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Research Article

CHARACTERIZATION AND DEGRADATION POTENTIAL OF ENGINE OIL DEGRADING BACTERIAL STRAIN, ISOLATED FROM BARMER REGION, INDIA

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ABSTRACT
World is highly dependent on petroleum oil and its products. A large amount of petrol is explored, processed and transported every day. Accidentally, these petroleum products have been added into the environment and produce soil and marine pollution. The presence of these pollutants in the terrestrial and aquatic systems affects public health and leads to socio-economic hazard. So treatment of these petroleum contaminants is our necessity. Bioremediation is claimed to be an efficient, economic and versatile alternative to physico-chemical treatment of pollutants. In the present research, soil samples were collected from the Barmer district of Rajasthan, India. Soil samples were characterized on the basis of physico-chemical analysis. Bacterial strains were isolated on Minimal Salt Medium (MSM) and one of the potent engine oil degrader was identified as a *Pseudomonas* strain. The reduction in TPH (Total Petroleum Hydrocarbon) content of engine oil uninoculated (Control) and inoculated with bacterial strain indicates, this strain is able to break down of engine oil content. TPH reduction up to 83% was recorded after 8 weeks remediation. It clearly establishes the potentiality of this strain.

Keywords: *2T engine oil, TPH (Total Petroleum Hydrocarbon), Soil contamination, Pseudomonas spp, Bioremediation, Minimal Salt Medium (MSM)*

INTRODUCTION
The environment has been heavily contaminated with hydrocarbon pollutants, since the beginning of oil exploration and the development of petroleum industry. These enters in the environment through several routes. The most common route is spillages that occur during routine operations and accidents during transportation. The illegal dumping of used motor oil is an environmental hazard with global ramification (Blodgett, 2001). Soil contamination by petroleum products is considered as one of the most serious problems throughout the world (Lu *et al.,* 2015; Pant *et al.,* 2016; Cachada *et al.,* 2018). The presence of these pollutants in the terrestrial and aquatic environments constitutes public health and socio-economic hazard problems (Adelowo and Oloke, 2002; Okerentugba and Ezeronye, 2003; Edewor *et al.,* 2004). So, now these pollutants have reached an alarming stage and recognized as the most significant environmental problem (Timmis *et al.,* 1998; Snape *et al.,* 2001).
The currently used physical and chemical treatments are effective for the degradation of petroleum contaminants but they lag behind in the desired properties and also produce many hazardous compounds which are potent immunotoxicants and carcinogenic for living beings (USEPA, 2001; Lageman *et al.,* 2005). Incineration or burial in secure landfills can become much expensive when the amounts of contaminants are large (USEPA, 2001; ITOPF, 2006). Though these treatments are effective but after burning, soil loses most of its nutritional value and structure. These methods do not remove the contamination but only relocate the problem (Lageman *et al.,* 2005).
Microbial biodegradation of pollutants has intensified in recent years as mankind strives to find sustainable ways to clean up contaminated environments (Diaz, 2008). Biodegradation of oil contaminated soils, which exploits the ability of microorganisms to degrade and/or detoxify organic contamination, has been established as an efficient, economic, versatile and environmentally sound treatment (Margesin and Schinner, 1997; Yuniati, 2018). Biodegradation of petroleum hydrocarbon

pollutants and petrochemicals by bacteria have been extensively investigated (Sanni and Ajisebutu, 2003; Oboirien *et al.*, 2005, Coste *et al.*, 2013).

