Chapter V

DISCUSSION

Assam has been explored for its various natural resources from since time back. Crude oil is one of these valuable natural resources. But with the advent of crude oil exploration and allied activities, environmental components had been found to pollute by the hydrocarbon compounds present in crude oil. In this context, bioremediation has become the first choice for the environmentalist to get rid of the problem of petroleum pollution.

The findings of the present investigation presented in the results have been discussed here; which is based on the support of a few reports in this field and other allied areas.

5.1 MOISTURE CONTENT

The moisture content was found to differ in control sites and oil contaminated sites for all the five locations. It was observed that in all the five crude oil contaminated sites, soil moisture was found to reduce as compared to the respective control sites. This result is in conformity with the findings of Akomeo (1981). Akomeo explained that it was possible because the volatile portion of the oil tend to evaporate while the heavier components migrate downward under the force of gravity. During its downward movement, the oil displaces and pushes the water down into the soil. The oil therefore occupies the soil interspaces resulting in reduced moisture content.

5.2 SOIL pH

In the present investigation it was found that hydrocarbon contamination always lead to a decrease in soil pH. The pH reaction of all the control sites was recorded to be slightly alkaline (pH 7.7-8.6). But with petroleum oil contamination the P^H values showed a shifting towards the acidic to neutral range (pH 6-7.5). Hydrocarbon biodegradation in soil has an optimum around pH 6.5–8.0 (Morgan and Watkinson, 1989). But substantial hydrocarbon mineralization activity has been reported in polar
soils at pH >8.8 (Aislabie et al. 1998; Whyte et al. 1999). The decrease in the pH of oil contaminated soils may be due to production of acidic intermediates such as phenolic acid, organic acid, esters and fatty acids through the degradation of alkanes as described by Fenchal and Blackburn (1979). Similar explanation for decrease in pH in oil polluted soils was also recorded by Odu (1981).

5.3 SOIL NUTRIENTS

The amount of total nitrogen and phosphorus was found to decrease in oil contaminated sites as compared to control sites, whereas the total carbon content was found to increase in oil polluted soil. Nutrients are very important ingredients for successful biodegradation of hydrocarbon pollutants especially nitrogen, phosphorus and in some cases Iron as described by Cooney (1984). Some of these nutrients could become limiting factor thus affecting the biodegradation process. Atlas also reported that when a major oil spill occurred in marine and freshwater environments the supply of carbon was significantly increased and the availability of nitrogen and phosphorus generally become the limiting factor for oil degradation. Deni and Penninckx (1999) also reported that an oil polluted soil is a nitrogen limited environment. Similar kind of observation was recorded in the present study. John et al. (2011) also reported that the total phosphorus and nitrogen content were impeded in the crude oil contaminated wetland soil.

5.4 C/N RATIO

The carbon to nitrogen ratio is an important determinant of optimal plant growth and microbial degradation of hydrocarbon in soil. It was found that oil contamination always impede this ratio. Very high ratios of C/N were recorded in all the five oil contaminated sites. In the present investigation, C/N ratio was recorded as high as 276:1. It was reported that hydrocarbon contamination results in high soil carbon/nitrogen ratio (e.g. >250:1, McLeod and Fraser, 1998) which may limit the optimum plant growth and also the growth of microorganisms used for degradation of different oil hydrocarbons in contaminated soil (Leahy and Colwell, 1991). Trindale et al. (2005) suggested that optimal ratio of carbon to nitrogen was 100:1.25 for bioremediation of hydrocarbon contaminated soil. The optimum C/N ratio of normal fertile soil to support plant growth
is around 25-30:1. Observation of the present study supports the fact that hydrocarbon pollution increases the C/N ratio many folds. Therefore, it can be concluded that hydrocarbon contamination results in extremely high carbon to nitrogen ratio, which will definitely hamper the growth of plants as well as the growth of hydrocarbonoclastic microorganisms.

5.5 METAL LOAD

The present study on the metal load in the oil contaminated soil revealed that contamination of soil with crude oil increases the amount of lead, cobalt, nickel, zinc, iron and manganese, which may affect both the plant and microorganisms. Keith and Telliard (1979) reported that the presence of heavy metals such as of lead, cobalt, nickel, zinc, cadmium, vanadium in crude oil may be mutagenic and carcinogenic for living organisms. Deni and Penninckx (1999) also reported the similar findings. John et al. (2011) observed the elevated level of these six metals in crude oil contaminated wetland soil. Thus it can be concluded that crude oil contamination always results in increase of metals like Lead, cobalt, nickel and zinc, manganese etc. which in turn may be detrimental for living organisms and therefore, needs urgent attention.

