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Research Article

BIOCHEMICAL CHARACTERIZATION OF 4T ENGINE OIL DEGRADING MICROORGANISM ISOLATED FROM POLLUTED SOIL WITH PETROLEUM HYDROCARBONS

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ABSTRACT
There are many industrial areas which produce a large amount of Poly Aromatic Hydrocarbons which represent many hazardous effects on the ambient environment. Bacterial strains were isolated from contaminated site at Sitapura industrial area Jaipur. Microorganisms used in present investigation were isolated by selective enrichment technique. MSM broth was used in enrichment technique supplemented with 1% v/v hydrocarbon substrate (4T engine oil). Five microbial strains were isolated and showed biodegradation potential for 4 T engine oil. The data shows that isolated microorganisms were belongs to *Pseudomonas* Sp., *Enterobacter* Sp., *E.Coli* Sp., *Bacillus Subtilis* Sp. *and P.Fragi* Sp.

Key Words*: Enrichment technique, MSM, 4T engine oil*

INTRODUCTION
Soil and water represent the first lines of recipients of oil pollution. Petroleum-based products are the major source of energy for industry and daily life. In the last few years, large numbers of ecosystems have changed by the significant influence of human activity and about five million tons of crude oil and refined oil enter the environment each year (Hinchee and Kitte, 1995). Petroleum hydrocarbons are toxic and potent carcinogenic to human health as well as environment (Propst *et al.*, 1999). Petroleum products are used as fuels, solvents and feed stocks in the textile, pharmaceutical and plastic industries. Petroleum is a viscous liquid mixture that contains thousands of complex mixture of hydrocarbons and other organic compounds, including some organometallo-constituents.

Polyaromatic hydrocarbons in the environment originate from anthropogenic source like mineral oil. Numerous bacteria, fungi and algae have been isolated for the breakdown of aromatic hydrocarbons as carbon and energy sources (Cerniglia, 1992; Lal *et al.*, 2004; Pathak *et al.* 2008). The degradation pathways have been -elucidated PAHs present as natural constituents in fossils fuels, are formed during the incomplete combustion of organic materials (Lee *et al.*, 1981; Wang *et al.*, 1999; Desche Anes *et al.*, 1996; Wang *et al.*, 1999). PAHs can exert toxic effects or possess mutagenic, teratogenic, or carcinogenic properties (Cerniglia and Heitkamp, 1987; International Agency for Research on Cancer, 1972; Phillips, 1983). Bioremediation is defined as the use of microorganisms to detoxify or remove pollutants owing to their diverse metabolic capabilities and it is believed to be noninvasive and relatively cost-effective.

MATERIALS AND METHODS
Physical Analysis of Soil
Physical properties of soil like soil texture, bulk density, moisture content, pH, heavy metal detection, calcium carbonate, carbonate and bicarbonate, available phosphorous and water holding capacity of soil samples were determined. Soil texture was determined by using sieves of different sizes and bulk density was determined gravimetrically activity. Moisture content and water holding capacity was evaluated by flooding the soils with water and the weight of flooded soil was recorded after 24 hours of drying at 100˚C temperature then the amount of soil remained was weighted and the percentage of water evaporated was calculated. Soil chemical properties like pH, available phosphorus, available carbonate

and bicarbonate, chloride, and calcium carbonate were determined by using standard titration methods (Pandey and Sharma, 2003).

Isolation of Microorganisms

4T engine oil (mainly composed of medium chain length of hydrocarbon) obtained from Bharat petrol pump. MSM (Minimal Salt Media) media containing K_2HPO_4 1 g, NH_4NO_3 1g, KH_2PO_4 1 g, $CaCl_2$ 0.02 g, $FeCl_3$ 0.05g, MgSO4 0.2 g was used for serial dilutions to determine viable cell counts. Microorganisms used in all experiments were isolated by selective enrichment technique. MSM broth was used in enrichment technique supplemented with 1% v/v hydrocarbon substrate (4T engine oil). After one week of incubation on rotary shaker at 27ºC, and 200 rpm, 10 ml of sample from primary enrichment was transferred to a fresh MSM broth. The initial number of total viable cells in the original sample was determined by serial dilution agar plating procedure.

Biodegradation of 4t Engine Oil

MSM (Minimal Salt Media) media was used in this experiment. Bacterial growth was recorded at 600nm. In this experiment 1 ml of bacterial inoculums was added to media. 1% 4T engine oil was added as hydrocarbon substrate. These test tubes were kept for incubation at 35±2ºC for 24 hr, 48 hr, 72 hr, 96 hr, 120 hr and 144hr.

Biochemical Characterization

A number of biochemical tests were performed as Gram staining, Amylase production, Urease, Catalase, Indole production, Methyl red, Citrate utilization and Voges-Proskauer test. Gram staining is used to differentiate between gram positive and gram negative. In amylase production microbes shows the ability to degrade starch in which a dark pink colouration and a yellow zone around a colony indicates amylolytic activity. In urease test phenol red is a pH indicator (pH 6.8) and during incubation, microorganisms possessing urease will produce ammonia.

