INTERNATIONAL RESEARCH JOURNAL OF PHARMACY
www.irjponline.com
ISSN 2230 – 8407

Research Article

ISOLATION AND IDENTIFICATION OF PETROL DEGRADING MICROORGANISMS FROM CONTAMINATED SOIL AND COMPARISON OF THEIR BIOREMEDIATION POTENTIAL

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Article Received on: 06/01/17 Approved for publication: 18/02/17

DOI: 10.7897/2230-8407.080221

ABSTRACT

The present work was aimed to isolate bacterial strains capable of metabolizing engine oil in the studied environments which can later be used in biotechnology for environment de-pollution. Petrol contaminated soil samples were collected from eighteen filling stations at Dehradun, Uttarakhand, India. Nine strains were isolated from various petroleum contaminated sites using enrichment technique. All the isolated strains were identified by staining and biochemical tests. They included *Enterobacter sp., Klebsiella sp., Enterococcus sp., Streptococcus sp.,* and *Serratia sp*. Biosurfactant activity of the isolated strains was detected using drop collapsing test, oil spreading method and emulsification stability test. *Enterococcus sp.* produced maximum biosurfactant activity followed by *Serratia sp.* and *Klebsiella sp.* while *Enterobacter sp.* produced 61.11% emulsification index followed by 50.00% by *Klebsiella sp.* and 47.36% by *Serratia sp*. The percentage of petroleum hydrocarbon degradation was determined by gravimetric assay at 7 days interval for a total period of 35 days. *Streptococcus* sp. degraded 95.45% of oil in 35 days followed by 90.09% of oil by *Enterobacter* and *Klebsiella* sp. and 81.81% by *Enterococcus* sp.

Keywords: Biodegradation, Biosurfactant activity, Gravimetric assay, Petrol degradation.

INTRODUCTION

Hydrocarbon contamination is one of the most significant source of pollution around the world.[1] Petroleum is used as a conventional energy source even though it has prominence as a global environmental pollutant.[2] The increase in automobiles, number of gasoline stations and auto mobile service is ever increasing. During transfer and service, oil is spilled by which soil is contaminated.[3,4] Contamination causes soil to lose its useful properties such as binding capacity and fertility. Oil released into the environment affects many plants, animals, micro-organisms and humans within the oil impacted environment. Prolonged exposure to oil as well as high concentration of oil could cause the development of liver or kidney disease, possible damage to the bone marrow and an increased risk of cancer.[5,6,7] The clean-up of soil and groundwater pollution caused by hydrocarbon compounds is the utmost challenge in environmental remediation. Fortunately, the degradation of these oils in the environment is possible through several techniques: physical, chemical or biological. The technology commonly used for the soil remediation includes mechanical, burying, evaporation, dissolution, dispersion and washing. However, these technologies are expensive and can lead to incomplete decomposition of contaminants. To overcome these problems, microbial bioremediation is the only way to preserve our nature. The presence of different substrates and metabolites in hydrocarbon contaminated soils has provided an environment for the development of a quite complicated microbial community.[8,9] Some microorganisms can utilize the hydrocarbons as sole carbon sources for getting their energy and metabolic activities.[10] Among them *Bacillus* sp., *Rhizobium* sp., *Microbacterium oxydans* and *Arthrobacter* sp., *Micrococcus* sp., *Corynebacterium* sp., *Flavobacterium* sp., *Pseudomonas* sp. have been reported.[11] The success of oil biodegradation technology depends on the ability to establish and maintain conditions that favour enhanced oil biodegradation rates in the contaminated environments.[3] The objectives of this study were to isolate and identify petroleum hydrocarbon degrading bacterial strains from petrol contaminated sites at Dehradun, Uttarakhand, India, and to assess the biodegradation potential of the selected strains.

MATERIALS AND METHODS

Collection of Samples

Soil samples were collected from eighteen petrol pumps at Dehradun, Uttarakhand. The soil at the sites of sample collection had a black colour due to continous spillage and the soil surfaces were hard. The samples were collected in sterile ziplock bags, brought to the laboratory and stored at 4^0C till further analysis.

