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DEGRADATION OF KEROSENE HYDROCARBON BY INDIGENOUS DIAZOTROPHIC BACTERIA ISOLATED FROM CRUDE OIL CONTAMINATED SOIL

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Abstract: Hydrocarbon contamination significantly increases supply of carbon, but the availability of nitrogen and phosphorus generally become the growth limiting factors for indigenous oil degraders. This nitrogen imbalance selectively favors the development of the soil asymbiotic dinitrogen fixing microorganisms. The present study was undertaken to isolate such diazotrophic bacteria capable of degrading petroleum hydrocarbons. Two bacterial strains were isolated, 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. Hydrocarbon degradation by the isolates was studied under Gas chromatography-Mass spectroscopy (GC-MS) and the nitrogenase activity of the isolates was measured by Acetylene Reduction Assay. 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.

Key words: Bioremediation; Crude Oil Contamination; Diazotrophic Bacteria; Gas Chromatography; Hydrocarbon

INTRODUCTION

With the rapid industrial and economic growth, there has been a significant increase of crude oil contamination throughout the world. A major problem associated with petroleum hydrocarbon contamination is high soil carbon/nitrogen (C/N) ratio (eg. >250:1, McLeod and Fraser, 1998). Though the hydrocarbon degrading microorganisms are ubiquitous in nature but are found at relatively higher densities in petroleum contaminated sites (Bragg *et al.*, 1994; Harayama *et al.,* 2004; Head *et al.,* 2006). But a higher C/N ratio limits the growth of these indigenous microorganisms (Leahy & Colwell, 1991). Again nutrients are very important for successful biodegradation of petroleum pollutants; especially nitrogen, phosphorus and in some cases Iron by microorganisms (Cooney, 1984). However, the nitrogen imbalance created by oil spills selectively favors the development of the soil asymbiotic dinitrogen fixing microorganisms (Odu, 1977). Therefore, heterotrophic nitrogen fixing activity in contaminated soil may be important for natural attenuation of oil spills by providing combined nitrogen fixing to hydrocarbon degrading microbes, indirectly enhancing bioremediation of such sites. Some strains of the bacterial genera, namely, *Pseudomonas, Azospirrilum, Azotobacter* are capable of both hydrocarbon degradation as well as nitrogen fixation (Eckford *et al.,* 2002, Thavasi *et al.,* 2006).

The present study was undertaken with an objective to isolate such indigenous diazotrophic bacteria capable of degrading petroleum hydrocarbons from crude oil contaminated sites which could be efficiently used to remediate oil polluted croplands and to recover soil fertility.

MATERIALS AND METHODS

Soil Samples

Crude oil contaminated soil samples were collected from various oil drilling sites of Assam aseptically in sterile polythene bags. Samples (500g each) were collected from subsurface (depth ranging from 5cm to 25cm) as the hydrocarbon degrading bacterial population has been reported to be maximum in summer and in subsurface area (Atlas, 1984). The samples were stored at -4°C for further analysis.

Isolation of Crude Oil Degrading Nitrogen Fixing Bacteria

For the isolation of hydrocarbon degrading bacteria, M3 enrichment media was used (Baruah and Deka, 1995). Constituents for the M3 media per litre of deionized water are as follows: 4 g of NH_4NO_3, 2 g of $NaNO_3$, 4 g of KH_2PO_4, 4 g of Na_2HPO_4, 0.5 g of $MgSO_4$, 0.01 g of $CaCl_2$, 0.01 g of $FeSO_4$, 0.007 g of $ZnSO_4$, 0.002 g of Na_2MoO_4, 2% (v/v) Light Liquid Parafin (LLP), pH 6.8. 0.5 g of soil sample was transferred to a conical flask containing 50ml of M3 medium. The flasks were incubated at 36° C for 7 days in a rotary shaker at 150 rpm. After incubation, the enrichment cultures were serially diluted and plated on the M3 media containing 1.5% agar and 2% (v/v) LLP. The plates were then incubated at 36° C for 72 hours. The successfully growing strains were then transferred in Nitrogen Deficient Media (Eckford *et al.,* 2002) and incubated at

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28°C for 72 hours in a rotary shaker at 150 rpm. The successfully growing strains were transferred to slants prepared by Nitrogen Deficient Media (NDM) enriched with 2% (v/v) light liquid paraffin. The constituents of NDM are as follows per liter of deionised water: 0.5 g of K_2HPO_4, 0.2 g of $MgSO_4.7H_2O$, 0.1 g NaCl, 0.02 g of $CaCl_2$, 0.01 g of $FeCl_3$, 0.002 g of $Na_2MoO_4.2H_2O$, 0.01 g of $MnSO_4$, pH 6.8-7.0.

