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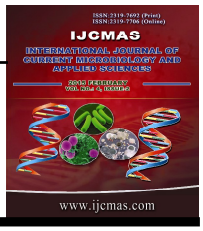

Original Research Article

Identification and Characterization of Intrinsic Petrophilic Bacteria from Oil Contaminated Soil and Water

Manzoor Ahmad[1], Wasim Sajjad[1]*, Zia Ur Rehman[2], Muhammad Hayat[1], and Imran Khan[1]

[1]Department of microbiology Quaid-I-Azam University Islamabad - 45320, Pakistan
[2]Department of microbiology Kohat University of science and technology
KUST Kohat - 26000, Pakistan
**Corresponding author*

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A B S T R A C T

Crude oil contamination is an important global problem. The conventional methods are uneconomical and produce residues that harm the surrounding biota. Therefore, microbial biodegradation exhibit effective, safe and eco-friendly practise for crude oil contaminants remediation. The intrinsic microbial population degrade crude oil effectively. The aim of this study was to identify the intrinsic petrophilic bacterial isolates from oil contaminated regions of Karak, Khyber Pakhtunkhwa Pakistan. Twenty four bacterial strains were isolated from oil contaminated regions and 15 strains were selected for further study. A rapid screening (redox indicator 2, 6 dichlorophenol indophenol) technique was used for the selection of effective crude oil degrading bacteria technique. The isolates were identified morphologically, microscopically and biochemically. The identified isolates were mainly gram positive. However, in the current study S7 (*Pseudomonas* species) and W5 (*Acinetobacter* species) obtained indigenously from the soil and water contaminated with crude oil in the vicinity of oil drilling well were found to be most efficient crude oil utilizers as turbidity observed by spectrophotometrically.

Introduction

Petroleum hydrocarbons are significant environmental contaminants (Ruberto *et al.*, 2005; Shukor *et al.*, 2008). Oil spills are major threats predominantly in industrial and underdeveloped countries (Mittal and Singh, 2009) that cause a pronounced hazard to the adjoining environments (Head *et al.*, 2006; Emtiazi *et al.*, 2009). Such hazards cause soil infertility, harm soil born microbes and damage vegetation (Nweke and Okpokwasili, 2004). On the other hand persistent contact to high oil concentration might cause the hepatic or kidney diseases, probable destruction to the bone marrow and high risk of cancer (Lloyd *et al.*, 2001); Mishra *et al.*, 2001; Propst *et al.*, 1999; Mandri and Lin, 2007) due to the presence of harmful and mutagenic substances. Therefore, the Environmental Protection

Agency (EPA) classified Crude oil as significant pollutants (Liu *et al.*, 2010).

Oil pollution might occurs accidently or operationally during its processing, transportation or storage (Head *et al.*, 2006). Biodegradation by intrinsic microbial populations is the key and reliable system through which thousands of xenobiotic contaminants, comprising crude oil are eradicated from the environment (Cappello *et al.*, 2007).

Numerous microorganisms known as petrophiles are capable of hydrocarbons utilization, can inherently degrade large hydrocarbons (Mandri and Lin, 2007) into nontoxic, biodegradable, and environmentally friendly products (Bharti and Irafan, 2011). Various microbial populations that are capable of degrading different petroleum products including species of *Pseudomonas*, *Flavobacterium*, *Arthrobacter*, *Alcaligenes*, *Nocardia*, *Micrococcus*, *Corynebacterium* and *Mycobacterium* have been isolated from soil (Malik and Ahmed, 2012). Similarly the bacterial species like *Pseudomonas*, *Arthrobacter, Sphingomonas, Rhodococcus, Ochrobactrum, Psychrobacter, Pseudoalteromonas*, *Acinetobacter* and *Bacillus* have been isolated from marine environment (Patel *et al.*, 2013; Hou *et al.*, 2012; Song *et al.*, 2011; Arulazhagan and Vasudevan, 2009; Chen *et al.*, 2008).

Nowadays, the petroleum exploitation is common in the sea due to fulfilling the increased demand of petroleum oil. Simultaneously, nearly 50% of petroleum transportation is through sea. Pakistan is situated in geographic location that is in proximity to Central Asia and Arabian Gulf. Pakistan can provide a short and better way for oil transportation to Central Asian Republics. Oil spills can occur which cause a threats to marine organisms as well as to human. Therefore a better way of bioremediation must be taken into accounts. There is no known published data about oil degradation in Pakistan. Therefore, the current study was designed to isolates and characterize bacterial isolates capable of oil degrading from soils and water polluted with crude oils.