Isolation of Bacteria

The oil degrading bacteria were isolated by the enrichment technique. Nutrient agar plates enriched with 1.0 % petrol were prepared. 5 gm of oil spill contaminated soil sample was weighed aseptically and added to 45 ml of sterile distilled water. The flasks were placed in a rotary shaker for about 30 min at 30^0C. Serial dilutions of the samples from 10^{-1} to 10^{-5} were prepared. 0.1 ml of each dilution was plated on duplicate nutrient agar plates using spread plate method. The Petri plates were then incubated at 37^0C for 24 to 48 hours. The colonies so obtained on the plates were marked and numbered. They were then streaked onto nutrient agar plates for purification and identification. Bacterial pure cultures were also maintained on nutrient agar slants and broth, stored at 4˚C and sub cultured every month.

Characterization of Bacterial Isolates

Isolated pure strains were identified on the basis of morphological and physiological characteristics and by biochemical tests.[12] Simple and Gram staining was carried out. Catalase test, oxidase test, citrate utilization test, indole production test, methyl red-Voges Proskeur (MR-VP) test, urease test, gelatinase test, hydrogen sulphide production test and amylase tests were carried out for the identification. Organisms were identified according to the Bergey's Manual of Systemic Bacteriology.[13]

Screening for Biosufactant Production

Screening for biosurfactant production was performed using drop collapsing test, oil spreading method and emulsification stability test.

Drop Collapsing Test

Biosurfactant production was screened using the qualitative drop-collapse test.[14] 2µl Petrol was applied to the well regions delimited on the covers of 96-well micro plates and these were left to equilibrate for 1 hour at 37°C. 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 1 minute. Collapsed drop indicated positive result for presence of biosurfactant and intact drop indicated negative result. Distilled water was used as a 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 cultures were introduced at different spots on the petrol which is coated on the water surface. The occurrence of a clear zone indicates positive result.[15]

Emulsification Index (E_{24})

The emulsifying capacity was evaluated by an emulsification index. E_{24} of the culture samples was determined by adding 2 ml of petrol and 2 ml of the culture supernatants in test tubes. The test tubes were vortexed for 2 minutes to obtain maximum emulsification and allowed to stand for 24 hours.[16] The emulsion index (E_{24}) is the height of the emulsion layer (in cm) divided by total height (in cm), multiplied by 100. Percentage of emulsification index was calculated using the formula:

$$E24 = \text{Height of emulsion formed} / \text{Height of total solution} \times 100$$

Percentage Biodegradation of Petrol by Gravimetric Analysis

Inocula of 0.1 mL aliquots of overnight nutrient broth cultures was washed twice in physiological saline solution (0.87% NaCl, pH 7.2) and suspended in the same to optical density of 0.1 ($OD_{600\ nm}$). Log phase cultures were inoculated to 100 mL of sterile mineral salts medium with 0.2% v/v petrol, in duplicate, with uninoculated media consituting as control.[17] All flasks were incubated at 22^0C for time intervals of 7, 14, 21, 28 and 35 days. The content of each flask was taken at the end of the determined incubation period to assess residual concentrations of crude oil by gravimetric analysis. 3 part Sample:1 part Chloroform was placed in a separating funnel with continuous shaking. The contents were allowed to settle. Watery layer and chloroform layer containing the residual hydrocarbons were formed. After chloroform evaporation, the residual oil was quantified gravimetrically as given by the formula below.[18]

$$\text{Percentage petrol degraded} = \text{Weight of petrol degraded} / \text{Weight of petrol present originally} \times 100$$

