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# Isolation, Identification and Characterization of Petroleum Degrading Bacteria from Oil Contaminated Soils

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Hydrocarbon degraders, oil spills, Bioremediation, Enrichment culture technique, Petroleum polluted soils.

**A B S T R A C T**

The wide spread use of petroleum products leads to contamination of soil and aquatic environments thereby possess a serious threat to all life forms counting humans. Hydrocarbon degrading bacteria were isolated from petrol contaminated soil to determine their biodegradation capabilities of aromatic hydrocarbon. A study was conducted in order to decipher the microorganisms from oil contaminated sites for oil degradation abilities. Five soil samples were screened for oil degrading bacteria in Nutrient agar medium. Among those samples totally 10 isolates were screened to determine their biodegrading activities on hydrocarbon.

## Introduction

Nowadays pollution is considered as one of the major problem of the world which could be either organic or inorganic in nature. In this modern world the need for petroleum products has highly increased for the contribution of modern lifestyles. Petroleum products are complex mixtures which are mainly derived from crude oil and they are processed in oil refineries (Rafik *et al*., 2013). The most common petroleum derivatives which include alkanes and aliphatic and aromatic compound and other minor constituents (Chikere *et al*., 2011). Oil contamination is one of the most dangerous pollution factor known today.

It can cause a threat to the environment. it is very feared by environmentalist and it's very hard to control if it gets hand out.

Hydrocarbons enter into the environment through waste disposal, accidental spills, as pesticides and via losses during transport, storage and use and their accumulation in the environment cause serious problem.

Current physical and chemical treatment of wastes are generally expensive and are not able to remove trace qualities of pollutant (Shukor *et al*., 2009).

Bioremediation is the application of biological treatment to clean up hazardous chemicals. This process involves detoxification where the pollutant may be converted to less toxic substances and mineralization, where the waste material is converted into organic compounds such as carbon di oxide, water, methane and sometimes fatty acids (Martello, 1991). Contamination of the environment with petroleum hydrocarbon has caused critical health defects and therefore increasing attention has been focused on developing and implementing innovative technology for cleaning up this contamination (Rahman *et al*., 2002).

Bioremediation methods therefore come in handy and have correctly received favorable publicity as promising environmentally friendly technique for the remediation of hydrocarbon contaminated ecosystem (Descai *et al*., 1997). This is possible because microorganisms have enzymes system to degrade and utilize different hydrocarbons as a source of carbon and energy. A number of gram positive and negative microbes have been reported to be capable of utilizing a wide variety of hydrocarbons as carbon and energy (Fought *et al*., 1987).

The ability of microorganism to utilize hydrocarbon in oil contaminated environments has been documented (Atlas *et al*., 1972). Microbial degradation process aids the elimination of spilled oil from the environment after critical removal of large amount of oil by various physical and chemical methods. This is possible because microorganisms have enzymes system to degrade and utilize diesel oil as a source of carbon and energy (Ljah *et al*., 1998; Antai *et al*., 1993)

The present study has been focused on this approach, aiming to isolate novel bacterial strains capable of petroleum hydrocarbon degradation in situ conditions in this study, we report isolates capable of degrading a wide spectrum of hydrocarbon efficiently. Degradation studies to be carried out with different isolates at varying interval of time will help to find out the most potent hydrocarbon degrading strains, which can be used for any bio augmentation studies during bioremediation.

## Materials and Materials

### Sterilization

Media and glassware were sterilized in an autoclave at 121°C with 15 lbs pressure for 20 mins.

### Soil Sample Collection

Several surface soil samples were collected from various oil contaminated sources in Chennai including CPCL, IOC, Avadi Yard, korukupettai crude oil station, thiruvetriyoor yard. Using sterile spatula at a tillage depth of 1-2 cm, from different places.

The soil samples were collected into sterilized bottles, properly sealed, labelled and wrapped with foil to prevent any further light reactions. All collected soil samples were temporally stored in an icebox at 4°C and then transferred to the laboratory for further analysis. Temperature of collected soils ranged from 35-36°C.

### Culture Media

Microbes were isolated from oil contaminated soil samples by using Nutrient Agar medium by adding Diesel oil, Petrol &2T Engine oil in various concentration. Bacteria were maintained on both liquid and solid medium with diesel oil as sole carbon source for isolation and screening of hydrocarbons degrading bacteria.

### Enrichment of Microorganisms

10gm of each soil samples were suspended in 100ml of Nutrient broth separately in 20 conical flasks. Each soil samples are subjected with three carbon source (diesel, petrol&2Tengine oil and 1 control. Flask were mixed completely and incubated for 7 days at 37°C (Latha, 2012).