Studies on Biodegradation Activity

For this purpose, a set of two screw capped culture flasks (500 ml) containing 100 g of sterilized oil free soil were prepared for each isolate. 10 ml of sterile kerosene (purchased from local gas station, sterilized by filter sterilization) was added to each flask inside a laminar flow hood. One ml inoculum (Bacterial cell number was adjusted to give initial number 1 X 10^8 CFU ml^{-1}) of the isolate was added to only one conical flask. The other conical flask served as control. All the conical flasks were then incubated at 36°C in an incubator for a period of 60 days.

After desired interval of time, 20 g of soil was taken out from the conical flask and mixed with equal volume of anhydrous Na_2SO_4. Then the mixture underwent soxhlet extraction with dichloromethane as extracting solvent for 24 hours. The extract was then concentrated with a rotary evaporator. The extracts were sealed in dry glass vials and stored at 4°C until analysis.

Gas Chromatography Analyses of Kerosene Biodegradation

Gas chromatography analyses were performed by Thermo Trace GC-MS Ultra equipped with flame ionization detector. Nitrogen was used as carrier gas and the speed was maintained at 1ml/min. One µl sample was injected with a injection port maintained at 300° C. Column temperature was maintained at 60° C for one min and then raised to 180° C at the rate of 5° C per min, then raised to 280° C for 10 min. Auto sampler AS-3000 was used for sample injection.

Estimation of Nitrogen Fixation

The acetylene reduction assay was used for examining the nitrogen fixing activity of the bacterial isolates. 15 ml glass vials filled with 5 ml of culture suspension were stoppered with air-tight rubber septa (subaseals), incubated under a gas mixture (which had been substituted with 10% acetylene, i.e.1 ml of air was removed using a syringe with needle, and 1 ml of acetylene was injected in the 10 ml gas space).Such vials were placed under standard growth conditions for 7 days. One ml aliquots of gaseous phase were removed after 24 hours interval for 7 days from each vial and injected into a preconditioned Bruker Gas Chromatograph model GC 650, housing a two meter long Porapak R stainless steel column and a flame ionization detector. The column temperature was maintained at 100°C and injector and detector at 110°C. A flow rate of 35 ml min^{-1} of N_2 served as the carrier gas. Standard ethylene gas was used for calibration and calculations. Positive nitrogen fixation activity of bacterial cultures was demonstrated by increased ethylene concentration over time.

The amount of nitrogen fixed by individual isolate was estimated under laboratory condition from cell free culture broth by adopting micro kjeldhal method. 1 liter of Nitrogen Deficient medium was inoculated with bacterial isolate and incubated at 28°C for 3 days. After incubation the broth was centrifuged at 12000 rpm for 10 minutes. The supernatant was then used to determine the amount of fixed nitrogen. Parallel control was run without any isolate.

Identification of the Isolates

Isolates were identified by using their 16S r DNA. Phylogenetic tree was constructed using MEGA 4.

Statistical Analysis

Statistical analysis was carried out by SAS 9.3 software. Three observations were taken for each of the characters and data were subjected to Fisher's method of analysis of variance. Once significant difference was noticed, data was subjected to Post Hoc range tests.

RESULTS

Isolation of Hydrocarbon Degrading and Free Living Nitrogen Fixing Bacteria

In the present study, bacterial strains were isolated based on the ability to grow in hydrocarbon enrichment and nitrogen deficient media. Two such bacterial strains (named AM05 and AM10) were isolated from soil sample SS1 and SS2 respectively. The density of hydrocarbon degrading bacteria in the soil samples from where AM05 and AM10 were isolated, was found to be 8.2X10^7 and 6.7X10^7 CFU g^{-1} respectively. Comparatively the numbers of nitrogen fixing bacteria were lower which was recorded to be 4.3X10^6 and 2.7X10^6 CFU g^{-1} respectively (Figure 1). In case of the crude oil contamination free soil, the nitrogen fixing bacterial population was very high, up to 9.2X10^7 and 12.7X10^7 CFU g^{-1} respectively. But the hydrocarbon degrading bacterial population was extremely low in such soil (Table 1).

