



## **Bioremediation of complex hydrocarbons using microbial consortium isolated from diesel oil polluted soil**

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### **ABSTRACT**

*In the present work we have studied degradation of diesel oil in oil-polluted soil. Oil-polluted soil culturing showed presence of variety of microorganisms. Hydrocarbon degrading potential of these microbes was confirmed and was identified. Acinetobacter sp, Bacillus sp., Micrococci sp., Pseudomonas sp. Streptomyces sp., and fungi Aspergillus sp, Trichoderma sp, Penicillin sp.were found part of hydrocarbon degrading microbial consortium. Analysis of extracted diesel oil was done using FTIR, HPLC, 1H NMR and GCMS techniques. It confirmed biodegradation of diesel oil confirming naphthalene and it's derivatives as major pollutants which are able to dissolve in water. This further confirmed by hydrocarbon dominated water table.*

**Keywords**-Biodegradation, Diesel oil, Microbial consortium, GCMS, naphthalene.

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### **INTRODUCTION**

The advent of modern industrialization and development in transportation, the use of petroleum based energy products has been increased [Gary and Handwerk, 2001] and leads to water and soil pollution [Kamble et al., 2011]. The pollution may occur due to spillage of oil through storage tank, accident during transportation by truck, ship or oil pipelines [Castro-Gutierrez et al., 2012]. Anthropogenic activities have been extensively releasing petroleum based pollutants in environment which leads soil, water and air pollution [Castro-Gutierrez et al., 2012]. The last century was of machines. The maximum machines requires petroleum hydrocarbon as a source of energy. This resulted in tremendous increase in use of petroleum hydrocarbon in automobile industry . Petrol and diesel are one of the most used petroleum hydrocarbons for energy sources giving rise to inevitable spillage during routine operations .

Diesel fuel is principle end product of gas oil obtained during fractional distillation of petroleum as the portion boiling off between 25<sup>0</sup>C and 36<sup>0</sup>C . [Atlas, 1981]. Diesel oil is a medium distillate of petroleum containing: n- alkanes, branched alkanes, olefins and small concentrations of aromatic polycyclic compounds [Baker and Herson, 1999]. However the biological method has been practiced to abatement of oil pollution. Bioremediation processes have been found to be an efficient method for remediation of petroleum by-products, pesticides and other potential harmful chemical [Castro-Gutierrez et al., 2012]. Bioremediation is being used or proposed as a treatment option at many hydro-Carbon-contaminated sites [Braddock et al., 1997]. Bioremediation processes are significantly affected by the inherent capabilities of the microorganisms, their ability to overcome the bioavailability limitations in multiphase environmental scenarios [oil–water–soil] and environmental factors such as temperature, pH, nutrients and electron acceptor availability [Mukherji and Vijay, 2002]. Environmental Microorganisms with the ability to degrade crude oil are ubiquitously distributed in soil and marine environments, [Venkateswaran and Harayama, 1995]. Diesel oil spills on agricultural land generally reduce plant growth and reasons for the reduced plant growth in diesel oil contaminated soils range from direct toxic effect on plants [Baker, 1982] and reduced germination [Udo

and Fayemi, 1975] Microorganisms have enzyme systems to degrade and utilize diesel oil as a source of carbon and energy [Ijah and Antai, 1988; Ezeji et al., 2005; Antai and Mgbomo,

1993]. Biostimulation is considered as a most appropriate remediation technique for diesel removal in soil and requires the evaluation of both intrinsic degradation capacities of the autochthonous microflora and the environmental parameters involved in the kinetics of the in situ process [Molina-Barahona et al., 2004].

This study was designed to isolate, characterize and identify diesel oil-degrading micro-organisms. Diesel oil degradation by the isolates was further monitored to determine their potentials as bioremediation agents.

In this present work the characterization of the microbial fauna from the contaminated sediments and its bioremediation potential of diesel contaminated soil have studied. Occasional spillage contaminate soil above ground and percolate in soil. Surface spreading and infiltration of diesel result into adsorption of diesel on soil particles. When the water flows through river basin, the sediment contaminated with diesel is being carried with water flow and become a potential form aquatic life. When soil surface aeration is good and temperature moisture conditions are appropriate, rate of microbial degradation increases.

