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Isolation and Identification of Oil Degrading Bacteria From Oil Contaminated Soil and Comparison of Their Bioremediation Potential

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ABSTRACT

Petroleum refineries around the world have generated the solid wastes during the refining process and stocking of crude oil. environment. The ecology of hydrocarbon degradation by microbial populations in the natural environment is reviewed, emphasizing the physical, chemical, and biological factors that contribute to the biodegradation of petroleum and individual hydrocarbons. Oil contaminated soil samples were collected from five different places (Salem Railway shed, Kanyakumari, Tirunelveli, Indian Oil Corporation (IOC) and Chennai Petroleum Corporation Limited (CPCL) of Tamil Nadu. These samples were screened for bacterial oil degradation using 1 % diesel in Nutrient agar medium. Samples were incubated separately in shaking orbital incubator at 37º C at 125 rpm up to 48 hours. Eleven isolates were isolated from five different places, the hydrocarbon degrading bacterial species such as five isolates of Pseudomonas, four isolates of Bacillus and two isolates of Micrococcus were isolated from the oil spilled contaminated soil. The level of petroleum hydrocarbon degradation was determined by gravimetric assay at each

5 days interval. After 25 days of incubation period, Pseudomonas sp. found to degrade oil better than other isolated species. Pseudomonas species degraded 92.3% of oil in

25 days followed by 83.7% of oil degraded by Bacillus species and 35.5% of oil degraded by Micrococcus species. The present investigation shows that Pseudomonas sp. isolated from Kanyakumari sample can be effectively used to degrade oil contaminated soils

KEYWORDS : Bioremediation, Oil spills, Oil degradation, Pseudomonas sp.

INTRODUCTION

Oil contamination is one of the most dangerous pollution factors known today. It can cause a threat to the environment. It is very feared by environmentalists and it's very hard to control if it gets out of hand. Oil spill have become a global problem in industrialized and developing countries. Attention has been focused on the marine environment, because of the largest and most dramatic spills (Cooney, 1984).

Oil spills have been a major issue across decades. Recent oil spill was in Mumbai (India) and caused due to the leakage in Mumbai-Uran pipeline dated January 21, 2011 and about 55 tons of oil was leaked in Arabian Sea. Various such accidents occur throughout the years and it causes damage to our surrounding(Jahir Alam Khanand& Syed Hasan Abbas Rizvi ,2011).Diesel engine oil, which is one of the major products of crude oil, constitutes a major source of pollution in our environment. Diesel oil spills contaminated soils on agricultural land generally reduce soil fertility and plant growth. Baker (1982) and reduced germination to unsatisfactory soil condition due to insufficient aeration of the soil because of the displacement of air from the space between the soil particles by diesel engine oil (Zahir, 2001).

Many indigenous microorganisms in water and soil are capable of degrading hydrocarbon contaminants. The amount of natural crude oil seepage was estimated to be 600,000 metric tons per year with a range of uncertainty of 200,000 metric tons per year Kvenvolden and Cooper (2003) of hydrocarbons into the environment whether accidentally or due to human activities is a main cause of water and soil pollution (Holliger, Gaspard & Glod ,1997). The technology commonly used for the soil remediation includes mechanical, burying, evaporation, dispersion, and washing (Alvarez & Vogel, 1991). However, these technologies are expensive and can lead to incomplete decomposition of contaminants. The process of bioremediation, defined as the use of microorganisms to detoxify or remove pollutants owing to their diverse metabolic capabilities is an evolving method for the removal and degradation of many environmental pollutants including the products of petroleum industry (Medina-Bellver, Marín & Delgado ,2005).

Contamination of the soil by oil causes it to lose its useful properties such as soil fertility, water-holding capacity, permeability and binding capacity. The contamination of groundwater is also a potential problem, which receives a lot of untreated effluent from service stations containing oil and grease (Vasanthavigar, Srinivasamoorthy, Rajiv Ganthi& Vijayaraghavan,2010). To overcome these environmental problems, microbial bioremediation is only way to preserve our nature. The purpose of the present study was to investigate a possible ex–situ method to enhance the rate of biodegradation of diesel contaminated soil sites. The main objectives of the study were to isolate a potential strain which could be used in bioremediation of oil contaminated sites and to find out the efficiency of the isolate in preliminary screening of bioremediation. This study also aimed to investigate the use of biosurfactant in soil cleanup methods and to prove that biostimulation can be effectively employed in the remediation of crude oil polluted soil ecosystem.