Materials and Methods

Collection of samples

Total 10 samples including soil and waste water from garages, oil wells and service stations contaminated by oil were collected. Both soil and water samples were collected in appropriately labelled pre-sterilized bottles. Soil samples were taken with the help of sterile spatula from the depth of 0.5 to 1.0 cm surface and subsurface. All the samples were then carefully transferred to laboratory and stored at 4±1°C before analysis.

Crude oil sample

The crude oil was collected from the oil drilling site of Oil and Gas development company limited (O.G.D.C.L) (Nashpa Karak). This crude oil was used as a sole source of carbon in media during entire study. It was collected in sterilized air tight bottle and stored at cool and dark place.

Growth Medium

Bushnell-Haas medium (BHM) was used for the isolation of hydrocarbon degrading bacteria. Bushnell-Haas medium was prepared by adding (g/L): KH_2PO_4, 1; K_2HPO_4, 1; NH_4NO_3, 1; Cholesterol, 0.3; $MgSO_4.7H_2O$, 0.2; $FeCl_3$, 0.05; and $CaCl_2.2H_2O$, 0.02; and 2% sterile crude oil. Crude oil was sterilized separately by

autoclaving at 121°C for 15 minutes in sealed Erlenmeyer flasks.

Isolation and screening

Oil contaminated samples were inoculated in BHM used 250 ml Erlenmeyer flasks with 2% crude oil as sole source of carbon. The flasks were incubated on rotary shaker at 150 rpm for 7 days at 30°C. After 7 days incubation, inoculum were transferred to fresh media with crude oil as the sole carbon source and incubated for three days. After three subsequent transfers, liquid culture were serially diluted each dilution were culture on nutrient agar plates by pour plate method in order to obtain isolated colonies (Santhini *et al.,* 2009).

Identification of pure isolates

Each isolate was examined for its cultural (colony size, shape, margin, opacity, elevation, pigmentation) morphological (gram reaction, cell morphology, motility) as described in Bergey's Manual of Determinative Bacteriology (Holt, 1994).

Various biochemical tests were performed including indole, catalase, oxidative fermentation of sugar, methyl red test, Voges Proskauer test, citrate utilization test, gelatin utilization, starch utilization test, Casein utilization test as previously performed by (Santhini *et al.,* 2009).

Estimation of crude oil degradation

For estimation of crude oil degradation a shake flask experiment was performed. Each isolates were cultured in 250ml Erlenmeyer flasks containing BHM with 2% crude oil. The flasks were incubated in a rotator shaker at 150rpm at 30°C. Degradation of crude oil is determined in term of increase in cell biomass. Increase in cell biomass was assessed with spectrophotometer at 600nm. Increase in cell biomass indicates degradation of crude oil. Flasks were incubated for five days and after each 24 hrs UV absorbance was determined (Santhini *et al.,* 2009).

Confirmative Screening of hydrocarbon degraders by 2,6- Dichlorophenol Indophenol (2,6-DCPIP) oxidation test

Method as described by Hanson *et al.* (1993) was used for hydrocarbon degradation. An electron accepter dye such as 2, 6-dichlorophenolindophenol (DCPIP) was incorporated into medium the change in colour from blue to colourless (reduced) indicates the degradation of crude oil by microorganism.

Result and Discussion

In this study a total of 24 crude oil degrading bacteria were isolated from both samples. Among them, 15 best oil degrading bacterial isolates named S1-S10 from soil and W1-W5 from water samples were selected and characterized culturally, microscopically and biochemically.

Morphologic and Microscopic Identification

All the bacterial isolates were categorized on the basis of morphology like size, colour, shape, opacity and margins of colony. Microscopic analysis revealed the presence of both gram negative and gram positive isolates with various arrangement Table 1.

Biochemical characterization

After accomplishment of various tests for intracellular and extracellular enzymes, the isolates were recognised culturally, morphologically and biochemically by using

Bergey's manual of systematic bacteriology (Holt, 1994). Isolates S1, S4, S8, W1, W2 and W3 were identified as *Bacillus* species, S2, S3, S9 and W4, as *Micrococcus* species while, S5, S10, and W5 as *Acinetobacter* species, S6 as *Staphylococcus* species and S7 as *Pseudomonas* species (Table 2).

Oil Degrading Ability

The ability of these isolates to utilize crude oil as carbon source is estimated in terms of increase in turbidity that indicate a variety of results ranging from profuse growth to moderate growth by using spectrophotometer. The increase in cell mass in terms of turbidity directly indicates the utilization of crude oil as the sole source of carbon. The samples were analysed after 24 hours intervals for 4 days and diluted if necessary (Table 3).

