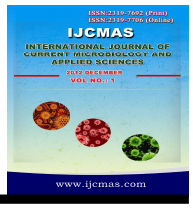
International Journal of Current Microbiology and Applied Sciences
ISSN: 2319-7706 **Volume 1 Number 1 (2012) pp.12 -16**



Original Research Article

An experimental study for crude oil biodegradation in contaminated soil

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Keywords

Crude oil; biodegradation; soil pollution and Gas chromatography.

A B S T R A C T

Petroleum forms a major source of raw material for many chemical products such as plastics, paints and cosmetics. It is also a source of energy for Industry and daily life. When it is transported oil spills occur leading to oil pollution which causes serious problem in marine and soil environment. So Biodegradation plays a vital role in removing oil spills. Different sources of microorganisms such as bacteria and fungi degrade crude oil. Bacteria such as *Bacillus*, *Pseudomonas* and *Cornyebacterium* and fungi such as *Aspergillus*, *Penicillium* and *Trichoderma* degrades a variety of hydrocarbons. In this present study oil degrading bacterial strains were isolated from Soil samples collected from the Oil spill near the drilling site. The isolates were identified as *Pseudomonas* sp. and *Bacillus* sp. with reference to Bergey's manual of Determinative Bacteriology. The Gas chromatography analysis reveals that the petroleum fraction is subjected to biodegradation by oil degrading bacteria which paves way for biodegradation process.

Introduction

Crude oil is constituted from thousands of components such as saturated hydrocarbons, aromatic hydrocarbons, resins and asphaltenes. Saturated hydrocarbons are the major pollutants; especially those of smaller molecular weight are readily biodegraded in soil environment. The aromatic hydrocarbons with one, two or three aromatic rings are also efficiently biodegraded in soil environment. However, those with four or more aromatic rings are quite resistant to biodegradation. The asphaltene and resin fractions contain higher molecular weight, whose chemical structures have not yet been resolved. The biodegradability of these compounds is not yet known.

Petroleum-based products are the major source of energy for industry and daily life. Moreover, petroleum is the major source of raw material for many chemical products such as plastics, paints and cosmetics. The transport of petroleum across the world is frequent. The oil spill is heavily concentrated around offshore production sites, major shipping routes, and refineries and frequently exceeds the self purification capacity of the receiving waters. Oil floating on water is technically difficult to contain and collect. Oil pollution is destructive to birds and various forms of marine life. Oil spills pollute ground water and are destructive to vegetation due to lack of oxygen and evolution of H_2S, which kills the roots of most plants (Oudot 1994, Prince *et al*.,1994 and Obire *et al*., 2001).

Crude oil (Petroleum) is a rich source of organic matter, many microorganisms readily attack and

utilize crude oil as substrate and this results in the biodegradation of crude oil. Microorganisms such as bacteria and fungi degrade a variety of hydrocarbons(Ding-KeQiang *et al*., 2002). Microbial biodegradation of hydrocarbons depends on the following factors such as potential of the indigenous microorganisms, temperature, pH, oxygen and nutrient availability and hydrocarbon concentration (Kerry *et al*., 1993). However, bacterial degradation of oil is more significant (80%) than fungi (20%) (Wrenn *et al*., 1986, Van Hamme *et al*., 1999 and Leathy *et al*.,1990).

The genus *Pseudomonas* and *Bacillus* in particular have been the subject of numerous studies. The *Pseudomonas sp* is one of the best crude oil degraders. *Pseudomonas* is termed as "Oil Eating Bugs" in crude oil industry. An interesting and useful characteristic of many *Pseudomonas sp* is their ability to utilize a wide variety of organic substrates for growth. The bacterium invades the crude oil compounds which consists of rich source of organic compounds suitable for the growth of this bacterium, which also produces Biosurfactants that cleans up the crude oil (Kilbane *et al*., 2000). *Pseudomonas sp* utilizes crude oil organic compounds as sole source of nutrients which includes saturated and aromatic compounds (Genthner *et al*., 1989, Britton, L.N. 1984 (Madigan *et al*., 1997). Laboratory pilot study were also conducted to evaluate the capacity of three strains of fungi, indigenous fungus *Fusarium* sp. *Penicillium chrysosporium Phanerochaete chrysosporium* and *Coriolus versicolor* in crude oil degradation *(*Ding-KeQiang *et al*., 2002)

Bacillus on the other hand has the ability to grow on different substrates. They have the ability to degrade different hydrocarbons by the production of Bio surfactants (BIO), which facilitates the hydrocarbon degradation. They also produce a variety of enzymes, which is involved in Biodegradation. This characteristic property was applied in Biodegradation process.

This present study aims to ascertain the biodegradation of crude oil using *Pseudomonas* and *Bacillus sp* in the laboratory condition. This present study, aims to isolate and characterize the hydrocarbon degrading organisms such as Pseudomonas and Bacillus sp from the soil samples collected from the Oil spill near the drilling of Oil and Natural Gas Commission (ONGC) Tamilnadu.

Materials and methods

Isolation of hydrocarbon degrading organisms

Soil samples were collected from the Oil spill near the drilling of oil and Natural Gas Commission (ONGC) Tamilnadu. About 1 g of the soil samples were taken in 10 ml of sterile distilled water and was subjected to shaking condition (150 rpm) for 1 hour in order to achieve uniform distribution of cells. These samples were serially diluted and 0.1 ml of each dilution were plated on Bushnell-Hass agar (Composition in g/L: Magnesium sulphate 0.20 g, Calcium chloride 0.02 g, Monopotassium phosphate 1.0 g, Dipotassium phosphate 1.0g, Ammonium nitrate 1.0g, Ferric chloride 0.05g, Petroleum(crude oil) 10.0 ml, Agar 15g, Distilled water 1000ml, pH - 7.2$\pm$ 0.2) by spread plate method using L-rod . The plates were incubated at 37^{o}C for 24-48 hrs. The oil degrading bacterial colonies were identified by a zone formation on the media. The isolates were characterized and classified taxonomically according to Bergey's manual of systematic bacteriology. The results of various biochemical tests of the isolates were tabulated.

