pseudomonas* isolates. Fourteen and eleven isolates reacted positively with LcH and PHA, indicating the binding with α-glucose and α-D-glucosyl residues present on the cell surfaces of the isolates. The occurrences of N-acetyl-β-D glucosaminyl and N-acetyl-neuraminic acid, N-acetyl-β-D-glucosamine and N-acetyl-α galactosaminyl were found to be rather common as a large proportion of the *Pseudomonas* isolates (numbering 27, 28 and 16 respectively) reacted positively with the three corresponding lectins *viz.,* WGA, STA and HPA specific for these carbohydrate moieties. The remaining three lectins *i.e.,* ConA, BSL and SBA reacted positively with 14, 22 and 25 number of isolates respectively. Nine isolates showed binding activities with all members of the panel of the lectins used. On the other hand, five isolates *viz.,* AB89, AB52, AB64, AB86 and AB73 did not show binding activity with any one of the lectins used. The type strain used *P. aeruginosa*, MTCC1034 showed

agglutination which is similar to the one found in the case of strain *P. aeruginosa* AB94 (Fig.6.1 & Table 6.1).



a b

Fig.6.1 Representative results of lectin agglutination assay
(a) Positive result; (b) Negative result

Table-6.1 Results showing the response of the *Pseudomonas* species to the panel of lectins. (+ = positive; - = negative)

Type	Lectin 1	Lectin 2	Lectin 3	Lectin 4	Lectin 5	Lectin 6	Lectin 7	Lectin 8	Lectin 9	Control
AB11	+	+	-	+	+	-	+	-	+	-
AB13	+	+	-	+	+	-	+	-	+	-
AB15	+	+	-	+	+	-	+	-	+	-
AB23	-	+	+	-	-	-	-	-	-	-
AB25	-	+	+	-	-	-	-	-	-	-
AB37	-	+	+	-	-	-	-	-	-	-
AB41	-	+	+	-	-	-	-	-	-	-
AB43	-	+	+	-	-	-	-	-	-	-
AB44	-	+	+	-	-	+	-	-	+	-
AB46	+	+	-	+	+	-	+	-	+	-
AB48	+	+	-	+	+	-	+	-	+	-
AB49	-	+	+	-	-	-	-	-	-	-
AB52	-	-	-	-	-	-	-	-	-	-
AB53	+	+	-	+	+	-	+	-	+	-
AB54	+	+	-	+	+	-	+	-	+	-
AB55	-	+	-	+	+	-	+	-	+	-
AB56	+	+	+	+	+	+	+	+	+	-
AB57	+	+	+	+	+	+	+	+	+	-
AB58	-	+	-	+	+	-	+	-	+	-
AB59	-	+	+	-	-	+	-	-	+	-
AB62	-	+	+	-	-	+	-	-	+	-
AB63	+	+	+	+	+	+	+	+	+	-
AB64	-	-	-	-	-	-	-	-	-	-
AB65	-	+	-	+	+	-	+	-	+	-
AB67	-	+	-	+	+	-	+	-	+	-
AB68	+	+	+	+	+	+	+	+	+	-
AB69	+	+	+	+	+	+	+	+	+	-
AB71	+	+	+	+	+	+	+	+	+	-
AB73	-	-	-	-	-	-	-	-	-	-
AB78	+	+	+	+	+	+	+	+	+	-
AB79	+	+	+	+	+	+	+	+	+	-
AB81	+	+	+	+	+	+	+	+	+	-
AB82	-	+	-	-	+	-	+	-	-	-
AB83	-	+	-	-	+	-	+	-	-	-
AB84	-	+	-	-	+	-	+	-	-	-
AB85	-	+	+	-	-	+	-	-	+	-
AB86	-	-	-	-	-	-	-	-	-	-
AB87	-	-	-	-	-	+	-	+	-	-
AB89	-	-	-	-	-	-	-	-	-	-
AB91	+	+	-	-	-	-	-	-	-	-
AB92	+	+	-	+	+	-	+	-	+	-
AB93	+	+	-	+	+	-	+	-	+	-
AB94[a]	+	+	-	-	-	-	-	-	-	-