RESULTS AND DISCUSSION

Utilization of chemical contaminant present in the soil as source of carbon and energy by different bacterial communities. Prevailing environmental conditions are among the most important limiting factors for optimum bioremediation. The factors affecting the success and rate of microbial bioremediation are nutrient availability, moisture content, soil reaction (pH), temperature and soil texture etc. The present study was carried out to assess the quality of contaminated soils. These qualities determine the capability of bacterial isolates to biodegrade petroleum compounds. All soil microorganisms require moisture for growth and functioning. Moisture affects diffusion of water and soluble nutrients into and out of the microbial cells. In the present investigation the moisture content recorded during the experiments ranged from 10.10 to 15.02 %.A better degradation of PAH was recorded in soil sample PCS-1 having moisture content 11.12 % as compared to PCS-2 with 10.10% moisture content. Excess moisture, in saturated soil is undesirable because it reduces the amount of available oxygen for aerobic respiration. The water holding capacity of the contaminated soil samples was 58.40 % in PCS-1 and 59.30 % in PCS-2 i.e. in the range suggested to be optimal for bioremediation (US-EPA, 2006). It is also difficult to control moisture content in fine soil because their small pores and high surface area allow better water retention. Soil reaction (pH) is a critical factor for microbial growth and survival. A pH value of near neutral is suitable for growth of diverse bacterial populations. The most appropriate range for bioremediation has been suggested to be pH 6-8 (US-EPA, 2006). The results show that PCS-1 Cu, Zn and As were present and Fe, Cr and Lead were absent, however all these heavy metals were present in normal soil. This results show that PCS-II (1.32 gm/ml) has high bulk density as comparison to PCS-I (0.98 gm/ml) and Normal Soil. The amount of Organic matter was high in PCS-I (37.08%) as comparison to PCSII (31.80%) soil samples. Both the soil samples show negative response for Bicarbonate, Chloride and Nitrate test whereas for Calcium Carbonate and Carbonate show positive results. The amount of available phosphorous is high in PCS-II as comparison to PCS-I soil samples.

Table 1: Physico- chemical properties of petroleum contaminated soil

S No.	Parameters	Petroleum contaminated soil I	Petroleum contaminated soil II
1.	pH	8.35	8.00
2.	Bulk density	0.93	1.32
3.	Moisture content	11.12%	10.10%
4.	Water holding capacity	58.4	59.3
5.	Available phosphorous	-ve	-ve
6.	Calcium chloride	+ve	+ve
7.	Chloride	-ve	-ve
8.	Carbonate and Bicarbonate	+ve	+ve
9.	Organic matter	37.8%	31.8%
10.	Cu	+ve	-ve
11.	Fe	-ve	+ve
12.	Zn	+ve	-ve
13.	Cr	-ve	+ve
14.	Lead	-ve	-ve
15.	As	+ve	-ve

Isolation of Microorganisms

Appreciable numbers of bacteria up to 10^{10} colony forming units (CFU) have been found. Indigenous organisms isolated by selective enrichment culturing technique. As the result, a significant increase in hydrocarbon degrading microorganisms in PCS–1, after the first and second week of enrichment in 4T engine oil.

Table 2: Isolation and cell count of microorganisms

Samples	Enrichment Substrate	Cell Count
PCS	Original sample	0.9×10^{10}
	2^{nd} Enrichment	3.3×10^{10}
	3^{rd} Enrichment	5.4×10^{10}

Table 3: Analysis of Biochemical Tests

S.No.	Tests	*Pseudomonas* Sp.	*Enterobacter* Sp.	*E.Coli*	*P.Fragi*	*B.Subtilis*
1.	Amylase	-ve	+ve	+ve	+ve	-ve
2.	Catalase	+ve	-ve	-ve	-ve	-ve
3.	Gram+ve/-ve	-ve	+ve	-ve	-ve	+ve
4.	Urease	-ve	-ve	+ve	+ve	+ve
5.	Indole	-ve	-ve	+ve	+ve	+ve
6.	MR	-ve	+ve	+ve	+ve	+ve
7.	VP		-ve	-ve	-ve	-ve
8.	Citrate	-ve	+ve	-ve	-ve	-ve

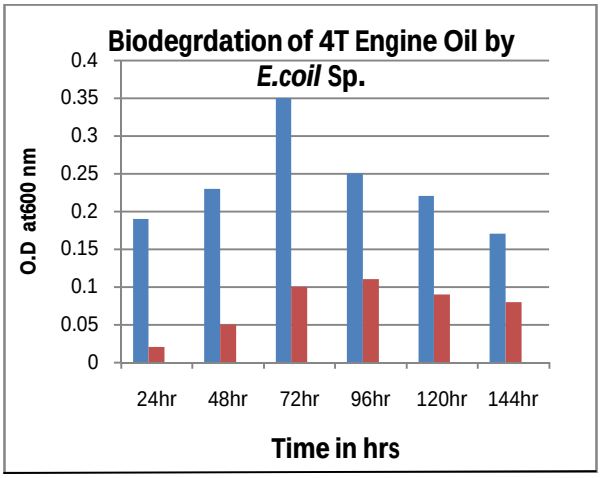

Figure 1: Showing Biodegradation of 4T engine Oil by *E.Coli* Sp.