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

Table 1: Biochemical Characterization of Isolates

Acc. No.	Gelatinase	Urease	MR	VP	Indole	Citrate utilisation	Catalase	Oxidase	H_2S	Genus
DS01	-	+	-	+	-	+	+	-	-	*Enterobacter*
DS02	-	+	-	-	-	+	+	-	-	*Klebsiella*
DS05	-	-	-	+		-	-	-	-	*Enterococcus*
DS07	+	-	-	+	-	+	+	-	-	*Serratia*
DS08	-	-	-	+	-	+	-	-	-	*Enterobacter*
DS11	-	-	-	+	-	+	+	-	-	*Enterobacter*
DS13	-	-	-	+	-	-	-	-	-	*Enterococcus*
DS17	-	+	-	-	-	+	+	+	-	*Klebsiella*
DS19	-	+	+	-	-	-	-	+	-	*Streptococcus*

Table 2: Screening of Isolates for Biosurfactant Production

Bacterial isolates	Drop collapsing test	Oil spreading test	Emulsification index (%)
Enterobacter sp.	++	+	61.11
Klebsiella sp.	+	++	35.29
Enterococcus sp.	+	+++	35.00
Serratia sp.	+	+++	47.36
Enterobacter sp.	+	++	44.44
Enterobacter sp.	+	++	27.77
Enterococcus sp.	+	++	33.33
Klebsiella sp.	++	+++	50.00
Streptococcus	+	++	45.45

Table 3: Percentage of Petrol Degradation of Isolates After 35 days of Incubation

Bacterial Isolates	Before treatment	After Treatment (35 Days)	Oil Degradation (%)
Enterobacter sp.	2.2	2.2	Nil
Klebsiella sp.	2.2	2.2	Nil
Enterococcus sp.	2.2	0.8	63.63
Serratia sp.	2.2	2.2	Nil
Enterobacter sp.	2.2	2.2	Nil
Enterobacter sp.	2.2	0.2	90.09
Enterococcus sp.	2.2	O.4	81.81
Klebsiella sp.	2.2	0.2	90.09
Streptococcus sp.	2.2	0.1	95.45