A large number of bacteria for example *Pseudomonas* spp., (Johnson *et al.*, 1996; Pathak *et al.*, 2008), *Yokenella* spp., *Stenotrophomonas* spp., *Alcaligens* spp., *Roseomonas* spp., *Flavobacter* spp., *Corynebacterium* spp., *Streptococcus* spp., *Providencia* spp., *Sphingobacterium* spp., *Capnocytophaga* spp., *Moraxella* spp., *Bacillus* spp. (Bhattacharya *et al.*, 2002), *Enterobacter* spp. (Jain *et al.*, 2010, Ausama *et al.*, 2014), *Escherichia* spp., *Acinetobacter* spp. (Shiri *et al.*, 2014), *Burkholderia xenovorans* (Bhattacharya and Khare, 2018), *Rhodobacter sphaeroides* (Peng *et al.*, 2018), *Hafnia* spp. (Diaz *et al.*, 2001) and have been reported in the literature for hydrocarbon degradation. A considerable fraction of petroleum oil entering marine system is eliminated by the hydrocarbon degrading activities of the bacteria and these are known as hydrocarbonoclastic bacteria (HCB) (Martins, 2008). A generalized sequence of petroleum component in order of decreasing biodegradability is represented as follows (Huesemann, 1995): n-alkanes > branched chain alkanes > branched alkenes > low molecular weight n alkyl aromatics > monoaromatics > cyclic alkanes > polynuclear aromatics > asphaltenes. To perform a correct assessment, it is necessary to consider various microorganisms having a variety of genomes and expressed transcripts and proteins. However, several high-throughput techniques originally developed for medical studies can be applied to assess bioremediation in confined environments (Watanabe and Kasai, 2008).

The present study focus on the Barmer district of Rajasthan state, India. It is located in the western part of the state forming a part of the Thar desert. The district is located between 24, 58′ to 26, 32′ N latitudes and 70, 05′ to 72, 52′ E longitudes. The Barmer district's population is 2,60,4453 (Census-2011). Presently the Barmer district is in news due to its large oil basin. The Mangala, Bhagyam and Aishwariya (MBA) fields together with their Enhanced Oil Recovery (EOR) potential are being developed in sequence and when complete, MBA production is expected to rise to at least 1,75,000 barrels of oil per day. The main developed area is 1,858 km^2, which includes Mangala, Aishwariya, Saraswati and Raageshwari discovered by the British exploration company **(**Cairn Energy) in 2004. The high-permeability Fatehgarh sandstone is the basin's main hydrocarbon reservoir. The Cairn Energy has started the production in August, 2009 on the large scale. In March 2010, Cairn increased oil potential from this field to 6.5 billion barrels of oil (Cairns India annual report, 2014).

Environmental and safety concerns mean that oil refineries are sometimes located some distance away from major urban areas. Nevertheless, there are many instances where refinery operations are brought close to the populated areas and have posed health risks, such as- Mangala, Bhagyam and Aishwariya are the major oil fields located near the city of Barmer. Therefore, in the present research, attempts were made to isolation and characterization of bacterial strains from oil contaminated sites of Barmer region which can be used to reclaim petroleum oil contaminated sites.

METHODOLOGY

Isolation and Characterization of soil samples

Collection of samples

Soil samples were collect nearby oil contaminated area, after removal of surface litter to a depth of about 5 cm in an approximately 2 m^2 area after survey of different locations of petroleum contaminated sites of Barmer region. Soil samples were processed immediately upon arrival at laboratory.

Physico-chemical characterization of samples

The soil samples were analyzed for soil texture, soil reaction (pH), electrical conductivity (EC), soil moisture content, soil organic carbon (SOC), total nitrogen (N), C/N ratio, available phosphorus and total petroleum hydrocarbon content (TPH).

Microbiological characterization of samples

The population of total bacterial count (TBC) and total petroleum degrading bacteria were enumerated on nutrient agar containing crude oil as a carbon source.

Source enrichment and isolation of bacteria

A defined minimal salt medium (MSM) (Lal and Khanna, 1996) was used for isolation and enrichment of oil degrading bacteria.

The isolated 2T engine oil degrading bacteria were identified by morphological, cultural and biochemical tests (Balows *et al.*, 1992).

Characterization of the degradation potential and its growth patterns

Growth of the isolated bacterial strain was verified by the increase in optical density (OD) and decrease in 2T engine oil. A single colony of the isolate was inoculated into 10 ml nutrient broth at 37ºC overnight. The overnight culture was centrifuged for 10 minutes at 4000 rpm. The cell pellet was washed and suspended in the medium until OD600 was equivalent to one. 1 ml of bacterial inoculum (1OD equivalent) was transferred into 250 ml MSM (Minimal Salt Medium) with 5 ml 2T engine oil and incubated at 37ºC at 170 rpm. A control devoid of the bacterial isolate was prepared for each set of experiment. Different pHs (5.0, 7.0 and 9.0) and different temperatures (15, 30 and 42ºC) were used to test the optimal conditions of each isolates. The growth pattern was obtained by measuring OD at 600 nm.

