Chapter-1

Introduction

Crude oil is one of the major fossil fuel of the world and its discovery can be trace back to more than four thousand years ago. Subsequently, Assam is the second place in the world (after Titusville in the United States) where petroleum was discovered and is one of the leading states of India in crude oil production. Subsequently, large number of oilfields namely Amguri, Baghjan, Borhulla, Dirok, Gelaky, Hapjan, Jorajan, Kumchai, Khoraghat, Lakhmani, Lakowa, Rudrasagar, Shalmari etc. have been discovered in Assam (http://www.dghindia.org/3.aspx) and both intensive and extensive exploration were taken up till today. Simultaneously, few refineries came up to process crude oil namely Digboi, Bongaingaon, Guwahati, Numaligarh under oil, India and Oil and natural gas corporation (ONGC). But the major problem associated with processing of crude oil is the environmental contamination and degradation. The environmental degradation and contamination of different environment arise due to abandonment crude oil drilling sites, tank failure, flooding, accidental spillage etc (Mashreghi and Marialigeti, 2005; Saikia et al., 2009, 2010). In addition to this the drilling process itself produces vast quantity of oil, which contaminate the neighboring soil and other environment carried by rain water and flooding. Due to raining and flooding the spilled crude oil and oily sludge may spread to the nearby cultivated fields causing soil pollution (Gogoi et al., 2003). Subsequently, oil pollution very often results in enormous disturbances of both the biotic and abiotic components of the ecosystem (Mueller, 1992). Considering the large quantity of oil exploration, the soil and water bodies of the areas nearby oilfields of upper Assam lead to contaminate various types of lands which needs to clean up.
There are many technologies accessible for remediation of sites contaminated with petroleum hydrocarbons. Several physical and chemical processes have been used to remove oil contaminant from the environment; but remediation with conventional techniques has some drawbacks. Such kind of methods is suitable for decontaminating relatively small areas and also requires site restoration (Chekol et al., 2004; Escalante-Espinosa et al., 2005). In contrast to these bioremediation is the mostly preferred method because of its eco-friendly nature and complete cleanup of the soil. Earlier reports have demonstrated that a number of microorganisms have broad range of oil eating and tolerable capacity that enable them to degrade many chemicals (Chen et al., 1999; Kanaly and Harayama, 2000; Watanabe, 2001). Bioremediation technology uses such micro-organisms to reduce, eliminate or transform contaminants present in soils, sediments or water (Pandey and Fulekar, 2012; Gogoi et al., 2013). These beneficial microbes break down different kinds of organic (carbon containing) compounds found in crude oil, and other oil contaminated environment. Therefore, screening and experimentation on use of such bacteria from different environment are being continued.

1.1. Crude oil degrading bacteria

The use of biological material like bacteria, fungi gained impetus in remediation of crude oil contaminated soil since its discovery in oil degradation and mineralization. But the screening and its use is varied and depends on the nature of the crude oil contamination (Yenn et al., 2014; Roy et al., 2013). Therefore, screening of microbes capable in degrading crude oil for bioremediation purpose is being continued. Studies has reported different oil degrading bacteria belong to the genus *Bacillus, Bravibacillus, Achromobacter, Pseudomonas* etc (Lal and Khanna, 1996 ; Roy et al., 2013). As a result screening of vernicular crude oil degrading bacteria for use in bioremediation depends on nature of isolated source. Since, Assam is the potential source for different crude oil contamination environment hence it is predicted that this environment could be good habitats for potential crude oil utilizing microbes.
Many heterotrophic bacteria are able to utilize hydrocarbons as a source of carbon and energy (Nichols et al., 1996). The most commonly used heterotrophic soil bacteria are *Pseudomonas* and *Arthrobacter*. They are capable of breaking down hydrocarbons through various metabolic pathways (Morelli et al., 2005) and breakdown of the hydrocarbon compounds depends on solubility and concentration of hydrocarbons. At low concentrations of hydrocarbon, all fractions are to be expected of being attacked by bacteria. However, at high concentrations, only those fractions most susceptible to degradation will be broken down. Also the concentration of contaminants will affect the number of organisms present.

