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ISOLATION AND CHARACTERIZATION OF DIESEL DEGRADING BACTERIA FROM OIL POLLUTED SOIL

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Introduction

Petrol and diesel are one of the most used petroleum hydrocarbons for energy sources giving rise to inevitable spillage during routine operations. The advent of modern industrialization and development in transportation and the use of petroleum based energy products have been increased (Gary and Handwerk, 2001). The pollution may occur due to spillage of oil through storage tank, accident during transportation by truck, ship or oil pipelines (Castro-Gutierrez et al., 2012). Oil contamination is one of the most dangerous pollution factors known today. It can cause a threat to the environment. Petroleum exploitation, exploration, transportation, consumption, attendant spills and disposal often lead to release of hydrocarbon (HC) pollutants into the environment with serious ecological problems (Okoh, 2006). Petroleum pollutants are not only toxic to biological components of the environment, some are indeed carcinogenic. Mechanical and chemical methods to reduce hydrocarbon pollution are often expensive, time consuming and not environment friendly (Mandri and Lin, 2007). Diesel oil is one of most widely used refined petroleum (Yuswono, 2008). Continues low-level input of diesel oil accidentally from pipeline, tanker, or storage tank during refuelling creates an acute pollution problem. Among several clean–up techniques available to remove petroleum hydrocarbons from the soil and groundwater, bioremediation processes are gaining ground due to their simplicity, higher efficiency and cost–effectiveness when compared to other technologies (Mariano et al., 2007). The best biological way to mitigate the pollution load is to utilize existing oil degrading microorganisms in the environment. This way does not damage the environment and it is cost-effective. Bioremediation is a popular approach of cleaning up petroleum hydrocarbons because it is simple to maintain, applicable over large areas, cost-effective and leads to the complete destruction of the contaminant (Frankenberger J., 1992). When microorganisms grow in environment rich in hydrocarbon, they undergo many adaptations. One such adaptation is biosurfactant production which is a frequently encountered feature in hydrocarbon degrading bacteria or sometimes even a prerequisite for growth on hydrocarbons (Pirnik et al., 1974). Hence our present study was aimed to isolate fuel oil degrading bacteria from native environment (Petrol and Diesel polluted soil collected from in and around Coimbatore, Tamil Nadu) and study the biodegradation ability of Diesel by the isolated strains. Finally the potential strains were identified using suitable biochemical tests.

Materials and Methods

Sample Collection

Soil samples were collected from 24 different oil contaminated sites in sterile bottles and Polythene bags (Work shop, Petrol pump, Service Centre). Samples were collected in 5 cm depth from the surface of soil to avoid surface contamination. Collected samples were transferred to the laboratory under sterile condition and stored at 4°C until processed for analysis.

Isolation and Enumeration of Total Heterotrophic Bacteria (THB)

1g of the soil was taken from each sample and it was serially diluted from 10^{-2} to 10^{-8}. 100µl from each sample was spreaded onto nutrient agar medium and incubated at 30ºC for 24-48 hours. The colonies on each plate were counted and tabulated

Analysis of Total Hydrocarbon Present in Soil

About 10 g of soil samples was taken and transferred into a 50-ml flask and the hydrocarbon content in oil polluted soil was extracted using 10 ml of n-hexane. The mixture was shaken vigorously on a magnetic stirrer for 30 min and allowed to stand for 10 min until the hexane extract completely separate the oil from the soil sample. The solution was then filtered using a Whatman filter paper and the liquid phase extract (filtrate) diluted by taking 1 ml of the extract into 50 ml of hexane. The absorbance of this solution was measured spectrophotometric at a wavelength of 420 nm spectrophotometer using n-hexane as blank (Akpan and Usuah. 2014).

Acclimatization Study

1g of soil (highly polluted) was transferred into sterile LB broth and incubated. After incubation the broth was centrifuged and the collected pellet was washed with saline (1.0 OD at 620 nm) and introduced into Bushnell – Hass (BH) medium (Magnesium sulphate 0.2 g, Calcium chloride 0.02 g, Monopotassium phosphate 1 g, Dipotassium phosphate 1 g, Ammonium nitrate 1 g and Ferric chloride 0.05 g per litre) containing 1% of Diesel separately. The flasks were kept in rotary shaker for 15 days with100 rpm. After specified time, flasks were taken out. The ability of the consortium to degrade Diesel was studied by determining the following parameters

pH Estimation

Sample from the culture medium was checked for pH after 0, 5, 10, and 15 days of treatment with the pH meter.