[a] This group included the type strain of *P. aeruginosa*, MTCC 1034

6.2 Data analyses

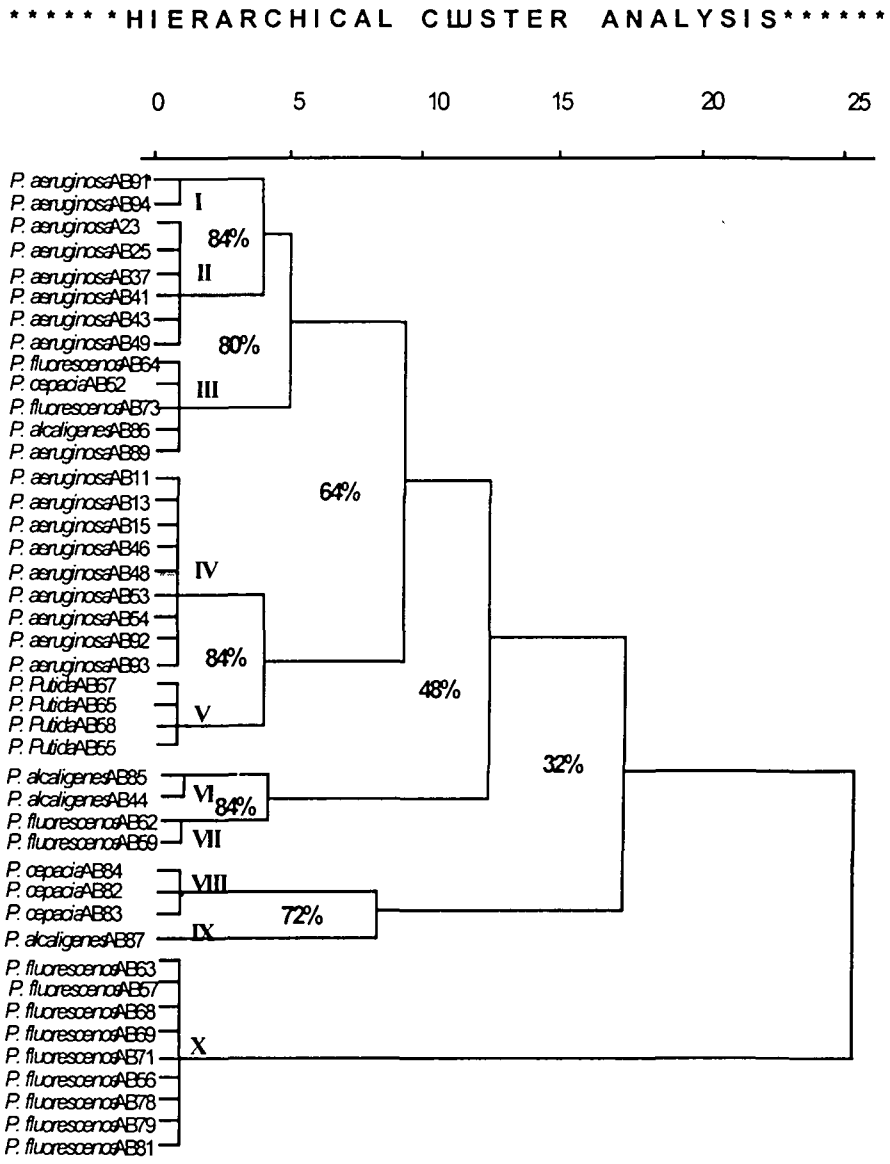
The extent of similarity among the *Pseudomonas* isolates on the basis of their reaction to the panel of lectins used was measured using SPSS - 11.0 software following Jaccard's similarity coefficient analysis. The 43 *Pseudomonas* isolates belonging to the five species were divided into 10 different clusters on the basis of their lectin agglutination patterns as shown in the dendrogram (Fig.6.2).

Analysis of the dendrogram reveals that the 18 isolates of *P. aeruginosa* isolates could be clustered into three major lectin types. On the basis of their overall binding activities with the panel of 9 different lectins used in the present study, it is seen that the species under cluster I has 84 % similarity with the isolates of same species categorized under cluster II. The isolates belonging to *P. aeruginosa* in cluster II and I on the other hand display 64% similarity with those in cluster IV. Almost all *P. fluorescence* has formed a single cluster (Cluster X). The isolates belonging to cluster X are peculiar in showing binding activities with the entire panel of lectins used. All four isolates of *P. putida* constitute a single cluster.

Various molecular typing techniques have advanced the idea of large amounts of genetic diversity existing between strains of diverse range of bacteria. Large numbers of studies were carried out using RAPD, RFLP and ribotyping and these yielded results, which highlighted this genetic diversity. This genetic diversity was unquestioned until it was found that these techniques have low resolution and may have overestimated this diversity (Hynes *et. al.*, 1999).

While various molecular approaches like RAPD, RFLP, ribotyping *etc.*, are available to determine the genetic diversity and to establish the phylogeny of microbes, these methods are relatively expensive and time consuming.

Fig.6.2 Dendrogram of 43 *Pseudomonas* species based on the results of lectin typing



Therefore, for rapid typing of the isolated bacteria, lectin typing was the preferred option. There are reports on discrimination of several pathogenic bacteria by lectin assay, *e.g., Campylobacter* sp., *Bacillus* sp., *Staphylococcus aures, Streptococci sp. Pseudomonas* sp., *Helicobacter pylori* etc. (Chatterjee *et. al.,* 1989; Cole, *et. al.,* 1984; Pistole, 1981; Aabenhus *et. al.,* 2002; Slifkin and Doyle, 1990).

Autoagglutination sometimes makes lectin interactions difficult for detecting agglutination reaction. A pre-treatment method was developed to eliminate this autoagglutination ensuring reproducible and reliable results. The pre-treatment uses a wash in an acidic buffer and subsequent enzymatic treatment to eliminate non-specific binding interactions between bacteria. In this way, an enriched LPS extract can be obtained after pre-treatment. This can be reacted in an agglutination assay with a panel of lectins. Since the extract contains predominantly LPS, a panel of lectins were chosen to detect novel lectin reaction sites. An initial study using this system and optimised panel of nine of these lectins has been successful as no strain proved untypable due to autoagglutination. A group of 43 species of *Pseudomonas* could be divided into 10 separate groupings of common lectin reaction patterns as described above. This system could be exploited for examining environmentally distinct strains among the species of *Pseudomonas* for use in preparation of suitable consortium for bioremediation operation. Although no relationship has been found between degradation and the lectin types, a larger population of strains needs to be tested before any conclusive inferences can be drawn. The main advantages of the system are that it requires a very simple treatment system, which can be easily adapted for applied purposes. The lectin types produced are stable and reproducible.

