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ISOLATION AND DEGRADING OF ENGINE OIL BY PSEUDOMONAS AERUGINOSA

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Abstract

The soil sample was collected at the field near truck workshop at Namakkal district that was disposed with used motor oil discharged from truck and uncontaminated soil sample also collected in same field. The pH of the spent engine oil contaminated soil is slightly acidic when compared to that of the uncontaminated soil. The spent engine oil contaminated soil had a higher conductivity, total organic carbon and phosphorus when compared to the uncontaminated soil. The spent engine oil degradation bacteria was isolated and identified as *Bacillus* sp.HC-1 and HC-3. and *Pseudomonas* sp. HC-2 and HC-4. Among the four isolates, *Pseudomonas* sp. HC-2 exhibited the maximum degradation of spent oil. *Pseudomonas* and *Bacillus* isolates were degraded upto 1 and 2 % concentration respectively. The 3 % and 4 % inoculum size was optimum for *Pseudomonas* sp. and *Bacillus* sp. respectively. The degradation was increased with increase the incubation periods. Among the 4 isolates, HC-2 was efficient for degradation of spent engine oil. Based on 16SrRNA technique, HC-2 strain was designated as *Pseudomonas aeruginosa* HC-2 which involved in spent oil degradation.

Keywords: Engine oil, Hydrocarbon, Petroleum, Microorganisms, 16s rRNA

Introduction

Petroleum is a complex mixture of hydrocarbon and other organic compounds including some organometallo constituents, most noticeably vanadium and nickel (Van Hamme *et al*.,2003). Petroleum hydrocarbon can be divided into four classes: saturates, aromatics, asphaltenes (phenols, fatty acid, ketones, esters and porphyrins) and resins (pyridines, quinolines, carbazoles, sulfoxides and amides) (Leahy and Colwell, 1990). Hydrocarbons are the world's most widely used primary energy and fuel resources, due to the energy they produce.. Crude oil and its products are vital to modern society and thus vast quantities are consumed each year. As the necessity increases, the rate of hydrocarbon contamination also increases day by day. Nowadays, due to the highly increasing volume of transportation (roadway, railway, air and water), the number of roadway vehicles and other transport vehicles increased significantly and the volume of growth is going to still intensify in the future. (Emodi, 2003). Between 1970 and 2010 passenger transportation as per passenger kilometer will triple, furthermore, the growth of transport volume will be even more than that. Naturally, this tendency is also true for the whole world, but in less motorized regions, in so called developing countries, the growth is even more intensified.

Spent engine oil a brown-to-black liquid mixture of heavy metal contaminants such as zinc, lead, and chromium that come from engine parts as they wear down, including low to high molecular weight (C15 to C18) aliphatic and aromatic hydrocarbons, polychlorinated biphenyls, chlorodibenzofurans, lubricative additives, and decomposition products (Wang *et al*., 2000).

Most motor oils are made from a heavier, thicker petroleum hydrocarbon base stock derived from crude oil with additives to improve certain properties. The bulk of typical motor oil consists of hydrocarbons with between 18 and 34 carbon atoms per molecule. Prior to its use, motor oil consists of a complex mixture of hydrocarbons that make up 80 to 90 percent of its volume and performance-enhancing additives that make up 10 to 20 percent of its volume (Chris, 2007). On land, the release of used motor oil into the environment can have great negative impact on food productivity by its effects on soil fertility. The scale of impact would however depend on the quantity of oil spilled. Substantial quantities of petroleum hydrocarbons can thus 'sterilize' the soil and prevent crop growth and yield for a long period of time (Onwurah *et al*., 2007).

Hydrocarbon-degrading bacteria, yeast and fungi are widely distributed in marine, fresh water and soil habitats. Bacteria and yeast appear to be the dominant degraders in aquatic ecosystems while fungi and bacteria are the main degraders in soil environments (Cooney and Summers, 1976; Hanson *et al*., 1997; Balba *et al*., 1998). Previous reports elucidated that the oil spilled area is rich with oil degrading bacteria include *Psuedomonas, Micrococcus, Staphylococcus, Bacillus, Flavobacterium, Acromobacter, Klebsiella, Actinomycetes, Acetobacter, Rhodococcus* etc which can utilize hydrocarbons as primary carbon source and hence they are called Hydrocarbon utilizing bacteria (Bhuvaneswar *et al*., 2012). Although there are

several reports on bioremediation of pollutants by the action of different bacterial strains, only very few studies reported on biodegradation of spent oil.

