Exploration of crude oil degrading potent microbial consortium from oil polluted sites

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ABSTRACT

Polycyclic aromatic hydrocarbons (PAHs) are a class of toxic environmental pollutants that have been accumulated in the environment due to a variety of anthropogenic activities. Hydrocarbon compounds such as petroleum are essential elements of life. Fuel and lubricating oil spills have been become a major environmental hazard to-date. Biodegradation plays a vital role in removing oil spills. Scientists have been conducted research on cost-effective clean-up techniques with minimal long-term damage to the environment. Biodegradation reveals an efficient biological ecofriendly and safe treatment process to remediate crude oil contamination. Naturally spread bacterial strains have capacity to degrade crude oil. In this present study bacterial strains were isolated and identified from oil contaminated areas. Screening method was used for efficient crude oil degrading bacteria by using redox indicator 2,6 dichlorophenol indophenol method and bacteria having high crude oil degrading potential it turns the medium to become colorless. Mixed bacterial consortium was used for testing the effect of those bacterial species on the biodegradation of crude oil. Individual bacterial cultures showed less growth and degradation than the mixed bacterial consortium. The efficiency was measured by optical density and further screened by gravimetric analysis. The genera *Pseudomonas* sp. and *Bacillus* sp. found to be best potential crude oil degraders and can be used to remediate petroleum polluted environment.

KEY WORDS:

Polycyclic aromatic hydrocarbons, Crude oil, Screening, Gravimetric analysis, *Pseudomonas* sp. and *Bacillus* sp.

1. INTRODUCTION

Pollution is the entry of contaminants in the natural environment and leads to cause instability, harm to the ecosystem. Soil pollution is caused by the presence of xenobiotic chemicals or other sources in the natural soil environment. Many compounds of oily sludge are toxic, mutagenic and carcinogenic and classified as priority environmental pollutants by the US Environmental Protection Agency (Liu *et al*., 2010). Petroleum components can be divided into four fractions: Saturated, aromatic, resin and asphaltene fractions determined by absorption chromatography (Shigeaki *et al.,* 1999). These occurs when chemicals are released by oil spill or underground leakage. Many microorganisms have the ability to utilize hydrocarbons as a sole source of energy and carbon that are widely distributed in nature; such organisms are called as petrophiles. Petrophiles are very unique organisms that can naturally degrade large hydrocarbons (Mandri *et al.*, 2007).

Diverse bacterial population can metabolize the hydrocarbons found in the crude oil in to non-toxic, non-hazardous, biodegradable and ecofriendly end products (Bharti *et al.*, 2011). The biodegradation of crude oil by microorganisms is one of the primary ways to remove crude oil from contaminated area. It has been studied that bacterium grow in oil contaminated soil are much capable of degrading oil when compare with other bacteria which are found on non-contaminated soil. The natural biodegradation process can be enhanced by addition of nutrients and optimizing the growth parameters.

Bioremediation is also environmentally friendly, it does not produce waste products and is cost effective. Microorganisms with potentials for oil degradation are widespread in nature. It can be combined with other technologies and naturally occurring process when the conditions are suitable for the growth of microorganisms (Singh and Chandra, 2014) and isolated from oil contaminated sites for bioremediation purposes (Ogunbayo *et al*., 2012). There are more than 175 genera of bacteria, which biodegrade hydrocarbons solely or in consortia (Malik and Ahmed, 2012).*Aeromonas, Alcaligenes, Acinetobacter, Arthrobacter, Bacillus, Brevibacterium, Mycobacterium, Pseudomonas, Rhodococcus, Sphingomonas* and *Xanthomonas* are known as potential hydrocarbon degraders (Kulkarni *et al*., 2012).

In order to remediate the crude oil pollution, crude oil biodegradation is necessary to isolate and characterize unique microbial species for evolution and its efficacy to utilization of crude oil before application of the contaminated sites. The objective of this study is to isolate, identify and screening of efficient oil degrading bacterial strains and to study the growth bacterial consortium on crude oil under different concentration.

2. MATERIALS METHODS

2.1. Sample collection

Petroleum hydrocarbon contaminated soil samples were collected from motor vehicle workshops, water service stations and vehicle parking areas located in and around Chennai, Tamil Nadu. Soil samples were collected randomly 5-10 cm beneath the surface using spatula and packed in sterile container. The samples were transported to the laboratory in an ice box and stored at 4^{o}C for analysis. The collected soil samples were serially diluted from 10^{-1} to 10^{-6}, spreaded on nutrient agar plates and incubated at 37^{o}C for 24 hours. The obtained cultures were purified by quadrant streaking on sterile nutrient agar plates (Khan and Rizvi, 2011).