Chapter VII

PCR-RFLP analyses of 16S-23S rRNA intergenic spacer region (ISR) of *Pseudomonas* species

7.1 Results and Discussion

In this study, a molecular method was adopted for differentiating the 43 closely related *Pseudomonas* species isolated from petroleum rich soil of Assam based on PCR-RFLP of the 16S-23S rRNA intergenic spacer region. The method has been found to be useful for differentiating all strains belonging to five species of *Pseudomonas* at the inter as well as intraspecies level accurately and reliably.

7.1.1 PCR and nested PCR amplification of the ISR of 16S-23S rRNA

The ribosomal DNA region between the 16S and the 23S has been used often to cluster different closely related bacteria (Pace *et. al.* 1986). The amplified intergenic region of 16S-23S rDNA was found out to be ~ 800 base pair in all forty three *Pseudomonas* isolates. To confirm that the amplified product is indeed the rDNA, a nested PCR was carried out on the primary PCR product. The nested PCR product was ~550 bp which is in concordant with the estimated size (Fig.7.1), suggesting that the nested PCR product is the amplified rDNA of the *Pseudomonas* species and not an artifact or product of unspecific primer binding. It is also pertinent to note that all *Pseudomonas* belonging to different species have ISRs with similar size.

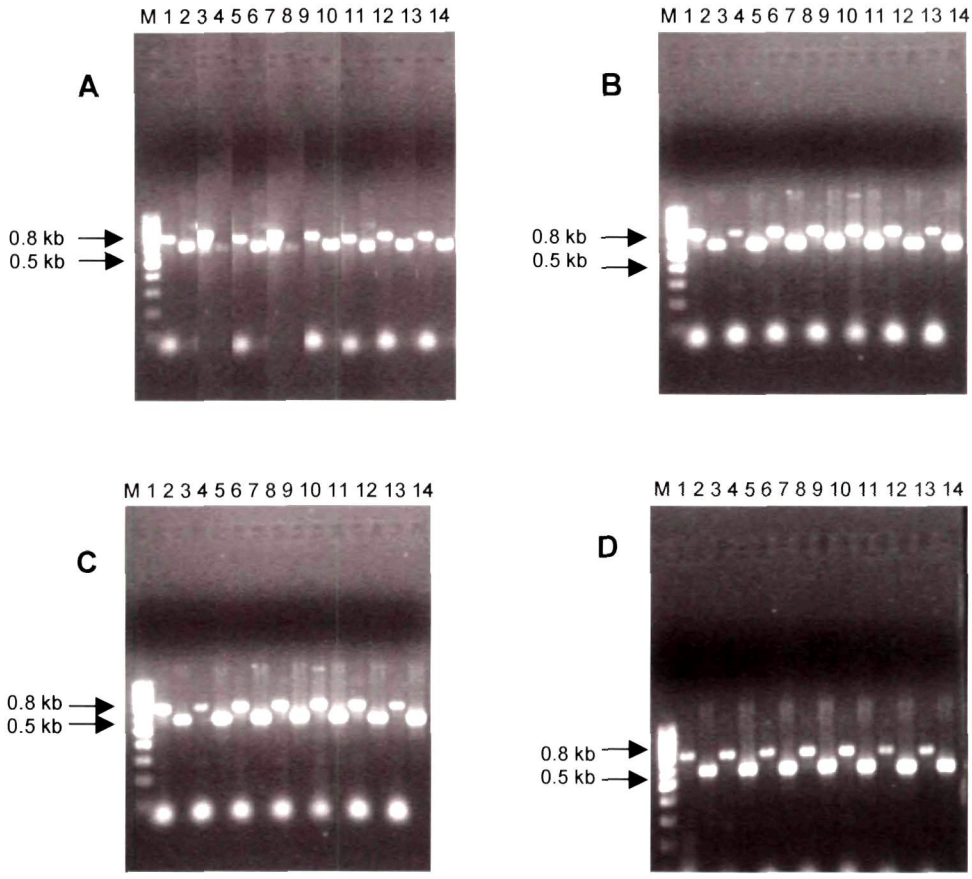


Fig.7.1 PCR (first lane) and nested PCR (second lane) amplification of ISR of 16S-23S rRNA gene of *Pseudomonas* species.

(A) Lane M: Molecular weight marker (100 bp ladder); Lane1 & 2: AB11; Lane 3 & 4: AB13; Lane 5 & 6: AB15; Lane 7 & 8: AB23; Lane 9 & 10: AB25; Lane 11 & 12: AB37; Lane 13 & 14: AB41.

(B) Lane M: Molecular weight marker (100 bp ladder); Lane1 & 2: AB43; Lane 3 & 4: AB44; Lane 5 & 6: AB46; Lane 7 & 8: AB48; Lane 9 & 10: AB49; Lane 11 & 12: AB53; Lane 13 & 14: AB54.

(C) Lane M: Molecular weight marker (100 bp ladder); Lane1 & 2: AB55; Lane 3 & 4: AB56; Lane 5 & 6: AB57; Lane 7 & 8: AB58; Lane 9 & 10: AB59; Lane 11 & 12: AB62; Lane 13 & 14: AB63.

(D) Lane M: Molecular weight marker (100 bp ladder); Lane1 & 2: AB64; Lane 3 & 4: AB65; Lane 5 & 6: AB67; Lane 7 & 8: AB68; Lane 9 & 10: AB69; Lane 11 & 12: AB71; Lane 13 & 14: AB73.

