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Research Article

DOMINANT PETROLEUM HYDROCARBON DEGRADING BACTERIA IN THE CONTAMINATED SOIL OF ALWAR REGION, RAJASTHAN, INDIA

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ABSTRACT

In the present investigation the bioremediation of hydrocarbon in contaminated soils by the cultures of hydrocarbon-degrading bacteria was investigated. Petrol and diesel oil are non degradable. In our study we have identified the *Bacillus* and *Pseudomonas* spp as a great potential for hydrocarbon degradation. The *Bacillus* and *Pseudomonas* spp. were isolated from hydrocarbon contaminated soil and various morphological and biochemical test were applied for their identification. In optimization studies, the best results observed for *Bacillus* and *Pseudomonas spp* .were 2T engine oil as the suitable carbon source, Optimum pH and temperature is 7 and 37°C respectively. The present study reveals the fact that *Bacillus* and *Pseudomonas spp*. have high potential for hydrocarbon degradation and can be used especially for microbial enhanced oil recovery and bioremediation of hydrocarbons in near future.

INTRODUCTION

Bioremediation is one of the most efficient technique, by which we can clean oil spills. Bioremediation is a treatment that uses naturally occurring organisms to breakdown hazardous substances into less toxic or non-toxic substances. Bioremediation of waste materials which contain hydrocarbons and their derivatives is based on the ability of microorganisms growing on these substrates to increase their biomass and to degrade them to non-toxic products, such as H_2O and CO_2 (Toledo *et al.*2006). Unlike solid wastes, further procedures are required to remove these pollutants from the soil. Various crude oil fractions are used in these garages and end up being dumped on soil. Different chemical contaminants are introduced into the soil and mostly require diverse bioremediative processes to curate the pollution. Contaminants usually found at the garages include brake fluid, engine/dirty oil, petrol, kerosene and bitumen (Lang *et al*., 2001). Soil contaminants can be organic; such as pesticides, biocides, petroleum hydrocarbons and chlorinated solvents; or inorganic; such as heavy metals, radionuclides, nitrate and chloride. Many of these anthropogenic compounds are, fortunately, degradable by microorganisms in soil (Alexander 1999, Philp *et al.* 2005).

Das *et al*. (2007) reported to influence the biodiversity, distribution and pollution of Microorganisms in an environment (Latha *et al*. 2012). There have been increased public concerns on the adverse effect of oil exploration on the environment. The toxic effects of crude oil and refined petroleum oils on plants, animals, humans and the environment are devastating (Elliot *et al*.1997). Environmental monitoring of petroleum hydrocarbons pollution ranges from specific methods, such as the use of radioactive labeled compounds to general methods including quantifying gross contamination and evaluating the extent of change caused in the environment by the presence of the pollutant (Farmer *et al.*1997).

These microorganisms are directly involved in biogeochemical cycling of many carbon sources, including petroleumhydrocarbons. The application of bacterial isolates in degrading oil involves the manipulation of environmental parameters to allow microbial growth and degradation to proceed at a faster rate (Vidali *et al*. 2001). The intensity of oils biodegradation is influenced by several factors, such as nutrients, oxygen, pH, composition, concentration and bioavailability of the contaminants, chemical and physical characteristics and the pollution history of the contaminated environment (Al-Darbi *et al.*2005).

MATERIALS AND METHODS

Soil samples were collected from different motor workshop situated at Nimrana industrial area. Petroleum oil used in this study was obtained from Hindustan oil petroleum ltd. Sitapura,

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Jaipur city and sterilized by filter sterilization. All other chemicals used in the present study were of highest purity in grade.

Isolation, screening and characterization

Hydrocarbon degrading bacterial population was isolated from petroleum contaminated soil from Nimrana, Alwar region, Rajasthan, India. Therefore for their isolation selective media as Bushnell Hass Agar media (BHA) containing 2T oil as sole source of carbon and energy was used and incubated for 3 days at 37^0C. The pure isolated bacterial colonies were subcultured, maintained and identified by morphological and biochemical characteristics.