2.2. Primary screening of crude oil degrading bacteria

Bushnell Hass media (BHM) along with redox indicator 2, 6-dichlorophenol indophenol was prepared, 1% of sterile crude oil was added. The isolated strains were inoculated into broth and incubated at 37°C for 7 days (Ibrahim *et al*., 2013). Ten ml of broth was taken, centrifuged and the supernatant was used to measure the optical density at 640nm for degradation ability of the isolates. About 0.1ml of each 7 days old BH broth culture was spread over to the nutrient agar plates to count bacterial population.

2.3. Secondary screening by gravimetric analysis

The bacterial strains (15 nos) showed more efficiency on crude oil degradation and increased growth in primary screening was selected for secondary screening. About 100 ml of Bushnell Hass broth media was prepared with this one gram of crude oil was added in the broth. Oil degrading isolates were aseptically added as an inoculum. The flask was incubated at 30°C for 7 days in a rotary shaker at 120 rpm. After incubation, the flask was added with diethyl ether solvent and transferred to the separating flask. The setup was left for 30 minutes for oil and broth separation. The broth was separated in the lowest portion. Diethyl ether was added to the remove complete oil from separating flask. Oil along with solvent was collected in a preweighed petriplates. After the complete evaporation of the solvent the plate was weighed. The estimation of residual oil left after degradation was made by the amount of oil in a preweighed plate (Anupama *et al*., 2009). The percentage of oil degradation was calculated as {1-(Xo-X1)/Xo}100%(%)< where Xo- initial amount of crude oil, X1- amount of crude oil after degradation (Jayashree *et al.*, 2012).

2.4. Antagonistic activity for selected bacterial isolates

Antagonistic activity of potential bacterial strains was studied by well diffusion assay to eliminate the strains which hold secretions of harmful extracellular compounds like antibiotics, cell wall degrading enzymes. Based on the inhibition of the consortium formulation was made to find the efficiency on crude oil degradation. Similarly all the test organisms were inoculated on to sterile Muller hinton agar plates by swabbing. 4 mm in diameter wells were punctured by using well cutter in each plate and filled with 100 µl of remaining individual bacterial broth by using micropipette and the plates were incubated at 37°C for 24 hours. The level of compatibility was visually observed by the presence of clear zone around the culture loaded on the wells (Das and Mukherjee, 2008).

2.5. Bioremediation of crude oil by bacterial consortium

Bacterial consortium containing 5 mL of 10^4 to 10^5 cfu/mL was added to the Bushnell Hass medium and supplemented with 1% of crude oil containing as sole carbon source. The conical flask was kept in shaker at 150 rpm at 37 ºC for 7 days incubation. After growth of bacteria 5 mL of enrichment culture was transferred to a fresh medium and incubated under the same conditions to enrich the bacterial consortium. Crude oil content was estimated by solvent extraction and gravimetric analysis (Arulazhagan *et al*., 2010).

3. Results

3.1. Sample collection and isolation of bacteria from soil sample

There are 14 crude oil contaminated soil samples were collected for obtaining efficient crude oil degrading bacteria isolates from in and around Chennai. Totally 135 bacterial strains were isolated from crude oil contaminated samples and identified by various biochemical tests according to the Bergey's manual of determinative bacteriology. Among the 135 bacterial strains the major genera present in petroleum contaminated sites were *Micrococcus*, *Pseudomonas*, *Bacillus*, *Moraella* and *Enterobacteriaceae* sp.