Bioremediation of soil

The contaminated soil samples were incubated with various bacterial strains to study the bioremediation efficacy. The soil samples prior and after incubation were analyzed for soil reaction (pH), soil moisture content, soil organic carbon (SOC), total nitrogen (N), C/N ratio, available phosphorus (P), total petroleum hydrocarbon content (TPH), total bacterial count (TBC) and total petroleum degrading bacteria.

RESULTS AND DISCUSSION

The Normal soil of Barmer is sandy loam. The percentage amount of sand, slit and clay were 73.4%, 24.6% and 1.8%. recorded whereas it was 64.3%, 33.6% and 2.1% in petroleum contaminated soil sample. pH of petroleum contaminated soil sample was 7.5 and in normal soil it was normal. Electric conductivity (EC) of Petroleum contaminated soil sample was 185 µS/cm recorded. It was higher than the normal soil sample (97.8 µS/cm). The Nitrogen and available phosphorus content in normal soil sample was 0.06 and 1.1 mg/kg respectively. These contents were higher in Petroleum contaminated soil sample (0.1 and 1.6). The soil organic carbon was higher in Petroleum contaminated soil sample (6.7%) rather than normal soil sample (2.9 %). The C/N ratio was also high in Petroleum contaminated soil sample.

The Total Petroleum Bacterial Count (TBC) in Petroleum contaminated soil sample was $5.8x10^7$ at 37 ºC which was lower than normal soil sample. Total petroleum degrading bacteria was lower ($1.8x10^7$) as compare to normal soil sample ($5.5x10^6$).

A defined minimal salt medium (MSM) was used for isolation and enrichment of oil degrading bacteria. Total six bacteria were isolated and out of these two bacteria were potent degrader of petroleum oil. Out of these one bacteria was motile, non-spore forming, bacilli, Gram negative rods. It formed mucoid colonies which were blue green in color. Methyl red (MR) reaction test and Voges-Proskauer (VP) reaction test were negative. Urea hydrolysis and nitrate reductase test were negative for this bacterium. Fermentation of lactose and sucrose could not be carried out by this bacterium. Positive catalase test and urease test were observed. The results of other biochemical tests are given in Table 1. Optimum pH and temperature for growth of this bacterium were 7.0 and 42ºC respectively. Figure 1a, 1b and1c shows the growth pattern of this bacterium. By the above tests the isolated bacterial species was identified as *Pseudomonas aeruginosa*.

During bioremediation process gradual changes in pH, soil moisture content, SOC, total N, C/N ratio, available P, TPH, TBC and total petroleum degrading bacteria in soil samples at different time intervals were observed. Values obtained revealed that there was a clear modulating effect of bacteria on these determinations (Table 2).

The results indicate that this bacterium was competent to degrade PAH's as compared to other bacterial strains studied here. Oil degradation up to 83% was recorded in 30 days' incubation period with