Staining Characteristics (Clark, 1973)

By the use of different staining techniques described that one bacteria were gram negative while other five bacteria were gram positive. Quatrini *et al.* (2008) and Navdeep *et al.* (2013) have been isolated the hydrocarbon degrading bacteria based on the gram staining technique. The gram positive bacteria dominate in oil contaminated areas. The isolated bacterial colonies were studied for their cell wall composition (gram staining).

Biochemical Characteristics

The biochemical tests are applied to identify various bacterial isolates upto their generic level which includes Indole production test, Methyl Red-Voges Proskauer (MR-VP), Catalase production test, Lipase production test, Nitrate reduction test, Starch hydrolysis, Urea hydrolysis, Carbohydrate fermentation test, Gelatin hydrolysis, Phenylalanine deaminase activity was done using the established protocol of Cappuccino & Sherman (1998).

Bacterial degradation of the petroleum oil (2T Engine oil)

This was done using the protocol of Mittal and Singh (2009). The Luria Bertani (LB) Broth (pH 7.5) media and then inoculate the bacterial culture into the LB Broth media at 37 °C for 48 hours. Further the Mineral Salt Media containing pH 5.6$\pm$ 0.2 was prepared. Afterwards 1% 2T engine oil was introduced into the MS Media .Thereafter inoculated 1% of the isolated inoculum from LB Broth into the respective flasks. Finally Gravimetric analysis was done on Day 0, Day 7 and Day 14.

Gravimetric analysis 1% 1N HCl: added in 25 ml media into each flask. 25 ml Petroleum ether and Acetone (in 1:1 ratio) was added and mixed properly. Then 1 ml Acetone was added and the funnel remain still for 15-20 minute. After 15-20 minutes different layers (3 layers) was observed. The 1st and 2nd layers were discarded and the 3rd layer was collected in the weight beaker and kept at water bath at 100 C^o for 10-15 minutes for evaporation. Once the evaporation is complete, clean the beaker from outside properly to remove any water on the outer side and the again weigh the beaker (final weight).

Optimization of growth condition

The optimization condition at pH and temperature was followed using established protocol. (Mahalingam and Sampath, 2014; Triupti. and Dave 2007).

The Effect of hydrogen ion concentration on the growth of the bacterial isolate and their ability to degrade oil at different time intervals were determined using BUSHNELL-HASS medium and Nutrient Broth Media with the different pH i.e. 5, 7and 9 supplemented with 1% oil as carbon source at 37°C. The bacterial isolates were determined spectrophotometrically at 600nm.

The influence of temperature 25°C and 37°C on the growth of the bacterial isolate and their ability to degrade oil at different time intervals were studied using BUSHNELL-HASS medium and Nutrient Broth Media with pH7 supplemented with 1%2Toil.

RESULTS

Isolation and screening of indigenous bacteria from petroleum contaminated soil samples

Hydrocarbon degrading bacterial population utilize hydrocarbons as a sole source of carbon and energy. Therefore for their isolation selective media containing 2T oil as sole source of carbon and energy was used. Based on varied colony morphology, 3 different types of isolates were screened. They were designated by numbers as isolate no.1, 2 and 3 for further characterization (table-A). Some of these populations were found capable of breaking down complex hydrocarbon molecules by adaption of their degradation machinery and enzyme system (Sohal and Srivastava, 1994). The success of bioremediation strategies is dependent on the presence of appropriate pollutant-degrading microorganisms as well as environmental conditions which are conducive to microbial metabolism (Khan *et al*., 2004).

