Chapter-2

REVIEW OF LITERATURE

This chapter deals with the regional, national and international statuses of microbial bioremediation process of petroleum hydrocarbon with special emphasis on use of biosurfactant producing microbes and microbial consortium.

2.1. Microbial bioremediation of petroleum hydrocarbon: International status

The process of using of microbes for degradation of toxic hydrocarbon contaminants known as microbial bioremediation which has become an attractive technology (Hamdi et al. 2007). Barathi and Vasudevan (2001) stated that petroleum compounds bind to soil components, and they are problematic to be eliminated or degraded. Petroleum pollution in soil consequences an imbalance in the carbon-nitrogen ratio at the spilled site, because crude oil is fundamentally a complex mixture of carbon and hydrogen. This motives a nitrogen deficiency in oil saturated soil, thus hindering the growth of microorganisms and the consumption of carbon sources. Additionally, large concentrations of decomposable organic matters in the top layer soil reduce oxygen reserves of the soil and delays the rates of oxygen diffusion into deeper levels of soil. Many aboriginal microbes in water and soil are proficient of degrading petroleum contaminants (Jain et al. 2010, Dhaker and Jain 2011).

The concept of bioremediation by using biosurfactant producing microbes is not new. In one of the preliminary reports of application of biosurfactant or biosurfactant producing microorganisms in hydrocarbon remediation by Itoh and Suzuki (1972), it was revealed that the growth of glycolipid biosurfactant producing Pseudomonas aeruginosa was stimulated when grown in hydrocarbon containing culture media. Since then many authors have been reported about the effectiveness of the biosurfactants and biosurfactant producing microbes in bioremediation of oil contaminated soil in successive manner. Chakrabarty (1985) described that an emulsifier obtained from Pseudomonas aeruginosa SB30 was able to rapidly disperse oil into fine droplets and concluded that it might be suitable in removing oil from polluted seashores.
A mixed soil population was used by Oberbremer et al. (1990) to evaluate hydrocarbon degradation in an artificial model oil field. When sophorose-lipids biosurfactant were supplemented to the model system containing 10% soil and 1.35% hydrocarbon mixture of tetradecane, pentadecane, hexadecane, pristane, phenyldecane and naphthalene in mineral salt medium a statistically significant enhancement of hydrocarbon degradation was observed. In the absence of sophorose-lipids surfactant, 81% of the hydrocarbon content was degraded in 114 h, while in the application of biosurfactant, up to 90% of the hydrocarbon contents was degraded within 79 h.

Enhancement in the biodegradation of tetradecane, pristane, and hexadecane in a silt loam with 2.1% organic matter was achieved by Jain et al. (1992) by amending biosurfactant obtained from Pseudomonas sp. Likewise, enhanced octadecane dispersion and lightened biodegradation by the use a Pseudomonas rhamnolipid surfactant was reported by Zhang and Miller (1995).

The elimination of polycyclic aromatic hydrocarbon from the crude oil contaminated mud flats was achieved by Kosaric (2001) and the author stated that the results were obtained due to wave action and to microbial degradation. The author also specified that addition of trehalose lipid biosurfactant stirred the biodegradation rate and caused the completed elimination of PAHs within six months of time. In a soil microcosms study for treatment of waste crude oil with Halomonas biosurfactants Calvo et al. (2002) achieved a selective enhancement in the total count of indigenous hydrocarbon degrader strains. The study suggests the usefulness of biosurfactants as a potent bio-stimulating agent for uplifting microbial remediation process.

Significantly enhanced biodegradation of naphthalene in a soil slurry reactors by the use of sophorolipid biosurfactant was reported by Norman et al. (2002). Again, Norman et al. (2002) have clearly demonstrated that rhamnolipid biosurfactants can also stimulate various processes occurred in degradation of hydrocarbon pollutants. The efficacy of the biodegradation process and the specific mechanism of action of rhamnolipid may vary from substrate to substrate. They have showed that various biosurfactants stirred the degradation of hexadecane when it was captured in soil matrices with pore-sizes higher than 300 nm rather than in matrix with smaller pore-sizes or in sea
sand. The authors also specified that the degradation rate of hydrocarbons can be improved by surfactant only when the process is under rate limiting conditions.

Then again Aislabie et al. (2006) reported that biosurfactants play a role of paramount importance in remediation of hydrocarbon contaminated soils in very low temperature conditions such as those on polar areas. This is because biosurfactants or emulsifiers can alter the increased viscosity and decreased water solubility of the hydrocarbons at lower temperatures (Aislabie et al. 2006).

Biodegradation potentiality of biosurfactant producing strain Brevibacterium sp. PDM-3 were confirmed by Reddy et al. (2010). They described that this strain could degrade 93.92% of the phenanthrene and also had ability to degrade other PAHs such as anthracene and fluorene.