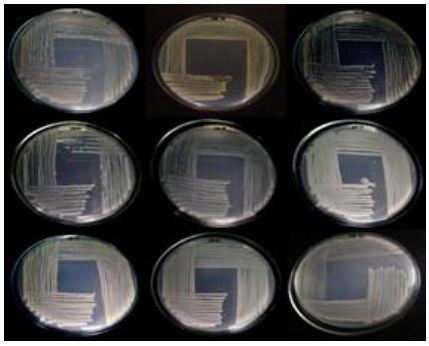

Figure 1: Master plates of bacterial isolates

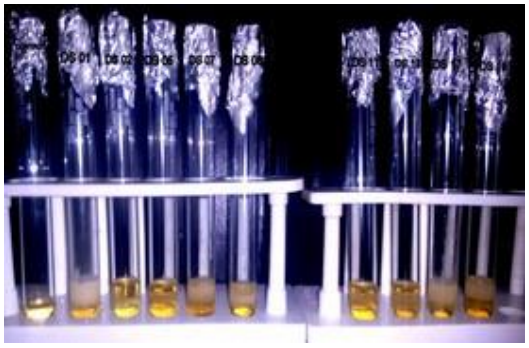

Figure 2: Emulsification index

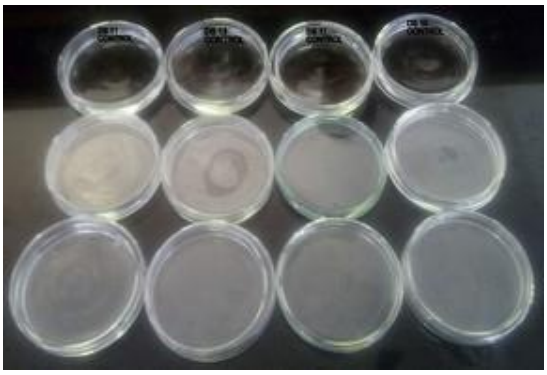

Figure 3(a): Oil spreading method

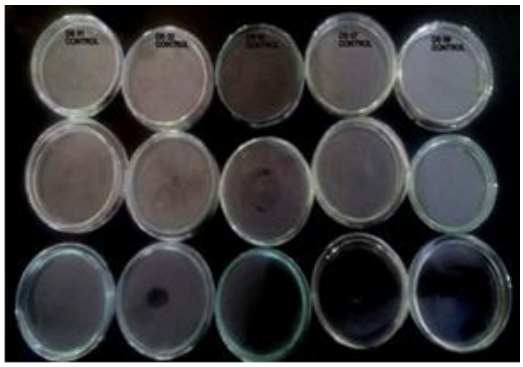

Figure 3(b): Oil spreading method

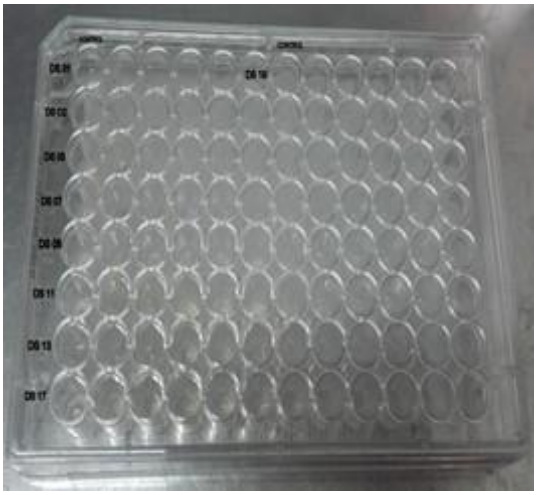

Figure 4: Drop collapsing test

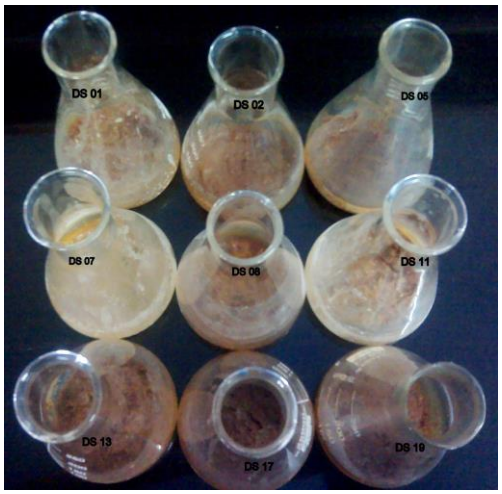

Figure 5: Degradation of petrol by gravimetric analysis

RESULTS AND DISCUSSION

Isolation of Bacterial Strains

Hydrocarbon degrading bacteria were isolated from the oil contaminated soil from 18 petrol filling stations at Dehradun on nutrient agar medium. A total of 9 isolates showing different morphological characteristics on Nutrient Agar plates were selected for further characterization. Isolates which showed different morphology on plates were purified and preserved as shown in Figure 1.

Characterization of Bacterial Isolates

The isolates were characterized for their colony morphology on nutrient agar plates and nutrient agar slants. Biochemical characterization was done for their tentative identification. Results obtained are depicted in Table 1.

The various isolates were identified up to the genus level (*Enterobacter, Klebsiella, Enterococcus, Serratia, Streptococcus).* In an earlier study, bacteria were isolated and characterized from diesel polluted soil by Gram staining, endospore staining, sugar fermentation, sodium chloride test and other biochemical tests and the isolates were identified as *Staphylococcus*, *Micrococcus*, *Klebsiella* and *Bacillus* species.[19] In another study the capability of bacterial strains to utilize petroleum oil as the sole carbon source was evaluated under *in vitro* conditions. A total of four isolated bacterial strains from oil spill contaminated areas were assessed for their oil degradation efficiency. Two biodegradation experiments were performed in low and high (1% and 10%) concentration of crude oil for 21 days using selected bacterial cultures. At temperature 22^0C, out of the four strains, only one demonstrated maximum oil degradation capacity i.e. 66% and 58% respectively, for the two concentrations of crude oil, after 21 days of incubation. Based on biochemical characterization, the isolate was identified as *Bacillus* sp.[20]