5.6 MICROBIAL LOAD IN CRUDE OIL CONTAMINATED SOIL

The current study revealed that heterotrophic bacterial isolates were dominant both in oil polluted and unpolluted soil compared to other physiological groups of microorganisms, probably because heterotrophic bacteria are more numerous in soil. Some of them are fast growing and capable of utilizing a wide variety of organic compounds including hydrocarbons for survival (Stainer et al. 1976). Again the population of hydrocarbon degrading bacteria in oil polluted soils increased many folds as compared to unpolluted soils. This result is in accordance with Atlas (1981) who described that hydrocarbonoclastic microbial population normally increase following the addition of oil to soil. Bragg et al. (1994), Harayama et al. (2004), Head et al. (2006) also separately reported that a typical soil, sand or ocean sediment contains significant numbers of hydrocarbon degrading microorganisms and their number increase considerably in oil polluted sites.
On the other hand, in the present investigation, it was observed that the oil pollution results in decrease in the number of nitrogen fixers in soil. Though crude oil affects the multiplicity of the diazotrophs, some tolerant species may come up in such polluted soils (John et al. 2011). Fenchal and Blackburn (1979) also reported similar kind of observation. They reported a decrease in the population of diazotrophs in the oil polluted soil as compared to unpolluted soil, which might be due to the fact that nitrogen fixing bacteria grow best in soils with neutral to alkaline pH (6.6-8.0) and may be retarded at pH of 4.0-4.5. Therefore, decrease in the pH of the soil caused by oil pollution may be attributed to the reduction in number of diazotrophic bacteria.

5.7 ISOLATION OF HYDROCARBON DEGRADING CUM NITROGEN FIXING BACTERIA

The numbers of hydrocarbon degrading microbial population naturally increases in oil polluted soil than that of unpolluted soil (Bragg et al. 1994, Harayama et al. 2004, Head et al. 2006). Hence for the present investigation, samples were collected from sites which were exposed to petroleum pollutants since a prolonged time. Hydrocarbonoclastic bacteria were isolated using a hydrocarbon enriched media, where light liquid paraffin (LLP) served as the single source of energy. The use of hydrocarbon enriched media for isolation of hydrocarbonoclastic bacteria from different samples had already been reported by several workers (Baruah and Deka, 1992).

A total of 18 strains of hydrocarbon utilizing bacteria were isolated which can grow successfully in LLP enriched media.

These 18 isolates were further subjected to grow on nitrogen deficient media (NDM). But only five isolates exhibited luxuriant growth in NDM. Rest of the isolates were incapable of utilizing atmospheric nitrogen to support their growth in NDM. Eckford et al. (2002) used the similar composition of NDM to isolate hydrocarbon degrading diazotrophs from jet fuel oil contaminated samples. They could isolate five such isolates.
5.8 BIODEGRADATION STUDIES

5.8.1 Bacterial density in kerosene amended soil

The population pattern of the five isolates varied from time to time when grown in kerosene hydrocarbon enriched soil. In case of the isolate AM02, AM05 and AM10, the individual bacterial cell number increased from day 1 to 45 days of inoculation. But after 45 days their cell numbers gradually decreased. However, in AM07 and AM14, the cell numbers kept on increasing upto 60 days. However, the increase in cell numbers was very fast upto 45 days, but the rate became slower after 45 days of incubation. This may be due to the fact that the three isolates AM02, AM05 and AM10 had utilized the nutrients at the maximum during the first 45 days after inoculation resulting in very fast growth in their population. But after 45 days the available nutrients and energy source got exhausted and consequently the bacterial population came down. The increase in population of AM07 and AM14 till 60 days of incubation may be attributed towards their slow growth with slow uptake of nutrients.

5.8.2 Biodegradation by individual isolate

Biodegradation was carried out by individual isolates as well as by mixed cultures. The five isolates namely AM02, AM05, AM07, AM10 and AM14 were found to be efficient hydrocarbon degrader. Mixed culture of the isolates could deplete all the major hydrocarbon present in kerosene oil. All the five isolates were identified through 16s r DNA sequencing. Based on the results of 16s r DNA analyses the isolate AM02 was identified as *Achromobacter* sp., Isolate AM05 as *Achromobacter* sp., AM07 as *Alcaligenes faecalis*, AM10 as *Arthrobacter* sp. and AM14 as *Acinetobacter junii*.