Franzetti et al. (2010) defined a proposed roles for biological surfactants with respect to their interactions between microbes and hydrocarbons in the content of modulation of cell surface hydrophobicity. Higher cell-hydrophobicity permits microbes to straight with contact oil drops and solid hydrocarbons while low cell hydrophobicity allows their adhesion to emulsified oils. They proposed three mechanisms of interaction between microbes and pollutants: access to water-solubilized hydrocarbons, direct interaction of cells with bigger oil droplets and contact with pseudo-solubilized, emulsified oil. The authors also stated that in various growth stages of microbes, biosurfactants can alter hydrocarbon accession modes. According to their experimental finding, Gordonia sp. strain BS 29 cultured in hexadecane containing media produced glycolipid type of biosurfactant and extracellular bioemulsifier, and during the various growth phases on hexadecane containing media changes in the cell surface hydrophobicity were observed.

Cameotra and Singh (2008) put forwarded more information on the uptake mechanism of hydrocarbon by Pseudomonas aeruginosa and the significant role of rhamnolipids in the process. They have reported a new and exciting investigation for hydrocarbon uptake connecting internalization of hydrocarbon pollutants inside the cell for subsequent degradation. The action of biosurfactant dispersed hexadecane into micro-droplets, increasing the bio-availability of the hydrocarbons to the bacterial cells. This electron microscopic studies, the occurrence of the uptake of the biosurfactant-layered hydrocarbon droplets was established. Fascinatingly the internalization mechanism of
biosurfactant covered hydrocarbon droplets by the microbes was similar to active pinocytosis. This mechanism of internalization was not previously reported in bacterial modes for hydrocarbon uptake visually. Even though much work has been primed by many research groups to explain the ultimate role of biosurfactants in the degradation of water immiscible substrates, most processes still remain unclear.

Kang et al. (2010) investigated the use of sophorolipid on biodegradation of aliphatic and aromatic hydrocarbons and Iranian light, crude oil under laboratory conditions. Addition of the sophorolipid to soil increased biodegradation of tested hydrocarbons with the rate of degradation extending from 85% to 97% of the entire amount of hydrocarbon mixture. The results specified that sophorolipid may have potential for facilitating the bioremediation of sites polluted with hydrocarbons having inadequate water solubility and increasing the bioavailability of microbial consortia for biodegradation.

Over the years, numerous studies have described the application of microbial consortia for crude oil degradation throughout the world. Majority of these studies also reported the vital importance of biosurfactant producing microbes in a consortium used for crude oil remediation.

Crude oil degradation using a continuous flow fermenter employing a mixed bacterial community isolated from seawater was investigated by Mattei et al. (1986) and they have reported an enhanced degradation rate of crude oil by using that bacterial community.

A bacterial consortium of Pseudomonas sp. strain JHK having biphenyl-degrading ability, Pseudomonas putida PaW1 having the ability to transform chlorinated benzoates and Pseudomonas sp. B13 having the ability degrade chlorocatechols were investigated for degradation of Aroclor 1221 (a mixture of polychlorinated biphenyls) in soil microcosm by Havel and Reineke (1992). Because of diverse biochemical activities of applied bacterial consortium they achieved complete mineralization of various congeners of Aroclor 1221 in the treated sterile soil. Nevertheless, in non-sterile soil the indigenous microflora harmfully affected degradation due to the formation of harmful compounds 4-chlorobenzoate (4-CB).
The study by Ghazali et al. (2004) throws more light on the applicability of bacterial consortium in remediation purpose. Hydrocarbon-degrading bacterial consortia were applied to remediate soils polluted with diesel, crude oil and engine oil. They employed two consortia which were made by mixing pure bacterial strains isolated from hydrocarbon-contaminated soil. The first included two Pseudomonas aeruginosa strains S4.1 and S53 with Bacillus sp. S3.2, whereas the second consortium additionally contained Micrococcus sp. S, two Bacillus sp. 113i and O63. Their investigation proved that the tested consortia varied in the practicality for bioremediation and the second consortium was more effective in removing medium and long chained alkanes in both diesel and engine oil contaminated soils.

Likewise, Yu et al. (2005) reported the higher efficiency of PAH degradation in artificially contaminated sediments by a bacterial consortium. The biodegradation of a mixture of fluorene, phenanthrene and pyrene by a bacterial consortium made up of three strains of Acinetobacter sp., Rhodococcus sp. and Pseudomonas sp. was studied in this effort. Results obtained in this study showed that by adding the bacterial consortium into sediments, the biodegradation rate of fluorene and phenanthrene can be significantly boosted but not case of pyrene. The total degradation rate reached the values of 97% and 99% for phenanthrene and fluorene, respectively after 2 weeks of incubation. However, in case of pyrene only about 10% was degraded. Total elimination of all PAHs was attained after 4 weeks.