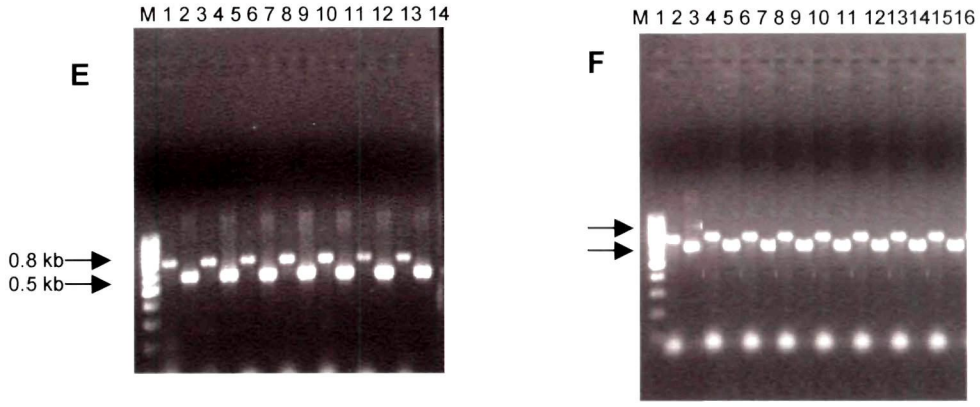


Fig.7.1 PCR (first lane) and nested PCR (second lane) amplification of ISR of 16S-23S rRNA gene of *Pseudomonas* species.

(E) Lane M: Molecular weight marker (100 bp ladder); Lane1 & 2: AB78; Lane 3 & 4: AB79; Lane 5 & 6: AB81; Lane 7 & 8: AB85; Lane 9 & 10: AB86; Lane 11 & 12: AB87; Lane 13 & 14: AB89.

(F) Lane M: Molecular weight marker (100 bp ladder); Lane1 & 2: AB91; Lane 3 & 4: AB92; Lane 5 & 6: AB93; Lane 7 & 8: AB94; Lane 9 & 10: AB84; Lane 11 & 12: AB82; Lane 13 & 14: AB52; Lane 15 & 16: AB83.

7.1.2 Restriction analyses of the PCR products

The PCR product derived from each strain was digested separately with *Mbo*I and *Alu*I. Considering the relatively small size of the amplicon, 4 base cutter restriction enzymes have been used in the RFLP analyses. Digestion with both *Mbo*I and *Alu*I generated seven different genotypes. Each PCR-RFLP experiment was done in triplicates to ensure reproducibility.

The observed RFLP patterns of the all the 43 *Pseudomonas* species as well as of the type strain *P. aeruginosa* MTCC 1034 with *Mbo*I revealed bands, which can be clustered into seven different groups. These patterns comprise of the following: two bands of ~ 150 and ~220 bp for first group; three bands at ~130, ~160 and ~390 bp for second group; two bands at 390 and 500 bp for third group; only single bands at ~390 bp for fifth group; two bands at ~160 and ~310 bp for sixth group; and three bands at ~120, ~200 and ~290 bp for the seventh group respectively. The type strain *P. aeruginosa* MTCC 1034 displayed restriction fragment that conform to the bands in the seventh group (Fig.7.2).

Similarly the PCR-RFLP with *Alu*I revealed seven different genotypes with well resolved bands of the following patterns. Group I with ~220 bp and ~ 300 bp. The type strain *P. aeruginosa* MTCC 1034 belongs to this group. The second group has bands of the size ~250 and ~300 bp. There are four bands with ~60, ~80, ~120 and ~260 bp that form the third group. The remaining groups have fragments of the size ~280 and ~320 bp (fourth group); ~180, ~200 and ~360 bp (fifth group); ~160 and ~400 bp (sixth group) and ~80, ~140 and 380 bp (seventh group) (Fig.7.3).