Among these isolates, S7 (*Pseudomonas* species) and W5 (*Acinetobacter* species) were obtained indigenously from the soil and water contaminated with crude oil in the vicinity of oil drilling well were found to be the most efficient crude oil utilizers.

For screening of crude oil degradation, an electron acceptor dye 2, 6 dichlorophenolindophenol (DCPIP) was used. All the isolates turned the blue colour of dye into colourless and showed that all these isolates were able to degrade crude oil. The combined effects of bacterial consortia were study on crude oil degradation. Among the isolates, 4 best oil degrading isolates S7, W5, S1 and W1 were selected for the combine effect on oil degradation.

Crude oil is essentially, a mixture of different hydrocarbons. The releases of crude oil products are of particular dread in the environment. One of the most considerable impacts linked with garages, oil wells, service stations and automobile workshops used engine oil include go down of water holding capacity in soil, soil texture and fertility (Sathiya Moorthi *et al.,* 2008). Microorganisms resides in the contaminated sites utilizing crude oil as an energy source which lead to the increase in cell number during the degradation processes. Since all the bacteria in the present study were isolated from crude oil contaminated soil and water samples, these microorganisms survived and adopted very easily to the oil contaminated solid/liquid environment as reported by Rahman *et al.* (2003) and Sugiura *et al.* (1997). A total of 24 bacterial isolates were isolated from 10 samples of oil contaminated soil and water by serial dilution and agar plating method as reported by Ojo, (2006) and Okoh, (2003). Soil and water samples were collected from three oil contaminated places as done previously by Ojo (2006) and Okoh (2003). Among them, 15 best oil degrading bacterial isolates named S1-S10 from soil and W1-W5 from water samples were selected and characterized culturally, microscopically, biochemically and was compared with Bergey's manual as done prior by Udeani *et al.* (2009). Among the 15 isolates *Bacillus* spp, *Pseudomonas* spp, *Micrococcus* spp and *Acinetobacter* spp were best oil degradation abilities. Among these isolates, S7 (*Pseudomonas* spp) and S4 (*Bacillus* spp) obtained indigenously from the soil and water contaminated with crude oil in the vicinity of oil drilling well were found to be most efficient crude oil utilizers and turbidity observed as a result of organic contaminants degradation and increase of microbial cell numbers. Few studies by (Sepahi *et al.,* 2008) have been reported on the roles of *Bacillus* spp and *Pseudomonas* spp in hydrocarbon bioremediation. It was proposed by Thomas *et al.* (1992) that *Bacillus* strains may play an important role for the more extensive biodegradation in the

oil containing soil in a combination with *Pseudomonas* spp. In the current study the role of *Pseudomonas* spp and *Bacillus* spp in the process of oil degradation were predominant but very few studies in the literature suggested that microbial consortia including *Micrococcus* spp, *Corynebacterium, Citrobacter, Klebsiella* and *Acinetobacter* spp also have a potential role in the bioremediation of pollutants. It was reported by Ijah and Antai (2003) that *Bacillus* spp are more resistant to high level of hydrocarbons present in soil due to their resistant endospores.

Current study provides an explanation to the role of various indigenous bacteria individually, in consorted form as well as enhanced biodegradation of crude oil contents. It also suggests the use of exogenous supplies of minerals along with organic sorbents to boost bacterial growth and hence their ecological role of removing pollutants.

Table.1 Morphologic and microscopic characteristics of isolates

	Isolates	Morphological characteristics	Microscopic characteristics
1	S1	Large, Round, Irregular, Flat, Milky, Smooth, Opaque	Small, Gram Positive, Rods
2	S2	Small, Round, Entire, Slightly Raised, White, Smooth, opaque	small, Gram Positive, Cocci
3	S3	Pin Point, Round, Entire, Flat, Greenish, Smooth, Opaque	Small, Gram Positive, Cocci
4	S4	Small, Round, Entire, Flat, Creamy, Smooth, Opaque	Small, Gram Positive, Rods
5	S5	Small, Round, Entire, Flat, Creamy, Smooth, Opaque	Small, Gram Negative, Rods
6	S6	Small, Round, Irregular, Flat, Greenish, Smooth, Translucent	Small, Gram Positive, Staph
7	S7	Pin Point, Round, Entire, Flat, Creamy, Smooth, Opaque	Small, Gram Positive, Rods
8	S8	Large, Round, Entire, Flat, Brownish Creamy, Smooth, Opaque	Big, Gram Positive, Rods
9	S9	Large, Irregular, Flat, Browny, concentric, Opaque	Small, Gram Positive, Cocci
10	S10	Large, Irregular, Lobate, Flat, Greenish, Rough, Opaque	Small, Gram Negative, Cocco-bacilli.
11	W1	Large, Round, Irregular, Flat, Milky, Rough, Opaque	Big, Gram Positive, Rods
12	W2	Large, Round, Irregular, Flat, Milky, Smooth, Opaque	big, Gram Positive, Rods
13	W3	Large, Round, Mucoid, Entire, Raised, Milky, Smooth, Opaque	Small, Gram Positive, Rods
14	W4	Small, Round, Entire, Raised, Yellowish, Smooth, Opaque	Small, Gram positive, Cocci
15	W5	Small, Round, Entire, Flat, Greenish, Smooth, Transparent	Small, Gram negative, Rod

