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Biodegradation Potentials of Petrol Degraders from waste-Lubricating oil-Spilled Soils

*Sadhana valmiki, Swati Singh, Mamta Tiwari, Swati Jain
Lords Universal College, Goregaon, Mumbai

Abstract: *Petroleum refineries around the world have generated the solid wastes during the refining process and stocking of crude oil. 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. The potential of bacterial isolates as hydrocarbon degraders was investigated. A total of 8 bacterial isolates were able to grow on mineral salt medium by enrichment procedure. 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. Two isolates were isolated, the hydrocarbon degrading bacterial species such as two isolates of Pseudomonas were isolated from the oil spilled contaminated soil. The level of petroleum hydrocarbon degradation was determined by gravimetric assay. After 25 days of incubation period, Strain 1 found to degrade oil better than other isolated species. Strain 1 degraded 80% of oil in 25 days followed by 85.7% of oil degraded by Pseudomonas species. In addition, emulsification test was carried out for the two organisms, and the results showed that Pseudomonas. has the highest emulsification ability at 1% spent oil. The present investigation shows that Pseudomonas sp isolated can be effectively used to degrade oil contaminated soil.*

Keywords: *Bioremediation, Oil spills, Oil degradation, Bacillus sp*

I. Introduction

The quality of life on earth is linked inextricably to the overall quality of the environment. Releases of persistent bio accumulative and toxic chemicals have a detrimental impact on human health and the environment (Onuoha et al., 2011).Contamination with petroleum hydrocarbons pose a significant threat to terrestrial and marine ecosystems, tourism and recreation activities (Zhang et al., 2012). Oil spill have become a global problem in industrialized and developing countries. 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).In Mumbai, oil spills at auto mechanic workshops have been left uncared for over the years and its continuous accumulation is of serious environmental concern, because of the hazard associated with it. For instance the spent motor oil disposed off improperly contains potentially toxic substances such as benzene (carcinogens), lead, arsenic, zinc and cadmium, which can seep into the water tables and contaminate ground water (Igwe et al., 2008; Shah et al., 2009). It consequently results in serious health hazard such as anemia and tremor, which can cause death. Attention has been focused on the marine environment, because of the largest and most dramatic spills (Cooney, 1984).

Awareness of this reality has led to international efforts to remediate many of these sites, either as a response to the health risks or to control the detrimental effects on the environment caused by contamination aiming the recovery of the contaminated sites. Over the years, many cleanup methods have been developed and applied (Pawar, 2012). The removal of petroleum hydrocarbons contamination can be carried out by physical and chemical treatments,which allows the recovery of the adsorbent and adsorbed, though it is a technique that requires a lot of expenses (Daifullah and Girgis, 2003). Various conventional methods like land filling, incineration, air spurging, etc. have been applied to remove these hydrocarbons since long time for remediation of oily waste. It is observed that none of the conventional methods is environment friendly solution (Sood et al., 2009). Nevertheless, biological treatment is an efficient, environment-friendly, and cost-effective technology for both ex-situ and in-situ remediation of environments contaminated by hydrocarbons (Liu et al., 2011).The present work 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 hydrocarbons 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 bioaugmentation studies during bioremediation.

II. Materials & Methods

The petrol fuel used in this experiment was purchased from a local oil filling station and stored in dark at ambient temperature throughout the study. Oil contaminated Soil sample was (500g) collected from a local garage which was used for isolation of hydrocarbon utilizing microorganisms. The samples were collected in pre-sterilized sample bottle following aseptic conditions. The samples duly labeled were stored at 4^0C for further analysis.

Medium used for Screening and isolation of petrol degraders

The bacterial isolates used in this study were isolated from soil collected nearby different gas stations by enrichment cultivation. Nutrient agar plates enriched with 1.0 % petrol 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. 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. The selected bacterial isolates based on the phenotypic variations were cultivated overnight in LB broth. The washed bacterial cells were used to inoculate 300 ml flasks containing 25ml of LB supplemented with 1ml crude oil. All flasks were incubated at 30^oC with checking at 150 rpm/min for 13 day. The estimation of crude oil degradation was determined gravimetrically (Sakalle and Rajkumar, 2009) and the best crude oil degraders were selected for further investigations.