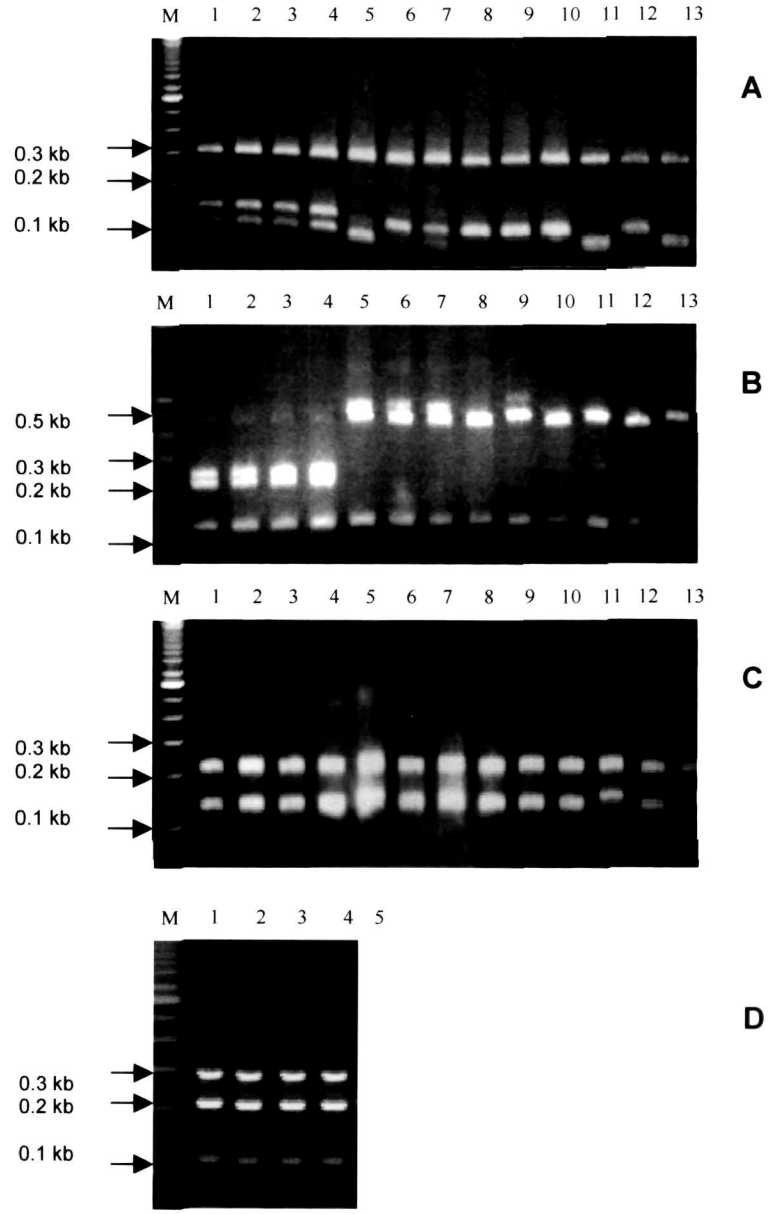


Fig.7.2 Analysis of the *Mbo*I digested ISR region between 16S-23S rRNA genes (PCR-RFLP).

(A) Lane M: Molecular weight marker (100 bp ladder); Lane1: AB65; Lane2: AB67; Lane3: AB55; Lane4: AB58; Lane5: AB93; Lane6: AB94; Lane7: AB89; Lane8: AB64; Lane9: AB57; Lane10: AB62; Lane11: AB91; Lane12: AB63; Lane13: AB92.

(B) Lane M: Molecular weight marker (100 bp ladder); Lane1: AB86; Lane2: AB85; Lane3: AB87; Lane4: AB44; Lane5: AB79; Lane6: AB81; Lane7: AB59; Lane8: AB73; Lane9: AB78; Lane10: AB69; Lane11: AB71; Lane12: AB68; Lane13: AB56.

(C) Lane M: Molecular weight marker (100 bp ladder); Lane1: AB53; Lane2: AB54; Lane3: AB11; Lane4: AB48; Lane5: AB49; Lane6: AB43; Lane7: AB46; Lane8: AB37; Lane9: AB41; Lane10: AB23; Lane11: AB25; Lane12: AB13; Lane13: AB15.

(D) Lane M: Molecular weight marker (100 bp ladder); Lane1: AB84; Lane2: AB82; Lane3: AB52; Lane4: AB83.

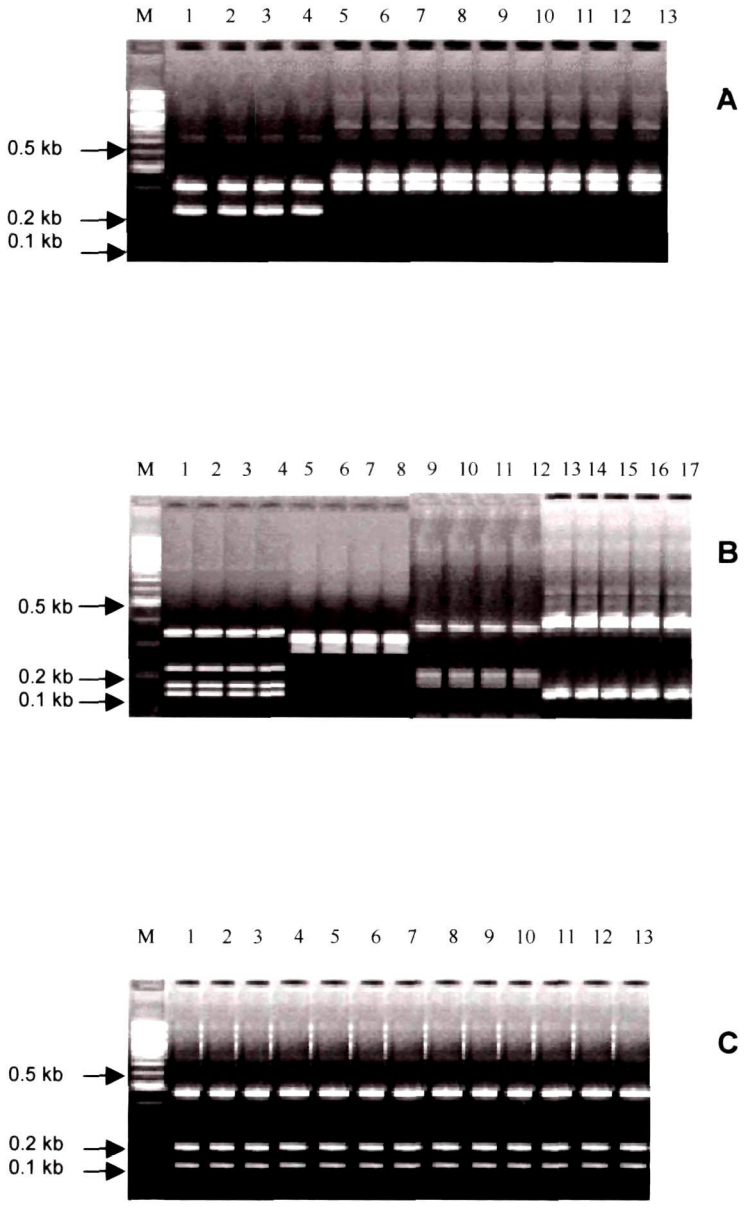


Fig.7.3 Analysis of the *AluI* digested ISR region between 16S-23S rRNA genes

(A) Lane M: Molecular weight marker (100 bp ladder); Lane1: AB59; Lane2: AB62; Lane3: AB56; Lane4: AB57; Lane5: AB79; Lane6: AB81; Lane7: AB63; Lane8: AB73; Lane9: AB78; Lane10: AB69; Lane11: AB71; Lane12: AB64; Lane13: AB68.