Table.2 Biochemical characterization of isolates

S.No	Isolates	Starch hydrolysis	Gelatin hydrolysis	Phenylalanine	Casein hydrolysis	Triple Sugar Iron(TSI) (Glucose, sucrose, lactose)			Citrate	Urease	Indole	H_2S	Methyl red	Voges-Proskaur	Oxidase	Catalase Test	DNase	Mannitol Salt Test	Motility	Spore Formation	Identified organism
						Slant	Butt	Gas													
1	S1	+	+	-	+	K	A	-	-	-	-	-	+	-	-	+	+	-	+	+	*Bacillus* species
2	S2	-	+	-	+	K	A	-	+	-	-	-	-	+	-	+	+	-	-	-	*Bacillus* species
3	S3	-	+	+	+	K	K	-	+	+	-	+	+	-	+	+	+	-	-	-	*Micrococcus* species
4	S4	-	-	-	-	K	A	-	-	-	-	-	-	-	-	+	+	-	-	-	*Bacillus* species
5	S5	-	+	-	-	K	K	-	+	-	-	-	-	-	-	+	-	+	-	-	*Acinetobacter*
6	S6	-	-	-	-	K	K	-	+	-	-	-	-	-	-	+	-	+	-	-	*Staphylococcus* species
7	S7	-	-	-	+	K	K	-	+	-	-	-	-	-	+	+	-	-	+	-	*Pseudomonas*
8	S8	+	+	+	+	K	A	-	-	-		-	-	-	-	+	-	-	+	-	*Bacillus* species
9	S9	-	+	+	+	K	K	-	+	-		-	-	-	+	+	-	-	-	-	*Micrococcus* species
10	S10	-	-	+	+	K	K	-	+	-		-	-	-	+	+	-	-	-	-	*Acinetobacter* species
11	W1	+	+	-	+	K	K	-	+	-	-	-	+	-	+	+	+	-	+	-	*Bacillus* species
12	W2	+	+	-	+	K	A	-	-	-	-	-	+	-	-	+	-	-	+	-	*Bacillus* species
13	W3	+	+	-	+	K	K	-	-	-	-	-	+	-	-	+	-	+	+	-	*Bacillus* species
14	W4	-	+	+	+	K	K	-	+	-		-	-	-	-	+	-	-	-	-	*Micrococcus* species
15	W5	-	+	+	-	K	K	-	+	-		-	-	-	+	+	-	-	-	-	*Acinetobacter* species

Key: **+ = Positive Results** **A = Yellow Colour (Acidic)**
- = Negative Results **K = Red Colour (Alkaline)**

Table.3 Optical Density (O.D) values of isolates grown in crude oil as carbon source

Isolates	OD values(600nm)				
	Day0	Day1	Day2	Day3	Day4
S1	0.101	0.124	0.170	0.298	0.538
S2	0.102	0.117	0.153	0.238	0.282
S3	0.102	0.124	0.149	0.291	0.507
S4	0.102	0.141	0.182	0.204	0.268
S5	0.103	0.142	0.261	0.449	0.493
S6	0.101	0.134	0.212	0.346	0.388
S7	0.102	0.217	0.675	0.747	0.812
S8	0.103	0.145	0.187	0.321	0.386
S9	0.10	0.138	0.176	0.236	0.398
S10	0.101	0.138	0.242	0.446	0.482
W1	0.102	0.176	0.251	0.394	0.498
W2	0.104	0.184	0.246	0.328	0.385
W3	0.102	0.146	0.214	0.284	0.368
W4	0.101	0.140	0.182	0.224	0.462
W5	0.102	0.212	0.469	0.619	0.780
Consortia of S7, W5, S1 and W1	0.33	1.04	1.79	1.91	2.01

Figure.1 Sampling sites contaminated with oil

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