### Isolation of Oil Degrading Bacteria

After the second enrichment of microbes for 7days of incubation, the soil samples were serially diluted. 0.1 ml of the sample was taken from 10-4 & 10-6 tubes and was spread onto Nutrient agar plates with Diesel, petrol and 2T engine oil as sole carbon source. The plates were kept at 37°C until visible colonies were taken for culture in nutrient agar plates. Pure culture of bacterial isolates was identified on the basis of their colony morphology and biochemical techniques. Then pure colonies were streaked on to the slant and preserved for further studies.

### Identification of Isolates

Pure colonies were identified and characterized on the basis of their morphological characteristics and biochemical properties according to the identification scheme of Bergey's manual of determinative bacteriology (Chaudhary *et al*., 2011; Khan, 2011). Gram reaction, motility, shape and color of colony and acid and gas production from carbohydrates and sugars fermentation were performed. Biochemical tests catalase, urease, indole, oxidase, VP, nitrate reduction was tested (Smibert, 1994). Based on the test results the preliminary identification of the isolated bacterial strain was done.

### Oil Degradation Studies

For examining the degradation of oil, nutrient agar medium was supplemented with diesel, petrol and 2 T engine oil. About 50 ml medium was dispensed in 250 ml conical flask. The media was inoculated with 0.1ml of oil degrading bacteria and incubated at 37°C for 20 days in different flask.

### Gravimetric Analysis

Oil degradation was studied by gravimetric analysis. After desired interval of time, the flask was taken out and bacterial activities were stopped by adding 1% 1N-HCL. For extraction of oil, 50ml of culture broth was mixed with 20ml petroleum ether: acetone (1:1) in separating funnels and was shaken vigorously to get a single emulsified layer. Acetone was then added to it and shaken gently to break the emulsification, which resulted in three layers (Bharti *et al*., 2011). Top layer was a mixture of petroleum ether, oil and acetone; clumping cells make a middle layer and bottom aqueous layer contains acetone, water and bio surfactant in soluble form. The lower two layers were spread out while top layer containing petroleum ether mixed with oil and acetone was taken in a pre-weighed clean beaker. The extracted oil was passed through anhydrous sodium sulphate to remove moisture the petroleum ether and acetone was evaporated on the water bath. The gravimetric estimation of residual oil left after biodegradation was made by weighing the quantity of oil in a tarred beaker. The percentage of degradation was calculated as

Weight of residual oil = weight of beaker containing extracted oil – weight of empty beaker.

Amount of crude oil degraded = weight of oil added -weight of residual oil

% degradation = amount of oil degraded media x 100

## Result and Discussion

Poly aromatic hydrocarbon(PHAs) have been identified as hazardous chemicals by different state and central pollution control boards, because of their toxic, carcinogenic and tetra genic effects on living body. At present, hydrocarbon fuels (diesel) contain an excessive quantity of PHAs causing abundant distribution of the same in the ecosphere. In order to protect environment from such PAH emission from diesel oil, a straight EURO III standard has recently been enforced. This specifies that the maximum allowable concentration of PAH in diesel oil to be used as automobile fuel should be 11% by weight.

As the usage of petroleum hydrocarbon products increases soil contamination with diesel and engine oil is becoming one of the major environment problem. There are so many bacterial strains that can degrade or transform the component of crude oil products to the nontoxic, nonhazardous, biodegradable and environmentally friendly compounds. This is known as biodegradation. Many oil degrading bacteria have been isolated and their degradation potential is investigated. Most of bioremediation studies have been carried out using pure culture and the role of these bacteria in a natural environment remain substantially unknown (Harayama *et al*., 1999). In the present study, the soil samples were gathered from the garage and yard because the capability of native bacterial population to mineralize crude oil hydrocarbon in oil contaminated sites was confirmed before by many scientists (Akhavansepahi *et al*., 2008). These soil samples are serially diluted and results are tabulated table 1.

From these soil samples two bacterial species was identified and pure cultured separately. Among these two *Pseudomonas* was the predominant one in all the samples. The bacterial characterization of pseudomonas was tabulated in table 2. Oil content of the soil sample can be estimated by using gravimetric analysis. Gravimetric analysis was done to extract the oil degraded by bacterial culture using petroleum ether and acetone in the 1:1 ratio of 20ml. the results were tabulated based on initial weight and final weight of the soil sample. The results are tabulated as follows in table 3.