To carry out the bioaugmentation successfully, it would be necessary to select bacteria having a high capacity and the versatility to degrade the many components of petroleum products. Kerosene was known to contain short to middle size hydrocarbons in the range of C$_8$ to C$_{19}$, which include n-alkanes, iso-alkanes, cycloalkanes and aromatics (Bartha, 1986 and Solomons, 1990). It contains approximately 80% aliphatic and 20% aromatic fractions (Bacosa *et al.* 2010).

Consequently, bacteria able to grow on this carbon source would easily acquire the ability to degrade a wide variety of hydrocarbon components of different petroleum
products. Therefore, to investigate the hydrocarbon degrading ability of the bacterial isolates, kerosene was used as a sole carbon source for the present study.

The target hydrocarbon i.e., kerosene was run as standard in GC-MS. Non inoculated flasks mixed with kerosene were included as control for abiotic losses. It helped to determine the actual fractions of kerosene hydrocarbons acted upon by the isolates. Petroleum hydrocarbons contain many volatile components towards carbon chain length \(< C_9\), which can be lost by physical or abiotic factors such as volatilization etc. In the present study, it was found that the lower hydrocarbons (below chain length \(C_9\)) were almost 100% depleted in the control kerosene. This observation is in conformity with the findings reported by Malik and Ahmed (2012). They explained that the lower alkanes \(C_8\), \(C_9\) and \(C_{10}\) present in crude oil depleted 100% in 12 days without inoculum, indicating abiotic removal by physical factors such as volatilization and leaching effect. Luzi et al. (2006) also explained that the disappearance of significant quantities of short chain n-alkanes (\(C_8\)-\(C_{11}\)) of crude oil during the experiment could also be related to abiotic factors, such as evaporation and dispersion. Again according to Choi et al. (2002), 94-98% loss of kerosene from the upper 5 cm layer of soil within 2 days was evidently a result of physicochemical processes such as leaching deeper into the sediment and evaporation. In the present study it was recorded that all the \(C_8\) and \(C_9\) compounds present in standard kerosene were missing in control. Gas chromatographic studies of control kerosene revealed that the major hydrocarbons fractions constituted of \(C_{10}\) to \(C_{19}\). The first prominent peak appeared in RT 9.31, which was detected as n-undecane (\(C_{11}H_{24}\)). At the RT 8th to 9th minutes, some very insignificant peaks developed which were found difficult to identify. These peaks might be of short chain hydrocarbons of carbon length \(C_9\) to \(C_{10}\). Though numerous peaks emerged within ninth minute till twenty sixth minutes in the chromatogram, only 22 prominent peaks were chosen for the current study. This is because of the fact that the unselected peaks were very small and could not be identified (area coverage <0.1%). The highest percentage area in the chromatogram was given by the peak emerged in the 9.31th minutes (6.974% area coverage). The compound was detected as n-undecane (\(C_{11}H_{24}\)). It was followed by the peak at RT 22.20 having area coverage of 6.576 % and detected as heptylcyclohexane (\(C_{13}H_{26}\)). Out of the 22 peaks, the smallest peak was detected in
16.81th minutes (0.518 % area coverage) and the component was 2,6,8,10 Tetramethyldecane (C\textsubscript{14}H\textsubscript{30}).

Therefore, from the findings of the present investigation, it can be concluded that the short chain hydrocarbon (C\textsubscript{8} to C\textsubscript{9}) fraction of kerosene oil were disappeared by abiotic means.

When kerosene was treated by the isolate AM02, after 60 days of incubation, the total numbers of peaks decreased. A total of 170 peaks appeared within RT 5\textsuperscript{th} to 26\textsuperscript{th} minute. However, only 11 peaks were very much prominent and the corresponding compounds to these peaks were identified. These 11 peaks were completely new peaks, found to be absent in the control. It was found that hydrocarbons of carbon chain length C\textsubscript{10} and C\textsubscript{11} were prominent after 60 days of biodegradation. The peak emerged in RT 7.31\textsuperscript{th} minutes was recorded to have the highest percentage area coverage (4.920). The compound corresponding to this peak was detected as n-Decane (C\textsubscript{10}H\textsubscript{22}). Whereas the least area peak was detected in the 14.74\textsuperscript{th} minute (0.319 %). The compound was 2-Methylnaphthalene (C\textsubscript{11}H\textsubscript{10}). The chromatogram also revealed the appearance of some new peaks within RT 5.24\textsuperscript{th} to 7.31\textsuperscript{st} minutes. These peaks consisted of hydrocarbon components of carbon chain length C\textsubcript{9} to C\textsubscript{10}. These newer compounds assumed to appear as a result of biodegradation of high molecular weight compounds or gathering of fractions resulted from biodegradation of compounds which experienced peak area declining. The similar observation was also documented by Basuki \textit{et al.} (2011). They observed that biodegradation of used engine oil resulted in generation of some new compounds of low molecular weight.