MATERIAL AND METHODS:-

Sample collection

The soil sample were collected from truck workshop at Namakkal district that was disposed with used motor oil discharged from truck and uncontaminated soil sample also collected in same filed. The soil samples collected from a depth of 10cm in sterilized. Plastic containers and store at 40ºC for the farther studies.

Analysis of soil physicochemical characteristics

Physicochemical characteristics, such as pH, Moisture content, organic carbon, total Nitrogen, Phosphorous and Potassium were determined in the contaminated soil and uncontaminated soils.

Isolation of hydrocarbon degrading bacteria:

The bacteria were isolated by inoculating the soil samples on enrichment medium that contains the autoclaved mineral salt medium (MSM) supplemented with single hydrocarbon compound as sole carbon source (1% liquid petrol and diesel). The medium contains K2HPO4 (1.8 g/L); NH4Cl (4 g/L); MgSO4.7H2O (0.2 g/L); NaCl (0.1 g/L); Na2SO4.7H2O (0.01 g/L); agar (20 g/L); carbon source (1% petrol , diesel); and distilled water (1L) with pH 7.2. The medium without hydrocarbons was sterilized by autoclaving at 121°C for 15 min. The medium was supplemented with 1% (v/v) sterilized used engine oil to serve as the only source of carbon and energy The medium was incubated at 37°C for 5-10 days. After the incubation period the bacterial colonies that were grown on the medium were identified by Gram's staining and biochemical characterization according to Bergy's manual of systemic bactriology.

Determination of bacterial biodegradative activity

Fifty ml of minimal salt media was prepared and 2 g of used engine oil was added, whereas 2 mL of individual culture broth was aseptically added in the flasks. The degradation capability of the bacterial species was checked after 5days, 10days, 15 days, and 20days for degradation efficiency of organisms.

Effect of spent engine oil concentration for degradation (nikhil *et al*., 2013)

Ten gm sterilized contaminated soil mixed with 1 to 5 % of spent engine oil with 0.3 ml 24 h old bacterial isolates were inoculated. The plates were incubated at room temperature for 15 days. After incubation degradation percentage was calculated.

Effect of inoculam size for degradation of spent engine oil(nikhil *et al*., 2013)

Ten gm sterilized contaminated soil mixed with optimum level of spent engine oil with 1 to 5 % 24 h old bacterial isolates were inoculated. The plates were incubated at room temperature for 15 days. After incubation degradation percentage was calculated.

Effect of incubation periods for degradation of spent engine oil (nikhil *et al*., 2013)

Ten gm of sterilized contaminated soil mixed with optimum level of spent engine oil with 24 h old bacterial isolates were inoculated. The plates were incubated at room temperature for 5 to 30 days. After incubation degradation percentage was calculated.

PCR amplification

PCR amplification of 16S rDNA was performed using both universal and specific primers: forward primer (5'-AAGCAACGCGAAGAACCTTA-3') and reverse primer (5'-CGTAAGGGCCATGATGACTT-3'). The reaction mixture was prepared in a total volume of 50 µL containing 10X PCR reaction buffer (5µL), $MgCl_2$ (2 mM), Taq DNA polymerase (1.5U), dNTP (200 µM), forward primer (2 µM), reverse primer (2 µM) and template DNA (2 µL). The amplification was performed using a thermal cycler (ABI2720). The PCR programme was started with an initial denaturation at 94°C for 5 min. Then followed by 35 cycles of PCR programmed *viz.* denaturation at 94°C for 30 sec, annealing at 55°C for 30 sec and extension at 72°C for 2 min, and a final extension at 72°C for 5 min and cooled to 4°C. The PCR product was detected by 1.5% agarose gel electrophoresis and visualized on UV-transilluminator after staining with 0.01% ethidium bromide and photographed (Roy and Chakraborty, 2009). Then, the PCR product was sequenced using an ABI 3500 XL Genetic Analyzer.

Molecular identification of selected strain

The gene analysis of 16S rRNA was performed for confirmation of the isolate. The methodology included standard molecular techniques.

1. Statistical analysis

The data were expressed as the mean value ± the standard error (SE) using IBM SPSS Statistics 20 (SPSS Inc., IBM Company Chicago, IL, USA, 2010).

RESULT AND DISCUSSION

Physiochemical properties of spent engine oil contaminated soil and uncontaminated soil

The physiochemical properties of spent engine oil contaminated soil and uncontaminated soil were tabulated in (Table-1). The pH of the spent engine oil contaminated soil is slightly acidic when compared to that of the uncontaminated soil, which is neutral. The spent engine oil contaminated soil had a higher conductivity when compared to the uncontaminated soil. However, the uncontaminated soil had high moisture content than the spent engine oil contaminated soil. The total organic carbon and phosphorus content of the pent engine oil contaminated soil were higher than those of the uncontaminated soil; however, the uncontaminated soil had higher nitrogen content than the spent engine oil contaminated soil.