Table1: Morphological, Biochemical and Cultural tests of isolated strains

S. No.	Morphological Test	Characteristics
	Colony morphology	
1.	Margin	Irregular
2.	Elevation	Flat
3.	Pigmentation	+ (Light cream)
4.	Gram reaction	-
5.	Size	Rods
6.	Motility	+
	Growth Characteristics at temperature	
7.	4°C	-
8.	15°C	+
9.	20°C	+
10.	25°C	+
11.	30°C	+
12.	37°C	+
13.	42°C	+
14.	52°C	-
	Growth Characteristics at pH	
15.	pH 4.0	-
16.	pH 5.0	-
17.	pH 6.0	+
18.	pH 7.0	+
19.	pH 8.0	+
20.	pH 9.0	+
	Growth Characteristics on NaCl (%)	
21.	2	+
22.	5	-
23.	10	-
24.	15	-
	Biochemical test	
25.	Indole test	-
26.	Methyl Red test	-
27.	Voges Proskauer test	-
28.	Citrate utilization	+
29.	Gas from glucose (TSI test)	+
30.	Acid from glucose (TSI test)	+
31.	Acid from lactose (TSI test	-
32.	Casein hydrolysis	+
33.	Gelatin hydrolysis	-
34.	Starch hydrolysis	-
35.	Nitrate reduction	-
36.	Catalase test	-
37.	Oxidase test	
38.	O/F test	-
	Acid production of carbohydrate	
39.	Arabinose	-
40.	Maltose	-
41.	Sucrose	-
42.	Xylose	+
43.	Lipid hydrolysis	-
44.	Litmus milk reaction	Alkaline

Table 2: Modulations in soil composition at different incubation period

S. No.	Soil Parameters	Unit	Control (Uninoculated)	2 weaks (Inoculated)	4 weaks (Inoculated)	6 weaks (Inoculated)	8 weaks (Inoculated)
1.	pH	-	7.50	7.25	7.00	6.96	6.37
2.	Moisture content	%	09.91	10.28	12.41	14.10	14.20
3.	Organic carbon	%	6.76	5.48	4.9	3.96	3.48
4.	Total nitrogen	%	0.10	0.26	0.67	1.15	1.59
5.	C/N ratio	-	67.6	21.07	7.3	3.44	2.18
6.	Available P	mg/kg	1.60	2.31	2.88	3.27	4.43
7.	Total petroleum hydrocarbon (TPH)	mg/kg	11823	9009	5021	4506	2013
8.	Total bacterial count (TBC)	CFU/g	$5.8x10^7$	$7.2x10^7$	$11.5 x10^7$	$15.5 x10^7$	$17.5x10^7$
9.	Total petroleum degrading bacteria	CFU/g	$1.8x10^7$	$3.6x10^7$	$4.95x10^7$	$7.3 x10^7$	$11.3x10^7$

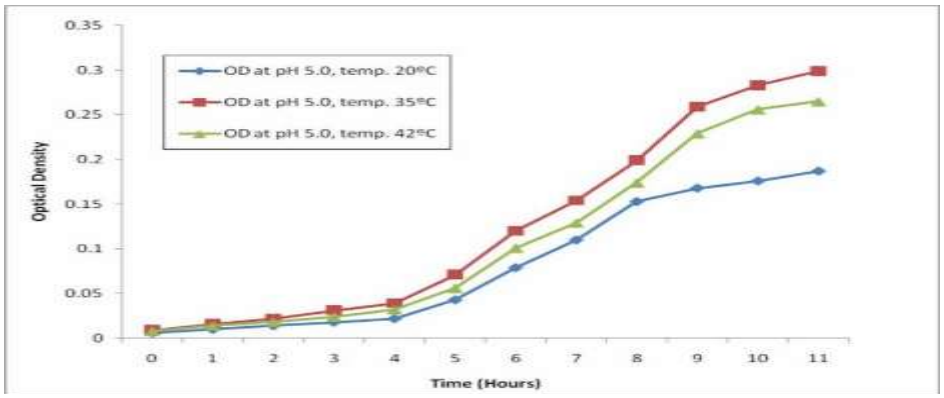

Fig. 1a: Growth pattern of *P. aeruginosa* on 2T engine oil at pH 5.0 and 20, 35 and 42ºC temperature

