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 H₂, CH₄, or S²⁻ (lithoautotrophy). The metabolic reactions involved in these processes include oxidation of organic matter to CO₂ and reduction of Fe(III) or Mn(IV) to Fe(II) or Mn(II), and of SO₄²⁻ to S²⁻. 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 H₂ or CH₄ outgasing from the mantle or produced \textit{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, Micrococcus, 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).
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.