The neutral pH recorded for the control soil, the contaminated soil sourced from the vicinity of the automobile workshop was acidic. This observation was similar to a report by Ipeaiyeda *et al*., (2007) which stated pH values which ranged from 6.1 to 7.2 for top soil samples obtained from five auto mechanic workshops located at Iwo town, Osun state. Iembayo and Kolade, (2008) stated that high pH might reduce the mobility

of some metal species down the soil strata while low pH values usually enhance metal distribution and transport in the soil.

Table-1

Physiochemical properties of spent engine oil contaminated soil and uncontaminated soil

S.No	Soil samples	Spent engine oil Contaminated Soil	Uncontaminated Soil
1	pH	6.7	7.2
2	EC (ds/m)	1.16	0.42
3	Moisture content (%)	74.2	86.5
3	Organic Carbon (%)	2.68	0.74
4	N (Kg ha^{-1})	67	212
5	P (Kg ha^{-1})	516	27
6	K (Kg ha^{-1})	345	286

Microbial load of spent engine oil contaminated soil and uncontaminated soil

The results shown in table 2 revealed that the mean bacterial, fungi and actinomycetes counts of soil samples contaminated with spent engine oil from the auto mechanic workshop and that of soil which had no contamination. The mean aerobic bacterial counts were 4.8 x 10^3 CFU/g and 2.7 x10^4 CFU/g for the contaminated and non-contaminated soils respectively. Mean fungi counts were 6.7 x10^4 CFU/g cfu/g and 3.48 x 10^4 CFU/g cfu/g for contaminated and non-contaminated soils respectively. Mean actinomyces counts were 1.7 x10^3 CFU/g and 1.1 x10^4 CFU/g for contaminated and non-contaminated soils respectively.

Table-2

Microbial load of spent engine oil contaminated soil and uncontaminated soil

SNo	Isolates name	Gram staining	Cell shape	Endo spore	Motility	Catalase	Oxidase	Tentative bacterial genus
1	HC-1	+	Rods	+	+	+	+	*Bacillus* sp.
2	HC-2	-	Short rods	-	+	+	+	*Pseudomonas* sp.
3	HC-3	+	Rods	+	+	+	+	*Bacillus* sp.
4	HC-4	-	Short rods	-	+	+	+	*Pseudomonas* sp.

Isolation of bacteria from spent engine oil contaminated soil

The isolation of bacteria from spent engine oil contaminated soil was tabulated in table-3. Among the four bacterial isolates, two isolates namely HC-1 and HC-3 were gram positive rods, motile, endospore forming with catalase and oxidase positive bacteria which identified tentatively as *Bacillus* sp.HC-1 and HC-3. HC-2 and HC-4 isolates were gram negative, short rods, gram negative, non-spore forming with catalase and oxidase positive bacteria, which identified tentatively as *Pseudomonas* sp. HC-2 and HC-4. Jaboro *et al*. (2013) reported that bacterial isolates identified from the soil samples were: *Klebsiella oxytoca*, *Bacillus subtilis*, *Strepto*coccus *sp.*, *Pseudomonas aeruginosa*, *Bacillus megaterium, Staphylococcus epidermidis, Enterobacter aerogenes, Escherichia coli, Arthrobacter* sp., *Nocardia* sp. and *Corynebacterium* sp.

Table-3

Isolation of bacteria from spent engine oil contaminated soil

S.No	**Soil samples**	Total Bacterial Count (cfu/g)	Total fungal count (cfu/g)	Total actinomycetes count (cfu/g)
1	Used engine oil contaminated soil	4.8 x 10^3	6.7 x10^4	1.7 x10^3
2	Uncontaminated soil	2.7 x10^4	3.48 x 10^4	1.1 x10^4

Out of the 10 bacterial isolates three predominant bacterial isolates such as Bacillus sp, Pseudomonas sp and Micrococcus sp were identified as hydrocarbon degraders possessing higher efficiency to grow on diesel (Mahalingam and Nithya Sampath, 2014).