Although numerous bacteria are able to metabolize organic contaminants, a single bacterium does not have the metabolic potential to degrade all or even most of the organic compounds in a polluted soil. The genetic information of more than one organism is necessary to degrade the complete mixtures of crude oil compounds present in the contaminated region, for this reason mixed microbial community have the most influential degradation potential than pure culture (Fritsche and Hofrichter, 2008). As reported by Bouchez et al. (1995) mixed culture of microorganisms may possibly enhance PAH utilization since the intermediatory biotransformation products of one microorganism may serve as substrate for catabolism and growth for other bacteria. As mentioned above degradation of crude oil seems to involve a consortium of microorganisms, including both eukaryotic and prokaryotic such as *Acinetobacter, Actinobacter, Arthrobacter, Bravibacillins, Berijerinckia, Cellulosimicrobium, Flavobacterium, Microbacterium, Mycobacterium, Mycococcus, Nocardia, Pseudomonas* etc. Among the crude oil mineralizing organisms usually the gram negative bacteria, most of them belong to the genus *Pseudomonas* are common in degradation of polycyclic aromatic hydrocarbon like Naphthalene. Besides these, studies have also been reported the bacteria from the genera *Corynbacterium, Aeromonas, Rhodococcus* and *Bacillus* (Neilson and Allard, 1998; Annweiler et al., 2000). On the other hand, a few organisms are capable of degrading
fluoranthene which includes mainly bacteria from the genera *Mycobacterium* and *Alcaligenes*. Studies have also been conducted on strains from *Pseudomonas, Aeromonas, Arthrobacter, Sphingomonas, Mycobacterium* and *Nocardia* on phenanthrene degradation (Cerniglia, 1984; Pinyakong et al., 2000).

1. 2. Bioremediation

By definition, bioremediation is the use of living organisms, chiefly microorganisms, to degrade the environmental contaminants into less toxic forms. It uses naturally occurring bacteria and fungi or plants to degrade or detoxify material which are harmful to the environment. Bioremediation depends on the breakdown of target pollutant compounds in the soil through microbial degradation, by using either *ex situ* or *in situ* techniques (Stroud et al., 2007).

1.2.1. *Ex situ* bioremediation

These techniques involve the excavation or removal of contaminated soil from ground. Among the different *ex situ* bioremediation methods land farming is a simple technique in which contaminated soil is excavated and spread over a prepared bed and periodically tilled until pollutants are degraded. Composting is the method which involves treatment of contaminated soil with nonhazardous organic amendment such as manure or agricultural wastes. Biopiles is a technique where contaminated soil is placed on a bed of sand through which piles are placed. The resulting pile is sealed with plastic and covered by fishing net to keep the plastic in place. Then air or water with nutrients is injected into the piles. Bioreactors are used in bioremediation technique where processing of contaminated solid material (soil, sediment, sludge) or water through an engineered containment system. A slurry bioreactor may be defined as a containment vessel and apparatus used to create a three-phase (solid, liquid, and gas) mixing condition to increase the bioremediation rate of soil bound and water-soluble pollutants as a
water slurry of the contaminated soil and biomass (usually indigenous microorganisms) capable of degrading target contaminants.

1.2.2. In situ bioremediation

This technology avoids excavation and transport of contaminants. It is a simple, low cost and safe method. There are 3 major techniques commonly employed namely biosparging, bioventing and injection recovery. Biosparging technique used in remediation where contamination occurs at or below the water table boundary. Here air is introduced through pipes sunk down in to the contaminated area and in the ground water bubbles are formed. As a result the available additional oxygen gets dissolved in to the water. Another technique bioventing is used to remediate contamination above the water table boundary. This technology uses microorganisms to biodegrade organic constituents adsorbed in the groundwater. Bioventing simulates the natural in situ biodegradation of hydrocarbon by inducing air or oxygen flow into the unsaturated zone.

While injection recovery involves remediation of both, within the ground water and also externally to it. The technique involves two-well system sunk in to the ground, the ‘Injection Well’ and the ‘Recovery Well’, the former being located upstream of the later. Nutrients and air are forced down the injection well and as they flow the contaminants, they stimulate the growth and activity of the indigenous microorganisms, which begin the process of bioremediation.

1.2.3. Factors responsible for bioremediation

In bioremediation different factors which play critical role are:

1. Metabolism of organic contaminants

2. Adaptation and simultaneous multiplication of microbial population capable of degrading the pollutants

3. The availability of contaminants to the microbial population
4. The environmental factors

1. Metabolism of organic contaminants

Microbes which are able to mineralize contaminants in different ecological niche must enable to metabolize it. To metabolize, microbes use the substrate as a source of (i) primary and secondary substrate (ii) co-metabolism (iii) for energy transformation.