Biodegradation studies
Inoculum development

For inoculum development, a loopfull of culture from the agar slants were transferred to a 20ml of Bushnell-Haas broth in 100ml conical flask .About 1.0 ml of crude oil was added. The flasks were incubated at 28^{o}C in an rotary shaker set at 150rpm for one month. After a month of growth, Samples were removed and centrifuged at 10000rpm for 10minutes. The supernatant was used for Column Chromatography

Column chromatography
Packing the column

The glass column was clamped into a ring stand so that it is in a vertical position. A small piece of glass wool was tamped carefully down into the column using a rod. The alumina was heated in an oven at temperature 180°C for 2 hours then cooled to room temperature in a dessicator. This activated alumina was added slowly to the column till the mark 50 on the column. The column was then gently tapped by a glass rod to settle the alumina properly in the column. A 100ml beaker was placed under the column.

Applying the sample

With the help of a syringe 1ml of the petroleum was taken and loaded on the alumina column. It was kept undisturbed for some time till the absorbent (alumina) absorbs the sample.

Elution

50ml of Hexane was taken in a measuring jar and slowly poured from the edges into the column, to separate out the saturate fraction from the petroleum.

Recovering the separated compounds

After 3-4 hours of running a colorless compound i.e saturated hydrocarbons was collected in the beaker, along with the solvent. The columns chromatographically obtained saturated hydrocarbons were further analyzed by using Gas chromatography.

Gas chromatography analysis

GC Column: SE 30, Carrier gas: N_2, Injector temperature: 220°C, Injection solvent: Hexane, Injection Volume: 1µl, FID temperature: 220°C, Oven Temperature: 1500°C, Temperature program: Iso thermal mode.

Sample preparation

The sample was mixed with a little amount of solvent (Hexane). Then 1µl of the sample was taken in the 5µl Syringe and with the tissue paper the syringe needle was wiped clean.

Procedure

SE 30 column was selected and fitted to the GC. The carrier gas supply was switched on the flow rate was checked. The power was switched and the temperature settings for columns, injector and detector were adjusted. As FID was used the hydrogen gas flow was switched on then the flame was lit. The chart recorder in the computer was switched on and was set to zero. When, the temperatures were stable, a trail sample (solvent) was injected and adjusted the attenuator to produce a satisfactory peak size. The samples were then injected and recorded.

Result and Discussion

Petroleum forms a major source of raw material for many chemical products such as plastics, paints and cosmetics. It is also a source of energy for Industry and daily life. When it is transported oil spills occur leading to oil pollution which causes serious problem in marine and soil environment. So Biodegradation plays a vital role in removing oil spills. (Alexander, 1981, Mohamed *et al*., 1993; Atlas *et al*., 1981) The inability of natural environment to clear the oil spilled pollution is being overcome by biological weapons such as biodegrading organisms.(Thavasi *et al*., 2011) These biodegrading organisms were isolated and a remedy for oil spilled pollution was brought about by biodegradation.

Screening of oil degrading bacteria

In this present study oil degrading bacterial strains were isolated from Soil samples collected from the oil spill near the drilling site of oil and Natural Gas Commission (ONGC). The isolates were identified as *Pseudomonas* sp. and *Bacillus* sp**.** with reference to Bergey's manual of Determinative Bacteriology.

Table. 1 Biochemical characterization of the isolates

Bio-chemical Test	Results
Catalase	Positive
Oxidase	Positive
Indole	Negative
Methyl red	Negative
Voges-Proskauer	Negative
Citrate	Positive
Urease	Positive
Gelatinase	Negative
Oxidative-Fermentative	
Glucose	Oxidative with gas
Sucrose	Oxidative with gas
Dextrose	Oxidative with gas

From the above result it was concluded that the isolate might be partially identified as *Pseudomonas* Sp**.**

Table. 2 Biochemical characterization of the isolate 2

Bio-chemical Test	Results
Catalase	Positive
Oxidase	Negative
Indole	Negative
Methyl red	Positive
Voges-Proskauer	Negative
Citrate utilization	Negative
Urease	Negative
Gelatinase	Positive
Oxidative – fermentative	
Glucose	Fermentative
Sucrose	Fermentative
Dextrose	Fermentative

From the above result it was concluded that the isolate might be a partially identified as *Bacillus* sp.

Studies of Biodegradation of crude oil

The Gas Chromatography analysis shows appreciable amount of biodegradation in the test samples when compared to that of sterile control. The Gas Chromatography analysis data profiles shows that the peak level is high in the case of control sample, whereas in the case of Isolate 1(*Pseudomonas* sp) and Isolate 2 (*Bacillus* sp) the peak value is low due to biodegradation of both the isolates as shown in chromatograph (Fig 1, Fig 2 and Fig 3). This shows that biodegradation of hydrocarbon was exhibited by these isolates (Bacillus sp. and Pseudomonas sp.) because the compound which is having the retention time (RT) 1.23 and 1.31 in the control was completely absent in the test sample in which the bacteria was grown, Whereas in the control the peak value was distinct and have the area % of 0.1198% and 75.1550%. (Thavasi *et al*., 2011) But this study was having lacunae regarding the nature of hydrocarbon involved for biodegradation was not identified.

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