Materials and Methods

Sample collection

Soil samples were collected from contaminated sites of Salem Railway shed, Kanyakumari, Tirunelveli, Indian Oil Corporation (IOC) and Chennai Petroleum Corporation Limited (CPCL). The soil at the sites had a characteristic black colour due to continuous oil spillage and the soil surfaces were hard and collected samples were packed in sterile polybags and brought to the laboratory. The entire sample was stored at refrigeration temperature before the experimental work.

Isolation of oil degrading Bacteria

The oil degrading bacteria was isolated by enrichment technique. Nutrient agar plates enriched with 1.0 % diesel were prepared (Jayashree, Evany Nithya, Rajesh Prasanna& Krishnaraju, 2012).1g of oil spill contaminated soil sample was weighed aseptically and added to the 99ml of sterile distilled water. The flask was placed in a rotary shaker for about 30 minutes at 30 ºC. Serial dilutions of the 5 samples were performed separately. Serially diluted samples from 10^{-1} to 10^{-7} were plated on nutrient agar using spread plate method. A sterile micropipette tip was used to dispense 0.1 ml from each dilution onto duplicate nutrient agar plates. A glass spreader dipped in alcohol, flamed and cooled was used for spreading the plates. The petriplates were then incubated at 37°C for 24 to 48 hours. After incubation period the isolated colonies were streaked in to the nutrient agar plates for purification and identification. The isolated colonies were transformed to nutrient agar slants and stored for further studies.

Growth and maintenance of Bacterial Isolates:
A fresh single pure colony of each bacterial isolates was transferred aseptically from agar plate into Nutrient Agar broth medium using a sterile loop. The inoculated medium was then incubated at 37°C at 100 rpm in orbital shaker. All pure isolates were maintained in liquid and solid media. They were regularly sub cultured into fresh medium for short-term storage.

Screening for Biosurfactant Activity:
Biosurfactant activity of isolated bacteria was detected by using Drop Collapsing Test, oil spreading method and emulsification stability test in three different oils namely vegetable oil, petrol and diesel.

Drop Collapsing Test:
Biosurfactant production was screened using the qualitative drop-collapse test described by (Youssef,Duncan, Nagle, Savage, Knapp and McInerney, 2004). Diesel oil (2µl) was added to 96-well microtitre plates. The plate was equilibrated for 1 h at 37°C d 5 µl of the culture supernatant was added to the culture supernatant was added to the surface of the oil in the well. The shape of drop on the oil surface was observed after 1min. The culture supernatant makes the drop collapsed was indicated as positive result for biosurfactant presence and if the drops remains intact indicates negative result. Distilled water was used as negative control.

Oil Spreading Method:
The petriplate base was filled with 50 ml of distilled water. On the water surface, 20 µl of diesel and 10 µl of culture supernatant were added respectively. The culture was introduced at different spots on the diesel which is coated on the water surface. The occurrence of a clear zone was an indicates of positive result (Rodrigues, Teixeira& Mei, 2006).

Emulsification Index (E24)
The emulsifying capacity was evaluated by an emulsification index. The E24 of the culture samples was determined by adding 2 ml of diesel and 2 ml of the culture supernatant in a test tube and mixing with a vortex for 2 minutes to obtain maximum emul -sification and allowed to stand for 24 hours. (Priya & Usharani, 2009)

$$E24 = \frac{\text{Height of the emulsified layer (cm.)}}{\text{Total height of the column(cm.)}} \times 100$$

The percentage of the E24 index is calculated by the following formula:

Estimation of oil using Gravimetric method
The estimation of oil in oil spill contaminated soil samples were studied by gravimetric analysis (Chang, 1998), (Marquez-Rocha, Hernandez-Rodriguez & Lamela ,2001). One gram of the soil was taken from each sample site. Petroleum ether and acetone were taken in the ratio 1:1 and was mixed with the soil sample in a separating funnel. The mixture was shaken for about 45 minutes and then was left undisturbed for about 10 minutes. The upper solvent along with oil was separated from the lower soil extract. The solvent with the oil layer was then kept in the hot air oven at 50° C until the solvent gets evaporated. After the complete evaporation, the oil residue obtained was weighed and taken as the gravimetric value for further calculation. Analysis of soil before and after treatment was done using this Gravimetric method. The percentage of diesel oil degraded was determined from the following formula:

Percentage of diesel oil degraded =

$$\frac{\text{Weight of diesel oil degraded}}{\text{Weight of diesel oil present originally}} \times 100$$

where,

The weight of diesel oil degraded = original weight of diesel oil - weight of residual diesel oil obtained after evaporating the extractant.

Extraction of lipase enzyme
The extra cellular Lipase enzyme production was studied by standard method (Kumar, Maheswaran, Kartheek& sharmila Banu,2011). The extra cellular Lipase enzyme is extracted from the production medium (isolates cultures were inoculated on Nutrient medium) after desired incubation time (48 h) by centrifugation at 10000 x g for 30 min in a refrigerated centrifuge. The resulting supernatant contained extra cellular lipase enzyme.

Screening for lipase activity - Tributyrin Plate Assay:
Lipolytic organisms (extra cellular Lipase supernatant) were screened by qualitative plate assay. Isolates were grown on tributyrin agar (Media containing 0.5% peptone, 0.3% yeast extract, 1% Tributyrin and 2% agar was prepared, pH was adjusted to 7.3 ± 0.2) base plates and the zone size were measured after 24 h of incubation at 37°C. Zone of clearance was observed due to hydrolysis of tributyrin.

Quantitative analysis Lipase activity:
The quantitative enzymatic assay of lipase activity was done using olive oil as the substrate. One ml of Tris HCl buffer was taken along with 3ml of olive oil substrate, mixed by swirling and equilibrated at 37°C. The pH was adjusted to 7.7. To this One ml of the extra cellular Lipase supernatant enzyme solution was added. It was mixed and incubated at 37°C for nearly 30 minutes. Then, 3ml of 95 % ethanol was added to the mixture. 4 drops of 0.9 % Thymolphthalein indicator solution was added later. It is immediately titrated with standardized 50 mM Sodium Hydroxide (NaOH) solution. Appearance of pale blue colour served as the end point. The procedure was repeated for concordant values and burette reading was noted. The blank was also titrated in the same manner and the readings were tabulated.

The quantitative activity of the enzyme can be calculated by the following formula:

$$\text{Units / ml of enzyme} = \frac{(\text{NaOH})\,(\text{Molarity of NaOH})\,(1000)\,(2)\,(\text{df})}{\text{Volume (in milliliter) of enzyme used}}$$

Where, 'df' is the Dilution Factor

Seed Germination tests in treated and untreated soil samples
In the present study, *Vigna mungo* (L.) Hepper was chosen for the seed germination test since it is a common fast growing leguminous plant vailable locally. Healthy, viable, uniformly sized seeds of Black gram (*Vigna mungo*) were taken and the seeds were surface sterilized by cleaning thoroughly under running tap water for 10 min, washed with a solution of Tween 20 (two drops in 100 ml of water) for one min ,and again washed with sterile distilled water.

The cleaned seeds were finally treated with (0.1%) mercuric chloride (HgCl) for four min under aseptic conditions and washed under aseptic conditions and washed five times with sterile distilled water to remove traces of HgCl . After surface sterilized seeds, seeds were then sowed in trays which contained treated and untreated soil samples. After a few days of watering, the germination of the seeds was noted.