## MATERIALS AND METHODS

### 2.1 SAMPLING, COLLECTION AND PHYSICO-CHEMICAL PROPERTIES OF SOIL SAMPLES

Pravara river bed around Sangamner city, western India was selected as soil sampling area. Samples were collected in sterile conical flasks, and were immediately transported by icebox and brought to laboratory. All collected soils were air dried, homogenized, sieved to 2 mm mesh size and stored at 4°C prior to experiment.

### 2.2 ISOLATION OF MICROORGANISMS

For selection of potential diesel degrading microorganisms, the ability to degrade hydrocarbon substrate was analyzed [Mariano et al., 2008].

Enumeration of fungal and bacterial population was done by plating 0.1 ml of serially diluted soil sample on potato dextrose agar [pH –acidic] containing streptomycin [1 mg/ mL] and nutrient agar. The identification of actinomycetes was done on starch casein agar. Ashbey's mannitol agar was used for determination of nitrogen fixing microorganisms. Fungi and actinomycetes were incubated at 30°C, bacteria and nitrogen fixing bacteria were incubated at 37°C. The incubation period was 48 h, 96 h, 120 h and 168 h respectively for bacteria, fungi, actinomycetes and nitrogen fixers respectively.

### 2.3 CONFIRMATORY TEST FOR HYDROCARBON DEGRADING MICROORGANISMS AND THEIR IDENTIFICATION

For confirmation of hydrocarbon degrading ability of microorganisms Hanson method was used. Each isolated microorganism was inoculated in BH broth. Each well of microtitre plate was filled with 250 µl of BH medium containing Dichlorophenol indophenols [DCPIP], 10 µl of diesel oil and 25 µl of each microbial suspension [10<sup>8</sup> CFU/ mL]. The color change from blue [oxidized] to colorless indicate the ability of microorganism to utilize hydrocarbon substrate.

Bacterial colonies were identified based on the taxonomic schemes of Cowan and Steels. Fungal isolates were classified based on hyphal mass, nature of fruiting bodies, morphology of cells and spores.

### 2.4 BIOMASS AND PH MEASUREMENT

Three sets of conical flask were taken. Each set containing 5 flasks [500 mL]. In one set 100 ml BH broth, 10 ml diesel and 5 ml inoculum were added. In another set 100 ml nutrient broth, 10 ml diesel and 5 ml inoculum were added. A third set is maintained as a control containing BH broth, 10 ml diesel without inoculums. Fourth set was maintained as nutrient broth control containing nutrient broth, 10 ml diesel without inoculums. From each set, after 3 days interval, sample was collected and analyzed for pH and temperature. Biomass and pH was measured after every 3rd day as a dry weight. For biomass measurement 25 ml fraction of sample was filtered through a microbial membrane [0.45 µm] and dried overnight.

### 2.5 EXTRACTION OF OIL FROM SOIL SAMPLE

10 gm of soil sample was mixed with 20 ml of carbon tetrachloride. The solution was placed in a separating flask was shaken vigorously for 5 min and allowed to settle for overnight. The liquid was separated and the moisture was removed by filtering through whatman no 1 paper already coated with

anhydrous sodium sulphate. The liquid phase was collected in 50 ml conical flask and used for further analysis of diesel degradation .

## 2.6 ANALYSIS OF DIESEL DEGRADATION

Degraded diesel was analyzed by Fourier transform infrared spectroscopy [FTIR]. The FTIR study spectrum was taken [Shimadzu IR solution] in the mid IR region of 400–4000  $\text{cm}^{-1}$  with 16-scan speed. The UV visible spectra [Elico BL 198 Bio spectrophotometer] were taken in the range of 190 to 900 nm. The HPLC analysis of untreated and treated diesel was carried out [by using column C-18um250 mm 4.6 mm, solvent methanol: water 45:55 at ambient temperature. For HPLC analysis pure diesel was considered as control and extracted oil was considered as sample. Pure and contaminated oil were dissolved in methanol: water [45:55] solvent system at 25 C temperatures. For separation C 18 column with 5um x 250 mm x 4.6 mm was used with 2.5 ul of injection volume. Chromatographic and mass spectrometric analyses were performed by GC-MS [Shimadzu™, Model QP2010A] using a DB-5 chromatographic column.. The temperature was programmed to vary linearly from 40°C to 270°C at the rate of 10°C and maintained for 22 min. Injection volume was 1ul. Helium was the carrier gas and the interface temperature was 280°C. Injection of samples and control into a GC-MS system was carried out in triplicate. The highest resolution chromatographic peaks were scanned to find their corresponding mass fragmentation and were characterized based on similarities between their mass spectrum and those presented by NISIT library.