Fig 1 Pure culture of isolated bacteria

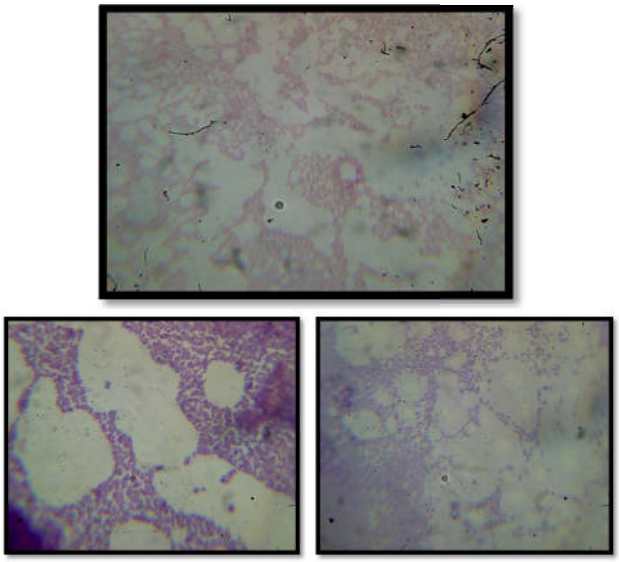

Fig 2 Gram staining of bacterial isolates

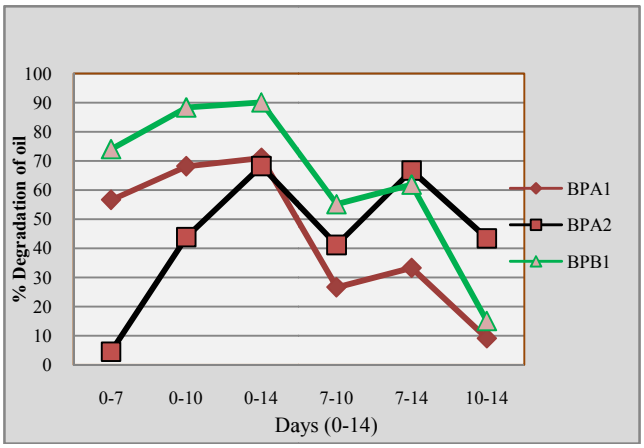

Fig 3 Plot Between %Degradation of oil and 0-14 Days. It represents maximum growth of BPB1 isolate at 0-14 days intervals and at last during the period of 7-14 BPA2 isolate shows highest growth

Optimization of growth condition: For 2T oil Best degrading bacteria*Bacillus spp.* (BPB1) at different pH and temperature:

Table A Colony morphology and gram staining of the isolated bacteria

S.No.	Source	Media	Sample Code	Form	Margin	Elevation	Surface	Color	Gram Staining
01.	Soil	BHA WITH OIL	A	Circular	Entire	Flat	Smooth	White with green centre	Negative
				Circular	Raised	Moist	Opaque	Yellow	Positive
02.	Soil	BHA WITH OIL	B	Circular	Entire	Flat	Smooth	Off-White	Positive

Table B Biochemical cultural characteristics of the isolated bacteria

Isolates	Indole	SIM	MR	VP	G	H2S	Catalase	Citrate	Urease	Oxidase	Phe A	Nitrate reduction	Carbohydrate Fermentation	Starch Hydrolysis	Identification
BPA1-	+		–	–	+	–	+	+	–	+	–	+	–	–	*Pseudomonas spp.*
BPA2	±		–	–	+	–	+	±	+	–	–	+	–	–	*Micrococcus spp.*
BPB3		+	+	–	–	–	+	+	+	–	+	+	+	+	*Bacillus spp.*

Bacterial degradation of the 2T Engine Oil:First calculate the amount of oil left in the beaker after evaporation as follows:
Amount of oil = Final weight of beaker – Initial weight of beaker

Table C Weight of oil

Bacterial Isolates	Sample code	0 Day	7 Day	10 Day	14 Day
Pseudomonas spp.	BPA1	0.728	0.316	0.232	0.211
Micrococcus spp.	BPA2	0.529	0.505	0.297	0.168
Bacillus spp.	BPB1	1.202	0.312	0.140	0.119

Degradation can then be calculated by the following method:-
Degradation = (Initial weight – Final weight)/ Initial weight × 100