(a) Primary and secondary substrates

The contaminant is referred to as primary substrate when metabolism of that contaminant provides energy for cell maintenance. When an organic contaminant undergoes metabolism and provides the cell with energy but does not have affect on cell growth, such type of organic contaminant is called secondary substrate (Fulekar, 2007).

(b) Co-metabolisms

Co-metabolism is defined as the metabolism of an organic compound in the presence of a growth substrate without nutritional benefit which is used as the primary carbon and energy source. Microorganisms growing on a particular substrate undeservedly oxidize a second substrate (co-substrate). The co-substrate is not assimilated, but the product may be obtainable as substrate for other organisms of mixed culture (Fritsche and Hofrichter, 2008).

(c) Energy biotransformation

Sometimes metabolic process that occurs under anaerobic condition some contaminants is capable of act as terminal electron acceptors. In such case energy for bacterial growth is not obtained from the contaminants itself, but its transformation is a component of metabolic processes that provide energy to the cell for growth (Pandey and Fulekar, 2012).

2. Existence of microbial population capable of degrading the pollutants
In crude oil contaminated soil, changes of microbial population occurs along with the change of oil composition during degradation, because the products formed by certain organisms serve as substrates for others (Bausum and Taylor, 1986). Such hydrocarbon degrading organisms may be native to the crude oil contaminated area or they may be isolated from another place and transferred to the polluted soil. Indigenous bacteria in the soil can degrade a broad range of target constituents of the oily sludge, but their population and efficiency affected when any toxic contaminant is present at high concentrations (Olivera et al., 1997; Eriksson et al., 1999). In such case bioaugmentation is to be adopted to restore the degraded soil. Bioaugmentation is the introduction of a group of natural microbial strains or a genetically engineered variant to treat contaminated soil or water.

3. The availability of contaminants to the microbial population

For contaminants to be available for microbial uptake, it must be available for degradation and mineralization by in aqueous phase. Thus contaminants that exist as non aqueous phase liquids or are sequestered within a solid phase may not be readily metabolized. For degradation it is necessary that the microorganisms and contaminants be in contact.

4. Environmental factors

The optimum environmental factors for degradation of contaminants are essential for microbial growth. The basic growth requirements are: (a) Chemical requirements and (b) Physical requirements

(a) Chemical requirement

(i) Carbon source

Carbon contained in many organic contaminants may serve as a carbon source for cell growth. Almost all chemical substances in microorganisms contain carbon in some form, whether they
are proteins, fats, carbohydrates, or lipids. More than 50 percent of a bacterium's dry weight is carbon. Carbon can be obtained from organic materials in the environment, or it may be derived from carbon dioxide.

(ii) Nutrient

Nutrient should be made available for proper growth and maintenance of microorganism. Among the other elements required by microorganisms are nitrogen and phosphorous. Nitrogen is used for the synthesis of proteins, amino acids, DNA, and RNA. Phosphorus is an essential element for nucleic acid synthesis and for the construction of phospholipids.

(iii) Energy source

Organic contaminant is the energy source during primary metabolism. When the metabolism of a contaminant provides energy for cell maintenance, the contaminant is referred to as primary substrate.

(iv) Electron acceptor

Oxygen is used by aerobic bacteria during the process of cellular respiration as a final electron acceptor. For aerobic organisms, oxygen is an absolute requirement for their energy-yielding properties. Certain microorganisms grow in oxygen-free environments and are described as anaerobic. NO$_3^-$, SO$_4^{2-}$, Fe$^{3+}$ and CO$_2$ may serve as electron acceptor in case of anaerobic respiration (Fulekar, 2007). Other chemical requirements for microbial growth include trace elements like iron, copper, and zinc.

(b) Physical requirements

(i) Temperature
Temperature affects the rate of microbial growth. Microbial enzymatic activity depends on the temperature of the environment, and microorganisms are classified in three groups according to their temperature preferences: psychrophilic organisms (psychrophiles) prefer cold temperatures of about 0°C to 20°C; mesophilic organisms (mesophiles) prefer temperatures at 20°C to 40°C; thermophilic organisms (thermophiles) prefer temperatures higher than 40°C. Generally, mesophilic conditions are best suited for most bioremediation application (Fulekar, 2007)

(ii) pH

Soil pH is another factor responsible for bioremediation process. The optimum pH ranges is from 6.5-7.5 to accomplish the process. Degradation of contaminants can be enhanced by changing pH by adding more microbes from outside (Saikia et al., 2009).