Screening of Isolates for Biosufactant Production

The nine bacterial species isolated from oil contaminated soil were screened for their biosurfactant activity by drop collapsing test, oil spreading technique and emulsification stability test. The results obtained were as shown in table 2 and figure 2 to 4. Biosurfactants are biological amphipathic compounds produced by various bacteria, fungi and molds. They are lipid compounds with hydrophobic end consisting of hydrocarbon and hydrophilic end consisting of carbohydrate, amino acid, cyclic peptide, phosphate and carboxylic acid or alcohol. They enhance the emulsification of hydrocarbons, solubilize hydrocarbon contaminants and increase their availability for microbial degradation. Due to their advantages, such as lower toxicity, high biodegradability, higher foaming, better environmental compatibility, the ability to act in high temperatures, low pH, different salinity levels and low production costs, biosurfactants are preferred to synthetic and chemical surfactants. These components have extended applications in petrochemical and oil industries, pharmacy, medical, cosmetics, food and pharmaceutical.

The flat drop appearance in micro titer plate confirmed positive result for drop collapse test as suggested by Jain[21], proving the use of drop collapse method as a sensitive and easy method to test for biosurfactant production. *Enterococcus* produced maximum biosurfactant activity followed by *Serratia* and *Klebsiella* while *Enterobacter* produced 61.11% emulsification index followed by 50.00% by Klebsiella and 47.36% by Serratia. In a similar study twenty-one oil-degrading bacteria were isolated from bilge water, of which seven strains were selected for further studies. They were found to belong to *Bacillus, Pseudomonas* and *Halomonas* Genera. Based on a high growth rate on crude oil and hydrocarbon degradation ability, *Psedomonas* showed a high Biosurfactant Activity and *Bacillus* showed high Emulsification Index.[22]

Percentage Biodegradation of Petrol by Gravimetric Analysis

The degradation capability of isolated bacterial species was determined by gravimetric assay after 35 days of incubation in which *Streptococcus* species was found to degrade oil better than other isolated species. *Streptococcus* species degraded 95.45% of oil in 35 days followed by 90.09% of oil by *Enterobacter* and *Klebsiella* species and 81.81% by *Enterococcus* species. The results obtained are as shown in Table 3 and Figure 5.

Earlier the level of petroleum hydrocarbon degradation at 5 days interval by gravimetric assay was determined by Chithra.[23] They found that *Pseudomonas* species degraded 92.3% of oil in 25 days followed by 83.7% by *Bacillus* species and 35.5% by *Micrococcus* species.

CONCLUSION

Oil contamination affects the environment and in return, the environmental conditions impact its bioremediation processes. In this study, nine oil-degrading bacterial strains were isolated from long-term petroleum contaminated soil from Dehradun, Uttarakhand, India. The isolates were identified as *Enterobacter sp., Klebsiella sp., Enterococcus sp., Streptococcus sp.,* and *Serratia sp.* Further, the species need to be confirmed. This work indicates that the studied environments have the ability to undergo natural attenuation and clean-up of engine oil over time. Our research shows that oil contaminated soil can be a principle source for potent oil degrading bacteria. Also, the bacterial isolates obtained from this study could be exploited for oil spill clean-up in similar environments.

ACKNOWLEDGEMENTS

The authors are thankful to Dolphin (PG) Institute of Biomedical and Natural Sciences for providing the necessary laboratory requirements and facilities to carry out this work.

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Cite this article as:

Deepti Gulati and Srishty Mehta. Isolation and identification of petrol degrading microorganisms from contaminated soil and comparison of their bioremediation potential. Int. Res. J. Pharm. 2017;8(2):34-38 http://dx.doi.org/10.7897/2230-8407.080221

Source of support: Nil, Conflict of interest: None Declared