(B) Lane M: Molecular weight marker (100 bp ladder); Lane1: AB65; Lane2: AB67; Lane3: AB55; Lane4: AB58; Lane5: AB86; Lane6: AB87; Lane7: AB44; Lane8: AB85; Lane9: AB84; Lane10: AB82; Lane11: AB52; Lane12: AB83; Lane13: AB93; Lane14: AB94; Lane15: AB89; Lane16: AB91; Lane17: AB92.

(C) Lane M: Molecular weight marker (100 bp ladder); Lane1: AB53; Lane2: AB54; Lane3: AB11; Lane4: AB48; Lane5: AB49; Lane6: AB43; Lane7: AB46; Lane8: AB37; Lane9: AB41; Lane10: AB23; Lane11: AB25; Lane12: AB13; Lane13: AB15.

Considering the preponderance of *Pseudomonas* species in the oil rich or in the hydrocarbon contaminated ecological habitats, determination of the diversity among the various species was expected to generate interesting data pertaining to the microbial community structure. The primary objective of the present study was to select the *Pseudomonas* strains that have the genetic potential to degrade the polluting components of the petroleum hydrocarbons. Secondly, there is a necessity for developing an approach for rapid identification of the selected strains for the eventual development of a consortium to be tried in bioremediation operations at various petroleum-contaminated sites. Phylogenetic placement of the microorganisms helps in the understanding of the bioremediation process for developing bacterial consortium (Derek and Lovley, 2003).

7.1.3 Data analyses

In the context of this a dendrogram was constructed using the polymorphism of the ISRs between 16S-23S rDNA as revealed in the PCR-RFLP analyses. As evident from the dendrogram, the 43 isolates have been clustered into seven different groups. In the dendrogram constructed on the basis of PCR-RFLP by *Mbo* I reveals that all the *P. aeruginosa* have been grouped into 2 clusters (Cluster I & IV). *P. aeruginosa* strains that are able to degrade aromatic compounds like benzene and pyrene are grouped together in cluster I. Rest of the strains of *P. aeruginosa* that are able to degrade aliphatic hydrocarbons (both n-hexadecane and paraffin wax) are grouped together forming a large cluster (Cluster IV). Interestingly, the intraspecific variations at the molecular level are reflected in these two clusters that contain the *Mbo* I digested PCR products of the ISRs regions of the same species *viz., P. aeruginosa.* Cluster II that consists of all the four *P. putida* are showing