(iii) Soil moisture

Moisture is a critical environmental variable. Moisture level affects soil respiration and as such the environment must contain sufficient water for maximum microbiological action (Osuj et al., 2006). The water holding capacity recommended for bioremediation process may range from 25-28%.

(iv) Time

Time of growth of bacteria is another important factor responsible for initiation of bioremediation process. Generally lag phases are often observed prior to the beginning of the activity.

1.3. Metabolic machinery of hydrocarbon degrading bacteria
Microorganisms are equipped with metabolic machinery to use hydrocarbon as a carbon and energy source. The metabolic pathways that hydrocarbon degrading heterotrophs use can be either aerobic or anaerobic.

(a) Aerobic degradation

Aerobic degradation usually proceeds more rapidly because aerobic reactions require less free energy for initiation and yield more energy per reaction and is considered to be more effective than anaerobic degradation. It involves breakdown of hydrocarbons by a series of enzyme mediated reaction. Oxygen serves as an external electron acceptor, while an organic component of the contaminating substance functions as the electron donor or energy source. The enzymatic key reactions are oxidations catalyzed by oxygenases and peroxidases. Oxygenases are oxidoreductase that use \( \text{O}_2 \) to incorporate oxygen into substrate. Microorganisms capable of degrading hydrocarbons require oxygen at two metabolic sites, at the initial attack of the substrate and at the end of the respiratory chain.

(b) Anaerobic degradation

Oxygen is not available in all environments such as deep sediments, flooded soil and oil reservoirs etc. where hydrocarbons occur. In such condition to accomplish the metabolic pathway, bacteria utilize an alternative electron acceptor such as nitrate or sulfate. Studies have confirmed that these bacteria activate organic compounds by special biochemical mechanisms that differ completely from those employed in aerobic hydrocarbon metabolism (Riser-Roberts, 1992). Anaerobic photosynthetic bacteria, or in syntrophic consortia of proton-reducing and methanogenic bacteria can mediate such reactions under Fe (III)-reducing, denitrifying and sulfate reducing conditions.

1.4. Role of plants in bioremediation of hydrocarbon contaminated soil
Hydrocarbon degrading bacteria can degrade organic contaminants, while metals present in soil due to crude oil contamination need immobilization or physical removal. The plants may bio-concentrate or bio-accumulate some toxic components in the crude oil including heavy metals and other hydrocarbon contaminants in their structures and systems (Agbogidi et al., 2007). Certain plants not only accumulate metals in the roots but also translocate from roots to the leaves or shoots (Baker et al., 2000). Therefore, besides the use of hydrocarbon degrading bacteria, role of plant is also vital in complete remediation of soil contaminated with hydrocarbon compounds along with heavy metals. A number of reports are available on use of different plant species to remediate crude oil contaminated soil (Dominguez-Rosado and Pichtel, 2004; Okoh, 2006; Saikia et al., 2008; Essiett et al., 2010; Khokhar et al., 2012 and Yenn et al., 2014). Saikia et al. (2009) reported germination study of some seeds in crude oil contaminated soil after treatment with bioformulation. Bordoloi et al. (2010) reported 6 plant species that show degradation of Total petroleum hydrocarbon (TPH) in soil supplemented with 0.2-20% crude oil. Nevertheless, for successful phytoremediation, both plants and microbes must survive and able to proliferate in crude oil contaminated soil (White et al., 2005).

The present study involves characterization of efficient hydrocarbon degrading bacteria isolated from different crude oil contaminated sites of Assam namely Amguri, Borhulla, Gelaky, Lakowa and Rudrasagar. Stress was given to application of indigenous hydrocarbon utilizing bacteria in recovery of crude oil contaminated sites which follow the successive plantation of indigenous plant species. The integration of bacterial treatment along with the subsequent plantation deciphers complete restoration of crude oil contaminated sites. The study involves the following objectives.

### 1.5. Objectives

- Biochemical characterization of hydrocarbon contaminated soil.
- Isolation and screening of hydrocarbon degrading bacteria and evaluation of their potential of degradation under control condition.
- Study on bacterial degradation of known hydrocarbon compounds.
- Verification of the degradation ability of hydrocarbon degrading bacteria in degradation of crude oil present in soil with respect to time under *in situ* condition.
- Assessment of effect of crude oil on seed germination, survivability and plant growth.
- Analysis of different plant parts to assess the accumulation pattern of metals.
- Evaluation of effect of crude oil on physiological and biochemical changes of screened plant.
- Evaluation of biochemical changes of the soil after treatment with efficient hydrocarbon degrading bacteria.