Heinaru et al. (2005) used a bacterial consortium consisting Pseudomonas mendocina PC1 and three strains of Pseudomonas fluorescens namely, PC18, PC20 and PC24 for biodegradation of phenolic compounds in leachate and hydrocarbon contaminated microcosm. They showed that pollutants present in contaminated microcosm impacts on the presence and action of specific microorganisms. For instance, P. fluorescens PC20 and PC18 prevailed in oil-amended soil while P. mendocina PC1 and P. fluorescens PC24 prevailed in phenolic-leachate microcosm. The study also detected that leachate microcosm possessed high extents of bacteria keeping meta and ortho pathways for phenol and p-cresol degradation. Dominance of PC20 and PC18 strains was accustomed by the ability to break down naphthalene and salicylate. The change observed in microbial populations in the microcosms was explained by the fact that diverse pathways of catabolism of aromatic compounds controlled under defined
conditions and bacteria accustomed the response to pollutants present in their surrounding environment.

Fig. 2.1. Schematic diagram of basic pathway of microbial hydrocarbon degradation (Das and Chandran, 2011).

A study Jacques et al. (2008) assessed the capability of an artificially developed microbial consortium consisting of *Bacillus cereus*, *Mycobacterium fortuitum*, *Gordonia polysoprenivorans*, *Microbacteriaceae* bacterium, *Microbacterium* sp. and *Fusarium oxysporum* to degrade and mineralize various PAHs compounds namely, anthracene, phenanthrene and pyrene in soil condition. Within a treatment of 70 days each PAH was degraded on average from 96% to 99% at initial doses (250, 500 and 1000 mg kg\(^{-1}\)) by this consortium. Within the same incubation time the consortium was able to mineralize diverse concentrations of PAH mixture by 70% while non-inoculated control soil did not show any significant utilization of PAHs. The key finding of this study was that the microbial consortium was more effective than the same bacterial and fungal isolates applied separately to the soil.
Nievas et al. (2008) examined the biodegradation of oily bilge wastes by a biosurfactant producing microbial consortium. As the result for both levels of oily wastes, 136 g·kg\(^{-1}\) of resolvent pollutants and 406 g·kg\(^{-1}\) of unsolvent mixture, it was found that all of the hydrocarbon types showed an important reduction in concentrations from their initial values. They specified that the degree of biodegradation followed the order \(n\)-alkanes > resolved total hydrocarbon > unsolvent complex mixture. The biosurfactant producing microbial consortium used for biodegradation of bilge wastes exhibited reduction of \(n\)-alkanes, resolvent hydrocarbons and unsolvent mixture nearby 85%, 75% and 58%, respectively.

The effect of microorganisms and nutrients on the overall degradation of TPH over a 12 week study period was comparatively studied by Bento et al. (2004) by using hydrocarbon contaminated soil. The research was designed to compare the process of combination therapy with bio-augmentation. They have studied the degradation of diesel oil mainly in the light (C\(_{12}\)-C\(_{23}\)) and heavy (C\(_{23}\)-C\(_{40}\)) range. It was reported that a reduction of 63-84% of the light portion using bacterial consortium and 72% in the light fraction using a combination of both the treatments (biostimulation and bioaugmentation). A reduction of 19% and 31% was reported for heavy fractions with combination treatment and bioaugmentation respectively. Remarkably, they have stated that the addition of nutrients did not significantly influence the number of diesel consuming microbes and heterotrophic population and recognised a major limitation in a lack of complete site specification and characterization. They have also claimed that it is very necessary to decide the technique to be adopted for bioremediation previously. Finally, the researchers promoted that nutrient facility for remediation was more a less not as significant as application of efficient microbial strains with proven bio-degradative qualities; although the alteration in these degradation rates appeared not to be statistically significant.