Percentage of spent engine oil degradation in minimal salt medium by bacterial isolates

The percentage of spent engine oil degradation in minimal salt medium by bacterial isolates were presented in figure-1. The spent oil degradation was increased with increase the incubation periods. The maximum degradation was observed at 20th day of incubation. Among the four isolates, *Pseudomonas* sp. HC-2 exhibited the maximum degradation of spent oil followed by *Pseudomonas* sp. HC-4, *Bacillus* sp. HC-3 and *Bacillus* sp.HC-1. This was probably due to the exponential phase of the cell growth but after that the rate of degradation was slightly decreased. It was possibly because of cells of the *Pseudomonas* sp. were near to its stationary phase of cell growht (Nikhil *et al*., 2013). Current research reveals that in aquatic and terrestrial environments microorganisms are the chief agents for the biodegradation of molecules of environmental concern, including petroleum hydrocarbons (Swanell and Head, 1994; Balba et al., 1998). The most common genera known to be responsible for oil degradation or breakdown comprise mainly *Nocardia, Pseudomonas, Acinetobacter, Flavobacterium, Micrococcus, Arthrobacter, Corynebacterium, Achromobacter, Rhodococcus, Alcaligenes, Mycobacterium, Bacillus, Aspergillus, Mucor, Fusarium, Penicillium, Rhodotorula, Candida and Sporobolomyces* spp. (Atlas, 1981; Atlas and Bartha, 1992; Sarkhoh *et al*., 1990).

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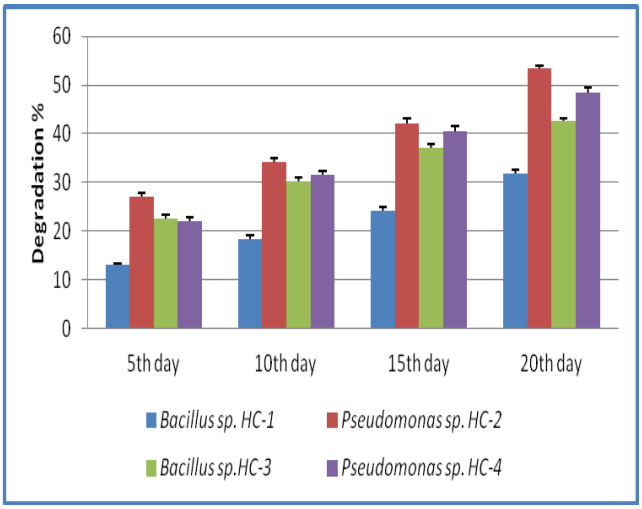

Figure-1: Percentage of spent engine oil degradation in minimal salt medium by bacterial isolates

Effect of spent engine oil concentration for degradation in soil

The effects of spent engine oil concentration for degradation in soil were presented in figure-2. The degradation was increased with low concentration of substrate while beyond the saturated level, the degradation ability was decreased. *Pseudomonas* and *Bacillus* isolates were degraded upto 1 and 2 % concentration respectively after that degradation capacity was reduced. *Pseudomonas* sp. HC-2 isolate had ability to degrade significantly upto 3% concentration of spent engine oil.

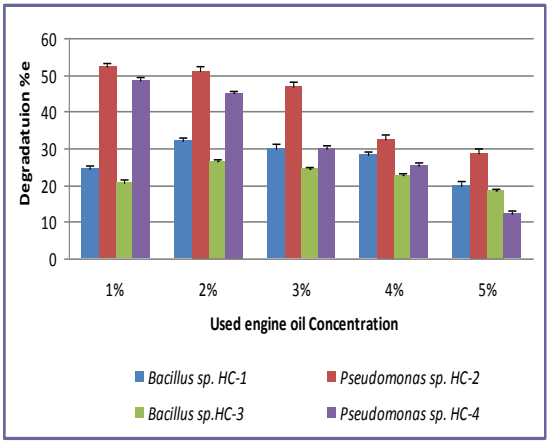

Figure-2: Effect of spent engine oil concentration for degradation in soil

Effect of inoculum size for degradation of spent engine oil in soil

The effect of inoculum size for degradation of spent engine oil in soil was presented in figure-3. The degradation of spent engine oil in soil increased with increased the inoculum size (%) beyond the optimal density of bacterial load the degradation was reduced. The 3 % inoculum size was optimum for *Pseudomonas* sp. HC-2 (48.58± 0.59387 %) and *Pseudomonas* sp. HC-4 (38.59± 0.53183%). The 4% inoculum size was optimum for *Bacillus* sp. HC-1 (36.40± 0.68226%) and *Bacillus* sp. HC-3 (37.28± 1.00127).