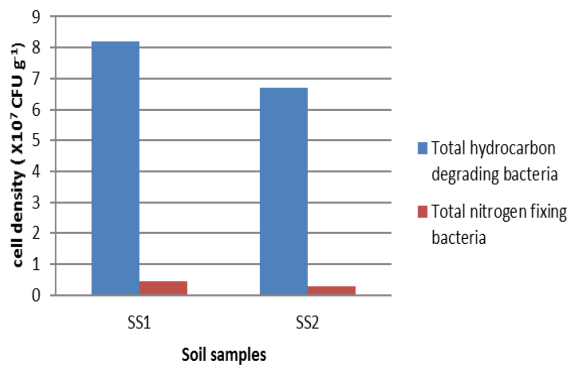

Figure1: Total hydrocarbon degrading and nitrogen fixing bacterial density in crude oil contaminated soil

Table 1: Microbial load in soil

Soil sample	Total bacteria ($CFUg^{-1}$)	Total hydrocarbon degrading bacteria ($CFUg^{-1}$)	Total nitrogen fixing bacteria ($CFUg^{-1}$)
SS1	2.4 ±0.42 x 10^9	8.2 ±0.35x10^7	0.43±0.17x10^6
SS2	5.8 ±0.5 x10^9	6.7 ± 0.23x10^7	0.27±0.22x10^6
Crude oil free soil 1	7.2 ± 0.12 x10^9	2.4 ± 0.16x10^4	9.2±0.57x10^7
Crude oil free soil 2	9.6 ±X0.33 10^9	10.0± 0.46x 10^4	12.7±0.31x10^7

Values are the mean of 3 replicates ± SE of mean

Identification of the Isolates

The results of 16S rDNA sequences confirmed that strain AM05 has similarity (99%) with *Achromobacter sp.* (Figure 2) and culture AM10 has 99% similarity with *Arthrobacter sp* (Figure 3)

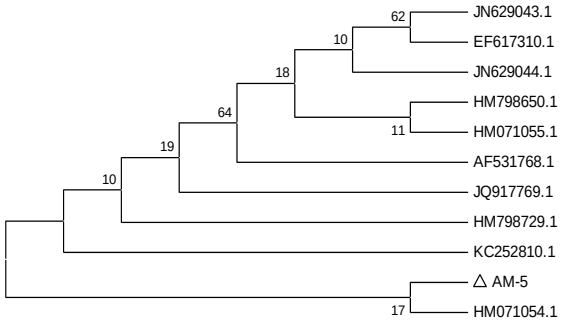

Figure 2: Phylogenetic analysis was inferred by Neighbor joining method using MEGA 4

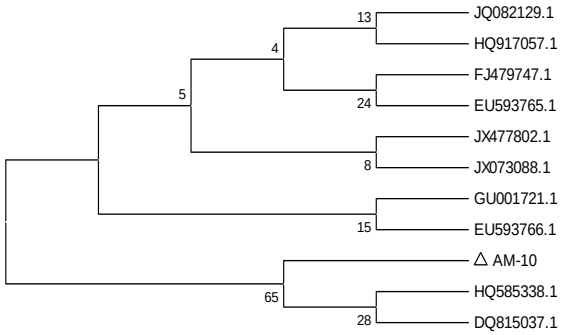

Figure 3: Phylogenetic analysis was inferred by Neighbor joining method using MEGA 4

GC Analyses of Kerosene Biodegradation

Figure 4 shows the gas chromatogram of kerosene oil before biodegradation, which composed of 22 prominent peaks. The main components peak appeared from retention time (RT) ninth minute to twenty seventh minutes. The main classes of hydrocarbons in kerosene before biodegradation were n-alkanes, iso-alkanes and aromatics ranging carbon chain length from 10 to 19. Figure 5 explains the biodegradation of kerosene by AM05 after 60 days of incubation. A total of 13 new peaks were observed which were found to be absent in kerosene before biodegradation. Alkanes consisting of 13 carbons were the main component. In case of biodegradation by AM10 (*Arthrobacter* sp.), only 7 peak components were detected after 60 days of biodegradation (Figure 6). Carbon length of these hydrocarbons ranged from 10 to 13. Mainly straight chain saturated hydrocarbons were prominent.