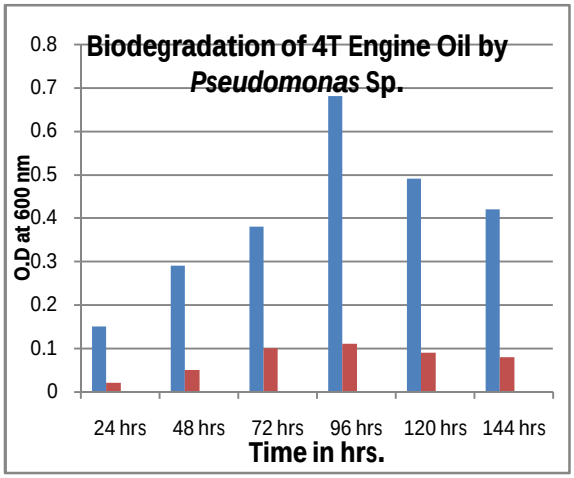

Figure 2: Showing Biodegradation of 4T engine Oil by *Pseudomonas* Sp.

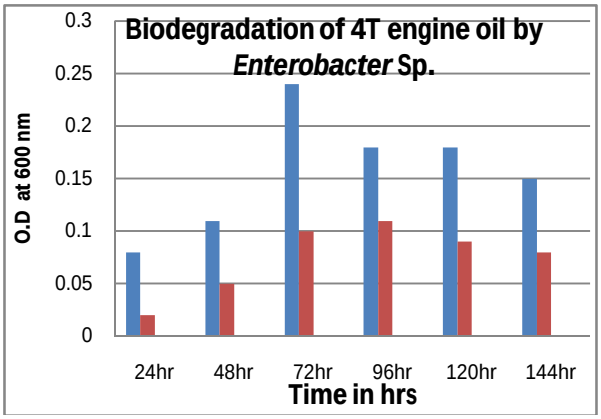

Figure 3: Showing Biodegradation of 4T engine Oil by *Enterobacter* Sp.

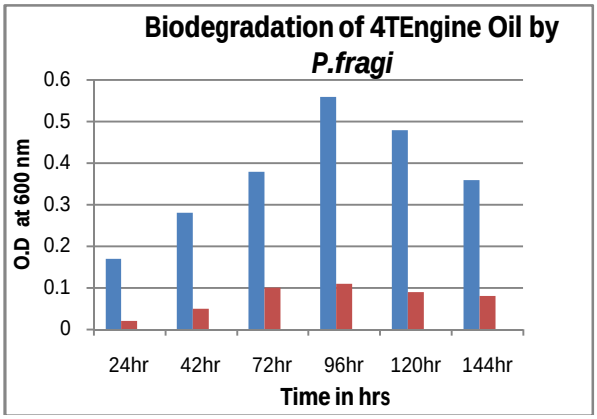

Figure 4: Showing Biodegradation of 4T engine *P. fragi*

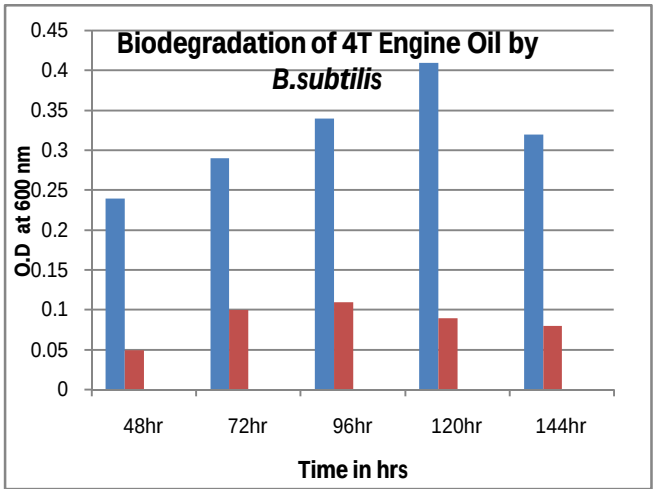

Figure 5: Showing Biodegradation of 4T engine Oil by *B. subtillis*

Biodegradation of 4T Engine Oil by Microbial Strain on MSM Broth

Effective method to study the degradation of hydrocarbon by microorganisms is to measure the growth of microorganisms during utilization of organic compound. Growth of microorganisms at 600nm on different were measured at different time intervals as 24hr, 48hr, 72hr, 96hr, 120hr and 144hr.

According to graphs microorganisms were grown well till 72hrs to 96hrs after that the growth of microorganisms was slightly decrease.

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