Table D % Degradation of oil

Bacterial Isolates	Sample code	0 to 7 Day	0 to 10 Day	0 to 14 Day	7 to 10 Day	7 to 14 Day	10 to 14 Day
Pseudomonas spp.	BPA1	56.5934	68.1319	71.0165	26.5823	33.2278	9.05172
Micrococcus spp.	BPA2	4.53686	43.8563	68.242	41.1881	66.7327	43.4343
Bacillus spp.	BPB1	74.0433	88.3527	90.0998	55.1282	61.859	15.00

Table E Effect of different pH for growth of *Bacillus spp*. by using 1% 2T Oil in Bushnell Hass Broth media. (Values in optical density)

(Duration)	pH5	pH7	pH9
24 HOURS	0.005667	0.003333	0.006
48HOURS	0.008333	0.009333	0.013333
72 HOURS	0.016	0.018	0.031
7th Day	0.032333	0.038667	0.05

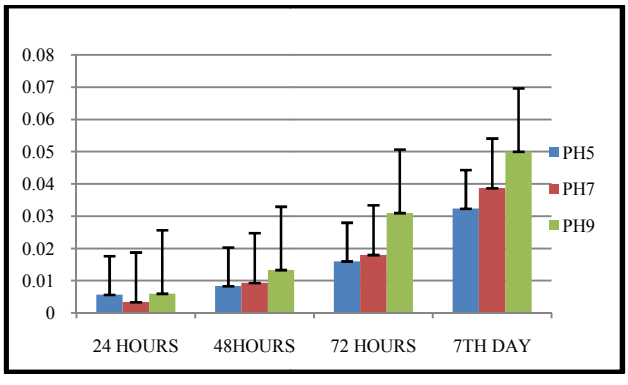

Fig 4 Plot represent that on pH 9 *Bacillus spp.* shows maximum growth at every intervals using BHB media

Table (F) Effect of different pH for growth of *Bacillus spp.* by using 1% 2T Oil in Nutrient Broth media

Duration	pH5	pH7	pH9
24 HOURS	0.104	0.095	0.322333
48HOURS	0.468667	0.382667	0.332333
72 HOURS	0.352	0.596	0.226
7TH DAY	0.081	0.348	0.962

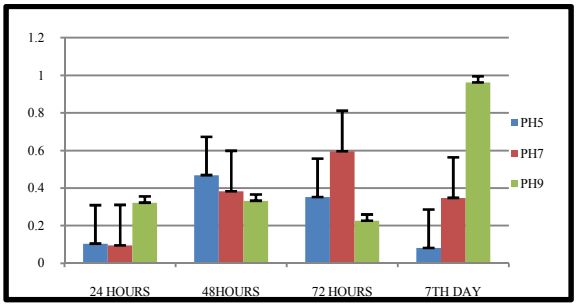

Fig 5 growth is maximum on 7th day but on 3rd day bacterial growth is maximum at pH7. Bacterial growth is different at different pH in separate time periods.

Table G Effect of different temperature for growth of *Bacillus spp.* by using 1% 2T Oil in Bushnell Hass Broth media.

Duration	TEMP 25° C	TEMP 37° C
24 HOURS	0.004	0.004
48 HOURS	0.01433333	0.013
72 HOURS	0.01633333	0.015
7TH DAY	0.029	0.012

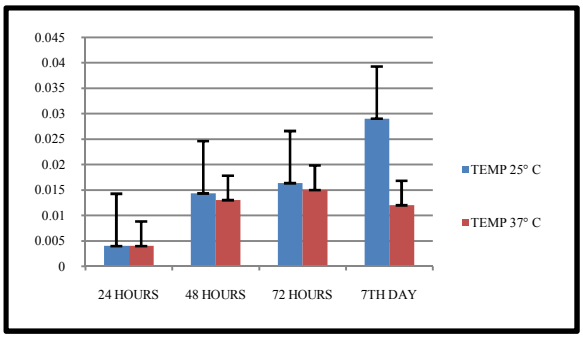

Fig 6 Bacterial growth is highest at 25 degree Celsius temperature at each interval in BHB media