The release of contaminant to the environment, including petroleum and petroleum derived products is one of the main cause of soil and water contamination which cause a risk for humans and animal health, since many of these contaminant have demonstrated to be toxic and carcinogenic hydrocarbon molecule that are released into the environment are hard to remove as they absorb to surfaces and are trapped by capillary in water immiscible phase. Bio surfactant is biological surface active microorganism in order to metabolize water immiscible substrates, allowing its adsorption, emulsification or dispersion. Hydrocarbon contaminated sites can be considered as enrichment environment for isolation of hydrocarbon degrading bio surfactant producing microbial strains.

In recent years, many microbial ecologists have identified various microbial species that are effective degraders of hydrocarbon in natural environments.

Pseudomonas appears to be the most ubiquitous bacteria found in oil contaminated soils and soil in general. This bacterium is able to adapt too many different hydrocarbons. They are responsible for degrading most of aromatics in gasoline, although the efficiency in degrading aromatics hydrocarbons can vary among strains.

**Table.1** Different soil samples collected from oil surface.

| S.No | Soil Source | Carbon Source | $10^{-4}$ | $10^{-6}$ |
|---|---|---|---|---|
| 1 | CPCL | Diesel | B | B |
| | | petrol | P | B |
| | | 2T engine oil | B | P |
| 2 | IOC | diesel | P | P |
| | | petrol | P | B |
| | | 2t engine oil | B | B |
| 3 | THIRUVATTIYOR YARD | Diesel | P | B |
| | | Petrol | B | B |
| | | 2t engine oil | P | B |
| 4 | KORUKUPETAI OIL STATION | Diesel | B | B |
| | | Petrol | B | P |
| | | 2t engine oil | P | B |
| 5 | AVADI YARD | Diesel | B | B |
| | | Petrol | P | B |
| | | 2t engine oil | P | B |

**Table.2** Characteristics of bacteria

| S.No | Character | Results |
|---|---|---|
| 1 | Gram stain | Negative |
| 2 | Morphology | Rods |
| 3 | Arrangements | Solitary |
| 4 | Motility | Sluggishly motile |
| 5 | Indole | Negative |
| 6 | Methyl red | Negative |
| 7 | VP | Negative |
| 8 | Citrate | Positive |
| 9 | Urease | Negative |
| 10 | Catalase | Positive |
| 11 | Oxidase | Positive |
| 12 | Gelatin | Positive |
| 13 | Triple sugar iron | Ak/no gas/no $H_2S$ |
| 14 | Nitrate reduction | Negative |
| 15 | Glucose fermentation | No fermentation |
| 16 | Lactose fermentation | No fermentation |
| 17 | Sucrose fermentation | No fermentation |
| 18 | mannitol fermentation | No fermentation |
| 19 | Organism | Pseudomonas |

**Table.3** Gravimetric analysis of soil sample

| S. No | Soil sample | Initial weight | Final weight | Amount of oil content in 10 gm of soil |
|---|---|---|---|---|
| 1 | CPCL | 54.1732 | 53.6261 | 0.5472 |
| 2 | IOC | 53.7652 | 52.2563 | 1.5089 |
| 3 | Thiruvattiyoor yard | 55.093 | 54.764 | 0.329 |
| 4 | Korukkupettai crude oil station | 63.9073 | 63.3162 | 0.5911 |
| 5 | Avadi yard | 54.7316 | 54.5273 | 0.2043 |

Reference (Lies, 2007) have showed that autochthonous microorganism present in contaminated soils are more efficient in degrading petroleum compounds than microbes existing in non-contaminated soils. Since many naturally occurring microorganisms have the ability to utilize hydrocarbons as the sole source of carbon and are widely distributed.

Bacterial degradation of petroleum has been known for over 50 years, responsible bacteria have mostly been isolated from areas, such as soils, petroleum storage tanks and oil spills (Rehm *et al*., 1981). There are numerous report on isolation of petroleum hydrocarbon degrader bacteria from oil exposed areas. The ability to isolate high number of certain oil degrading microorganisms from petroleum contaminated environment is commonly taken as evidence that these microorganisms are the active degraders of that environment (Tazaki *et al*., 2005).

Bioremediation is not new to human but new approaches that advances in molecular biology and process engineering are emerging microbe's bio remediates the environment as they biodegrade the pollutant to obtain carbon and energy, biodegradation specifically refers to chemical breakdown or mineralization of materials facilitated by biological organisms or products (Onaventure *et al*., 1997).

The present study reveals that the bacterial species (Pseudomonas sp.) richness in the petroleum oil contaminated soil favored the degradation of hydrocarbons by their enzyme activities.

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