One of the important finding was that all the peaks detected in the control kerosene were not completely degraded by the isolate AM02. The peaks in RT 12.13, 14.08, 16.51, 18.95, 19.15 and 24.38\textsuperscript{th} minutes appeared in control were found to degrade partially in treated kerosene. A maximum of 84% area depression was observed in the peak appearing at RT 16.51\textsuperscript{th} minutes. The area depression for those peaks ranged from 58% to 84%. Similar findings on various hydrocarbons were reported by several workers worldwide. Lal and Khanna (1996) reported 58% overall degradation of Indian crude oil samples by \textit{Acinetobacter calcoaceticus} and \textit{Alcaligen odorans} in combination over a 15
day period. Ijah (1998) reported that bacteria and yeast isolates from tropical soils capable of degrading 52% and 69% of crude oil in 16 days, respectively, where; the isolates primarily degraded the alkanes over specific carbon number ranges. According to Dott et al. (1989), degradation percentages of fuel oil ranged from 24 to 64% from commercially available inocula for oil degradation. In a similar study, Sugiura et al. (1997) reported 19 to 34% degradation of total extracts of different crude oils by non-defined bacterial culture obtained by enrichment in oil. Pallitopongarnpim et al. (1998) reported 26 to 63% biodegradation of Tapis crude oil by three different single bacterial isolates.

The bacterial strain AM05 was recorded to deplete all the major hydrocarbon components of kerosene oil after 60 days of inoculation. The products of biodegradation of kerosene by AM05 resulted in the formation of some short chain hydrocarbons of chain length ranging from C₁₀ to C₁₃. A total of 13 new hydrocarbons were detected after biodegradation. No partially degraded kerosene compounds were recorded after treatment.

The isolate AM07 was also found efficient in the degradation of the major kerosene components, mainly the aliphatic fractions. However 100% attack on the components was not achieved. Four components namely n-undecane and one of its methyl derivative, n-dodecane and n-tridecane were found to be degraded partially. The compounds formed by degradation of kerosene were mainly some short chain hydrocarbons (C₉ to C₁₂ compounds).

The bacterium AM10 could degrade various kerosene components except n-undecane, n-dodecane, n-tridecane, n-tetradecane, n-pentadecane and heptylcyclohexane. Again in case of isolate AM14, four compounds namely n-undecane, n-dodecane, n-tridecane, n-tetradecane were not fully degraded by the strain. Whereas, the rest of the compounds were depleted completely.

Therefore, it can be concluded that the three hydrocarbons n-undecane, n-dodecane, n-tridecane are difficult to be biodegraded completely by the bacteria AM07, AM10 and AM14. Isolate AM07 could degrade n-undecane 73.8%, whereas AM10 and AM14 could do 62.4% and 92.4% respectively. The degradation was found 82.8%,
55.6% and 78% for n-dodecane by the isolates AM07, AM10 and AM14 respectively. Whereas AM02 could degrade 80-84% of dodecane and its methyl derivatives. For n-tridecane, the degradation was found 83.6%, 76.8% and 58.2% for AM07, AM10 and AM14 respectively. The compound n-tetradecane present in kerosene oil was not fully degraded by any of AM02, AM10 and AM14. The degree of depression was 76.5%, 62.3% and 86.3% respectively. So, it can be established from the results of the study that the degree of depression of the same compound by different bacteria can vary significantly.

The five isolates studied in the present work were found to be efficient degrader of various hydrocarbon components of kerosene oil. All these five isolates are reported previously as potent hydrocarbon degraders by many workers separately worldwide.