Bioremediation of benzene has been investigated by employing a micro-flora isolated from cow dung in bioreactor scale by Singh and Fulekar (2009). The bioremediation of benzene under the effect of cow dung micro-flora was found to be 100% and 67.5%, at preliminary concentrations of 100 mg/l and 250 mg/l at 72 h and 168 h respectively. However, at higher concentration (500 mg/l), benzene was found to be inhibitory. Therefore they have designed and developed a two phase partitioning bioreactor (TPPB) to carryout biodegradation at higher concentration. By using the
bioreactor the contaminant found to be degraded at 5000 mg/l concentration up to 50.17% over a period of 168 h. Further the *Pseudomonas putida* MHF 7109 strain was isolated from cow dung micro-flora as efficient benzene degrader and its ability to degrade benzene at various concentrations was assessed. The data specifies that 100%, 81% and 65% degradation at the concentrations of 50 mg/l, 100 mg/l and 250 mg/l was achieved within the time period of 24 h, 96 h and 168 h respectively. The GC-MS data also confirms the presence of catechol and 2-hydroxymuconic semi-aldehyde (degradation intermediates), which authorises the established pathway of benzene biodegradation. The research proved the potential of cow dung microflora as a source of biomass for biodegradation of benzene in bioreactor (Singh and Fulekar 2009).

Elevated temperature can enhance the solubility of hydrophobic contaminants, reduce their viscosity, increase their diffusion, and facilitate the transport of long-chain n-alkanes from solid phase to liquid phase (Margesin and Schinner 2001, Feitkenhauer *et al.* 2003). Collective effect of thermophiles and production of emulsifying agents by *Bacillus* strain NG80-2 has been described by Wang *et al.* (2006) with great outcome for long-chain n-alkanes degradation. Yet again, petroleum compounds differ in their vulnerability to microbial attack and normally degrade in the subsequent order of decreasing susceptibility: n-alkanes > branched alkanes > low molecular weight aromatics > cyclic alkanes, > polycyclic aromatic hydrocarbons > polar compounds (Ulrich 2000).

Diaz *et al.* (2002) have reported the enrichment of microbial consortia, MPD-7 and MPD-M from Cormorant oil fields of North Sea and the sediments associated with mangrove roots, separately. These consortia could degrade aliphatic and aromatic hydrocarbons of crude oil. Total oil degradation by MPD-7 ranged from 20 to 38%, while MPD-M degraded comparatively higher amount of crude oil extending between 45 and 48%.

Riis *et al.* (2003) revealed the degradation of diesel fuel by microbial communities obtained from Argentinean saline soils. Furthermore, the authors also isolated several halotolerant bacteria of the genera *Bacillus, Cellulomonas, Dietzia*, and *Halomonas* with the ability to utilize crude oil as the carbon source.

The isolation of several strains of hydrocarbon-degrading bacteria of the genera *Gordonia, Rhodococcus, Dietzia*, and *Pseudomonas* from oil and striatal waters of
Tatarstan, western Siberia, and Vietnam oil fields was reported by Borzenkov et al. (2006). These isolates could oxidized n-alkane fractions of crude oil. The Bacillus sp. strain DHT, isolated from oil contaminated soil, could produce biosurfactant when cultivated in the presence of various hydrocarbons including crude oil, hexadecane, naphthalene, dibenzothiophene, diesel oil, pyrene, catechol, salicylate and phenanthrene as the sole carbon sources at a temperature range of 30–45°C. However, no significant growth was observed on toluene, 2-hydroxyquinoline, phenol and carbazole.

The biodegradation of phenanthrene by a halophilic bacterial consortium formulated from soil samples collected from the Shengli Oilfield of China was investigated by Zhao et al. (2009). Phenanthrene was entirely degraded by the halophilic bacterial consortium in 8 days. Molecular identification of the halophilic bacterial consortium specified the presence of various alpha and gamma-proteobacteria including members of the genus Chromohalobacter, Halomonas, Marinobacter, Alcanivorax, Idiomarina and Thalassospira.

A stable carbazole-degrading microbial consortium consisting of Chryseobacterium sp. NCY and Achromobacter sp was isolated by Guo et al. (2008). Zhao et al. 92011) employed a positive end dilution technique for the selection of crude oil degrading functional consortium from contaminated soil. The particular consortium was consisted of Pseudomonas sp., Rhizobiales sp., Bacillus sp., Rhodococcus sp., Brucella sp., Microbacterium sp. and Roseomonas sp. and could remove approximately 52.1% of crude oil at initial concentration of 10,000 mg L^{-1} in 7 days, with elimination of aliphatic fractions by 71.4% and aromatic fractions by 36.0%, respectively. The efficiency of the consortium for bioaugmentation was estimated with microcosm test by contaminated soil obtained from Karemary Oilfield, China. The degradation efficiency of crude oil was enhanced to more than 50% in microcosms by the consortium as compared to 8.13% in controls over a 60 day period. According to the authors, the crude oil degradation reaction was possibly first order reaction and the rate was significantly enhanced by bioaugmentation. Addition of nitrogen and phosphate sources showed limited effect on the oil removal in this treatment.