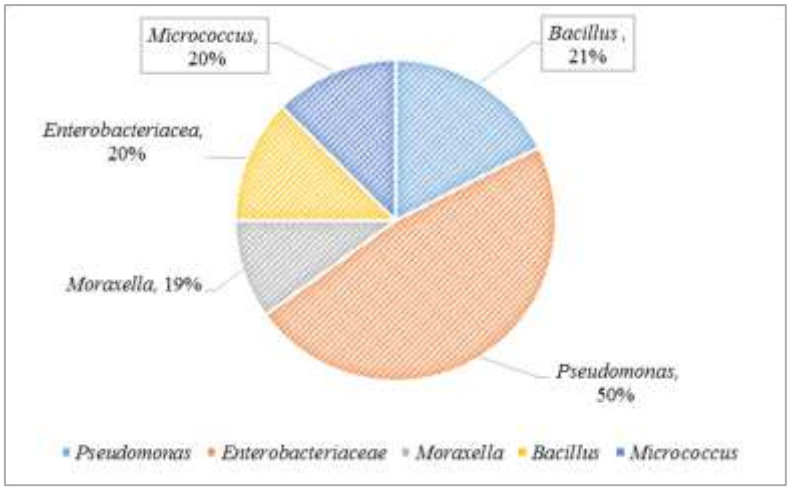

Fig. 1. Bacterial genera isolated from oil contaminated soil samples

3.2. Primary screening of by DCPIP test

Among the 135 bacterial isolates, 15 isolates were screened for degradation of petroleum hydrocarbons by primary screening method using DCPIP test. Ability of the isolates to degrade the hydrocarbon was confirmed by the colour change from blue to colourless. The bacterial strains were identified based on optical density and colony forming units as shown Table 1.

Table 1 - Colony forming units and optical density of DCPIP test

S. No	Strain No	Genus	CFU/ml	OD at 640 nm
1.	2	*Enterobacteriaceae*	231	0.116
2.	5	*Bacillus*	255	0.140
3.	10	*Bacillus*	233	0.142
4.	11	*Bacillus*	264	0.137
5.	15	*Pseudomonas*	248	0.154
6.	16	*Pseudomonas*	255	0.009
7.	17	*Enterobacteriaceae*	241	0.014
8.	18	*Bacillus*	101	0.127
9.	19	*Pseudomonas*	199	0.144
10.	30	*Micrococcus*	282	0.018
11.	31	*Pseudomonas*	230	0.011
12.	32	*Pseudomonas*	242	0.155
13.	33	*Bacillus*	241	0.025
14.	34	*Bacillus*	211	0.110
15	35	*Pseudomonas*	272	0.010

3.3. Secondary screening by gravimetric analysis

The rate of degradation was confirmed by gravimetric method. Among the 15 bacterial isolates only two bacterial genera namely *Bacillus* sp. 18 and *Pseudomaonas* sp. 35 showed higher degradation ability was confirmed in secondary screening. The bacterium *Bacillus* sp 18 degraded 37% and *Pseudomaonas* sp 35 degraded 38% within 7 days (Table 2 and figure 2).

Table 2 - Secondary screening of crude oil degradation

Isolates	Initial (mg)	Final (mg)	Degradation %
Control	1000	1000	0
2	1000	689	31.1
5	1000	699	30.1
10	1000	690	31.0
11	1000	796	20.4
15	1000	724	27.6
16	1000	678	32.2
17	1000	747	25.3
18	1000	622	37.8
19	1000	701	29.9
30	1000	760	24.0
31	1000	730	27.0
32	1000	625	37.0
33	1000	653	34.7
34	1000	735	26.5
35	1000	620	38.0