Ebrahimi et al. (2012) reported hydrocarbon degrading efficiency of some isolated bacteria from oil polluted sites, such as *Pseudomonas stutzeri*, *Achromobacter* sp. and *Acinetobacter* sp. Leathy and Colwell (1990) explained the biodegradation of petroleum oil by some more bacterial genera such as *Achromobacter*, *Arthrobacter*, *Alcaligenes* etc.

Two of the potent hydrocarbon degraders AM02 and AM05, isolated from different crude oil contaminated locations, belonged to the genus *Achromobacter*. But the hydrocarbon degradation efficiency of the two isolates was not similar. AM05 was proved more powerful degrader than AM02. AM02 could not fully degrade some of the compounds of carbon length C15 and above. However the rest of the components were broken down to short length carbon compounds. Basuki et al. (2011) reported the generation of some new hydrocarbon compounds of low molecular weight and short chain length as a result of biodegradation of used engine oil. Whereas the isolate AM05 was capable of complete degradation of all the kerosene components to lighter fragments. The resultant lighter fractions ranged from C10 to C13 hydrocarbons. Very little literature was available to document in the present context of the involvement of *Achromobacter* sp. in petroleum hydrocarbon degradation. However similar organism was reported to degrade some complex fractions of crude oil by Guo et al. (2008). They
reported the involvement of *Achromobacter* sp. in degradation of carbazole, a PAH present in petroleum hydrocarbon.

When AM 07 was studied for evaluating its petroleum oil degradation capacity, it was found that except Undecane to Tridecane compounds, this organism could degrade rest other hydrocarbons fully present in kerosene oil. The percent degradation of undecane, dodecane and tridecane by AM07 varied from 50 to 84%. The findings are similar to that of Lakshmi *et al.* (2011). They reported *Alcaligenes faecalis* to have the ability to biodegrade phenanthrene, a model PAH. The strain metabolized 98 % of phenanthrene which implied *A. faecalis* as a promising candidate for bioremediation. *Alcaligenes sp.* has been investigated and documented as a potential degrader obtained from crude oil contaminated soil (Vinas *et al.* 2005) and solid waste oil samples (Weissenfels *et al.* 1990, Khanna *et al.* 1996). The same bacterium was also mentioned by Chuma *et al.* (2010). They established that *Alcaligenes* sp. after 40 days of exposure to untreated produced water reduced 80% of the oil and grease content. Okaro *et al.* (2010) used pure culture of *Alcaligenes* sp. to treat hydrocarbons in produce water from an oil production facility in Nigeria. They found the bacterium to be highly efficient in degradation of alkanes and aromatic hydrocarbon components.

Kayode- Isola *et al.* (2008) found the genus *Alcaligenes* as one of the efficient members in biodegradation of diesel oil.

The isolate AM10 (*Arthrobacter* sp.) was also found as a good petroleum oil degrading agent from the results of current investigation. It can also degrade the main kerosene components and can utilize as a source for carbon and energy. This result is similar with the findings of Seo *et al.* (2006). They explained that the genera *Arthrobacter* is involved in degradation of the PAHs phenanthrene, dibenzothiophene and carbazole. Similar organism was also reported by Casellas *et al.* (1997) and Jones *et al.* (1983) to degrade several alkyl aromatic component of crude oil.

However, this organism was not reported anywhere to involve in degradation of aliphatic fractions of crude oil.
The results of biodegradation of petroleum hydrocarbons by the strain AM14 \((\textit{Acinetobacter junii})\) is similar with the observation by Basuki \textit{et al.} (2011). They reported the organism \textit{Acinetobacter junii}, isolated from soil contaminated with used engine oil to have capability to use hydrocarbons in used engine oil as the sole carbon source and energy, also it significantly degraded almost all hydrocarbon compounds in used engine oil. In the present study it was also observed that \textit{Acinetobacter junii} can degrade almost all the major components of kerosene hydrocarbon, but can partially degrade the range from undecane to tetradecane. Same organism was found to be capable of utilizing n-alkanes of chain length \(\text{C}_{10}-\text{C}_{40}\) as a sole source of carbon (Holst \textit{et al.} 2007).