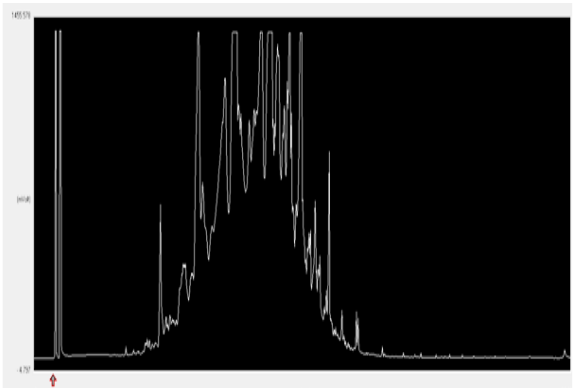

Figure 4: Gas Chromatogram of Kerosene before biodegradation

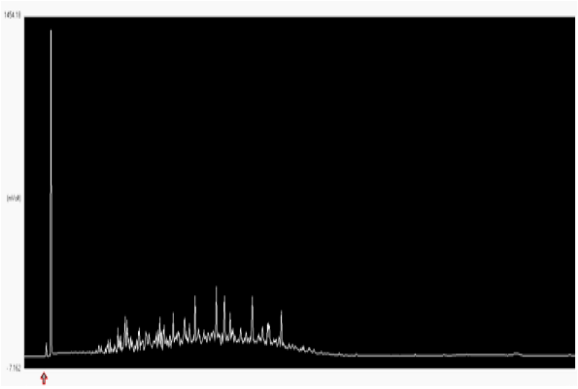

Figure 5: Gas Chromatogram of Kerosene after biodegradation by AM05

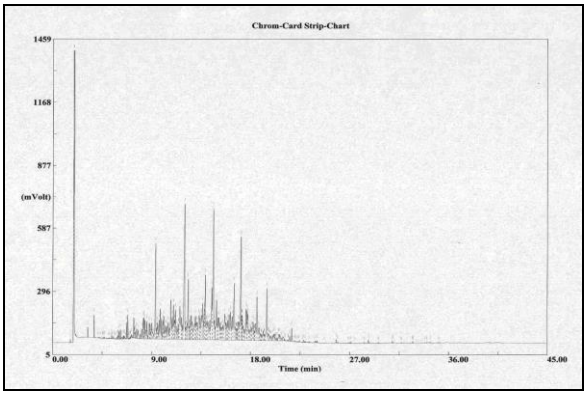

Figure 6: Gas Chromatogram of Kerosene after biodegradation by AM10

Acetylene Reduction Assay

Isolates AM05 and AM10 revealed positive result in the acetylene reduction assay. In case of both the isolates, ethylene production increased from day1 to day7 (Figure 7). In the present investigation, out of the two isolates studied, AM05 (*Achromobacter* sp.) showed maximum nitrogenase activity throughout the course of experimentation. After 24 hours of incubation with acetylene, AM05 produced 48.10±0.55 ppm of ethylene, which increased upto 162.40±0.28 ppm in the 7th day. Likewise, in case of AM10 the value was 43.1±0.25 ppm in the first day which increased to 133.63±0.26 ppm in the 7th day.

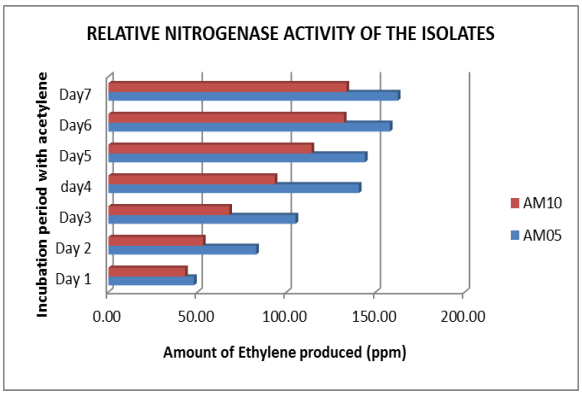

Figure 7: Relative nitrogenase activity of the three isolates

After two months of incubation in NDM, AM05 could fix nitrogen on an average of 12.06±0.67 mg l^{-1} whereas AM10 showed a relatively higher amount of fixed nitrogen i.e., 16.64±0.60 mg l^{-1} (Figure 9). In the present study, the rate of nitrogen fixation of isolates significantly increased till 30 days of incubation and then the rate declined upto 45 days (figure 8). Fixation remained stagnant for all the isolates after 45 days. This may be due to the fact that the nutrients used in the culture media were exhausted or the organisms had reached a stationery phase by the end of 45 days of incubation.