Malik and Ahmed (2012) prepared a mixed consortium combining 15 bacterial strains isolated by enrichment technique from the sample obtained from a hydrocarbon
contaminated site. To investigate the metabolic capability of bacteria, the consortium was incubated with crude oil. The degradation efficacy of the isolates of the consortium was tested by growing in 2% crude oil containing mineral salt medium in shake flask condition at 37 °C for 24 days. Overall removal of aliphatic and aromatics fractions were 94.64% and 93.75% respectively. Among the different components of the crude oil degraded by the bacterial consortium, the removal of alkanes was maximum, 90.96% for tridecane (C13) followed by pentadecane (C15) at 77.95%, octadecane (C18) at 74.1%. Whereas, other alkanes showed 56 to 69% degradation after 24 days of incubation. The aromatics fractions (benzene, toluene and xylene) were evaporated in the 4th day of incubation. Although the degradation efficiency on PAHs fractions (anthracene, phenanthrene and pyrene) was 46.17 to 55.3% after 24 days.

A mixed culture consisting of *Halomonas* sp. and *Marinobacter* sp. was isolated by Dastgheib *et al.* (2012) from oil contaminated saline soil obtained from five different areas of Iran. These microorganisms could degrade several PAHs including naphthalene, anthracene, phenanthrene, fluorine, fluoranthene, pyrene, benz[a]anthracene and benzo[a]pyrene as the sole carbon sources.

Bioremediation of PAHs-contaminated soil by a microbial consortium and connected changes in microbial community was reported by Mao *et al.* (2012). They have tested the efficacy of the applied consortium in soil which contained 9362.1 mg kg⁻¹ of PAHs (almost 90% of those PAHs were having 4- and 5-rings). In subsequent incubation for 56 days, 20.2% and 35.8% of total PAHs were degraded from the soil with the addition of 10% and 20% of a bacterial consortium suspension. They have indicated that cultivation with a bacterial consortium may be a promising technique for reclamation of PAH-contaminated soils.

Moscoso *et al.* (2012) investigated the biodegradation of three polycyclic aromatic hydrocarbons (PAHS) such as Phenanthrene, Pyrene and Benzo[a]anthracene. The bacterial consortium consisting of two strains (*Staphylococcus warneri* and *Bacillus pumilus*) was used based on initially attained promising biodegradation data. Degradation values higher than 85% were found for each single PAH when operated at flask scale. However minimum levels of 90% of PAHS elimination were obtained only after just 3 days of cultivation at bioreactor scale. The procedure of co-metabolic conditions led to
highest levels about 75% and 100% at flask and bioreactor scale, respectively. For the purpose of better understanding of the biodegradation process by *S. warneri* and *B. pumilus* all the experimental data were analyzed in the light of logistic and Luedeking and Piret type models.

Microbial degradation of crude oil using an efficient bacterial consortium in a simulated marine environment was investigated by Bao *et al.* (2012). *Brevibacillus parabrevis* N2, *B. parabrevis* N3, *Ochrobactrum* sp. N1 and *Brevibacillus parabrevis* N4 were selectively combined for preparation of mixed bacterial consortium based on their efficacy of crude oil utilization. A crude oil degradation rate of the microbial consortium extended upwards of 79% at 25 °C in a 3.0% NaCl solution in the shake flask condition. In microcosm study the microbial consortium could effectively remove over 51.1% of the crude oil from the simulated water body.

Likewise, Mnif *et al.* (2011) have described the isolation of numerous strains of thermophilic and mesophilic hydrocarbon degrading organisms from Tunisian oil fields having biosurfactant producing ability. Among the isolates, *Pseudomonas* sp. C450R and *Halomonas* sp. C2SS100 were able to degrade 93–96% of the aliphatic fraction of crude oil (C_{13}–C_{29}) with the production of biosurfactants.

Degradation of crude oil by one artificially created consortium of microalgae and bacteria was reported by Tang *et al.* (2012). The consortium which was assembled by algal strain *Scenedesmus obliquus* GH2 and four oil degrading bacteria with known harmonizing degradative capabilities, including *Burkholderia cepacia* GS3C, *Pseudomonas* GP3A, *Sphingomonas* GY2B and *Pandoraea pnomenusa* GP3B. These bacterial strains were testified to completely remove alkanes, alkyl-cycloalkanes and alkyl-benzens within a period of 7-10 days. The newly formulated consortium preferentially degraded high molecular weight PAHs such as phenanthrene and methyl-phenanthrenes along with the isomers of naphthalene. Some additional lower molecular substances (degradation intermediates) were formed during the PAHs degradation.