### 5.9 NITROGEN FIXATION BY INDIVIDUAL ISOLATE

The nitrogen deficient environment in hydrocarbon contaminated sites always favors the development of atmospheric nitrogen fixing microorganisms. In the present study the five bacterial strains isolated from various crude oil polluted sites were tested for the presence of nitrogenase enzyme. Nitrogenase activity of individual isolate was measured at 24 hours interval for a period of 7 days. The ethylene concentration significantly increased from day 1 to day 7 in all the isolates. The highest value of ethylene after seven days incubation with acetylene was recorded in isolate AM 07, whereas that of the lowest was found in isolate AM14. Ethylene produced by the isolates at different interval of time varied in concentration amongst the strains. Determination of nitrogenase activity by ARA in hydrocarbon degrading bacterial species has been reported by various workers. Eckford \textit{et al.} (2002) isolated the bacterium \textit{Pseudomonas stutzeri} from fuel contaminated Antarctic soil. They stated that this bacterium was capable of utilizing jet fuel as well as a significant nitrogen fixer. John \textit{et al.} (2011) reported seven nitrogen fixing bacterial species from the rhizosphere region of two legumes \textit{Calopogonium muconoides} and \textit{Centrosema pubescens} grown in crude oil contaminated soil. Similar result was also observed by Laguerre \textit{et al.} (1987). Their bacterial isolates BPD1 and BPD2 also showed an elevated level of ethylene concentration during the first week of incubation which continued till the fourth week. Nitrogenase activity measurement through ARA had also been reported in bacteria isolated from sites other than oil contamination. Doty \textit{et al.} (2009) also studied the
nitrogenase activity of five bacterial isolates from plant through acetylene reduction assay. They studied the level of ethylene produced for a period of 72 hours. In the present investigation *Alcaligenes faecalis* (isolate AM07) revealed relatively higher nitrogenase activity. The same organism was also previously isolated by Prantera *et al.* (2002) from oil contaminated soil and found to degrade gasoline aromatic hydrocarbon and capacity to fix nitrogen.

Goerz *et al.* (1961) isolated a species of the genus *Achromobacter* actively involved in the atmospheric nitrogen fixation activity. Wedhastri *et al.* (2012) reported free living nitrogen fixing *Achromobacter* sp from coffee rhizosphere from East Java which was the best nitrogen fixer amongst sixty isolates.

Nitrogen fixation by *Arthrobacter* sp. had been reported by many workers worldwide (Abbas, 2008). Efroymson (1991) explained the hydrocarbon degradation as well as nitrogen fixing capacity of *Arthrobacter* sp.

A relatively lower nitrogenase activity was detected in isolate AM14, (identified as *Acinetobacter junii*). The nitrogen fixing capacity of this bacterium is not previously documented in any literature published nationally and internationally. Therefore, this microorganism can become a very potent agent for bioremediation and biofertilization of oil polluted cropland.

From the above discussion it can be concluded that all the five bacterial strains isolated from crude oil contaminated soil have the potentiality to fix the atmospheric dinitrogen. However, the nitrogenase activity varied from strain to strain under in vitro condition.

Successful stimulation of nitrogen fixation and petroleum hydrocarbon degradation in indigenous microorganisms may decrease exogenous nitrogen requirements and reduce environmental impacts of petroleum pollution. This study explored the nitrogen fixing capacity of the hydrocarbon degrading bacteria isolated from petroleum enriched soil. Five different strains were able to grow successfully and sufficiently in NDM out of the ten isolates tested. It was obtained from the present investigation that all these five bacterial isolates were efficient nitrogen fixers. The
amount of fixed nitrogen varied amongst the isolates. AM02 was found to have the highest nitrogen fixing capacity (17.45 mg l\(^{-1}\)) and AM14 showed the least (9.47 mg l\(^{-1}\)). Similar observation was reported by Davis et al. (1964) on different bacterium. They explained that *Pseudomonas methanitrificans* capable of utilizing methane as a sole source of energy can fix 70 mg l\(^{-1}\) nitrogen in an average when incubated for a period of two months. In large scale experiments, the maximum nitrogen fixed was 53 mg l\(^{-1}\) by Davis and his coworkers. But in the current study the value of fixed nitrogen by the isolates were low as compared to that of Davis et al. (1964). This may be due to the fact that the culturing conditions provided to nitrogen fixing strains were not optimum or had not occur any cell autolysis during the two months of incubation. The findings of the present study are in conformity with Thavasi et al. (2006). They could isolate the bacterium *Azotobacter chroococcum* from crude oil contaminated marine environment and found that the organism could fix 4.2 mg nitrogen per liter of nitrogen deficient culture broth in 96 hours.