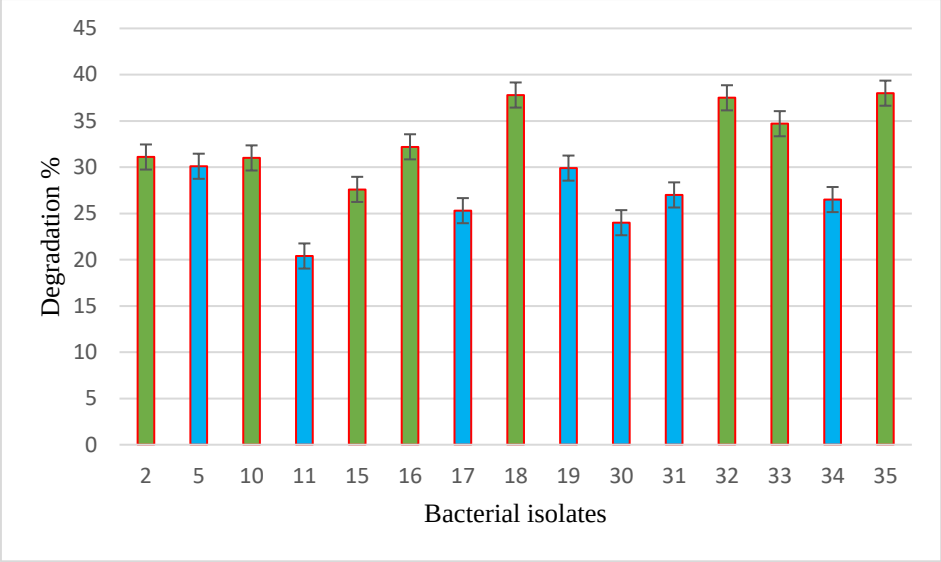

Figure. 2. Secondary screening of bacteria in crude oil degradation

3.4. Antagonistic activity and consortium formation

In this antagonistic study the strain *Bacillus* sp 18 and *Pseudomaonas* sp 35 had an antagonistic activity against all the other strains and used as effective bacterial consortium formulation. Antagonistic activity was carried out to bacterial consortium and the results are given in table 3.

Table 3 - Antagonistic activity of selected bacterial strains

Strains	**A**	**B**	**C**	**D**	**E**	**F**	**G**	**H**
A		AB	AC	AD	AE	AF	AG	AH
B	BA		BC	BD	BE	BF	BG	BH
C	CA	**CB**		CD	CE	CF	CG	CH
D	DA	**DB**	**DC**		DE	DF	DG	DH
E	EA	EB	EC	ED		EF	EG	EH
F	FA	**FB**	**FC**	**FD**	FE		FG	FH
G	GA	**GB**	**GC**	**GD**	GE	**GF**		GH
H	HA	**HB**	**HC**	**HD**	HE	**HF**	**HG**	

3.5. Crude oil treatment by selected microbial consortium

In this study three set of flask was used and 1% of crude oil contaminated BH broth were treated with optimized bacterial consortium (*Bacillus* strain 16 and *Pseudomonas* Strain

35). The concentration of oil was analyzed by gravimetric method. The first experimental setup is containing 1% crude oil was treated with deionized water. This treatment setup is maintained as control. The second flask contains crude oil was treated with bacterial consortium *Bacillus* strain 18 and *Pseudomonas* Strain 35. The third set of flask contains 1% crude oil was treated with optimized bacterial. After the treatment the 78% of crude oil degradation was observed. The results were showed in and figure 3.

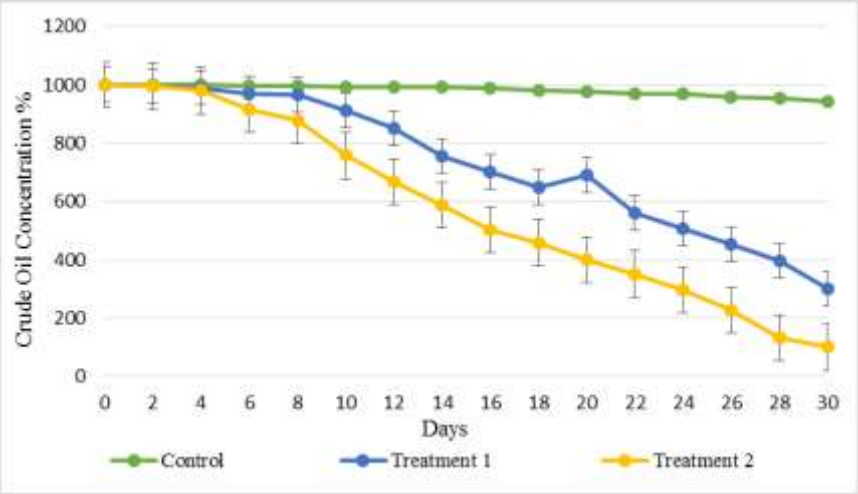

Fig. 3. Crude oil treatment by selected bacterial consortium

4. DISCUSSION

An environmental contaminant acts on the indigenous biota of the ecosystem, eliminating or selecting microorganisms in accordance sensitivity in the presence of the toxic agent. (McNaughton *et al.* 1999). Microorganisms are extremely diverse and are capable of utilizing the contaminants as energy and carbon source to survive in natural environment (Singh *et al*., 2011). The soil, groundwater and superficial waters contain microorganisms are able to degrade the crude oil and used as energy source, there by eliminate them from polluted environments (Pedrozo *et al*. 2007). Applications of microorganisms play a major role in the removal of contaminants and took advantage of the astonishing catabolic versatility of microorganisms to degrade or convert such compound.