Carbon Dioxide Estimation

One ml of sample was taken after 5, 10, 15 and 20 days of treatment and titrated against 0.05 N NaOH solution. Phenolphthalein was used as the indicator and appearance of stable pink colour was considered as the end point. The amount of CO2 was calculated using the following equation:

$$\text{Free } Co_2 \text{ (mg } L^{-1}) \quad \frac{\text{Titre value} \times \text{Normality of NaOH} \times 1000 \times 44}{\text{Volume of the sample}}$$

Emulsification Index

About 2 ml of the medium was centrifuged at 3000 rpm for 15 min to separate the cells. The supernatant was collected in the test tube while the cells were discarded. The emulsification stability of the isolate was determined by adding 2 ml of crude oil to the test tube containing the spent medium. The tubes were vortexed for 2 min and allowed to stand for 24 h. the emulsification index was calculated as the height of the emulsion divided by the total height of supernatant with added oil multiplied by 100 adapted from Abbasi and Amiri, 2008.

Emulsification Activity

Emulsification measurement: Emulsification activity was measured according to the method of Cooper and Goldenberg (1987) with a slight modification. To 4 ml of culture supernatant or biosurfactant crude extract (0.5%, w/v), 1ml of benzene was added and vortexed at high speed for 10 min. The mixture was allowed to stand for 1hr prior to measurement. The emulsification activity was determined by taking OD at 610 nm.

Isolation of Hydrocarbon Degrading Bacteria

The bacteria (obtained from acclimatization study) were isolated from the consortium by spread plate technique on Bushnell - Hass (BH agar) supplemented with single hydrocarbon compound as sole carbon source (1% diesel). The plates were incubated for 7-10 days and observed by zone formation

Biochemical Test

The following test was performed in order to identify the strains. The tests were Lactose Fermentation, Glucose Fermentation & Gas Production, Sucrose Fermentation, Indole Test, Methyl Red/Voges-Proskauer (MR/VP), Citrate Utilization test, Nitrate reduction test, Motility Test, Catalase and oxidase test.

Results

Collection of Samples

The samples were collected from 24 different places as listed out in the table .The Collected samples were transferred to the laboratory under sterile condition and stored at 4°C until processed for analysis. The microbial load was found to be in the range of 20-100 CFU/ml.

Table 1: Total Heterotrophic Bacterial Population Count

Sample	10^{-6}	10^{-8}
1	100	20
2	80	65
3	50	21
4	35	28
5	90	20

Analysis of Total Hydrocarbon Present in Soil

Total hydrocarbon present in the collected sample was determined by using hexane as a solvent. The absorbance range was found to be in the range of 0.011 -2.269. We chosen 5 samples based on the absorbance.

Table 2: Analysis of Total Hydrocarbon Present in Soil

Sample	Location	λ Max	Absorbance
S1	Mechanical shop -Neelambur	234	0.277
S2	Mechanical shop -Neelambur	234	0.744
S3	Mechanical shop -Neelambur	234	0.176
S4	Mechanical shop – Trichy road	234	0 .234

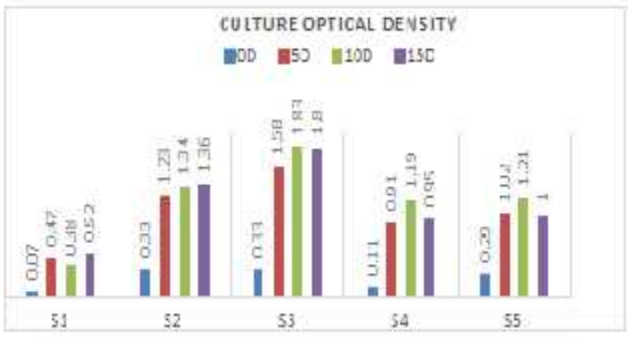
S5	Petrol bank-Trichy road	234	1.108
S6	Petrol bunk - Kalapatti road	234	0.656
S7	Service station -Sulur-trichy road	234	0.059
S8	Mechanical shop-SITRA	234	0.011
S9	Petrol bunk -Kalapatti road	234	0`023
S10	Petrol bunk –M.K.P palayam	234	**1.175**
S11	Service station-Avinashi road	234	**2.269**
S12	Mechanical shop-Sulur	234	**1.647**
S13	Petrol bunk-Sungum	234	1.106
S14	Petrol bunk -Kalapatti road	234	**1.357**
S15	Petrol bunk -Chinniyampalayam	234	0.236
S16	Petrol bunk -Chinniyampalayam	234	0.004
S17	Petrol bunk- Sulur	234	0.116
S18	Mechanical shop- Singanallur	234	0.035
S19	Mechanical shop -Singanallur	234	0.462
S20	Mechanical shop –Avinasi road	234	0.150
S21	Mechanical shop- Ramanathapuram	234	0.184
S22	Petrol bunk -Sulur	234	1.150
S23	Mechanical shop-Trichy road	234	0.287
S24	Mechanical shop Chinniyampalayam	234	**2.612**