## RESULTS AND DISCUSSION

### 3.1 SOIL SAMPLE PROPERTIES

Collected soil samples were analyzed for physical and chemical properties of soil. The particle size was measured in polluted soil and the clay was found 0.24 %, sand 12.83 % and slit 86.42 %. The soil nature was silty. The water holding capacity was 10.5%. The pH of diesel polluted soil was 6.74 while of unpolluted soil [control] pH of same area was 7.54. Soil conductivity was noted 1.767 mS/cm.

### 3.2 IDENTIFICATION OF MICROORGANISMS

The bacteria were analysed and enlisted in table 1 and fungal analysis was shown in table 2. Study dealing with screening of the microorganism resulted in isolation of bacteria and fungi capable of degrading diesel oil. The sample collection from diesel impregnated soil indicates the natural adaptation of these microorganisms to survive in the presence of diesel oil. The culture was able to degrade diesel oil at a wide range of temperature 30-40°C and at acidic pH optimum at neutral pH.

**Table 1. Bacterial identification**

| Character  | Bacterial isolate 1 | Bacterial isolate 2 | Bacterial isolate 3 | Bacterial isolate 4 | Bacterial isolate 5 |
|------------|---------------------|---------------------|---------------------|---------------------|---------------------|
| Gram stain | -                   | +                   | +                   | -                   | +                   |
| Shape      | Cocco bacillary     | rod                 | cocci               | rod                 | rod                 |
| Spore      | Nonspore forming    | Spore forming       | Non spore forming   | Nonspore forming    | Spore forming       |
| Motility   | Nonmotile           | motile              | Nonmotile           | motile              | motile              |
| Catalase   | +                   | +                   | +                   | +                   | +                   |
| Oxidase    | -                   | -                   | +                   | +                   | +                   |
| Citrate    | +                   | +                   | -                   | +                   | -                   |
| Indole     | -                   | -                   | -                   | -                   | -                   |
| Glucose    | -                   | +                   | +                   | -                   | +                   |
| Lactose    | -                   | +                   | -                   | -                   | +                   |
| Organism   | Acinetobacter       | Bacillus sp.        | Micrococci          | Pseudomonas sp.     | Streptomyces sp     |

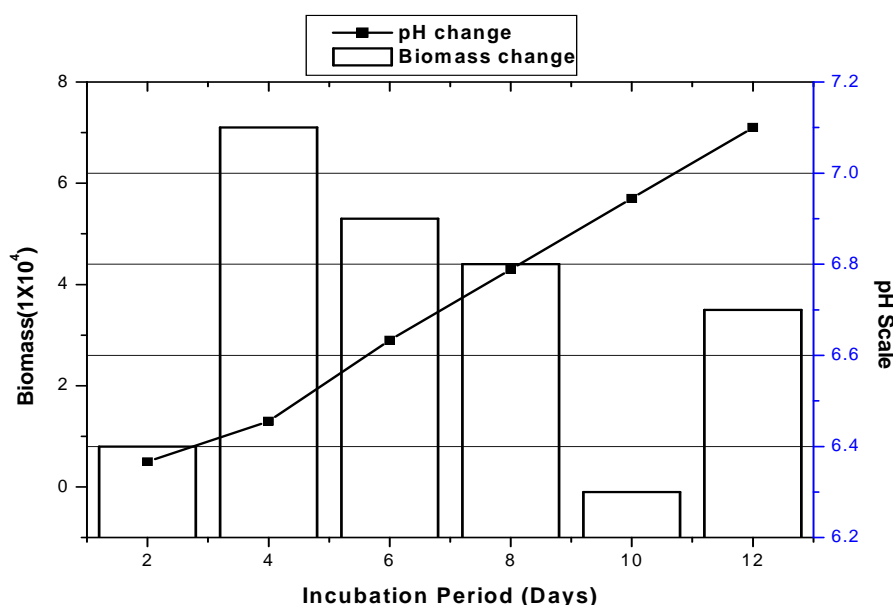
**Table 2. Fungal identification**

| Character         | Fungal isolate 1            | Fungal isolate 2               | Fungal isolate 3                      |
|-------------------|-----------------------------|--------------------------------|---------------------------------------|
| Microscopy        | Conidiophores with branches | Hyaline branched conidiospores | Branched conidiospores with phialides |
| Colony morphology | Yellow-green colony         | Wooly green colony             | Cottony Blue green colony             |
| Organism          | Aspergillus niger           | Trichoderma                    | Penicillin                            |