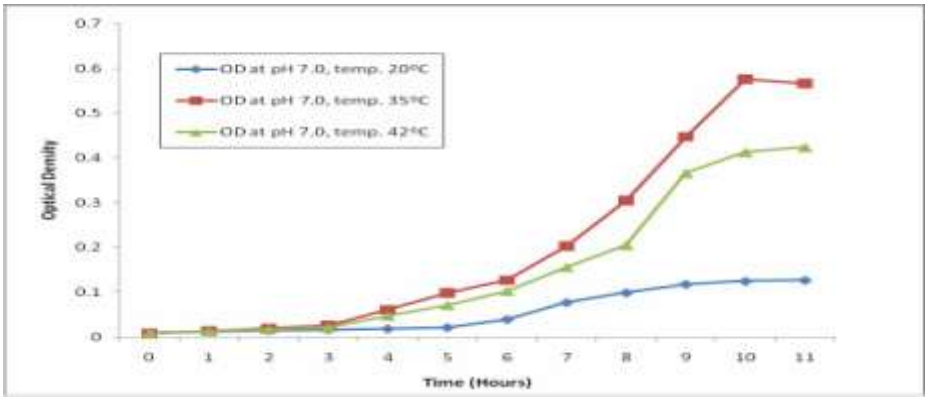

Fig. 1b: Growth pattern of *P. aeruginosa* on 2T engine oil at pH 7.0 and 20, 35 and 42ºC temperature

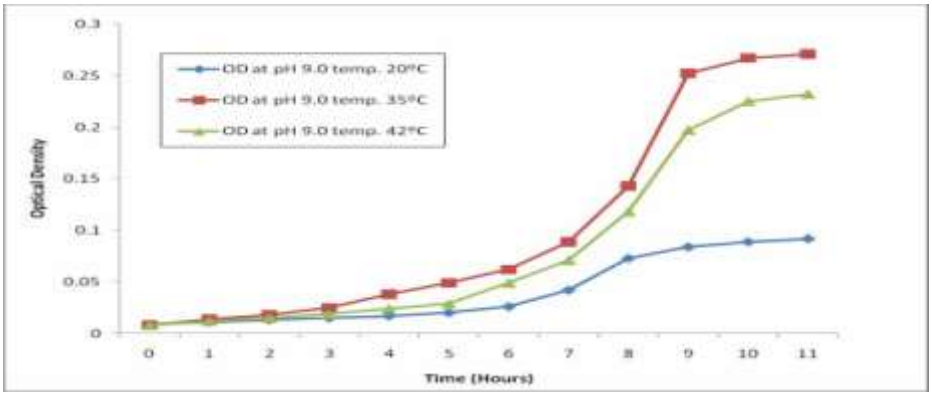

Fig. 1c: Growth pattern of *Pseudomonas aeruginosa* on 2T engine oil at pH 9.0 and 20, 35 and 42ºC temperature

Pseudomonas aeruginosa clearly establishing the potentiality of this spp. Similar bacteria have also been reported for their efficiency in bioremediation by several workers (Plohl *et al.*, 2002; Emtiazi *et al.*, 2005; Adelowo *et al.*, 2006; Madri and Lin, 2007; Pathak *et al.*, 2008; Shokrollahzadeh *et al.*, 2008). The similar bacterial strain was also isolated by Mukherjee and Das (2005) from a petroleum oil contaminated soil in north east India (ONGC-oil field). They observed that this strain was capable of growing on large number of hydrocarbon compounds when provided as sole source of carbon and energy. The total ion chromatogram of 2T engine oil from the soil samples incubated with *Pseudomonas* spp. established that this bacterium was more efficient in degradation of PAHs as compared to other strains studied here. The similar results were also put forward by Madri and Lin (2007). However, they achieved 90% degradation of oil component. Madri and Lin (2007) carried out the bioremediation at pH 9.0 and 30°C temperature. The possible reason of relatively more biodegradation may also be the production of more bio-surfactants in hydrocarbon rich culture media (Das and Mukherjee, 2007).

Above results suggest that the isolated strain is able to utilize engine oil as a sole source of carbon and energy. This make this strain as a potential organism for utilization of engine oil contaminated sites. However, individual strain can degrade a limited range of hydrocarbon substrates. Consequently, a mixed population of bacteria and other organisms are required to provide all metabolic capabilities for the complete degradation of complex mixture of hydrocarbons (Leahy and Colwell, 1990). However, microorganisms able to degrade hydrocarbon pollutants in culture medium but fail to function when inoculated into the natural environment due to lack of nutrients, contaminant concentrations below threshold for organism survival, organisms may feed on alternative substrates, predation or competition. Therefore, further exploration is required to make such process practically.

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