Biodegradation of organic compounds is more efficient when the microorganisms in the inoculum are selected and thus become potentially more adapted to target pollutants. There are several reports on bioremediation of pollutants by the action of different bacterial strains have been capable of degrading hydrocarbons (Morais and Tornisielo, 2009). Few studies (Meckenstock *et al*., 2000; Rahman *et al.*, 2002; Varjani *et al.,* 2015) have been reported on the roles of *Bacillus* sp. and *Pseudomonas* sp. in hydrocarbon bioremediation.

Only a few studies have reported that some bacterial consortium were able to catalyzing the conversion of crude oil as a source of carbon and energy (Parameswarappa *et al*., 2008). Bioremediation exploits the natural process by promoting the growth of microbes that can effectively degrade specific contaminants (Kiran *et al*., 2010). A rapid primary screening procedure was performed to assess the indicator dye (2, 6-DCPIP) decolourization efficiency of selected strains for confirmation of crude oil biodegradation. Bushnell Hass medium is an excellent growth media for isolation of heterotrophic microorganisms which provide all nutrient sources except carbon source by our study crude oil used as a sole source of carbon. To ascertain microbial ability to utilize hydrocarbon substrate by simple observing the color change of DCPIP in which the quickest decolourization time represents the best oil biodegradation is a major breakthrough in biodegradation studies (Bidoia *et al*., 2010). Total bacterial count was determined by standard plate count method (Selvakumar *et al*., 2014). Increase in oil degradation is directly proportional to an increase in cell count indicating that bacterial isolates were capable for oil degradation (Rahman *et al*., 2002).

Secondary screening of purified culture is also done by recovering oil from the flask and the estimated amount of oil is left after degradation. This is one of the few reports on this method of quantifying oil degradation ability of the strain (Bano *et al*., 2017). *Pseudomonas* sp. is an outstanding and natural crude oil degrader reported in the literature which is wide spread in nature and can degrade wide range of xenobiotics (Singh *et al.*, 2011). It has been postulated that *Bacillus* sp. are predominant and more tolerant to high level of crude oil contaminated soil due to their ability of the resistant endospore which may protect them from the toxic effect of the hydrocarbon (Usman *et al*., 2012).

In the field of bioremediation process, most of the results substantiated microbial consortium was suitable for complete remediation of many kind of environmental pollutants compare to single strain. Microbial consortia antagonistic relationship of each strain with other strains was important. Antagonism is the relationship between two organisms in which one is inhibiting the growth of the other (or both). In natural ecosystem, certain bacteria are used as antagonists, it will suppress (inhibit) the growth of other bacteria (Gana *et al*., 2011). In present study the bacterial consortium (*Bacillus* strain and *Pseudomonas* strain) that degrading crude oil in BH medium was identified and effect of bacterial consortium and results are revealed that the degradation rate of crude oil was much higher than other reported bacterial species (Kim and Lee 2011). The same result was reported by Batista *et al.,* (2006) and found an 80% recovery rate of residual crude oil adsorbed to sand. It is possible to use

Pseudomonas sp. and *Bacillus* sp. as dominant strains for bioremediation and resolve the pollution by crude oil treatment of soils contaminated with crude oil.

5. CONCLUSION

Microbes play a vital role in the weathering process microbial degradation is the major mechanism for the elimination of spilled oil from the environment. The different microbial genera isolated from petroleum contaminated soil suggests that the pivotal role in the transformation of hydrocarbon. In this study, hydrocarbon utilizing *Pseudomaonas* sp. and *Bacillus* sp. bacteria was isolated from contaminated soil. Screening of crude oil degrading bacteria was performed by DCPIP redox indicator spectrophotometric technique. *Pseudomaonas* sp. and *Bacillus* sp. both are predominant bacterial strains have more ability to degrade the petroleum oil contamination was proved. Bacterial consortium used for crude oil degradation process and obtained good results for oil degradation. It was further investigated for degradation of hydrocarbon by gravimetric analysis revealed 78% of degradation. In view of this, under optimized conditions bacterial consortium *Pseudomaonas* sp. and *Bacillus* sp. efficient strains and ecofriendly for degradation of hydrocarbon. Using microbial consortium process is successful and safe way to enhance environment health in particular with low cost, technique and high public acceptance to cleaning up aquatic ecosystems from oil spills. From the study, it was concluded that microbial consortium degradation can be considered as a key component in cleanup strategy in oil contamination.

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