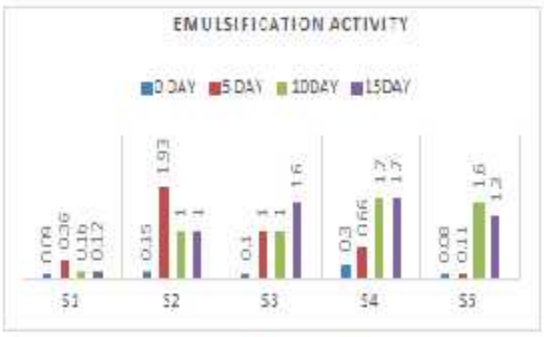
Analysis of Various Parameters

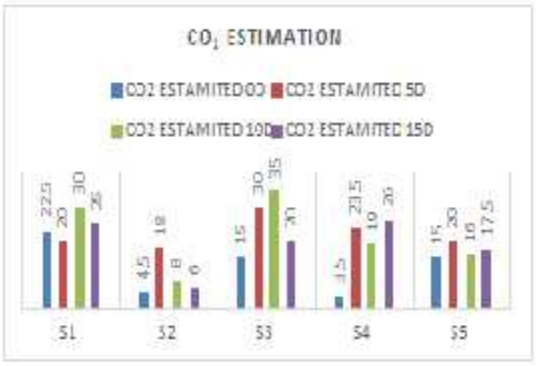
In order to determine the degradation of the diesel oil, various parameters were analyzed. Biosurfactant or bioemulsifiers play a key role in emulsifying hydrocarbons. Biosurfactant and bioemulsifiers are thought to be very suitable alternatives to chemical surfactants. The carbon dioxide analyses were used to estimate the total amount of hydrocarbons mineralized during biodegradation experiments.