The germination percentage can be calculated using the following formula:

$$\text{Germination Percentage (\%)} = \frac{\text{Number of seeds germinated}}{\text{Total number of seeds}} \times 100$$

RESULTS AND DISCUSSION
The microorganisms which could be employed for the degradation of petroleum and its derivatives in minimising contamination due to oil leak and spill, has promoted a number of investigators to study the process in the laboratory (Zo Bell ,1946). Oil contaminated soil samples were collected from five different places namely Salem Railway shed, Kanyakumari, Tirunelveli, Indian Oil Corporation (IOC) and Chennai Petroleum Corporation Limited (CPCL) of Tamil Nadu. Three different bacterial species were isolated from the oil contaminated soil samples and standard biochemical tests were done to identify

the organisms (Table 1). The three bacterial species were identified as *Pseudomonas sp., Bacillus sp.,* and *Micrococcus sp.*

Table 1: Gram stain and Biochemical test results

Biochemical Test	Microorganisms		
	Pseudomonas sp.	Bacillus sp.	Micrococcus sp.
Gram staining	Gram Negative, Rod	Gram Positive, Rod	Gram Positive, Cocci
Catalase	Positive	Positive	Positive
Oxidase	Positive	Positive	Negative
Indole	Negative	Negative	Negative
Methyl Red	Negative	Negative	Negative
Voges proskauer	Negative	Positive	Negative
Citrate	Positive	Negative	Negative
Urease	Negative	Negative	Positive
Nitrate Reduction	Negative	Positive	Negative

The three different bacterial species isolated from oil contaminated soil were screened for their biosurfactant activity by Drop Collapsing Test, oil spreading technique and emulsification stability test in three different oils namely vegetable oil, petrol and diesel. In oil spreading test the organisms *Pseudomonas, Bacillus* and *Micrococcus* produced clear zone (Table 2) and in the drop collapse test the samples were collapsed. This clearly indicated that the three organisms produced biosurfactant.

Similarly the three organisms were able to form stable emulsions for 24 h (Table 3). These emulsification results showed that, biosurfactant produced from a substrate can emulsify different hydrocarbons to a greater extent which confirmed its applicability against different hydrocarbon pollution (Thavasi, Jayalakshmi & Banat, 2010.). Among the three oils the higher E24 value was observed in diesel and *Pseudomonas* sp. showed the highest biosurfactant activity compared to *Bacillus* sp. and *Micrococcus* sp. The results are shown in Table 2 and 3.

Table 2: Oil Spreading Test for Pseudomonas sp., Bacillus sp., and Micrococcus sp.

Micro-organism	Zone formation in various oils tested (diameter in mm)		
	Vegetable oil	Petrol	Diesel
Bacillus sp.	11	17	24
Pseudomonas sp.	18	26	32
Micrococcus sp.	9	14	19

Table 3: Emulsification Stability Test for Pseudomonas sp., Bacillus sp., and Micrococcus sp.

Micro-organism	E24 value (%)		
	Vegetable oil	Petrol	Diesel
Bacillus sp.	45	52	66
Pseudomonas sp.	57	68	75
Micrococcus sp.	34	49	60

Biosurfactants or microbial surfactants are surface-active biomolecules that are produced by a variety of microorganisms. The results obtained are in accordance with the reports of Priya and Usharani, (2009) where *Pseudomonas aeroginosa* recorded higher biosurfactant activity than *Bacillus subtilis.* Oil contaminated environment contain large amount of hydrocarbons and biosurfactant producing microorganisms were naturally present in the oil contaminated soil. Biosurfactants have gained importance in the fields of enhanced oil recovery, environmental bioremediation, food processing and pharmaceuticals owing to their unique properties such as high biodegradability and lower toxicity.

The degradation capability of isolated bacterial species was determined by gravimetric assay after 25 days of incubation period in which *Pseudomonas sp.* found to degrade oil better than other isolated species. *Pseudomonas* species degraded 92.3% of oil in 25 days followed by 83.7% of oil degraded by *Bacillus* species and 35.5% of oil degraded by *Micrococcus* species (Table 4).

Table 4: Percentage of Oil Degradation of isolated bacterial species after 25 days of incubation

Micro-organism	Before Treatment Oil Content of soil (g)	After Treatment Oil Content of soil (g)	Oil Degradation (%)
***Bacillus* sp.**	1.5	0.2445	83.7
***Pseudomonas* sp.**	1.5	0.1155	92.3
***Micrococcus* sp.**	1.5	0.9675	35.5

The oil degradation by *Pseudomonas* sp. was not surprising not only because it was isolated from oil spilled soil but also because it is known to possess a more competent and active hydrocarbon degrading enzyme system than *Micrococcus* sp. It is known to be fast growing and is capable of degrading a wide variety of organic compounds (Ijah & Okang, 1993). In the case of *Micrococcus* sp. which is also known to possess the considerable efficiency to use it as an oil degrader, but it requires more time compared to that of *Pseudomonas* sp. The results were in accordance with the findings of Van hamme,Singh, & Ward (2003) in which *Pesudomonas* degraded 90.2 % of oil in 30 days followed by 82.3 % of oil degraded by *Bacillus,* 78.8% of oil degraded by *Serratia* and 25.5% of oil degraded by *Staphylococcus*.