It was found that there was difference in the amount of fixed nitrogen in NDM and in soil amongst the isolates. In soil, the highest amount of nitrogen was fixed by AM10, followed by AM02, AM07, AM05 and AM14. Whereas in the NDM, that of the maximum was given by AM02, followed by AM10, AM07, AM05 and AM14. A considerable degree of difference was also found in the nitrogen fixing rates between soil and nitrogen deficient media. For every isolate the fixed nitrogen content was less in nitrogen deficient media than in soil. The strain AM02 fixed nitrogen at the rate of 59.84 mg/kg in soil, but the amount was found 17.45mg/l in NDM. Likewise, AM10 was found to fix 63.96 mg/kg in soil, but a lower amount of 16.65mg/l in NDM. This result is in conformity with the findings of Kizilkaya (2009) on his experiment with nitrogen fixing *Azotobacter* spp. He explained that the nitrogen fixing capacity of *Azotobacter* sp. was better when inoculated to soil than inoculated to nitrogen deficient Ashby media.

### 5.10 HYDROCARBON DEGRADATION AND NITROGEN FIXATION BY THE CONSORTIUM

Successful stimulation of N\(_2\) fixation and petroleum hydrocarbon degradation in indigenous microbial consortia may decrease exogenous nitrogen requirements and
reduce environmental impacts of bioremediation following petroleum pollution. This
study explored the biodegradation of petroleum pollution by indigenous nitrogen fixing
microbial consortia.

Crude oil is made of a mixture of compounds. A pure culture of a microorganism
metabolizes only a limited range of hydrocarbon substrates (Britton, 1984). Biodegradation of crude oil requires a mixture of different bacterial groups to degrade a
wider range of hydrocarbon (Bordenave et al. 2007, Cagnon et al. 2011). Hence in terms
to achieve successful bioremediation of petroleum contaminants, microbial consortia
with the metabolic ability to degrade a wide range of hydrocarbons are preferred over a
pure culture (Ghazali et al. 2004). For this reason, biodegradation experiments were
directed towards understanding the roles of individual isolates in a mixed culture and to
develop a formulation that can be directly employed into a contaminated area. In the
present work, five bacterial strains were tested separately for their biodegradation
activity. Finally the five strains belonging to several different genera having diverse
range of oil degrading capacity were mixed at equal proportion and their combined effect
on biodegradation of kerosene was studied. The consortium was capable enough for
degradation of all the major hydrocarbon constituents of kerosene oil. The nine
prominent and new peaks appeared after 60 days was a result of biodegradation of
kerosene components by the consortium. In case of biodegradation carried out by
individual isolate, the resultant products were mainly branched alkanes and aromatics.
The aromatic fractions are however more resistant and difficult to degrade further to
innocuous products as compared to other groups of hydrocarbons (Wang et al. 1998).
The aromatics are even more hazardous to the environment. The present consortium was
capable enough to degrade the kerosene components to some short chain and non toxic
alkanes of C_{10} to C_{12} chain length, which would be quite easier to degrade by some other
consortium already documented and available. Such a concept of formulation of
consortia comprising many bacteria with overall broad enzymatic capacities for total
degradation of petroleum pollutants are already tried and became successful to a large
extent. The observation of the present study is in conformity with the findings reported
by Malik and Ahmed (2012). They prepared a mixed consortium with fifteen bacteria
isolated by enrichment technology collected from crude oil contaminated site. This
consortium was checked with 10% kerosene oil for a period of 24 days. The consortium was capable of removing 94.64% aliphatic and 93.75% aromatic fraction of kerosene oil.

The findings of Chhatre et al. (1996) also supports the observation of the present study in the consortium. They reported a 95% degradation of the major peaks resolved in GC of Bombay high crude oil by a defined consortium constructed with four oil degrading bacterial strains for their capability to degrade saturates and aromatics.

The total amount of fixed nitrogen by the present consortium was also found higher as compared to the amount fixed by the isolates individually. In soil, the strain AM10 was recorded to fix maximum amount of nitrogen (63.96 mg/kg) out of the five isolates. But when the five strains were mixed in equal proportions and applied to soil, the total amount was recorded 81.50 mg/kg. Hence, it can be concluded that the five strains together have an enhanced effect on nitrogen fixation.

Therefore, based on the results of the present research, it is inevitable that the consortium formed by these five bacterial strains, which have hydrocarbon degrading and nitrogen fixation ability, can be used in oil contaminated croplands to overcome the problem of oil pollution and also to enhance fertility status of those sites.