Three bacterial isolates identified as *Rhodococcus* sp. M15-2 (UKMP-5T), *Pseudomonas aeruginosa* (UKMP-8T) and *Rhodococcus* sp. ZH8 (UKMP-7T) isolated by Hamzah *et al.* (2013) from the groundwater of a petroleum refinery plant. By combining the isolates, four bacterial consortia were designed by mixing the pure
bacterial cultures in the following ratios: \((P.\ aeruginosa\ UKMP-8T: Rhodococcus\ sp.\ M15-2, 1:1)\), \((P.\ aeruginosa\ UKMP-8T: Rhodococcus\ sp.\ ZH8, 1:1)\), \((Rhodococcus\ sp.\ M15-2: Rhodococcus\ sp.\ ZH8, 1:1)\), and \((P.\ aeruginosa\ UKMP-8T: Rhodococcus\ sp.\ ZH8: Rhodococcus\ sp.\ M15-2, 1:1:1)\), respectively. These bacterial isolates individually and various consortia exhibited differing preferences for nitrogen sources. When amended with the optimized nitrogen sources and cultured in minimal salt medium, all the three isolates individually and the four different consortia biodegraded 97.6–99.9% of Tapis Massa oil devoid of any significant differences within 7 days of incubation.

2.2. Microbial bioremediation of petroleum hydrocarbon: National status

India is a chief petroleum energy producer as well as consumer. India at present ranks as the world's eleventh highest energy producer, accounting for about 2.4% of the world's overall annual energy production and the world's sixth highest energy consumer, accounting for about 3.3% of the world's total yearly energy consumption (Kandasamy 2002). Being a crude oil producing country various environmental contamination by petroleum hydrocarbon is a very common phenomenon in different parts of the country. Therefore, microbial bioremediation practices are being performed by various researchers and research agencies throughout India.

In a laboratory scale bioremediation study of diesel contaminated soil by indigenous bacterial consortium containing \(Moraxella\ saccharolytica, Alteromonas\ putrefaciens, Klebsiella pneumonia\) (subsp. aerogenes) and \(Pseudomonas\ fragi\) were used (Sharma and Rehman 2009). The experiments were conducted in two bioreactor scale (having \(NH_4NO_3\) and control) and incubated for 30 days. On the GC-MS analyses for detection of degradation of hydrocarbons, it was observed that length of the long-chain hydrocarbon compounds were reduced to shorter length. It was also observed that degradation was better with the consortium than individual bacterial strains. They have concluded that the bacterial consortium could be a better tool for speedy and complete remediation of hydrocarbon contaminated soils.

Patel \textit{et al.} (2012) developed a naphthalene degrader bacterial consortium (DV-AL) through enrichment culture techniques from hydrocarbon contaminated sediments
collected from Gujarat, India. The molecular identification (16S rRNA gene based) revealed that the bacterial consortium contained four strains namely, *Pseudomonas* sp. DV-AL2, *Achromobacter* sp. BAB239, *Pseudomonas* sp. BAB241 and *Enterobacter* sp. BAB240. Under shaking conditions (150 rpm), the tested consortium was able to degrade 1000 ppm of naphthalene in Bushnell Haas medium within 24 h. The consortium was also able to utilize other aromatic and aliphatic hydrocarbon substrate such as benzene, carbazole, phenol, petrol, diesel and PAHs namely phenanthrene and 2-methyl naphthalene as sole carbon source. The consortium was also effective in degradation of naphthalene in the presence of other pollutants such as heavy metals and petroleum hydrocarbons.

The Energy and Resource Institute (TERI), India has isolated different very efficient bacterial strains from various petroleum contaminated sites and developed numerous bacterial consortia for field scale bioremediation of various refinery sludge contaminated sites (Mandal *et al.* 2012). They have treated a total of 48,914 tons of refinery sludge in groups at various oil refineries of the country. In the end of the treatment, bio-remediated soil has been found holding TPH content to the amount of < 10 g·kg⁻¹ of oily waste and was found to be not toxic to the environment. The time for bioremediation was within 2-12 months from the date of first presentation of microbes on the oily waste. An indigenous microbial consortium was developed by assemble of four species of bacteria. The bacterial strains were isolated from various oil contaminated sites of India. The isolates could utilize different fractions of petroleum hydrocarbon from the oily waste along with the generation of environment friendly end products.

A study dealing with the bio-treatment of waste water generated from petroleum refinery to evaluate the degradation potential of microbial species present in the waste water and their consortium to treat the water in a suspension form was performed by Singh *et al.* (2013). They have isolated four efficient bacterial strains, namely, *Bacillus subtilis*, *Alcaligenes odorans*, *Pseudomonas aeruginosa* and *Corynebacterium propinquum* and evaluated for biodegradation of crude oil and phenolic compounds present in the waste water inoculating as a consortium. The refinery waste water was collected from a refinery situated at Mathura, India. Degradation were estimated by gravimetric and spectrophotometric analysis. After inoculation of the consortium, it was
observed that oil and phenol pollutants of the waste water reduced up to 70% and 85% via bioremediation respectively.