### 3.3 BIOMASS AND pH MEASUREMENT

Significant increase in biomass was observed while pH showed variation from acidic to basic range. At 3day microbial culture became alkaline. As the incubation time increased culture showed acidic characteristics and further tend to become alkaline as incubation proceed. This variation in pH did not show significant effect on biomass as the maximum biomass was achieved at 11<sup>th</sup> day of incubation.

Fig. 1. Indicating change in biomass and pH



The systematic change in biomass and pH was shown in fig. 1.

### 3.4 ANALYSIS OF DIESEL DEGRADATION

#### FTIR analysis of diesel degradation

For IR spectroscopy pure diesel sample was used as control. Control showed characteristic bands at 3421.83, 3410.26, 2931.61 to 2673.43, 2281.87, 1753.35, 1606.76, 1456.30, 1373.36, 1292.35, 1184.33, 875.71, 740.84 indicating presence of phenol or amines alkanes or carboxylic acids or esters or aromatic rings.

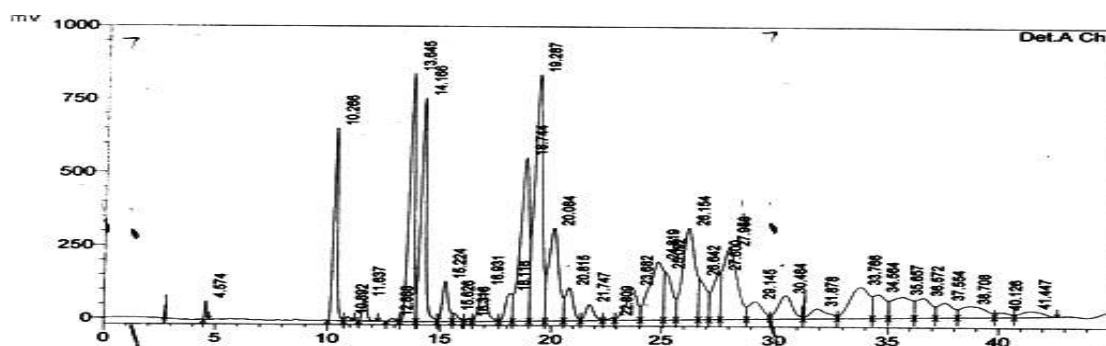
Extracted oil was analyzed by FTIR showed fingerprint pattern at 3422.98, 2922.25, 2828.60, 2727.44, 2675.36, 1042.68, 1610.68, and 1375.29.

Absence of characteristic peak in the region of 1610 to 2042 in extracted sample indicates absence of ester linkage.

### 3.5 HPLC ANALYSIS OF DIESEL DEGRADATION

HPLC analysis showed presence of 36 peaks in control diesel sample while 35 peaks were observed in extracted sample. Many components in sample showed shift in retention time and peak area indicating changes in molecular weight and quantity of respective components present in control [figure 2 and 3].

Figure 2 Control results from HPLC analysis.



### 3.6 GC - MS analysis of diesel degradation

The identified components present in pure diesel sample by GC MS was shown in Fig. 4. GC MS analysis of extracted sample was shown in Fig. 5. The extracted sample showed presence of naphthalene and its derivatives which were completely absent in pure diesel sample. The significant decrease in peak area of components was observed in extracted sample.