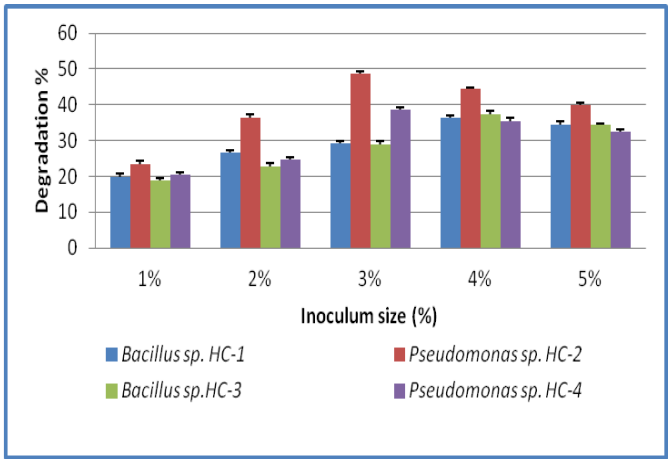

Figure-3: Effect of inoculum size for degradation of spent engine oil in soil

Effect of incubation periods for degradation of spent engine oil in soil

Effect of incubation periods for degradation of spent engine oil in soil were presented in figure- 4. The degradation was increased with increase the incubation periods. The maximum degradation was observed with *Pseudomonas* sp. HC-2 (57.05± 0.86377%), followed by *Pseudomonas* sp.HC-4 (53.42± 1.12295%), *Bacillus* sp. HC-3 (49.23± 0.80230%) and *Bacillus* sp. HC-1 (47.07±0.87178 %).

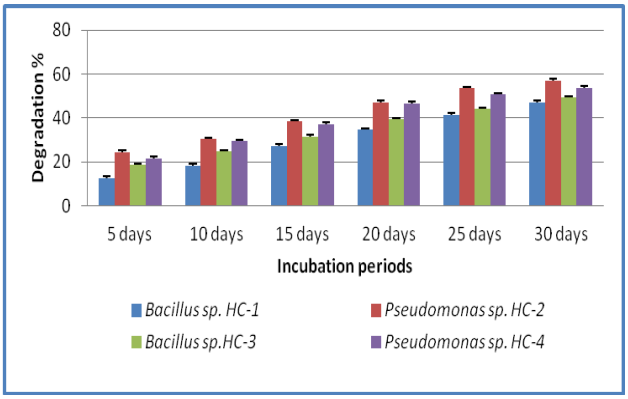

Figure-4: Effect of incubation periods for degradation of spent engine oil in soil

Analysis of 16s rrna gene sequence

The 16S rDNA gene of the strain HC-2 was PCR amplified and sequenced. A total of 1419 bp has been obtained (Fig.5). A BLAST search of the Gen Bank data base results showed that the new isolate had the highest similarity (99%) with *Pseudomonas aeruginosa* K-6 (GenBank entry: FJ972528), *Pseudomonas aeruginosa* S-3 (JX 090597), *Pseudomonas aeruginosa* NO3 (FJ972537) and *Pseudomonas aeruginosa* P60 (KF 670598). The phylogenetic tree generated by a weighted neighbor-joining (Fig.-7) method clearly revealed the evolutionary relationship of the strain HC-2 to a group of *Pseudomonas aeruginosa.* Thus, this strain was designated as *Pseudomonas aeruginosa* HC-2 which involved in spent oil degradation.

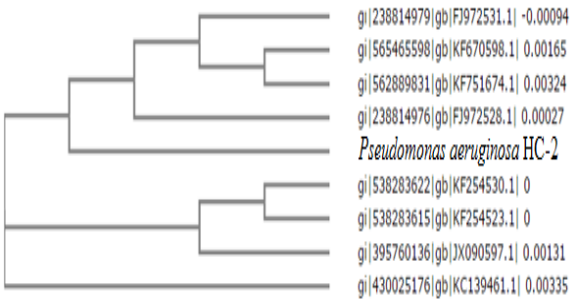

Figure-5: Phylogenetic tree based on weighted neighbor-joining method for the selected strain

The above experiment shows that bioremediation can be used effectively to treat oil contaminated soil. The remarkable rate of diesel oil degradation by bacterial isolates shown by this method allows for the safe and convenient use of this microorganism in the oil contaminated area. Moreover the results obtained from the comparison between the diesel oil degrading ability of Pseudomonas sp and mixture of both helps them to use in different bioremediation processes based upon their efficiencies. And the advantages of employing mixed cultures as opposed to pure cultures in bioremediation have been demonstrated.

Conclusions:-

The spent engine oil contaminated soil had a higher conductivity, total organic carbon and phosphorus when compared to the uncontaminated soil. The present study monitored the spent engine oil degradation bacteria was isolated and identified as Pseudomonas sp. The results show that all the isolated strains are capable to grow in mineral salt medium that were contaminated with spent engine oil.

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