Pramanik and Rajalakshmi (2013) studied the biodegradation of petroleum hydrocarbon pollutants using microbial consortium in soil condition. For this study, soil samples were obtained from oil contaminated places of Coimbatore. Three MTCC cultures reported as efficient hydrocarbon degraders, namely, Bacillus sp., Micrococcus sp., Pseudomonas sp. were supplemented to collected soil samples. The growth profiles were estimated by monitoring the optical density, dry weights and pH of the culture emended with lubricating oil as sole source of carbon and energy and incubated for one week at 30 °C. The gravimetric analysis revealed that the microbial consortium could utilize 80-90% the oil samples from Bushnell-Hass medium at 30 °C and 170 rpm under laboratory conditions. A positive correlation between optical density and pH (correlation coefficient = 0.735) was found through correlation analysis. After extraction of residual hydrocarbon from culture media, the samples were quantified using GC-MS. Gas chromatographic examination displayed the degradation for petrol, diesel, and kerosene are 48%, 78% and 57% respectively.

Gaikwad et al. (2014) recently reported that the microbial consortia comprise of Actinomycetes spp., Pseudomonas spp., Streptomyces spp., Bacillus spp. and Staphylococcus spp. was able to rationalize the physicochemical parameters of contaminated complex wastewater. The consortia exhibited high COD and BOD reduction up to 90.17% and 94.02% respectively as compared to individual bacterial species fluctuating from 42.11-59.76% in case of COD and 58.55-77.31% for BOD.

Prabhakaran et al. (2014) described about effective bioremediation of crude oil using an aerobic bacterial consortium in synthetic mineral salt medium. They have evaluated remediation employing bacterial consortium in coir waste through improving environmental parameters and absorption studies. They have isolated and screened different petroleum hydrocarbon degrading microbes and formulated a bacterial consortium containing three different strains of Bacillus sp. namely, Bacillus jeotagali (EW6), Bacillus subtilis (EW4), Bacillus foraminis (SSM2). Petroleum hydrocarbons contaminated soil samples were taken from oil contaminated regions of Namakkal and Salem districts. Suitable environmental parameters improve the remediation process up to 78% according to the first assessment. Successively, they have assessed that the
absorption of oil with coirpith. That amplified the remediation up to 98% as compared to the remediation attained through the effective bacterial consortium (54%).

Shankar et al. (2014) studied the efficacy of indigenous microbial consortia in bioremediation of oil-contaminated soils were oil-contaminated soils from north Chennai used as model soil samples. They have isolated a total of 32 efficient oil degrading isolates from 3 different locations, i.e., petrol filling stations, automobile workshops and oil refineries. They have found mainly bacteria belongs to *Pseudomonas* and *Bacillus* genera. They have designed four different consortia where the fourth consortium (consortium D) where the consortium contained all the isolated bacterial strains. Substrate utilization patterns of individual isolates and the various consortia sets were observed. The substrate oil utilized by consortia was taken for thin-layer and column chromatography which perfectly resulted in varied fractions of oil compared to the unused oil as control. The best consortium i.e. consortium D were further investigated for bioremediation of contaminate soil where three different oil-contaminated soils were considered. Set ‘D’ consortia were detected to be very effective in treating all kinds of experimental soils with different residual oil content. The rate of bioremediation differed within the three soil samples, nevertheless all soils were almost 100% reclaimed within 30 days. Additionally, the biodegradation potential of consortia was analysed through bioremediation kinetics which resulted in the first-order kinetic constants for different soils with different initial oil content (range 0.051–0.077/day). Thus, this study confirmed that microbes in a consortium serve as better treating agents in bioremediation of oil-contaminated soils than individual microorganisms.

Mandalaywala and Trivedi (2016) reported an effective microbial consortium of bacteria isolated from hydrocarbon polluted soils of Gujarat, India. Hydrocarbon utilizing bacteria were isolated from a hydrocarbon based polluted site and screened by series of tests to find out the most efficient degraders. Among the isolates, six isolates belonged to *Pseudomonas* sp. (*Pseudomonas aeruginosa* RRLP1, *Pseudomonas aeruginosa* AS-1, *Pseudomonas aeruginosa* SI5, *Pseudomonas* spp. 14-1, *Pseudomonas aeruginosa* RRLP1 and *Pseudomonas aeruginosa* JQ-41) have been found to be effective in hydrocarbon utilizing capability. The study represented that these *Pseudomonas* species isolated from hydrocarbon contaminated waste matter were able to degrade various petroleum derivatives at different concentration and also under the influence of various
physical conditions. Moreover different strains of *Pseudomonas* should be further studied for their hydrocarbon degradative properties at large scale. This work can be helpful to design consortia to biodegrade hydrocarbon pollution in natural environment and to have more insight in this area of research which needs more attention considering the prevailing pollution rate.