Figure 3 Biodegraded diesel sample results from HPLC analysis

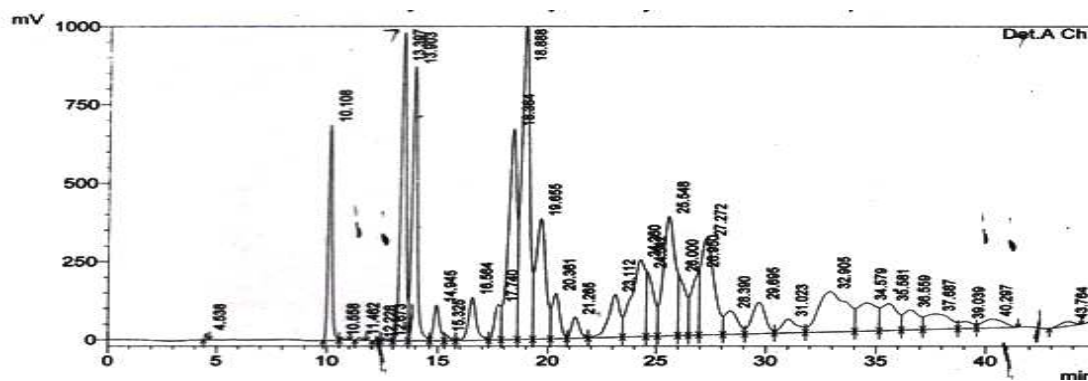


Figure 4 Control GC-MS analysis

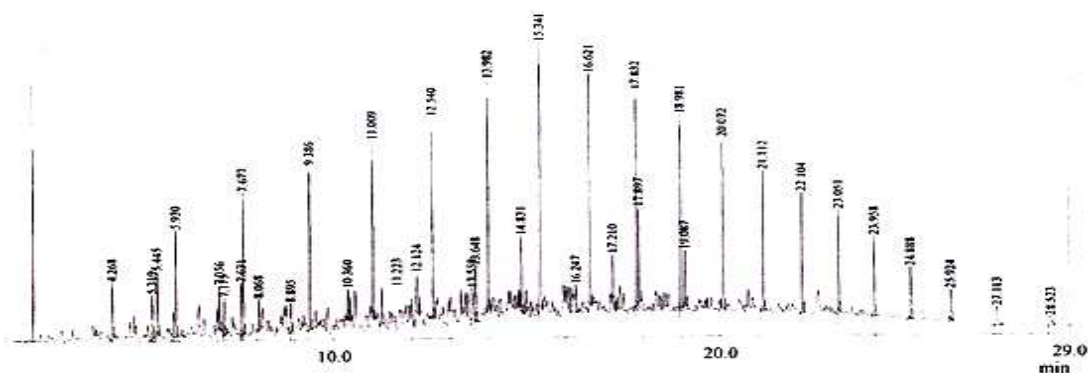
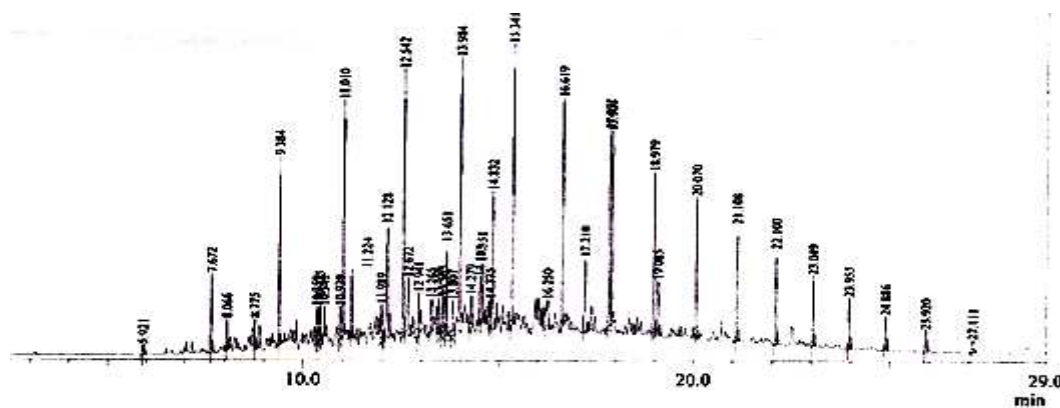


Figure 5 Biodegraded diesel sample GC-MS analysis



## CONCLUSION

Diesel oil polluted Soil from river bed was analyzed for presence of hydrocarbon pollutants. Result showed change in physical as well as chemical properties of soil. Microbial analysis of soil indicate presence of bacteria, fungi and nitrogen fixers. FTIR analysis of extracted oil showed absence of ester compared to pure diesel, GCMS analysis confirmed presence of naphthalene and its derivatives as hydrocarbon pollutant. The diesel pollutant was degraded by the existing microbial fauna of the soil and the resultant pollutant able to dissolve in river water resulting in the hydrocarbon dominated water table pollution.

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