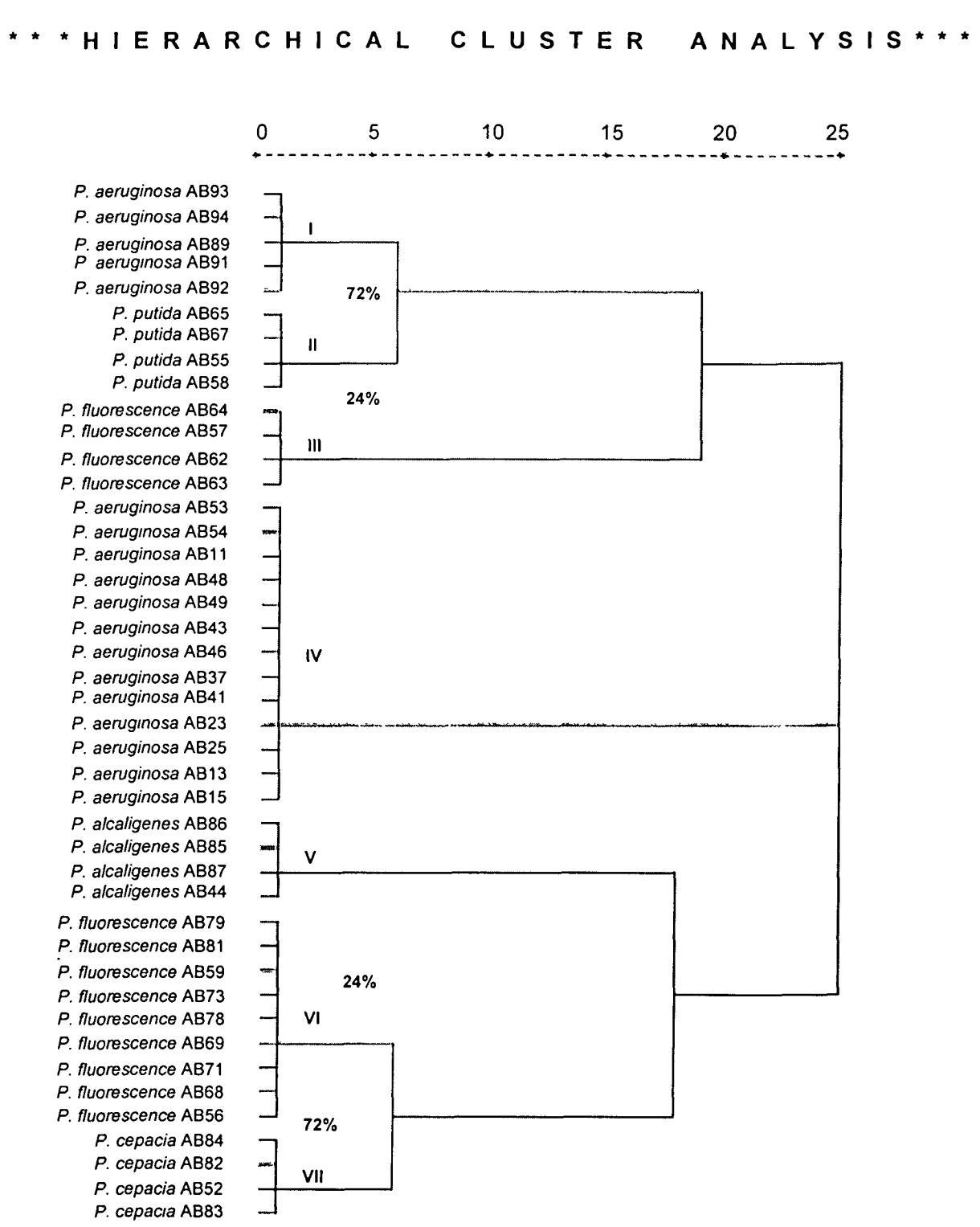


Fig.7.4 Dendrogram of 43 *Pseudomonas* species based on the PCR-RFLP of 16S-23 rRNA intergenic spacer regions with *Mbo*I.

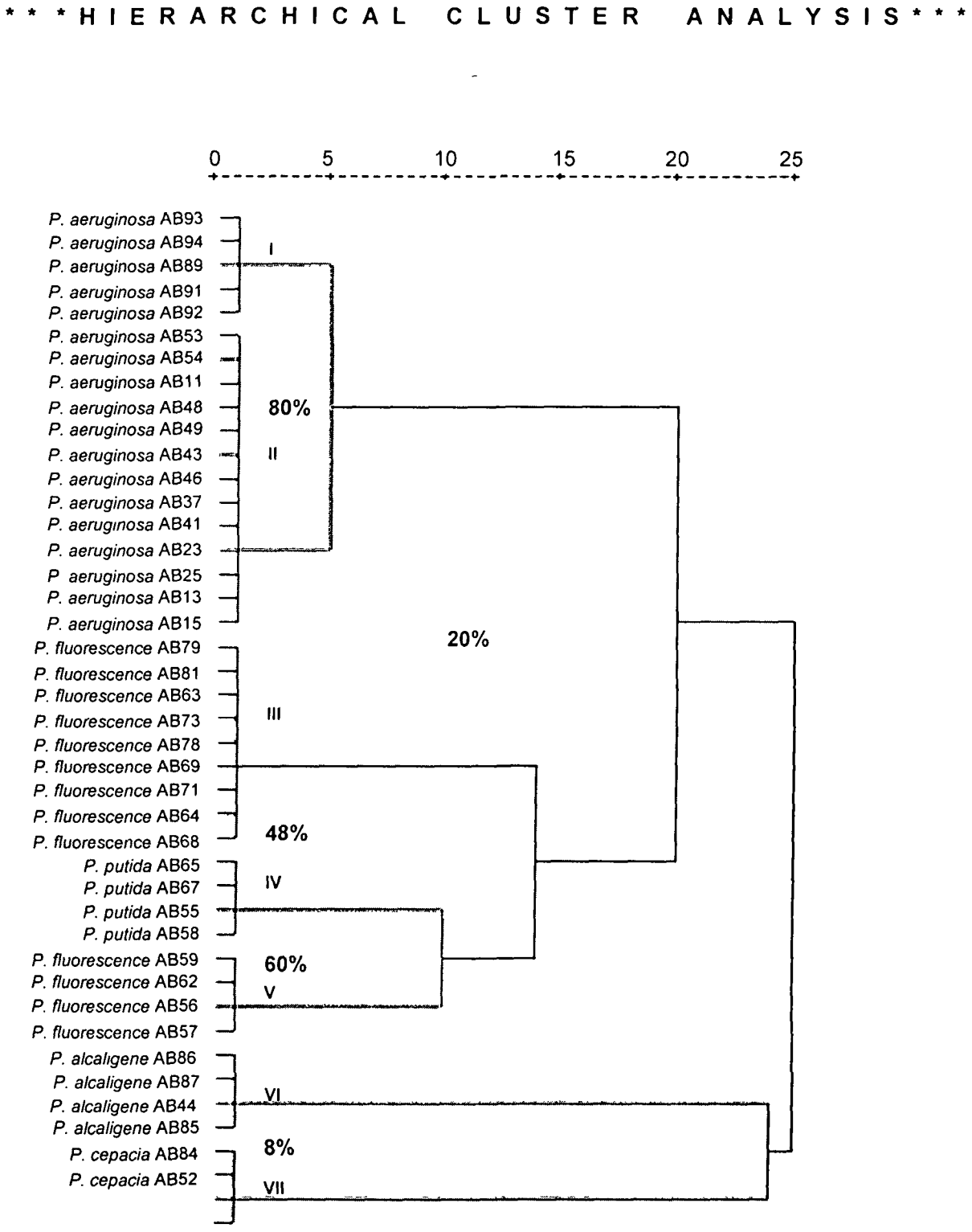


Fig.7.5 Dendrogram of 43 *Pseudomonas* species based on the PCR-RFLP of 16S-23 rRNA intergenic spacer regions with *Alu*I.