2.3. Microbial bioremediation of petroleum hydrocarbon: Regional status

As mentioned in the introduction (Chapter-1), Assam is a petro-chemically rich geographical location where environmental contamination (mainly soil contamination) by crude petroleum is very common. Therefore innovative bioremediation endeavour carries a very significant regional importance.

The superior utility of bacterial consortia with biosurfactant production ability in remediation of highly contaminated sites was established by Das and Mukherjee (2007). Three biosurfactant producing bacterial strains, namely, *Bacillus subtilis* DM-04, *Pseudomonas aeruginosa* M and *Pseudomonas aeruginosa* NM was applied for remediation of crude-oil contaminated soil samples. Aqueous solutions of biosurfactants obtained from the respective bacterial strains were applied along with their consortium. Furthermore, the tested soil samples were separately inoculated with the bacterial strains separately. To investigate the degree of biodegradation, the soil-phase total petroleum hydrocarbons (TPH) concentrations were examined after 120 days and compared to a control where the soil was treated with un-inoculated medium. Soil augmented with *P. aeruginosa* M and NM consortium and *B. subtilis* DM-04 strain showed that TPH levels were reduced from 84 to 21 and 39 g·kg⁻¹ of soil respectively.

In a laboratory scale investigation on bacterial biosurfactant for increasing solubility and metabolism of petroleum hydrocarbon, Bordoloi and Konwar (2009) highlighted that glycolipid biosurfactant produced by various strains of *Pseudomonas aeruginosa* could upsurge the solubilization of the PAHs molecules like fluorine, phenanthrene, pyrene in the growth medium. The strain *Pseudomonas aeruginosa* strains
also exhibited production of biosurfactants in the culture media through metabolism of the PAHs molecules.

In a pilot scale study on bioremediation and reclamation of soil contaminated with 20% (v/w) crude oil, Mukherjee and Bordoloi (2011) applied a bacterial consortium of three previously reported biosurfactant producing bacterial strain, namely, *Bacillus subtilis* DM-04, *Pseudomonas aeruginosa* M and *Pseudomonas aeruginosa* NM for 180 days. Bacterial consortium showed a significant reduction in total petroleum hydrocarbon level in contaminated soil (76% degradation) as compared to the control soil (3.6% degradation) 180 days post-inoculation. The GC analysis confirmed that bacterial consortium was more effective in degrading the alkane fraction compared to aromatic fraction of crude petroleum oil hydrocarbons in soil. The study supported the theory of application of bacterial consortium rather than individual bacterium for the effective bioremediation of soil contaminated with petroleum oil.

To formulate an active indigenous bacterial consortium for bioremediation of petroleum tar–polluted sites of Assam, India, and the potential of certain soil bacteria were assessed for the biodegradation of petroleum tar (Tanti and Buragohain 2013). In vitro enrichment cultures of five *Pseudomonas* spp. were found to metabolize petroleum tar. Complete degradation of low-molecular-weight hydrocarbons, and the consequent appearance of some additional peaks indicating the formation of intermediate metabolites during the degradation of petroleum tar was revealed through gas chromatography–flame ionization detection (GC-FID) analyses.

Hydrocarbon degradation ability of certain atmospheric nitrogen fixing bacteria was investigated by Mazumdar *et al.* (2015). In the study they have isolated nitrogen fixing (diazotrophic) bacteria capable of degrading petroleum hydrocarbons. Two bacterial strains were isolated from crude oil contaminated soil and identified by using their 16SrDNA as *Achromobacter* sp. and *Arthrobacter* sp. Both the isolates could degrade almost all major components of kerosene hydrocarbon within 60 days. Results of the present investigation revealed the potentiality of free living indigenous nitrogen fixing bacteria to degrade petroleum hydrocarbon and thereby contribute to bioremediation and bio-fertilization of crude oil contaminated soil.
Over the last decades, various studies with exciting outcomes have described the fruitful application of biosurfactant-producing, hydrocarbon-degrading bacterial consortia for reclamation of crude oil contaminated sites throughout the globe. However, except few, most of the studies were carried out in shake flask conditions at laboratory. The efficient hydrocarbon degrading microbes isolating from indigenous soil environment and their application in the fields for remediation of the hydrocarbon from contaminated soil or water is a thrust area of research. Keeping this in mind the present investigation was carried out, so that a method for remediation of contaminated sites of oil fields situated at upper Assam could be possible.