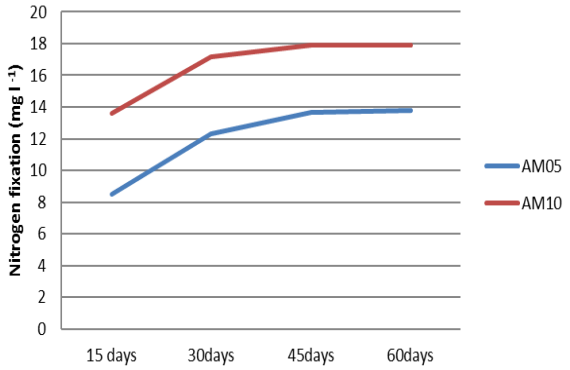

Figure 8: Nitrogen fixation by two isolates over a period of two months

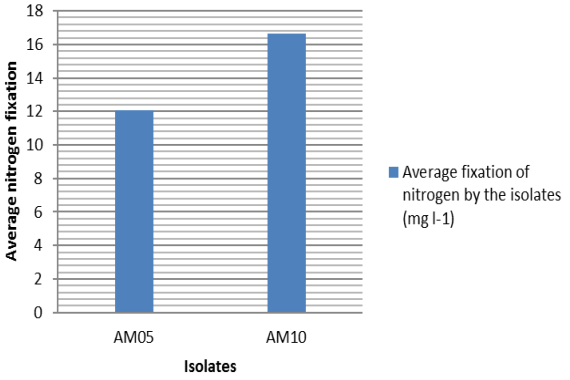

Figure 9: Average amount of nitrogen fixation (mg/l) by the two isolates

DISCUSSION

It is evident from the present study that oil pollution favors the growth of hydrocarbon degraders in soil. Bragg *et al.,* (1994) 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. Fenchal and Blackburn explained that the low counts of nitrogen fixing bacteria in oil contaminated soil may be due to the fact that these organisms grow best in soils with neutral to alkaline pH (6.6-8.0). Hence the low pH of oil contaminated soil resulted in the low counts of nitrogen fixing bacteria in the oil contaminated soil.

Analyses of hydrocarbon components by GC-MS is performed to know the changed composition of hydrocarbons done by biodegradation activity of an organism. AM05, an *Achromobacter* sp., however was reported previously to degrade some complex fractions of crude oil by Guo *et al.,* (2008). Isolate AM10 was identified as *Arthrobacter* sp. various aromatic hydrocarbons are degraded by the genus *Arthrobacter* (Seo *et al.,* 2006). In the present study, AM05 and AM10 could degrade the long chain complex hydrocarbons of kerosene to low carbon containing simple alkanes within 60 days of inoculation. These resultant short

chain alkanes can be further degraded to the simplest and environmentally safest form by treating with some already available microbial consortia. While degrading kerosene by the isolated bacterial strains, the newer compounds assumed to appear as a result of degradation of high molecular weight compounds or gathering of fractions resulted from biodegradation of compounds which experienced peak area declining.

The elevated level of ethylene in response to Acetylene Reduction Assay was also observed by Laguerre (1987) in case of bacterial isolates. Production of ethylene corresponds to positive nitrogenase activity of an organism. The isolates AM05 and AM10 could produce ethylene in an increasing order from day1 to day7. Wedhastri *et al.*, (2012) reported the presence of diazotrophic *Achromobacter* sp. in coffee rhizosphere region. Several workers had reported the occurrence of nitrogen fixing free living bacteria in hydrocarbon contaminated soil and as well as water. Eckford *et al.*, (2002) isolated the bacterium *Pseudomonas stutzeri* from fuel contaminated Antarctic soil. Isolates AM05 and AM10 could thus fix relatively good amount of atmospheric nitrogen both in culture broth and soil. Thus it can be concluded that the bacteria used for the present investigation do have the hydrocarbon degrading and nitrogen fixing capacity.

CONCLUSION

Though nitrogen inadequacy limits the growth and degradation capacity of hydrocarbon degrading microorganisms, meanwhile it also favors the development of some resistant microbial strains which could have the hydrocarbon degradation as well as nitrogen fixation capacity. The present study concludes that such microorganisms can be successfully isolated from crude oil contaminated sites and they have the potentiality to be used both in biostimulation and bioaugmentation processes. This will help the bioremediation and biofertilization of petroleum contaminated sites in an environmentally sound way.

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