Table H Effect of different temperature for growth of *Bacillus spp.* by using 1% 2T Oil in Nutrient Broth media

Duration	TEMP 25° C	TEMP 37° C
24 HOURS	0.013	0.018
48 HOURS	0.02166667	0.037666667
72 HOURS	0.02266667	0.081
7TH DAY	0.10266667	0.155

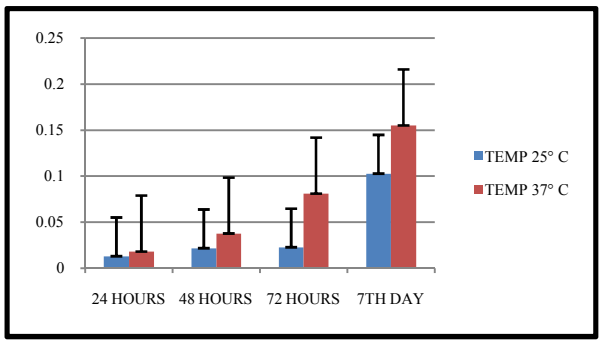

Fig 7 Here, bacterial growth are highest at 37° C at each interval in Nutrient Broth media

DISCUSSION

Recently extensive research has focused on oil bioremediation using pure cultures or mixed bacterial consortia isolated from oil spilled soils. However, only few studies have been reported on the different bacterial communities and diversity in soils, which were contaminated at different times of oil exposure.

Present study focused on the degradation of petroleum oil by a bacterial species isolated from petroleum contaminated site. To make the best isolation of bacterial species with special abilities from sites of pollution, enrichment method, is still an all-important process particularly where the target pollutants serve as the carbon and energy source (Nwinyi 2011; Soudi and Kolahchi 2011). The pattern of degradation varies for different degrading microorganisms because different microorganisms possess different catabolizing enzymes (Penetand Marchal, 2006). Initially a total of 18 bacteria were isolated from the 2 sites, but on the basis of their ability to grow on petroleum oil supplemented Bushnell Hass Agar (BHA) Medium and to degrade oil, 3 were selected for further studies. Two were selected from plot-A (PA-1, PA-2) and one from plot-B (PB-1). These were rod shape, gram negative and gram positive. The isolates 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* (Buchanan & Gibbons, 1976).

Sutton *et al* (2013) clearly underscored that the presence of oil contamination significantly influences bacterial community structure and diversity, regardless of the soil matrix type, and suggested that clean samples had higher diversity than contaminated soil.

By the biochemical cultural characteristics these organisms were identified as: *Pseudomonas spp*., *Micrococuus spp*., *Bacillus spp*. Bacteria belonging to *Bacillus spp*. are predominant bacteria isolated from the polluted soil samples (Ijah and Antai, 2003). This has been observed that *Bacillus spp.,* is more tolerant to high levels of petroleum hydrocarbons. This is attributed to the resistant endospores formation (Ijah and Antai, 2003). These enzymes play an important role in the hydrocarbon degradation and the respective genes that encode those enzymes were identified in recent studies (Whyte *et al*., 2002; Hassanshahian *et al*., 2012).

The success of oil bioremediation depends on our ability to optimize various physical, chemical, and biological conditions in the contaminated environment. Here, *Bacillus spp*. was able to degrade more than 90 % of petroleum. The present study may be applied for efficient removal of petroleum oil containing industrial effluents released from petroleum refineries. *Pseudomonas* and *Bacillus* have been reported to be among the most frequently isolated bacteria from hydrocarbon-polluted sites (Atlas 1992; Okoh and Trejo-Hernandez, 2006). Species of *Pseudomonas, Bacillus, Micrococcus* and *Proteus* isolated from hydrocarbon contaminated site have been found by several authors to utilize hydrocarbon through oxidation of DCPIP (Roy *et al.,* 2002; Joshi and Pandey, 2011; Patil *et al.,* 2013; Adegbola *et al.,* 2014).

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