72% similarity with those in Cluster I. Although both the clusters consist of two different types of species, there is similarity among the species with respect to degradation of aromatic compounds. Cluster III consists of *P. fluorescence* which shows 24% similarity with cluster II and I. Cluster V consists of *P. alcaligenes* showing 24% similarity with cluster VI and VII, whereas there is 72% similarity between cluster VI and VII that contain *P. fluorescence* and *P. cepacia* respectively (Fig.7.4).

Again, in the dendrogram constructed from PCR-RFLP by *Alu* I reveals that all the *P. aeruginosa* have been grouped into two clusters (Cluster I & II). But in this case both the clusters of the same species are showing 80% similarity in their ISR-RFLP banding patterns although cluster I can degrade aromatic and cluster II can degrade aliphatic hydrocarbons only. This is quite interesting in the context of the requirement for identifying species with distinct metabolic capabilities with respect to degradation of petroleum hydrocarbons. Both the clusters IV and V consist of *P. putida* and *P. alcaligenes* showing 60% similarity with one another. Collectively these two groups exhibit 48% similarity with cluster III with *P. fluorescence.* Cluster II and I display 20% similarity with the cluster III, IV and V. Cluster VI and cluster VIII consisting of the rest of the *P. fluorescence* and *P. cepacia* respectively show only 8% similarity with one another. These two clusters are not showing least similarity with the other clusters (Fig.7.5).

Chapter VIII

Conclusions

1. Ninety four bacterial isolates were obtained in pure cultures from environmental samples of subsurface soil in the oil fields of Upper Assam and various habitats known to be contaminated with petroleum hydrocarbons using nine different hydrocarbons belonging to aliphatic and aromatic compounds as sole sources of carbon and energy.
2. Forty three isolates have been identified as *Pseudomonas* species *viz., P. aeruginosa, P. cepacia, P. fluorescence, P. putida and P. alcaligenes.*
3. Five isolates of *P. aeruginosa viz.,* AB23, AB25, AB37, AB41 and AB43 have been found to degrade paraffin wax as revealed through FT-IR and GC-FID analyses.
4. These five isolates displayed higher degree of degradation of paraffin wax when present in a consortium possibly because of certain synergistic effect in co-cultivation.
5. Presence of a synthetic surfactant (0.1% Tween-80) in the culture of the consortium displayed enhanced biodegradation of paraffin wax. This enhancement is brought about most likely because of the increased mobilization of paraffin wax into the bacterial cells by the surfactant.
6. *P. aeruginosa* has been found to have wider occurrence and with diverse metabolic capabilities to cause degradation of a large number of petroleum hydrocarbons and therefore, may be considered as a strong candidate in formulation of a consortium for application in bioremediation.

7. *Pseudomonas* isolates belonging to the same species have been found to be capable of growing on different petroleum hydrocarbon compounds while isolates belonging to different species have been found to be capable of assimilating the same types of hydrocarbons.

8. A biochemical method based on lectin typing of the 43 *Pseudomonas* isolates enabled their differentiation into 10 distinct groups (lectin types).

9. PCR-RFLP of the ISRs between 16S-23S rDNA of the 43 isolates of *Pseudomonas* belonging to the five species *viz., P. aeruginosa, P. cepacia, P. fluorescence, P. putida and P. alcaligenes.* With *Mbo*I and *Alu*I generated distinct banding patterns. A dendrogram of these isolates has been constructed on the basis of these PCR-RFLP patterns.

10. Clustering of the isolates belonging to the same species into more than one groups reflected the occurrence of intraspecific variations within the species.

11. Clustering of the 43 isolates into seven groups each on the basis of the PCR-RFLP analyses of the ISRs between 16S-23S rDNA did not reveal any clear discrimination among the species on the basis of their hydrocarbon degrading abilities. However, *Pseudomonas* species belonging to different clusters of lectin typing and PCR-RFLP of the ISRs between 16S-23S rDNA did reveal some corelational tendencies with respect to their petroleum hydrocarbon degrading properties.

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Appendix

LB Medium (Luria – Bertain Medium)

Bacto-tryptone	10 g/l
Bacto-Yeast Extract	5.0 g/l
NaCl	10 g/l

Nutrient Agar

Peptone	5.0 g/l
NaCl	5.0 g/l
Yeast Extract	2.0 g/l
Beef Extract	1.0 g/l
Agar	15.0 g/l

Phosphate buffered saline (PBS)

NaCl	8.0 g/l
KCl	0.2 g/l
Na_2HPO_4	1.44 g/l
KH_2PO_4	0.24 g/l

Tris-borate (TBE) (5X)

Tris base	54 g/l
Boric acid	27.5 g/l
0.5 M EDTA	20 ml (pH 8.0)

GenBank

GenBank Accessions
Group T-10, Mail Stop K710
Los Alamos National Laboratory
Los Alamos, NM 87545
USA

EMBL

EMBL Data Library
Postfach 10.2209
6900 Heidelberg
Federal Republic of Germany

Ethidium Bromide Solution

250 ng/ml

Gel loading dye

Bromophenol blue	0.25%
Xylene cyanol FF	0.25%
glycerol	30% in water