Lipases are versatile biocatalysts that can perform innumerable different reactions. Their enantio-, chemo- and stereo-selective nature makes them an important tool in the area of organic synthesis. Unlike other hydrolases that work in aqueous phase, lipases are unique as they act at the oil/water interface. Besides being lipolytic, lipases also possess esterolytic activity and thus have a wide substrate range.

Among the three isolated bacterial species, *pseudomonas* showed maximum zone of clearance (21 mm) when plated on tributyrin agar base (Fig. 1). Maximum lipase enzyme activity was observed for *Pseudomonas* sp. (440 IU/mL) followed by *Bacillus* sp. (390 IU/mL) and *Micrococcus* sp. (170 IU/mL). The results are shown in Table 5 and Fig. 2.

Table 5: Qualitative and Quantitative analysis of isolated bacterial species for Lipase Activity

Micro-organism	Qualitative analysis Zone on Tributyrin agar (Diameter in mm)	Quantitative analysis of Lipase enzyme activity (IU/mL)
***Pseudomonas* sp.**	21	440
***Micrococcus* sp.**	12	170
***Bacillus* sp.**	15	390

Fig 1. Qualitative analysis of Lipase Activity - Tributyrin Plate Assay

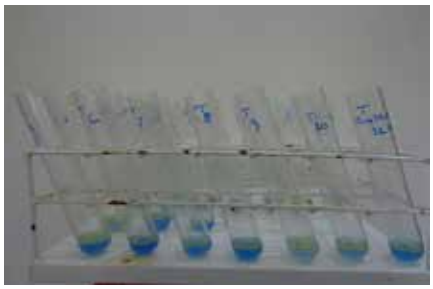

Fig 2. Quantitative analysis of isolated bacterial species for Lipase Activity

In seed germination study *Vigna mungo* (L.) Hepper was chosen for the seed germination test. The oil contaminated soil sample before and after treatment with the biosurfactant obtained from *Pseudomonas* sp. was used for the study. The clean surface sterilized seeds were inoculated in two different pots. The seed germination percentage (85%) was clearly greater than that of the untreated soil (Table 6). The results obtained clearly concluded that bioremediation mediated by *Pseudomonas* sp. has been very effective (Fig. 3).

Table 6. Germination of seeds in Treated and Untreated soil

Sample	Response of seed germination (%)
Soil (Untreated)	20
Soil (Treated)	75

Values are mean of tripicates

Fig 3. Seed Germination Test in Treated and Untreated soil

Cleaning up of petroleum hydrocarbons in the subsurface environment is a real world problem. A better understanding of the mechanism of biodegradation has a high ecological significance that depends on the indigenous microorganisms to transform or mineralize the organic contaminants. Microbial degradation process aids the elimination of spilled oil from the environment after critical removal of large amounts of the oil by various physical and chemical methods. This is possible because microorganisms have enzyme systems to degrade and utilize different hydrocarbons as a source of carbon and energy.

These results conclude that three different bacterial species were isolated from oil contaminated soil and their degradation capability was checked individually by various screening methods among which *Pseudomonas* sp. showed highest degradation efficiency followed by *Bacillus* sp. and *Micrococcus* sp. Thus the above experiment shows that bioremediation can be used effectively to treat oil contaminated soil. By using biological processes, as in the case of bioremediation, usually lowers the costs as compared to chemical treatment processes for various contaminated sites. It is also less disturbing to the environment. The toxicity and fertility of the soil before and after treatment was also assessed, thereby proving that biostimulation is an effective method of reducing environmental pollution. Therefore, based on the present study, it may be concluded that microbial degradation can be considered as a key component in the cleanup strategy for petroleum hydrocarbon remediation.

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