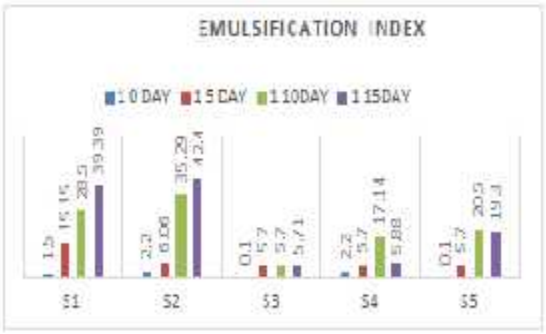
Table 3: Analysis of Various Parameters

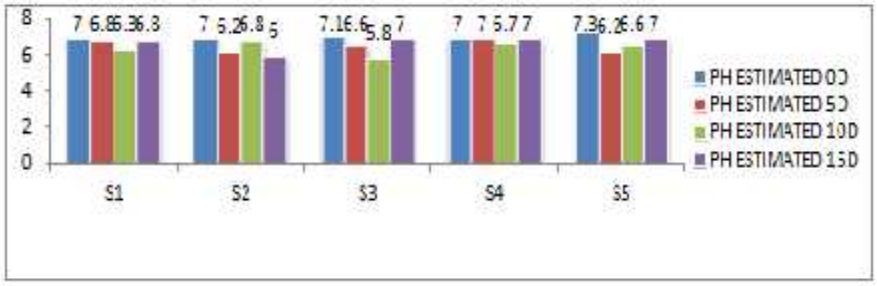
Sample	Sample 1				Sample2				Sample 3				Sample 4				Sample 5			
TESTS	0^{th}D	5^{th}D	10^{th}D	15^{th}D	0^{th}D	5^{th}D	10^{th}D	15^{th}D	0^{th}D	5^{th}D	10^{th}D	15^{th}D	0^{th}D	5^{th}D	10^{th}D	15^{th}D	0^{th}D	5^{th}D	10^{th}D	15^{th}D
PH	7.0	6.8	6.3	6.8	7	6.2	6.8	6	7.1	6.6	5.8	7	7	7	6.7	7	7.3	6.2	6.6	7
CO2 ESTIMATION(mg/l)	0.45	0.4	0.6	0.5	0.3	0.35	0.4	0.3	0.3	0.6	0.7	0.4	0.3	0.32	0.4	0.4	0.3	0.4	0.32	0.35
EMULSIFICATION ACTIVITY(OD)	0.09	0.36	0.16	0.12	0.15	1.93	1	1	0.1	1	1	1.6	0.3	0.66	1.7	1.7	0.08	0.11	1.6	1.3
EMULSIFICATION INDEX(cm)	1.5	15.15	28.57	39.39	2.22	6.06	35.29	42.24	0.5	5.7	5.7	5.7	2.22	5.71	17.14	5.88	0.2	5.7	20.58	19.35
CULTURE OPTICAL DENSITY(OD)	0.07	0.47	0.38	0.52	0.33	1.23	1.34	1.36	0.33	1.58	1.83	1.8	0.11	0.91	1.19	0.95	0.29	1.02	1.21	1











Isolation of Hydrocarbon Degrading Bacteria

The bacteria were cultured on BH mineral salt agar, and the hydrocarbon degrading bacteria produced clear zone.

Biochemical Test Result

The strains were identified as *Bacillus* sp , *Pseudomonas* sp *Psedomonas fluresence* and *Psedomonas aerogenosa* .These two strains are predominantly occur in oil contaminated soil because of the production of Biosurfactant.

Table 4-Biochemical Test Result

Test	S1		S2		S3		S4		S6	
	Result	Growth	Result	Growth	Result	Growth	Result	Growth	Result	Growth
Lactose	+	+	—	+	—	+	—	+	—	+
Glucose	+	+	+	+	+	+	+	+	+	+
Sucrose		+		+		+	+	+		+
Ethanol	—	+	—	+	—	+	—	—	—	+
Mannitol	—	+	—	+	—	+	—	—	—	+
Glycerol	—	+	—	+	—	+	—	—	—	+
Indole Production	—	+	—	+	—	+	—	+	+	+
MR	+	+		+		+	+	+		+
VP	+	+	—	+	–	+	+	+	–	+
Citrate-Utilization	+	+	+		+	+	+		+	+
Nitrate Reduction Test	–	–	–	+	–	+	–	+	–	+
Sim Agar		+			+	+			+	+
Strains identified as	Bacillus sp		Pseudomonas sp		Pseudomonas sp		P fluorescens		P aeruginosa	

Discussion

Out of 24 soil samples collected only 5 samples were utilized based on hydrocarbon analysis study. The consortium was used in degradation of diesel and various parameters also analyzed. Colonies of different morphology was obtained from consortium using spread plate technique. The isolated strains were identified as *Bacillus* sp and *Psedomonas* sp. Study showed the bacterial strains were isolated from contaminated soil samples of two petrol pumps of Karachi by selective enrichment culture technique. For that, Bushnell Hass medium was used that contained 1% diesel. These bacterial strains were identified on the basis of morphological and biochemical characteristics by using Bergey's Manual of Determinative Bacteriology (Khan JA and Rizvi SHA 2011).The enumeration is the best way to study the PAH degrading bacterial population . Therefore, hydrocarbon degrading bacterial strains were enumerated by spread plate method on nutrient agar plate.

In mineral salt medium, the strains showed maximum clearing zone. Clearing of crude oil in the medium showed the bacterial growth. It indicates the degradation, may be due to production of emulsifiers, surfactants etc. Among the 5 isolates, S1 and S5 formed maximum clearing zone on mineral salt medium. Based on various morphological, physiological and biochemical characterization, isolate S1 was identified as *Bacillus* sp and S5 as *Pseudomonas aeruginosa*, .Colony Morphology on nutrient agar plate, *B.subtilis* showed Creamy, big spreading, finely wrinkled and Slimy. Udochukwu *et al.*, (2014) stated that *P. aeroginosa* is the predominant species in petroleum degradation and always found in every oil pollution site analyzed. The genus Pseudomonas is capable of using different substrates, such as glycerol, mannitol, fructose, glucose, n-paraffins and vegetable oils, to produce rhamnolipid-type biosurfactants.