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 37ºC 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). Petrol (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 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 petrol and 10 µl of culture supernatant were added respectively. The culture was introduced at different spots on the petrol 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 petrol 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) The percentage of the E24 index is calculated by the following formula:

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

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 50º 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:

$$\text{Percentage of petrol degraded} = \frac{\text{Weightof petrol degraded}}{\text{Wight of petrol present originally}} * 100$$

where,The weight of petrol degraded = original weight of petrol - weight of residual petrol obtained after evaporating the extractant.

III. Results and Discussion

Isolation of petrol degraders

An enrichment culture has been widely accepted as the method of choice for isolating bacteria expressing specific phenotypes and has been used successfully to isolate bacteria capable of degrading hydrocarbons (Al-Wasify and Hamed, 2014). A total of 12 bacterial isolates were able to grow on mineral salt medium by enrichment procedure screening test of the isolates for hydrocarbon degradation shows that all the bacterial isolates showed different degree of degradation, but 2 out of all the isolates that have the highest degree of degradation were chosen for further studies and were characterized. The two isolates designated as, I and II, which showed the highest degree of degradation in mineral salt medium using spent oil as sole source of carbon were characterized and identified to the genus level on the basis of colony morphology, cultural, physiological and biochemical characteristics. They were identified as *Pseudomonas aeruginosa* and *Pseudomonas putida* (Table I) (Buchanan & Gibbons, 1976).

Table 1: Biochemical tests for identification of Strain I and II

Biochemical Tests	**Results (Strain I)**	**Results (Strain II)**
Gram staining	Grams –ve (Rods)	Grams –ve (Rods)
Catalase	+ve	+ve
Nitrate reduction	+ve	-ve
Indole	-ve	-ve
Citrate	+ve	+ve
H_2S gas production	-ve	-ve
Oxidase	+ve	+ve
Urease	-ve	+ve
Methyl Red	-ve	-ve
VP	-ve	-ve
Growth at 37^0C	+ve	+ve
TSI	-ve	A/K
	Pseudomonas aeruginosa	*Pseudomonas putida*

Screening for Biosurfactant Activity

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 sp produced clear zone (Table 2) and in the drop collapse test the samples were collapsed. This clearly indicated that the two organisms produced biosurfactant.

Table 2: Oil Spreading Test for Strain I and II

Micro-organism	**Zone formation in various oils tested (diameter in mm)**
Strain I	26
Strain II	17

The two different bacterial species isolated from oil contaminated soil. 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 (Table 3) (Thavasi, Jayalakshmi & Banat, 2010.).

Table 3: Emulsification Stability Test for Strain I and II

Micro-organism	**E24 value (%)**
Strain I	33.3
Strain II	34.2

A variety of microorganisms produce biosurfactants or microbial surfactants which are surface-active biomolecules. 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. In the fields of oil recovery, environmental bioremediation, food processing and pharmaceuticals owing to their unique properties such as high biodegradability and lower toxicity, biosurfactants have gained importance.

Estimation of oil using Gravimetric method

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. Strain I degraded 80.0 % of oil in 25 days followed by 85.7% of oil degraded by Strain II (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 (%)
Strain I	1.4	1.12	80.0
Strain II	1.4	1.21	85.7

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). The present studies also revealed that the organism exhibited varying ability to grow, utilize and emulsify the spent oil. Pseudomonas sp has the highest emulsification at 1% spent motor oil followed by other Pseudomonas sp. These organisms produced surface active agents (biosurfactants) during oil degradation. These organisms have earlier been associated with the production of biosurfactants when grown on petroleum hydrocarbons. Microbial biosurfactants are useful as soaps and detergents and thus, their application in simple cleaning, tertiary oil recovery and oil spill cleanup (Cooper, 1986). It therefore means that these organisms may be useful in treating oil spills in the environment.

IV. Conclusion

Pseudomonas sp exhibit the ability to bio-transform a wide range of organic compounds and are able to degrade various chemical pollutants such as simple hydrocarbons, aromatic hydrocarbons, nitroaromatics, chlorinated polycyclic aromatics etc. Therefore the present study was focused to isolate a novel petrol degrading bacteria from the oil spilled soil which can be useful for the remediation of oil contaminated soil.

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