Biodegradation has been widely received by the public. However a number of factors must be taken into consideration before in situ biodegradation can be applied. These includes, type and concentration of oil contaminated, prevalent climatic conditions, type of environment that has been contaminated and Nutrient content as well as pH of the contaminated site. The rate of crude oil biodegradation in the soil seems to be rapid. This may be due to the fact that the microorganisms in the soil have efficiency ability in utilizing the residual crude oil as a source of carbon and energy (Antai SP, 1990). In our study, a slight decrease in pH indicates the production of acids. Microbial utilization of hydrocarbons, often led to the production of organic acids (Amund and Adebiyi 1991; Okpokwasili and James 1995). The increase in pH towards the alkalinity reported could be due to the behavior of the bacterial cells in diesel oil. According to Wu *et al.*, (2013), a consortium of many different bacterial species is required to efficiently degrade polycyclic aromatic hydrocarbons (PAH) in oil-contaminated soil.

Conclusion

The biodegradative potential of the bacterial strains isolated from diesel contaminated soil was analyzed.. Five hydrocarbon degrading bacteria with the ability to degrade diesel oil were isolated and characterized using biochemical testes. Study on Characterization of biosurfactant is under process.

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Collected Soil Samples

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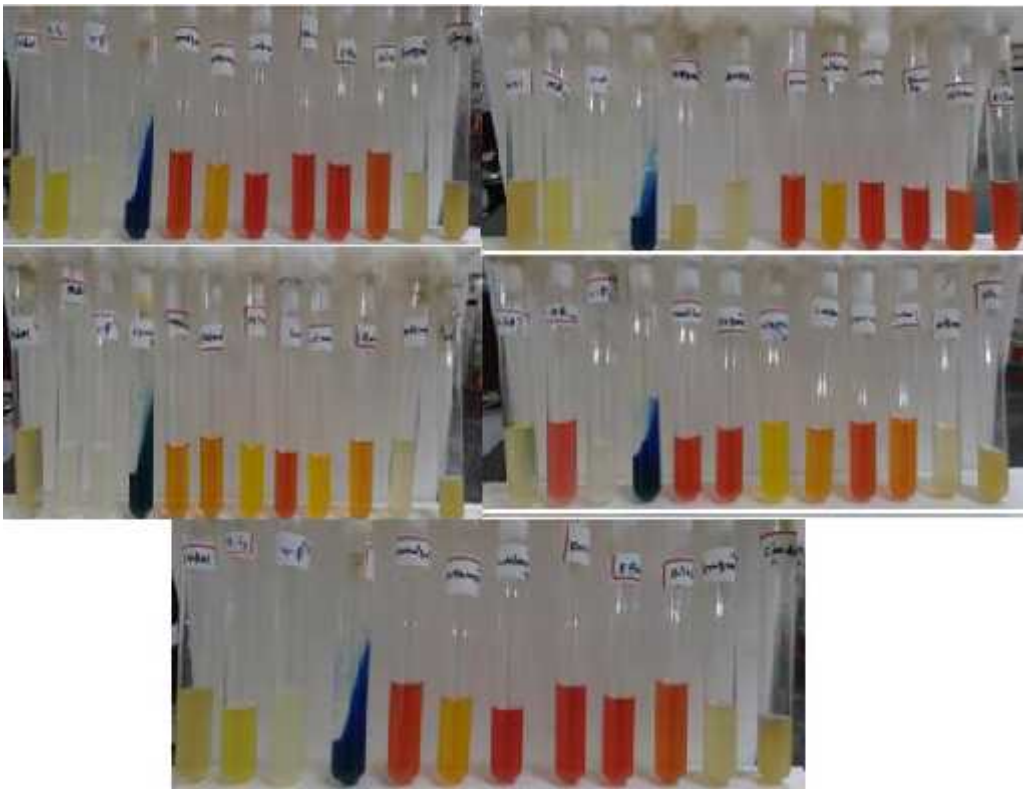
Zone Produced by Hydrocarbon Degrading Bacteria on the Bunshual Hass Media



Biochemical Test of 5 Bacterial Cultures